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Original Article

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Duloxetine Hydrochloride and Methylcobalamin

Alka Verma*, D.S. Rathor*, G.VidyaSagar, Bhupesh K.Verma***, Abhishek*, Poonam Parashar******

Abstract

This work is concerned with application of simple, accurate, precise and highly selective reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of duloxetine hydrochloride (DUL)and methylcobalamin (MCB) in combined dosage form. chromatographi separation was achieved by using a reverse phase C18 coloum (Hypersil BDS 250X 4.6X5µm). The mobile phase composed methanol : phosphate buffer in ratio 70:30 at flow rate of 1ml/min .The ph was adjusted to 4.5 with .1N NaOH. Detection was carried out using a UV-vis detector at 215 nm. The mean retention time of duloxetine hydrochloride (DUL)and methylcobalamin(MCB) was found to be 3.3 min 6.7 min respectively. The method was found to be linear in the range of 30 - 90 µg/ml and 2.5-6.75 µg/ml for DUL and MCB respectively, with mean recovery of 98.88 % DUL and 100.22% for MCB the correlation coefficient for both DUL and MCB were closed to one. The developed method was validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribe values thus proposed method was successfully applied for simultaneous determination of DUL and MCB in routine analysis of formulation.

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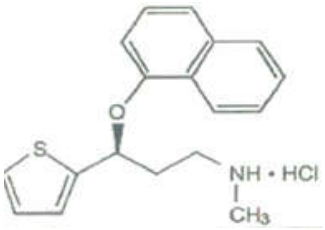
Introduction

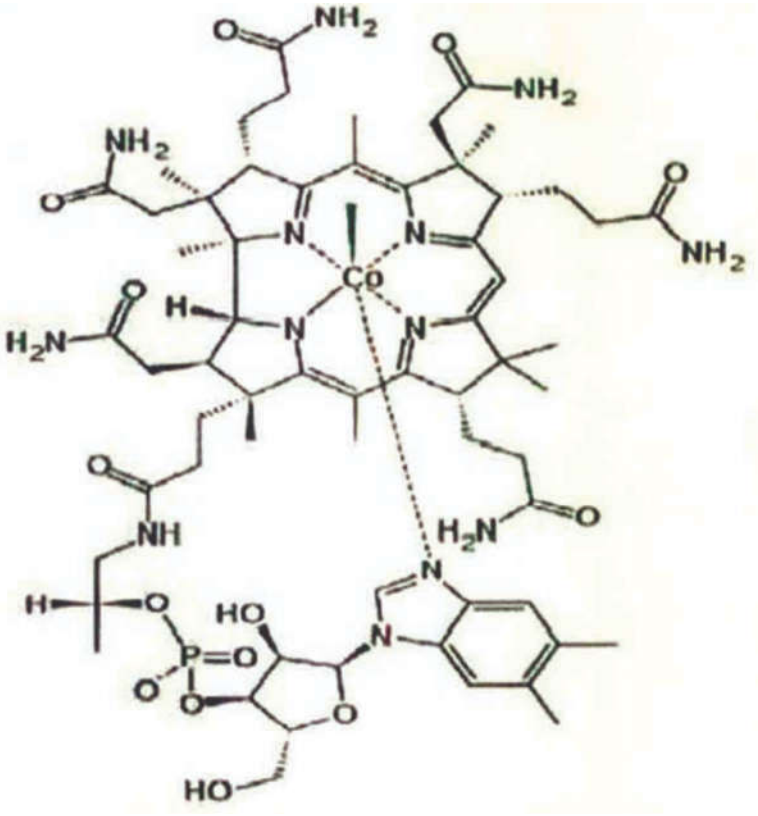
Duloxetine is used for major depression disorders and anxiety. It is a potent inhibitor of neuronal serotonin and nor epinephrine reuptake [1,2]. It produces its pain inhibitory action by potentiation of serotonergic and Noradrenergic [3,4]. Duloxetine hydrochloride (DUL) is chemically (3S)-N-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl) propan-1-amine hydrochloride [5,6].



Duloxetine Hydrochloride

Methylcobalamin used in the treatment of diabetic neuropathy, trigeminal neuralgia, megaloplastic anemia, and facial paralysis in Bell's palsy syndrome. It is chemically Coα-[α-(5,6-dimethylbenz-1H-imidazolyl)] Coâmethylcobamide.Chemically it is known as carbanidecobalt(3+)[5-(5,6-dimethylbenzimidazol-1-yl)-4-hydroxy-2(hydroxymethyl)oxolan-3-yl] 1-[3-[(4Z, 9Z,14 Z)-2,13,18-tris (2-amino-2-oxoethyl)-7,12,17-tris (3-amino-3-oxopropyl)-3, 5, 8, 8, 13, 15, 18, 19-octamethyl-2, 7,12, 17 tetrahydro-1H-corrin-21-id-3-yl] propanoylamino propan-2-yl phosphate and have

molecular formula.C63H91CoN13O14P[4]. It is a dark red crystalline powder soluble in water and ethanol[5]. Vitamin B12 is used in the body in two forms such as Methylcobalamin and 5-deoxyadenosyl cobalamin [7-12].



Methylcobalamin

The combined dosage forms of these drugs are used for the treatment of neuropathic pain associated with peripheral neuropathy especially diabetic polyneuropathy. It restores the balance of neurotransmitters in the brain like serotonin and norepinephrine [13-17].

Literature review revealed that analysis of DULO and MCA is mainly carried out on single or with other drugs combination by UV-Spectrophotometry, HPLC and HPTLC with derivatization. The present work describes simple, specific, rapid, accurate and precise chromatographic method based on Absorption correction method for simultaneous estimation of both drugs in their combined tablet dosage forms. Several methods has been reported for estimation for individual drugs. Dulo can be quantified by spectrophotometric method [14],RP-HPLC including solid phase extraction , GC-NPD and GC-MS [15-21], Similarly literature are available for the quantification of methylcobalamin by HPLC [22-32].

Experimental

Chemicals & Reagents

DUL and MCB were obtained as gift samples from Lupin pharmaceutical Ankeleshwar India. All chemicals and reagents were of analytical grade. HPLC grade methanol,water from Loba chem. A commercial sample of tablet of containing DUL and MCB in ratio of 20mg:1500mcg respectively was procured from local market.

Instrumentation and Chromatographic Conditions

The HPLC system consisted of a thermo separation products quaternary gradient system equipped with HPLC pump specter system Stationary phase used Hypersil BDS C-18 (250 x 4.6 mm), 5)l column . the data was acquired by a chromatographic module connected to a personal computer and processing was performed running DATA ACE software . The chromatographic conditions were optimized by varying the concentration and Ph of water and the percentage of organic solvent . The mobile phase consisted of Methanol: Phosphate Buffer (70/30 v/v) pH of mobile phase was adjusted to 4.5 using O.1N

NaOH ,Flow rate: 1.0 ml/min. The mean retention time for DUL and MCB was as 3.3min and 6.7min respectively.

Preparation of Standard Solution

Accurately weighed Duloxetine Hydrochloride (60 mg) was transferred to 100 ml volumetric flask and dissolved in Methanol: Phosphate Buffer (PH 4.5) and diluted up to the mark to give a stock solution having strength 1mg/ml (600 ug/ml).60 ug/ml of DUL working standard solution was prepared by diluting 1 ml of stock solution to 10 ml with Methanol: Phosphate Buffer (pH 4.5) Accurately weighed Methy1cobalamin (45 mg) was transferred to 100 ml volumetric flask and dissolved in Methanol: Phosphate Buffer (pH 4.5) and diluted up to the mark to give a stock solution having strength 1mg/ml (450ug/ml), Take 1 ml of secondary stock solution and diluted it up to 10ml with Methanol: Phosphate Buffer (pH 4.5) to Produce a stock solution having strength 1mg/ml (45ug/ml). 4.5ug/ml of Methylcobalamin working standard solution was prepared by diluting 1 ml of stock solution to 10 ml with Methanol: Phosphate Buffer (pH 4.5)

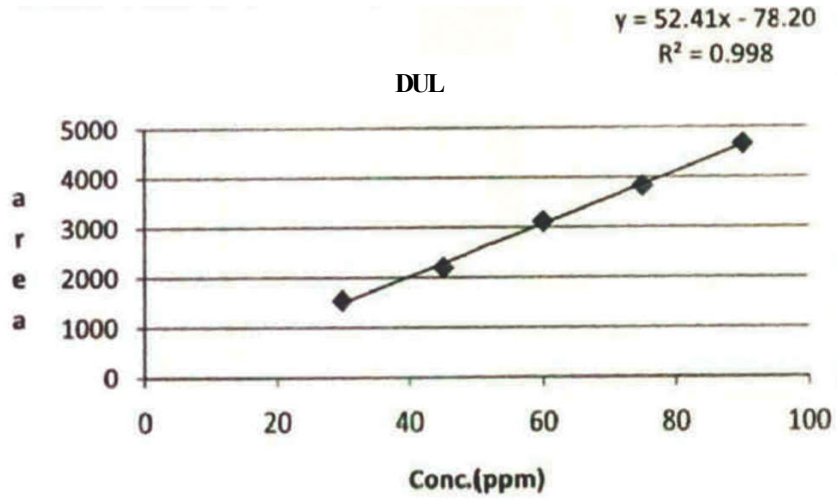


Fig. 1: Standard calibration curve for duloxetine hydrochloride

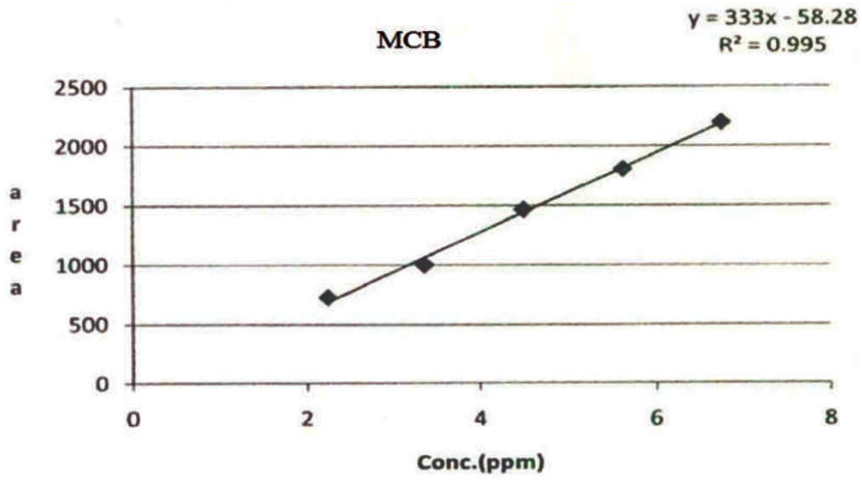


Fig. 2: Standard calibration curve for methylcobalamin

Sample Preparation

Accurately 20 tablets were weighed to determine average weight of tablets. Then tablets were finely crushed and tablet powder equivalent to 20 mg Duloxetine hydrochloride and 1500 mcg Methylcobalamin was transferred into 100 ml volumetric flask. Then 50 ml diluents was added to flask and sonicated for few minutes with intermittent shaking. Make up volume up to 100 ml. than solution was filtered and the final concentration of test sample solution was made up to 60ug/ml of Duloxetine Hydrochloride and 4.5 ug/ml of Methylcobalamin.

Results and Discussion

Method Development and Optimization of Chromatographic Condition

Chromatographic condition was achieved on Hypersil BDS C-18 (250 x 4.6 mm), 5) 1 column by varying the concentration of organic phase and water simultaneously pH was varied. Mobile phase was optimized for method advantage by making use of methanol instead of water hence in the process od optimization success was achieved by making use of Methanol: Phosphate Buffer (70/30 v/v).The pH of mobile phase was adjusted to 4.5 using O.1N NaOH , flow rate was 1.0 ml/min, Temperature: 25 ± 2^0C , Wavelength: 215nm , Run time: 20 min ,both DUL and MCB were will separated from each other with mean retention time for DUL and MCB as3.3min and 6.7min respectively.

Method Validation

Linearity

The method was linear in the range of 30 -90µg/ml to 2.25 to 6.75 µg/ml for DUL and MCB. Linear regression date was given in Table 1.

Precision

For the precision study ,repeatability study was carried out for 0.5, 1.0 and 1.5 ml of DUL & 0.5, 1.0 and 1.5 ml of MCB of combined working standard solutions (600 ug/ml of DUL and 45ug/rnl of MCB) were transferred into a 10 ml volumetric flasks and diluted up to mark with Methanol: Phosphate Buffer (pH 4.5) to get 30, 60 and 90µg/ml of DUL and 2.5, 4.5 and *6.75µg/ml* of MCB. Each concentration was prepared in triplicate. The absorbance of the each solution was measured at selected wavelengths and %C.V. was calculated The Intraday %C.V. for DUL and MCB were found to be 0.949-1.742 and0.611-1.348 respectively. Interday %C.V. for DUL and MCB were found to be 0.761-1.65 and1.135-2.426 respectively. from the date obtained the developed RP-HPLC Method was found to be precise.

Accuracy

Accuracy was determined over the range 50% to 150% of the sample concentration. Calculated amount of DUL and MCB was added in placebo to attain 80%, 100% and 120% of sample concentration. Each sample was prepared in triplicate at each level and each preparation was injected in duplicate. Blank and standard preparations were injected and the chromatograms were recorded. Mean % recovery for Duloxetine Hydrochloride and Methylcobalamin were found to be 99.88% and 100.22% respectively.

Table 1: Linear regression data for calibration curve

Parameter	Duloxetine Hydrochloride	Methylcobalamin
Linearity range (µg/ml)	30-90	2.25-6.75
Correlation coefficient	0.998	0.995
Intercept	78.20	58.28

Table 2: Repeatability data of duloxetine hydrochloride and methylcobalamine

Sr. No.	Statistical data	Duloxetine Hydrochloride	Methylcobalamin
1	Mean	3100.65	1455
2	S.D.	12.55	23.78
3	%C.V.	1.10	1.47

Table 3: Summary of precision data for duloxetine hydrochloride and methylcobalamine

Parameter	Statistical data	Duloxetine Hydrochloride			Methylcobalamin		
	Concentration(µg/ml)	30	60	90	2.25	4.5	6.75
Intra- day	Mean	1524.4	3092	4625.6	458.14	856.1	1259.02
	SD	17.26	10.33	14.6	2.90	7.56	16.98
	%C.V.	1.021	1.742	0.949	0.611	0.884	1.348
Inter-day	Mean	1528	3090	4631	463.01	857.42	1254.15
	SD	7.96	8.46	13.6	11.23	9.74	14.23
	%C.V.	1.650	0.761	0.828	2.426	1.136	1.135

Conclusion

RP-HPLC method was developed for estimation of Duloxetine Hcl and Methylcobalamin using methanol: phosphate buffer pH 4.5 (70:30 v/v) as a mobile phase. The plot of area versus respective concentrations of DUL and MCB were found to be linear in the concentration range of 30- 90lµg/ml and 2.25-6.75 µg/ml respectively with correlation coefficient 0.998 and 0.995. Repeatability was found to be 1.10 for DUL and 1.47 for MCB. Precision was calculated as interday and intraday variations and % CV was found to be less than 3% for both the drugs. The mean % recovery ranges were found to be 98.78-100.63% and 98.98-101.23% for DUL and MCB respectively.

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Original Article

Bioanalytical Method Development and Validation by Hyphenated Technique (LC-MS/MS)

Farah Iram*, Huma Iram*, Mohd Arif*, A.A. Siddiqui*, Asif Husain*

Abstract

Bioanalytical method development plays importance role in the preclinical and clinical studies. Pharmacokinetics of any drug and/or its metabolite can be recognised by bioanalytical studies. The quantitative analysis of drugs and their metabolites in the biological media is done by bioanalytical studies. Physico-chemical and biological techniques are used for these studies. Each bioanalytical method should be selective, sensitive and reliable for the quantitative evaluation in drug discovery process. Bioanalytical method development consists of sample preparation, chromatographic separation and detection by using proper analytical method. Each developed method should be validated as per the regulatory authorities, so as to give reliable and reproducible method for the intended use. Many analytical techniques can be use for bioanlysis, LCMS/MS is one of them. In Liquid chromatography-mass spectrometry (LC-MS/MS) the separation of analyte is done by LC and detection is carried out by MS. LC-MS/MS prominently used in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. This review also focused on various validation parameters such as: accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability, etc.

Keywords: Bioanalytical; Liquid Chromatography; Spectrometry; Bioanalysis; Validation.

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Introduction

Bioanalytical Methods are widely engaged for the quantitative analysis of the drugs. Bioanalysis is the method to investigate the concentration of drugs, their metabolites and/or endogenous substances in the biological matrices such as blood, plasma, serum, cerebrospinal fluid, urine, and saliva [1-3]. It also plays an important role in the evaluation of bioavailability, bioequivalence, pharmacokinetics studies [4-7]. The reason behind for new method of analysis is:

- Unavailability of suitable method for a particular analyte in the specific matrix. Already available method may have too pitfalls and poor in accuracy or precision.
- Present methods may be costly, laborious, and tedious.
- Poor in sensitivity and selectivity in samples of interest.
- If new instrumentation or techniques is incorporate in for developed method.
- There is a need for alternative methods to confirm, for legal or scientific reasons. [8-12].

Chromatographic techniques like, HPLC, Gas chromatography, LC-MS, GC-MS, Ligand binding assay, immunological and microbiological procedures are used for the bioanalysis purpose. The

method includes collection of sample, processing, storage in suitable conditions and finally analysis of a biological matrix for a drug. Method development consists of three essential parts sample preparation, chromatographic separation and detection by using proper analytical method. The documentation and verification of specific laboratory investigations, quantitatively of a drug substance in a given biological matrix is done by bioanlytical method validation. The basic parameters of validation comprises of various parameters such as selectivity, sensitivity, calibration curve, accuracy, precision, stability, lower limit of quantification (LLOQ), recovery, linearity, limit of detection, reproducibility, and ruggedness [13-17] .

This U.S. FDA guidance supports the sponsor of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs) and supplements in developing bioanalytical method validation. It also provides the assistance in the information used in human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation. The guideline also supports the bioanalytical methods used for non-human pharmacology/toxicology studies and preclinical studies [18].

The bioanalysis of drugs in plasma can be done by both HPLC and LCMS-MS method. Each analytical instruments has its own pros and cons. HPLC coupled with detector (UV, PDA or fluorescence) can evaluate many compounds and it offers a cost effective bioanalytical method. The demerit is poor sensitivity and selectivity for some of the potent compounds. Whereas low detection limits, good ability to generate structural information, minimal sample requirement and wider coverage of range of analytes differing in their polarities can be obtained by using LC-MS/MS. But some extent LC/MS/MS instruments are limited due to matrix-induced effect in ionization efficiencies and ion suppression or enhancement (due to presence of biological matrix). The integral use of LC-MS/MS can be seen from last few decade, as it provides high sensitivity, amazing selectivity, and rapid rate of analysis [19,20]. The review focuses on bioanalytical method development and validation using LC-MS/MS technology.

Bioavailability and Bioequivalence

The pharmaceutically equivalency between the test product and a reference product bioavailability/bioequivalence studies are required to done by regulatory bodies. Both objectives is on the release of drug substance from its dosage form and successive absorption into the systemic circulation. The equivalence can be assess by: comparative bioavailability/bioequivalence studies, comparative pharmacodynamic studies in humans, comparative clinical trials and In-vitro dissolution tests. For evaluation of two medicinal products containing the same active substance bioequivalence studies are done. The therapeutic equivalency should be present for two products marketed by different licensees, containing same active ingredient, in order to be considered interchangeable. Bioequivalence studies are a pivotal part of registering dossiers. Bioequivalence data is preliminary requirement for ANDA submission. The pharmacokinetic parameters such as area under the curve (AUC), peak concentration (Cmax), time to peak concentration (Tmax) can be evaluate by the plasma concentration data [20-22].

Method Development

A well organized method development is important in drug development. Analytical method development can be defined as the process of identifying the procedure to facilitate the identification and quantification of compound of interest in a matrix. Several methods can be used in identification of compound, analytical method involves in identification and characterisation depends on: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, and speed of the analysis, quantitative or qualitative measurement, precision required, the necessary equipment and on many other factors [4,9,23-30].

A Method development comprises of three components: sample preparation, separation of analyte and detection of analyte [31-33].

Collection of Sample and Its Preparation

The analyte are usually present in biological matrix that is blood, plasma, urine, serum etc. Hypodermic syringe (5 to 7 ml) is used to puncture the vein for blood collection. Further the venous blood is withdrawn into tubes, using an anticoagulant, e.g. EDTA, heparin etc is used. Finally centrifugation at 4000 rpm for 15 min is carried out by which plasma is separated out [34-36]. Sample preparation carried out to clean up the sample before analysis and to concentrate the sample. Much interference may occur

during analysis which may be due to proteins, salts, endogenous macromolecules, small molecules and metabolic by products. The sample preparations also allow the exchange of analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. Sample preparation can be done by solid-phase extraction (SPE), liquid/liquid extraction and protein precipitation [37-40].

Biological Samples Preservation

Biological fluids are highly susceptible to physicochemical changes. Processing or purifying biological samples is often time consuming therefore optimal storage conditions must be established for biological samples. Samples sensitive to oxidation can be protected by using air tight containers. Moisture sensitive drugs dehydration could be achieved largely by freeze-drying or lyophilisation [41,42].

Sample Pretreatment [43-44]

If the analyte is protein-bound. In such cases, one of the following pre-treatment can be followed:

- Using 0.1M or greater concentration of acids or bases make the pH of the sample to pH<3 or pH>9.
- Precipitate the proteins from biological fluid with a polar solvent such as acetonitrile, methanol, or acetone in 1:2 ratios.
- The biological fluid is then treated by acids or inorganic salts, such as formic acid, perchloric acid, trichloroacetic acid, ammonium sulphate etc.

If the analyte is not protein bound, the pre-treatment is done by centrifugation, homogenization and hydrolysis of conjugates.

Separation of Analyte

Extraction Procedures for Drugs from Biological Samples

Extraction of analyte from biological matrix is mainly carried out by three processes:

(i) liquid-liquid extraction (LLE),

(ii) solid-phase extraction (SPE) and

(iii) precipitation of plasma proteins (PP)

Liquid-Liquid Extraction (LLE)

It is based on the principle of differential solubility and partitioning equilibrium of the analytes between the aqueous and organic phases. It generally involves the extraction of analyte from one phase into another phase and the distribution of the analyte molecules between two immiscible phases. In LLE compounds separation is carried out in a mixture using water and an immiscible organic solvent. LLE method is simple, rapid, and relatively cost effective compared to other techniques. 90% of the drug can be recovered by multiple continuous extraction technique [9,38,45].

In LLE dissolve the component mixture in a suitable solvent and then add an immiscible solvent with the first solvent. Completely mix the content and allow the separation of two immiscible solvents into layers. Based on the partition coefficients of the solvents the components of the mixture will be scattered amongst the two immiscible solvents. Separate the two immiscible solvent layers, transfer and isolate the component from each solvent. After extraction the aqueous phase has hydrophilic compounds and hydrophobic compounds are found in the organic solvents. By evaporation the non polar analytes in organic solvents are recovered. Further the residue reconstituted with a small volume of an appropriate solvent preferably mobile phase. Whereas the analytes which are polar in nature can be extracted in to the aqueous phase and can directly inject into a reverse phase (RP) column [9,39,46]. Traditional LLE can be replaced with advanced and improved techniques like liquid phase micro extraction (LPME), single drop liquid phase micro extraction (DLPME) and supported membrane extraction (SME) [2,4].

Protein Precipitation (PP)

Protein precipitation is another important technique for extraction of the analyte from matrix. The principle behind PP is the precipitation (denaturation) of the proteins by using a range of reagents like acid (trichloroacetic acid and perchloric acid), organic solvents (methanol, acetone and acetonitrile) or by salts (ammonium sulphate). After denaturation the sample is centrifuged, that gives analyte into supernatant form. PP is less time consuming, smaller amount solvents are used. The samples often contain protein residues and it is a non-selective sample cleanup method. The limitation of PP is that it may clog the LC column. Of recently PP technique is combined with SPE to give clean extract. Methanol is generally preferred as solvent and can produce the appropriate for direct injection into LC-MS/MS [4,9,47,48].

Solid Phase Extraction (SPE)

SPE is frequent and effective technique for isolation of analyte in trace amounts in sample matrices. With SPE the level of interferences can be reduced. The final sample volume is minimized to maximize analyte sensitivity. SPE provide higher recovery of analyte. In SPE small plastic disposable column or cartridge packed with 0.1 to 0.5 g of sorbent which is commonly RP material (C18 or C8) is used. The analyte may either preferentially adsorbed to the solid, or they may remain in the liquid phase. The analyte can desorb by washing with an appropriate solvent, if the analyte is adsorbed. If the component of interest remains in a liquid phase, it can be recovered through concentration, evaporation and or recrystallization [4,9,46,49-51].

Solid phase consists of four steps: conditioning, sample loading, washing and elution.

- *Conditioning*:Conditioning is basically activation of the column. Organic solvent that acts as a wetting agent on the packing material are used for conditioning of the column. The solvents solvates the functional groups of the sorbent. For proper adsorption, water or aqueous buffer is added to activate the column.
- *Sample Loading:* The sample is loaded on the column, after the adjustment of pH
- *Washing*: Washing is done in which interferences from the matrix are removed and the analyte will retain.
- *Elution:* For elution a suitable solvent or buffer is used, which elutes the analyte from the SPE bed for analysis [52,53].

Types of Solid Phase Extraction Cartridges: [54-57]

1. *HLB Cartridge:* HLB is Hydrophilic-Lipophilic Balanced water-wettable reversed phase sorbent. Two monomers hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbezene are present in specific ratio in HLB cartridg . It is available in various particle sizes such as (60ìm, 30ìm, 15ìm etc.)
2. *MCX Cartridge:* It is a mixed mode cation exchange, water-wettable, polymeric sorbent. It is a water-wettable, mixed-mode polymeric sorbent, to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups.
3. *MAX Cartridge:* MAX (mixed-mode anion-exchange) is usually intended to overcome the drawback of silica-based mixed-mode SPE sorbents. This cartridge has a mixed-mode anion-exchange, water-wettable, polymeric sorbent which is stable from pH 0 to 14.
4. *WCX Cartridge:* WCX (mixed-mode weak cation-exchange) is usually intended to provide sample preparation for strong bases and quaternary amines. It has a water-wettable polymeric sorbent.
5. *WAX Cartridge:* WAX cartridge is for strong acids. WAX is mixed-mode weak anion-exchange reversed phase sorbent.
6. *Bond Elute Plexa:* It has non polar retention mechanism. It gives clean extracts which minimizes matrix interference

Strategy of LC-MS/MS Method Development

In many diverse ways method of analysis are being routinely developed, improved and validated. Type of sample will decide the required chromatographic conditions. So the knowledge of sample and chromatographic procedure is a must for systematic approach to LC-MS/MS method development [55,57-59].

- Physicochemical properties of drug molecules from literature
- Determine solubility profile
- MS scanning and optimization
- Mobile phase selection
- Selection of extraction method and optimization
- Selection of chromatographic method (based on solubility study, retention of compound)

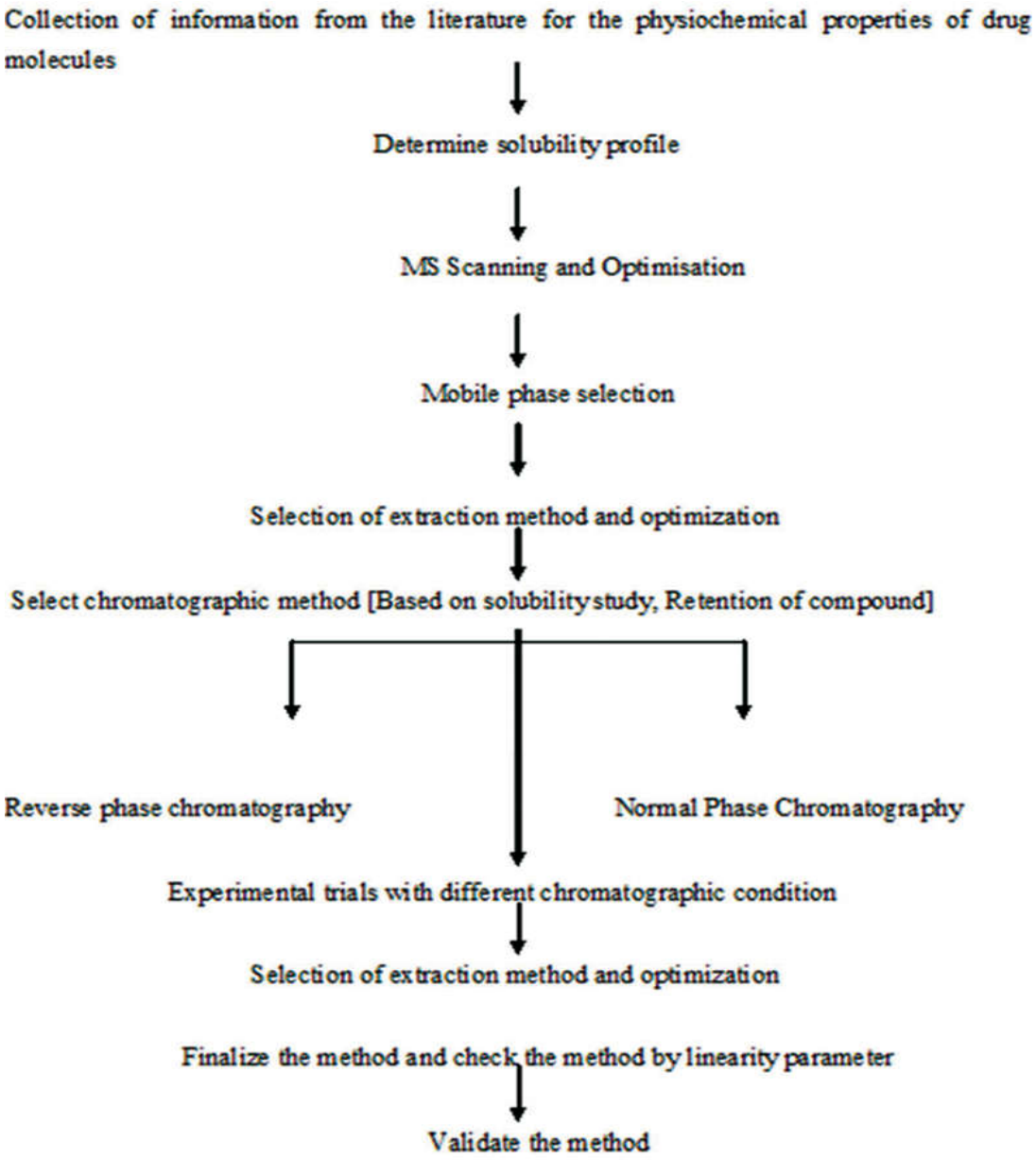
Flow chart for Method Development

During method optimization, the initial problems that have arise from the first stages of development are enhanced in terms of resolution and peak shape, retention time, limit of quantitation, and overall ability to quantify the specific analyte of interest. In method development various parameters need to be optimized: [4,9,60].

- Separation mode
- Stationary phase selection
- Mobile phase selection
- Selection of detector

Mode of Separation Technique

Most of the pharmaceutical compounds are polar



in nature so reverse phase chromatography is preferred first in which a non polar stationary phase and non polar mobile phase is used. The mobile phase comprises of water or buffer and organic phase (acetonitrile/methanol). Hence polar compounds get eluted first and non-polar compounds are retained for a longer time. The stationary phases used in reverse phase chromatography are n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, cyano, diol and hydrophobic polymers. It is the first choice for most samples; especially neutral or un-ionized compounds that dissolve in water-organic mixtures. Normal phase is try if reverse phase fails where the sample may be strongly retained with 100% acetonitrile as mobile phase. In reversed-phase chromatography the retention mechanism is between the column's stationary phase and sample analytes [61].

Selection of Stationary Phase

Selection of stationary phase is based on following parameter.

Column

The column is considered as the heart of separation process. The development of an accurate, precise, rugged and reproducible method is possible with the availability of a stable, high performance column. Commercial columns can differ widely. These differences can have a serious impact on method

development. The columns differ usually in plate number, band symmetry, retention, band spacing and lifetime.

Column Selection

Column selection should be based both upon knowledge of the sample and goals of the separation. Including sample knowledge and the goals of separation the following factors also considered for column selection:

Column Internal Diameter

Generally wider diameter columns are chosen for greater sample loading, whereas narrow columns are preferred for more sensitivity.

Particle Size

For complex mixture with similar components smaller particle (3-4 μm) are considered. Whereas bigger particle (5-10 μm) for sample with structurally different compounds [62,63].

Selection of Mobile Phase

Mobile phase composition plays significant role in improving peak resolution and peak sensitivity. By choosing the appropriate match between the stationary phase and mobile phase composition, the developed method simplifies the procedure and significantly decrease total analysis time as well as increase peak height.

In case of reverse phase chromatography, mobile phase with polar characteristic is used while as for normal phase a non polar mobile phase is used. More polar solvents cause increased retention in RPC or reduce retention in NPC. If the buffer pH is close to the pKa of the analytes, then selectivity altered. Subsequently, the mobile phase is modified by decreasing the proportion of water, and by increasing the addition of an organic solvent such as MeOH or acetonitrile, which causes the retained analyte to elute off the stationary phase. Changing the mobile phase composition in this way is the most efficient way of achieving chromatographic resolution.

Following parameters shall be taken into consideration while selecting and optimizing the mobile phase [60,64].

Buffer

Buffer and its strength play an essential role in deciding the peak symmetries and separations. The retention times also depend on the molar strengths of the buffer. Molar strength is proportional to retention times.

pH of the Buffer

pH plays an essential role for good chromatographic separations by controlling the ionization characteristics. It is essential in maintaining the pH of the mobile phase in the range of 2.0 to 8.0, columns does not withstand to the pH which are outside this range. The reason behind this is that the siloxane linkage area cleaved below pH 2.0, while pH valued above 8.0 silica may dissolve.

Mobile Phase Composition

Selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions in mobile phase. Methanol and acetonitrile are widely used solvents in reverse phase chromatography [24,47,65].

Estimation of Drugs by LC-MS/MS

LC/MS is a hyphenated technique, combining the HPLC separation ability and detection ability of mass spectrometry. The charged particle in LC-MS/MS passes through a magnetic field, which further deflected along a circular path on a radius that is proportional to the mass to charge ratio, m/e. In a mass spectrometer, an electron is displaced from the organic molecule. Too unstable molecular ions get fragmented. The ions is then focused into a beam and accelerated into the magnetic field. According to the masses of the ions, they get deflected along circular paths. The ions are further focused on the detector and finally recorded [47,66-68].

A MS detector consists of three main parts: the ***interface*** where the ions are generated, the ***mass analyser*** (separation) and ***the electron multiplier*** (detector).

Ionization Modes

Electrospray Ionization (ESI): From ESI needle the sample solution enters, the needle was at relatively higher voltage which sprays the sample solution into a fine mist of droplets that are electrically charged at their surface. At the surface of the droplets the electrical charge density increases as solvent evaporates from the droplets. From the very small, highly charged droplets, sample ions are ejected into the gas phase by electrostatic repulsion. The sample ions enter the mass spectrometer and finally analyzed [69].

Atmospheric Pressure Chemical Ionization (APCI)

In APCI, ions are produced when the sample solution in the form of fine mist of droplets enters to the APCI nozzle sprays. In high temperature tube droplets are vaporized. A high voltage is applied to a needle located near the exit end of the tube. This voltage creates a corona discharge that forms reagent ions through a series of chemical reactions with solvent molecules and nitrogen sheath gas. Further the reagent ions react with sample molecules and the sample ions enter the mass spectrometer and further they are analyzed [70].

Matrix Assisted Laser Desorption/Ionization (MALDI)

High molecular weight compounds with high sensitivity are ionised by MALDI. The ionization beam is laser light and matrix is solid. The pulsed laser beam is directed on a sample which is suspended or dissolved in matrix ions [71].

Mass Analyzers

Mass analyzer is the most important part of the LCMS-MS. There are different types of analyser based on their mechanism e.g. Electric sector (Kinetic Energy) Magnetic sector, Quadrople/Ion trap, Time of flight Flight time, FT-ion cyclotron resonance [47,72].

Scan Types

Full Scan: Each analyte is provided with full mass spectrum. Full scan is done to conclude or confirm the identity of unknown compounds or for the identification of each component in a mixture of unknown compounds.

Selected Ion Monitoring (SIM): In SIM a particular ion or set of ions is monitored. SIM experiments are applied when the mass spectrum of target compound is known in detection of small quantities of a target compound in complex mixture.

Selected Reaction Monitoring (SRM): A particular reaction or set of reactions, such as the fragmentation of an ion or the loss of a neutral moiety is monitored is determined in SRM [20,73]

Detectors

The ions from the analyser enter to the detector, where they get detected separately. Electron multipliers, dynolyte photomultiplier and microchannel plates are the different type of detectors used in LC-MS/MS [28,74].

Method Validation

Regulatory agencies have mandated the method validation. Selective and sensitive bioanalytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful biopharmaceutics and clinical pharmacology studies [75]. Bioanalytical method validation includes all procedures that display that a particular developed method for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use [76]. Bioanalytical method undergoes many modifications during a drug development process. Each modification should be validated to ensure suitable performance of the bioanalytical method. The objective of method validation is to exhibit the reliability of a particular method developed for the quantitative determination of an analyte in a specific biological matrix [30,77-81]

Goals

1. Well distinguish and completely validated bioanalytical methods should be used to yield reliable results that can be adequately interpreted.
2. To recognized the changes in the bioanalytical methods.
3. To highlight that each bioanalytical technique has its own features and characters, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyte
4. When sample analysis for a given study is carried out at more than one site, it is necessary to validate the bioanalytical method(s) at each site and provide suitable validation information for different sites to establish inter-laboratory reliability [82-85].

Types of Bioanalytical Method Validation

Full Validation: Full validation is done when developing a bio-analytical method for the first time. It is important for a new drug entity and if metabolites are new to an existing assay for quantification [86-90].

Partial Validation: Partial validations are modifications of previously validated bioanalytical methods. Partial validation can range from one intra-assay accuracy and precision determination to a nearly full validation. Bioanalytical method changes that fall into this category include [91-93]:

- Bioanalytical method transfers between laboratories or analysts
- Change in bioanalysis methodology (e.g., change in detection systems)
- Anticoagulant changes
- Within species if matrix changes (e.g., human plasma to human urine)
- If Sample processing procedures change
- Change in species within matrix (e.g., rat plasma to mouse plasma)

Cross Validation

Cross-validation is a evaluation and comparison of two bioanalytical methods; where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator [91,94-97].

Validation Parameters

System Suitability Test [97- 99]

System suitability test was carried out to verify that the analytical system is working appropriately and give accurate and precise result .It is perform prior to initiation of each analytical run instead of at the start of project. Circumstances where system suitability test is needed are as follows:-

1. After completion of repair of malfunction of chromatographic system in middle of project
2. On change of column in middle of project.
3. Interchange of system component in middle of project.

Method

System suitability test of the LC-MS system, to be used for validation is done by giving

a. One injection of reconstitution solution.

b. After that 6six injection of drug dilution.

c. Finally one injection of reconstitution solution was given.

Acceptance Criteria

%CV for peak area response ratio should be within 4%

%CV for retention should be within 5%

Selectivity [93,98,100]

Selectivity is the capability of an analytical method to discriminate and measure the analyte in the presence of other components in the sample. In selectivity, analyses of blank samples of the suitable biological matrix (plasma, urine, or other matrix) should be acquired from at least six sources. Each blank sample must be tested for obstruction, and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix consist of endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is planned to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

Method

- Six lot of blank plasma were processed and run without addition of internal standard.
- Blank matrix with no or minimum peak area response at RT of all peaks of interest were selected.
- Analyte was spiked in selected blank matrix at LOQ concentration
- Six aliquot of spiked LOQ were processed. Response of interesting peak at retension time of the drug and internal standard in blank matrix was calculated.

Acceptance Criteria

» Response of interfering peak at the RT of analyte in blank matrix must be ≤ 20% of mean peak area response of analyte in LOQQC.

» Response of interfering peak at the RT of internal standard in blank matrix must be ≤ 5% of mean peak area response of internal standard in LOQQC.

» Atleast 80% screened matrix batches should pass.

» %CV should be ≤ 20% for both analyte and internal standard.

Sensitivity [101-103]

Sensitivity can be articulated as the slope of linear regression in the calibration curve, and it is calculated at the time in the linearity test. A technique is said to be sensitive if small changes in concentration cause large change in the response

function. The sensitivity achievable with an analytical method depends on the nature of the analyte and the revealing technique employed. The sensitivity required for a detailed response depends on the concentration to be calculated in the biological specimens generated in the specific study.

The lowest standard should be accepted as LOQ of the method if:-

1. Between batches precision (%CV) at LOQQC is ≤ 20%.
2. Between batches accuracy (% nominal) at LOQQC is 80-120%.
3. Analyte response at LOQQC is at least 5 times the response compared to blank matrix response.
4. S/N ratio of LOQQC sample should be at least 5 times of mean S/N ratio of black matrix samples.

S/N ratio was calculated by taking 4 replicate of spiked LOQQC samples and 4 replicate of pooled blank matrix samples. It was found greater than 5%.

Method

1. Process and analyze four replicate of spiked LOQQC samples and four replicates of pooled blank matrix sample.
2. Report S/N ratio for all and calculate mean of S/N ratio for pooled blank matrix sample
3. Compare the S/N ratio of each LOQQC with mean S/N ratio of blank matrix, it should be ≥ 5 for all LOQQC samples.

Precision and Accuracy [104-107]

Precision

The precision of an analytical method describe the closeness of individual measures of an analyte when the method is applied repetitively to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be considered using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

% CV = (Standard deviation/Mean value) x 100

Two Types of Precision

Inter-Day Precision

The ability to repeat the similar method under altered conditions, e.g. change of analyst, reagent, or equipment; or on subsequent occasions, e.g. over several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay precision.

Intra-Day Precision

This is also known as repeatability i.e. the ability to repeat the same method with the same analyst, using the same reagent and equipment in a short interval of time, e.g. within a day and obtaining similar results.

Acceptance Criteria: The precision resolute at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LOQQC, where it should not exceed 20% of the CV.

Accuracy

The accuracy express as the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. By replicate analysis of samples containing known amounts of the analyte accuracy can be calculated. Accuracy should be calculated using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

% Nominal = (Mean concentration/Nominal concentration) x 100

Acceptance Criteria

The mean value must be within 15% of the actual value except at LOQQC, where it should not diverge by more than 20%. The difference of the mean from the true value serves as the measure of accuracy.

Method

Precision and accuracy of the method was evaluated by running three analytical batches. Determine within batch, between batch, inter-batch accuracy and precision. Each batch contained the following samples

- Reference standard solution (one sample, mixture with internal standard)
- Blank Matrix
- Blank Matrix with internal standard
- Spiked calibration standards (1 set of 8 non-zero concentration)
- LOQQC (6 samples)
- LQC (6 samples)

- ·MQC (6 samples)
- HQC (6samples)

Recovery [81,100,108]

The recovery can be define as the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure reliable standard. Recovery pertains to extraction efficiency of an analytical method. Recovery of the analyte need not to be 100% but extent of recovery of an analyte and of the internal standard is supposed to be consistent, precise and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, high) with unextracted standards that represent 100% recovery. The percentage recoveries for the drugs and the internal standard were determined by comparing the peak areas of the response of drug extracted with that of the peak areas of unextracted aqueous standard samples containing the same concentration of the drug and the internal standard. The percent recoveries were calculated at each QC concentration by the following equation:

% Recovery = Mean peak response of non-extracted samples/ Mean peak response of extracted samples X 100

Acceptance Criteria

The recovery is acceptable if CV is ≤ 20% for % mean recovery between low, middle & high QC concentrations.

Calibration/Standard Curve (Linearity) [89,102,109]

A calibration curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve is generated for each analyte in the sample. Adequate number of standards is used to adequately define the relationship between concentration and response. The biological matrix is same as the samples in the intended study by spiking the matrix with known concentrations of the analyte for calibration curve. The predictable range of analytical values and the nature of the analyte/ response relationship was the function of calibration curve.. A calibration curve be supposed to consist of a blank sample which has matrix sample processed without internal standard, a zero sample which has matrix sample processed with internal standard, and six to eight non-zero samples covering the expected range, including LLOQ.

The simplest model that sufficiently describes the concentration-response correlation should be used. Selection of weighting and use of a complex regression equation should be acceptable. The following conditions should be met in developing a calibration curve for particular analyte:

- ±20% deviation of the LLOQ from nominal concentration.
- ±15% deviation of standards other than LLOQ from nominal concentration.

At least four out of six non-zero standards must meet the above criteria, including the LLOQ and the calibration standard at the highest concentration.

Lower Limit of Quantification (LLOQ) [90,102,110]

The lowest standard on the calibration curve be supposed to be established as the limit of quantification. The analyte response at the LLOQ should be at least 5 times the response compared to blank response. Analyte peak (response) must be identifiable, distinct, and reproducible with a precision of 20% and accuracy of 80-120%.

Matrix Effect [94,105,111]

Direct or indirect alteration or intervention in response due to the existence of unintended analytes or other interfering material in sample is called matrix effect .Matrix effect calculated by comparing the response of extracted samples spiked before extraction with the response of the extracted blank matrix to which analyte has been added at the same supposed concentration just previous to injection. Matrix effect evaluated for six lots of plasma with aqueous dilution of LQC, MQC and HQC along with internal standard.

Method

Spike low and high QC samples into minimum six different batches of accepted blank matrix. Take 2 aliquot of LQC and HQC from each batch of blank matrix, add IS and process as per method SOP. Also prepare and process freshly spiked calibration standards .Inject CC standards and QC samples. The assessment of QC samples is back calculated against a calibration curve.

Acceptance Criteria

Mean concentration is inside ± 15% of nominal concentration at LQC and HQC level.

%CV should be ≤ 15% for LQC and HQC level

Matrix factor [103,108,112]

A quantitative evaluation of the matrix effect due to suppression or enhancement of ionization in a mass spectrometric detector is called as matrix factor.

Method

Prepared reference combination of analyte and internal standard at conc. representing 100% extraction of analyte and internal standard at LQC, MQC and HQC conc., used as reference samples. 12 aliquots of pooled plasma were taken and processed as per manner SOP without IS. For method not involving terminal drying step ,pipette out 12 processes pooled plasma samples and spike four aliquot each with IS and Analyte dilution to get concentration demonstrating 100% extraction of IS and Analyte at low, middle and high QC concentration. These samples serve as matrix samples reconstituted with reference sample. Inject these 12 samples along with 4 replicate of each reference mixture of IS and analyte at low, middle and high QC concentration .Tabulate peak area ratio of each .Calculate Matrix factor at each LQC, MQC, HQC concentration the following formula:

M.F = Mean peak area ratio of matrix samples reconstituted with reference samples/ Mean peak area ratio of reference samples X 100

Acceptance Criteria

% CV of matrix factor among LQC, MQC, and HQC level should be within ±15

Ruggedness [89,106,113]

It can be define as the degree of reproducibility of test results achieve by the analysis of the same samples under a range of background, which may be different laboratories, analysts, instruments, reagent lots.

Method

The ruggedness of the extraction process and the chromatographic process was calculated by analysis of a batch of six sets of quality control samples (including LOQQC) and a set of calibration standards using a new column by a changed analyst.

Acceptance Criteria

Same as precision accuracy batch

Stability [92,108,113-115]

Drug stability is a function of the storage situation, the chemical properties of the drug, the matrix and the container system. The stability of an analyte in a definite matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term and short-term storage and after going through freeze and thaw cycles and the analytical procedure. Setting used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The method should also include an assessment of analyte stability in stock solution.

The stability purpose must use a set of sample organized from a freshly made stock solution of the analyte in the suitable analyte free, interference free biological matrix .Stock solution of the analyte for stability estimate must be prepared in an suitable solvent at identified concentrations

Stock Solution Stability

Stock solution stability is the capability of a product maintain its composition and reliability after an intentional period of time

Method

Prepare analyte and IS solution and maintain aliquots of the same at refrigerated temperature. These shall serve as stability stock solutions. Following a particular storage period, prepare fresh stock solution of the analyte(s) and IS, these shall serve as evaluation stock solution. Inject 6 replicate from the vials of the stability stock dilutions and replicate from the comparison stock dilution. Tabulate the peak area response obtained from the stability and comparison stock dilution .Calculate the mean response, SD, %CV and %Stability.

% Stability = Mean peak area response of stability samples/ Mean peak area response of comparison samples X 100 X C.F

C.F = Concentration of stability samples/ Concentration of comparison samples

Acceptance Criteria

%Stability should be within the range of 85-115%

Freeze Thaw Stability

It is the ability of a product to uphold its

composition and reliability after repetitive cycles between freezing and ambient temperature levels. Even minor temperature variation can cause minor thawing of liquid within a product. Those ice crystals freeze at a big size, causing the breakdown of a products structure. Analyte stability should be resolute after three freeze and thaw cycles.

Method

Minimum of four aliquot at each of the low and high concentrations must be stored at the intended storage temperature for 24hrs and thawed unassisted at room temperature. When totally thawed, the sample should be refrozen for 12 to 24 hrs under the identical conditions. The freeze-thaw cycle should be repeated two or more times and the stability should be then analyzed on the third cycle .The QC concentrations are tabulated and the mean concentrations, SD, %CV and %nominal values are determined at low and high QC levels. If an analyte is not stable at the planned storage temperature, the stability sample should be frozen at -70^{0}C through the three freeze and thaw cycles

%Stability= Mean concentration of stability sample/ Mean concentration of comparison sample x C.F X 100

$$C.F.= \frac{\text{Stability Samples concentration}}{\text{Comparison Samples concentration}}$$

Acceptance Criteria: %Stability should be within the range of 85-115%

In-Injector Stability

The in injector stability duration is calculated as the time of injection of last QC sample less the time of their placement in auto injector.

Method

At least four aliquot at every low and high concentrations must be processed. The processed QC samples are placed in auto injector. The time of residency of QC samples must be recorded. Following the stability period four replicate of recently spiked LQO and HQC must be processed along with recently spiked calibration standard. Analyze the contrast QC, freshly spiked calibration standard along with stability QC samples. The in-injector stability was determined at LQC and HQC samples by analyzing 4 replicates at each level. The stability of the drug was resolute by back calculating the concentration of the stability samples against freshly processed calibration curve standards.

Acceptance Criteria: %Stability should be within the range of 85-115%

Bench Stop Stability

Stability of the drug in plasma at room temperature was examined by observance 4 sets LQC and HQC samples at room temperature for 4-24hrs. The concentration of the stability samples were considered and stability was evaluated by using a freshly prepare calibration curve and also by comparing against the set of QC samples at LQC and HQC level.

AcceptanceCcriteria: %Stability should be within the range of 85-115%

Re-Injector Stability

Any one of the three PA batch analyzed and meeting the acceptance criteria can be used for establishing reinjection reproducibility .Re-inject all LQC, MQC and HQC samples of the selected batch. Establish in injector stability duration for the intended period of time for which samples remained in auto injector. Calculate the re-injected QC concentration and determine the mean concentration, SD, %CV and %nominal values are determined at low and high QC concentration

Calculate % difference for each QC concentration.

$$\%\ \text{Difference} = \frac{\text{Absolute (original value} - \text{re-injected value)}}{\text{Original value}} \times 100$$

Acceptance Criteria: All the re-injected QC samples must meet the acceptance criteria of PA batch .The Reinjection Reproducibility is satisfactory if % difference of 80% of all QCs re-injected is within ±15%.

Conclusion

This review describes various aspects of the hyphenated technique; LC MS/MS, used for the bioanalysis. Bioanalytical method development and validation are required for the information of bioavailability and bioequivalence. These studies provide pharmacokinetic, toxicokinetic and metabolic data of drugs. Bioanalytical method development consists of sample preparation, chromatographic separation and detection by using LC MS/MS. Validation of a bioanalytical method comprises of various validation parameters which includes accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability, etc. These studies can be carried

out by many analytical techniques including HPLC, UPLC, GC and LC MS/MS. LC MS/MS is most widely used analytical technique for bioanalytical method development and validation. Bioanalysis by LC MS/MS provides low detection limits, good ability to generate structural information, minimal sample requirement and wider coverage of range of analytes differing in their polarities.

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Original Article

Hepatoprotective Activity of Ethonolic Extract of *Allophylus Serratus* Root

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Abstract

In the present study we selected a plant namely *allophylus serratus belonging* to the family (sapindaceae) commonly known as Triputa (in Sanskrit). It is a large shrub or small tree grows up to 10 meters in height. It contains beta-sitosterol, phenacetamide, two flavonoid glycosides like luteolin-7-o-[beta]-D-glucopyranoside and apigenin-4'-o[beta]-D-glucoside, quercetin, pinitol, rutin etc. It is useful in bone fractures, dislocations, Inflammations, ulcers, wounds, dyspepsia, anorexia and diarrhoea. The fruits are sweet cooling and nourishing tonic. Roots of widely grown plant *Allophylus serratus* reported to possess a very high amount of polyphenols and gallic acid which are well known potent antioxidants. Evaluation of the hepatoprotective activity was done by estimating the biochemical marker like SGPT, SGOT, ALP, LDH, total bilirubin, total protein in serum. EEAS significantly reversed the above parameters to near normal levels and the activity of EEAS was comparable to that of standard silymarin. The present studies indicated that the EEAS possess potent hepatoprotective activity comparable to standard LIV 52. The possible hepatoprotective mechanism of roots of *Allophylus serratus* may be via its antioxidant property.

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Introduction

The liver is the major site of xenobiotic metabolism and excretion. Liver injury caused by xenobiotics such as toxic chemicals, drugs, herbal products, environmental chemicals and virus infiltration from ingestion or infection represents the leading cause of acute liver failure. Thus liver diseases remain one of the serious health problems. Drugs have been estimated to cause around 15-20% of all cases of fulminant and 10% of all cases of sub-fulminant in Western countries and 10% of all cases in Japan. Traditionally, various plants are being used to treat hepatic patients. It is believed that herbal medicine has little side effects as well, as it requires no cost in few cases. So, the herbal medicine can solve the economic problem for the poor. *Allophylus serratus* has been claimed to be contain flavanoids useful in hepatotoxicity. CCl_4, Paracetamol and Anti-TB drugs cause ROS mediated cellular damage especially in liver where these drugs are metabolized. Polyphenolic compounds like flavonoids, tannins are useful as antioxidants and organ protectants. The liver is the key organ which regulates homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction [1]. The liver is expected not only to perform physiological functions but also to protect against the hazards of harmful drugs and chemicals [2]. Liver diseases posses a serious health problem. In the absence of reliable liver protective drugs in allopathic medical practices, herbs play a major role

in the management of various liver disorders. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices and in traditional system of medicine in India. However, we do not have satisfactory remedy for serious liver disease; most of the herbal drugs speed up the natural healing process of liver. So the search for effective hepatoprotective drug continues [3].

Many diseases of the liver are accompanied by jaundice caused by increased levels of bilirubin in the system. The bilirubin results from the breakup of the heamoglobin of dead red blood cells; normally, the liver removes bilirubin from the blood and excretes it through bile. The carbon tetrachloride(CCl4) induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. Liver diseases remain one of the serious health problems. In the absences of reliable liver protective drugs in allopathic medical practices. Herbs play important role in the management of various liver disorders . However, in ayurveda many indigenous plants have been used as hepatoprotective agents.

In the present study we selected a plant namely *allophylus serratus* belonging to the family (sapindaceae) commonly known as Triputa (in Sanskrit). It is a large shrub or small tree grows up to 10 meters in height. It contains beta-sitosterol, phenacetamide, two flavonoid glycosides like luteolin-7-o-[beta]-D-glucopyranoside and apigenin-4'-o[beta]-D-glucoside, Quercetin, Pinitol, Rutin. The plant is astringent, bitter, sweet, analgesic, anti-inflammatory, vulnerary, digestive, Carminative and constipating. It is useful in bone fractures, dislocations, Inflammations, ulcers, wounds, dyspepsia, anorexia and diarrhoea. The fruits are sweet cooling and nourishing tonic. However, no scientific report has been carried out on the leaves of *allophylus serratus* to prove the hepatoprotective activity. So the aim of present study is to evaluate hepatoprotective activity of allophylus serratus against CCL4 induced hepatotoxicity in experimental rats.

Materials and Method

Chemicals and Reagents

CCL_4 was obtained from Sigma-Aldrich, Bangalore. LIV 52 was obtained from local medical hall. And all other reagents used were of analytical grade. SGPT, SGOT, alkaline phosphatase, total cholesterol and HDL, total bilirubin and Total protein estimation kits were procured from S.V Biological agency, Kadapa.

Collection of Plant Material

Allophylus serratus was obtained from local area of kadapa & authentified by Sri madhava chetty taxonomist S.V University Tirupathi.

Preparation of Extract

The collected plant material *Allophylus serratus* was washed thoroughly in water, cut into small pieces and air dried for two weeks at 35-40^{0}C temperature. Extraction was done by using soxhlet apparatus with 70% ethanol (hydro alcoholic) as solvent. The extracts were concentrated under reduced pressure dried and stored at 4^{0}c temp in air tight containers for further studies.

Experimental Animals and Ethical Clearance

Experimental animals of either sex weighing 150-170 g were obtained from Raghavendra enterprises (Bangalore). The animals were housed in stainless steel cages at a controlled room temperature of 24^{0}C, under a 12 h light and 12 h dark cycle. After one week of acclimatization, the experimental animals were divided randomly in to 5 groups (n=6). The experimental protocol was approved by the Institutional Animal Ethical Committee of R R S college of Pharmacy UP.

Experimental Design

Treatment schedule for assessing the hepatoprotective activity of Ethanolic extract of *Allophylus serratus* (EEAS) (Table 1)

Assessment of Hepatoprotective Activity

All the animals were killed on day 21 under light ether anaesthesia. The blood samples were collected separately by carotid bleeding into sterilized dry centrifuge tubes and allowed to coagulate for 30 min at 370 C. The clear serum was separated at 2500 rpm for 10 min and biochemical investigations were carried out to assess liver function viz., total bilirubin, total protein, serum transaminases and serum alkaline phosphatase .

Measurement of Biochemical Parameters

The parameters *viz.* Estimation of SGPT (Serum glutamyl pyruvate transaminase) *(Modified IFCC UV-*

kinetic method), Estimation of SGOT (Serum glutamyl oxalacetic acid transaminase) *(Modified IFCC UV-Kinetic method),* Estimation of Alkaline phosphatase *(Kind and King's Method)*, Estimation of Total cholesterol, *(Chod-Pod/ Phosphotungstate Method),* Estimation of HDL, Estimation of Bilirubin *(Modified Jendrassik & Grof's Method)* were determined for experimental rats.

Collection of Blood Sample

The blood samples were withdrawn on 0^{th}, 7^{th}, 14^{th}, and 21^{st} day from the retrorbital venous plexus of rats without any coagulant for the separation of serum. These were then allowed to clot at room temperature for half an hour and centrifuged at 4000 r/min for 15 min using a WIFUNG centrifuge LABOR-50M. The clear straw colored serum was then collected from the upper part of the tubes in vials with a Pasteur pipette.

Histopathology

After draining the blood, liver samples were excised, washed with normal saline and processed separately for histological observations. Initially, the materials were fixed in 10% buffered neutral formalin for 48 h and then with bovine solution for 6 h. Paraffin sections were taken at 5mm thickness, processed in alcohol- xylene series and were stained with alum hematoxylin and eosin.The sections were examined microscopically for histopathological changes.

Statistical Analysis

All the data was expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one way ANOVA followed by the Tukey test using computer based fitting program (Prism graph pad.). Statistical significance was taken as $P< 0.05$.

Results

Rats treated with carbon tetrachloride showed a significant hepatic damage as observed from elevated serum level of hepatospecific enzymes as well as severe alteration in different liver parameters.

Morphological Observation

Morphological observations showed an increased size and enlargement of the liver in CCl_4 treated control group. These changes were reversed by treatment with LIV-52 and also EEAS at the two different doses in tested groups.(Table 2, Figure 1)

Biochemical Estimations

Biochemical estimations was studied on 14^{th} day and the results were presented (Table 3 & Figure 2-8)

Table 1: Dosing pattern

S. No.	Groups	No of animals	Treatment	Purpose
I	Normal	6	OLIVE OIL	To serve as negative control
II	Control	6	CCL4(1ml/kg)+ OLIVE OIL (1:1)	To serve as positive control
III	Standard	6	LIV-52 Syrup (4 mL/kg, p.o.)	To serve as standard
IV	Treatment-1	6	EEAS (250 mg/kg, p.o.)+ CCL4(1ml/kg)	To assess the hepatoprotective activity of EEAS. (250mg/kg,p.o).
V	Treatment -2	6	EEAS (500 mg/kg, p.o.)+ CCL4(1ml/kg)	To assess the hepatoprotective activity of EEAS. (500mg/kg,p.o.)

Table 2: Hepatoprotective activities of EEAS on Liver weights

S. No.	Groups	Treatment	Liver Weights
I	Normal	olive oil	5.783 ± 0.13^{ns}
II	Control	CCl_4 (1ml/kg)+ olive oil (1:1)	$8.167 \pm 0.19^{###}$
III	Standard	Liv 52 syrup (4 ml/kg, p.o.)	$5.717 \pm 0.09^{***}$
IV	Treatmaent 1	EEAS (250 mg/kg, p.o.)+ CCl_4 (1ml/kg)	$7.117 \pm 0.19^{***}$
V	Treatment 2	EEAS (500 mg/kg, p.o.)+ CCl_4 (1ml/kg)	$6.217 \pm 0.14^{***}$

Table 3: Hepatoprotective activities of EEAS on all biochemical parameters

Group	SGOT	SGPT	ALP	Total Protein	Total Bilirubin	Cholesterol	HDL Cholesterol
Group – I (Control)	116.7±4.24	125.3±3.42	288.7±8.96	5.08±0.76	6.74±0.42	165±21.51	0.66±0.03
Group – II (Normal)	45.22±0.62	43.17±2.7	173.7±4.35	7.81±0.44	1.66±0.8	75.33±4.98	0.36±0.02
Group – III (Standard)	56.18±2.00	47.67±1.3	189.8±4.68	8.01±0.51	2.04±12.6	78.67±4.31	0.37±0.02
Group – IV (EEAS 250 mg/kg)	80.33±3.19	86.00±9.8	230.7±22.86	6.76±0.46	3.06±0.26	86.17±3.40	0.38±0.02
Group – V (EEAS 500 mg/kg)	56.83±2.55	45.17±3.0	191.7±2.76	7.43±0.39	2.03±0.22	76.01±5.24	0.42±0.03

All values are shown as mean ± SEM and n=6.
indicate $p<0.001$ when compared to normal group.
*** indicate $p<0.001$ when compared to control group.

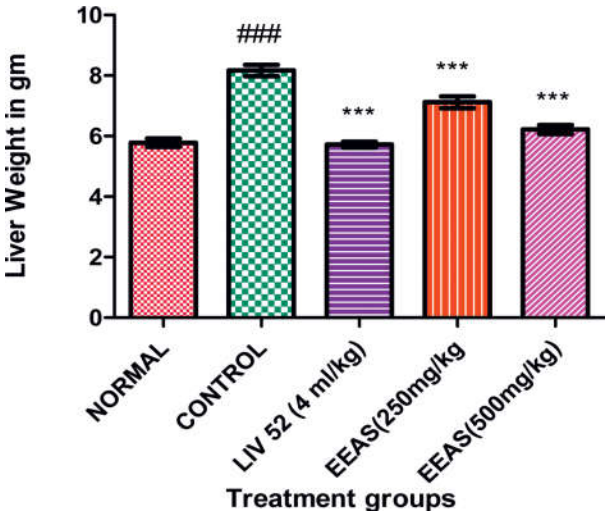

Fig. 1: Effect of EEAS on Liver weights

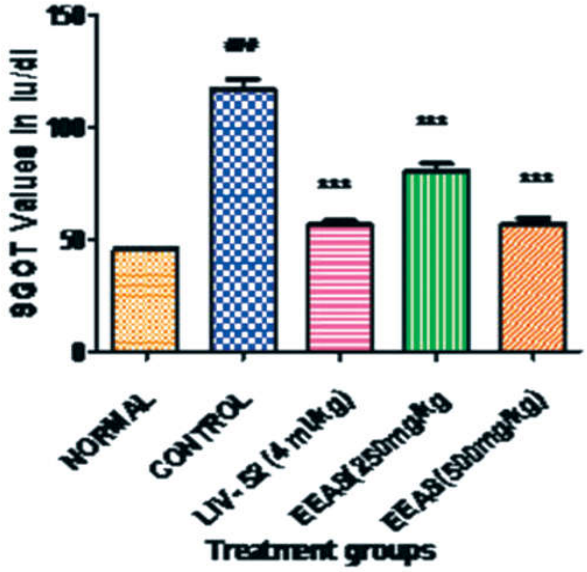

Fig. 2: Effect of EEAS on SGOT levels

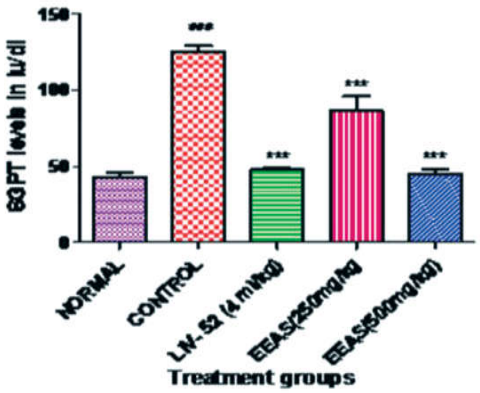

Fig. 3: Effect of EEAS on SGPT levels

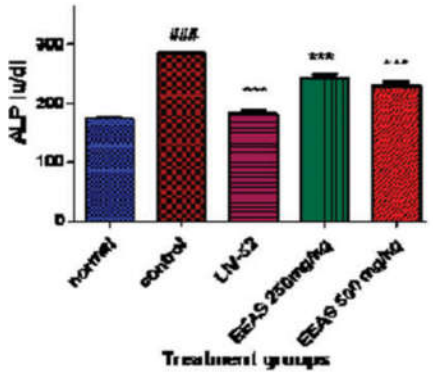

Fig. 4: Effect of EEAS on ALP levels

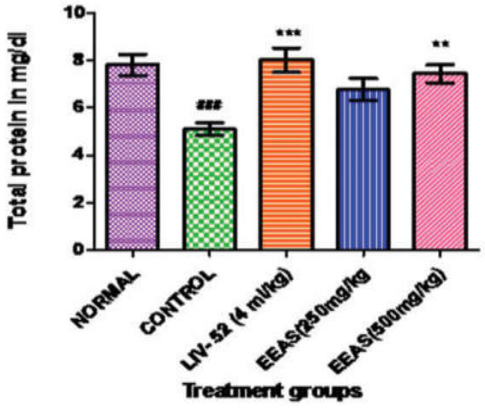

Fig. 5: Effect of EEAS on total protien levels

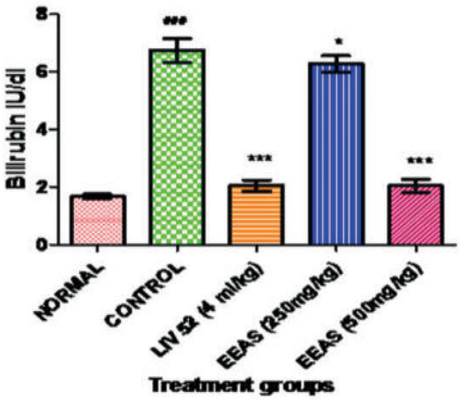

Fig. 6: Effect of EEAS on total bilirubin

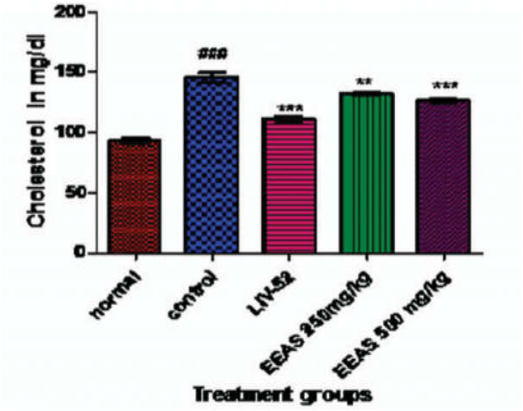

Fig. 7: Effect of EEAS on cholesterol

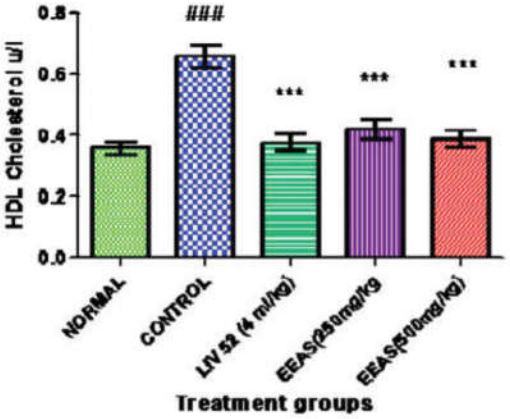

Fig. 8: Effect of EEAS on HDL cholesterol

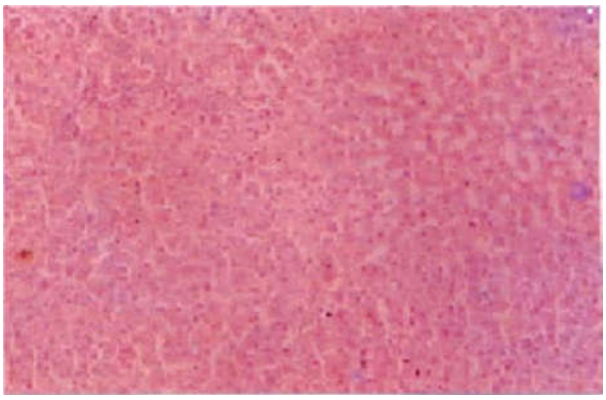

Fig. 9(a): Normal group hepatic cell

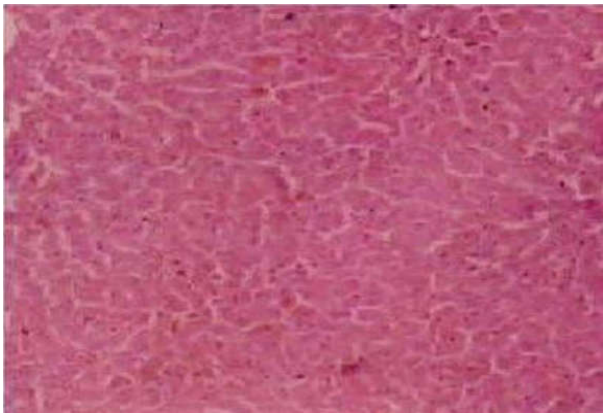

Fig. 9(b): Low dose group hepatic cell

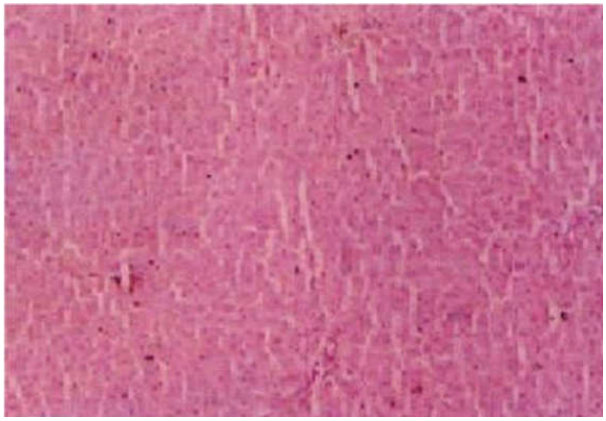

Fig. 9(c): High dose group hepatic cell

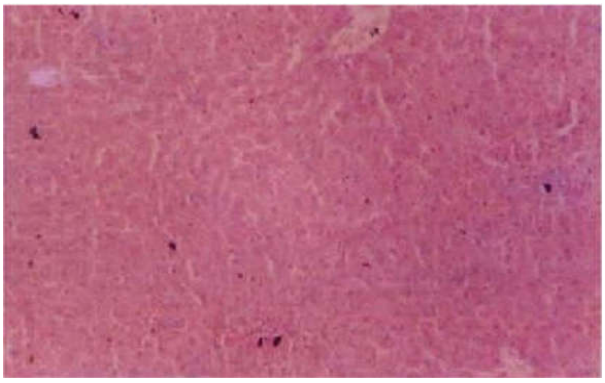

Fig. 9(d): Control group hepatic cell

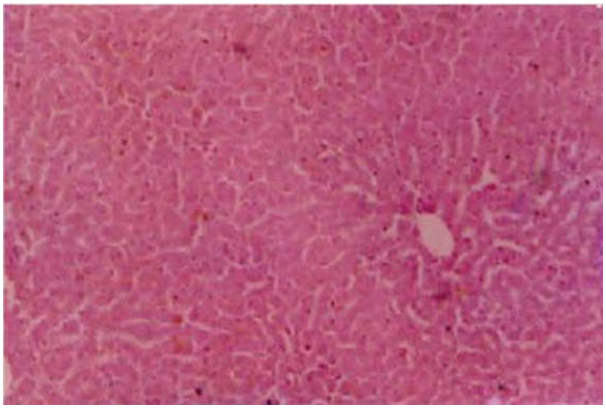

Fig. 9(e): Standard group hepatic cell

Histopathological Observations

Histopathological Findings in the Liver

It was found from the histopathological studies that CCl4-intoxication caused centrilobular hepatocyte necrosis, fatty changes, vacuolization and inflammatory changes. Treatment EEAS either reversed or prevented the changes in these histopathological parameters of the liver indicating that the fractions showed remarkable hepatoprotective activity

- The normal histological liver structure showed in Figure (9a).
- Control group (group II) shows marked inflammatory changes associated with fatty changes are seen in liver sections of CCl_4 treated group II, in Figure 9(e).
- The liver sections of EEAS extract treated group (group IV & Group V) showed periportal lymphocytic and neutrophilic infiltration without any lesions in the hepatocytes. Figure (9b)(9c).
- Lesser degree of inflammation was seen in the LIV-52 treated group when compared with control (Group III). Figure (9d)

All these results indicate a hepatoprotective potential by the ethanol extract of *Allophylus serrates* (EEAS).

Discussion

CCl_4 cause ROS mediated cellular damage especially in liver, the site of metabolism of toxins. During the regular physiological functioning the cells/tissues/organs use oxygen and various nutrients to generate energy. The free radicals are also generated in this process as the reaction intermediates. These free radicals may be very useful because they may promote beneficial oxidative processes. However the higher quantities of such radicals like superoxide anion (O_2^-), NO^- radical, and hydroxyl ion radical (^-OH), NOO^-, etc. may interact with the membrane lipids leading to lipid peroxidation and attack the DNA resulting DNA strand breaks. The lipid peroxidation also damage cell membrane resulting in the leakage of enzymes into the blood stream. Therefore the elevated biochemical levels are treated as biochemical markers of tissue damage. In addition the extent of lipid peroxidation is directly proportional to the tissue damage [4].

There are certain inbuilt protective mechanisms, tissue enzymes GSH, SOD, CAT etc. which are involved in the process of combating free radical induced tissue damage. Over powering the inbuilt protective mechanism due to excessive generation of free radicals may lead to destruction of the tissues/ organs [5].

Antioxidants are the chemical constituents, which are used for inhibiting the tissue damage by countering the free radicals; most of the antioxidants available in the markets are from natural origin e.g. - vit-E, vit-C, tocopherol, quercetine, b-carotene etc. In addition there are reports that polyphenolic compounds like flavonoids are useful as antioxidants and organ protectants. Therefore many researchers are attempting to screen the herbs and herbal preparations containing polyphenolic compounds for organ protective properties.

SGOT is a mitochondrial enzyme released from heart, liver, skeletal muscle and kidney. Liver toxicity elevated the SGOT level in serum due to the damage to the tissues producing acute necrosis, such as severe viral hepatitis & acute cholestasis. Alcoholic liver damage and cirrhosis can also associate with mild to moderate elevation of transaminases [6]. EEAS reversed these enzyme levels indicating stabilization of cell membrane by preventing the damage due to free radicals generated by CCl_4.

SGPT is a cytosolic enzyme primarily present in the liver. The level of SGPT in serum increases due to leakage of this cellular enzyme into plasma by hepatic injury [7]. Serum levels of SGPT can increase due to damage of the tissues producing acute hepatic necrosis, such as viral hepatitis and acute cholestasis. Alcoholic liver damage and cirrhosis also can associate with mild to moderate elevation of transaminase. EEAS effectivey reduced the SGPT levels by preventing the damage of hepatocytes due to free radicals.

In case of liver toxicity, ALP levels are very high, which may be due to defective hepatic excretion or by increased production of ALP by hepatic parenchymal or duct cells [6]. EEAS decreased these enzyme levels indicating stabilization of cell membrane by preventing the damage due to free radicals generated by CCl_4.

A reduction in total serum protein observed in the CCl_4 treated control rats may be associated with the decrease in the number of hepatocytes which in turn might result in decreased hepatic capacity to synthesize protein. when the EEAS was administered along CCl_4 a significant increase in protein content was observed indicating the hepatoprotection of EEAS.

In case of liver toxicity, bilirubin levels are elevated. Hyperbilirubinemia can result from impaired hepatic uptake of unconjugated bilirubin. Such a situation can occur in generalized liver cell injury. CCl_4 interfere with the net uptake of bilirubin by the liver cell and may produce a mild unconjugated hyperbilirubinemia [8]. Bilirubin level rises in diseases of hepatocytes, obstruction to biliary excretion into duodenum, in haemolysis and defects of hepatic uptake and conjugation of bilirubin pigment such as in Gilbert's disease [6]. Significant reversal of elevated bilirubin level in CCl_4 treated animals by EEAS indicated the strong hepatoprotective activity of EEAS.

Cells have a number of mechanisms to protect themselves from the toxic effects of the ROS. SOD removes superoxide (O_2) by converting it to H_2O_2, which can be rapidly converted to water by CAT and Glutathione peroxidase. In addition, a large reserve of reduced glutathione is present in hepatocytes and red blood cells for detoxification of xenobiotics or free radicals. However, oxidative stress results in toxicity when the rate of which the ROS are generated exceeds the cell capacity for their removal. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. MDA is one of the end products in the lipid peroxidation process. In order to elucidate the protection mechanism of EEAS in CCl_4 induced rat liver, lipid peroxide levels and anti-oxidative enzymes activities were analyzed.

GSH is widely distributed in cells. GSH is an intracellular reductant and plays a major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. GSH is a naturally occurring substance that is abundant in many living creatures. It is well known that a deficiency of GSH within living organisms can lead to tissue disorders and injury. For example, liver injury included by consuming alcohol or by taking drugs like Paracetamol, lung injury by smoking and muscle injury by intense physical activity, all are known to be correlated with low tissue level of GSH.

In our study, elevation in the levels of end products of lipid peroxidation in liver of rat treated with ccl_4 was observed. The increase in MDA level in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of anti-oxidant defense mechanism to prevent formation of excessive free radicals. Treatment with EEAS significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of EEAS is due to its antioxidant effect.

It is well known that there are significant elevations in the levels of serum GPT, GOT and ALP in liver diseases and disorders and in hepatocellular damage caused by a number of agents. An increase in these enzyme levels is also observed with cardiac damage due to myocardial infarction and with liver disorders [9]. Biochemical measurements of these parameters in normal mice treated with ASC showed some extent of increase due to little hepatotoxicity during treatment period but they become normal after completion of treatment schedule. The slight host toxic effects observed in mice during treatment time are mostly reversible. This means that, the treatments of the compound do not cause any acute or permanent damage to the liver. But in case of tumor bearing mice, these parameters were found to be increase more drastically with time due to the acute and permanent toxicities induced by EAC cells on host. After treatment with ASC in the EAC bearing mice these values remain near the normal range in the treated group. From this it follows that the damage generated by EAC was prevented by ASC supplementation.

The development of hypoglycaemia and hyperlipidaemia in experimental animals with carcinoma has been previously reported [10-12]. In this experiment, the reduced glucose level and elevated cholesterol, triglycerides and serum urea were returned to more or less normal levels in ASC-treated mice, thereby indicating a potent antitumour efficacy of ASC.

The histopathology studies of major organs also revealed the relatively less toxic nature of ASC as compared to control group when viewed under microscope. The histopathology of kidney tissues of ASC treated mice did not show any cellular and glomerular infiltration, and there is no sign of tubular necrosis, casts and glomerular congestion. Tissues from brain and lung did not shown any cellular degeneration or regeneration in the treated mice and this is why they have no signs of neurotoxicity and pulmonary toxicity. Treated mice also have not any change in the splenic architecture. The histology of liver showed very little infiltration (inflammation) with no central vein dilation, fatty generation or nodule formation and due to this mild hepatotoxicity some biochemical parameters were deteriorate during treatment period which become normal after closing treatment whereas tissues from EAC bearing mice showed major abnormalities and it is interesting that the hepatic damage induced by EAC cells were nullified by ASC supplementation. All these slight host toxic effects observed in normal mice during

treatment time are mostly reversible and so treatment with ASC do not cause any acute or permanent damage to the host.

The aim of this study was to determine the hepatoprotective effects and sub-acute toxicity of the compound to find out less host toxic potential anticancer agents and did not attempt to identify the specific mechanism involved. This study revealed some interesting features have been presented here. Almost in all cases the effects of EAC cells on biomolecules have been found to be nullified by such treatment. In most cases antagonistic effects have been found instead of additive effects. Further elevation of glucose levels of EAC bearing mice by the treatment of the compound probably indicates their partial recovery from tumour growth.

As the major organs of the treated mice do not show any histopathological abnormalities, these findings in conjunction with those obtained from the measurement of serum biomolecules definitely give positive support to conclude that ASC is an effective antineoplastic agent with comparatively less toxic effects in our experimental model. However, further chronic toxicological studies and its anti-tumor activity should be carried out against other tumor cell lines which may bring promising results in cancer chemotherapy.

Rats treated with carbon tetrachloride showed a significant hepatic damage as observed from elevated serum level of hepatospecific enzymes as well as severe alteration in different liver parameters. It can be concluded that EEAS posses marked hepatoprotective activity with minimal toxicity and thus has a promising role in the treatment of acute hepatic injury induced by hepatotoxins. Further the present investigation provides a scientific base for the use of the *Allophylus Serratus* plant in treatment of jaundice in Indian folklore medicine.

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Original Article

Hypoglycemic Effect of Dichloromethane Extract of *Caeselpinia bonducella* leaves in Rats with High Fructose Diet Induced Diabetes

Rachana V. Katbamna*, Mayuri M. Thummer*

Abstract

Insulinresistancehasbeen considered as the most important component of type 2 diabetes mellitus (DM2). Plants used infolkmedicine to treat diabetes mellitusre present aviable alternative forthe control ofthis disease. This study was aimed to examine the antidiabetic effects of dichlorome thane extract of *Caesalpinia bonducella* in an animal model of DM2. Diabetes was induced in male Wistarrats (6-8 week sold) by feeding 21% fructose in drinking water for 8 weeks. They were treated with dichlorome thane extract of leaves of *Caesalpinia bonducella* (DCM 200, DCM 400) for 2 weeks. After diabetes induction and the last day of the experiment, body weight, fasting blood glucose, plasmainsulin, urinevolume and urine glucose were assayed. Blood glucose, plasmainsulin, urine gluco sean durin evolume were in creased significantly after 8 weeks of high fructose feeding (P<0.005); DCM 200 reduced body weight, plasma insulin andurineglucose (p<0.05)and no significant difference in blood glucose and urine volue. While DCM 400 significantly decreasedblood glucose (p<0.05), urineglucose (p<0.01) and plasma insulin (p<0.01), body weight (p<0.05)andurine volume (p<0.05) when compared with that of 8th week values. The study showed hypoglycemia effects and improvement of insulin resistance of dichloromethane extract of leaves of *Caeslpinia bonducella*. These result scan bee xtra polated to human sand these extracts might be useful in the treatment of insulin resistance.

Keywords: Hypoglycemia; Insulin Resistance; Fructose; *Caesalpenia bonducella.*

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Introduction

The current trend suggests that there is a substantial consumption of fructose per capita as a sweetener in the food industry, primarily in the form of sucrose (a disaccharide consisting of 50% fructose) and high-fructose corn syrup (HFCS; 55–90% fructose content) [1].

It is widely accepted that excessive intake of fructose can promote metabolic changes such as hyperlipidemia, hyperinsulinemia, insulin resistance, hyperuricemia, hypertension, glucose intolerance and non-enzymatic fructosylation of proteins resulting in advanced glycated end products and associated complications [2,3]. In addition, excessive fructose consumption may be responsible in part for the increasing prevalence of obesity, diabetes mellitus, non-alcoholic fatty liver disease and cardiovascular diseases [4].

Studies have reported that rats fed with a high-fructose diet form a model of diet-induced insulin resistance, associated with hyperinsulinemia, hypertriglyceridemia and glucose intolerance [5]. Feeding of a high fructose diet to normal Wistar rats provides a dietary model of type 2 diabetes associated

with insulin resistance, hyperinsulinemia, hypertriglyceridemia [1] andhypertension [6].The precisemolecular mechanisms that high fructose diet induces the abnormalities in liver carbohydrate metabolism are not fully understood. Thus, fructose has been implicated as the useful tool to induce insulin resistance in animals [7]. Recently, antioxidants are found to be effective in preventing metabolic disturbances and resultant diseases induced by high-fructose diet [8,9].

Caesalpiniabonducella(L.) Fleming (Syn. *Caesalpinia bonduc* (L.)Roxb, Syn. *Caesalpinia crista*Linn.), belonging to the family Fabaceae /caesalpiniaceae, is a prickly shrub widely distributed all over the world specially, in India, Sri Lanka and Andaman and Nicobar Islands, in India specially found in tropical regions [10,11]. All parts of the plant have medicinal properties so it is a very valuable medicinal plant which is utilized in traditional system of medicine [12]. The plant has been reported to possess anxiolytic, antinociceptive, antidiarrhoeal, antioxidant, hypoglycemic, antidiabetic, antimicrobial, antiproliferative, antiestrogenic, antimalarial, antitumor, antipsoriatic, larvcidal and antifilarial activities [13]. Phytochemical analysis of seeds of *Caesalpiniabonducella* has revealed the presence of alkaloids, flavonoids, glycosides, saponins, tannins and triterpenoids [14,15].

Materialsand Methods

Plan Collection and Processing

Leaves of *C.bonducella*were collected from Rajkot (Gujarat). The plant was identified by comparing it morphologically and microscopically with description given in different standard texts and floras. The plant was identified and authenticated by prof. VISHAL MULIYA, Christ College, Rajkot and a voucher specimen was deposited. The leaves material was cleaned and dried in shade, powdered and stored in air tight container at room temperature.

Plantmaterial& Preparationof Extracts

The powdered leaves wereextracted using dichloromethane by Soxhlet extractionat controlled temperature (40 °C) for 72 hours. Resulting solutions were filtered through Whatman filter paper (No.42). The filtrates so obtained were concentrated in a water bath at low temperature (40 °C). Later, dichlorome thane was evaporated off and dry the residue in vacuum oven to remove solvent completely from extract. The extracts so prepared were subjected for further studies.

Preliminary Phytochemical Investigation

All four crude extracts were subjected to qualitative phytochemical screening for the presence of various secondary metabolites [16].

Animals

Male Wistar rats(6-8 weeks old weighing approximately 170–200g) were housed in stainless steelcages (three animals per cage). The animals were kept 1 week prior to the experiment for acclimatization in an air-conditioned animal room (22±2^{0}C) under a 12 hlight/darkcycle with free access to standard pellet diet and water. The study was approved by the ethics committee, and the rats were maintained in accordance with guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals.

Induction of Hyperglycemia

21% Fructose solution was used as inducing agent for induction of hyperglycemia and insulin resistance [17].

Experimentaldesign

The animals were randomly divided into four groups containing six rats in each group as given below: Group I – Normal Control (NC) Received 0.5% CMC, po, and normal drinking water, group II – Diseases Control (DC) received 0.5% CMC, po, and 21%fructose solution as drinking water, group III – (DCM – 200) Received 200 mg /kg body weight dichloromethane extract of *Caesalpinia bonducella* leaves, po, and 21% fructose solution as drinking water, group IV – (DCM – 400) received 400 mg /kg body weight dichloromethane extract of *Caesalpiniabonducella* leaves, po, and 21% fructose solution as drinking water.

All animals group were given 21% fructose solution as drinking water for 8 weeks. After 8 weeks of fructose feeding, animals with fasting serum glucose greater than 135 mg/dl were considered as diabetic and received investigational drug treatment. From the 9th week, drug interventions were initiated for next 2 weeks. Body weight of animals was measured at weekly intervals. Extracts were suspended in 0.5% CMC and administered by gastric gavage tube once a day for 2 weeks and rats were fed standard pellet diet throughout the study.

Sample Collection

From overnight fasted animals, blood samples

were collected from retro-orbital plexuses on 0, 56th (8th week), 70thday(10th week) under the influence of light ether anesthesia and subjected to centrifugation at 6000 rpm for 15 min to obtain serum.

Biochemical Measurements

Fasting glucose and insulin levels were measured in serum using span diagnostics kit, Surat, Gujarat, India and Insulin ELISA kit, Kamiya Biomedical Company, USA respectively. Similarly urine samples were collected and urine volume was measured at 6 hr intervals on 0, 56th and 70th day and urine glucose levels were measured using span diagnostic kit, Surat, Gujarat, India.

Statistical Analysis

Values are given as mean ± standard error of mean (SEM). The statistical analysis of biochemical parameters were conducted using prism 5.03. Within-group comparisons were performed by paired test. In all analyses, *P*<0.05 was considered significant.

Result

Preliminary Phytochemical Investigation

Qualitative phytochemical screening of different crude extracts of *Caesalpiniabonducella* (Table 1) showed the presence of secondary metabolites such as alkaloids, glycosides, saponins, flavonoids, triterpenoids, phytosterols, phenolic compounds/ tannins, in variable proportions.

Effects of *C.Bonducella* on Body Weight, Blood & Urine Glucose, Serum Insulin and Urine Volume

Values for blood glucose, urine glucose, plasma insulin and urine volume increased significantly ininduced diabetes groups (DC, DCM-200, DCM-400) after 8 weeks of high fructose feeding (P<0.05), while no significant difference was observed in these parameters in NC group during this period of time (Table 2 & 3). In the NC group in the 10th week no significant differences was observed in the studied parameters compared to those of 8th week, however in DC group the changes were no table. The mean difference of body weight of rats in DC group was 126.36g (p<0.001) and 158.94g (p<0.001) after 8 and 10 weeks of fructose feeding, respectively when compare with 0 day values and the difference was significant.

At the end of 10th week (Table 2 & 3), no significant difference was detected inblood glucose and urine volume in the DCM 200 group,while body weight(p<0.05), urine glucose(p<0.01) and plasma insulin(p<0.05)showed significant decline when compared with that of 8th week values. Whereas, DCM 400 significantly decreased blood glucose (p<0.05), urine glucose(p<0.01) and plasma insulin (p<0.01), body weight(p<0.05)and urine volume (p<0.05) when compared with that of 8th week values.

Table 1: Extract yield* of *C. Bonducella* leaves in dichloromethane

Class	
% *Yield	7.5
Alkaloid	+
Carbohydrates	-
Phytosterols	+
Tannins	+
Proteins & Amino Acids	-
Flavanoids	+
Glycosides	+
Saponins	+
Gum/Mucilage	-

*Yield (%) is a percentage of the weight of the extract in relation to the weight of the raw material. +: Present, -: Absent

Table 2: Effects of *C.Bonducella* on body weight, blood & urine glucose, serum insulin and urine volume

GROUP (n=6)	Body Weight (g)			Serum Insulin (ng/ml)			Urine volume (ml)		
	0	8	10	0	8	10	0	8	10
NC	178.68±5.39	235.13±6.6≠	254.46±6.83ns	1.07±0.06	1.62±0.09≠	1.61±0.07ns	3.57±0.37	2.73±0.09*	3.07±0.21ns
DC	181.56±3.87	307.92±7.69Φ	340.50±7.72*	1.15±0.05	2.75±0.06Φ	2.92±0.14ns	2.83±0.16	7.38±0.34Φ	7.27±0.32ns
DCM 200	175.89±4.84	312.06±7.96Φ	286.38±6.22*	1.19±0.05	2.70±0.07Φ	2.50±0.07*	3.12±0.19	7.05±0.35Φ	6.12±0.33ns
DCM 400	175.49±4.22	315.57±10.24Φ	268.54±10.17*	1.13±0.10	2.79±0.06Φ	2.17±0.05≠	3.28±0.22	6.63±0.38Φ	5.22±0.43*

Values are expressed as Mean±SEM and 8th week values were compared with 0 day values and 10th week values were compared with 8th week value using paired t test.ns= not significant,*= P<0.01,≠= P<0.05,Φ= P<0.001NC: Normal control, DC: Diease control, DCM 200 & 400: Dichloromethane extract of C.bonducella 200 & 400 mg/kg body weight

Table 3: Effects of *C.Bonducella* on body weight, blood & urine glucose, serum insulin and urine volume

GROUP (n=6)	Urine Glucose (mg/dl)			Blood Glucose (mg/dl)		
	0	8	10	0	8	10
NC	NA	NA	NA	96.98±3.70	88.20±4.04^{ns}	93.55±4.47^{ns}
DC	NA	53.62±5.78	48.16±6.39	98.48±2.31	138.78±3.45$^{\Phi}$	147.34±6.71$^{\Phi}$
DCB 200	NA	55.21±6.45	21.35±3.29$^{\neq}$	97.05±3.31	133.48±4.76$^{\neq}$	122.47±6.91$^{\Phi}$
DCB 400	NA	54.19±7.90	8.27±1.89$^{\neq}$	95.79±3.48	140.98±4.20$^{\neq}$	107.37±8.43*

Values are expressed as Mean±SEM and 8th week values were compared with 0 day values and 10th week values were compared with 8th week value using paired t test. NA= Not Applicable, ns= not significant, * = P<0.01, ≠ = P<0.05, Φ = P<0.001

Discussion

Primary treatment goals in diabetes include restoration and maintenance of normogly caemia, avoidance of diabetic complications and prevention of cardiovascular events.Inadditiontoglycemic control, management of hyper insulinemia is also essential for limiting the complications of type 2 diabetes mellitus(Type 2 DM) [18].

Fructose is a potent reducing sugar that promotes the formation of toxic advanced glycation end-products. Excessive fructose consumption may be responsible in part for the increasing prevalence of obesity, diabetes mellitus, and non-alcoholic fatty liver disease characterized by an impaired glucose tolerance test. In the present study clearly shows, high fructose feeding resulted in significant increase in the body weight, fasting hyperglycemia, hyperinsulinemia and urine glucose leading to the development of insulin resistance.

Chronic fructose feeding in experimental animals is reported to produce glucose tolerance and increase in body weight associated with hyperinsulineamia and loss of normal *in vivo* sensitivity to insulin [19,20]. Our results are consistent with previous studies which found that consumption of high-fructose diets markedly induces an increase in body weight, glycemia associated with hyperinsulinemia.

Exposure of liver to such large quantities of fructose leads to rapid stimulation of lipogenesis with the accumulation of triglycerides (TGs), which contributes, in turn, to reduced insulin sensitivity and hepatic insulin resistance / glucose intolerance [21]. Animal studies have shown that high fructose diet-fed rats display hepatic insulin resistance and altered lipid metabolism due to hepatic lipid accumulation as a result of the burden of fructose metabolism [22]. This cluster of disorders is similar to those observed in human multimetabolic syndrome or syndrome X or insulin resistance syndrome, which is observed in prediabetic patients that progresses to type 2 diabetes mellitus and cardiovascular diseases [23].

In the present study *C.bonducella*treatment significantly reduced the body weight, plasma glucose, urine glucose, urine volume and insulin level. This therapeutic effect could be attributed to the presence of alkaloids, flavonoids, glycosides, saponins, tannins and triterpenoids[24,25]. Parameshwar *et al* and Mandal *et al* have already reported the antioxidant and reactive oxygen species scavenging activity of Methanolic extract of*Caesalpiniabonducella* leaf. The antioxidantswith ROS scavenging ability may have great relevance in the prevention of diabetes linked oxidative stress [26,27]. The antihyperglycemic action of the extracts may be due to blocking of glucose absorption [28]. The aqueous ethanolic seed extract of *C.bonducella* has shown hypoglycemic activity in streptozotocin induced diabetic rats and produced increase in secretion of insulin. Moreover, the phytochemical of *C.bonducella*- Bargenin, caesalpinine A, α and β amyrinlupeol have demonstrated increase in insulin release from pancreatic cells [29]. Liu IM *et al* have reported that *Abelmoschusmoschatus*stimulates insulin-signalling cascades and improves insulin-signalling transduction by modification of Ser/Thr phosphorylation of IRS-1 [30]. In addition, *Brassica juncea,* Brassicaceae and *Calotropisgigantean,* Apocynaceae restored the pre-diabetic state of insulin resistance [31,32] in rats fed fructose-enriched diet. Besides, *Tinosporacordifolia* Menispermaceae has been found to activate various hepatic enzymes such as hexokinase, phosphofructokinase, pyruvate kinase, glucose-6-phosphatase, fructose-1,6bisphosphatase, and glucose-6-phosphate dehydrogenase [33,34] and significantly ameliorated diabetic state in high-fructose diet rats. Previously published reports also suggest that bonducin (a Homoisoflavone) [35] has a potential to improve insulin resistance. The plant under investigation is also reported to have bonducin as one of the phytochemicals. Therefore the observed insulin sensitivity improving effect of *C. bonducella* in present study could be due to bonducin.

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Original Article

Stability Indicating Chromatographic Method Development and Validation for the Simultaneous Estimation of Nifedipine and Lidocaine in Cream Dosage Form by HPLC

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Abstract

A simple, novel, rapid, precise, accurate, specific and cost effective High performance thin layer chromatographic method has been developed and validated for Simultaneous Estimation of Nifedipine and Lidocaine in Cream Dosage Form. The Stationary Phase used was C_{18} Hypersil BDS column and the Mobile Phase used was Mixture of Buffer (pH 3.0): Methanol (50:50). The Developed Method was Validated As per International Conference on Harmonization (ICH) guidelines. Calibration Curve was found to be Linear and the Correlation Coefficient was found to be 0.998 and 0.999 for Nifedipine and Lidocaine respectively. The Limit of Detection for Nifedipine and Lidocaine was found to be 0.18µg/ml and 0.59µg/ml respectively. The Limit of Quantization for Nifedipine and Lidocaine was found to be 0.55µg/ml and 1.79µg/ml respectively. The Degradation Study Preformed are Oxidative, Acidic, Basic, Thermal and Photolytic.

Keywords: Validation; HPLC; Nifedipine; Lidocaine; Cream Dosage Form.

Introduction

Nifedipine (3,5 dimethy l 2,6 dimethyl 4(2nitrophenyl)1,4dihydropyridine3,5dicarboxylate) is a dihydropyridine calcium channel blocker that primarily blocks L-type calcium channels. Its main uses are as an antianginal and antihypertensive, although a large number of other indications have recently been found for this agent, such as Raynaud's phenomenon, premature labor, and painful spasms of the esophagus such as in cancer and tetanus patients. Lidocaine (2 diethylamino) N (2,6dimethylphenyl) acetamide) is a local anesthetic and cardiac depressant used as an antiarrhythmia agent.

Chronic anal fissure is the most common cause of anal pain associated with internal anal sphincter hypertonia. Reduction of hypertonocity is a special treatment for fissure healing. For this purpose chronic anal fissures were conventionally treated by anal dilatation or by lateral sphincterotomy. Hence the Combination of Nifedipine and Lidocaine in the form of Cream is used to Treat Anal Fissure. Various methods have been developed for the estimation of these two drugs separately, no method is developed for combination estimation.

Hence efforts are made to develop a HPLC method which used to find them in combination and to validate the HPLC method which is developed as per the ICH guideline, to get a better, reliable, accurate and easy method for the estimation of these two drugs.

Materials and Method

Instrumentation and Chromatographic Condition

Analysis was performed with RP-HPLC Instrument (Model: LC 10-AT) equipped with

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Software Spinchrom. The C_{18} (25 cm × 0.46 cm) Hypersil BDS column is used. It is having a fixed 20µL loop. Different combinations of mobile phases were tested so that to find a suitable mobile phase which can detect both the drugs at the same time. The appropriate mixture of mobile phase which can detect both the drug was found to be Buffer (pH 3.0): Methanol (50:50). The flow rate was adjusted at 1.0 ml/min with the run time of 10 min. The sample was injected with the injector into the fixed loop of 20µL and the system is run to find the specific wavelength. Standard solution of Lidocaine HCl (15µg/ml) and Standard solution of Nifedipine (3µg/ml) in Methanol were scanned between 200-400 nm using UV-visible spectrophotometer. Both solutions were scanned between 200 - 400 nm. Wavelength was selected from the overlay spectra of above solutions. The specific wavelength were both drug intersect each other was found to be 233nm. The identity of the compounds was established by comparing the retention times of compounds in the sample solution with those in standard solutions.

Materials and Reagents

Nifedipine was procured from RPG Life Science and Lidocaine from Oasis Laboratory. The other reagents used are Acetonitrile, water, Methanol, Acetic acid. All are HPLC grade reagents. The other materials used are UV Spectrophotometer (Shimadzu 1800), Analytical balance, PH meter, Ultrasonicator. The combined drug was found in Cream dosage form which is having name Anobliss which is manufacture by the Samarth Life science.

Preparation of Standard and Sample Solutions

Lidocaine HCl standard stock solution: (150µg/mL) A 15 mg of Lidocaine HCl was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with methanol.

Nifedipine Standard Stock Solution: (30µg/mL)

A 30 mg of Nifedipine was weighed and transferred to a 100 mL volumetric flask. and volume was made up to the mark with methanol and Take 1 ml from this solution and transfer to 10 ml volumetric flask and made up the to the mark.

Preparation of standard solution of binary mixtures of Lidocaine HCl (15 µg/mL) and Nifedipine (3 µg/mL)

Take 1 mL from the Lidocaine HCl stock solution and 1mL from Nifedipine stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

Procedure for Analysis of Formulation

Take Cream equivalent to 15 mg Lidocaine HCl and 3mg of Nifedipine was transferred to a 100ml volumetric flask, shake for 15 minutes than put this solution on water bath for 15 minutes at 60 ^{o}C, Allow to cool the solution and made up volume up to the mark with mobile phase. The solution was filtered through Whatman filter paper no.42 and first few drops of filtrate were discarded. 10 ml of this solution was diluted to 100ml with mobile phase. The solution was injected 10 µl. The areas of resulting peak were measured at 233 nm.

Degradation Study

The Drug was employed for Oxidative, Thermal, Photolytic, Acidic and Basic Degradation condition. After the Degradation treatment were completed the solution were cooled at room temperature and were diluted to get the concentration of Lidocaine HCl (15 µg/mL) and Nifedipine (3 µg/mL). These solution is then run into the system and the chromatogram were recorded to assess the Stability of Sample. Specific Degradation conditions are as follows:

Oxidative Degradation

Oxidative decomposition studies were performed by refluxing One ml of stock solution was transferred in to 10 ml of volumetric flask. Two ml of 3% H_2O_2 solutions was added and mixed well and put for 3 hrs at 70 ^{o}C 250 ml Round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluent to get 15µg/ml for Lidocaine HCl and 3µg/ml for Nifedipine.

Thermal Degradation

Thermal Degradation studies were performed One ml of stock solution was transferred in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 110°C for 4 hrs. Then the volume was adjusted with diluent to get 15µg/ml for Lidocaine HCl and 3µg/ml for Nifedipine.

Photo Degradation

Photo Degradation studies were performed One ml of stock solution was transferred in to 10 ml of

volumetric flask. The volumetric flask was keep in presence of Sunlight for 3 hrs. Then the volume was adjusted with diluent to get 15µg/ml for Lidocaine HCl and 3µg/ml for Nifedipine.

Acid Degradation

Acid decomposition studies were performed by Refluxing One ml of stock solution was transferred in to 10 ml of volumetric flask. Two ml of 0.1 N HCl solutions was added and mixed well and put for 4 hrs at 70 °C 250 ml Round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluent to get 15µg/ml for Lidocaine HCl and 3µg/ml for Nifedipine.

Base Degradation

Basic decomposition studies were performed by refluxing One ml of stock solution was transferred in to 10 ml of volumetric flask. Two ml of 0.1 N NaOH solutions was added and mixed well and put for 4 hrs at 70 °C 250 ml Round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluent to get 15µg/ml for Lidocaine HCl and 3µg/ml for Nifedipine

Method Development

Several Test were Performed in order to get a satisfactory separation of Nifedipine and Lidocaine with the different mobile phase and ratios. The appropriate mobile phase was found to be the mixture of Buffer (pH 3.0): Methanol (50:50). This mobile phase gives a proper separation and Resolution of Nifedipine and Lidocaine. The retention time of Nifedipine and Lidocaine on the analytical column was evaluated at a flow rate of 1 ml/min. The injection volume was 20µL. The retention time of standard and sample for Nifedipine and Lidocaine were satisfactory with good resolution. These mobile phase condition were the optimized to find whether is there any interference due to solvents.

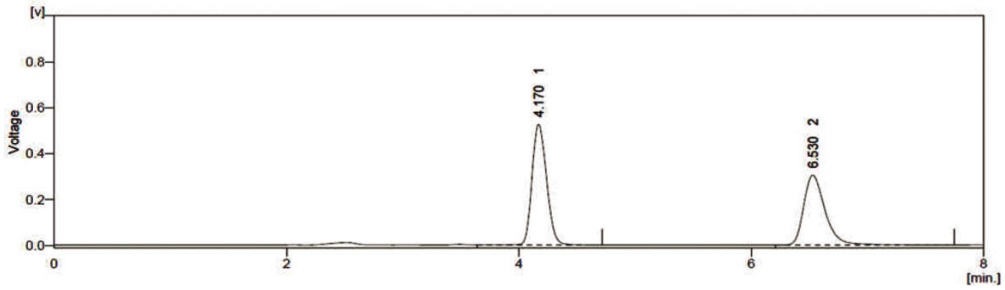

Fig. 1: Chromatogram for lidocaine (15µg/ml) and nifedipine (3µg/ml)

Method Validation

The method was validated for specificity, linearity, accuracy, intra-day and inter-day precision and robustness, in accordance with ICH guidelines.

Linearity

The linearity for Lidocaine HCl and Nifedipine were assessed by analysis of combined standard solution in range of 7.5-22.5 µg/ml and 1.5-4.5 µg/ml respectively, 5,7.5,10,12.5,15 ml solutions were pipette out from the Stock solution of Lidocaine HCl (150 µg/ml) and Nifedipine(30 µg/ml) and transfer to 100 ml volumetric flask and make up with mobile phase to obtain 7.5, 11.25, 15, 18.75 and 22.5 µg/ml and 1.5, 2.25, 3, 3.75 and 4.5 µg/ml for Lidocaine HCl and Nifedipine respectively

In term of Slope, Intercept and Correlation Co-efficient value. The graph of peak area obtained verses respective concentration was plotted.

Table 1: Linearity data for lidocaine

S. No.	Concentration (µg/ml)	Area
1	7.5	2319.102
2	11.25	3361.305
3	15	4592.486
4	18.75	5633.077
5	22.5	6823.593

Table 2: Linearity data for nifedipine

Sr. No.	Concentration (µg/ml)	Area
1	1.5	1957.077
2	2.25	2836.708
3	3	3748.038
4	3.75	4825.087
5	4.5	5790.415

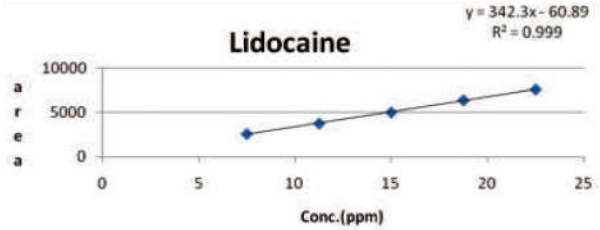

Fig. 2: Linearity curve for lidocaine

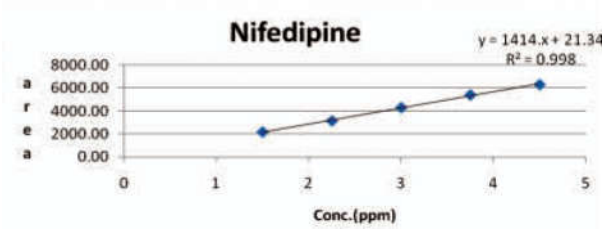

Fig. 3: Linearity curve for nifedipine

Precision

Results should be expressed as Relative standard deviation (RSD) or coefficient of variance.

A. Repeatability

Standard solution containing Lidocaine HCl (15µg/ml) and Nifedipine (3µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

B. Intra-Day Precision

Standard solution containing (7.5, 15, 22.5µg/ml) of Lidocaine HCl and (1.5, 3, 4.5µg/ml) of Nifedipine were analyzed three times on the same day and % R.S.D was calculated.

Inter-Day Precision

Standard solution containing (7.5, 15, 22.5µg/ml) of Lidocaine HCl (1.5, 3, 4.5µg/ml) of Nifedipine were analyzed three times on the different day and % R.S.D was calculated.

Table 3: Repeatability data for lidocaine

Sr. No.	Conc (µg/ml)	Lidocaine HCl Area	Mean ± S.D (n=6)	% R.S.D
1.	15	5090.69 5065.13 5070.01 5044.56 5040.07 5009.23	5053.28±28.32	0.56

Table 4: Repeatability data for nifedipine

Sr. No.	Conc (µg/ml)	Nifedipine Area	Mean ± S.D (n=6)	% R.S.D
1.	3	4293.54 4271.93 4240.94 4254.71 4155.87 4224.92	4240.32 ±47.77	1.13

Table 5: Intraday precision data for estimation of lidocaine HCl and nifedipine

S. No.	Conc. (µg/ml)	Lidocaine HCl Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Nifedipine Area Mean ± S.D. (n=3)	% R.S.D
1	7.5	2569.81 ± 3.87	0.151	1.5	2143.86± 24.94	1.16
2	15	5084.05± 15.91	0.31	3	4291.4.3± 19.20	0.45
3	22.5	7570.16± 15.22	0.20	4.5	6356.46±50.55	0.79

Table 6: Interday precision data for estimation of lidocaine HCl and nifedipine

S. No.	Conc. (µg/ml)	Lidocaine HCl Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Nifedipine Area Mean ± S.D. (n=3)	% R.S.D
1	7.5	2542.27±19.04	0.75	1.5	2141.09±21.41	0.99
2	15	5003.10±23.83	0.48	3	4205.55±40.79	0.97
3	22.5	7625.79±31.98	0.42	4.5	6431.53±26.92	0.42

Table 7: Recovery data for lidocaine HCl

S. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	7.5	6	6.00	99.99	99.55 ± 0.45
2		7.5	6	5.97	99.54	
3		7.5	6	5.95	99.10	
4	100 %	7.5	7.5	7.46	99.52	98.54± 0.86
5		7.5	7.5	7.37	98.26	
6		7.5	7.5	7.34	97.86	
7	120 %	7.5	9	8.97	99.66	98.99 ± 0.86
8		7.5	9	8.94	99.30	
9		7.5	9	8.82	98.03	

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. Percentage recovery for Lidocaine HCl was 99.72-100.02 %, while for Nifedipine, it was found to be in range of 99.87-100.17 %.

LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Where,

SD = Standard deviation of intercepts Lidocaine HCl Nifedipine

LOD = 3.3 x (SD / Slope)= 3.3 x (61.36/342.36)
= 0.59 µg/ml

LOD = 3.3 x (SD / Slope) = 3.3 x (77.75/1414)
= 0.18 µg/ml

Lidocaine HCl Nifedipine

LOQ = 10 x (SD / Slope) = 10x(61.36/342.36)
= 1.79 µg/ml

LOQ = 10 x (SD / Slope) = 10 x (77.75/1414)
= 0.55 µg/ml

Table 8: Recovery data for nifedipine

Sr. No.	Conc. Level (%)	Sample Amount	Amount Added	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	1.5	1.2	1.20	99.92	98.69 ± 1.74
2		1.5	1.2	1.19	99.46	
3		1.5	1.2	1.16	96.70	
4	100 %	1.5	1.5	1.49	99.58	98.28 ± 1.13
5		1.5	1.5	1.46	97.48	
6		1.5	1.5	1.47	97.78	
7	120 %	1.5	1.8	1.79	99.62	97.92 ± 1.72
8		1.5	1.8	1.73	96.18	
9		1.5	1.8	1.76	97.97	

Table 9: LOD lidocaine and nifedipine

Lidocaine HCl	Nifedipine
LOD = 3.3 x (SD / Slope) = 3.3 x (61.36/342.36) = 0.59 µg/ml	LOD = 3.3 x (SD / Slope) = 3.3 x (77.75/1414) = 0.18 µg/ml

Table 10: LOQ lidocaine and nifedipine

Lidocaine HCl	Nifedipine
LOQ = 10 x (SD / Slope) = 10 x (61.36/342.36) = 1.79 µg/ml	LOQ = 10 x (SD / Slope) = 10 x (77.75/1414) = 0.55 µg/ml

Robustness

As defined by ICH, The robustness of an analytical procedure describes to its capability to remain unaffected by small and deliberate variations in method parameters. Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1. Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.
2. pH of Mobile phase was changed (± 0.2) 3.2 and 2.8
3. Ratio of Mobile phase was changed(±2) Buffer: Methanol (48:22) and Buffer: Methanol (52:48)

Table 11: Robustness data for lidocaine

Sr. No.	Area at Flow rate (-0.2 ml/min)	Area at Flow rate (+0.2ml/min)	Area at pH (- 0.2)	Area at pH (+ 0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	5583.07	4563.41	4937.97	5125.37	5385.93	4805.39
2	5571.80	4554.17	4931.63	5120.19	5380.44	4776.65
3	5560.59	4531.30	4974.74	5111.43	5396.44	4786.02
% R.S.D	0.20	0.36	0.47	0.14	0.15	0.31

Table 12: Robustness data for nifedipine

Sr. No.	Area at Flow rate (-0.2 ml/min)	Area at Flow rate (+0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	4708.81	3848.82	4164.74	4322.82	4542.53	4052.92
2	4637.77	3841.16	4173.02	4318.46	4537.96	4028.60
3	4689.90	3807.21	4195.90	4287.20	4520.69	3987.59
% R.S.D	0.79	0.58	0.39	0.45	0.25	0.82

Table 13: Forced degradation studies of lidocaine

Parameter	Lidocaine HCl			
	Standard		Sample	
	Area	%Degradation	Area	%Degradation
Acid	3991.13	24.91	3946.00	25.76
Base	4304.57	19.01	4270.81	19.65
Thermal	4332.13	18.49	4455.23	16.18
Oxidation	3922.27	26.20	3936.41	25.94
Photo	4584.30	13.75	4686.55	11.82

Table 14: Forced degradation studies of nifedipine

Parameter	Nifedipine			
	Standard		Sample	
	Area	%Degradation	Area	%Degradation
Acid	3980.91	13.10	3884.50	15.20
Base	3702.77	19.17	3692.24	19.40
Thermal	3352.58	26.81	3328.94	27.33
Oxidation	3934.61	14.11	3801.56	17.01
Photo	3962.53	13.50	3979.14	13.13

Conclusion

The validated HPLC method employed here proved to be simple, fast, accurate, precise and robust, thus can be used for routine analysis of Lidocaine and Nifedipine in combined Cream dosage form.

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Original Article

Investigation into Mechanism of Action of Antiulcer Activity of Polyherbal Formulation in Experimentally Induced Gastric Ulcers in Rats

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Abstract

Background and Objective: The polyherbal formulation (PHF) consisted of *Piper betel* leaves and *Acacia catechu*. The leaves of *Piper betel* has major active constituents as Hydroxychavicol and eugenol which are readily extracted by water. The bark of *Acacia catechu* mainly contains catechin, and epicatechin. The extract of *Piper betel* have been tested for Antiplatelet activity against various pro aggregating substances, like PAF, TXB2, PGD2, and ADP, collagen and arachidonic acid. Anti-cancer activity was evaluated by MTT (tetrazolium) assay using human breast cancer cell line (MCF-7). Anti-arthritic activity of Hydroxychavicol was evaluated at graded doses which significantly decreased the expression of IL-1β, PGE2, LTB4, and nitric oxide levels. Antioxidant and anti-inflammatory activities of Hydroxychavicol was assessed using hyaluronidase (HYA), xanthine oxidase (XOD) and lipoxygenase (LOX) inhibition assays. The extracts of *Piper betel* and *Acacia catechu* also exhibited significant inhibition in XOD and LOX assays. Antimutagenic activity was exhibited by dose dependent suppression of dimethylbenzanthracene induced mutagenesis in *S. typhimurium* strain TA98 with metabolic activation and also showed that Hydroxychavicol was more potent than eugenol in this respect. The anticoagulant activity of the compound was assayed by the activated partial thromboplastin time (APTT), prothrombin time (PT), and these assays were compared with the anticoagulant heparin which showed that it acted on the intrinsic as well as extrinsic pathways of the blood coagulation systems. It could be a potential therapeutic agent for prevention and treatment of Atherosclerosis and other cardiovascular diseases.

Literature search reveals the traditional use of *Piper betel* leaves as an aromatic, stimulant, carminative, astringent and antiseptic (Kirtikar and Basu, 1987 and Panda, 2004). The Acacia catechu has antiviral, anti-inflammatory, hepatoprotective and spasmolytic (Dahanukar et al, 2000). To confirm the traditional claim of antiulcer activity of *Piper betel* leaves and *Acacia catechu* scientifically, the study was undertaken to investigate the antiulcer and antisecretory activity of Poly Herbal Formulation (PHF) consisting of *Piper betel* leaves and *Acacia catechu* in experimentally induced gastric ulcer models in rats. The study supported that PHF has anti-ulcerogenic activity as it decreased ulcer index, volume of gastric acid secretion, total acidity, total acid output, pepsin output, TC/PR ratio, protein content and MDA content while increasing total carbohydrates as compared to disease control group. Thus, the antisecretory and cytoprotective activity of PHF are confirmed and further studies are warranted to investigate the efficacy of the same. *Methods:* Wistar albino rats of either sex were treated with PHF in dose of 100mg/kg and 200mg/kg for 7 days p.o. once a day in Aspirin + pylorus ligation induced gastric ulcer model and 200mg/kg in ethanol induced gastric ulcer model in rats. The physical parameters, acid secretory parameters, dissolved

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mucosubstances and MDA content were analyzed. *Results and Discussion:* When compared to disease control group, PHF treated group showed significant decrease in ulcer index ($p<0.001$), Volume of gastric acid secretion ($p<0.01$), Total Acidity ($p<0.01$), Total Acid Output ($p<0.01$), Pepsin output ($p<0.01$) and Protein content ($p<0.01$) while significant increase in Total carbohydrates ($p<0.01$) and total carbohydrate: Protein (TC:PR) ratio ($p<0.01$) in Aspirin + Pylorus ligation induced gastric ulcer model. Significant decrease in ulcer index ($p<0.01$) and MDA content ($p<0.001$) was seen in ethanol induced gastric ulcer model in rats. *Conclusion:* The present study demonstrated that PHF possess significant antiulcer activity against experimentally induced gastric ulcer in rats which might be attributed to its antioxidant activity of major phytochemical constituent Hydroxychavicol and catechin.

Keywords: Hydroxychavicol; Catechin; Acacia Catechu; *Piper Betel* Leaves; Anti Ulceractivity.

Introduction

Peptic ulceration is one of the common diseases affecting nearly 10% of the world population[1]. Ulcers are identified as ulcerative colitis and peptic ulcers depending on the location in the GI tract. Peptic ulcer disease (PUD) affects substantial number of people worldwide. It develops due to imbalance between the 'aggressive factors' i.e. infection by Helicobacter pylori, gastric acid, pepsins, NSAIDs, bile acids, hypoxia, ischemia, smoking and alcohol intake; and 'protective factors' i.e. bicarbonate, mucus layer, mucosal blood flow, PGs and growth factors at the gastric epithelial lining [2].

The existing treatments involve use of antacids (aluminum hydroxide, magnesium trisilicate), acid suppressive agents (Anti-secretory drugs) like proton pump H+/K+ ATPase inhibitors (omeprazole, lanzoprazole), histamine H2 receptor antagonist (cimetidine, ranitidine) and anticholinergic (M1) (pirenzepine), cytoprotective agents (sucralfate and prostaglandin analogues (misoprostol), antimicrobials for eradication of H. pylori (amoxicillin, clarithromycin) and Triple therapy (one week triple therapy consisting of a proton pump inhibitor such as Omeprazole and the antibiotics Clarithromycin and Amoxicillin) [2,3].

A widespread search has been launched to identify new natural anti-ulcer therapies to replace currently used drugs of doubtful efficacy and safety. There is a rich heritage of plants reputed in traditional medicine known to possess antiulcer properties. These can be a valuable source of new molecules which after chemical manipulation can provide new and improved anti-ulcer treatment [1].

Piper betel is a twining plant cultivated in hotter and damper parts of India. The major active protective phyto constituents are Hydroxychavicol and eugenol which are found in its aqueous extract [4]. Hydroxyclavicol has been reported for its antioxidant, anti-inflammatory, anti-arthritic, anti-enteropathogenic, anticancer, superoxide radical and hydroxyl radical scavenging activity, COX-1/COX-2 inhibiting, antimicrobial, antimutagenic and antifungal activities [4-6].

Acacia catechu Willd .(Family: Mimosaceae) is a perennial tree and is found throughout India. It has antiviral, anti-inflammatory, hepatoprotective and spasmolytic activity (7 Dahanukar et al, 2000). Aqueous extracts of Acacia catechu are rich source of catechin and epicatechin (gallic acid derivatives), with smaller amounts of flavonoids. Potent antioxidant activity has been well established in both in vitro and in vivo studies. This antioxidant activity is believed to be responsible for the anti-inflammatory, tissue protectant, antineoplastic, and analgesic activities that have been demonstrated and clearly established in animal and cell culture systems. Furthermore, antihyperglycemic, antidiarrheal, antinociceptive, and antipyretic activities have been demonstrated in animal studies (8 Stohs and Bagchi, 2015).

Thus, it was deduced from literature survey that due to its antioxidant and free radical scavenging ability, the PHF may show anti-ulcerative effect. Thus this study was undertaken to evaluate the antiulcer activity of a PHF in experimentally induced gastric ulcer in rats.

Materials and Method

Plant Extract Preparation

The *Piper betel* leaves and heartwood of *Acacia catechu* were authenticated at Department of Botany, Urban Science College, Mehsana. The aqueous extract of the *Piper betel* dried leaves was used for the study. Accurately weighed 100 gm of fresh *Piper betel* leaves were grinded by mixing with 500ml of distilled water. The mixture was heated at 80°C for about 30 minutes and filtered using vacuum filter assembly. Then the filtrate was concentrated[9]. The dry powder was prepared by spray drying. The clumpy dry powder obtained was scraped off and fine powder

was packed in air tight plastic container [10].

The fresh heartwood of *Acacia catechu* was dried in shade, cut and crushed. The cut and crushed Acacia catechu wood was plant material (20 g) and was extracted in hot ethyl alcohol by Soxhlet extraction method. The extract was concentrated and the solvent was evaporated off. A part of the residue was used for further studies.

Experimental Animals

Wistar albino rats weighing between 250 to 300 g were housed in groups of four at temperatures (25-28°C), under a standard light/dark cycle. The rats were fed with a standard pellet diet and water. The animals were utilised after acclimatization period. The experimental procedures were performed in accordance with the Institutional Animal Ethical Committee (IAEC) constituted as per the directions of the Council for Medical Research and the Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA), New Delhi, India. (SSPC/IAEC/17/07/2013)

Treatment

Polyherbal formulation (PHF) consisted of *Piper betel* and *Acacia catechu* extracts in equal proportion. PHF (aqueous extract) was administered in two different dose regimes- 100 mg/Kg and 200 mg/Kg, orally, once daily for 6 days to study the effect of drug. The efficacious dose of PHF 200mg/Kg was further tested in Ethanol induced gastric ulcer model in rats.

Experimental Protocols

Aspirin + Pylorus Ligation Model

Rats were divided into four groups of six animals each. Group I animals were treated as Disease control animals (Aspirin + pylorus ligation and Vehicle). Group II animals received standard drug treatment of Ranitidine (50mg/kg). Group III and IV animals were treated with PHF 100 mg/kg and 200mg/kg dose respectively. From days 4 to 6, animals of all the groups received aspirin orally as an aqueous suspension at a dose of 200 mg/kg. Two hours after the administration of respective drug treatment, animals in all the groups were fasted for eighteen hours and anaesthetized with ether. Pyloric ligation was performed by ligating the pyloric end of stomach. After four hours of pyloric ligation the animals of all the groups were sacrificed and gastric contents were collected for analysis for Ulcer Index, Volume of gastric acid secretion, Total Acidity, Total Acid output, Pepsin output, Total Carbohydrates(TC), Protein content (PR) and TC/PR ratio. The stomachs were removed and opened along the greater curvature to determine the ulcer index [11,12].

Ethanol-Induced Gastric Ulcers in Rats

Animals were treated by respective treatments for 7 days. Group I animals were treated as Disease control animals and were administered 1 ml of 80% Ethanol p.o. Group II animals received standard drug treatment of Ranitidine (50mg/kg). Group III animals were treated with PHF (200mg/kg). On 7th day the animals were fasted for 36 hours before the experiment. 1 ml of 80% ethanol was administered p.o. in the fasted animals [13–15]. In treated group, drug was administered p.o., 1 h before the administration of ethanol. After 2 h of ethanol administration, animals were sacrificed and stomach was removed, and opened along the greater curvature and subjected to measurement of ulcer index. After that, the stomach was homogenised for thiobarbituric acid reactive substances assay (MDA content). The following parameters investigated:

1. Physical parameters- (Ulcer index [1] and Volume of gastric acid secretion [16]),
2. Acid secretory parameters- (Total acidity [16], Total acid output [16], Pepsin output [17]),
3. Dissolved mucosubstances- (Total carbohydrates (TC) [18], Protein content (PR) [19], TC/ PR ratio)
4. Thiobarbituric acid reactive substances assay (MDA content) [20].

Results

Gastric ulcers were induced by Aspirin + pylorus ligation model to assess the efficacy of PHF at 100 mg/Kg and 200 mg/Kg. The physical parameters i.e. ulcer index, volume of gastric acid secretion, total acidity and total acid output showed reduction by PHF treatment and by the standard treatment Ranitidine as compared to disease control animals (Table 1). The biochemical parameters involved estimation of pepsin output which was found to be decreased as compared to diseased animals. Pepsin output by PHF extract at 200 mg/Kg dose was comparable to the standard treatment ranitidine (Graph 1). Total carbohydrate content was found to

be increased in the treated animals in comparison with diseased animals. PHF extract (100 mg/Kg) treated animals showed similar increase in total carbohydrate content as Ranitidine (Graph 2). The protein content found to be decreased in all the treatment arms when compared to disease control animals (Graph 3). TC/PR ratio showed significant difference in disease treated with aqueous extract of PHF 200 mg/kg dose as compared to 100 mg/kg dose and the ranitidine treatment which had comparable effects (Graph 4).

The previous study was suggestive that the efficacy of PHF was found maximum at 200 mg/Kg and thus was further analysed for cytoprotection using Ethanol-induced gastric ulcer model. Ulcer index in treatment groups was found to be reduced as compared to disease control group. The aqueous extract showed equivalent effect in reduction of ulcer index as in ranitidine treatment arm (Graph 5). MDA content was higher in disease control group as compared to PHF treated group. Reduction in MDA content in PHF was comparable to ranitidine treated group.

Table 1: Physical parameters of aspirin + pylorus ligated gastric ulcer model

Groups	Ulcer Index	Volume of Gastric acid (ml)	Total Acidity (mEq/L)	Total Acid Output (mEq/L/100g.b.w.)
Disease control	2.25 ± 0.087	8.03 ± 0.39	61.4 ± 0.65	493.04 ± 15.52
Ranitidine (50mg/kg)	1.21±0.14***	4.96±0.11**	32.87±0.56**	163.02 ± 5.68**
PHF (100mg/kg)	2.10±0.086**	6.88 ± 0.14*	45.0± 0.63*	309.67 ± 5.71*
PHF (200mg/kg)	0.97±0.099***	5.85±0.22**	28.73±0.54**	168.07 ± 6.044**

n=6, Values are expressed as mean± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with disease control group

Table 2: Acid secretory parameters of aspirin + pylorus ligated gastric ulcer model

Groups	Pepsin Output	Total Carbohydrates	Protein Content	TC/PR Ratio
Disease control	20.5 ± 0.057	602.03 ± 0.49	325.4 ± 0.45	1.85±0.55
Ranitidine (50mg/kg)	7.1±0.17**	1004.6±0.21***	210.57±0.56**	4.77±.033**
PHF (100mg/kg)	15.20±0.076*	1002.68 ± 0.24***	245.0± 0.63**	4.09± 0.71**
PHF (200mg/kg)	0.57±0.08***	1200.43±0.12***	154.23±0.14***	7.78±0.14***

n=6, Values are expressed as mean± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with disease control group

Table 3: Parameters for ethanol induced gastric ulcer model

Groups	Ulcer Index	MDA Content
Disease control	4.48±0.34	18.27±0.46
Ranitidine (50mg/kg)	2.98±0.11*	5.56±0.23**
PHF (200mg/kg)	3.01±0.23*	6.02±0.8**

n=6, Values are expressed as mean± SEM, * $p < 0.05$, ** $p < 0.001$ when compared with disease control group

Discussion

With the associated side effects of the modern medicine, traditional medicines are gaining importance and are now being studied to find the scientific basis of their therapeutic actions [21]. Ulcers mainly result from an imbalance between aggressive factors and the maintenance of the mucosal integrity through the endogenous defence mechanism [3]. To regain the balance, different therapeutic agents including herbal preparations are used to inhibit the gastric acid secretion or to boost the mucosal defence mechanism by increasing mucus production [22]. Keeping insight the above mentioned facts, the present investigation was designed to evaluate anti-ulcerogenic potential of aqueous extract of *Piper betel* leaves.

HPTLC analysis of aqueous extract of *Piper betel leaves* at 366 nm showed presence of Hydroxychavicol (rf value = 0.44) and Eugenol (rf value = 0.96). Our results are in concordance with the findings of Pin et al. (2010)[23]. This insight provided the basis for our study that Hydroxychavicol present in Aqueous extract of *Piper betel* leaves might be responsible for antiulcer activity by its free radical scavenging and antioxidant activity. In our study the % yield of hydroxychavicol was found to be 1.5. Hydroxychavicol and Eugenol both were detected in the aqueous extract but they differed in amounts. Aqueous extraction of Hydroxychavicol was carried out by Pin KY et al. The extraction yield of water was significant compared with other solvents. This indicated that the major phytochemical constituents in betel leaves

are mostly high in polarity and soluble in water. The chemical profile of water extract varied significantly from other extracts because it contained only two major peaks, one for hydroxychavicol and one for Eugenol [9]. Our results are in concordance with it and confirmed that Hydroxychavicol is the major active principle in aqueous extract of *Piper betel* leaves which might be responsible for antiulcer activity.The TLC of the alcoholic plant extract of *Acacia catechu* conducted using CEF (chloroform ethyl acetate formic acid, 5:4:1) as mobile phase and DPPH as spray reagent, gave yellow spots indicating presence of antioxidant constituents in the extract.

Acute toxicity test was performed by Katedeshmukh et al and Pingale for *Piper betel* leaves and *Acacia catechu* respectively. They showed that mice were free of any toxicity as per acceptable range given by the OECD guidelines up to the dose of 2000mg/kg and 6000mg/kg respectively [24,25].

NSAIDS like aspirin causes gastric mucosal damage by decreasing prost aglandins levels through inhibition of prostaglandins synthesis, increasing acid secretion, decreasing mucin activity and back diffusion of H+ ions and thus leading to breaking up of mucosal barrier. Furthermore, it results in generation of free radicals, imbalance in gastric secretion, elevated pepsin, protein content and back diffusion of H+ ions into gastric mucosa leading to necrosis and ulceration [26]. The increased protein content of the gastric juice suggests the mechanism of leakage of plasma protein into gastric juice. Pylorus ligation induced ulcers are due to auto digestion of gastric mucosa and breakdown of the gastric mucosal barrier. The PHF extract showed significant reduction in ulcer index and protein content. It also reduced volume of gastric acid secretion, total acid output and pepsin concentration indicating its anti-secretory effect. Decrease in protein content in gastric juice also signifies decrease in leakage from mucosal cells indicating mucosal resistance. Mucin secretion is a crucial factor in the protection of gastric mucosa from the gastric lesions and has been regarded as an important defensive factor in the gastric mucus barrier. Decrease in synthesis of mucus glycol proteins has been implicated in etiology of gastric ulcer[27]. The increase in carbohydrate: protein (TC:PR) ratio is the direct reflection of mucin activity.

Cytoprotection has been defined as the ability of pharmacological agents (PGs) to prevent or reduce gastric mucosal injury by mechanism other than inhibition of gastri cacid secretion. The mechanism behind cytoprotection may be mainly to maintain physico-chemical properties and integrity of the gastric mucosal barrier. Ethanol is reported to damage mucosa, alteration in permeability and free radical production. This is attributed to release of superoxide anion and hydroperoxy free radicals and have found to be involved in the mechanism of acute and chronic ulceration in gastric mucosa. Alcohol rapidly penetrates the gastric mucosa apparently causing cell and plasma membrane damage leading to increased intracellular membrane permeability to sodium and water. The massive intracellular accumulation of calcium represents a major step in pathogenesis of gastric mucosal injury. This leads to cell death and exfoliation in the surface epithelium. It was observed that the PHF extract significantly reduced ethanol induced ulcer. This may be due to cytoprotective effect of extract which was measured in terms of the inhibition of lipid peroxidation (MDA Content). The PHF extract showed protection against characteristic lesions produced by ethanol administration which might be attributed to both reduction in gastric acid secretion and increase in gastric cytoprotection.

In summary, our data suggests that the PHF extract seems to be effective against gastric ulcers induced by Aspirin together with pylorus ligation model and Ethanol model. The antiulcer effect of the drug could be attributed to its free radical scavenging property, inhibition of gastric acidity leading to prevention of H+ ion back diffusion from stomach mucosa and strengthening of the gastric mucosal barrier.

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Original Article

Development and Characterization of Self- Microemulsifying Drug Delivery System for Improvement of Bioavailability of Cefdinir

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Abstract

Oral route is the most common and preferred route for the drug administration due to convenience and ease of administration. Technology Catalysts International reported in 2002 that approximately 35-40% of all new chemical compounds suffer from poor aqueous solubility. Therefore, enhancing drug dissolution became one of the major challenges for pharmaceutical scientists over the past decade. Lipid formulations and in particular SMEDDS/ SNEDDS Self-Micro emulsifying Drug Delivery Systems can induce a considerable increase in dissolution rate Class II-IV drugs are considered the best candidates for intervention by formulation e.g. in self-emulsifying dosage forms. *Aim:* Cefdinir is a poorly water-soluble drug with varying bioavailability. The main purpose of present work was to develop self-micro emulsifying drug delivery system (SMEDDS) for enhancing solubility and bioavailability of Cefdinir is indicated for the treatment of bronchitis as well as for the treatment ofear, nose, throatdisorder. *Materials and Method:* Cefdinir had highest solubility in labrafac with comparison to other lipid vehicles. Emulsification study results were shown that tween 20 has highest solubility capacity of oil was higher (0.8528 ± 0.4075mL) than other surfactant.Sotween 20 was selected as surfactant. From the result were shown that PEG 400 has highest solubility capacity of oil (2.65 ± 1.801 mL). So PEG 400 was selected as co-surfactant. The formulation of Cefdinir SMEDDS was optimized by a simplex lattice design. The optimal formulation of SMEDDS was comprised of 20% oil (Labrafac), 60% surfactant (Tween-80) and 20% co-surfactant (PEG-400). *Results and Discussion:* Pseudo-ternary phase diagrams were constructed to identify the efficient self-emulsification region. Optimal ratio of surfactant to co-surfactant was selected to be 4:1. A suitable SMEDDS formulation should have a minimum self emulsification time, maximum% Transmittance, maximum time require to 20% of drug release.The individual desirability for each response was calculated and batch F2 showed the highest overall desirability therefore this batch considered to be the best batch. In order to obtain both high %Transmittance and high Cumulative %release, the appropriate ratio of components was chosen for optimized formulation, which consisting of oil (20%), surfactant (60%), co-surfactant (20%).The average globule size of SMEDDS containing Cefdinir was about 87.60 nm when diluted in water. No significant variations in globule size and In vitro diffusion studies showed remarkable increase in dissolution of drug. Order of drug release was F-2> F-4> F-1 > F-7> F-3> F-6 > F-5. *Conclusion:* The data suggest use of SMEDDS to provide great potential as an alternative to traditional oral formulations of Cefdinir.

Keywords: Cefdinir; Self-Micro Emulsifying Drug Delivery System (SMEDDS); Simplex lattice Design; Globule Size.

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Introduction

Introduction of Self Micro-Emulsifying Drug Delivery System

It is generally accepted that many of today's new chemical entities are poorly water-soluble and pose a challenge in developing an optimum solid oral dosage form. Oral route has been the major route of drug delivery for the treatment of various chronic diseases like cancer. However, oral delivery of approximately 40% of the drug compounds is limited because of low aqueous solubility, which leads to limited oral bioavailability, high intra and inter subject variability and lack of dose proportionality. To overcome the above discussed drawbacks, various other formulation strategies have been adopted including the use of, nanoparticles, solid dispersions and permeation enhancers. In recent years, much attention has focused on lipid-based formulations to improve the oral bioavailability of poorly water-soluble drug compounds. In fact, the most popular approach is the incorporation of the drug compound into inert lipid vehicles such as oils and surfactant dispersions, self-emulsifying formulations, emulsions and liposomes with particular emphasis on self-microemulsifying drug delivery systems (SMEDDS).

Cefdinir is a BCS class-IV compound it has low solubility and low permeability. Oral bioavailability of cefdinir is 16-25%. In current work cefdinir solubility will be enhanced by using SMEDDS which include various oils, surfactants/co-surfactants, solvents/ co-solvents [1-2].

Nevertheless, oral delivery of over one-half of the drug compounds through gastrointestinal (GI) tract gets diminished owing to their high lipophilicity and consequently poor aqueous solubility. Oral bioavailability of such drugs, being primarily a function of their solubility and dissolution, tends to exhibit inadequate magnitude with high intra- and inter-subject variability. Besides, oral bioavailability also depends upon a multitude of other drug factors such as stability in GI fluids, intestinal permeability, resistance to metabolism by cytochrome P450 family of enzymes present in gut enterocytes and liver hepatocytes, and interaction with efflux transporter systems like P-glycoprotein.Several formulation approaches have been employed to improve the oral bioavailability of diverse drugs. Amongst these, oral lipid-based SMEDDS have proved their immense potential in improving the poor and inconsistent drug absorption of many poorly water-soluble drugs, especially following their administration after meals [3-10].

Composition of SMEDDS

Drugs

Generally, SMEDDS are prepared for drugs possessing poor water-solubility.

Surfactant

Surfactants are having amphiphilic character. They help in solubilisation of lipophilic drug compounds. In GI lumen, this prevents precipitation of drug. So that the drug exists in solution form in lumen for prolonged time. Nonionic surfactants possessing high HLB value are widely employed. The role of surfactant is to enhance absorption of drug, because of induction of permeation changes in biological membrane. It is reported that a cationic emulsion show greater absorption than an anionic emulsion. To form a stable SMEDDS, 30-60% concentration of surfactant is used.

Lipids/Oils

Vegetable oil, mineral oil, lanolin, silicon oil, fatty acids, animal oil etc are utilized in SMEDDS. Mono-/di-/tri-glycerides are widely used in SMEDDS formulation because they enhance the dissolution rate of drug in the intestinal medium. It is also to be assumed that this glyceride form a droplet which carry drug, C *In vitro* dissolution studies are carried out for so that the metabolism of drug is protected. Polyethylene glycol and polyglycolyzed glycerides in along with vegetable oils have been utilized to solubilise lipophilic drugs. Galactolipids show good emulsifying properties, similar to those of phospholipids. The main difference between phospholipids and galactolipids include the former possess charge, while later is non-ionic and regarded as being safe for long-term use [11].

Co-Solvents

Various organic solvents are used as cosolvents such as ethanol, propylene glycol and polyethylene glycol, which may help to dissolve large amounts of drug in liquid base.

Viscosity Enhancers

The viscosity of the emulsions can be altered by the use of additional material such as acetyl alcohol, tragacanth, beeswax and stearic acids etc.

Polymers

Polymer matrix (inert) present in 5 to 40% w/w,

which is not ionizable at physiological pH and able to form matrix. Examples are hydroxyl propyl methyl cellulose, ethyl cellulose, etc [12].

Mechanism of self-emulsification

Self-emulsification occurs when the entropy change that favors dispersion is greater than the energy required to increase the surface area of the dispersion. The free energy of the conventional emulsion is a direct function of the energy required to create a new surface between the oil and water phases and can be described by the equation:

DG=SNipri2s

Where, DG is the free energy associated with the process (ignoring the free energy of mixing), N is the number of droplets of radius r and s represents the interfacial energy. The two phases of emulsion tend to separate with time to reduce the interfacial area, and subsequently, the emulsion is stabilized by emulsifying agents, which form a monolayer of emulsion droplets, and hence reduces the interfacial energy, as well as providing a barrier to prevent coalescence [13-17].

Emulsification Process

The emulsification process may be associated with the ease with which water penetrates the oil-water interface with the formation of liquid crystalline phases resulting in swelling at the interface thereby resulting in greater ease of emulsification. However, for system containing co- surfactant, significant partitioning of components between the oil and aqueous phases may take place leading to a mechanism described as "diffusion and stranding", where by the oil is solubilized, leading to migration in to the aqueous phase.

Dilution Phases

Upon dilution of a SMEDDS formulation, the spontaneous curvature of the surfactant layer changes via a number of possible liquid crystalline phases. The droplet structure can pass from a reversed spherical droplet to a reversed rod-shaped droplet, hexagonal phase, lamellar phase, cubic phase and various other structures until, after appropriate dilution, a spherical droplet will be formed again (Figure 1).

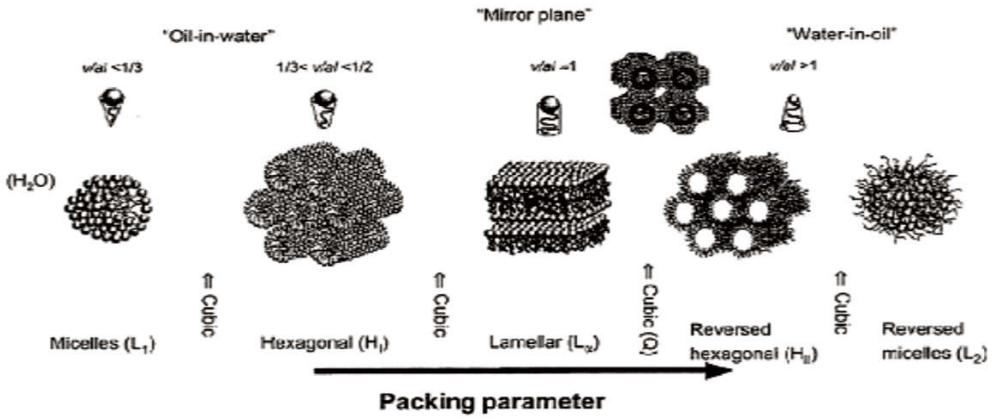


Fig. 1: Representation of the commonly encountered phases upon addition of water to an oil surfactant combination

Many roles have been ascribed to the occurrence of liquid crystalline phases upon aqueous dilution of a lipid formulation. Early work of Groves and Mustafa related the emulsification behavior to the phase behavior of the surfactant-oil mixtures with systems forming liquid crystals showing shorter emulsification times. The authors suggested that the ease of emulsification could be associated with the passage of water into the droplet, more precisely the ease with which the solvent may penetrate into the liquid crystalline phases formed on the surface of the droplet. The structures formed upon dilution have been ascribed an important role in the stability of the diluted micro emulsion and the rate of drug release [18].

Advantages of SMEDDS

Improvement in Oral Bioavailability

Dissolution rate dependant absorption is a major factor that limits the bioavailability of numerous poorly water soluble drugs. The ability of SMEDDS to present the drug to GIT in solubilised and micro emulsified form (globule size between 1-100 nm) and

subsequent increase in specific surface area enable more efficient drug transport through the intestinal aqueous boundary layer and through the absorptive brush border membrane leading to improved bioavailability. E.g. In case of halofantrine approximately 6-8 fold increase in bioavailability of drug was reported in comparison to tablet formulation [19].

Ease of Manufacture and Scale-up

Ease of manufacture and scale- up is one of the most important advantages that make SMEDDS unique when compared to other drug delivery systems like solid dispersions, liposomes, nanoparticles, etc., dealing with improvement of bio-availability. SMEDDS require very simple and economical manufacturing facilities like simple mixer with agitator and volumetric liquid filling equipment for large-scale manufacturing. This explains the interest of industry in the SMEDDS.

Reduction in Inter-Subject and Intra-Subject Variability and Food Effects

There are several drugs which show large inter-subject and intra-subject variation in absorption leading to decreased performance of drug and patient non-compliance. Food is a major factor affecting the therapeutic performance of the drug in the body. SMEDDS are a boon for such drugs. Several research papers specifying that, the performance of SMEDDS is independent of food and, SMEDDS offer reproducibility of plasma profile are available [20].

Ability to Deliver Peptides that are Prone to Enzymatic Hydrolysis in GIT

One unique property that makes SMEDDS superior as compared to the other drug delivery systems is their ability to deliver macromolecules like peptides, hormones, enzyme substrates and inhibitors and their ability to offer protection from enzymatic hydrolysis. The intestinal hydrolysis of prodrug by cholinesterase can be protected if Polysorbate 20 is emulsifier in micro emulsion formulation [21]. These systems are formed spontaneously without aid of energy or heating thus suitable for thermo labile drugs such as peptides.

No Influence of Lipid Digestion Process

Unlike the other lipid-based drug delivery systems, the performance of SMEDDS is not influenced by the lipolysis, emulsification by the bile salts, action of pancreatic lipases and mixed micelle formation. SMEDDS are not necessarily digested before the drug is absorbed as they present the drug in micro-emulsified form which can easily penetrate the mucin and water unstirred layer.

Increased Drug Loading Capacity

SMEDDS also provide the advantage of increased drug loading capacity when compared with conventional lipid solution as the solubility of poorly water soluble drugs with intermediate partition coefficient are typically low in natural lipids and much greater in amphilic surfactants, co surfactants and co-solvents.

Disadvantages of SMEDDS

One of the obstacles for the development of SMEDDS and other lipid-based formulations is the lack of good predicative *in vitro* models for assessment of the formulations.

- Traditional dissolution methods do not work, because these formulations potentially are dependent on digestion prior to release of the drug.
- This in*vitro* model needs further development and validation before its strength can be evaluated.
- The drawbacks of this system include chemical instabilities of drugs and high surfactant concentrations in formulations (approximately 30-60%) which irritate GIT.
- Moreover, volatile co solvents in the conventional self-microemulsifying formulations are known to migrate into the shells of soft or hard gelatin capsules, resulting in the precipitation of the lipophilic drugs [22].

Factor of SMEDDS

Nature and dose of the Drug

Drugs which are administered at very high dose are not suitable for SMEDDS unless they exhibit extremely good solubility in at least one of the components of SMEDDS, preferably lipophilic phase. The drugs which exhibit limited solubility in water and lipids (typically with log P values of approximately 2) are most difficult to deliver by SMEDDS. The ability of SMEDDS to maintain the drug in solubilised form is greatly influenced by the solubility of the drug in oil phase. As mentioned above if surfactant or co-surfactant is contributing to

the greater extent in drug solubilization then there could be a risk of precipitation, as dilution of SMEDDS will lead to lowering of solvent capacity of the surfactant or co-surfactant. Equilibrium solubility measurements can be carried out to anticipate potential cases of precipitation in the gut. However, crystallization could be slow in the solubilising and colloidal stabilizing environment of the gut. Pouton's study reveal that such formulations can take up to five days to reach equilibrium and that the drug can remain in a super-saturated state for up to 24 hours after the initial emulsification event. It could thus be argued that such products are not likely to cause precipitation of the drug in the gut before the drug is absorbed, and indeed that super-saturation could actually enhance absorption by increasing the thermodynamic activity of the drug. There is a clear need for practical methods to predict the fate of drugs after the dispersion of lipid systems in the gastro-intestinal tract.

Polarity of the Lipophilic Phase

The polarity of the lipid phase is one of the factors that govern the drug release from the microemulsions. The polarity of the droplet is governed by the HLB, the chain length and degree of unsaturation of the fatty acid, the molecular weight of micronized for their propensity to inhibit crystallization and, thereby, generate and maintain the supersaturated state for prolonged time periods. A supersaturable drug delivery system (S-SMEDDS) of paclitaxel was developed selfmicroemulsifying employing HPMC as a precipitation inhibitor with a conventional SMEDDS formulation. *In-vitro* dilution of the S-SMEDDS formulation resulted in formation of a microemulsion, followed by slow crystallization of paclitaxel on standing. This result indicated that the system was supersaturated with respect to crystalline paclitaxel, and the supersaturated state was prolonged by HPMC in the formulation. In the absence of HPMC, the SMEDDS formulation underwent rapid precipitation, yielding a low paclitaxel solution concentration. A pharmacokinetic study showed that the paclitaxel SMEDDS formulation produced approximately a 10-fold higher maximum concentration (Cmax) and a 5-fold higher oral bioavailability (F~9.5%) compared with that of the orally administered Taxol formulation (F~ 2.0%) and the SMEDDS formulation without HPMC (F~1%). Applying the supersaturable SMEDDS approach, a reduced amount of surfactant can be used with HPMC in order to produce a temporarily supersaturated state with reduced solubilization. Thus a high free drug concentration would be obtained through generating and maintaining a supersaturated state in vivo and to increase the driving force for absorption. It is worth emphasizing that the significantly reduced amount of surfactant used in the S-SMEDDS formulation approach provides a better toxicity/safety profile than the conventional SMEDDS formulations. However, the underlying mechanism of the inhibited crystal growth and stabilized super saturation by means of these polymers is poorly understood even although several studies have been carried out to investigate this [23].

Biological Relevance of Solubility

In the oral route drugs must enter the systemic circulation to exert a therapeutic effect. Figure 1 illustrates the steps that a solid oral formulation passes through in order to get into the blood stream. First, the drug in its solid dosage form disintegrates. Then the solid drug particles dissolve within an aqueous environment (gastrointestinal tract) into drug molecules. The extent and rate at which drug molecules go into solution is determined by the drug solubility and dissolution rate, respectively. This is then followed by permeation of the drug molecules into the bloodstream through the intestinal membrane [24].

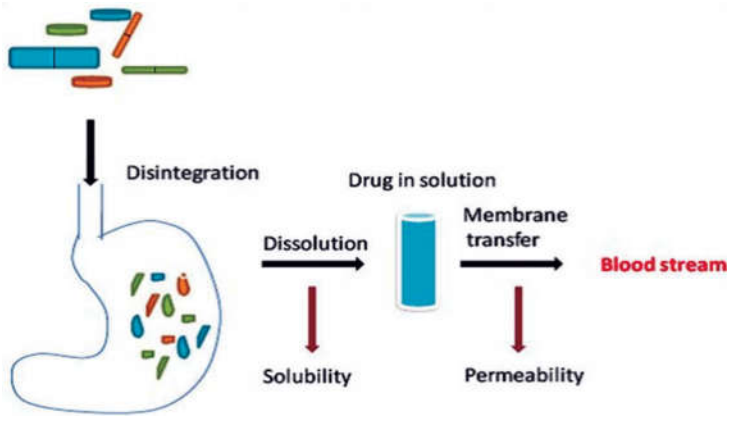


Fig. 2: Pathway of dosage form to reach blood stream

Two critical rate determining steps in the absorption of orally administered drugs are [25]

- Rate of dissolution
- Rate of drug permeation through biomembrane

Therapeutic effectiveness of a drug depends upon the bioavailability which is mostly dependent on the solubility of drug molecules [26]. Solubility behavior of drugs remains one of the most challenging aspect in formulation development. Because of their low aqueous solubility, up to 40% of new chemical entities fail to reach market despite exhibiting potential pharmacodynamic activities. Poorly aqueous soluble drugs are associated with slow drug absorption leading eventually to inadequate and variable bioavailability [27,28]. Oral absorption of a drug can be influenced by variety of factors, such as the physicochemical properties (e.g., pKa, solubility, stability, diffusivity, lipophilicity, polar-nonpolar surface area, presence of hydrogen bond functionalities, particle size and crystal form), physiological conditions (e.g., gastrointestinal pH, blood flow, gastric emptying, small intestinal transit time, colonic transit time and absorption mechanisms) and type of dosage form (e.g., tablet, capsule, solution, suspension and emulsion).

Biopharmaceutical Classification System (BCS)

The BCS system classifies immediate release solid oral dosage forms on the basis of solubility and permeability parameters. Fundamentally, the BCS is a scientific framework for classifying drug substances according to their aqueous solubility and their intestinal permeability. The BCS also takes account of the dissolution of the drug product and hence covers the three main factors which govern the rate and extent of drug absorption from immediate release (IR) solid oral dosage forms (e.g. tablets, capsules):

- Dissolution rate
- Solubility &
- Permeability

Table 1: Biopharmaceutical classification system for drugs

BCS class	Solubility	Permeability
Class I	High	High
Class II	Low	High
Class III	High	Low
Class IV	Low	Low

Table 2: Descriptive solubility profile [31]

Descriptive term	Parts of solvent required
Very soluble	Lessthan1
Freely soluble	From1to10
Soluble	From10to30
Sparingly soluble	From30to100
Slightly soluble	From100to1000
Very slightly soluble	From 1000to10000
Practically in soluble	10000 or more

Class I category drugs are defined as the drugs with the highest solubility and permeability, and therefore are readily absorbed when administered orally. The remaining classes II-IV suffer from poor solubility, permeability, or both and in turn affect the amount of absorption or bioavailability of the drug [29]. Solubility is a predetermined and rate limiting step for absorption, especially for class II drugs. According to Lipinksi, solubility is a much larger issue for drug discovery than permeability [30].

On the molecular level, solubility involves dissolution; the breaking of intermolecular attractions between solute-solute, solvent-solvent, and the formation of new interactions between solute-solvent [31]. It is these interactions, which are identified as ionic, van der Waals, and hydrogen bonding, which govern solubility. The first step is to free a solute molecule from its cavity. Next is to create a cavity in the solvent. Several factors play a role in determining the solubility of a compound. These include compound structure, pH, and temperature, physical state of the compound when placed in solution either solid or liquid, composition and physical conditions of solvent.

Solubility Measurements

Commonly measurements are taken by the traditional shake- flask method. Excess drug is added to solvent at desired temperature and shaken for 24 h, or for 7 days. The excess drug is removed from filtration, and the dissolved amount is detected by high pressure liquid chromatography or ultra-violet

spectroscopy or mass spectrometry detection [33].

Formulation Approaches to enhance solubility

There are different approaches available and reported in literature to enhance the solubility of poorly water soluble drugs [33]. The techniques are chosen on the basis of certain aspects such as properties of drug under consideration, nature of excipients to be selected and nature of intended dosage form.Approaches to enhance solubility are commonly based on chemical or physical modifications. Here different most exploited approaches for enhancement of solubility are illustrated below.

Physical Modification

- Particle size modification
- Micronization
- Nanosuspension

Modification of the Crystal Habit

- Polymorphs
- Pseudopolymorphs

Drug Dispersion in Carriers

- Eutectic mixtures
- Solid dispersions
- Solid solution
- Complexation
- Solubilization by surfactants
- Nanotechnology based approaches
- Chemical modification
- Formation of soluble prodrug
- Formation of salt of the compound
- Preparation of covalent drug conjugates

Dosage Forms from Self-Emulsifying System

Self-Emulsifying Capsule

It is a capsules containing liquid or semisolid form of self-emulsifying system. In the GIT, the capsules get dispersed to SES uniformly in the fluid to micron size, enhancing bioavailability. Second type of self-emulsifying capsule is solid SES filled into capsule.

Self-Emulsifying Tablets

S.nazzal et al developed self nanoemulsified tablet dosage form of Ubiquinone. The main objectives of this study were to study effect of formulation ingredients on the release rate of Ubiquinone and to evaluate an optimized self nanoemulsified tablets formulation. The first prepared self nanoemulsion system containing Ubiquinone was prepared as nanoemulsion, this nanoemulsion was adsorbed by granular materials and then compressed to form tablets. The optimized formulation of coenzyme Q10 self nanoemulsified tablet dissolution profile showed that 80 90% drug release took place in 45 minute [35].

Self-Emulsifying Beads

Self-emulsifying system can be formulated as a solid dosage form by using less excipient. Patil and Paradkar discovered that deposition of SES into microporous polystyrene beads was done by solvent evaporation. Porous polystyrene beads with complexinternal void structures were typically produced by co-polymerising styrene and divinyl benzene. It is inert and stable over a wide range of pH, temperature and humidity. Geometrical features, such as bead size and pore architecture of PPB, were found to govern the loading efficiency and in vitro drug release from SES loaded PPB [36].

Self-Emulsifying Microsphere

You et al. formulated solid SE sustained release microspheres using the quasi emulsion solvent diffusion method for the spherical crystallization technique. Zedoary turmeric oil release behavior could be controlled by the ratio of hydroxypropyl methylcellulose acetate succinate to Aerosil 200 in the formulation. The plasma concentration time profiles were achieved after oral administration of such microspheres into rabbits, with a bioavailability of 135.6% with respect to the conventional liquid SMEDDS [37].

Self-Emulsifying Nano Particle

Nanoparticle technology can be applied to the formulation of self-emulsifying nanoparticle. One of the solvent was injection; in this method the prepared molten lipid Mass contained lipid, surfactant and drug. This lipid molten mass was injected drop wise

into a non-solvent system. This is filtered and dried to get nanoparticles. By these method 100 nm size particles with 70 75% drug loading efficiency was obtained. Second technique is sonication emulsion diffusion evaporation; by this method co load 5 flurouracil and antisense EGFR plasmids into biodegradable PLGA/O CMC nanoparticles. The mixture of PLGA and O CMC had a SE effect; with no additional surfactant required. Trickler et al. developed a novel nanoparticle drug delivery system consisting of chitosan and glycerylmonooleatefor the delivery of paclitaxel. These chitosan/GMO nanoparticles, with bioadhesive properties increased cellular association and were prepared by multiple emulsion (o/w/o) solvent evaporation methods.

Application of Submicron Emulsion

Cosmetics

Submicron emulsion has recently become increasingly important as potential vehicles for the controlled delivery of cosmetics and for the optimized dispersion of active ingredients in particular skin layers. Due to their lipophilic interior they are more suitable for the transport of lipophilic compounds than liposomes. Similar to liposomes they support the skin penetration of active ingredients and thus increase their concentration in skin. Another advantage is the small sized droplet with its high surface area allowing effective transport of the active to skin.

New Jersey-Based TRI

K Industries and its parent company Kemira have launched a new nano-based gel aimed at enhancing the efficacy of a wide range of skin care products. Kemira Nano Gel is said to be a unique submicron emulsion carrier system that has been designed around easy formulation, combined with the added benefits brought about by its nanotechnology properties. Antimicrobial- Antimicrobial submicron emulsions are oil-in-water droplets that range from 200 to 600nm. They are composed of oil and water and are stabilized by surfactants and alcohol. The submicron emulsion has a broad-spectrum activity against bacteria, enveloped virus, fungi and spores. The submicron particles are thermodynamically driven to fuse with lipid containing organism. The fusion is enhanced by the electrostatic attraction between the cationic charge of emulsion and anionic charge on pathogen. When enough nanoparticles fuse with pathogens, they release part of energy trapped within emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lyses and death.

Bio-Terrorism Attack

Based on their antimicrobial activity, research has begun on use of submicron emulsion as a prophylactic medication, a human protective treatment, to protect people exposed to bio-attack pathogens such as anthrax and ebola.

Mucosal Vaccines

Submicron emulsions are being used to deliver either recombinant proteins or organisms to a mucosal surface to produce an immune response. The first application, an influenza vaccine and an HIV vaccine, can proceed to clinical trials. The submicron emulsion causes proteins applied to the mucosal surface to be adjuvant and it facilitates uptake by antigen-presenting cells.

Non-Toxic Disinfectant Cleaner

A breakthrough nontoxic disinfectant cleaner for use in commercial markets that include healthcare, hospitality, travel, food processing, and military applications has been developed by *invitro* systems, Inc. that kills tuberculosis and a wide spectrum of viruses, bacteria and fungi in 5-10 min without any of the hazards posed by other categories of disinfectants. The product needs no warning labels. It does not irritate eyes and can be absorbed through the skin, inhaled, or swallowed without harmful effects.

Cell Culture Technology

Cell cultures are used for *in vitro* assays or to produce biological compounds, such as antibiotic or recombinant proteins. To optimize cell growth, the culture medium can be supplemented with a number of defined molecules or with blood serum. Up to now, it has been very difficult to supplement the media with oil-soluble substance that are available to the cells, and only small amounts of these lipophilic compounds could be absorbed by the cells. Submicron emulsions are a new method for the delivery of oil-soluble substances to mammalian cell cultures. The delivery system is based on a nanoemulsion which is stabilized by phospholipids. These nanoemulsions are transparent and can be passed through 0.1 mm filters for sterilization. Nanoemulsion droplets are easily taken up by the cells. The encapsulated oil-soluble substances therefore have a high bioavailability to cells in culture. The advantage of using nanaoemulsions in cell culture technology are batter uptake of oil-soluble supplements in cell culture, improve growth and vitality of cultures cells, and allowance of toxicity

studies of oil-soluble drugs in cell cultures.

Cancer Therapy

The effects of the formulation and particle composition of gadolinium (Gd)-containing lipid NE (Gd-nanoLE) on the bio-distribution of Gd after its intravenous (IV) injection in D1-179 melanoma-bearing hamsters were evaluated for its application in cancer neutron-capture therapy. Bio-distribution data revealed that Brij 700 and HCO-60 prolonged the retention of Gd in the blood and enhanced its accumulation in tumors [38].

Material and Method

Table 4.1: Material and reagent used in present work

Materials	Background/Role
Cefdinir	API
Oleic acid	Oil
Castor oil	Oil
Cod liver oil	Oil
Sun flower oil	Oil
Soybean oil	Oil
Palm oil	Oil
Corn oil	Oil
Labrafac	Oil
Cremophore	Surfactant
Labrafil 1944	Surfactant
Span-20	Surfactant
Tween-80	Surfactant
Propylene glycol	Solvent
Poly Ethylene Glycol-400	Solvent
Transcutol	Solvent

Table 4.2: Instruments and apparatus used in current work

Sr. No.	Instruments/Apparatus	Company
1	Digital balance	Shimadzu
2	pH meter	Labtronics
3	Dissolution test apparatus	Electrolab dissolution test
4	UV-Visible Spectrophotometer	Labtronics
5	Brookfield viscometer	Brookfield viscometer
6	Sonicator	Ultrasonic bath

Spectrophotometric Estimation of Cefdinir

In the present study cefdinir will quantitatively analyze by UV-Visible spectrophotometer in dissolution fluid. Standard curve of Cefdinir will be generated in Methanol and 0.1N HCl.

Preparation of 0.1 N HCl

8.5ml of hydrochloric acid diluted with distilled water to produce 1000 ml.

Determination of ëmax of Cefdinir

Weigh accurately require amount of drug will dissolve in 0.1 N HCl. A stock solution will be prepared by withdrawing 10 ml of the above solution and made up to 100 ml. Make a serial dilution up to appropriate microgram. Then ëmax of Cefdinir will be measured by using UV spectroscopy.

Preparation of Standard Curve for Cefdinir in Methanol

Weigh accurately require amount of Cefdinir and transfer in 100 ml of volumetric flask and volume will be made up with methanol to the mark. Then standard curve of Cefdinir will be measured by using UV spectroscopy.

Preparation of Standard Curve for Cefdinir in 0.1 N HCl

Weigh accurately require amount of Cefdinir and transfer in 100 ml of volumetric flask and volume will be made up with 0.1N HCl to the mark. Then standard curve of Cefdinir will be measured by using UV spectroscopy.

Preliminary Study

Solubility Studies

Solubility studies will be conducted by placing an excess amount of cefdinir in a 2mL micro tube containing 1mL of the vehicle, and the mixture will be heated at 60^0C in a water bath to facilitate the solubilization using a vortex mixer. Mixtures will be equilibrated at 25^0C for 48h in a water bath. The

equilibrated samples will be centrifuged at 3000×*g* for 15min to remove the un dissolved cefdininr. The supernatant will be taken and diluted with methanol for quantification of Cefdinir by UV Spectro photometer [67].

Emulsification Study for Surfactant and Co-Surfactant

The surfactant and co-surfactant evaluated on the basis of their potential to emulsify the selected oil phase. In this study 10% solutions of different surfactant and co-surfactants will be prepared with vigorous vortex. If a uniform clear solution will be visually obtained, the addition of oil will be continued until the solution became cloudy and the total amount of oil added will be recorded [68].

Formulation of Self-Microemulsifying Drug Delivery System Containing Cefdinir

Construction of Pseudo-Ternary Phase Diagrams

Pseudo ternary phase diagrams of oil, surfactant/co-surfactant (S/Co-S), and water will be developed using water titration method. The mixtures of oil and S/Co-S at certain weight ratios will dilute with water in a drop wise manner. For each phase diagram at a specific ratio of S/Co-S (i.e. 1:1, 1:3 and 3:1 wt/wt), a transparent and homogenous mixture of oil and S/Co-S will be form by vortexing for 5 minutes. Then each mixture will be titrated with water and visually observed for phase clarity. The concentration of water at which turbidity-to-transparency and transparency-to-turbidity transitions occurred will be derived from weight measurements. These values will be used to determine boundaries of microemulsion domain corresponding to chosen value of oils, as well as S/Co-S mixing ratio. To determine effect of drug addition on micro emulsion boundary, phase diagrams will be also constructed in presence of drug using drug-enriched oil as hydrophobic component. Phase diagrams will be constructed using Tri plot v1-4 software.

Preparation of SMEDDS Formulations

A series of SMEDDS formulations will prepare using S/Co-S combination and selected oil by using Simplex Lattice Design. The actual concentrations of oil, surfactant and co-surfactant will be transformed based on the simplex lattice design so that minimum concentration corresponds to zero and maximum concentration corresponds to one. Briefly, accurately weighed cefdinir will place in a glass vial, and require quantity of oil, surfactant, and co-surfactant will add. Then all components will be mixed by gentle stirring and vortex mixing and will be warmed at 40°C on a magnetic stirrer, until cefdinir will be perfectly dissolved. The mixture will be store at room temperature until further use [69].

Formulation Optimization

Simplex lattice design will be used to optimize the formulation of SMEDDS containing cefdinir. The concentrations of oil (X1), surfactant (X2) and co-surfactant (X3) will be chosen as the independent variables. The emulsification time, % Transmittance and cumulative %release in 20 minute will be taken as responses (Y), respectively. The equation for simplex lattice model is described as follows:

$$Y=\beta_o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{123}X_1X_2X_3$$

Where *Y* is the dependent variable and *âi* is the estimated coefficient for the factor *Xi*. The major effects (*X1, X2,* and *X3*) represent average results of changing one factor at a time from its low to high value, the interactions *X1X2, X2X3, X1X3,* and *X1X2X3*, and polynomial terms show how the responses change when two or three factors change simultaneously.

According to simplex lattice design and the selected concentration ranges of oil, surfactant and co-surfactant, seven different formulations of SMEDDS containing cefdinir is constructed.

The responses for seven formulations will be used to fit an equation for simplex lattice model [70-71]. Which then can predict properties of all possible formulations. With the aid of Microsoft Excel, the model equation will develop to represent the relationship between the self-emulsification time, %transmittance and cumulative %release in 20 min the measured characteristics.

Characterization

Refractive Index and Turbid metric Evaluation

The Self-Micro emulsifying system (SMEDDS) will add to 0.1N hydrochloric acid (250 ml) and purified water (250 ml) under continuous stirring (50 rpm) on a magnetic plate at ambient temperature. Then Refractive index of system will be measured by using an Abbe's Refracto meter and turbidity will be measured by measuring % transmittance at 286.4 nm in UV-Visible spectrophotometer [67].

Measurement of Droplet Size and Zeta Potential

Droplet size distribution and zeta potential of

SMEDDS will be determined using Zetatrac. Zetatrac utilizes a high frequency AC electric field to oscillate the charged particles. The Brownian motion power spectrum is analyzed with Modulated Power Spectrum (MPS) technique, a component of power spectrum resulting from oscillating particles. Samples will be diluted to 250 ml with purified water. Diluted samples will be directly placed into cuvette and measure particle size and zeta potential. Zetatrac is controlled by Microtrac FLEX Operating Software.

Drug Content

Cefdinir from pre-weighed SMEDDS will be extracted by dissolving in 25 ml methanol. Then methanolic extract will be separated out and cefdinir content in methanolic extract will analyze spectrophotometrically (UV-Visible spectrophotometer) at 286.4 nm, against standard methanolic solution of cefdinir [72].

Measurement of Viscosity

Viscosity of cefdinir SMEDDS will be measured by using Brookfield viscometer at 25°C temperature. Spindle S-61 will be selected for measurement of viscosity of various SMEDDS formulation. Viscosity of SMEDDS will be measured at 30 rpm before dilution and after dilution with aqueous phase (250 ml) [73].

Measurement of PH

pH of cefdinir SMEDDS will be measured by using pH meter at room temperature. pH of SMEDDS will measure before dilution and after dilution with aqueous phase (250 ml) [73].

Self-Emulsification and Precipitation Assessment

Evaluation of the self-emulsifying properties of SMEDDS formulations will be performed by visual assessment as previously reported. In brief, different compositions will be categorized on speed of emulsification, clarity and apparent stability of resultant emulsion. Visual assessment will be performed by drop wise addition of pre-concentrate (SMEDDS) into 250 ml of distilled water. This will be done in a glass beaker at room temperature, and contents will gently stirr magnetically at 50-100 rpm. Precipitation will be evaluated by visual inspection of resultant emulsion after 24 hours. The formulations will be categorized as clear (transparent or transparent with bluish tinge), nonclear (turbid), stable (no precipitation at the end of 24 hours), or unstable (showing precipitation within 24 hours) [36].

In Vitrodiffusion Studies

In vitro diffusion studies were carried out for all formulations using dialysis technique. One end of pre-treated dialysis membrane tubing was with thread and then diluted SMEDDS was placed in it. The other end of tubing was also secured with thread and was allow to rotate freely in dissolution vessel of USP 24 type II dissolution test apparatus (Electrolab TDT-06P, India) that contained 250 ml dialyzing medium maintained at 37 ± 0.5 °C and stirred at 50 rpm. Aliquots were collected periodically and replaced with fresh dissolution medium. Aliquots, after filtration through whatman filter paper (No. 41), were analyzed spectrophotometrically at 286.4 nm for Cefdinir content. The data was analyzedusing the software.

Accelerated Stability Tests: Centrifugation and Freeze–Thaw Cycle

Cefdinir SMEDDS were diluted with 250 ml and 900 ml aqueous phase (distilled water and 0.1 N HCL) and centrifuged at 5000 rpm for 30 min. In addition, it was subjected to freeze–thaw cycle by storing it at -20°C for 24 hour and then for another 24 hourat 40°C. Micro emulsions were observed visually for phase separation and drug precipitation.

Result and Discussion

Spectrophotometric Estimation of Cefdinir

In the present study Cefdinir has been quantitatively analysed by UV-Visible spectrophotometer in dissolution fluid. Standard curve Cefdinir has been generated in methanol and 0.1N HClandphosphate buffer (pH 6.8).

Determination of ëmax of Cefdinir

100 µg/ml solution of Cefdinir in methanol was scanned in UV range of 200 to 400nm. Cefdinir showed maximum absorbance at 286.4 nm. Thus 286.4nm was taken as ëmax. Similar procedure was adopted with 0.1N HClandëmax in 0.1N HCl was also found out to be 286.4 nm. Figure 5.1, 5.2 and 5.3 shows absorbance spectra of Cefdinir in methanol, 0.1 N HCl and phosphate buffer (pH 6.8) respectively.

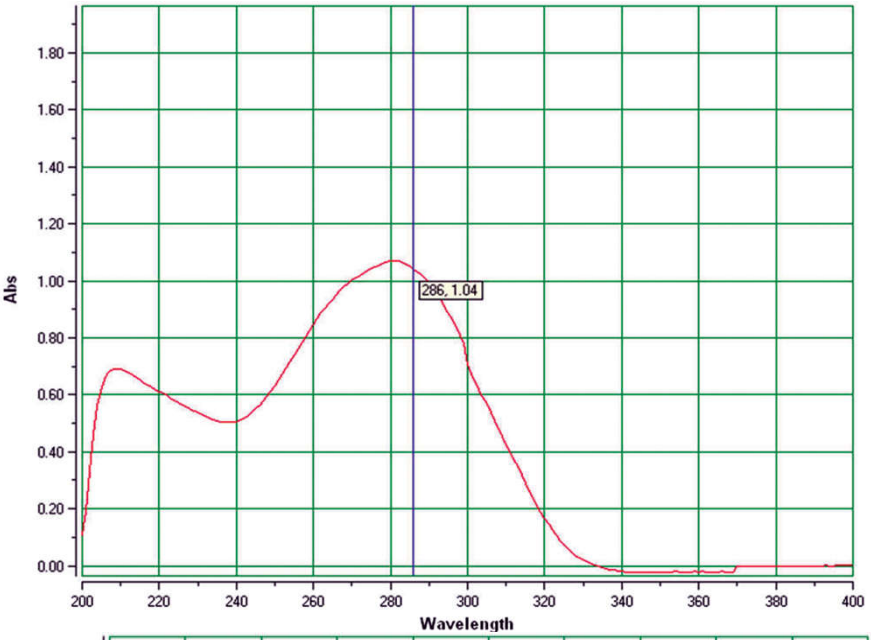


Fig. 5.1: UV spectrum of cefdinir in 0.1 N HCl

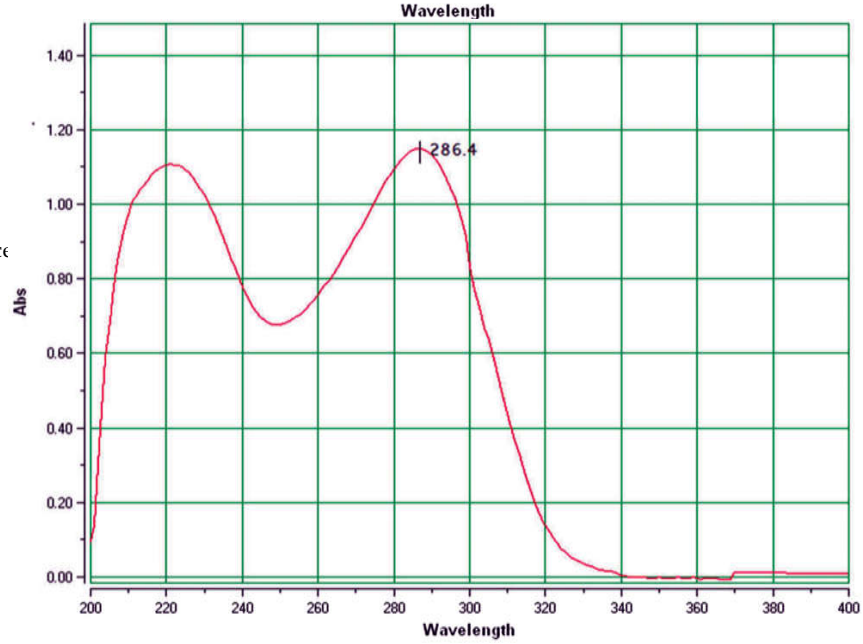


Fig. 5.2: UV spectrum of ce in methanol)

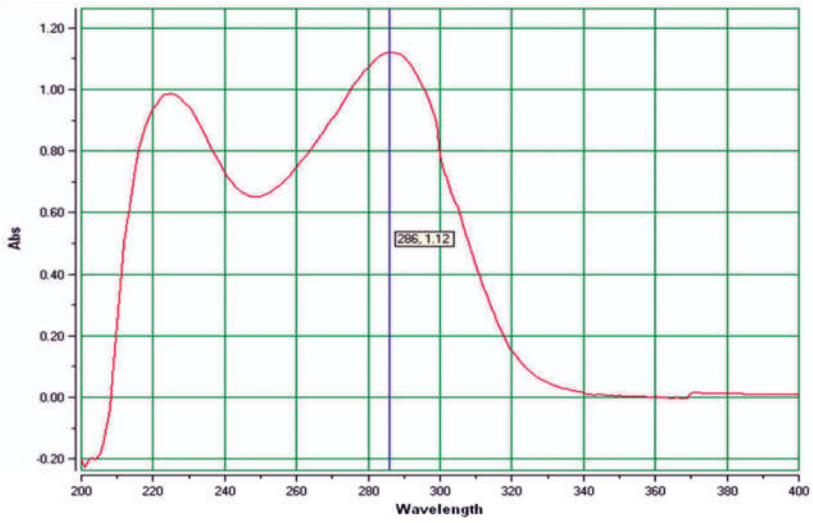


Fig. 5.3: UV spectrum of cefdinir in phosphate buffer (pH 6.8)

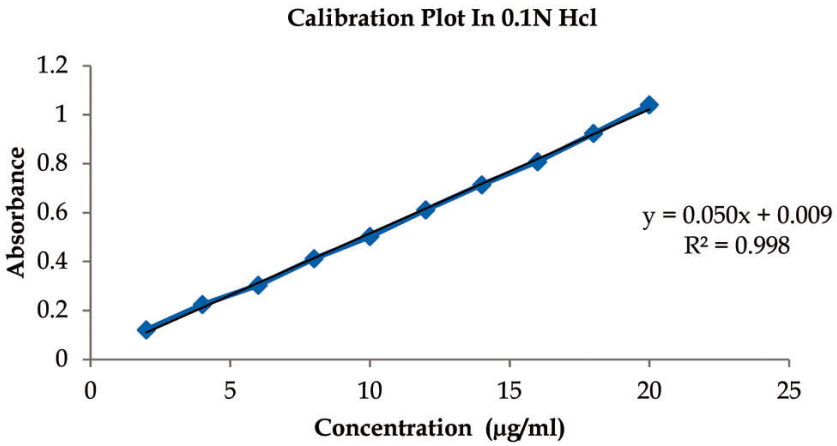


Fig. 5.4: Standard curve of cefdinir in 0.1 N HCl)

Table 5.1: Standard curve data of cefdinir in 0.1 N HCl)

Sr. no	Concentration (µg/ml)	Absorbance I	II	III	Average absorbance	Standard deviation
1	0	0	0	0	0	0
2	2	0.134	0.121	0.119	0.12466667	0.008144528
3	4	0.263	0.224	0.22	0.23566667	0.023755701
4	6	0.336	0.303	0.313	0.31733333	0.016921387
5	8	0.403	0.412	0.424	0.413	0.010535654
6	10	0.54	0.502	0.522	0.52133333	0.01900877
7	12	0.624	0.611	0.591	0.60866667	0.016623277
8	14	0.751	0.714	0.699	0.72133333	0.026764404
9	16	0.857	0.808	0.855	0.84	0.027730849
10	18	0.949	0.923	0.949	0.94033333	0.015011107
11	20	1.057	1.04	1.053	1.05	0.008888194

Preparation of standard curve for cefdinir in 0.1 N HCl

Weigh accurately 10 mg ofCefdinir and transfer in 100 ml of volumetric flask and volume was made up to the mark with 0.1 N HCl. Aliquots were taken from prepared stock solution and were appropriately diluted to prepare 2, 4, 6, 20 µg/ml and then absorbance were taken at 286.4 nm, keeping 0.1N HCl as blank solution. Data and Figure of standard curve were shown in Table 5.1 and Figure 5.4 respectively.

Results of Weighted Linear Regression Analysis

R square 0.998

R square Y=0.050x + 0.009

Linearity was observed between 0-10 µg/ml concentrations of Cefdinir, therefore the drug obeys beer's law.

Preparation of Standard Curve for Cefdinir in Methanol

Weigh accurately 10 mg of Cefdinir and transfer in 100 ml of volumetric flask and volume was made up to the mark with methanol. Aliquots were taken from prepared stock solution and were appropriately diluted to prepare 2, 4, 6, 20 µg/ml and thenabsorbance were taken at 286.4 nm, keeping methanol as blank solution. Data and Figure of standard curve were shown in Table 5.2 and Figure 5.5 respectively.

Results of Weighted Linear Regression Analysis

R square 0.997

R square Y=0.05x + 0.105

Linearity was observed between 0 -10 µg/ml concentrations of Cefdinir, therefore the drug obeys beer's law.

Preparation of Standard Curve for Cefdinir in Phosphate buffer (pH 6.8)

Weigh accurately 10 mg ofCefdinir and transfer in 100 ml of volumetric flask and volume was made up to the mark with phosphate buffer(pH 6.8). Aliquots were taken from prepared stock solution and were appropriately diluted to prepare 2, 4, 6, 20 µg/ml and then, absorbance were taken at 286.4 nm, keeping phosphate buffer as blank solution. Data

Table 5.2: Standard curve data of cefdinir in methanol

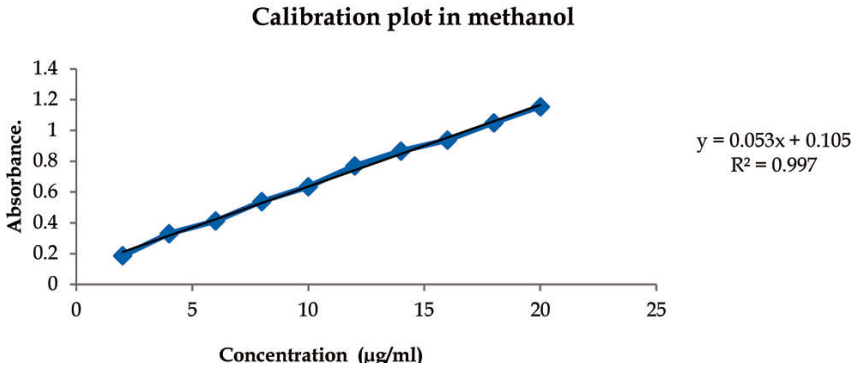


Fig. 5.5: Standard curve of cefdinir in methanol

Table 5.2: Standard curve data of cefdinir in methanol

Sr. no	Concentration (µg/ml)	Absorbance 1	2	3	Average absorbance	Standard deviation
1	0	0	0	0	0	0
2	2	0.187	0.155	0.17	0.17066667	0.016010413
3	4	0.33	0.3	0.302	0.31066667	0.016772994
4	6	0.412	0.44	0.414	0.422	0.015620499
5	8	0.539	0.512	0.53	0.527	0.013747727
6	10	0.635	0.633	0.63	0.63266667	0.002516611
7	12	0.77	0.733	0.755	0.75266667	0.018610033
8	14	0.867	0.855	0.868	0.86333333	0.007234178
9	16	0.938	0.955	0.944	0.94566667	0.008621678
10	18	1.05	1.02	1.03	1.03333333	0.015275252
11	20	1.154	1.125	1.13	1.13633333	0.015502688

Table 5.3: Standard curve data of cefdinir in phosphate buffer

Sr. no	Concentration (µg/ml)	Absorbance I	II	III	Average absorbance	Standard deviation
1	0	0	0	0	0	0
2	2	0.166	0.153	0.123	0.14733333	0.022052967
3	4	0.274	0.25	0.221	0.24833333	0.026539279
4	6	0.369	0.36	0.311	0.34666667	0.031214313
5	8	0.413	0.452	0.415	0.42666667	0.021962089
6	10	0.532	0.552	0.501	0.52833333	0.025696952
7	12	0.634	0.649	0.613	0.632	0.018083141
8	14	0.701	0.761	0.72	0.72733333	0.030664855
9	16	0.839	0.856	0.813	0.836	0.021656408
10	18	0.955	0.923	0.922	0.93333333	0.018770544
11	20	1.128	1.01	1.05	1.06266667	0.06001111

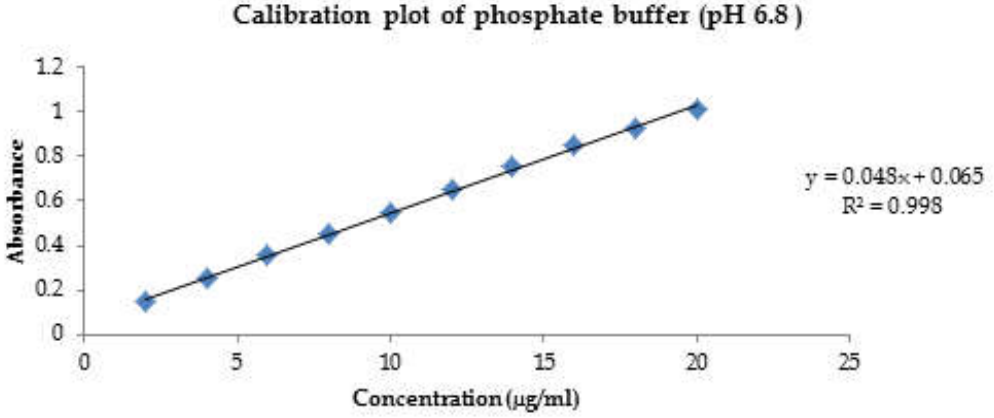


Fig. 5.6: Standard curve of cefdinir in phosphate buffer pH 6.8)

and Figure of standard curve were shown in Table 5.3 and Figure 5.6 respectively.

Results of Weighted Linear Regression Analysis

R square 0.998

R square Y = 0.048x + 0.065

Linearity was observed between 0 -10 µg/ml concentrations of Cefdinir, therefore the drug obeys beer's law.

Preliminary Study

Selection Cefdinir SMEDDS Components

Solubility Study

Screening of SMEDDS formulation involves formulation composition should be simple, safe, non-toxic and compatible. It should possess good solubility and large efficient self-micro emulsification region which should be found in pseudo-ternary

Table 5.4: Solubility of cefdinir in various oils, surfactants and co-surfactants

All Excipiants solubility data			
Sr. no.	**Name of Excipiants**	**Type of Excipiants**	**Solubility (mg/ml)***
1	soya been oil	Oil	0.568 ± 0.5685
2	palm oil	Oil	0.0835 ± 0.0473
3	castor oil	Oil	0.431 ± 0.3146
4	cod liver oil	Oil	0.236 ± 0.3949
5	oleic oil	Oil	0.542 ± 0.5388
6	sunflower oil	Oil	0.166 ± 0.0449
7	Olive oil	Oil	0.0331 ± 0.0057
8	Labrafac	Oil	1.087 ± 0.159
9	Tween-20	Surfactant	0.8528 ± 0.4075
10	Tween-80	Surfactant	0.68 ± 0.5600
11	Cremophore RH 40	Surfactant	0.71 ± 0.014
12	Labrafil M 1944	Surfactant	0.721 ± 0.007
13	Span-80	Surfactant	0.8065 ± 0.811
14	PEG	Co-Surfactant	1.4495 ± 0.5551
15	PEG-400	Co-Surfactant	2.65 ± 1.801
16	Transcutol	Co-Surfactant	0.547 ± 0.057

Note= *Data presented as a mean value ± standard error, n = 2
Abbreviation = PEG (Poly ethylene glycol)

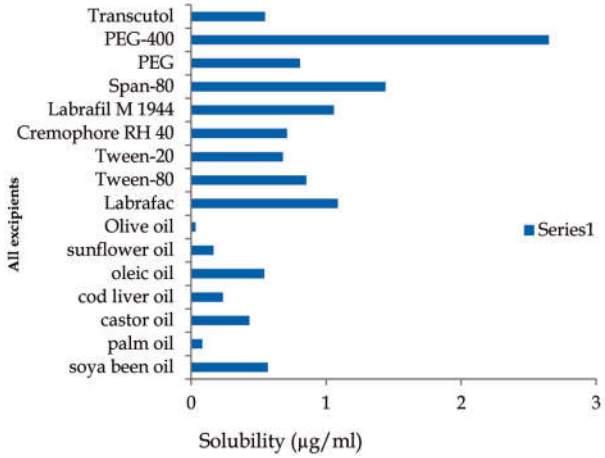


Fig. 5.7: Solubility studies of cefdinir in various oils, surfactants and co-surfactants data expressed as mean ± SD (*n*= 2)

phase diagram, and have efficient droplet size after forming micro emulsion.

Vehicles should have good solubilizing capacity of drug substance, which is essential for composing SMEDDS. The results of solubility of Cefdinir in various vehicles were shown in Table 5.3 and Figure 5.4. Cefdinir had highest solubility in labrafac, tween-80 and polyethylene glycol-400 as liquid vehicle, surfactant and co-surfactant respectively with comparison to others. So, labrafac as oil, tween-80 as surfactant and polyethylene glycol-400 as co-surfactant was selected for optimal SMEDDS formulation resulting in improved drug loading capabilities. Furthermore, with respect to its safety, labrafac, tween-80 and PEG-400 are included in the FDA Inactive Ingredients Guide.

Emulsification Study

The surfactant chosen must be able to lower the interfacial tension to a very small value to aid the dispersion process during the preparation of SMEDDS that can provide the correct curvature at interfacial region for SMEDDS.

Emulsification study results were shown that tween 80 has highest solubility capacity of oil was higher than other surfactant. So, tween 80 was selected as surfactant then selection of co-surfactant this study performed again. From the result were shown that PEG 400 has highest solubility capacity of oil. There tween 80 and PEG 400 were selected as surfactant and co-surfactant respectively.

Table 5.5: Emulsification study

Surfactants*	mL of oil**	Co-Surfactants*	mL of oil**
Tween 80	0.68 ± 0.5600	PEG	1.4495 ± 0.5551
Tween 20	0.8528 ± .4075	PEG-400	2.65 ± 1.801

*10 %v/v surfactant aqueous solution, **mL of labrafac oil

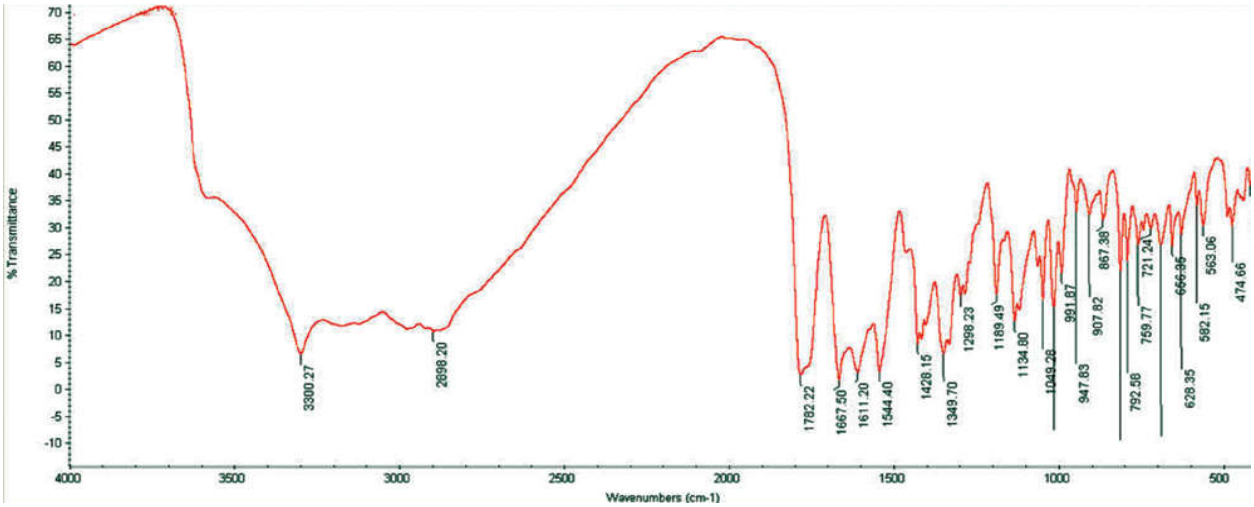


Fig. 5.8: FTIR spectra of cefdinir

FT-IR spectra of Cefdinir figure 5.8 exhibited principal peaks at 3300.27 cm-1 (= CH- H-), 2898.20 cm-1 (C-H stretching), and 1782.22 cm-1 presence of ester group.

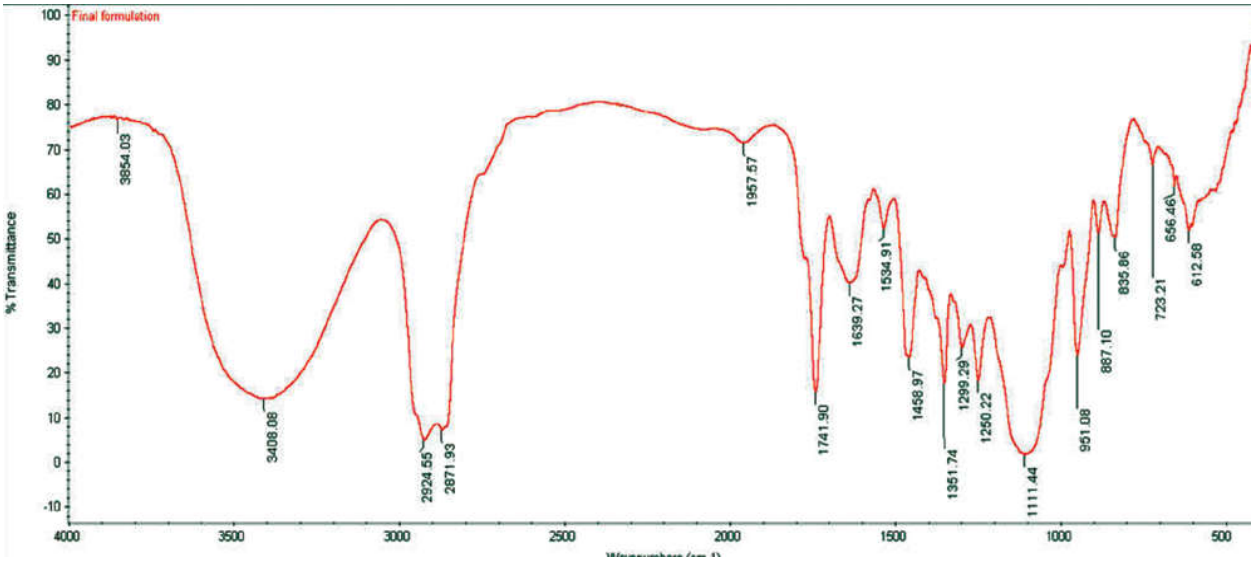


Fig. 5.9: FTIR spectra of SMEDDS formulation

FTIR spectra of SMEDDS formulation (figure 5.7) exhibited principal peaks at 3408.08 cm-1 (-OH{broad peak}), 2924.55 cm-1 (C-H streaching), and 1741.90 cm-1 presence of ester group

Fourier Transforms Infrared Spectroscopy (FT-IR) Studies

FT-IR study was carried out to determine possible drug interaction with excipients whichutilised in the formation of Cefdinir SMEDDS.

All these peaks clearly indicate that they are very much closely similar to the peaks of pure drug.

Formulation of Self-Micro emulsifying Drug Delivery System (SMEDDS)

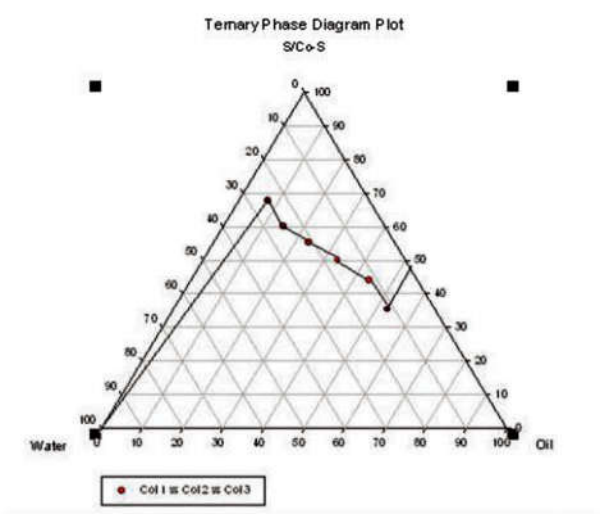
Containing Cefdinir

Pseudo-Ternary Phase Diagrams

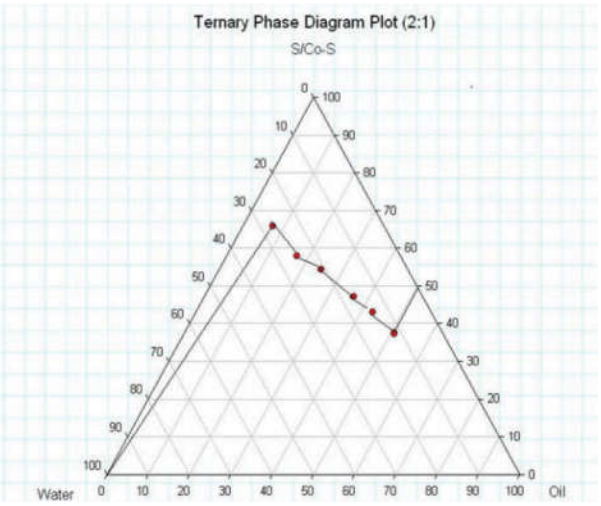
Phase diagrams were constructed to obtain optimum concentrations of oil, water, surfactant, and co-surfactant. SMEDDS form fine oil–water emulsions with only gentle agitation, upon its introduction into aqueous media.

Phase behavior investigations of this system demonstrated suitable approach to determining water phase, oil phase, surfactant concentration, and co-surfactant concentration with which transparent, one phase low-viscous micro emulsion system was formed.

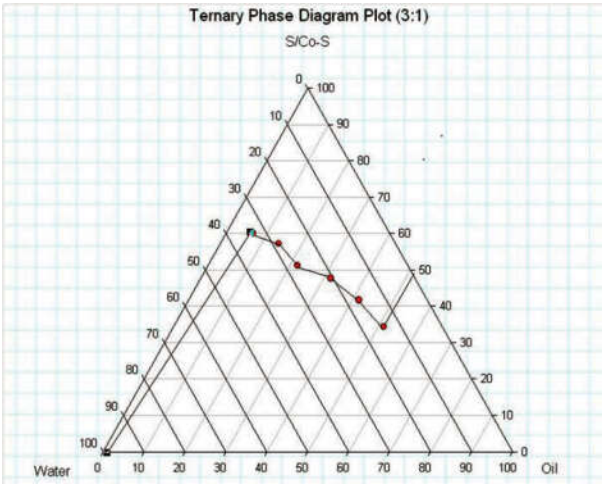
Since free energy required to form an emulsion is very low, formation is thermodynamically spontaneous. Surfactants form a layer around emulsion droplets and reduce interfacial energy as well as providing a mechanical barrier to coalescence. The visual test is measured apparent spontaneity of emulsion formation.



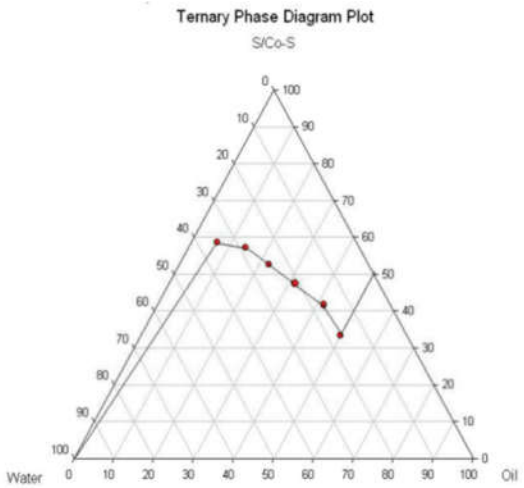
(a) S/Cos ratio 3:1



(b) S/Cos ratio 2:1



(c) S/Cos ratio 3:1



(d) S/Cos ratio 4:1

Fig. 5.10: Pseudo-Ternary Phase Diagrams (a) S/Cos ratio 1:1 (b) S/Cosratio 1:3 (c) S/Cos ratio 3:1 (d) S/Cos ratio 4:1)

The series of SMEDDS were prepared and their self-micro emulsifying properties were observed visually. Pseudo-ternary phase diagrams were constructed to identify the self-micro emulsifying regions and to optimize concentration of oil (Figure 5.10).

It was observed that increasing concentration of surfactant such as tween-80 in SMEDDS formulation increased spontaneity of self- emulsification region. Therefore, much higher concentration of surfactant, much higher self-emulsifying region in phase diagrams. The ratio of surfactant to co-surfactant was very effective to a stable and efficient SMEDDS formation. The phase diagrams were constructed at ratio of surfactant/co-surfactant 1:1, 2:1, 3:1, 4:1 (w/w). However, stability of self-emulsifying droplets from ratio of S/Co-S = 1:1, 2:1, 3:1 (w/w) was decreased because of precipitation after a few hours. So, ratio of S/Co-S = 4:1 was chosen in formulation. Figure shows phase diagrams which identify area of stable micro emulsion in presence of Cefdinir when diluted with aqueous media.

However, excessive amount of co-surfactant will cause system to become less stability for its intrinsic high aqueous solubility and lead to droplet size increasing as a result of expanding interfacial film. Hence, optimal ratio of surfactant to co-surfactant was selected to be 4:1.

Preparation of SMEDDS Formulations

A series of SMEDDS formulations were prepared using Tween 20 and PEG 400 as S/Co-S combination and labrafac as oil by using Simplex Lattice Design. The actual concentrations of oil, surfactant and co-surfactant were transformed based on the simplex lattice design so that minimum concentration corresponds to zero and maximum concentration corresponds to one (Shown in Table 5.6). Briefly, accurately

weighedCefdinir was placed in a glass vial, and require quantity of oil, surfactant, and co-surfactant was added. Then all components were mixed by gentle stirring and vortexmixing and were warmed at 40°C on a magnetic stirrer, until Cefdinir was perfectlydissolved. The mixture was store at room temperature until further use.

Table 5.6: Developed formulations with their actual and transformed value as per simplex lattice design

Formulation code	Dose of Cefdinir	Components (in ml)		
		Labrafac	Tween-80	PEG 400
F-1	10 mg	0.1	0.65	0.25
F-2	10 mg	0.2	0.60	0.20
F-3	10 mg	0.3	0.55	0.15
F-4	10 mg	0.4	0.50	0.10
F-5	10 mg	0.5	0.45	0.05
F-6	10 mg	0.6	0.30	0.10
F-7	10 mg	0.7	0.20	0.10

Formulation Optimization

Simplex lattice design was used to optimize the formulation of SMEDDS containing Cefdinir. The concentrations of oil (X1), surfactant (X2) and co-surfactant (X3) were chosen as the independent variables. The emulsification time and Cumulative %release in 20 minute was taken as responses (Y), respectively. The equation for simplex lattice model is described as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{23}X_1X_3 + b_{123}X_1X_2X_3 \quad (1)$$

Where *Y* is the dependent variable and βi is the estimated coefficient for the factor *Xi*. The major effects (*X1, X2,* and *X3*) represent average results of changing one factor at a time from its low to high value, the interactions *X1X2, X2X3, X1X3,* and *X1X2X3*, a polynomial term.

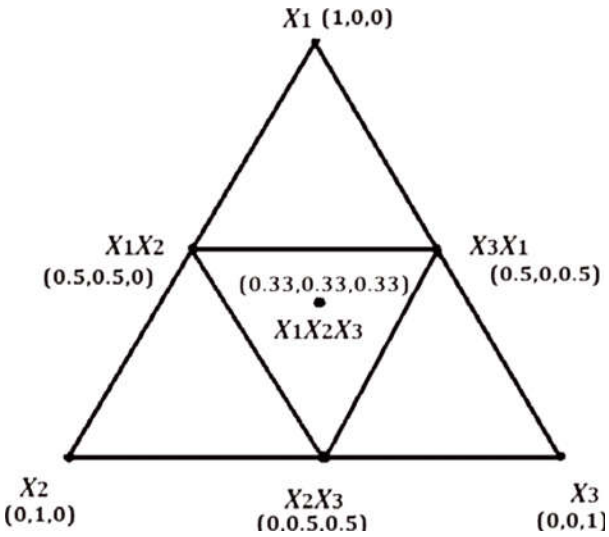


Fig. 5.11: Equilateral triangle representing simplex lattice design for three components

Table 5.8: Code representation of formulation by simplex lattice design

Formulation code	Code Representation	Concentration (Transformed value)		
		Oil	Surfactant	Co- Surfactant
F-1	X1	1	0	0
F-2	X2	0	1	0
F-3	X3	0	0	1
F-4	X1X2	0.5	0.5	0
F-5	X2X3	0	0.5	0.5
F-6	X1X3	0.5	0	0.5
F-7	X1X2X3	0.33	0.33	0.33

Table 5.9: Emulsification time (*Y1*) and cumulative percent release in 20 minute (*Y2*) of seven different formulations as per simplex lattice design

Formulation code	formulation component			Emulsification time sec (Y1)	Cumulative Percent release in 20(Y2)
	Oil	Surfactant	Co- Surfactant		
F-1	1	0	0	39	24.12
F-2	0	1	0	25	30.24
F-3	0	0	1	47	10.15
F-4	0.5	0.5	0	50	28.15
F-5	0	0.5	0.5	32	9.16
F-6	0.5	0	0.5	36	10.15
F-7	0.33	0.33	0.33	49	19.21

According to simplex lattice design and the selected concentration ranges of oil, surfactant and co-surfactant, seven different formulations of SMEDDS containing Cefdinir was constructed. The results of their emulsification time, % Transmittance and cumulative % release in 20 minute were given in Table 5.9.

With the help of Microsoft Excel, the fitted results are shown in Equations (2), (3):

$$Y_1 = 39X_1 + 25X_2 + 47X_3 + 72X_1X_2 + 8X_2X_3 - 28X_1X_3 + 168\ X_1X_2X_3 \quad (2)$$

$$Y_2 = 24.12X_1 + 30.24X_2 + 10.15X_3 + 3.92X_1X_2 - 44.76X_2X_3 - 27.94X_1X_3 + 142.56X_1X_2X_3 \quad (3)$$

Equations (2) and (3) can be used to calculate the predicted values for other formulations in the design space. Equation 2 showed β2 < β1 that means X1 has highest effect on emulsification time. Additionally β12 and β23 had positive value which showed synergistic effect of emulsification time. Whereas, β13 had positive value which showed synergistic effect of emulsification time. Equation-3 showed β1<β2 that means X2 has highest effect on amount of Cefdinir release. Additionally β12 had positive value which showed synergistic effect of Cefdinir release in 20 minutes. β23and β13had antagonistic effect on Cefdinir release because â23 and â13had negative value. The chosen concentrations of surfactant, co-surfactant and oil were introduced into above Equations (2) and (3).

A ternary contour plot can be used to examine relations between four dimensions where three of those dimensions represent components of a mixture (i.e., the relations between them is constrained such that values of three variables add up to the same constant). One typical application of this graph is when the measured responses from an experiment depends on relative proportions of three components (Oil, Surfactant and Co-surfactant) that are varied in order to determine an optimal combination of those components.

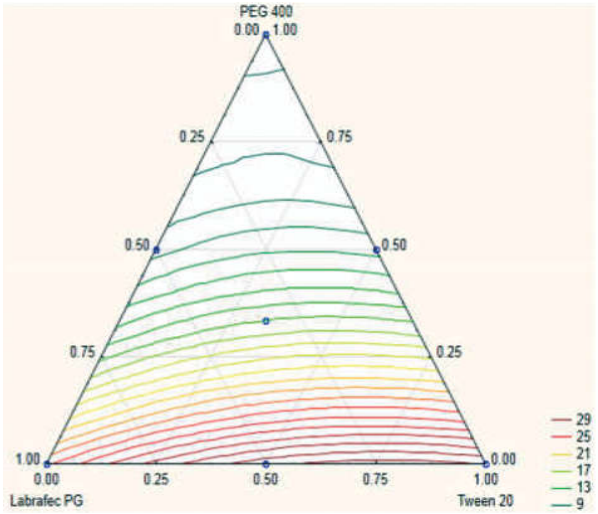
Graphics of Emulsification time and Cumulative % release in 20 minute were constructed in form of Ternary contour plots (Stastetica 12.o version), and optimized formulation was chosen by superimposing ternary contour plots of three responses, which were shown in Figure 5.7. combination of responses in one desirability requires the calculation of individual functions. A suitable SMEDDS formulation should have a minimum self emulsificationtime,maximum time required to 20% of drug release. The individual desirability for each response was calculated and batch F2 showed the highest overall desirability therefore this batch considered to be the best batch.

As shown in Table 5.8 and Figure 5.10, as the emulsification time of SMEDDS formulation was decreases,Cumulative %release increase. In order to obtain low emulsification time and high Cumulative %release, the appropriate ratio of components was chosen for optimized formulation, which consisting of oil, surfactant and co-surfactant.

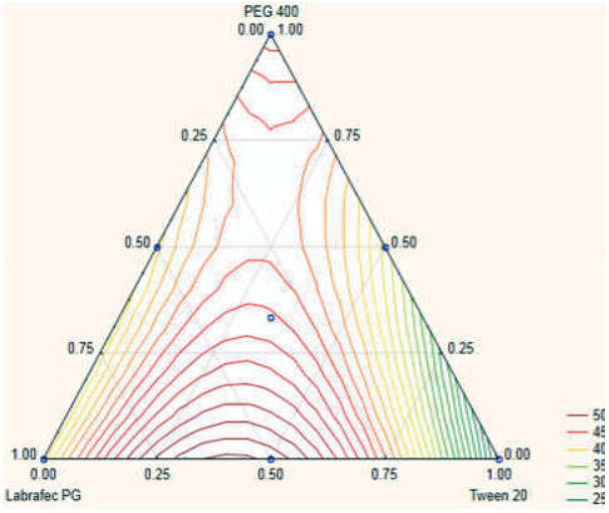
Characterization

Refractive Index and Turbidimetric Evaluation

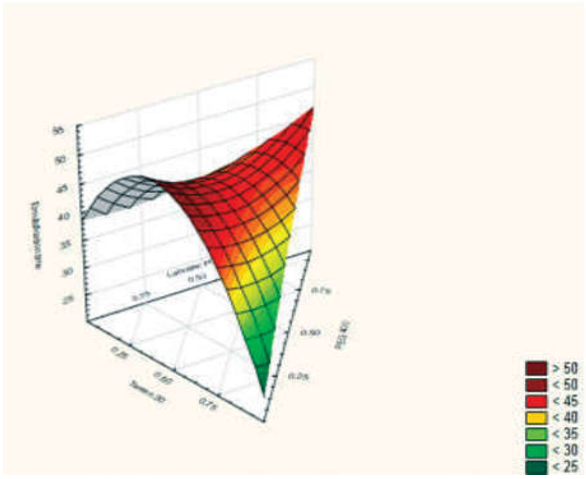
The refractive index and % transmittance of various formulations were shown in Table 5.10.



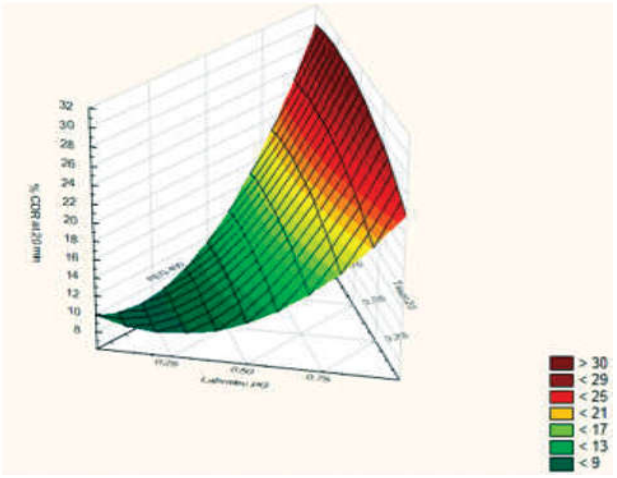
(a) Ternary Contour plot for % CD



(B) Contour Plot for Emulsification time



(c) Surface plot for emulsification time



(d) Surface plot for % CDR

(e) Overlay between % CDR and Emulsification Time

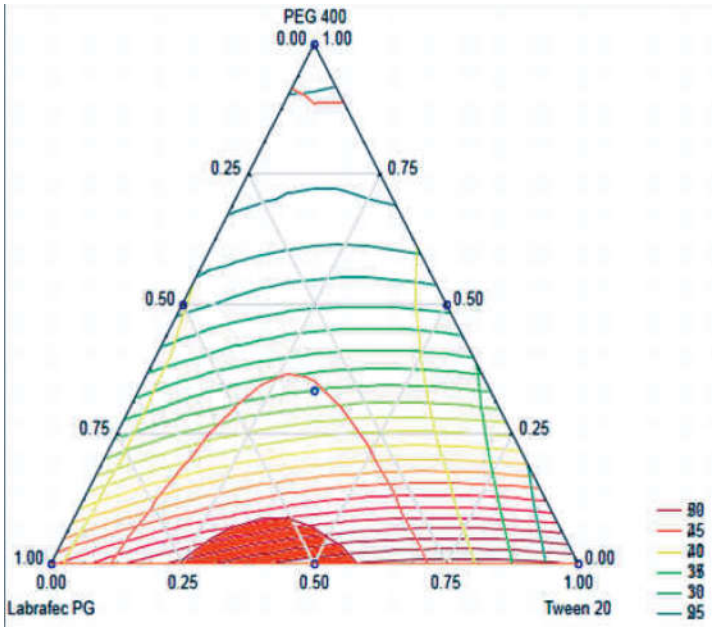


Fig. 5.12: Ternary contour plots (a) Ternary Contour plot for % CDR (B) Contour Plot for Emulsification time (c) Surface plot for emulsification time (d) Surface plot for % CDR

Optimized formulation (F-2) had refractive index similar to refractive index of water (1.333) and percent transmittance >97%. The refractive index and percent transmittance data prove that transparency of system.

Measurement of Droplet Size and Zeta Potential

Droplet size distribution following self-micro emulsification is a critical factor toevaluate self-micro emulsion system. Droplet size is thought to have an

Table 5.10: Refractive index and %transmittance of various SMEDDS formulations

Formulation code	Refractive Index		% Transmittance	
	0.1 N HCL (250 ml)	Water (250 ml)	0.1 N HCL (250 ml)	Water (250 ml)
F-1	1.492	1.516	80.23	85.23
F-2	1.246	1.335	97.35	99.26
F-3	1.43	1.405	80.22	87.56
F-4	1.116	1.126	70.86	76.75
F-5	1.22	1.293	60.95	68.95
F-6	1.12	1.22	58.75	59.73
F-7	1.1	1.14	50.34	55.56

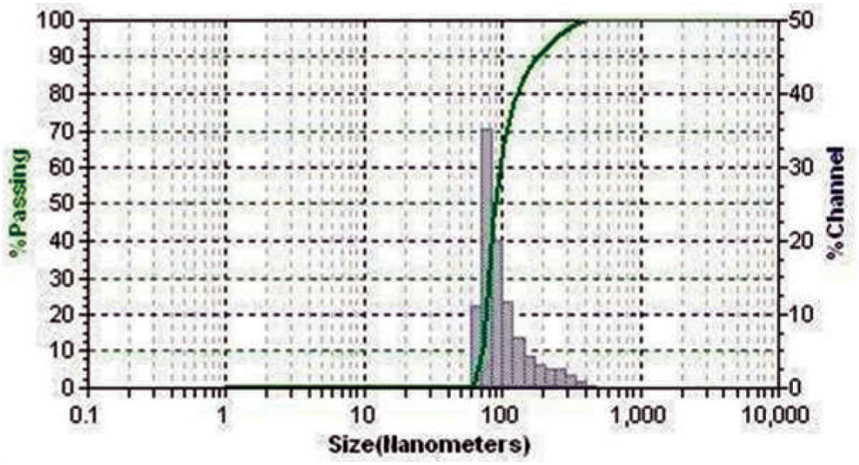


Fig. 5.13: Droplet size analysis of SMEDDS formulation F-2

effect on drug absorption as has been illustrated in several papers. The smaller droplets have largerinterfacial surface area will be provided for drug absorption.

The optimized formulation (F-2) have droplet size 87.60 nm (% passing is 50percent).

Droplet size analysis graph were shown in Figure-5.13.

Cefdinir SMEDDS (F-2) was diluted with distil water, and resulted zeta potential was 24.12mV. Several studies have reported that the zeta potential played an important role The charge of oil droplets of SMEDDS is another property that should be assessed for increased absorption. The charge of oil droplets in SMEDDS was negative due to presence of free fatty acid, the zeta potential of optimized formulation was 24.12 mV. In general the zeta potential value of ±30mV is sufficient for the stability of microemulsion.In our formulation, it is -30.92 which means complies with requirement of zetapotential for stability [73].

Drug Content

Drug content of SMEDDS formulation can be found by methanolicextractof SMEDDS wasanalysed spectrophotometrically (UV-Visible Spectrophotometer, Shimadzu) at 286.4 nm, against standard methanolic solution of Cefdinir. Drug content of various for mulations shown in Table 5.11.

Measurement of Viscosity and pH of Cefdinir SMEDDS

Viscosity of Cefdinir SMEDDS was measured by using Brookfield viscometer at 25°C temperature. Spindle S-61 was selected for measurement of viscosity of various SMEDDS formulation. Viscosity measurement was done at 30 rpm before and after dilution with water. PH of Cefdinir SMEDDS formulation was measured by using pH meter at room temperature. pH of SMEDDS formulations were also measured before and after dilution with 0.1 N HCl. Viscosity and pH data of SMEDDS formulation was shown in Table 5.12.

Table 5.11: Drug content in various SMEDDS formulations

Formulation code	Drug Content		Average
	I	II	
F-1	84.86	82.81	83.835
F-2	90.92	91.33	91.125
F-3	80.12	81.33	80.725
F-4	78.22	79.76	78.99
F-5	82.12	82.89	82.505
F-6	83.66	82.98	83.32
F-7	76.76	75.7	76.23

Table 5.12: Viscosity and pH of various SMEDDS formulations

	Dilution	Formulation code						
		F-1	F-2	F-3	F-4	F-5	F-6	F-7
Viscosity	Before	85.6	152	97.3	89	87	82.2	73.2
	After	1.35	1.88	1.78	1.35	1.25	1.22	1.12
pH	Before	7.831	7.5	7.2	7.01	7.21	7.05	7.03
	After	1.2	1.32	1.22	1.2	1.28	1.25	1.25

Table 5.13: Self-emulsification and precipitation of various SMEDDS formulations

Formulation code	Dispersion Time(Second)	Clarity	Precipitation
F-1	34	clear	Stable
F-2	28	clear	Stable
F-3	30	clear	Stable
F-4	33	Non clear	Stable
F-5	35	Non clear	Stable
F-6	40	Non clear	Stable
F-7	42	Non clear	Stable

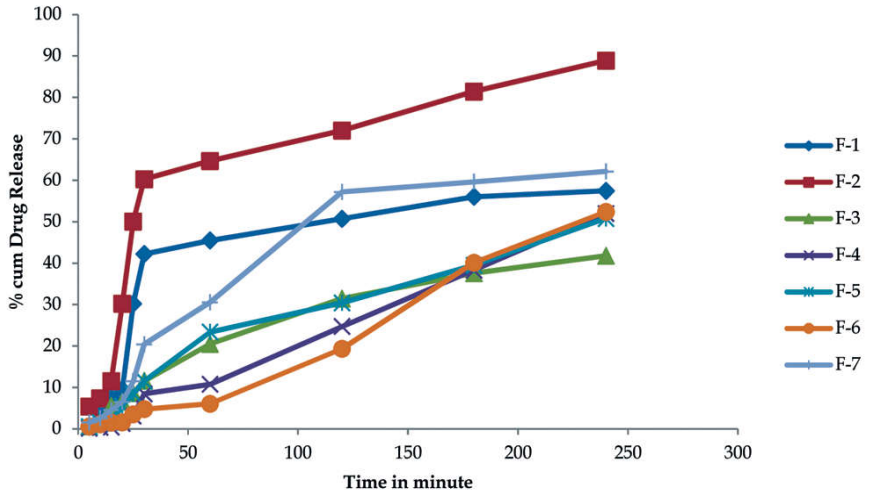


Fig. 5.14: Comparison of dissolution profile of various SMEDDS formulations

Self-Emulsification and Precipitation Assessment

The results of self-micro emulsification and precipitation studies were shown in Table 5.13.

Formulation F-2 and F-3 showed less dispersion time, clear and stable micro emulsion.

Other formulations were showed greater dispersion time as compare to optimizedformulation (F-2) and they were stable micro emulsion but not clear [74].

In VitroDissolution Studies

Alternatively, for evaluating the *in vitro* performance of SMEDDS, drug diffusion studies using the dialysis technique are well documented in literature [75-77]. The drug release profile was shown in Figure 5.14.

In case of SMEDDS (F-2), more than 30% of Cefdinir was released in 20 minute. The release percentage of Cefdinir from SMEDDS form was significantly higher than that of other Cefdinir Drug formulation.

It could suggest that Cefdinir dissolved perfectly in SMEDDS form could be released due to small droplet size, which permits a faster rate of drug release into aqueous phase and it could affect bioavailability. The release rate of Cefdinir from SMEDDS(F-2) was faster than SMEDDS than other formulation.

Accelerated Stability Studies of Cefdinir SMEDDS

The effect of centrifugation and freeze–thaw cycling on phase separation of Micro emulsion and precipitation of drug is shown in Table 5.14. Both accelerated tests are carried out to ascertain stability of Micro emulsion under stress conditions. Optimized formulation of Micro emulsion (F-2) did not exhibit any drug precipitation, phaseseparation after centrifugation confirming its stable nature. Similarly, Optimized formulation of Micro emulsion (F-2) survivedfreeze–thaw cycling as it was found to be reconstituted without anyphase separation, drug precipitation after exposure to freeze–thawcycling.

Table 5.14: Accelerated stability data of various SMEDDS formulations

Accelerated Study	Parameter	Formulation code						
		F-1	F-2	F-3	F-4	F-5	F-6	F-7
Centrifugation	phase separation	No	No	No	Slight	No	Slight	Slight
	Drug precipitation	No	No	No	Yes	No	Yes	Yes
Freeze-thaw cycle	phase separation	No	No	No	No	No	No	No
	Drug precipitation	No	No	No	No	No	No	No

Table 5.15: Stability data of SMEDDS formulation (F-2)

Time (Hours)	Storage condition	Observation
24	25 ± 3°C	No phase separation
	40 ± 2°C/75 ± 5%	No phase separation
48	25 ± 3°C	No phase separation
	40 ± 2°C/75 ± 5%	No phase separation
120	25 ± 3°C	No phase separation
	40 ± 2°C/75 ± 5%	No phase separation
240	25 ± 3°C	No phase separation
	40 ± 2°C/75 ± 5%	No phase separation
720	25 ± 3°C	No phase separation
	40 ± 2°C/75 ± 5%	No phase separation

Summery and Conclusion

Cefdiniris a BCS Class II drug and is water insoluble, with varying bioavailability. Cefdinir exhibits very low dissolution profile in the gastro intestinal fluid which might be attributed to its hydrophobic characteristic. The potential of SMEDDS was explored successfully for oral delivery of poorly water-soluble Cefdinir. SMEDDS are isotropic mixtures made up of oil, surfactant and sometimes co-surfactant or co-solvent.

Solubility study of Cefdinir was carried out in presence of various oil, surfactant and co-surfactant. Cefdinir had highest solubility in Labrafac PG, tween-80 and polyethylene glycol-400 as liquid vehicle, surfactant and co-surfactant respectively with comparison to others. So, Labrafac PG as oil, tween-80 as surfactant and polyethylene glycol-400 as co-surfactant was selected for optimal SMEDDS formulation resulting in improved drug loading capabilities.

Phase diagrams were constructed to obtain optimum concentrations of oil, water, surfactant, and co-surfactant. It was observed that increasing concentration of surfactant such as tween-80 in SMEDDS formulation increased spontaneity of self-emulsification region. Therefore, much higher concentration of surfactant, much higher self-emulsifying region in phase diagrams. The phase diagrams were constructed at ratio of surfactant/co-surfactant 1:1, 2:1, 3:1, 4:1 (w/w).

Simplex lattice design was used to optimize the formulation of SMEDDS containing Cefdinir. The concentrations of oil (X1), surfactant (X2) and co-surfactant (X3) were chosen as the independent variables. The emulsification time and Cumulative %release in 20 minute were taken as responses (Y), respectively. The optimal formulation of SMEDDS was comprised of 20% oil (Labrafac PG), 60% surfactant (Tween-20) and 20% co-surfactant (PEG-400).

Droplet size distribution following self-micro emulsification is a critical factor to evaluate self-micro

emulsion system. The smaller droplets have larger interfacial surface area will be provided for drug absorption. The optimized formulation (F-2) have droplet size 87.60 nm (% passing is 50).

In vitro release study was carried out to understand characteristics of drug release from SMEDDS. In case of SMEDDS (F-2), more than 99% of Cefdinir was dissolved. Order of drug dissolution was F-2> F-4> F-1 > F-7> F-3> F-6 > F-5.

Conclusion

The potential of SMEDDS was explored successfully for oral delivery of poorly water-solubleCefdinir. SMEDDS are isotropic mixtures made up of oil, surfactant and sometimes co-surfactant or co-solvent. In an aqueous environment a homogeneous, transparent, isotropic and thermodynamically stable dispersion will result, the formation of which is improved by gentle agitation, *in vivo* provided by gastrointestinal motility. The formulation of Cefdinir SMEDDS was optimized by a simplex lattice design. Solubility study was showed that highest solubility of Cefdinir in oleic acid as compare to other materials. The optimized formulation of SMEDDS was comprised of 20% oil (Labrafac PG), 40 % surfactant (Tween-20) and 20% co-surfactant (PEG-400). Pseudo-ternary phase diagrams were constructed to identify the efficient self-emulsification region. The average globule size of SMEDDS containing Cefdinirwas about 87.60 nm when diluted in water. SMEDDS had also shown that after dilution of formulation there was no precipitation and phase separation found. In vitro dissolution studies revealed that release of Cefdinirfrom SMEDDS was faster. Our studies illustrated potential use of SMEDDS for delivery of hydrophobic compounds, such as Cefdinir.

Experience with self-micro emulsifying drug delivery system (SMEDDS) reveals that this is a fruitful approach to improve the solubility and bioavailability ofCefdinir. Now, further study required to convert SMEDDS formulation in to powdered form which will either fill in capsule or compress the tablet. In vivo pharmacokinetic and pharmacodynamic study also required to be carried out to evaluate its efficiency in improving oral bioavailability of poorly water soluble drug.

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Original Article

Stability Indicating Chromatographic Method Development and Validation for the Simultaneous Determination of Ilaprazole and Domperidone in Its Pharmaceutical Dosage Form by RP- HPLC

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Abstract

A specific, accurate, precise and reproducible RP HPLC method has been developed and subsequently validated for the simultaneous estimation of Ilaprazole and Domperidone in pharmaceutical dosage form. The proposed HPLC method utilizes hypersil (Thermo scientific) C18 column (250 mm × 4.6 mm id, 5 ìm particle size), and mobile phase consisting of water: Acetonitrile: Acetic acid (30:70:0.1) at a flow rate of 1.0 mL/ min. Quantitation was achieved with PDA detection at 255 nm based on peak area with linear calibration curves at concentration ranges 5-15 µg/ ml for Ilaprazole and 15-45 µg/ml for Domperidone. The retention time of Ilaprazole and Domperidone were found to be 3.753 min and 6.120 min respectively. The stability studies were carried out and method was validated in terms of accuracy, precision, linearity, limits of detection, limits of quantitation and robustness. This method has been successively applied to marketed formulation and no interference from the formulation excipients was found.

Keywords: Ilaprazole; Domperidone; Stability Indicating Assay; HPLC.

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Introduction

For the development of stability-indicating assay method, the drug is subjected to Various ICH (International Conference on Harmonization) stress conditions such as photolytic, hydrolytic, thermal and oxidative. As per the ICH drug stability test guidelines Q1A (R2), validated stability-indicating assay method should be developed for the analysis of drug substance and drug product. Ilaprazole; 2-[[(4-Methoxy-3-methyl-2-pyridinyl)methyl]sulfinyl]-6-(1Hpyrrol-1-yl)-1H-benzimidazole is a new proton pump inhibitor used in the treatment of pepticulcer disease, dyspepsia, gastro esophageal reflux disease and duodenal ulcer which reduces acid secretion by inhibiting the parietal cell H+/K+ ATP pumpDomperidone (DOM), chemically is known as 5-chloro-1-{1-[3-(2-oxo-2,3-dihydro-1H-1,3-benzodiazol-1-yl)propyl]piperidin-4-yl}-2,3-dihydro-1H-1,3-benzodiazol-2-one, a specific blocker of dopamine receptors. It speeds gastrointestinal peristalsis, causes prolactin release, and is used as antiemetic and tool in the study of dopaminergic mechanisms. This combination of drugs will be used to treat peptic ulcers. In literatures, few analytical methods have been reported of analysis of Ilaprazole such as UPLC, LC-MS/MS, HPLC-ESI-MS/MS, however no stability-indicating HPLC method have been reported for the analysis of Ilaprazole and domperidone in its combined dosage form. Hence the aim of the present study was to develop and validate stability-indicating HPLC method for determination of Ilaprazole (IPZ) and Domperidone bulk drug as per the ICH guideline. The developed method is stability-indicating and was successfully

utilized for the determination of Ilaprazole in tablet formulation.

Materials and Methods

Apparatus and Instrument

The analysis was carried out on a HPLC system (WATERS Milford USA) equipped with PDA detector. Other apparatus and instruments used were a micro analytical balance (Shimadzu), Ultrasonic Cleaner (EIE Instruments Pvt. Ltd. Ahmedabad), Nylon Membrane Filters (0.22mcm, 47 mm D). All instruments and glass wares were calibrated.

Reagents and Materials

Ilaprazole and Domperidone were obtained as gratis sample from Accurate Pharmaceuticals, Godhra. Methanol HPLC Grade, Water HPLC Grade, A stock-standard solution of ILA and DOM was prepared by dissolving accurately weighed amount of pure drug in mobile phase.

Mobile Phase

The mobile phase water:Acetonitrile:Acetic acid (30:70:0.1) was filtered through Millipore filter paper type HV (0.45 µm) and degassed by sonication.

Chromatographic Conditions

Chromatographic analysis was carried out on an hypersil C-18 column, (5 µm, 250mm x 4.6mm i.d) LC-20 AT. The mobile phase consisted of water:Acetonitrile:Acetic acid (30:70:0.1). The mobile phase was filtered through Millipore filter paper type HV (0.45 µm) and degassed by sonication, was pumped at 1.0 ml/min flow rate. The column was thermostated at room temperature. Under these conditions the runtime was 10 min.

Preparation of Standard Stock Solution of Ilaprazole (100 mg/ml) and Domperidone (300mg/ml)

A 10 mg of standard Ilaprazole and 30 µg of standard Domperidone was weighed and transferred to a 100 ml volumetric flask each and dissolved in 25 ml mobile phase. The flask was shaken and volume was made up to the mark with mobile phase to give a solution containing 100 µg/ml Ilaprazole and 300 µg/ml Domperidone

Preparation of Combined Working Standard Solution Containing Ilaprazole and Domperidone in Ratio of 1:3

Accurately weighed 10 mg Ilaprazole and 30 mg of Domperidone were transferred to 100 ml volumetric flask, dissolved in sufficient amount of mobile phase and diluted up to mark with mobile phase to get concentration of 100 µg/ml Ilaprazole and 300 µg/ml Domperidone. This solution was diluted further to get the concentrations in range of 5, 7.5, 10, 12.5, 15 µg/ml of Ilaprazole and 15, 22.5, 30, 37.5, 45 µg/ml of Domperidone.

Degradation Studies

All stress studies for Ilaprazole and Domperidone were performed at concentration of 1mg/ml. The neutral (water) degradation study was performed by refluxing the drug solution at 800C for 8h. The alkaline degradation study was carried out by refluxing drug solution in 0.1N NaOHat 80C for 4h. The drug solution was refluxed with 0.1N HCl at 80C for 4h to conduct degradation study under acidic conditions. For degradation study in hydrogen peroxide (H2O2) drug solution was refluxed with 3% H2O2 at 80C for 8h. Photolytic stress degradation study was carried out by exposing the drug powder to UV light for 48h. Thermal degradation behavior of Ilaprazole and domperidone was studied by exposing the drug powder to dry heat in an oven at 80C for 24h. samples were withdrawn periodically and analyzed by HPLC after suitable dilution.

Method Validation

Precision

Repeatability

Precision of the method was studied by making repeated injections of the mixture of drugs on the same day for intraday precision. The % RSD after six determinations was determined at 10 µg/ml for ILA and 30 µg/ml for DOM.

Intraday and Inter-day Precision

Intraday and Inter-day precision for method were measured in term of %RSD. The experiment was repeated three times in a day for intraday and on three different days of same for inter-day precision by taking lower, middle and higher concentration of ILA(5, 10, 15 µg/ml) and DOM(15, 30, 45 µg/ml).

Linearity

The linearity of measurement was evaluated by

analyzing standard solutions of ILA and DOM in the range of 5–15 µg/ml and 15-45 µg/ml for both drugs respectively and calibration plot was constructed.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ of ILA and DOM were determined by calibration curve method. Solutions of Ilaprazole and Domperidone were prepared in the range of 5–15 µg/ml and 15-45 µg/ml for both drugs respectively and injected in triplicate.

Accuracy

Accuracy of the method was calculated by recovery studies at three levels by standard addition method i.e. spiking 80%, 100%, 120% of ILA and DOM to the standard solutions containing 5 µg/ml of ILA and 15 µg/ml of DOM.

Robustness

Influence of small changes in chromatographic conditions such as change in flow rate, that is, ± 0.2 ml/min, mobile phase composition ±2 ml and pH ± 0.2 was studied to determine the robustness of the method for the development of RP-HPLC method for the simultaneous estimation of ILA and DOM and their %RSD was determined.

System Suitability

The stock solution containing 10 µg/ml of ILA and 30 µg/ml of DOM was injected and repeated five times and the chromatograms were recorded. The resolution, number of theoretical plates, and peak asymmetry were calculated to determine whether the result complies with the recommended limit.

Results and Discussion

Method Development

The HPLC isocratic programming was utilized to analyze drug and its degradation products. The separation was achieved with mobile phase consisting of water: methanol initially in ratio 50:50 but no peak were observed so mobile phase water:Acetonitrile (30:70) was tried and Ilaprazole peak single was observed. Then again using isocratic conditions water:Acetonitrile:acetic acid were carried out. After considering the varying combinations of various mobile phases, Water: Acetonitrile: Acetic acid (30:70:0.1) was finalized as it was showing good peak shapes and a significant amount of resolution.

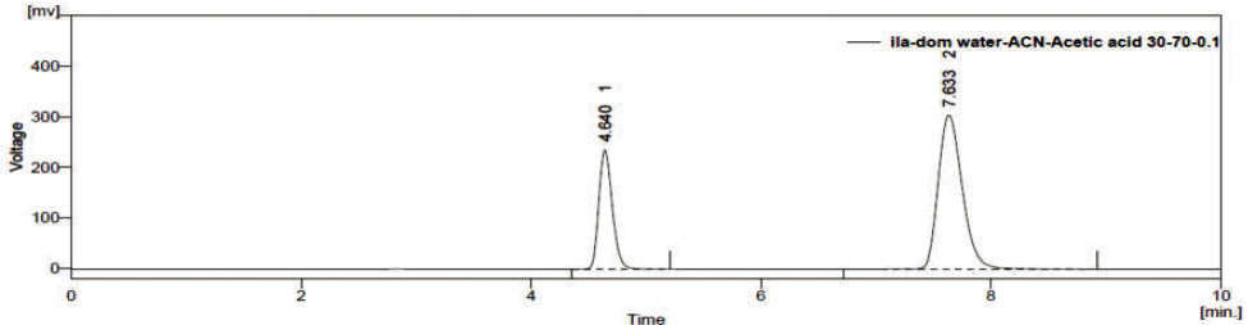

Fig. 3.1: Chromatogram of ilaprazole and domperidone in water: acetonitrile: acetic acid (30:70:0.1 v/v) (Flow rate-1.0 ml/min)

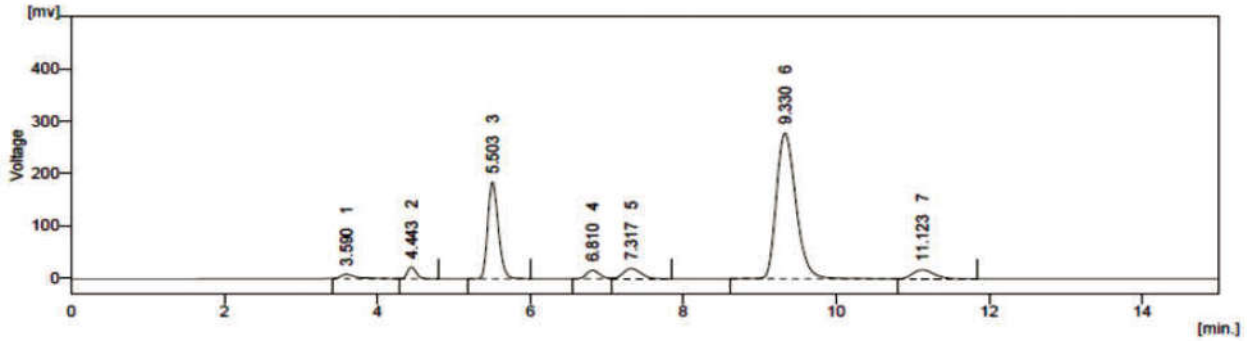

Fig. 3.2: Domperidone and ilapazole acid degradation

Degradation behavior of IPZ and DOM

Ilaprazole was subjected to above mentioned stress conditions and showed following degradation behavior.

Hydrolytic Studies

Acid decomposition studies were performed by refluxing the 1 ml of stock solution and transferred in to 10 ml of volumetric flask. 2 ml of 0.1 N HCl solutions was added and mixed well and put for 80°C for 4 hrs. Then the volume was adjusted with diluent to get Ilaprazole (10 µg/mL) and Domperidone (30 µg/mL) Basic decomposition studies were performed by refluxing the 1 ml of stock solution and transferred in to 10 ml of volumetric flask. 2 ml of 0.1 N NaOH solutions was added and mixed well and put for 4 hrs at 80°C. Then the volume was adjusted with diluent to get Ilaprazole (10 µg/mL) and Domperidone (30 µg/mL).

Oxidative Degradation

Oxidative decomposition studies were performed by refluxing the 1 ml of stock solution transferred in to 10 ml of volumetric flask. 2 ml of 3% H_2O_2 solutions was added and mixed well and put for 8hrs at 80°C. Then the volume was adjusted with diluent to get Ilaprazole (10 µg/mL) and Domperidone (30 µg/mL).

Thermal Degradation

Thermal Degradation studies were performed 1 ml of stock solution was transferred in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 80°C for 24 hrs. Then the volume was adjusted with diluent to get Ilaprazole (10 µg/mL) and Domperidone (30 µg/mL).

Photo Degradation

Photo Degradation studies were performed by taking 1 ml of stock solution and transferred in to 10 ml of volumetric flask. The volumetric flask was kept in presence of UV for 48 hrs. Then the volume was adjusted with diluent to get Ilaprazole (10 µg/mL) and Domperidone (30 µg/mL).

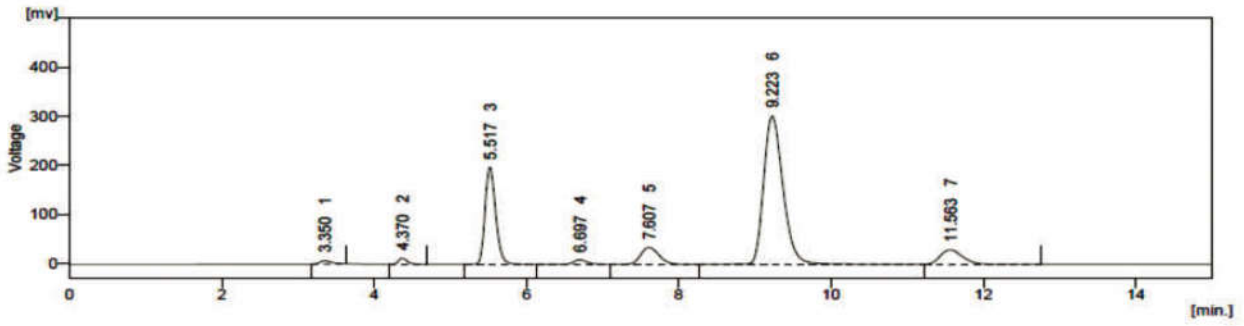

Fig. 3.3: Domperidone and ilapazole base degradation

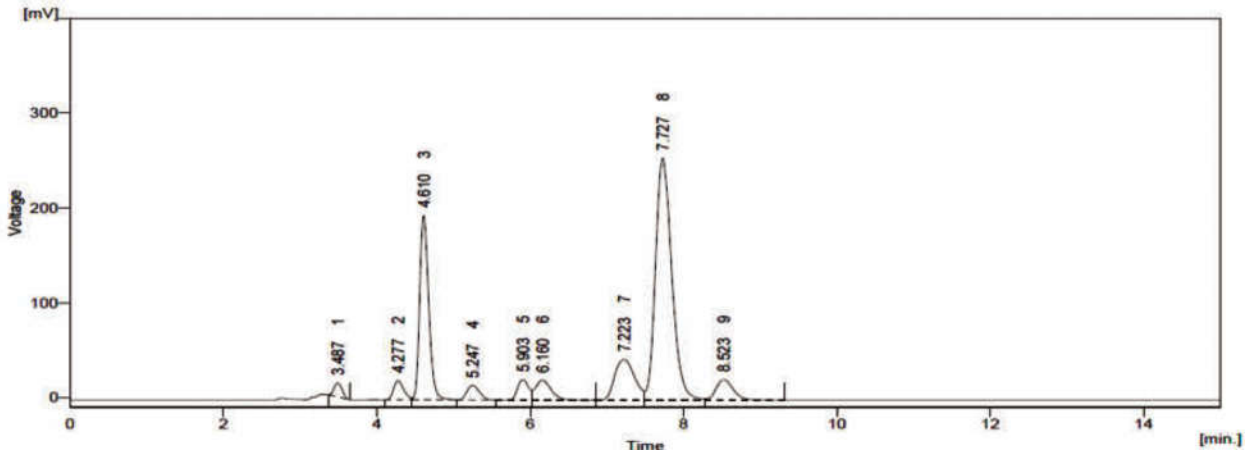

Fig. 3.4: Domperidone and ilaprazole oxidation degradation

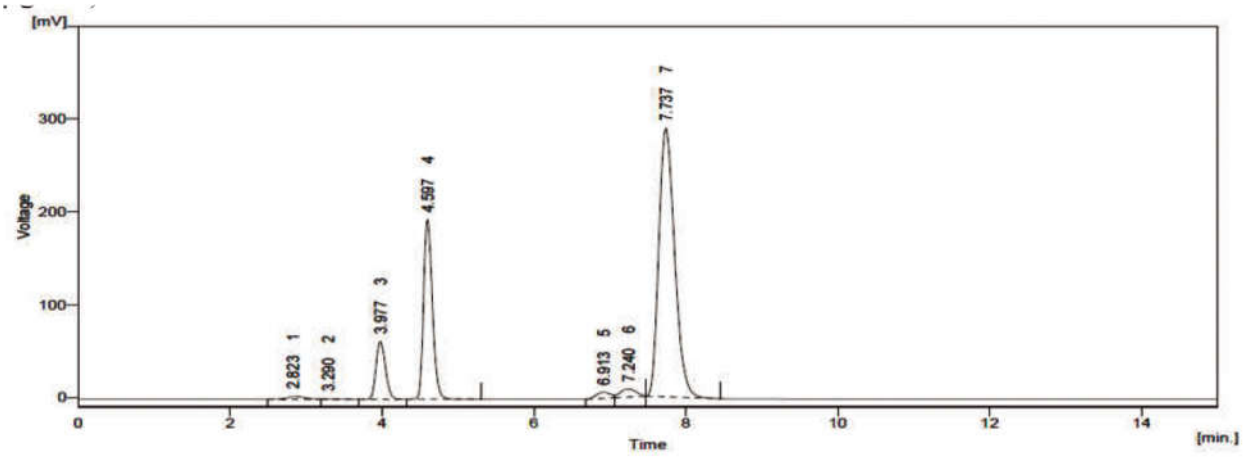

Fig. 3.5: Domperidone and ilaprazole thermal degradation

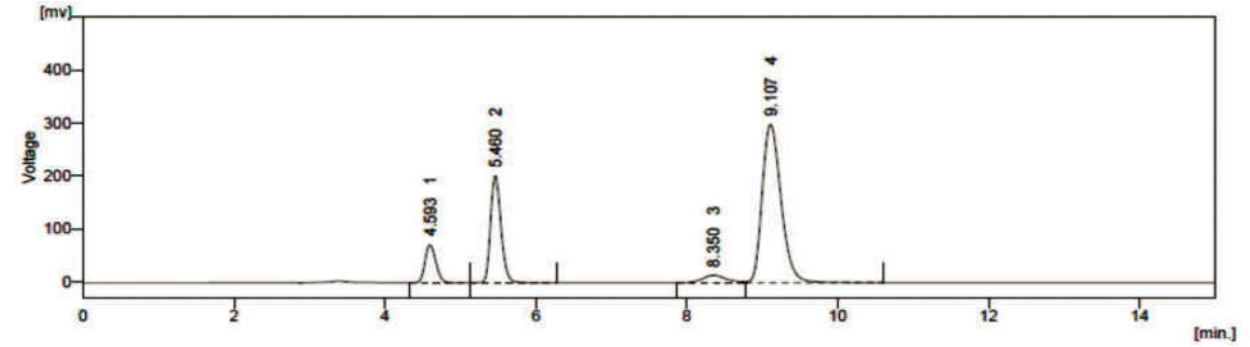

Fig. 3.6: Domperidone and ilapazole photo degradation

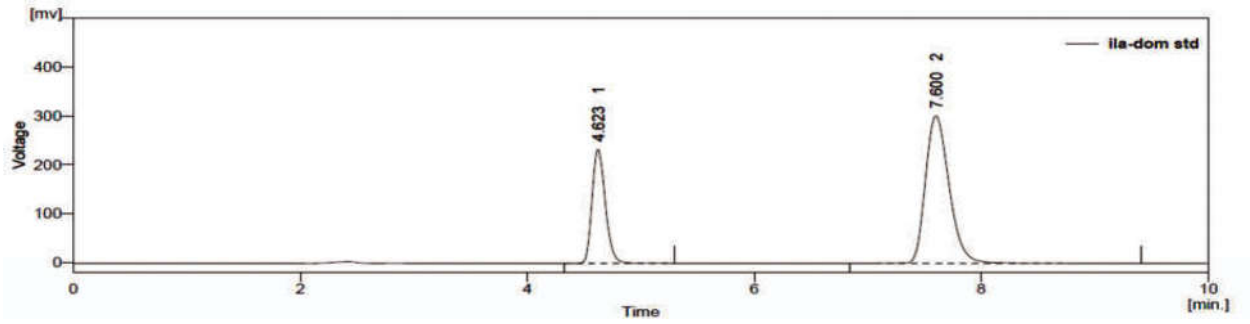

Fig. 3.7: Chromatogram of ilaprazole and domperidone standard

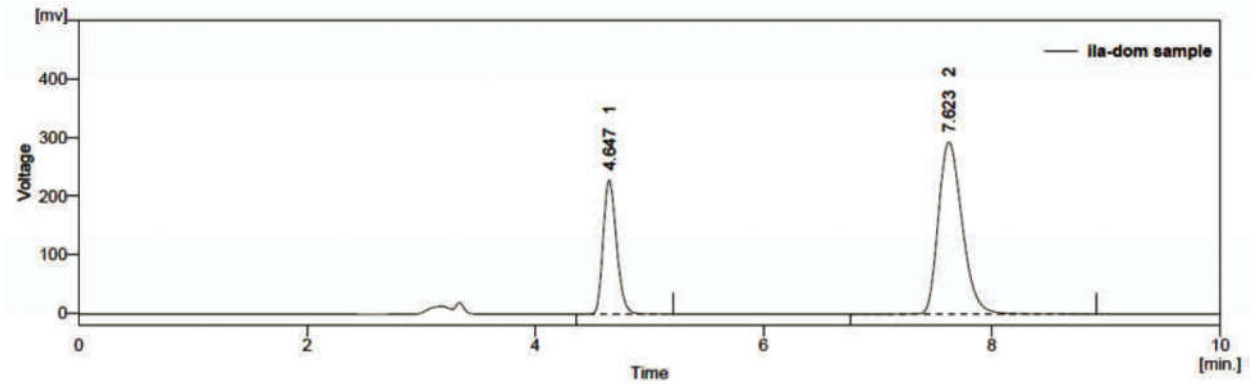

Fig. 3.8: Chromatogram of ilaprazole and domperidone sample

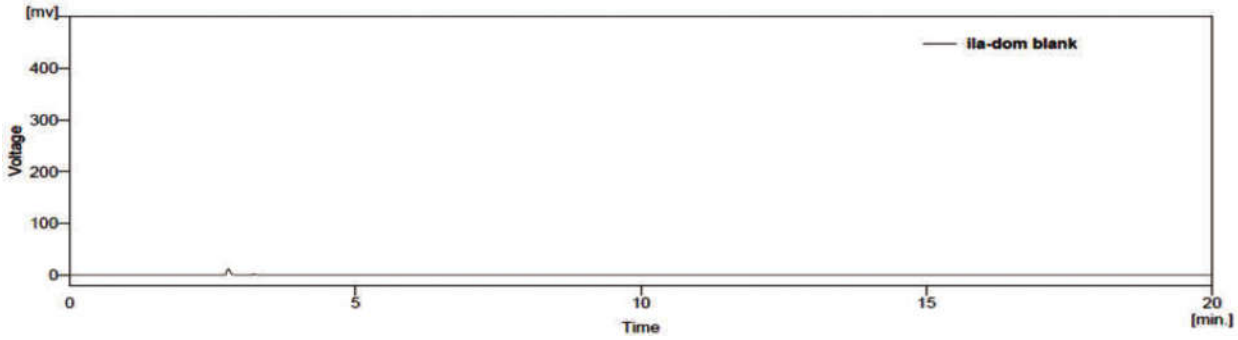

Fig. 3.9: Chromatogram of ilaprazole and domperidone blank

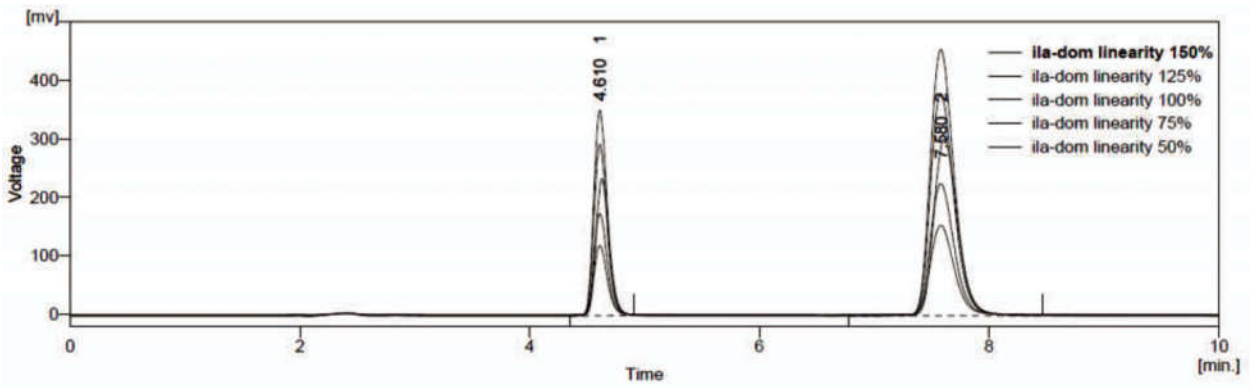

Fig. 3.10: Overlay chromatogram of different concentrations of binary mixtures of ilaprazole and domperidone

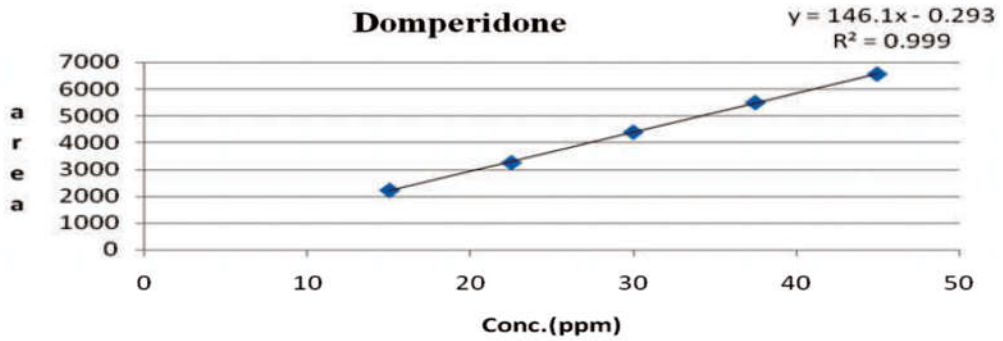

Fig. 3.11: Calibration curve of domperidone (15-45 ìg/ml)

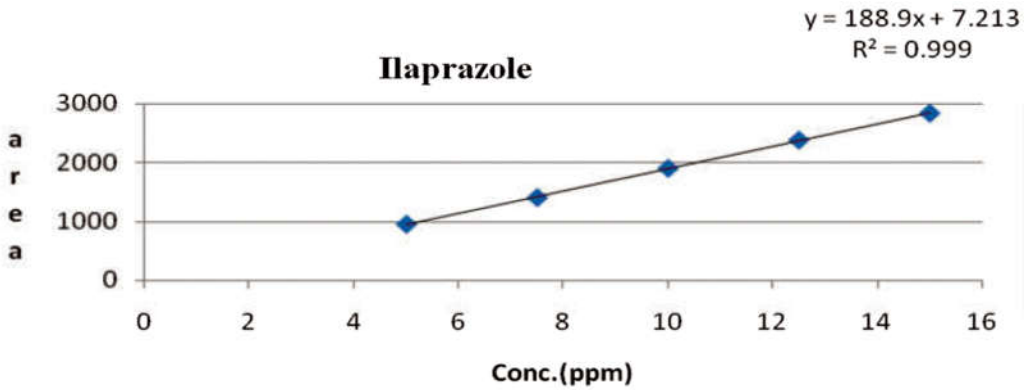

Fig. 3.12: Calibration curve of ilaprazole (5-15 ìg/ml)

Validation

Specificity

The Chromatograms of Ilaprazole and Domperidone standards and Ilaprazole and Domperidone sample show no interference with the Chromatogram of Ilaprazole and Domperidone Blank, so the Developed method is Specific.

Linearity and Range

The linearity for Domperidone and Ilaprazole were assessed by analysis of combined standard solution in range of 15-45 µg/ml and 5-15 µg/ml respectively. Correlation co-efficient for calibration curve Domperidone and Ilaprazole was found to be 0.999 and 0.999 respectively. The regression line equation For Domperidone: **y = 146.1x - 0.293** and For Ilaprazole : **y = 146.1x - 0.293.**

Table 1: Linearity data for domperidone

Sr. No	Concentration(µg/ml)	Area
1	15	2213.258
2	22.5	3247.411
3	30	4389.165
4	37.5	5500.228
5	45	6566.309

Table 2: Linearity data for Ilaprazole

Sr. No	Concentration(µg/ml)	Area
1	5	958.938
2	7.5	1408.078
3	10	1902.745
4	12.5	2380.279
5	15	2835.108

Precision

Repeatability

The data for repeatability of peak area measurement for Ilaprazole and Domperidone, based on six measurements of same solution of Ilaprazole and Domperidone are depicted in table 2 and 3. The % RSD for Ilaprazole and Domperidone was found to be 0.704 and 0.747 respectively.

Table 3: Repeatability data for domperidone

		Domperidone		
Sr. No.	Conc (µg/ml)	Area	Mean ± S.D (n=6)	% R.S.D
1.	30	5375.432	5341.141±39.900	0.747
		5380.794		
		5308.947		
		5311.033		
		5375.328		
		5295.311		

Table 4: Repeatability data for ilaprazole

SR. NO.	Conc. (µg/ml)	Domperidone Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Ilaprazole Area Mean ± S.D. (n=3)	% R.S.D
1	15	2675.239 ± 8.307	0.310	5	1158.236± 9.977	0.861
2	30	5314.804± 50.483	0.950	10	2317.879± 4.673	0.202
3	45	8001.386± 20.746	0.259	15	3469.973± 15.576	0.449

Table 5: Intraday precision data for estimation of ilaprazole and domperidone

Sr. No.	Conc. (µg/ml)	Domperidone Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Ilaprazole Area Mean ± S.D. (n=3)	% R.S.D
1	15	2675.239 ± 8.307	0.310	5	1158.236± 9.977	0.861
2	30	5314.804± 50.483	0.950	10	2317.879± 4.673	0.202
3	45	8001.386± 20.746	0.259	15	3469.973± 15.576	0.449

Table 6: Interday precision data for estimation of ilaprazole and domperidone

Sr. No.	Conc. (µg/ml)	Domperidone Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Ilaprazole Area Mean ± S.D. (n=3)	% R.S.D
1	15	2679.123±16.261	0.607	5	1163.900±7.726	0.664
2	30	5326.325±46.950	0.881	10	2315.576±12.951	0.559
3	45	7986.264±36.150	0.453	15	3450.463±26.217	0.759

Intraday Precision

The data for intraday precision for Ilaprazole and Domperidone is shown in Table 4. The % R.S.D. for Intraday precision was found to be 0.259-0.950. for Domperidone and 0.202-0.861 for Ilaprazole.

Interday Precision

The data for intraday precision for Ilaprazole and Domperidone is shown in table 5. The % R.S.D. for interday precision was found to be 0.893-1.753 for Domperidone and 0.706-1.055 for Ilaprazole.

Table 7: Recovery data for domperidone

Sr. No.	Conc. Level (%)	Sample Amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	15	12	12.21	101.75	100.11± 1.89
2		15	12	12.06	100.53	
3		15	12	11.77	98.05	
4	100 %	15	15	14.90	99.31	100.85 ± 1.08
5		15	15	14.94	99.57	
6		15	15	15.19	101.29	
7	120 %	15	18	18.15	100.81	101.06 ± 0.29
8		15	18	18.25	101.38	
9		15	18	18.18	100.99	

Table 8: Recovery data for ilaprazole

Sr. No.	Conc. Level (%)	Sample Amount	Amount Added	Amount Recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	5	4	4.05	101.35	100.24 ± 1.56
2		5	4	4.04	100.90	
3		5	4	3.94	98.45	
4	100 %	5	5	5.02	100.45	100.51 ± 0.30
5		5	5	5.01	100.25	
6		5	5	5.04	100.84	
7	120 %	5	6	6.02	100.36	100.18 ± 0.48
8		5	6	5.98	99.63	
9		5	6	6.03	100.54	

Table 9: Robustness data for domperidone

Sr. No.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	2482.653	2151.774	2130.257	2245.255	2527.745	2114.007
2	2485.057	2116.911	2125.967	2247.819	2530.193	2103.401
3	2472.598	2092.614	2119.684	2233.800	2517.503	2113.944
% R.S.D	0.266	1.402	0.250	0.333	0.267	0.289

Table 10: Analysis of marketed formulation

Capsule	mg/ Capsule powder		Assay (% of label claim*) Mean ± S. D.	
	Domperidone	Ilaprazole	% Domperidone	% Ilaprazole
Lupila-D	30	10	95.96±0.898 2.218124 2.218124 ± 1.5289	97.253 ± 0.572

LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Where,

SD = Standard deviation of intercepts

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The results are shown in table 6 and 7. Percentage recovery for Domperidone was 100.900-101.458 %, while for Ilaprazole, it was found to be in range of 100.365-100.837 %.

Robustness

The effect of changes was found to be within the acceptance criteria as shown in Table 8. The % RSD should Be less than 2%.

Analysis of Marketed Formulation by Developed Method

Applicability of the proposed method was tested by analyzing the commercially available

Capsule formulation Lupila-D. The results are shown in Table 9.

The assay results were comparable to labeled value of each drug in Capsule dosage form. These results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

Conclusion

The stability-indicating assay has been developed and validated for the determination of Ilaprazole and Domperidone in bulk drug and tablet dosage form. The degradation behavior of Ilaprazole and Domperidone was studied as per ICH recommended conditions. The proposed method is simple, precise, accurate, specific, and is able to separate drug from its degradation products. The developed method could also be extended to the analysis of stressed marketed formulation of Ilaprazole and Domperidone, as there is no interference from excipients or other components observed.

Acknowledgement

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Original Article

A Review on Bioanalytical Method Development and Validation by LC-MS/MS

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Abstract

The development of bioanalytical method is of paramount importance during the pre-clinical and clinical stage of drug development. Analytical method development where the appropriate bioanalytical method with its various parameters is developed and the assay is defined and application of the bioanalytical to actual analysis of sample from bioavailability, bioequivalence and pharmacokinetic studies. Bioanalytical methods are used for the quantitative analysis of drugs and their metabolites in the biological matrices like saliva, plasma, blood, serum, urine. In method development sample preparation and sample analysis are two important part. For sample analysis generally chromatographic technique are used like (HPLC, LC-MS/MS, GC, UPLC). After method development validation of that bioanalytical method is important. Method validation is a process that demonstrates that a method will successfully meet or exceed the minimum standard recommended in the USFDA or other guideline for accuracy, precision, selectivity, sensitivity, reproducibility and stability.

Keywords: Bioanalytical Method Development; LC-MS/MS; Bioanalytical Method Validation.

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Introduction

Method validation is a process that explain that a method will successfully meet or exceed the minimum standards recommend in the U.S.FDA , EMA or other regulatory guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability. This article discusses the validation of bioanalytical methods for small molecules with emphasis on chromatographic techniques. Bioanalytical methods are used for the quantitation of drugs and their metabolites in biological matrices like plasma, serum, saliva, urine. this type of bioanalytical methods are develop for analyte and some time for endogenous substance like levothyroxine sodium. for the purpose of analysis or quantification of analyte there is different type of analytical methods are available Hyphenated techniques in which LC-DAD (liquid chromatography-diode array detection), CE-MS(capillary electrophoresis- mass spectrometry), LC/MS (liquid chromatography-mass spectrometry), LC/MS/MS (tandem mass spectrometry), GC/MS (gas chromatography-mass spectrometry). Chromatographic methods HPLC(high performance liquid chromatography), GC(gas chromatography), UPLC(ultra performance liquid chromatography) Supercritical fluid chromatography. Ligand binding assays Dual polarization interferometry, ELISA (Enzyme-linked immune sorbent assay), MIA (magnetic immunoassay), RIA (radioimmunoassay) are used for method development and validation .In today's drug development environment, highly sensitive and selective methods are required such as high-performance liquid chromatography (HPLC) or

gas chromatography (GC) have been widely used for the bioanalysis with liquid chromatography coupled to triple quadrupole mass spectrometry (LC/MS/MS) is most commonly used technology [1]. Typically the combined increases in selectivity and sensitivity of LC-MS/MS based methods provide more than 10-fold improvement in limit of quantification (LOQ) relative to conventional methods using UV or fluorescence detection. Particular method used for quantitative measurement of analytes is reliable and reproducible for the intended use 1. Validation involves documenting, through the use of specific laboratory investigations, that the performance of characteristics of the method are suitable and reliable for the intended analytical applications. as increased number of therapeutics has promote the pharmaceutical industry to review and redefine aspects of development and validation of bioanalytical methods for the quantification of of this therapeutics in biological matrices in support of preclinical and clinical studies. A bioanalytical method is a set of procedures involved in the sample collection, sample processing, storage, and analysis of a biological matrix for a chemical compound.

Instrumentation

Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (*m/z*) ratios. Two key components in this process are the 1) ion source, which generates the ions, and 2) mass analyzer, which sorts the ions.

Ion Sources

Earlier LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermo spray) or if did so before ionization (particle beam). The analytes were then ionized under vacuum, often by traditional electron ionization. These approaches were successful only for a very limited number of compounds. In atmospheric pressure ionization, the analyte molecules are ionized first, at atmospheric pressure. The analyte ions are then mechanically and electro-statically separated from neutral molecules. Common atmospheric pressure ionization techniques are:

a. Electro spray ionization (ESI)
b. Atmospheric pressure chemical ionization (APCI)
c. Atmospheric pressure photo ionization (APPI)

a. Electrospray ionization

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas.

The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases.

Eventually, the repulsive force between ions with like charges exceeds the cohesive forces; ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer.

Electrospray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligonucleotides, but can also analyze smaller molecules like benzodiazepines and sulfated conjugates.

Mass Analyzer

Four types of mass analyzer used for LC/MS:

i. Quadrupole
ii. Time-of-flight
iii. Ion trap
iv. Fourier transform-ion cyclotron resonance (FT-ICR or FT-MS)

Quadrupole

A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time.

Quadrupole Mass Analyzers can Operate in Two Modes

- ❖ Scanning (scan) mode
- ❖ Selected ion monitoring (SIM) mode

In scan mode, the mass analyzer monitors a range of mass-to-charge ratios. In SIM mode, the mass analyzer monitors only a few mass to charge ratios. Scan mode is typically used for qualitative analyses or for quantitation when all analyte masses are not known in advance. SIM mode is used for quantitation and monitoring of target compounds [2].

Method Development

Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required and necessary equipment.the analytical process of method development includes sample collection, sample prepration, separation, detection and evalution of the results.

Sample Collection

According to specific time period sample/matrix is collected from human subject by vein puncture with a hypodermic syring (volume of sample is depending on the assay sensitivity and the total number of samples taken for study being performed).withdrawn sample is fill in the tube according to intended use (if sample is used as a plasma than blood is withdrawn in tube with anticoagulant EDTA. if serum is required than anticoagulant free tube used) Plasma is obtained by centrifugation at 4000 rpm for 15 min. serum is obtain by store sample at room temperature coagulation for 25-30 min. after the blood is coagulated centrifugation at 4000 rpm for 10 min. The purpose of sample preparation is to clean up the sample before analysis and to concentrate the sample [3].

Sample Preparation

Sample preparation technique is used to clean up a sample by removing endogenous material as well as to concentrate a sample before analysis to exclude errors in its detection, also Minimize interferences, Prevent clogging of column, Minimize matrix effect, Improve reproducibility and improve specificity .various methods used for sample preparation

Solid Phase Extraction

SPE employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed suitable sorbent. The first strategy is usually chosen when the desired sample component is present in high concentration. When components of interest are present at low levels, or multiple components of widely differing polarities need to be isolated, the second strategy is generally employed. The second strategy may also be used for trace enrichment of extremely low level compounds and concentration of dilute sample. A complex matrix may be treated by both elution strategies to isolate different target analyte. Solid phase consists of four steps; conditioning, sample loading, washing and elution. There two SPE methods

Off-Line Solid Phase Extraction (SPE)

Selective sample clean-up, Various SPE formats – phases, Sample pre concentration, Automated systems, Trend to 96-well micro SPE (avoids drying steps) these type of advantages for off-line SPE.

On-Line Solid Phase Extraction (SPE)

Disposable SPE cartridges, Minimal pre-treatment steps, Sample preparation at high pressure and controlled flow-rates, all samples are analyzed, sensitive assays, Systematic, automated method development, Fully automated and integrated process these are advantages of On-line SPE.

Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction is based on distribution of solutes between an aqueous phase and a water immiscible organic phase. Distribution of different solutes depends on their degree of solubility in different solvents. Analyte extracted into the organic phase can be evaporated to dryness and the residue reconstituted in a smaller volume of an appropriate solvent (preferably mobile phase), while analyte extracted in to the aqueous phase can often be injected directly on to a reversed-phase column. The technique is simple and rapid. Good quantitative recoveries are obtained through multiple continuous extractions. Solvents normally used are Ethyl Acetate, TerButyl methyl ether, Diethyl ether, n-Hexane and sometimes mixtures of the two or more solvent for efficient extraction.

Advantages: Low LODs are possible, Cost effective as compare to the On-line and Off line SPE techniques, Clean sample obtain as compare to protein precipitation technique.

Protein Precipitation: Protein precipitation is the simple method of extraction as compared to the LLE and SPE. It is utilized when high throughput of plasma and serum samples is Desired. Protein precipitation is very useful method in field of clinical toxicology, the drug discovery and the therapeutic drug monitoring in which high throughput is

required. Proteins are denaturated with acid, base, salts or organic solvents. (e. g. TFA, TCA, NaOH, ZnSO4, Acetonitrile, Methanol). Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents. After protein precipitation centrifugation is carried out and the supernatant obtained is used for analysis. The supernatant can be injected directly into the chromatographic column or it can be evaporated and reconstituted with the mobile phase. Simple, generic, easy to perform, just mix solvents centrifuge, No Method Development time, Fast sample preparation (96-well titer plate automation),Very low volume require for sample processing, Very few step to get final drug concentrate ready for inject into LC-MS/MS [4-6].

Bioanalytical Method Validation

Validation may be defined as documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes. Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. method validation To ensure that a particular method for quantitative measurement of an analyte in a biological matrix is reliable and reproducible. the possibility of a bioanalytical method not being used on a regular basis will require adequate revalidation data when needed to be used, in order to document and demonstrate that a method is still valid prior to analyses of samples in a study. there are different levels and types of method validations, including "Full Validation, Partial Validation, and Cross Validation". These different types of bioanalytical method validations are defined and characterized as follows:

Full Validation

Full validation is important when developing and implementing a bioanalytical method for the first time. Full validation is important for a new drug entity. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification. Generally, a full validation should be performed for each species and matrix (mainly plasma, serum, whole blood, or urine) to be analyzed.

Partial Validation

Partial validations are modifications of already validated bio-analytical Methods. Partial validation may be performed when minor changes are made to an analytical method that has already been fully validated. A set of parameters to be evaluated in a partial validation are determined according to the extent and nature of the changes made to the method.

Cross Validation

Cross-validation is a comparison of validation parameters when two or more bio-analytical methods are used to generate data within the same study or across different studies. When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different studies are included in a regulatory submission.

Need of Bioanalytical Method Validation

It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactory interpreted, It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology, It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria ma need to be developed for each analyte [7-8].

Validation Parameters

Parameters to validate method are include;

Specificity or Selectivity

Selectivity is ability of an analytical method to differentiate and quantify analyte in the presence of other components in sample. For selectivity, analysis of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics.

Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by method to the true value (concentration) of an analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15 % of actual value except at LLOQ, where it should not deviate by more than 20 %. The deviation of mean from true value serves as measure of accuracy.

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in range of expected concentrations is recommended.

Recovery

Recovery is a measure of efficiency at which an analytical method recovers analyte through sample comparing analytical results for extracted samples at three concentrations (low, medium, and high) and three replicates that represent 100 % recovery. Recovery of analyte need not be 100 %, but extent of recovery of an analyte and of internal standard should be consistent, precise, and reproducible.

$$\%\text{Mean recovery} = \frac{\text{Mean extracted peak area}}{\text{Mean un-extracted peak area}} \times 100$$

Matrix Factor

The ratio of analyte response in presence of matrix ions to response in absence of matrix ions. matrix factor is determined by comparing analyte response in presence of matrix with that in absence of matrix. Matrix factor may be normalized using an internal standard.

$$\text{Matrix factor} = \frac{\text{Peak are in presence of matrix ion}}{\text{mean aqueous peak area}}$$

$$\text{ISTD Normalized factor} = \frac{\text{Matrix factor of drug}}{\text{matrix factor of ISTD}}$$

Linearity

Linearity is determined by using mean of two calibration curve standards and it includes STD BL, standard zero and at least six calibration standards. r2 value should be more than ≥ 0.98.

Stability

Short Term Stock Solution Stability (STSS)

Stability of drug and ISTD stock solution should be evaluated for at least 06 hours. For STSS stock solutions are stored in refrigerator for minimum of 06 hours and after stability period retrieve it and make ULOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ and ISTD dilution.

Long Term Stock Solution Stability (LTSS)

Stability of drug and ISTD stock solution should be evaluated for relevant time period. For LTSS stock solutions are stored in refrigerator for 20 days and after stability period retrieve it and make ULOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ and ISTD dilution.

Bench Top Stability (BT) (Short Term Stability of Analyte in Matrix)

It should be performed at higher quality control and lower quality control level for six replicates. Prepare spiked sample of HQC and LQC and stored at room temperature for a specific time period. Generally, time period is about time required from spiking of sample to transfer in to vials. Use freshly spiked calibration curve and quality control standard for determination of stability samples. For BT kept spiked HQC and LQC for 06 hours at room temperature.

Stability of Dry Extract (DE)

evaporation step. DE stability was conducted by using previously processed and dried stability samples. Freshly spiked replicates of each LQC and HQC samples were prepared and processed as per sample preparation procedure. After drying, dry extract stability samples were stored at -20 ± 5 °C for a period of at least 24 hours or as per requirement.DE stability samples were analyzed along with freshly spiked CCs and freshly prepared QCs 6 replicates of each LQC and HQC samples as per procedure.

Freeze and Thaw Stability

QC samples (at high and low level) are stored and frozen in freezer at intended temperature and thereafter thawed at room or processing temperature. After complete thawing, samples are refrozen again applying same conditions. At each cycle, samples should be frozen for at least 12 hours before they are thawed. Number of cycles in freeze-thaw stability should equal or exceed that of freeze/thaw cycles of study samples. It is perform to demonstrate that accuracy and precision is not change upon freezing and thawing cycle. Freeze and thaw stability experiment is performing by processing n ≥ 5 sample (at high and low level) of freeze thaw stability along with freshly spiked calibration curve and quality control sample. Storage temperature: -20 ± 5 °C and -78 ± 8 °C.

Long Term Stability of Analyte in Matrix

It should be performed at higher quality control and lower quality control level which are analysed immediately after preparation (first day stability assessment) and after applied storage conditions that are to be evaluated. Prepare spiked sample of 2 set of HQC and LQC and stored at-70 °C and -20°C for a specific time period. Generally, time period is about time required from sample collection to last sample analysis. Use freshly spiked calibration curve and quality control standard for determination of stability samples. Stability is evaluated by 3 replicates of each quality control level Concentration [9-11].

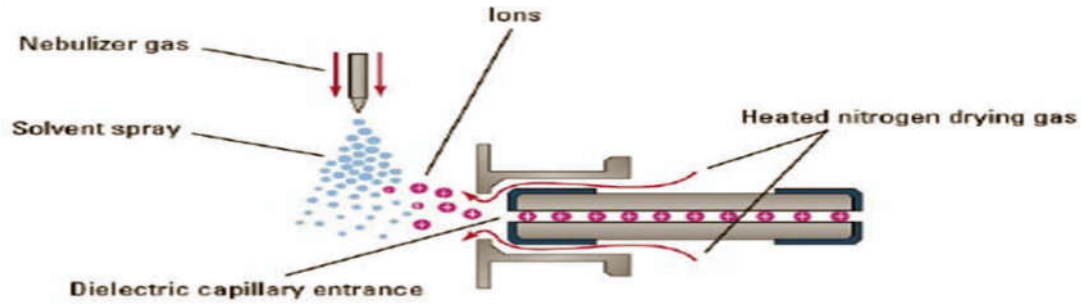

Fig. 1: Electrospray ionization

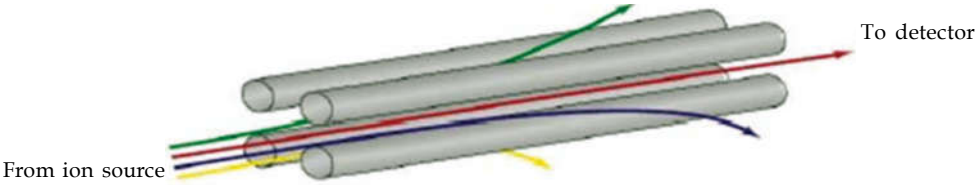

Fig. 2: Quadrupole

Fig. 3: off-line solide phase extraction (SPE)

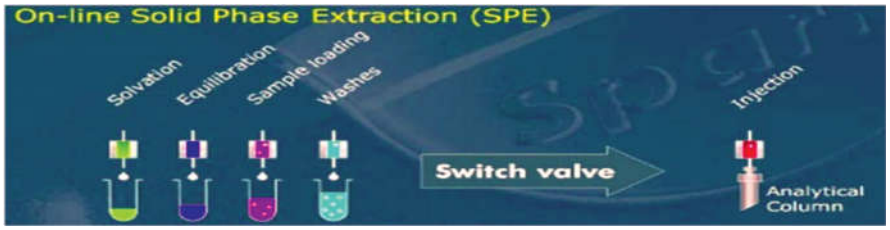

Fig. 4: on-line solid phase extraction (SPE)

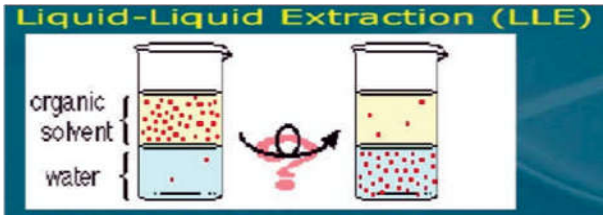

Fig. 5: Liquid-Liquid extraction (LLE)

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Review Article

Comparative Evaluation of Patent Term (Beyond 20 Years) in Developed and Developing Countries

Poonam S. Sukhramani*, Nilesh K. Patel, Ashok Patel***, Amit Vyas****, Ajay Patel*******

Abstract

Patent is an exclusive right granted for 20 years in exchange of complete discloser of the innovation. Different countries have their different patent laws. The main objective is comparatively evaluation of the patent term extensions granted in developed countries like US, Europe, etc and developing countries like India. Study the strategies used by these countries for extending the patent term. Study of patent laws and their amendments, different cases and conditions and thus analyse the facts and understand eligible conditions in which an extension can be granted. Extension of a patent term is to compensate the regulatory delay which occurs during the approval process of marketing authorisation for the patent, due to which the patentee is unable to enjoy the monopoly. Thus patent term extension encourages innovators to invest in the researches and bring out innovations. Here comparitive evaluation of patent term extension between developed and developing countries is discussed and all pros and cones are evaluated which would be helpful in drafting the policies of patent term extension for countries like India.

Keywords: Patent; Patent Term Adjustment; Patent Term Extension; Supplementary Protection Certificate.

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Introduction

Introduction To Intellectual Property Rights

Intellectual property (IP) pertains to any original creation of the human intellect such as artistic, literary, technical, or scientific creation. Intellectual property rights (IPR) refers to the legal rights given to the inventor or creator to protect his invention or creation for a certain period of time.[1]

Introduction to Patent

A patent is a legal right granted by a government that confers upon the creator of an invention the sole right to make, use, and sell that invention for a set period of time (usually 20 years)[1]. The purpose of patent system is to encourage inventions and promotion of technological innovation and to the transfer and dissemination of technology [2]. Patents are territorial and will only give the inventor rights in the country in which a patent is granted [3].

History of Pharmaceutical Patents

Until the TRIPS Agreement in 1994 many developing countries provided no patent protection for pharmaceutical products. The countries that have joined the WTO have obligated themselves to provide such protection; least developed countries are not required to meet this obligation until 2016. The continuing lack of patent protection for pharmaceutical products makes it very difficult to establish research-based industries in most developing countries. Most medical research in these countries takes place in the public sector. The lack of

any means of patenting these inventions and the related lack of experience in licensing them to the private sector, suppresses the development of commercial enterprises focused on alleviating the disease burdens common to developing countries. There are promising developments in countries such as India and Brazil that are beginning to use patents to develop commercial pharmaceutical industries that produce products directed at local diseases and available at price that patients in those countries can afford. These efforts show that developing countries have the capacity to build research-intensive pharmaceutical industries capable of operating profitably in the conditions of the local market. However, for such local industries to take root and grow, effective patent protection must be made available, the commercialization of publicly funded research must be encouraged, and compulsory licensing must be kept to a minimum. As a result of the GATT-TRIPs negotiations, the length of the patent term has been subsequently amended as twenty years from the first filing date of an application [2].

Patentability Criterias

- Novelty – The invention must never have been made public anywhere before the date on which an application for a patent is filed.
- Inventive Step – The invention must be sufficiently different from existing products or processes in a non-obvious way.
- Industrial application – It must be possible for the invention to be actually made or used [3].

Indian Patent System

The history of Patent law in India starts from 1911 when the Indian Patents and Designs Act, 1911 was enacted. The present Patents Act, 1970 came into force in the year 1972, amending and consolidating the existing law relating to Patents in India. The Patents Act, 1970 was again amended by the Patents (Amendment) Act, 2005, wherein product patent was extended to all fields of technology including food, drugs, chemicals and micro organisms. After the amendment, the provisions relating to Exclusive Marketing Rights (EMRs) have been repealed, and a provision for enabling grant of compulsory license has been introduced. The provisions relating to pre-grant and post-grant opposition have been also introduced. An invention relating to a product or a process that is new, involving inventive step and capable of industrial application can be patented in India. However, it must not fall into the category of inventions that are non-patentable as provided under Section 3(d) and 4 of the (Indian) Patents Act, 1970. In India, a patent application can be filed, either alone or jointly, by true and first inventor or his assignee [4].

Non Patentable Inventions as Per Section 3(d)

(a) An invention which is frivolous or which claims anything obviously contrary to well established natural laws;

(b) An invention the primary or intended use or commercial exploitation of which could be contrary to public order or morality or which causes serious prejudice to human, animal or plant life or health or to the environment;

(c) The mere discovery of a scientific principle or the formulation of an abstract theory (or discovery of any living thing or non-living substances occurring in nature);

(d) The mere discovery of a new form of a known substance which does not result in the enhancement of the known efficacy of that substance or the mere discovery of any new property or mere new use for a known substance or of the mere use of a known process, machine or apparatus unless such known process results in a new product or employs at least one new reactant ; Explanation- For the purposes of this clause, salts, esters, ethers, polymorphs, metabolites, pure form, particle size, isomers, mixtures of isomers, complexes, combinations and other derivatives of known substance shall be considered to be the same substance, unless they differ significantly in properties with regard to efficacy.

(e) A substance obtained by a mere admixture resulting only in the aggregation of the properties of the components thereof or a process for producing such substance;

(f) The mere arrangement or re-arrangement or duplication of known devices each functioning independently of one another in a known way;

(g) A method of agriculture or horticulture;

(h) Any process for the medicinal, surgical, curative, prophylactic,diagnostic, therapeutic or other treatment of human beings or any process for a similar treatment of animals to render them free of disease or to increase their economic value or that of their products.

(i) Plants and animals in whole or any part thereof other than micro-organisms but including seeds,

varieties and species and essentially biological processes for production or propagation of plants and animals;

(j) A mathematical or business method or a computer programme per se or algorithms;

(k) A literary, dramatic, musical or artistic work or any other aesthetic creation whatsoever including cinematographic works and television productions;

(l) A mere scheme or rule or method of performing mental act or method of playing game [4];

(m) A presentation of information;

(n) Topography of integrated circuits;

(o) An invention which in effect, is traditional knowledge or which is an aggregation or duplication of known properties of traditionally known component or components.

(p) Inventions relating to atomic energy and the inventions prejudicial to the interest of security of India [5].

Special Problems of Pharmaceutical Patents

Huge capital investment has to be done in pharmaceutical industry for laboratory research and clinical trials rather than the manufacturing of the final product, thus patent exclusivity seems to be the only effective way to protect and receive a return on that investment. This is because pharmaceutical industry is heavily regulated by government agencies to assure the safety and efficacy of products. In the United States, the Food and Drug Administration performs this function. Much of the investment in new drugs is in the clinical trials which are necessary to satisfy safety and efficacy regulators. The lengthy time period between patent filing and placing a product on the market means that pharmaceutical manufacturers receive far shorter periods of patent exclusivity than is the case for other patent dependent industries [2].

Different Wways for Extending Patent Term

Patent term Adjustment (PTA) (USA) (As per 35 U.S.C. 154)

It is the day-by-day adjustment of patent term due to delays caused by the US patent and trademark office during the Prosecution of a US patent application. The total PTA is an addition to the 20-year lifespan of a US patent [5,9]. Since the term of a design patent is not affected by the length of time prosecution takes place, there are no patent term adjustment provisions for design patents. Adjustment information appears on the face of a patent [6,7]. For example, US Patent No. 6,399,594 was subject to an adjustment of 18 days, as depicted in Figure 1.

Patent Term Extension (PTE):(As per 35 U.S.C. 156)

It often takes many years to develop a commercially available medicine from the initial chemical compound made in the laboratory. During this period, when the compound is under clinical testing and regulatory approval, the patent's life is ebbing away [7]. Often, by the time a drug is brought to market over half of the patent life has expired. To act as an incentive to develop and bring new drugs to market, many countries have implemented a legal framework that allows the extension of pharmaceutical patents. An originator can, under certain circumstances, gain a further period of patent exclusivity to exploit a product commercially [10]. Such extensions can be of enormous commercial value, protecting a product at the height of its revenue stream. These extensions are known as patent term extension. Patent term extension is available to compensate for regulatory delays. Maximum patent term extension granted is 5 years. An application for a patent term extension can be made within six months of the issue of the patent. Patent term extension is available in the US, Japan, Israel, Australia, Taïwan, Korea and in some other countries for products subject to a regulatory approval [11] (Figure .2).

Criteria for Patent Term Extension (For US):[8]

a) Patentee or its agent must file an application for PTE that includes detailed statements about the activities undertaken to secure FDA approval.

b) Applicant must establish that the product was subjected to regulatory review period before its commercial marketing or use.

c) The applicant must show that the product permitted is either the first permitted commercial marketing or the use of the product after such regulatory review

d) Patent has not yet expired.

e) Patent term has not been previously extended.

Supplementary Protection Certificate (SPC)(Europe)

It is same as Patent term extension. It is a sui generis intellectual property (IP) right available for various regulated, biologically active agents, namely human

or veterinary medicaments and plant protection products.[11] Created by, and are governed by, an EU Regulation. It has a lifetime of 5 years. One supplementary protection certificate per product. An application for a supplementary protection certificate can be made within six months of the issue of the patent. The duration of a supplementary protection certificate is determined on the basis of the period of time between the registration of the basic patent and the granting of the first licence for the product within the European Community. Five years are deducted from that period of time. For example, there is a period of ten years between the registration date and the date of issue of the licence; the supplementary certificate will be valid for five years [12] (Figure 3).

A Certificate shall be Granted if:[13]

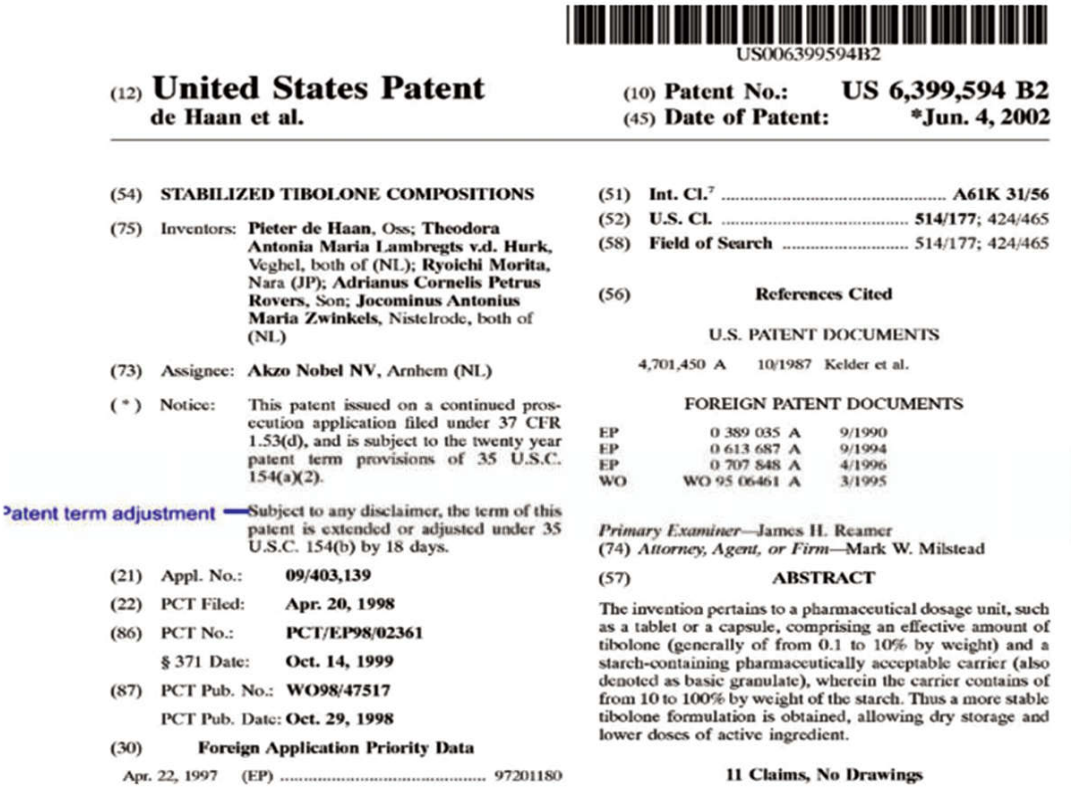
a. The product is protected by a basic patent in force;
b. A valid authorisation to place the product on the market as a medicinal product has been granted;
c. The product has not already been the subject of a certificate;
d. The authorisation referred to in point b) is the first authorisation to place the product on the market as a medicinal product.

Purpose of Extension of Patent Term

Capital investment in the pharmaceutical industry disproportionately related to laboratory research and clinical trials rather than the manufacture of the final product, patent exclusivity is the only effective way to protect and receive a return on that investment [13]. The purpose of the system for patent term extensions is to restore the period during which the patented invention was unable to be worked because it is necessary to obtain approvals or any other dispositions under a law [2].

Data Exclusivity (DE)

Data Exclusivity (DE) or exclusivity of registration data is the period of non-reliance and non-disclosure that is provided to new chemical entities, pharmaceutical compositions, and agrochemical registration data or test data. It is for a limited period of time when the drug regulatory authorities do not allow the test data of the originator to be used to register the generic version. Discovery and development of a new molecule takes about 8 to 10 years and costs millions of dollars, generating the test data takes about 50% of the time and expense. This data becomes very important at the time of obtaining marketing approval from regulatory authorities.



US006399594B2

(12) **United States Patent**
de Haan et al.

(10) **Patent No.: US 6,399,594 B2**
(45) **Date of Patent: *Jun. 4, 2002**

(54) **STABILIZED TIBOLONE COMPOSITIONS**

(75) Inventors: **Pieter de Haan**, Oss; **Theodora Antonia Maria Lambregts v.d. Hurk**, Veghel, both of (NL); **Ryoichi Morita**, Nara (JP); **Adrianus Cornelis Petrus Rovers**, Son; **Jocominus Antonius Maria Zwinkels**, Nistelrode, both of (NL)

(73) Assignee: **Akzo Nobel NV**, Arnhem (NL)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Patent term adjustment — Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 18 days.

(21) Appl. No.: **09/403,139**
(22) PCT Filed: **Apr. 20, 1998**
(86) PCT No.: **PCT/EP98/02361**
§ 371 Date: **Oct. 14, 1999**
(87) PCT Pub. No.: **WO98/47517**
PCT Pub. Date: **Oct. 29, 1998**

(30) **Foreign Application Priority Data**

Apr. 22, 1997 (EP) 97201180

(51) **Int. Cl.**[7] **A61K 31/56**
(52) **U.S. Cl.** **514/177**; 424/465
(58) **Field of Search** 514/177; 424/465

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Primary Examiner—James H. Reamer
(74) *Attorney, Agent, or Firm*—Mark W. Milstead

(57) **ABSTRACT**

The invention pertains to a pharmaceutical dosage unit, such as a tablet or a capsule, comprising an effective amount of tibolone (generally of from 0.1 to 10% by weight) and a starch-containing pharmaceutically acceptable carrier (also denoted as basic granulate), wherein the carrier contains of from 10 to 100% by weight of the starch. Thus a more stable tibolone formulation is obtained, allowing dry storage and lower doses of active ingredient.

11 Claims, No Drawings

Fig. 1: Front page of US6399594 illustrating patent term adjustment

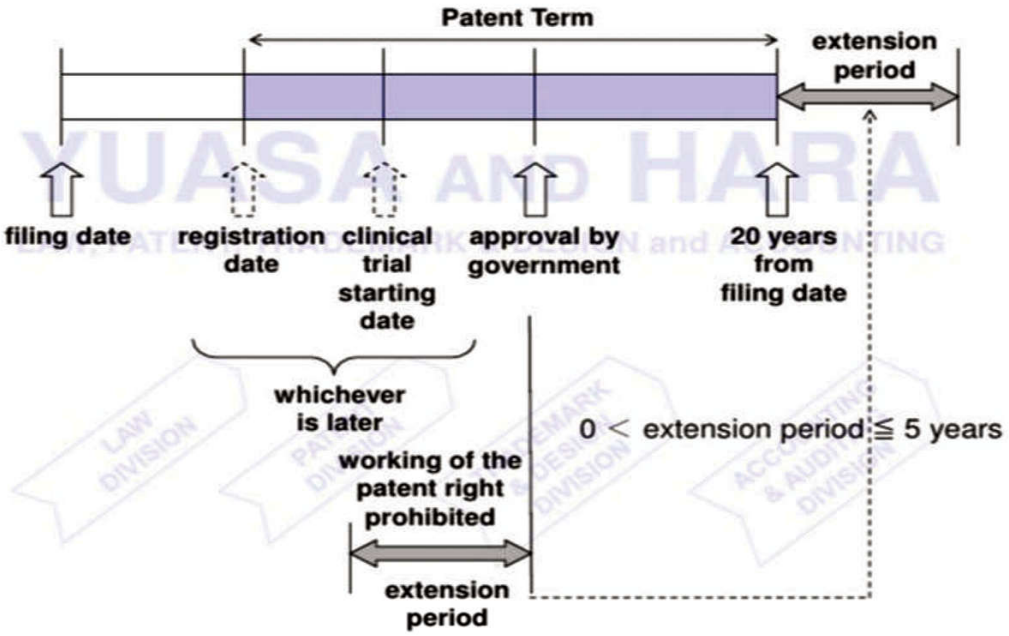

Fig. 2: Timeline of entire patent term with PTE

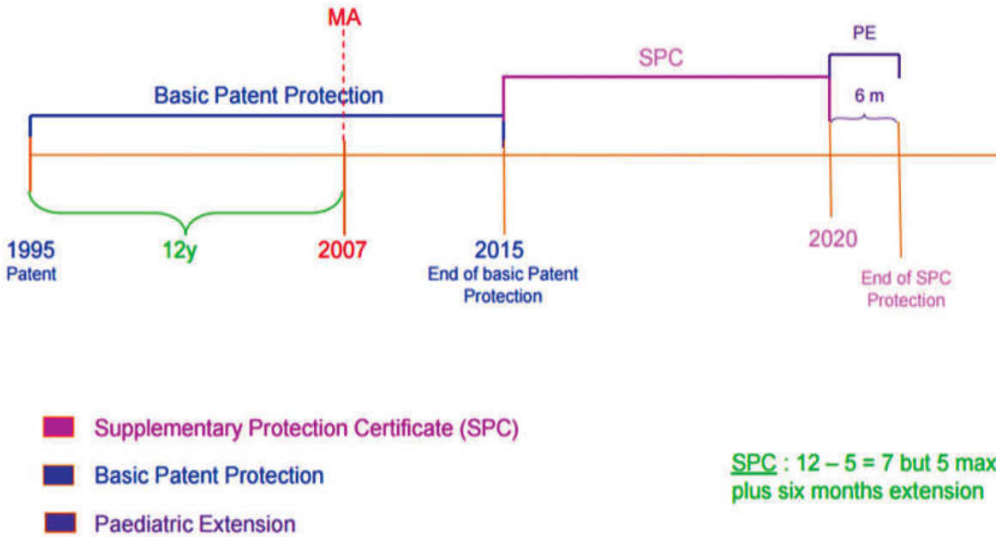

Fig. 3: Timeline of entire patent term with SPC

Data exclusivity provides the originator with rights to preclude third parties from relying on the data to obtain marketing approval for a specific period of time. However, it does not prevent third parties from generating their own data [14].

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Review Article

Oxazolones: A Review of Its Synthesis

Sajal Chhabra*, M. Shaqiqu Zamman*, M. Mumtaz Alam*, Mymoona Akhter*

Abstract

Oxazolones or oxazolidinone or azlactones are five membered heterocyclic lactams derived from oxazole, also known as (4*H*)-oxazol 5-ones. Oxazolones have been very widely explored as important synthons for the synthesis of several biologically active molecules. Oxazolones derivatives have been used as starting materials for the synthesis of modified á-amino acids, peptides, other heterocycles, and biosensors. This review preesnts the various methods of synthesis of oxazolone derivatives.

Keywords: Oxazolone; Hippuric Acid; Benzoylglycine.

Introduction

Oxazolones represent an important class of heterocyclic compounds with the molecular formula $C_3H_3NO_2$. It was named in-line with the Hantzsch–Widman nomenclature and is part of a large family of oxazole based compounds. There are a total of 5 structural isomers of oxazolone, three according to the location of the carbonyl group and two according to the location of the double bond C=X (with X= N or C) i.e. 2-(3*H*)-oxazolone, 2-(5*H*)-oxazolone, 4-(5*H*)-oxazolone, 5-(2*H*)-oxazolone, 5-(4*H*)-oxazolone [1].

Oxazolones has a special place in the synthesis of several organic molecules including amino acids [2], thiamine [3], amides [4], peptides [5] and polyfunctional compounds [6]. Oxazolone (Natural or synthetic) including benzoxazolone derivatives possess important biological activities; such as antimicrobial [7,8], anti-inflammatory [9], anticancer [10], antiangiogenic [11], pesticidal [12], cardiotonic [13], immunomodulator [14], and antioxidant [15] activity.

The synthetic ways for the preparation of oxazolone ring can be classified into several groups: cyclization reaction of benzoylglycine/acetylglycine with aromatic aldehydes in presence of acetic anhydride and different catalysts, cyclization reaction of benzoylglycine/acetylglycine with aromatic aldehydes in presence of different catalyst without acetic anhydride, synthesis from carbamates, reaction of benzoylglycine /acetylglycine with reagents other than aromatic aldehydes.

Cyclization Reaction of Benzoylglycine/ Acetylglycine with Aromatic Aldehydes in Presence of Acetic Anhydride and Different Catalysts.

Large number of catalyst have been used to accelerate the reaction ranging from sodium acetate through microwave irradiations to functionalized sphere SiO_2 nanoparticles.

Calcium acetate [16 (a,b)], bismuth (III) acetate [17], lead acetate [18], potassium phosphate [19] and ytterbium (III) triflate [20] have been used in place of sodium acetate for synthesis of substituted oxazolone derivatives.

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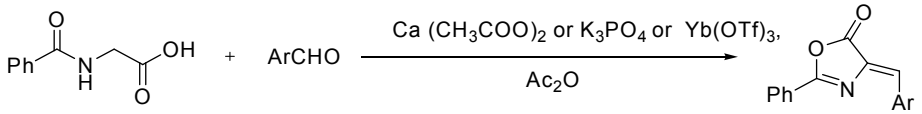
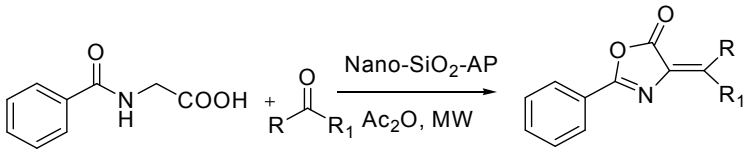
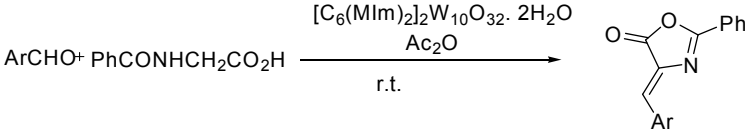


Akbar Mobinikhaledi *et. al.* have demonstrated effective use of microwave in presence of 2-aminopyridine-functionalized nano-sphere SiO_2 (nano-sphere SiO_2–AP) to synthesize oxazolone in good yield [21].

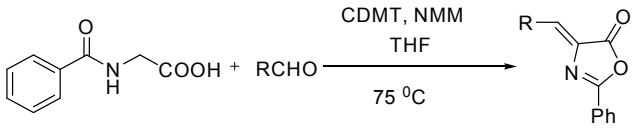


Use of $[C_6(MIm)_2]_2W_{10}O_{32}.2H_2O$ in ultrasonic conditions at room temperature has been explored by Mahboubeh Rostami *et. al.* [22] whereas Tikdari A. M. *et. al* has used three different catalyst (dodecatungstophosphoric acid ($H_3PW_{12}O_{40}$), samarium and ruthenium (^{222}) Chloride) to obtain substituted azalactones from hippuric acid and appropriate aldehydes or ketones under microwave irradiations [23].

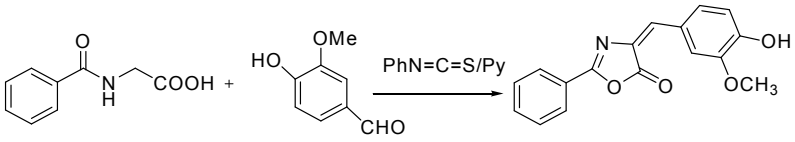


Cyclization Reaction of Benzoylglycine / Acetylglycine with Aromatic Aldehydes in Presence of Different Catalyst without Acetic Anhydride.

2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and N-Methylmorpholine (NMM) in tetrahydrofuran (THF) [24] has been utilized in place of sodium acetate and Acetic anhydride (Ac_2O) at room temperature (yield 65%) to obtain oxazolones from hippuric acid with aromatic aldehyde.

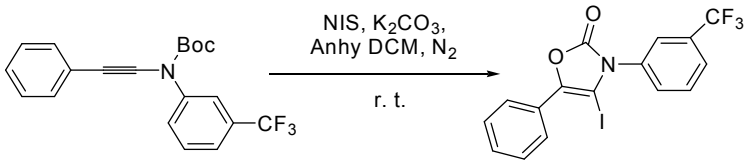


Isothiocyanate has also been utilized for cyclization of acetylglycine and aldehyde into oxazolones [25].

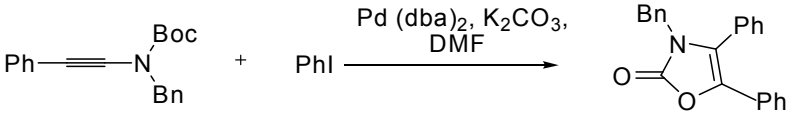


Synthesis from Carbamates

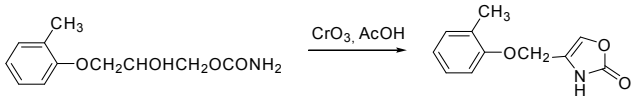
Oxazolones have been obtained from different carbamates using different catalyst in presence of base. *tert*-Butyl-*N*-phenyl-*N*-(phenylethynyl) carbamate and *tert*-butyl-2-phenylethynyl-(3-(trifluoromethyl) phenyl) carbamate has been used to obtain oxazolones by using palladium and *N*-iodosuccinimide respectively [26(a,b)].



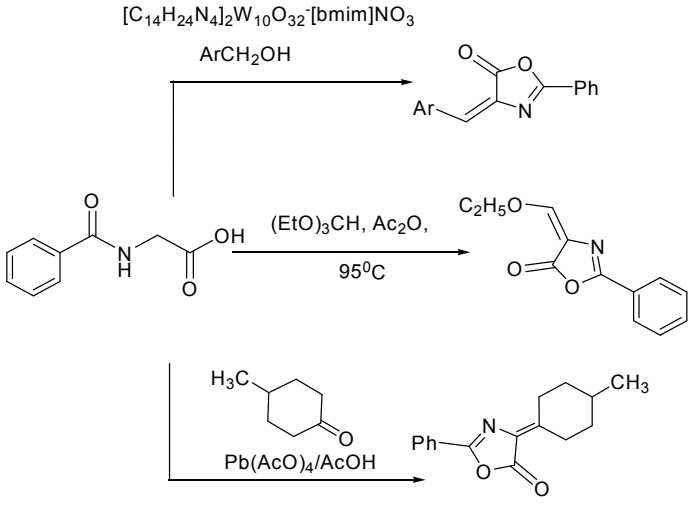
Zenghui Lu *et. al.* synthesized substituted oxazolones from *N*-alkynyl-*tert*-butyloxycarbamate with iodobenzene in presence of different Pd catalysts [$Pd(dba)_2$, $Pd(OAc)_2$, $Pd(PPh_3)_4$] along with different bases (Cs_2CO_3, Et_3N, *t*-BuOK, Na_2CO_3, K_2CO_3) and different ligands [(PPh_3), dppb, Xphos) in different solvents (DMF, DMSO, CH_3CN, dioxane, toluene). The combination of $Pd(dba)_2$, K_2CO_3, PPh_3 in DMF has been found to work best [27].



Mephenesin carbamate (2-hydroxy-3-(*o*-tolyloxy) propyl carbamate) a muscle relaxant has been converted to oxazolone by using CrO_3 and acetic acid [28].

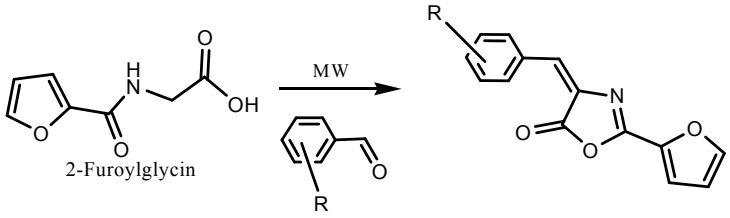


Reaction of benzoylglycine /acetylglycine with reagents other than aromatic aldehydes Oxazolones have been synthesized by replacing aromatic aldehyde with other reagents like triethyl-orthoformate with acetic anhydride [29] or aryl alcohol [30] or cyclic ketone [31] with immobilized $[(C_{14}H_{24}N_4)_2W_{10}O_{32}]$ on 1-*n*-butyl-3-methylimidazolium nitrate.



Microwave Assisted Synthesis [32]

Mariappan G et. al. has reported us of microwave to synthesize novel 4-(substituted benzyldiene)-2-furfurylidine oxazol-5-one and reported their antidiabetic activity.



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Review Article

Chac*toxi* Calc: A Computer Program for the Calculation of Chocolate Toxicity in Dogs

Sai Mahesh Reddy Avula*, Venkata Subba Reddy Avula**

Abstract

Assessment of chocolate toxicity is often carried out by veterinarians in pet clinics. The more reliable method used for this assessment is described here. The calculations involved in the assessment are complex, confusing, time consuming and not easily reproducible. Spreadsheet is a computer application in which data are arranged in rows and columns of a grid and can be manipulated and used in calculations. In today's era of smart phones, tablet PCs and netbooks, where a spreadsheet program in the form of Microsoft Excel [1] is readily accessible by most veterinarians, it should be possible to adapt the various laborious steps involved in the said method to a spreadsheet program by writing simple logical codes in the spreadsheets to do the calculations in a simple, clear, faster, reproducible, accurate and user friendly manner. This article describes a spreadsheet program (CHAC*Toxi* Calc) to calculate the chocolate toxicity

Keywords: Chocolate Toxicity Calculator; Chocolate Toxicity in Dogs; Toxicity Meter; Caffeine Poison in Dogs.

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Introduction

Assessment of chocolate toxicity is often carried out by veterinarians in pet clinics. The more reliable method used for this assessment is described here. The calculations involved in the assessment are complex, confusing, time consuming and not easily reproducible. Spreadsheet is a computer application in which data are arranged in rows and columns of a grid and can be manipulated and used in calculations. In today's era of smart phones, tablet PCs and netbooks, where a spreadsheet program in the form of Microsoft Excel [1] is readily accessible by most veterinarians, it should be possible to adapt the various laborious steps involved in the said method to a spreadsheet program by writing simple logical codes in the spreadsheets to do the calculations in a simple, clear, faster, reproducible, accurate and user friendly manner. This article describes a spreadsheet program (CHAC*Toxi* Calc) to calculate the chocolate toxicity [Figure 1].

Clinical signs usually occur within 6 to 12 hours of ingestion. Initial signs include polydipsia, vomiting, diarrhoea, bloating, and restlessness. Signs progress to hyperactivity, polyuria, ataxia, tremors, and seizures. Other effects include tachycardia, premature ventricular contractions, tachypnea, cyanosis, hypertension, hyperthermia, and coma. Less commonly, bradycardia and hypotension may occur. Hypokalaemia is possible late in the course of the toxicosis. Because of the high fat content of many chocolate products, pancreatitis is a potential sequel 24 to 72 hours after ingestion. Death is generally due to cardiac arrhythmias or respiratory failure.

Coding and Logical Data Used in this Program are as Follows;

Amount methyl xanthine in various chocolate types (mg/kg) is incorporated inC31 to C41 and D31 to D41

Compound	The obromine (mg/g)	Caffeine (mg/g)
White chocolate	0.00875	0.02975
Milk chocolate	2.03	0.21
Dark, sweet chocolate	4.55	0.7
Semi-sweet chocolate chips	4.83	0.77
Baker's (unsweetened) chocolate	13.755	1.645
Dry cocoa powder	25.795	2.45
Instant cocoa powder	4.76	0.525
Cocoa beans	21	NA
Coffee beans	0	21
Cocoa bean hulls	8.925	NA

Amount of Theobromine Can Be Calculated By Using the Logics As Shown Below

K18 = =D17*C32 (theobromineinWhite chocolate)

K19 = =D18*C33 (theobromineinMilk chocolate)

K20 = =D19*C34 (theobromineinDark, sweet chocolate)

K21 = =D20*C35 (theobromineinSemi-sweet chocolate chips)

K22 = =D21*C36 (theobromineinBaker's (unsweetened) chocolate)

K23 = =D22*C37 (theobromineinDry cocoa powder)

K24 = =D23*C38 (theobromineinInstant cocoa powder)

K25 = =D24*C39 (theobromineinCocoa beans)

K26 = =D25*C40 (theobromineinCoffee beans)

K27 = =D26*C41 (theobromineinCocoa bean hulls)

Amount of Caffeine Can Be Calculated By Using the Logics As Shown Below

L18 = =D17*D32 (caffeine inWhite chocolate)

L19 = =D18*D33 (caffeine inMilk chocolate)

L20 = =D19*D34 (caffeine inDark, sweet chocolate)

L21 = =D20*D35 (caffeine inSemi-sweet chocolate chips)

L22 = =D21*D36 (caffeine inBaker's (unsweetened) chocolate)

L23 = =D22*D37 (caffeine inDry cocoa powder)

L24 = =D23*D38 (caffeine inInstant cocoa powder)

L25 = =D24*D39 (caffeine inCocoa beans)

L26 = =D25*D40 (caffeine inCoffee beans)

L27 = =D26*D41 (caffeine inCocoa bean hulls)

Dose Theobromine received (mg/kg) can be calculated by the logics as shown below

F16 =SUM (K18:K27)/ (C14)

F17 (Dose Caffeine received (mg/kg)) =SUM (L18:L27)/ (C14)

F18 (Total Dose Methyl xanthine (mg/kg)) = F16+F17

Emergency Treatment Needed? Or not? Can be calculated by using the logic as shown below

=IF (OR (OR(F16>40, F17>40), (F16+F17)>40), "YES!!", "No")

Based on ASPCA Animal Poison Control Centre (APCC) experience, mild signs occur in animals ingesting 20 mg/kg of theobromine and caffeine, severe signs are seen at 40-50 mg/kg, and seizures occur at 60 mg/kg based on ASPCA/APCC Database: data (2).Doses from 20-40mg/kg may causing vomiting, diarrhoea. Doses > 40mg/kg should be decontaminated and then treated based on amount ingested and clinical signs.

One representative study is presented to help and understand the usefulness of the program. The findings of the experiment along with the calculation are presented as a screenshot of the program [Figure 1].

The difficulty which usually concerns veterinarians in clinic the toxicity assessment. They often encounter the following problems:

- The calculations involved in the assessment are time consuming
- The calculations appear confusing when reviewed at a later date

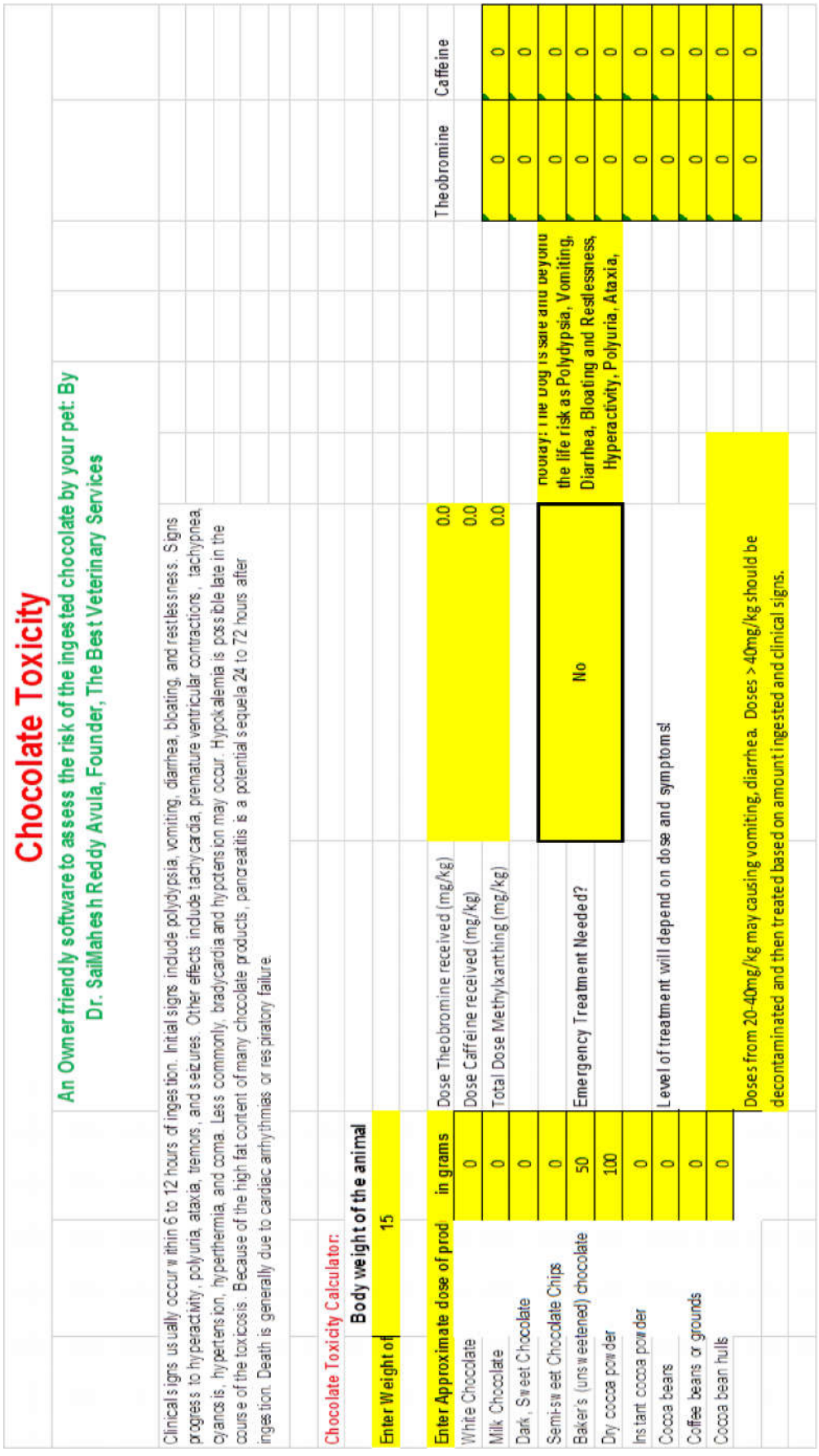

Fig. 1: Chocolate toxicity meter by using the developed spread sheet

- It is often difficult to explain in a report or communication how the final figure was arrived at.

The program described here can easily solve the above problems. It can be used to store, process, analyse and graphically represent data. A formula entered in a cell in the spreadsheet defines how the content of that cell is to be calculated from the contents of any other cell(s) each time the content of the other cell(s) is updated.

The program was thoroughly tested for the entire range of possible values. As the spreadsheet makes the whole process of assessment faster and user friendly, the program will be useful to veterinary professionals who are working in veterinary hospitals.

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Include summary of key findings (primary outcome measures, secondary outcome measures, results as they relate to a prior hypothesis); Strengths and limitations of the study (study question, study design, data collection, analysis and interpretation); Interpretation and implications in the context of the totality of evidence (is there a systematic review to refer to, if not, could one be reasonably done here and now?, What this study adds to the available evidence, effects on patient care and health policy, possible mechanisms)? Controversies raised by this study; and Future research directions (for this particular research collaboration, underlying mechanisms, clinical research). Do not repeat in detail data or other material given in the Introduction or the Results section.

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[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006; 35: 540-7.

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Article in supplement or special issue

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[5] Garoushi S, Lassila LV, Tezvergil A, Vallittu PK. Static and fatigue compression test for particulate filler composite resin with fiber-reinforced composite substructure. Dent Mater 2006.

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Chapter in book

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Reference from electronic media

[9] National Statistics Online—Trends in suicide by method in England and Wales, 1979-2001. www.statistics.gov.uk/downloads/theme_health/HSQ 20.pdf (accessed Jan 24, 2005): 7-18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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