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^{1}H-NMR based Metabolic Fingerprinting in Forensic Investigations

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Abstract

Nuclear Magnetic Resonance (NMR) spectroscopy plays a crucial role in forensic sciences by providing detailed chemical information about substances present in biological samples such as blood. NMR spectroscopy is a versatile tool in forensic sciences, offering non-destructive and high-resolution analysis of biological samples like blood. Machine learning tools are increasingly employed in forensic investigations to enhance data analysis, pattern recognition, and decision-making processes. These tools aid in processing vast amounts of forensic data efficiently and uncovering hidden insights from complex datasets. Its ability to provide detailed chemical information aids in substance identification, quantitative analysis, and understanding of NMR data interpretation, making it invaluable in criminal investigations and forensic pathology.

Keywords: Nuclear Magnetic Resonance spectroscopy; machine learning tools; Blood sample; Metabolites, 1D CPMG; 2D TOCSY.

INTRODUCTION

Forensic science is the application of several fields of science, such as biology, chemistry, physics, and medicine, to the domain of criminal investigation.[1] Through the forensic application and validation of scientific processes originally designed for diverse aims in a more confined framework, such as the legal medicine situation, it focuses mainly on providing evidence to be utilized in forensic science.[1]

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Since the scientific instruments available had been insufficient, forensic science has addressed several challenges in recent decades that were unfulfilled in the past.[1] The development of gas chromatography, liquid chromatography, and mass spectrometry (MS or MS/MS) methods for the quantification of several drugs and their metabolites in human biological fluids.[2] Otheradvanced imaging tools like computed tomography with or without contrast medium and magnetic resonance imaging or MRI in the identification of the cause of death, have all contributed to the solution of challenges such as human individuality, and toxicological analysis on different biological fluids.[3]

The qualitative and quantitative study of low molecular weight compounds found in biological systems is known as metabolomics, and it offers insights into the metabolic changes brought on by external stimuli.[4] Metabolism enables one to get a global profile of the metabolome or execute a specific study, depending on the information needed. One significant benefit of metabolomics for

forensics is the ability to combine data from several bodily compartments and biological fluids.[5-9]

NMR spectroscopy exploits the magnetic properties of atomic nuclei, particularly those of hydrogen atoms in the context of blood analysis. When placed in a magnetic field and subjected to radiofrequency pulses, nuclei such as protons within blood molecules absorb and re-emit electromagnetic radiation at characteristic frequencies.[10-11] By detecting these signals, NMR spectroscopy can generate detailed spectra that reveal the chemical composition and molecular structure of the sample under investigation. The data obtained from NMR spectroscopy is quantitative, allowing for precise measurements of concentrations and providing insights into the interactions between different components within the blood sample.[12-13] NMR spectroscopy can identify and quantify various drugs and metabolites present in blood samples, even at trace levels. This capability is particularly critical in cases involving drug-related offenses or incidents where substance abuse is suspected. By analyzing metabolic profiles, NMR spectroscopy can provide information about an individual's physiological state, including factors such as nutrition, health status, and exposure to environmental toxins.[14] NMR can complement traditional DNA profiling techniques by providing additional information about the chemical environment of nucleic acids, which can aid in confirming the identity of individuals from blood samples. NMR spectroscopy enables the identification and quantification of toxic substances in blood, such as heavy metals or poisons, which can be crucial in cases involving poisoning or environmental exposure.[15-17]

Advantages of NMR in forensic investigations include some important advantages Non-destructive Analysis using NMR spectroscopy minimal sample preparation and not consuming the sample, allowing for subsequent analyses if necessary.[1-2] Thus, **high sensitivity and Specificity with** NMR can detect substances at very low concentrations, enhancing its utility in forensic toxicology and drug detection. Furthermore, **quantitative analysis** to quantify concentrations of substances in blood samples makes NMR spectroscopy highly reliable for forensic applications. **The versatility of** NMR spectroscopy applies to a wide range of forensic analyses beyond blood, including other bodily fluids and tissue samples.[3-4]

Many studies areattentiveto the pathophysiological effects of acute and long-term alcoholic toxicity on the metabolomes profiling of humans.[1,18] Furthermore, several studies have focused on using NMR-based metabolomics of biological fluids or tissues to identify putative PMI biomarkers and track changes in endogenous metabolites brought on by death.[19-22]

We mainly investigated the potential use of NMR as an analytical platform for forensic blood metabolomic, or metabolic fingerprinting in the present study. Additionally, 1D and 2D NMR investigations were used to characterize and identify the metabolites in blood samples. This analysis was performed extensively for forensic purposes, where ^{1}H-NMR metabolic fingerprinting was presented as a new or alternative analytical approach.

Synopsis

NMR spectroscopy plays a critical role in forensic sciences by providing precise chemical analysis of biological samples, aiding in drug identification, toxicological screening, and determining causes of death. NMR spectroscopy was employed to screen blood samples collected fromindividuals. The technique detected and identified various metabolites simultaneously, providing comprehensive information vitalrole in metabolic fingerprinting. The integration of machine learning tools enhances the capabilities of forensic investigations by enabling advanced data processing, pattern recognition, and decision support. The referenced machine learning applications underscore the combined potential of NMR spectroscopy and machine learning in modern forensic science practices.

MATERIAL AND METHODS

Sample preparation

Blood samples were collected from each subject in the morning pre-prandial after overnight fasting in a heparinized vacutainer on the day of endoscopy. Blood plasma was immediately separated by centrifugation at 5000rpm for 10 minutes at 4°C and was stored at -80°C until NMR-based fingerprinting analysis. For NMR spectroscopy, 200 µl of plasma was diluted with 400 µl of D_2O, containing 0.5 mM of TSP and 0.5 mM of sodium formate. Blood samplesone-dimensional (1D) 1H NMR spectra were obtained using a CPMG pulse sequence that pre-saturated the H_2O peak. A spin-echo delay of 15 ms and a relaxation delay of 70 s was used for the experiment. A total of 64 scans were collected with 32K data points over a spectral width of 9124.1 Hz.A 70-second relaxation delay was used.

To facilitate chemical shift assignments, additional 2D NMR studies (2D TOCSY) were carried out. A typical parameter for the TOCSY experiments was acquired with a spectral width = 9124.1 Hz, data points = 2K in F2 dimension, 400 increments with 16 scans per increment, relaxation delay of 2.5 s, and mixing time of 80 ms.

NMR spectroscopy

All the NMR spectra were acquired at 700 MHz spectrometers (Agilent Technologies) at the Department of NMR, AIIMS, New Delhi. 1D (presat followed by CPMG) and 2D (TOCSY) NMR spectra were acquired for the assignment of metabolites.

The peaks observed in the 1D spectrum were assigned with the help of 2D NMR experiments. A conventional method was utilized to acquire a one-dimensional spectrum. Some important metabolites were assigned using 1D and 2D NMR.

NMR data analysis using machine learning tool

Free induction decay (FID) signals were processed by applying an exponential function with a line-broadening factor of 0.3 Hz before Fourier transformation. Then, the 1D [1]H-NMR spectra were manually phased and carefully corrected for baseline distortion. Chemical shift correction was referenced to the methyl group of TSP at 0.0 ppm. Each NMR spectrum was binned by 0.02 to 0.04 ppm and integrated using the Mestre Nova software (Version 9.0; Mestrelab Research S.L., La Coruña, Spain). Subsequently, the water resonance spectral area (δ5.5–4.5 ppm) was eliminated from the whole spectrum (δ10.0-0.5–0 ppm) to remove the skewed baseline caused by lacking water saturation.

The identification and characterization of blood metabolites and determined by comparing the intensity of metabolites isolated from peaks obtained by integration of the signal with that of formate.

The concentration of only those metabolites, that showed well-resolved peaks in the 1D spectrum of will be determined using the Chenomx software (Chenomx NMR Suite 7.1).

Statistical investigation was accomplished using SPSS (SPSS Inc., Chicago, IL, USA). The data were normalized and subjected to multivariate pattern recognition analysis using Metabo Analyst 6.0 software. Metabolites were considered significant at VIP >1.0 for further analysis of metabolomic data analysis. Partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-DA) loading plots were carried out for significant metabolite identification. Metabolites corresponding to significant spectral features were identified and characterized using the chemical shift and splitting patterns of metabolites reported in the literature,[24-29] two-dimensional NMR spectra.

RESULTS AND DISCUSSION

Spectral assignments and identification of metabolites of blood spectra

The resonance assignment of various metabolites detected in the blood plasma was carried out using 2D [1]H-NMR while the quantitative analysis of metabolites was carried out using peak integrals from the 1D [1]H-NMR spectra. Representative 1D [1]H-NMR spectrum of blood samples obtained from healthy individualsare shown in Fig. 1.

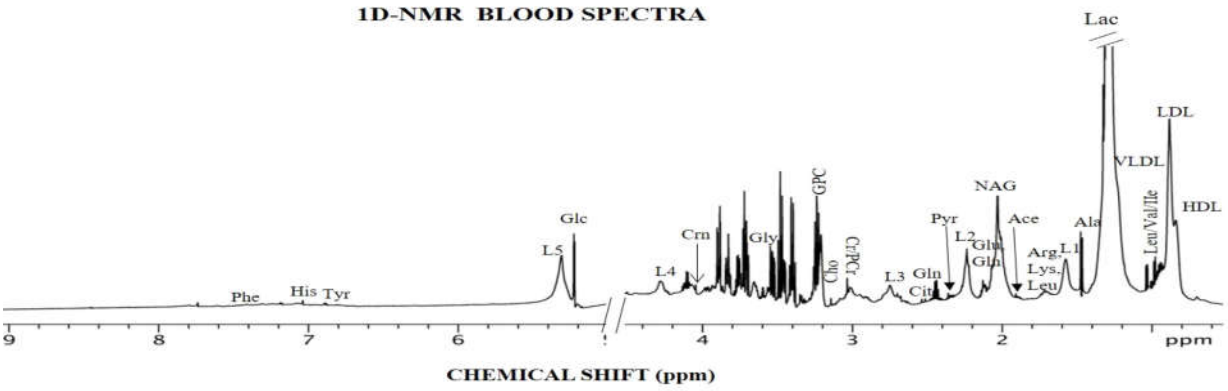

Fig. 1: Shows the representative of the one-dimensional (1D) Carr-Purcell-Meiboom Gill (CPMG) of [1]H-NMR (700 MHz) spectra in D2O at 25 °C of the blood sample. HDL: High-density Lipoproteins; LDL: Low-density lipoproteins; VLDL: Very low-density lipoproteins; Leu: Leucine; IIe: Isoleucine Val: Valine; Lac: Lactate; OHB; hydroxybutyrate; Ala: Alanine; Ace: Acetate; NAG; N-acetyl glycoproteins; Glu: Glutamate; Gln: Glutamine; Pyr: Pyruvate; Cit: Citrate; Cr: Creatine; PCr: Phosphocreatine; Cho: Choline; GPC: Glycerophosphocholine; Glc: glucose; Gly: Glycine; Crn: Creatinine; Tyr: Tyrosine; His: Histidine; Phe; Phenylalanine.

The resonance assignments were made based on coupling connectivity observed in 2D spectra (TOCSY) and compared well with the chemical shift values of metabolites in blood samples. As an advantage of TOCSY, cross-peaks are formed between every component of a connected network. In all, metabolites could be unambiguously assigned in the blood plasma sampleusing 1D and 2D TOCSY spectra.

The 1D [1]H-NMR spectrum of blood plasma showed peaks due to metabolites such as amino acids, sugars phospholipids, organic acids, and other compounds.[23] Representative 2D TOCSY spectra obtained from blood used for metabolite assignments are shown in Fig. 2.

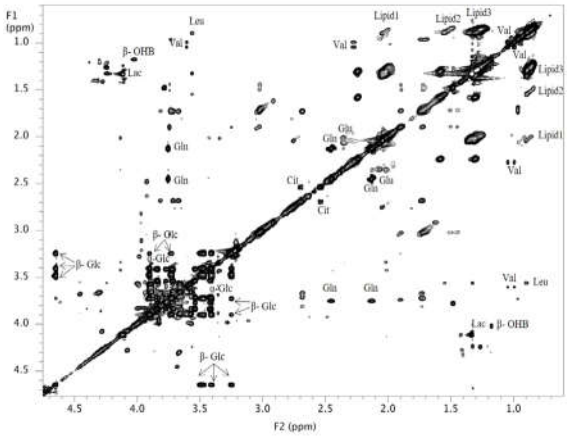

Fig 2: Shows representative region (0.7–4.6 ppm) of the two-dimensional total correlation (2D TOCSY) 1H NMR spectrum of the blood acquired at 700 MHz at 25 °C and assigned cross peak of various metabolites. VLDL: very low-density lipoproteins; Leu: Leucine; Ile: Isoleucine; Val: Valine; β-OHB: β-hydroxybutyrate; Lac: Lactate; Glu: Glutamate; Gln: Glutamine; Cit: Citrate; Glc: Glucose.

1D and 2D NMR spectra

Representative 1D proton NMR spectra of blood samples obtained from a healthy individual are shown in Fig. 1. In all, metabolites were assigned unambiguously using 1D and 2D TOCSY spectra are shown in Fig. 1 and Fig. 2, respectively, and by comparing with the chemical shift values reported in the literature. The identification of metabolites that gave well-resolved signals in the 1D proton NMR spectrum was determined. In the 1D NMR spectrum, chemical shift and spin multiplicity are the most important parameters for resonance assignment. In 2D experiments, the resonances assignment is primarily dictated by the observation of cross peaks between coupled protons.[30-37] 2D TOCSY experiments provide a particularly powerful combination that may be sufficientto identify most metabolites in the blood. Various metabolites assigned and respective chemical shift positions of resonance arising from chemical entities, and blood are presented in Table 1.

Resonance peaks arising from ten amino acids could be identified unambiguously in the spectra ofthe blood sample. Between 0.90 and 1.00 ppm, the methyl protons of isoleucine (Ile), leucine (Leu), and valine (Val) were identified. The other protons of Ile, Leu, and Val were assigned using cross-peaks observed in TOCSY. IsoleucineCH_3 protons at 0.95 ppm showed coupling with its Gamma γ-CH_2 at 1.25 and 1.46 ppm, respectively. The methyl protons of alanine (Ala) at 1.48 ppm showed connectivity to its CH at 3.76 ppm. The 1D spectrum shows the resonances caused by the CH2 protons of glutamate (Glu) and glutamine (Gln) as a multiplet at 2.36 and 2.45 ppm, respectively. At 3.57 ppm, a glycine (Gly)-related singlet was detected. Furthermore, resonance was found in aromatic amino acids, such as tyrosine (Tyr) and phenylalanine (Phe). To identify Tyr's resonances, an unambiguous cross peak between H3, H5 (6.91 ppm) and H2, H6 (7.18 ppm) was identified. Similarly, H2, H6 resonances of Phe which were degenerate at 7.33 ppm showed coupling with its H3, H5 protons resonating at 7.43 ppm.

Resonances due to various organic acids such as lactate (Lac) and pyruvate (Pyr) were also observed. The doublet corresponding to methyl protons of Lac at 1.33 ppm showed connectivity with itsCH proton at 4.12 ppm in TOCSY. The CH_3 proton of Pyr was detected at 2.37 ppm. The AB pattern of citrate (Cit) was identified in the 1D spectrum by characteristics doublet of doublets cantered at 2.54 ppm and Formate (For) singlet at 8.46 ppm was observed.

The N $(CH_3)_3$ of choline (Cho) wasdetected at 3.21 ppm. The cross peaks due to glycerophosphocholine (GPC) were seen clearly in the TOCSY. The N (CH_3) protons of GPC were observed at 3.23 ppm. A singlet peak at 1.91 ppm was assigned to the methyl protons of acetate (Ace). The signals due to hydroxybutyrate (βHOB) were observed at 1.20 ppm as a doublet. The connectivity between the CH_3 group at 1.20 ppm and CH resonance at 4.14 ppm of βHOB was observed.[30-37]

The H1'of-glucose(α-Glc) was assigned at 5.23 ppm which showed coupling to its H2' proton at 3.53 ppm in TOCSY. Similarly, the H1' of glucose (β-Glc) was observed as a doublet at 4.64 ppm. Resonances fromGlc were assigned following the connectivity pattern of H1' (4.64 ppm) with H2' (3.24 ppm), H4' (3.41 ppm) and H5' (3.47 ppm) protons. Other Metabolites Assignment Several resonances

Table 1: List of identified metabolites in the blood sample of healthy individuals using NMR spectroscopy and their chemical shift (ppm)position so fresonances1 H of metabolites. Code (identifiers) of the metabolites of data sets was searched indifferent metabolites Data bases (KEGGID and HMDB).

Metabolites	Multiplicity (δ) ppm	KEGG ID	HMDB
Isoleucine (Ile)	3.62 (α-CH), 1.97 (β-CH), 1.26 (γ CH2), 1.48 (γ′-CH2), 0.94 (δ-CH3)	C00407	HMDB0000172
Valine (Val)	3.52 (α-CH), 2.21 (β CH),1.00 (γ-CH3), 1.04 (γ′-CH3)	C00183	HMDB0000883
Leucine (Leu)	3.72 (α-CH), 1.72 (β-CH2), 1.69 (γ-CH2), 0.96 (δ-CH3)	C00123	HMDB0000687
Alanine (Ala)	3.77 (α-CH), 1.48 (β-CH3)	C00041	HMDB0000161
Glutamate (Glu)	3.65 (α-CH), 2.04 (β-CH2), 2.36 (γ-CH2)	C00025	HMDB0000148
Glutamine (Gln)	3.65 (α-CH), 2.08 (β-CH2), 2.45 (γ-CH2)	C00064	HMDB0000641
Methanol (MeOH)	3		
Glycine (Gly)	3.56 (CH2)	C00037	HMDB0000123
Histidine (His)	3.14 (β-CH2), 7.06 (H4), 7.83 (H2)	C00135	HMDB0000177
Tyrosine (Tyr)	7.20 (H2, H6), 6.88 (H3, H5)	C00082	HMDB0000158
Phenylalanine (Phe)	7.33 (H2, H6), 7.40 (H3, H5)	C00079	HMDB0000159
Choline (Cho)	3.21 N(CH3)3, 3.52 (NCH2), 4.07 (CH2OH)	C00114	HMDB0000097
Glycerophosphocholine (GPC)	3.23 N(CH3)3, 3.52 (NCH2)	C00670	HMDB0000086
Acetate (Ace)	1.92 (CH3)	C00033	HMDB0000042
Acetoacetate (AcAc)	2.23 (CH3)	C00164	HMDB0000060
Creatine (Cr)	3.03 (NCH3), 3.93(CH2)	C00300	HMDB0000064
Phosphocreatine (PCr)	3.04 (NCH3)	C02305	HMDB0001511
Creatinine (Crn)	4.06 (CH2)	C00791	HMDB0000562
Lactate (Lac)	4.12 (α-CH), 1.33 (β-CH3)	C00186	HMDB0000190
Pyruvate (Pyr)	2.37 (CH3)	C00022	HMDB0000243
Glucose (Glc)	5.23 (H1'), 4.64 (H1'), 3.53 (H2'), 3.72 (H3'), 3.42 (H4'), 3.24 (H2')	C00031	HMDB0000122

such as methyl, methylene, allylic and olefinic protons from different lipids and triglycerides were also observed in the 1D CPMG spectra of lipids in blood plasma. The resonances from the terminal CH_3 protons of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) overlapped at 0.90 ppm. In TOCSY, a prominent cross peak was seen between the CH_3 and CH_2 protons of LDL and VLDL. The resonance corresponding to CH_3 of HDL was assigned at 0.84 ppm. Similarly, the resonances of Gly, Tyr, histidine (His), and Phe were assigned using TOCSY.

CONCLUSION

In conclusion, NMR spectroscopy represents a powerful analytical technique that has revolutionized forensic investigations, particularly in the analysis of blood samples. Its ability to provide detailed chemical information with high sensitivity and specificity makes it invaluable in determining crucial aspects of criminal cases, ranging from drug detection to DNA analysis. As technology continues to advance, NMR spectroscopy is expected to play an increasingly prominent role in forensic science, contributing to enhanced accuracy and reliability in criminal investigations worldwide.

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