Available online on www.ijcpr.com
International Journal of Current Pharmaceutical Review and Research; 8(2); 79-84
doi: 10.25258/ijcprr.v8i02.9189
ISSN: 0976 822X

Review Article

Uses of Nuclear Magnetic Resonance Spectroscopy Technique in Pharmaceutical Analysis: A Review

Imad Hadi Hameed[1*], Abeer Fauzi Al-Rubaye[2], Mohanad Jawad Kadhim[3]

[1]*College of Nursing, University of Babylon, Iraq*
[2]*Department of Biology, College of Science for women, University of Babylon, Iraq*
[3]*College of Biotechnology, Department of Genetic Engineering, Al-Qasim Green University, Iraq*

Available Online: 25th April, 2017

ABSTRACT
Nuclear magnetic resonance (NMR) is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation. Many scientific techniques exploit NMR phenomena to study molecular physics, crystals, and non-crystalline materials through nuclear magnetic resonance spectroscopy. NMR phenomena are also utilized in low-field NMR, NMR spectroscopy and MRI in the Earth's magnetic field (referred to as Earth's field NMR), and in several types of magnetometers. Modern NMR spectroscopy has been emphasizing the application in biomolecular systems and plays an important role in structural biology. NMR spectroscopy is very important to identify a drug or an excipient, evaluate the level of impurities (and to elucidate the structure), observe the course of a decomposition, to evaluate residual solvents, determine the isomeric composition, i.e. the ratio of diastereomers and the enantiomeric excess by means of chiral additive, assess a single drug or drug composition, characterize a polymer mostly being a mixture and used as excipients, identify counter ions (if of organic origin and having protons), characterize an entire formulation, e.g. a tablet. Fundamentals of quantitative NMR spectroscopy NMR spectroscopy can be considered as a primary ratio method of measurement being characterized by the fact that the ratio of substances can be determined directly from the physical context of the measurement without referencing to another substance. NMR has become one of the most powerful and versatile spectroscopic techniques for the analysis of biomacromolecules, allowing characterization of biomacromolecules and their complexes up to 100 kDa. Together with X-ray crystallography.

Keyword: Nuclear magnetic resonance, spectroscopy, Applicacion, Metabolite analysis, Chemical analysis.

INTRODUCTION

NMR spectroscopy is known to be not very sensitive. However, for quality assessment of an API the sensitivity is sufficient as long as the signals considered are separated[1-3]. The sensitivity has been enhanced in the past years by the development of high-field spectrometers (>400 MHz) for routine purposes, invention of gradient shimming techniques, and inverse or cryo probes as well as the microcoil technology. There are innumerable examples reported where the structure of an impurity, being normally not present in a drug, is elucidated by means of NMR spectroscopy. Even though NMR spectroscopy is a powerful tool for quality control it is rarely used in international pharmacopoeias[4-6]. Nevertheless, the pharmaceutical industries, especially "big pharma", started to use NMR spectra more often in routine quality analysis. However, it will take quite some time till q NMR spectroscopy will find its way into the monographs of the international pharmacopoeias. Moreover, a large market has developed for herbal medicines (HMs) as a safe alternative to synthetic PDE-5 inhibitors. Indeed, in contrast to conventional pharmaceuticals, HM are regarded by many as being harmless and free from any side-effects because of their natural origin. However, their adulteration with conventional drugs is a growing trend and poses a health threat to patients who unwittingly consume a synthetic drug[7-11]. HM marketed for enhancement of sexual function are the most affected. NMR spectroscopy is one of the two leading technologies for the structure determination of biomacromolecules at atomic resolution[12].

Applications of NMR spectroscopy are listed below

Solution structure

The only method for atomic-resolution structure determination of biomacromolecules in aqueous solutions under near physiological conditions or membrane mimeric environments. Structure determination by NMR is an established technique and is routinely used to determine three-dimensional structures of biological macromolecules in solution with molecular weights up to 30 kDa[13-21]. The power of NMR over other spectroscopic techniques results from the fact that every NMR active nucleus gives rise to an individual signal (resonance line) in the spectrum that can be resolved by multi-dimensional NMR techniques[22-29]. NMR spectra of biological macromolecules contain hundreds or even thousands of resonance lines which cannot be resolved in a

**Author for Correspondence: imad_dna@yahoo.com*

conventional one-dimensional (1D) NMR experiment. However, for a detailed analysis resolved lines are a prerequisite. Further, the interpretation of NMR data requires correlations between different nuclei which are implicitly contained in 1D spectra but often difficult to extract[30-36]. Large molecules tumble slower which results in faster relaxation and consequently broader lines in the NMR spectrum. Thus, the corresponding spectra show poor resolution and sensitivity. In practice, it becomes very hard to determine structures from proteins that have molecular weights above 40 kDa[37].

Molecular dynamics

The most powerful technique for quantifying motional properties of biomacromolecules[38].

Protein folding

The most powerful tool for determining the residual structures of unfolded proteins and the structures of folding intermediates. An efficient structure determination by NMR requires a highly purified protein preparation. An inhomogeneous preparation and/or aggregation of the protein as well as low molecular weight protonated impurities may severely impair the structure determination. The first step in every protein NMR study therefore involves optimization of the measurement conditions[39-46]. The pH, ionic strength, and temperature can often be adjusted to mimic physiological conditions. The macromolecule under study should be stable in the chosen conditions for many weeks. Any buffers, co-solvents and additives (e.g. detergent molecules) used should be hydrogen-free or deuterated[47].

Ionization state

The most powerful tool for determining the chemical properties of functional groups in biomacromolecules, such as the ionization states of ionizable groups at the active sites of enzymes. Weak intermolecular interactions Allowing weak functional interactions between macrobiomolecules (e.g., those with dissociation constants in the micromolar to millimolar range) to be studied, which is not possible with other technologies[49].

Protein hydration

A power tool for the detection of interior water and its interaction with biomacromolecules[50].

Hydrogen bonding

A unique technique for the DIRECT detection of hydrogen bonding interactions.

Drug screening and design

Particularly useful for identifying drug leads and determining the conformations of the compounds bound to enzymes, receptors, and other proteins. Pharmaceutical and academic nuclear magnetic resonance (NMR) groups have implemented NMR screening techniques as a powerful approach to identify and to investigate protein/ligand interactions. Pharmaceutical groups in particular have incorporated NMR screening strategies into their drug discovery and drug design programs[51-58]. This stems from the fact that NMR screening is naturally synergistic with combinatorial or medicinal chemistry, high throughput screening (HTS), structure-based drug design, and genomics. Once the drug target is characterized, initial hits have to be found. Several NMR techniques are applicable for this purpose; some of them can be performed in a high-throughput manner. For a detailed discussion of the techniques, the reader is referred elsewhere. In this paragraph, only the basis of NMR screening is described[59-63]. The effects resulting from the binding of a ligand to a protein can be divided into two classes: global and local effects. The former are size-dependent and therefore are suited to the observation on the ligand, while the latter are restricted to the binding region and can be monitored on both the target and the ligand. Upon binding to a macromolecule, the apparent molecular weight and hydrodynamic radius of a small ligand change dramatically by several orders of magnitude. Those changes can be detected with several different NMR experiments[64-66]. Relaxation filtering and diffusion editing reveal binding, but cannot provide any structural information. In contrast, NOE-based methods (NOE pumping, reverse NOE pumping) or STD not only prove interactions, in addition they can be used to characterize the binding epitope of the ligand[67].

Native membrane protein

Solid state NMR has the potential for determining atomic-resolution structures of domains of membrane proteins in their native membrane environments, including those with bound ligands.

Metabolite analysis

A very powerful technology for metabolite analysis. Metabonomics is defined as ‘the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification’ and is concerned with the study of the metabolic response of organisms to disease, environmental change or genetic modification[68]. Metabonomics has many areas of application including biology and medicine with new developments such as pharmacometabonomics (the ability to predict drug responses prior to drug dosing) and the more general area of predictive metabonomics, emerging recently. Generally, the metabolic profile of a biological fluid is stable over a significant period of time at room temperature, and certainly stable enough for the acquisition of routine 1D and 2D 1HNMR data. However, there are exceptions. Some biological fluids are inherently unstable. A good example of this is human seminal fluid, where, post-ejaculation, enzymatic reactions take place that cause the biochemical transformation of some metabolites[69,70]. In addition, if a sample such as animal urine, has been in contact with animal faeces at any stage, it will be microbiologically contaminated and potentially unstable. Bacterial growth in a urine sample, for instance, will result in the transformation of certainmetabolites into newproducts, as the bacteria scavenge the biofluid for fuel sources.

Chemical analysis

A matured technique for chemical identification and conformational analysis of chemicals whether synthetic or natural. Material science a powerful tool in the research of polymer chemistry and physics. Nuclear magnetic resonance (NMR) is one of the most powerful tools in biology, chemistry, physics and medicine for the

investigation of structure, morphology and dynamics of various classes of compounds. Few analytical techniques in science match, in either breadth or depth, the impact achieved by nuclear magnetic resonance[71]. Polymer characterisation by Nuclear Magnetic Resonance Spectroscopy (NMR) provides detailed structural information for product development and quality control considerations (QC). Expert evaluation of polymers by specialist techniques is essential in order to ensure product integrity and for quality control demands. One of the challenges polymer scientists face is molecular weight (average chain length) determination of their materials. While membrane osmometry, gel permeation chromatography, viscosity analysis and mass spectrometry are typically used for molecular weight determination, the techniques can be time consuming, inaccurate for the molecular weight ranges involved, or require specialized instrumentation. End-group analysis by NMR offers an easy alternative method using an instrument commonly found in many analytical labs. In addition, NMR analysis can also be used to accurately determine monomer ratios for various copolymer[72].

CONCLUSION

NMR allows the observation of specific quantum mechanical magnetic properties of the atomic nucleus. The absolute amount of substances can be determined by using simple reference substances, which holds also true for coulometry, gravimetry or titrimetry. NMR is also routinely used in advanced medical imaging techniques, such as in magnetic resonance imaging (MRI). A key feature of NMR is that the resonance frequency of a particular substance is directly proportional to the strength of the applied magnetic field. NMR has become a sophisticated and powerful analytical technology that has found a variety of applications in many disciplines of scientific research, medicine, and various industries. In addition, NMR provides unique and important molecular motional and interaction profiles containing pivotal information on protein function.

ACKNOWLEDGEMENT

I thank Dr. Amean A. Al-yasiri, College of Nursing, for valuable suggestions and encouragement.

REFERENCES

1. U. Holzgrabe, I. Wawer, B. Diehl (Eds.), NMR Spectroscopy in Pharmaceutical Analysis, Elsevier, Amsterdam, 2008, pp. 157–180.
2. T. Beyer, U. Holzgrabe, Quantitative NMR spectroscopy of biologically active substances and excipients. *Bioanal. Rev.* 2011; 2: 1–22.
3. B.W.K. Diehl, F. Malz, U. Holzgrabe, Quantitative NMR spectroscopy in quality evaluation of active ingredients and excipients. *Spectrosc. Eur*. 2007; 19: 15–19.
4. T.J. Quinn, Primary methods of measurement and primary methods. *Metrologia*. 1997;34: 61–65.
5. F. Malz, H. Jancke, Validation of quantitative NMR. *J. Pharm. Biomed. Anal*. 2005; 38: 813–822.
6. U. Holzgrabe, Quantitative NMR spectroscopy in pharmaceutical applications. *Progr. NMR Spectrosc*. 57 (2010) 229–240.
7. U. Holzgrabe, Quantitative NMR, methods and applications, in: J. Lindon, G.Tranter, D. Koppendahl (Eds.), Encyclopedia of Spectroscopy and Spectrometry, vol. 3, 2nd ed., Elsevier, 2010, pp. 2331–2339.
8. G. Maniara, K. Rajamoorthi, S. Rajan, G.W. Stockton, Method performance and validation for quantitative analysis by 1H and 31P NMR spectroscopy. Applications to analytical standards and agricultural chemicals. *Anal. Chem*. 70 (1998) 4921–4928.
9. T. Beyer, C. Schollmayer, U. Holzgrabe, The role of solvents in the signal separation for quantitative 1H NMR spectroscopy, *J. Pharm. Biomed. Anal*. 52 (2010) 51–58.
10. U. Holzgrabe, S.K. Branch, 1H, 19F and 13C NMR data of fifteen gyrase inhibitors and some metabolites, *Magn. Res. Chem*. 32 (1994) 192–197.
11. G. Zheng, W.S. Price, Solvent signal suppression in NMR, Progr. NMR Spectrosc. 56 (2010) 267–288.
12. S. Michaleas, E. Antoniadou-Vyza, A new approach to quantitative NMR: fluoroquinolones analysis by evaluating the chemical shift displacements, J. Pharm. Biomed. Anal. 42 (2006) 405–410.
13. E.L. Eliel, S.H. Wilen, Stereochemistry of Organic Compounds, Wiley Interscience, New York, 1994 (Chapter 6.5).
14. U. Holzgrabe, I. Wawer, B. Diehl (Eds.), NMR Spectroscopy in Pharmaceutical Analysis, Elsevier, Amsterdam, 2008, pp. 269–289.
15. J.E. Tanner, Use of stimulated echo in NMR diffusion studies. *J. Chem. Phys*. 52 (1970) 2523–2526.
16. M.D. Pelta, H. Barjat, G.A. Morris, A.L. Davis, S.J. Hammond, Pulse sequences for high-resolution diffusion-ordered spectroscopy (HR-DOSY). *Magn. Reson. Chem*. 36 (1998) 706–714.
17. K.F. Morris, C.S. Johnson, Diffusion-ordered two-dimensional nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc*. 114 (1992) 3139–3141.
18. D.H. Wu, A.D. Chen, C.S. Johnson, An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses, *J. Magn. Reson. Ser. A* 115 (1995) 260–264.
19. T.K. Kishimoto, K. Viswanathan, T. Ganguly, S. Elankumaran, S. Smith, K. Pelzer, J.C. Lansing, N. Sriranganathan, G. Contaminated heparin associated with adverse clinical events and activation of the contact system, *N. Eng. J. Med*. 358 (2008) 2457–2467.
20. M. Guerrini, D. Beccati, Z. Shriver, A. Naggi, K. Viswanathan, A. Bisio, I. Capila, J.C. Lansing, S. Guglieri, B. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events, *Nat. Biotechnol*. 26 (2008) 669–675.
21. Kadhim MJ, Sosa AA, Hameed IH. Evaluation of anti-bacterial activity and bioactive chemical analysis of Ocimum basilicum using Fourier transform infrared

(FT-IR) and gas chromatography-mass spectrometry (GC-MS) techniques. *International Journal of Pharmacognosy and Phytochemical Research.* 2016; 8(6): 127-146.
22. Mohammed GJ, Kadhim MJ, Hussein HM. Characterization of bioactive chemical compounds from Aspergillus terreus and evaluation of antibacterial and antifungal activity. *International Journal of Pharmacognosy and Phytochemical Research.* 2016; 8(6): 889-905.
23. Hameed IH, Altameme HJ, Idan SA. Artemisia annua: Biochemical products analysis of methanolic aerial parts extract and anti-microbial capacity. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*. 2016; 7(2): 1843- 1868.
24. Hussein AO, Mohammed GJ, Hadi MY, Hameed IH. Phytochemical screening of methanolic dried galls extract of Quercus infectoria using gas chromatography-mass spectrometry (GC-MS) and Fourier transform-infrared (FT-IR). *Journal of Pharmacognosy and Phytotherapy*. 2016; 8(3): 49-59.
25. Sosa AA, Bagi SH, Hameed IH. Analysis of bioactive chemical compounds of Euphorbia lathyrus using gas chromatography-mass spectrometry and fourier-transform infrared spectroscopy. *International Journal of Pharmacognosy and Phytochemical Research.* 2016; 8(5): 109-126.
26. Altameme HJ, Hadi MY, Hameed IH. Phytochemical analysis of *Urtica dioica* leaves by fourier-transform infrared spectroscopy and gas chromatography-mass spectrometry. *Journal of Pharmacognosy and Phytotherapy*. 2015; 7(10): 238-252.
27. Mohammed GJ, Omran AM, Hussein HM. Antibacterial and Phytochemical Analysis of *Piper nigrum* using Gas Chromatography-Mass Spectrum and Fourier-Transform Infrared Spectroscopy. *International Journal of Pharmacognosy and Phytochemical Research*. 2016; 8(6): 977-996.
28. Hamza LF, Kamal SA, Hameed IH. Determination of metabolites products by *Penicillium expansum* and evaluating antimicobial activity. *Journal of Pharmacognosy and Phytotherapy.* 2015; 7(9): 194-220.
29. Jasim H, Hussein AO, Hameed IH, Kareem MA. Characterization of alkaloid constitution and evaluation of antimicrobial activity of *Solanum nigrum* using gas chromatography mass spectrometry (GC-MS). *Journal of Pharmacognosy and Phytotherapy*. 2015; 7(4): 56-72.
30. Hadi MY, Mohammed GJ, Hameed IH. Analysis of bioactive chemical compounds of Nigella sativa using gas chromatography-mass spectrometry. *Journal of Pharmacognosy and Phytotherapy*. 2016; 8(2): 8-24.
31. Hameed IH, Ibraheam IA, Kadhim HJ. Gas chromatography mass spectrum and fourier-transform infrared spectroscopy analysis of methanolic extract of *Rosmarinus oficinalis* leaves. *Journal of Pharmacognosy and Phytotherapy*. 2015; 7 (6): 90-106.
32. Shareef HK, Muhammed HJ, Hussein HM, Hameed IH. Antibacterial effect of ginger (*Zingiber officinale*) roscoe and bioactive chemical analysis using gas chromatography mass spectrum. *Oriental Journal of Chemistry*. 2016; 32(2): 20-40.
33. Al-Jassaci MJ, Mohammed GJ, Hameed IH. Secondary Metabolites Analysis of *Saccharomyces cerievisiae* and Evaluation of Antibacterial Activity. *International Journal of Pharmaceutical and Clinical Research*. 2016; 8(5): 304-315.
34. Mohammed GJ, Al-Jassani MJ, Hameed IH. Anti-bacterial, Antifungal Activity and Chemical analysis of *Punica grantanum* (Pomegranate peel) using GC-MS and FTIR spectroscopy. *International Journal of Pharmacognosy and Phytochemical Research.* 2016; 8(3): 480-494.
35. Al-Marzoqi AH, Hadi MY, Hameed IH. Determination of metabolites products by *Cassia angustifolia* and evaluate antimicobial activity. *Journal of Pharmacognosy and Phytotherapy*. 2016; 8(2): 25-48.
36. Altameme HJ, Hameed IH, Abu-Serag NA. Analysis of bioactive phytochemical compounds of two medicinal plants, *Equisetum arvense* and *Alchemila valgaris* seed using gas chromatography-mass spectrometry and fourier-transform infrared spectroscopy. *Malays. Appl. Biol*. 2015; 44(4): 47–58.
37. Hameed IH, Hamza LF, Kamal SA. Analysis of bioactive chemical compounds of *Aspergillus niger* by using gas chromatography-mass spectrometry and fourier-transform infrared spectroscopy. *Journal of Pharmacognosy and Phytotherapy.* 2015;7(8): 132-163.
38. Hameed IH, Hussein HJ, Kareem MA, Hamad NS. Identification of five newly described bioactive chemical compounds in methanolic extract of *Mentha viridis* by using gas chromatography-mass spectrometry (GC-MS). *Journal of Pharmacognosy and Phytotherapy*. 2015; 7 (7): 107-125.
39. Hussein HM, Hameed IH, Ibraheem OA. Antimicrobial Activity and spectral chemical analysis of methanolic leaves extract of *Adiantum Capillus-Veneris* using GC-MS and FT-IR spectroscopy. *International Journal of Pharmacognosy and Phytochemical Research*. 2016; 8(3): 369-385.
40. Hussein HJ, Hadi MY, Hameed IH. Study of chemical composition of Foeniculum vulgare using Fourier transform infrared spectrophotometer and gas chromatography - mass spectrometry. *Journal of Pharmacognosy and Phytotherapy*. 2016; 8(3): 60-89.
41. Kadhim MJ, Mohammed GJ, Hameed IH. In vitro antibacterial, antifungal and phytochemical analysis of methanolic fruit extract of Cassia fistula. *Oriental Journal of Chemistry*. 2016; 32(2): 10-30.
42. Altameme HJ, Hameed IH, Idan SA, Hadi MY. Biochemical analysis of *Origanum vulgare* seeds by fourier-transform infrared (FT-IR) spectroscopy and gas chromatography-mass spectrometry (GC-MS). *Journal of Pharmacognosy and Phytotherapy*. 2015; 7(9): 221-237.

43. Hussein HM. Determination of phytochemical composition and ten elements content (CD, CA, CR, CO, FE, PB, MG, MN, NI AND ZN) of CARDARIA DRABA by GC-MS, FT-IR and AAS technique. *Int. J Pharm Bio Sci*. 2016; 7(3): (B) 1009 –1017.
44. Hussein HM. Analysis of trace heavy metals and volatile chemical compounds of *Lepidium sativum* using atomic absorption spectroscopy, gas chromatography-mass spectrometric and fourier-transform infrared spectroscopy. *Research Journal of Pharmaceutical, Biological and Chemical Sciences.* 2016; 7(4): 2529 – 2555.
45. Hameed IH. A new polymorphic positions discovered in mitochondrial DNA hypervariable region HVIII from central and north-central of Iraq. *Mitochondrial DNA*. 2016; 27(5): 3250-4.
46. Jaddoa HH, Hameed IH, Mohammed GJ. Analysis of volatile metabolites released by Staphylococcus aureus using gas chromatography-Mass spectrometry and determination of its antifungal activity. *Orient J Chem*. 2016; 32(4).
47. Hameed IH, Salman HD, Mohammed GJ. Evaluation of antifungal and antibacterial activity and analysis of bioactive phytochemical compounds of *Cinnamomum zeylanicum* (Cinnamon bark) using gas chromatography-mass spectrometry. *Orient J Chem*. 2016; 32(4).
48. Altaee N, Kadhim MJ, Hameed IH. Detection of volatile compounds produced by Pseudomonas aeruginosa isolated from UTI patients by gas chromatography-mass spectrometry. *International Journal of Current Pharmaceutical Review and Research*. 2017; 7(6).
49. Altaee N, Kadhim MJ, Hameed IH. Characterization of metabolites produced by E. coli and analysis of its chemical compounds using GC-MS. *International Journal of Current Pharmaceutical Review and Research.* 2017; 7(6).
50. Al-Yaseri A, Kadhim WA, Hameed IH. Detection of volatile compounds emitted by Proteus mirabilis isolated from UTI patients and its anti-fungal potential. *Der Pharma Chemica*, 2016; 8(19): 671-678.
51. T. Beyer, B. Diehl, G. Randel, E. Humpfer, H. Schäfer, M. Spraul, C. Schollmayer, U. Holzgrabe, Quality assessment of unfractionated heparin using 1H nuclear magnetic resonance spectroscopy. *J. Pharm. Biomed. Anal.* 2008;48:13–19.
52. I. McEwen, B. Mulloy, E. Hellwig, L. Kozerski, T. Beyer, U. Holzgrabe, A. Rodomonte, R. Wanko, J.M. Spieser, Determination of oversulphated chondroitin sulphate and dermatan sulphate in unfractionated heparin by 1H NMR. *Pharmeur. Bio* 2008;1: 31–39.
53. S. Alban, Carbohydrates with anticoagulant and antithrombotic properties, in: Z.J. Witczak, K.A. Nieforth (Eds.), Carbohydrates in Drug Design, Marcel Dekker, New York, 1997, pp. 209–276.
54. T. Beyer, M. Matz, D. Brinz, O. Rädler, B. Wolf, J. Norwig, K. Baumann, S. Alban, U. Holzgrabe, Composition of OSCS-contaminated heparin occuring in 2008 in batches on the German Market. *Eur. J. Pharm. Sci*. 2010;40:297–304.
55. S. Alban, S. Lühn, S. Schiemann, T. Beyer, J. Norwig, C. Schilling, O. Rädler, B. Wolf, M. Matz, U. Holzgrabe, Comparison of established and novel purity tests for the quality control of heparin by means of a set of 177 heparin samples, *Anal. Bioanal. Chem*. 2011; 399:605–620.
56. G. Jackson, S. Arver, I. Banks, V.J. Stecher, Counterfeit phosphodiesterase type 5 inhibitors pose significant safety risks, *Int. J. Clin. Pract*. 2010; 64: 497–504.
57. W.T. Poon, Y.H. Lam, C.K. Lai, A.Y.W. Chan, T.W.L. Mak, Analogues of erectile dysfunction drugs: an underrecognised threat. *Hong Kong Med. J.* 2007;13: 359–363.
58. S. Balayssac, S. Trefi, V. Gilard,M.Malet-Martino, R.Martino,M.A. Delsuc, 2D and 3D DOSY 1H NMR, a useful tool for analysis of complex mixtures: application to herbal drugs or dietary supplements for erectile dysfunction. *J. Pharm. Biomed.Anal.* 2009;50:602–612.
59. Bernstein, F.C., T.F. Koetzle, G.J.B.Williams, E.F. Meyer, Jr., M.D. Brice, J.R. Rodgers, O. Kennard, T. Shimanouchi and M. Tasumi. The protein data bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* 1977;112:535–542.
60. Gardner, K.H., L.E. Kay. The use of H-2, C-13, N-15 multidimensional NMR to study the structure and dynamics of proteins. *Annu. Rev. Biophys. Biomol. Structure.* 1998;27:357–406.
61. Grzesiek, S. and A. Bax. 1992. Improved 3D triple-resonance NMR techniques applied to a 31 kDa protein. *J. Magn. Reson*. 1991;96:432–440.
62. Havel, T.F. An evaluation of computational strategies for use in the determination of protein structure from distance constraints obtained by nuclear magnetic resonance. *Prog. Biophys. Mol. Biol.* 56:43-78.
63. Viant MR. Improved methods for the acquisition and interpretation of NMR metabolomic data. *Biochem Biophys Res Commun* 2003;310(3):943–8.
64. Bain AD, Burton IW, ReynoldsWF. Artifacts in 2-dimensional NMR. *Prog Nucl Magn Reson Spectrosc* 1994;26:59–89.
65. Le Guennec A, Giraudeau P, Caldarelli S. Evaluation of fast 2D NMR for metabolomics. *Anal Chem* 2014;86(12):5946–54.
66. Davis DG, Bax AD. Assignment of complex 'H NMR spectra via wo-dimensional homonuclear Hartmann–Hahn spectroscopy. *J Am Chem Soc* 1985;107:2820–1.
67. Wishart DS. Computational strategies for metabolite identification in metabolomics. *Bioanalysis* 2009;1(9).
68. Lindon JC, Holmes E, Nicholson JK. Pattern recognition methods and applications in biomedical magnetic resonance. *Prog Nucl Magn Reson Spectrosc* 2001;39(1):1–40.
69. Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis. *Metabolomics* 2007;3(3):211–21.

70. Fiehn O, Robertson D, Griffin J. The metabolomics standards initiative (MSI). *Metabolomics* 2007; 3(3):175–8.
71. Salek RM, Steinbeck C, Viant MR, Goodacre R, DunnWB. The role of reporting standards for metabolite annotation and identification in metabolomic studies. *GigaScience* 2013; 2(1):13.
72. Creek DJ, DunnWB, Fiehn O, et al. Metabolite identification: are you sure? And how do your peers gauge your confidence? *Metabolomics* 2014; 10(3):350–3.
73. Sumner L, Lei Z, Nikolau B, Saito K, Roessner U, Trengove R. Proposed quantitative and alphanumeric metabolite identification metrics. *Metabolomics* 2014; 10(6): 1047–9.