Original Article

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Detection of Nerve Agent Metabolites in Plasma and Urine by ^{31}P NMR Spectroscopy

Mamta Sharma

Author Affiliation: Scientist 'E', Directorate of International Cooperation, DRDO, New Delhi, 110011, India.

Corresponding Author: Mamta Sharma, Scientist 'E', Directorate of International Cooperation, DRDO, New Delhi, 110011, India.

E-mail: reekana@rediffmail.com

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Abstract

A simple robust ^{31}P nuclear systems based NMR method is presented for the detection of alkyl methylphosphonic acids; metabolites of three organophosphorus nerve agents (GB or Sarin, GD or Soman, and GF or Cyclosarin) in the biological spiked samples of plasma and urine. The method gives linear response (correlation coefficients ~0.89) over the concentration range 10 μg/ml to 300 μg/ml in urine and plasma considering phosphorus nucleus. The quality control data demonstrated accurate identification of biomarkers of nerve agents at low concentration. NMR based method provides direct analysis of Biological samples without any sample pretreatment or sample preparation and sample tempering.

Keywords: Isopropyl methylphosphonate; Hydrolysis; 1H; 31P; NMR; Plasma; Urine.

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Introduction

Owing to the simple synthesis of nerve agents, these might be considered as one of the possible terrorist weapons in the present scenario. An increasing use of these agents has posed new challenges for the retrospective detection and identification of these chemicals in the environmental and biological samples.[1] The simplest way to decide their alleged use is detection of presence of their metabolites in the environmental and biological samples. G-agents were first introduced by Germany when Gerhard Schrader discovered the sarin, later GF and tabun were developed. Former Soviet Union and Britain developed VR (Russian V series nerve agents similar to VX also called Novichok agents) and VX (O-ethyl S-[2-ethyl] methylphosphonothioate) respectively.[2] So far besides the sulfur mustard other CW agents like nerve agents have also been used in the wars and terrorist attacks. SM (HD) and nerve agents were used by Iraq in Iraq-Iran war during 1983–1988, and against Kurdish people in 1988. Sarin and VX were also used by terrorist group in Japan in 1994 and 1995.[3] Series of reports reveal that Chemical weapons were also used in Syrian civil war during 2012 to 2017.[4,5,6]

Nerve agents are neurotoxins. These agents bind with serine moiety of Acetylcholinesterase enzyme in the nerve synapses and inhibit the hydrolysis of neurotransmitter acetylcholine. Due to this, excessive accumulation of acetylcholine occurs in nerve synapses, which causes over stimulation of nerves and ultimately paralysed various muscles. In later stage usually paralysis of respiration leads to death.[7]

As far as their detection is concerned, Nerve agents exposure is detected in the samples either by the direct presence of intact agent, or by their metabolites or by their adducts formed with tyrosine moiety of albumin protein or through the development of seizure related brain damages. Their presence in the samples depends upon the exposure concentration and time of sample collection of post exposure.[8]

It has been observed that various Mass based hyphenated analytical techniques like LC/MS/MS, GC/Ms [8] etc have been used for the analysis of biomarkers of nerve agents in the environmental and biological samples but less emphasis is been given on NMR based methods in detection field, owing to less sensitivity of NMR.[9] Mass spectrometric techniques are by far the most popular and sensitive, but these techniques require lengthy sample preparation methods like derivatization and extraction methods, while NMR analysis offers a direct, multinuclear and nondestructive method of analysis. So far Nuclear Magnetic Resonance Spectroscopy (NMR) is recognized as tool for characterization of molecules, kinetic studies of chemical reactions, metabolomics and for the detection of a wide range of small endogenous or exogenous molecules in the complex biofluids such as urine, plasma and tissues. Therefore, here NMR is utilized for direct monitoring of the hydrolysed products, without any issues associated with extraction, derivatization, chemical compatibility, background effect, manipulation of samples, and solvent effect.[7]

In this paper NMR based method is demonstrated for the detection of metabolites in urine (human) and serum (rat) spiked samples by the effective use of ^{31}P NMR active nucleus. Detection method is optimized by performing the number of experiments with various concentrations of hydrolysed products in buffered solutions at various pHs.

Materials and Methods

Urine samples were obtained and analysed using an IRB protocol approved for obtaining random healthy volunteers. Urine specimens were collected from eight individuals asked to join the study in a random anonymous fashion, to prevent any sort of bias.

Blood samples of male Harlan Sprague-Dawley rats were collected into heparinised tubes *via* the retro-orbital venous plexus using anesthesia. The plasma was immediately separated from red cells by centrifugation (2000 rpm, 20 min). Both urine and plasma samples were stored at - 80°C prior to analysis. This was done in accordance with the Helsinki Declaration of 1983. NMR solvent D_2O, Tritisol buffer capsules were procured from Merck. Deionized water was procured from a Millipore Milli-Q filtration system. Chemicals required for synthesis like thionyl chloride, triethyl amine, isopropyl alcohol, pinacolyl alcohol, cyclohexyl alcohol and Dimethyl methylphosphonate etc. were purchased from Merck and Aldrich.

NMR Spectroscopy

The internal standards TSP at 0 ppm (Trimethylsilylpropanoic acid) and TMP (Trimethyl phosphate) at 2.5 ppm were used for recording the ^{1}H and ^{31}P NMR spectra of standard samples of CW (Chemical warfare) agents and their metabolites respectively. Minimum detection limit of phosphorus nucleus was evaluated by setting the chemical shift scale externally using 85% phosphoric acid in D_2O at 0.00 ppm. Urine and plasma samples were also screened using external standard for ^{31}P nucleus. Thawed samples were centrifuged at 20,000 rpm, 4°C, for 20 min. 200 µl of D_2O was added to 400 µl of urine samples and 400 µl of D_2O was added to 200 µl of plasma samples for recording of spectra. The 1D ^{1}H and ^{31}P spectra were recorded at magnetic field 400.13 MHz and 161.97 MHz respectively on a Bruker DPX-400 MHz spectrometer at 300 K temperature using a standard pulse sequence comprising 5 µs pulse width with 2 µs delay and a pulse power of 3 db. The water resonance was suppressed by presaturation experiment using relaxation delay (2s) and 20µs power switching for irradiation delay for recording ^{1}H NMR of standard authentic samples and urine samples in water. A total of 64 FIDs were accumulated into 64k data points over a spectral width of 12019 Hz with 8 dummy scans. A line-broadening factor of 0.3 Hz was applied before Fourier transformation. Chemical shifts were referenced internally to the CH_3 signal of endogenous lactate doublet at 1.33 ppm in plasma sample. The creatinine singlet at 3.06 was taken as the reference for spectra of urine samples. ^{31}P NMR spectra of both the samples were recorded using 5.40 µs pulse width, 9 sec. delay, 64k FID data points, 32 k spectral data points, 64935.06 Hz sweep width with 1 Hz line broadening. For urine samples, a standard 1D spectrum with selective irradiation of the water resonance, with relaxation delay (RD), 2s and mixing time (tm), 0.1s was acquired. Additionally, for the plasma samples, a spin-echo (CPMG) spectrum acquired with a total echo time of 608 ms.

To obtain the accurate results in quantitative NMR, 90° pulse calibration and pulse delay were optimized to obtain the maximum recovery of magnetization.

Results and Discussion

Calibration Curve and Minimum detection limit

In the first set of experiments a concentration gradient consisting of seven solutions of IMPA (Isopropyl methylphosphonate) was prepared in urine containing buffer solution in concentration range of 5 µg/mL to 320 µg/mL for detector response calibration. Calibration curve was plotted between different concentrations of IMPA and signal height of ^{31}P nucleus. A straight line curve was obtained which represents linear detector response.

In the second set of experiments urine and human serum albumin solution (prepared in water), were prepared for elucidation of minimum detection limit of IMPA using ^{31}P NMR at neutral pH in D_2O. Here in place of plasma sample HBA was taken for experiment. Series of samples of IMPA with various concentrations ranging from 0.0 µg/mL (control), 1 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 30µg/mL and 60 µg/mL were prepared from the stalk solution of 200 ppm. The minimum detection limit was found 20 ppm and 30 ppm in plasma and urine respectively in D_2O solvent at neutral pH using ^{31}P nucleus. It was observed that IMPA present minimum detection limit 8 µg/ml in $CDCl_3$. Fig. 1 Shows the structures of organophosphorus CW agents and their metabolites.

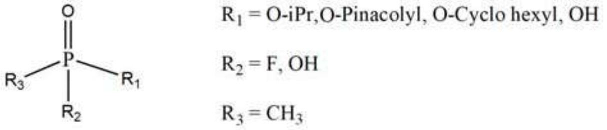
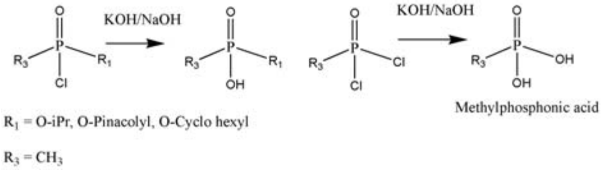


Fig. 1: General Structure of CW agents

Synthesis and NMR analysis of CW agents and metabolites

Nerve agents were synthesized by reported procedure[10] and their hydrolysed products were also prepared by the reaction of 30% NaOH (alkaline hydrolysis) as per the shown scheme 1. The methylphosphonic acid is synthesized directly from methylphosphonic dichloride using 30% NaOH solution. The analytes/metabolites those were aimed to detect using NMR analysis are: IMPA (Isopropyl methylphosphonic acid) of GB (Isopropyl methylphosphonofluoridate), PMPA (Pinacolyl methylphosphonic acid) of GD (Pinacolyl methylphosphonofluoridate) and CMPA (Cyclohexyl methylphosphonic acid) of GF-(Cyclohexyl methylphosphonofluoridate). The complete ^{1}H, ^{13}C, ^{31}P and ^{19}F chemical shifts values with coupling constant values for all the chemicals (nerve agents and hydrolysed products of nerve agents) were interpreted. There is great impact of polarity of Solvents used for analysis and pH of solution on the chemical shifts of the Phosphorus acids. Therefore the chemical shift of all the hydrolysed products were observed in different solvents $CDCl_3$, D_2O (pH= 2, 7 and 14) for phosphorus nucleus. These values are shown in the table 1.



Scheme 1: Hydrolysis of Nerve Agents

Hydrolysis of sarin into IMPA and MPA at various pHs

To monitor the hydrolysis reaction of sarin in NMR tube, Tritisol buffer capsules of pH 2, 7 and 14 were used to prepare solutions in water. Equal volumes of the sarin stock solution and buffer were added to NMR tubes along with deuterated water and vortexed. The final sarin concentration was 100 µg/ml in 0.5 M buffer. The chemical shift for ^{31}P signal of sarin was appeared as doublet at 30.891/37.344 ppm in D_2O solvent, because of coupling of phosphorus with fluorine. Analysis was performed within five minutes of sample preparation to observe the degradation path of sarin. The IMPA and MPA signals were observed at 31.9 and 31.4 ppm respectively at 1 pH after hydrolysis. Chemical hydrolysis of sarin into IMPA was rapid at pH 14, and slow at pH 1 and slowest at pH 7.0, reached 95% hydrolysis in about 24 hours. Methyl phosphonic acid was not found even after 2 days at pH 7 and 1. It was started forming in the basic solution after 08 Hrs, but complete hydrolysis into methyl phosphonic acid was not observed. Only 20% MPA was formed. The hydrolysis profile of sarin in plasma is shown in figure.

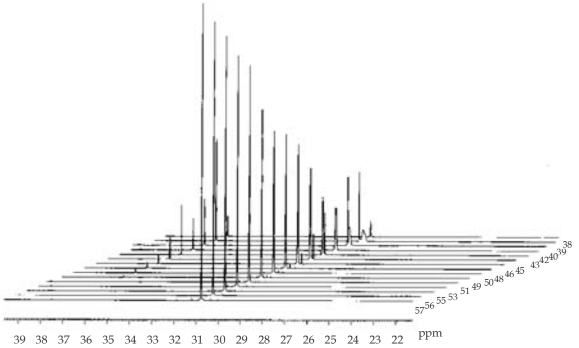


Fig. 2: Hydrolysis profile of Sarin

^{31}P NMR analysis of Sarin in Plasma and Urine

Sarin was taken in Plasma and Urine and solutions were mixed and kept under ambient temperature. The phosphorus NMR spectra was recorded after ten minutes which showed ^{31}P NMR chemical shift values for IMPA at 25.28 ppm in plasma (Fig 3) and 25.39 ppm in urine at 9 pH in D_2O solvent. Sarin hydrolysis was slow in the plasma and urine at neutral pH and took 48 hours for complete disappearance.

It has been observed that nerve agents produce characteristic signals as a metabolic response in proton and phosphorus NMR spectra with unique peak splitting information in the distinct chemical shift region. This factor helps in unambiguous detection of hydrolysed products in biological samples using ^{1}H NMR at high concentration of nerve agents.

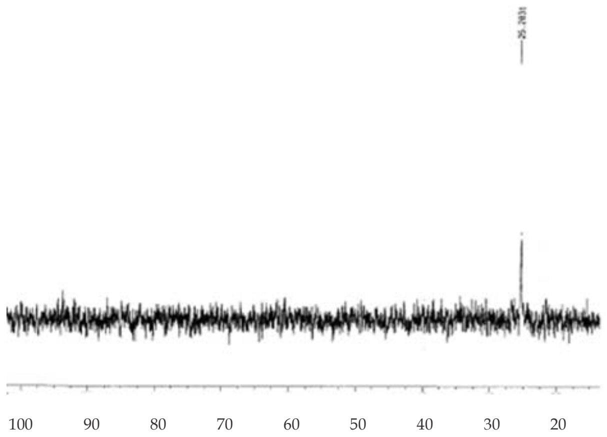


Fig. 3: ^{31}P NMR Spectrum of Isopropyl methyl phosphonic acid in Plasma.

However because of the low concentration of hydrolysed products of nerve agents, signals of

Table 1: ^{1}H, ^{31}P, ^{13}C and ^{19}F NMR Data of CW Agents and its Hydrolysed Products

S. No.	Name of the Chemicals	$^{31}P\{^1H\}$ NMR δ solvent	^{19}F δ* *J**	^{1}H NMR δ	^{13}C NMR δ
	Isopropyl methylphosphono fluoridate	29.52 Doublet ($CDCl_3$)	-57.38 1046.4Hz	1.60(dd,3H,CH_3), ($^2J_{H-P}$ =18.7) ($^3J_{H-F}$ =5.6 Hz); 1.36-1-39 (m,6H,-CH_3); 4.87 (m,H,CH)	11.06 (dd,C,CH_3-P), (J_{P-C} = 150); 71.50 (dd,C,CH-P), $^2J_{H-P}$=8) 23.84 (DD,3C, CH_3) ($^3J_{P-C}$= 4.49, $^3J_{F-C}$=2.7); 22.91(d,1C,-CH_3) (J_{F-C} =3.7)
	Isopropyl methylphos phonate	25.64(pH14), (D_2O), 27.18(DMSO), 30.56 (pH 1), (D_2O)	0	1.32(d,3H,CH_3), 4.69 (m,H,-CH); 1.32 (d,6H,CH_3)	27.92(d,C,CH_3), 76.23 (CH); 26.15 (CH_3)
	Methylphosphonic acid	20.32(pH14), (D_2O), 26.8(DMSO), 29.31 (pH 1), (D_2O)	0	1.27(d,3H,CH_3), ($^2J_{H-P}$ =17.7)	13.9(d,C,CH_3), (J_{H-P} =139)
	Pinacolylmethylphosphono fluoridate	27.92, 29.16 ($CDCl_3$)	-58.26, -55.39 1047.5Hz	1.62, 1.64 (dd,3H,CH_3), ($^2J_{H-P}$ =18.7) ($^3J_{H-F}$ =7.6 Hz);4.387, 4.41(m,H,CH); ($^3J_{H-F}$ =7.6, 8.9 Hz);1.32, 1.33 CH_3,($^3J_{H-F}$=0.6 Hz);0.98 (S,9H, CH_3)	10,05, 10.64 (CH3-P), (dd,C,CH_3-P), (J_{P-C} = 150);84.04, 83.75 (dd,C,CH-P), 16.60 (CH_3-CH), 25.4, 26.1$(CH_3)_3$
	Pinacolyl methylphosphonate	26.15 (pH 14), (D_2O), 28.31(DMSO), 32.26 (pH 1), (D_2O)	–	1.41 (d,3H,CH_3), ($^2J_{H-P}$ =6.7),4.38(m,H,CH);1.327 CH_3, 0.91 (s,9H, CH_3)	13.01 (d,3H,CH_3), ($^2J_{H=}$138), 81,CH, 16.4CH-CH_3, 25.83, $(CH_3)_3$, 33.97 C-$(CH_3)_3$
	Cyclo hexyl methylphosphono fluoridate	30.2 ($CDCl_3$)	-56.9 1046.7Hz	1.63(dd,3H,CH_3), ($^2J_{H-P}$ =18.7) ($^3J_{H-F}$ =5.8 Hz); 4.62(m,H,-CH) ; 1.69-2.03(m,4H,CH_2-(CH) CH_2) ($^3J_{H-H}$ =7.2,7.4); 1.16-1.64(m,6H,-CH_2-CH_2-CH_2)	10.5(dd,1C,CH_3), (J_{P-C} =147, $^2J_{P-F}$ =28.1; 77.48(d,1C,-CH); 33.45, 33.56(CH_2-CH-CH_2);,23.47, 23.49 (CH_2-CH_2-CH_2), 24.97(CH_2)
	Cyclohexyl methyl phosphonate	24.56 (pH 14), (D_2O), 32.28 (pH 1), (D_2O)	–	1.41(dd,3H,CH_3), ($^2J_{H-P}$ =17.7); 4.52(m,H,-CH) ; 1.19-195 {m,10H, (CH_2-(CH)-$CH_2)_2$}	12.56(dd,1C,CH_3), (J_{P-C} =149); 76.48(d,1C,-CH); 33.84, (CH_2-CH-CH_2),23.87 (CH_2-CH_2-CH_2), 26.47(-CH_2-)

* δ = Chemical Shift, J = Coupling Constant

interest are obscured by signals of metabolites present in biological samples in proton NMR. Therefore detection of metabolites using Phosphorus nucleus becomes significant at low concentration and present superiority of ^{31}P over the ^{1}H, in which no other signals of plasma/urine metabolites appear in the region of interest and chances of overlapping of signals are also disappeared.

Conclusion

The aim of the study is optimization of simple and robust NMR based analytical method for the detection of metabolites of nerve agents in biological samples plasma and urine. In this method no sample pretreatment or sample manipulation is required, as it is required in other spectroscopic analysis. Studies performed on the chemical inter conversion of nerve agents showed that hydrolysis is rapid at alkaline pH, slow and less complete at neutral pH and slowest at acidic pH. NMR is an ideal system for direct analysis of metabolites.

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Caution: Nerve agents are extremely toxic in nature. During Synthesis full extra precautions must be taken care of. Proper protective gears and detectors should be used for analysis and during synthesis. Washing of glassware using 40% NaOH is always recommended. Before synthesis operating and synthetic procedures are advised to be study carefully.

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