Determination by $^1$H NMR Spectroscopy of Paraquat in Urine from Acutely Poisoned Patients. Comparison with Second-Derivative Spectroscopy Method

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Abstract

The application of $^1$H nuclear magnetic resonance (NMR) spectroscopy to characterize and quantitate paraquat in urine is described. Characterization was performed taking advantage of two NMR spectroscopy parameters: chemical shifts and coupling patterns. Without any pretreatment of the biological samples, herbicide was detected by its aromatic doublets at 8.49 and 9.02 ppm. Quantitation of the xenobiotic was realized by relative integration of the dipyridyl protons to an internal standard. After a validation step using control urine samples, quantitation was performed in urine obtained from two poisoned patients. On admission, mean paraquat concentrations were 985 (patient 1) and 500 (patient 2) µmol/L. Results are compared and found to be in good agreement, using a second-derivative spectroscopy method.

Introduction

Paraquat is the 1,1’-dimethyl-4,4’-bipyridylidum ion (CAS registry number 4685-14-7). It is usually formulated as a dichloride under trade names such as Crisquat®, Dextrone®, Dexuron®, Esgram®, Goldquat 276®, Gramoxone®, Herbaxon®, Osaquat Super®, and Sweep® (1) or combined with diquat. These formulations are widely used for their nonselective herbicide properties. They are very toxic to humans in the available concentrations. The fatal adult dose ranges from 3 to 6 g (2) when ingested, and paraquat is considered a high-risk pesticide. Consequently, the accidental or deliberate ingestion of paraquat is often lethal and can constitute a very serious public health problem, even for survivors (3–5). Mortality from ingestion of paraquat preparations has been shown to reach 76% in Japan (4), 80% in Hong Kong (6), and 25% in the U.S. (7). Death can occur within several days after intake because a substantial proportion of the ingested dose is retained in the tissues. Toxicokinetic studies (1,5,8) have revealed that paraquat is not metabolized and that major urinary elimination occurs during the first 24 h. A considerable redistribution from tissues to blood circulation points out the need for suitable determination techniques. Moreover, intentional paraquat poisoning is not generally considered even when the physicians are faced with an atypical clinical course for inflammatory disease (9), unless it constitutes a common medical problem, such as in Hong Kong or in Taiwan (6,10).

Several methods have been described to detect or quantitate paraquat in biological fluids. Paraquat can be directly reduced with dithionite or glucose, with or without sample deproteinization or extraction, followed by spectrophotometric quantitation (11–15). Other determination protocols, including gas or liquid chromatography (16,17), capillary electrophoresis (18), and immunological methods (19), have been reported.

The high mortality rate encountered in poisoning cases led this laboratory to consider rapid and reliable determination methods. Colorimetric procedures appear to be the simplest of those mentioned. However, considering the stability problems of the free radicals formed and the relative complexity of time-consuming extraction protocols, the applicability of nuclear magnetic resonance (NMR) spectroscopy was evaluated.

Numerous studies have demonstrated the usefulness of NMR spectroscopy in the toxicological field (20,21). It is possible to show modifications of endogenous compounds or the appearance of xenobiotics or their metabolites in body fluids; this technique has been applied to overdose cases involving salicylates (22) and chloroquine (23), suggesting its usefulness as an alternative analytical method in clinical toxicology. Recently, a $^1$H NMR procedure was applied to specify renal injury in two
patients suffering from paraquat intoxication after overdose (24). In the present study, \( ^1H \) NMR spectroscopy was applied to detect and to quantitate paraquat directly in biological samples, and results were compared with the second-derivative spectroscopic method of Fuke et al. (14).

**Experimental**

**Reagents**

Standard paraquat was purchased, as tetrahydrated dichloride from Chem Service (West Chester, PA), sodium dithionite from Sigma-Aldrich, (St. Quentin Fallavier, France) and sodium hydroxide from Prolabo. Standard solutions of the herbicide were prepared from a stock solution of 1.07 mmol/L (352 mg/L as paraquat tetrahydrated dichloride, or 200 mg/L, as paraquat ion) prepared in distilled water. Dithionite solution for spectrophotometric measurements was 0.25 mol/L in 0.5N NaOH. For \( ^1H \) NMR spectroscopy, the internal standard was 3-(trimethylsilyl)-2,2,3,3-tetradeteropropionic acid or TSP-d4 (Eurisotop, St Aubin, France) in deuterium oxide (Eurisotop, St Aubin, France).

**Case histories**

In the emergency toxicological context of this study, no specimens were specifically collected, and procedures were therefore in accordance with the revised Helsinki declaration of 1983.

The first patient (a 41-year-old man) was hospitalized after the intake of one glass of pure Gramoxone plus® (containing 10% w/v paraquat concentration) in a suicide attempt. On admission to the hospital, clinical examination was normal, and a urine sample was collected. He received standard supportive treatment, gastric lavage and hemoperfusion, and another urine sample was collected after this treatment. After 24 h, moderate renal failure became evident as indicated by the following biochemical markers: 8.66 mmol/L (0.52 g/L) and 220 µmol/L (25 mg/L) for urea and creatinine, respectively. Progressively, he developed major pulmonary fibrosis, which was complicated by a septic shock. Hyperthermia was observed for a long time (39°C) despite multiantibiotic medication. Finally, he died from cardiac arrest one month after hospital admission.

The second patient (a 53-year-old woman) was suspected to have ingested both Gramoxone containing 20% (w/v) paraquat and acebutolol, a β-blocker used as medication. She was hospitalized in emergency. Clinical examination revealed modification in consciousness, cyanosis, and breathing difficulties; esophageic and gastric ulcerations were significant. A urine sample was collected at that time. She was ventilated and treated with vasoactive medication, gastric lavage, and hemoperfusion. On the third day, she developed renal failure: urea concentration was 7.50 mmol/L (0.45 g/L) on admission, 13.2 mmol/L (0.79 g/L) on the third day, and creatinine concentration increased from 132 µmol/L (15 mg/L) on admission to 238 µmol/L (27 mg/L) during the same period. Evolution included hypotension and hyperthermy, which, despite treatment, led rapidly to death from cardiac arrest four days after poisoning.

**Urine collection and sample preparation**

After collection, urinary samples were divided into two aliquots, immediately frozen, and stored at -20°C until analysis. Spiked control urine samples for colorimetric and \( ^1H \) NMR determinations were made from blank specimens previously tested to be free of paraquat. Blood samples were also obtained on hospital admission from the two patients, but no gastric liquid was available.

**Spectrophotometric quantitation**

A Kontron model Uvikon 860 double beam spectrophotometer was used with cuvettes of 1-cm pathlengths. Standard solutions of paraquat ranging from 5.35 to 53.5 µmol/L (1 to 10 mg/L of dication) were prepared from water and from spiked control urine samples previously tested to be free of paraquat. After the addition of 0.25 mL of dithionite solution to 1 mL standard solution or urine, each measurement was taken between 350 and 450 nm, within 1 min, as the blue radical ion is unstable beyond a 10-min period. The second derivative spectrum was calculated and calibration curves were constructed from the amplitude between 395 and 404 nm (14).

A very good linearity was observed in aqueous standard solutions from 0 to 10 mg/L expressed as paraquat dication. However, the linearity range was narrower for spiked control urines (from 0 to 5 mg/L), leading to a significant dependence of calibration slope on the considered biological sample. To solve this problem, a half-dilution step of urine had to be performed (data not shown). For the two urine samples from the poisoned patients that contained high levels of paraquat, a supplementary dilution step had to be performed to meet the linearity criterion of the Fuke et al. (14) method.

**Proton NMR spectroscopy**

Spectra were recorded at 300MHz on a Bruker (Bruker Spectroscopin, Wissembourg, France) AC 300 spectrometer at ambient temperature. The second aliquot of the collected samples was directly used for \( ^1H \) NMR analysis. Five-hundred microliters of each was introduced into a 5-mm NMR tube. A titrated solution of TSP-d4, used as the chemical shift reference (δ = 0.00 ppm) in deuterium oxide, was added into a capillary tube that was coaxially inserted into the NMR tube.

One-dimensional spectra were obtained by operating in the pulsed Fourier-transform mode with quadrature detection. Application of a gated secondary irradiation field at the water resonance frequency during the relaxation delay (2 s) sufficiently reduced the water signal. For each sample, 128 to 512 transients were collected into 16 K computer data points, with a spectral width of 3200 Hz and a 30° pulse. Prior to Fourier transform, an exponential apodization function was applied, corresponding to a 0.3 Hz broadening of the line.

**Quantitation by \( ^1H \) NMR spectroscopy**

TSP-d4 is known to bind with proteins in biological samples, and therefore cannot constitute a reliable internal standard for quantitation (25). Moreover, kidney damage can lead to marked disturbances in protein excretion. Consequently, to obtain the
relative determinations, a coaxial capillary tube containing a titrated solution of TSP-d$_4$ was placed into the NMR tube to be used as a quantitation standard, and a sufficient relaxation delay was respected. A deconvolution process making the peaks correspond to Lorentzian line shapes was applied to some resonances to determine relative areas compared to the TSP-d$_4$ signal in the integration step using the 1D WIN-NMR program from Bruker.

Calibration data for paraquat were obtained from aqueous standards and from spiked control urine samples. A working standard solution titrated at 107 µmol/L (20 mg/L as dication) was prepared from an appropriate dilution of the stock solution. Successive dilutions were performed to prepare 2.675, 5.35, 13.375, 26.75, and 53.5 µmol/L solutions (0.5 to 10 mg/L of dication).

Statistics
Within-run and between-run precision was determined by quality-control samples analyzed according to the described integration and quantitation processes. Calibration data for paraquat were subjected to linear regression analysis and significance of correlation coefficients was evaluated using Student's t-test. Detection limit was calculated as corresponding to twice the noise signal.

Results and Discussion

The second-derivative spectroscopic method (14) was applied to the two peaks of reduced paraquat observed at 395 and 404 nm. Within-run ($n = 5$) and between-run ($n = 5$) precisions were determined in spiked urine with 5.35, 13.375, 26.75, and 53.5 µmol/L paraquat concentrations (1, 2.5, 5, and 10 mg/L as dication). The mean corresponding variation coefficients were 1.8 and 2.1%. The quantitation limit was found to be 2.675 µmol/L (0.5 mg/L of dication).

In the $^1$H NMR spectrum of the first patient's urine, unusual resonances were observed near hippurate resonances in the aromatic proton region. Two doublets at 8.49 and 9.02 ppm with the same coupling constant of 6.3 Hz corresponded to the aromatic paraquat protons. The methyl resonance appeared as a singlet at 4.48 ppm, near the water peak (Figure 1).

Quantitation of paraquat was carried out by aromatic proton integration, after lorentzian deconvolution, with TSP-d$_4$ as reference. For spiked control urine samples, a significant linear relationship was observed with a correlation coefficient $r = 0.9887$ (Figure 2) from 25 µmol/L to 1 mmol/L paraquat concentrations. No disturbance from the biological fluid was to be observed because of the high specificity of NMR parameters. Within-run ($n = 5$) and between-run ($n = 5$) precisions were determined in spiked urine samples with the same paraquat concentrations as for the second-derivative spectroscopic method. The mean corresponding variation coefficients were 2.0 and 3.0%, respectively. The quantitation limit was found to be 25 µmol/L (4.67 mg/L of dication), and the detection limit was around 10 µmol/L (1.86 mg/L). The inherent relatively low sensitivity of $^1$H NMR spectroscopy is counterbalanced by its simplicity in analytical process, its specificity, and its potent application in clinical toxicology.

A comparison was made between paraquat concentrations determined on the biological samples by the two methods (Table I), and they were found to be very similar.

$^1$H NMR spectroscopy is a rapid determination technique without any hypothesis relative to the chemical species implicated in the poisoning, as demonstrated by the characterization of xenobiotics, such as paracetamol (20), salicylates (22), or chloroquine (23). A $^1$H NMR spectroscopic procedure has been applied for paraquat by Bairaktari et al. (24), but with some differences: (i) no comparison was made with another analytical technique, (ii) the relaxation delay was not specified (the integration step is dependent on this parameter), and (iii) another quantitation procedure was applied because TSP-d$_4$ was added to the crude urine. The binding of this compound with proteins leads to signal distortions, and this is of paramount importance as protein levels are disturbed by nephrotoxins. This laboratory quantitation process gives valuable variation coefficients, largely below 5%, and the linearity criteria are respected.

$^1$H NMR spectroscopic determinations in well-defined conditions may constitute a step forward in diagnosing acute paraquat poisonings since this method offers several advantages over the second-derivative spectrophotometric method. It provides a rapid diagnosis, recording the spectra takes 10-20 min, and only needs a small sample (500 µL) without any pretreatment such as extraction and derivatization necessary for the formation of the free radical. Serum or urine samples are

![Figure 1. 300 MHz $^1$H NMR spectrum of first patient's urine. The assignments of resonances are as follows: C, creatinine; H, hippurate; IS, indoxyl sulfate; HOD, residual water peak; *, paraquat (1081 µmol/L).]
directly introduced in the NMR tube, which is placed in the magnetic field. There is no chemical or thermal treatment, thus preserving its integrity. Moreover, samples used for NMR analysis can be kept and analysed later, by other classical techniques. This rapid diagnosis can be important in an emergency context because no dilution is needed to meet the linearity criterion of the spectrophotometric method (between 0 and 5 mg/L) and no a priori has to be supposed on the paraquat level. Moreover, in a complete experiment, besides the nature of the xenobiotic, disturbances in endogenous compounds not normally measured can be established, and the identification of the different chemical species can be reliably performed because their chemical shifts and coupling patterns are specific. Finally, qualitative and quantitative evaluation can be established in one spectrum, and this nondestructive technique should be considered as complementary to conventional laboratory methods.

The limitation of the NMR technique is that the quantitation process can be difficult with overlapping signals, as it is also with other techniques, but this could be minimized using two-dimensional experiments in which a new dimension is added to the usual chemical shift axis of the conventional spectrum. For overlapping signals, peak multiplicities will be presented on the second dimension of the J-resolved spectra, allowing a precise determination of coupling constant of each signal and leading to signal simplification (22).

$^1$H NMR analysis of biological fluids thus provides a convenient and very promising tool to assess several compounds in one analysis, without extraction or destruction of the biological sample, and regardless of chemical species. However, one limitation of NMR is that it only permits identification of the major compounds of biological fluids and consequently, only the major abnormalities. Obviously, some xenobiotics, when present at very low concentrations, will not be detected. Moreover, many compounds present have chemical shifts that are very dependent on the pH value of the biological fluids; this is a real advantage of NMR because this pH dependence can be an additional argument to characterize a compound. Nevertheless, it will be necessary to construct NMR data libraries, useful in clinical toxicology, as has been already done for mass and UV-visible spectrometries. Finally, the cost of a high field spectrometer could be considered a disadvantage, but it should be reduced as soon as NMR is more widely used in hospital laboratories. Despite this, many studies on xenobiotics and endogenous compounds should be concerned with NMR analysis. Clinical investigations in the field of diagnosis are at present being carried out in this laboratory with the help of $^1$H NMR spectroscopy.

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References


