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QUANTITATIVE DETERMINATION OF THE HERBICIDE PARAQUAT IN HUMAN PLASMA BY GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC METHODS

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SUMMARY

The gas chromatographic (GC) determination of the herbicide paraquat, the 1,1′-dimethyl-4,4′-dipyridyl cation in human plasma is described. In poisoning cases, plasma concentrations provide a necessary index of the severity of intoxication and a means of monitoring subsequent therapy. The methods may be extended to the specific trace analysis of paraquat in body fluids or *post-mortem* tissue. Reduction of fully ionised paraquat salts with sodium borohydride yields a hexahydro derivative, a diene, amenable to solvent extraction and GC. Employing 1,1′-diethyl-4,4′-dipyridyl dichloride as the internal standard, plasma concentrations of 0.1 μg/ml (± 6% S.D.) may be determined with flame ionisation detection and 0.025 μg/ml with nitrogen-selective flame ionisation. Further enhancement of specificity is achieved using selected ion monitoring mass spectrometry and the value of this technique in forensic analysis is illustrated.

INTRODUCTION

Paraquat, the 1,1′-dimethyl-4,4′-dipyridyl cation (I), is widely accepted as an effective contact herbicide[1]. As the dichloride salt, it is marketed in Great Britain under the trade names Gramoxone**, a 20% aqueous solution, and Weedol**, a granular preparation composed of 2.5% paraquat and 2.5% diquat (III). Paraquat does not represent a serious poisoning risk to man in the normal process of application or subsequently as an environmental contaminant. However, in the period from 1964 to 1974, there have been over 200 recorded deaths resulting from ingestion of paraquat in all countries of use. All aspects of paraquat poisoning have recently been reviewed[2]. While deaths are in part attributable to such misadventures as swallowing of concentrated solutions stored in unlabelled soft drink bottles, more than half appear to have

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** Trademark of Imperial Chemical Industries Ltd., Macclesfield, Great Britain.

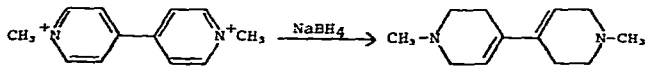
been with suicidal intent. Paraquat causes widespread organ damage, but typically where this phase is survived, death results from effects upon the lung. Our interests are in possible therapeutic approaches in paraquat poisoning[3] and in the mechanisms of its toxic effects. For studies in man, we required a reliable index of the severity of intoxication based on measurements made in plasma during several days after ingestion. New methods of analysis were required for this purpose and for related studies of the pharmacokinetics of the chemical in man.

The most widely used technique for the detection of paraquat is colorimetry after concentration by cation-exchange chromatography. This was originally developed for residue analysis in food crops[4] and is readily adapted for urine and tissue samples (*e.g.*, refs. 2 and 5). However, with a limit of detection of 5 μg per aliquot, it is inappropriate for serial determinations in blood. In our observations, even in severe poisoning cases, concentrations rapidly fall to the 0.1–0.5 μg/ml range and sample size is necessarily limited. High-speed liquid chromatography with UV spectrophotometric detection has been used for paraquat analysis in urine, but the limit of detection is around 100 μg paraquat per 1 urine[6].

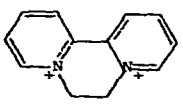
Several groups have reported gas chromatographic (GC) detection methods for paraquat. Thus, pyrolysis to 4,4′-dipyridyl in the injection port of the gas chromatograph has been introduced as a rapid technique for urine analysis in the 1-ppm range, with a probable limit of 0.1 μg/ml in 5-ml samples[7]. Mass spectrometry (MS) and nitrogen-selective flame ionisation have now also been used to determine the pyrolysis product and the method can be used for blood analyses in the range 0.02–0.5 μg/ml[8]. Reduction of both paraquat and diquat to volatile diamines amenable to GC has been applied to water and soil samples[9–11].

Of particular interest has been the reduction of paraquat in aqueous solutions of sodium borohydride to the diene (II)[10]. In our laboratory, the observation that this reduction procedure could be applied to biological extracts led us to explore the reaction as the basis of a quantitative GC assay from plasma. In this communication, we describe the use of flame ionisation (FID) and nitrogen-selective flame ionisation detection (NFID) suitable for routine analysis to 0.025 μg/ml. The necessary precision is achieved by the use of 1,1′-diethyl-4,4′-pyridyl dichloride as an internal standard. Extension to selected ion monitoring (SIM), MS in GC detection (otherwise "mass fragmentography") provides additional sensitivity and the specificity required for forensic analysis.

The methods described have been evaluated during a three year period and are now also in use in other centres in Great Britain. They have previously been reported only in the form of an abstract[12].



(I) (II)



(III)

EXPERIMENTAL

Gas chromatography and gas chromatography–mass spectrometry

For routine GC assay, a Pye model 104 gas chromatograph (dual FID head) was employed with 5 ft. × 2 mm I.D. glass columns. For NFID, the Hewlett-Packard model 5750 G gas chromatograph with a model 15161 B nitrogen detector was used. The glass columns were 5 ft. × 2 mm I.D. Combined GC–MS was carried out with an AEI (Manchester, Great Britain) Model MS12 mass spectrometer interfaced via a silicone membrane separator with a Varian Model 1400 gas chromatograph. The glass columns were 6 ft. × 2 mm I.D. This instrument had been modified for SIM analysis based on accelerating voltage switching[13]. Reference mass spectra were recorded from the gas chromatograph employing a Digital Equipment Corporation PDP 8/I computer for data reduction and background substraction (AEI, data system DS 30).

The GC packing employed routinely in FID and NFID assay was 0.75% Carbowax 20M, 5% KOH on Chromosorb G (80–100 mesh) (Phase Separations, Queensferry, Great Britain). Slight inter-batch differences in relative retention times were observed with this packing material and were inferred to be due to variation in the nominal KOH load. Within-batch variation was not encountered. The packing 3% OV-1 on Gas-Chrom Q (80–100 mesh) was used in GC–MS assay.

Reagents and reference compounds

Sodium borohydride and 4,4′-dipyridyl were obtained from BDH (Poole, Great Britain) and methyl iodide, ethyl iodide and methyl-d_3 iodide ($\geqslant$ 99 atom % D) from Koch-Light (Colnbrook, Great Britain). Solvents were AnalaR grade and were not redistilled. Paraquat dichloride (methyl viologen hydrate) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was dissolved in methanol, filtered, reprecipitated with acetone and dried before use as a reference standard.

[Methyl-^{14}C] paraquat dichloride (30 mCi/mmol) was supplied by the Radiochemical Centre (Amersham, Great Britain).

The diene II was synthesised by the following procedure. Sodium borohydride (100 mg) was added to a continuously stirred solution of paraquat dichloride (0.5 g) in 10 ml of water. An intense blue colouration due to the paraquat radical ion persisted for several minutes. After 30 min, the pH was adjusted to 12 and the reaction mixture extracted with three aliquots of 10 ml ether. The combined ether extracts were dried (sodium sulphate) and evaporated to dryness under nitrogen, yielding 297 mg of solid material (79% yield based on paraquat ion). Concentrated solutions rapidly darkened on standing. Recrystallisation was effected from chilled hexane after brief treatment with silicic acid to decolourise the solution. The diene II [m.p. 100.5° (uncorrected); $C_{12}H_{20}N_2$: calculated C 74.95%, H 10.48%, N 14.57%; found C 74.85%, H 10.35%) was characterised by NMR, UV and mass spectrometry.

1,1′-Diethyl-4,4′-dipyridyl dichloride and 1,1′-di(trideuteromethyl)-4,4′-dipyridyl dichloride (paraquat-d_6) were prepared from the appropriate alkyl halides and 4,4′-dipyridyl. The following procedure was used for the diethyl derivative. Ethyl iodide (1 g) and 4,4′-dipyridyl (0.5 g) were dissolved in 6 ml of dimethylformamide. After standing for 18 h at room temperature, the resulting red precipitate was filtered and washed with dimethylformamide and acetone. The diiodide was dissolved in 5 ml of water and an excess of a saturated aqueous solution of picric acid added. The

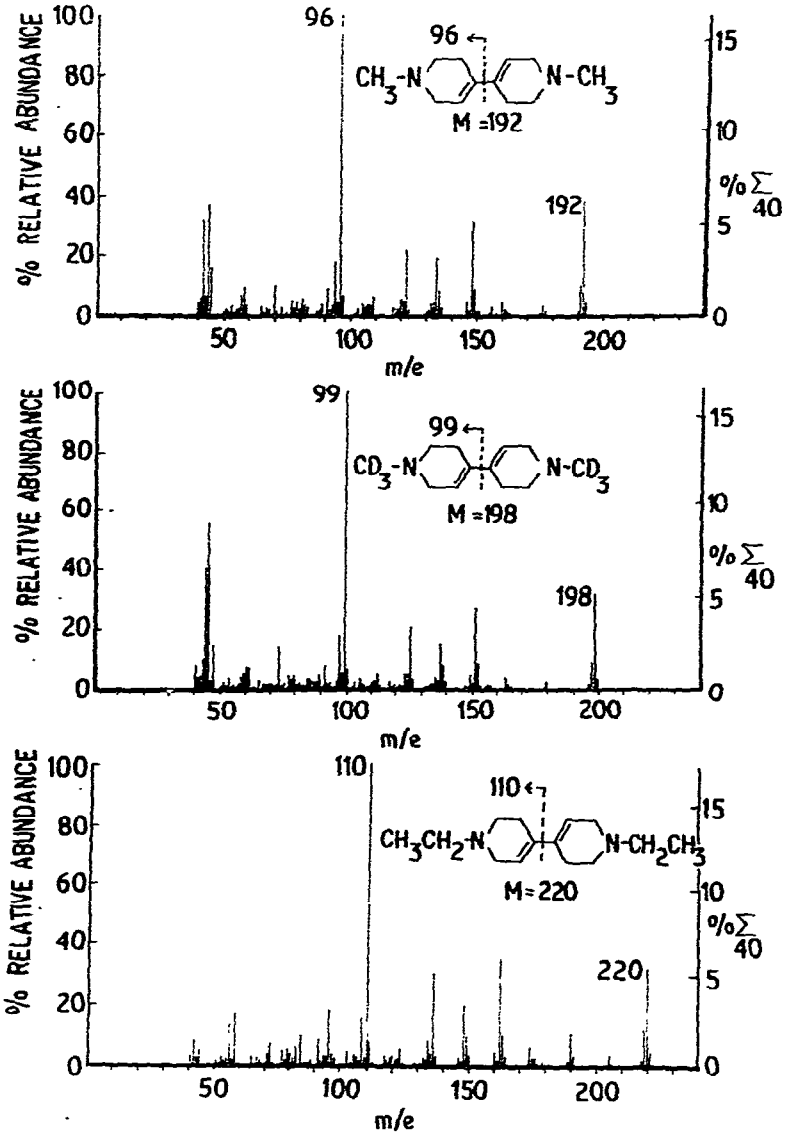


Fig. 1. Electron impact mass spectra of dipyridyl reduction products at 70 eV.

resulting fine light yellow precipitate was filtered and washed consecutively with water and acetone. The picrate salt was suspended in 100 ml of acetone, and 1 ml of concentrated HCl added with stirring, resulting in conversion to the colourless chloride. After 30 min, the precipitate was filtered, washed with acetone and dried *in vacuo*. A further purification could be obtained by solution in a minimum volume of methanol and reprecipitation by the slow addition of acetone. The product 1,1′-diethyl-4,4′-dipyridyl dichloride was characterised by GC–MS after sodium borohydride reduction. Paraquat-d_6 was similarly synthesised (from methyl iodide-d_3) and characterised by GC–MS; mass spectra are presented in Fig. 1.

Analytical procedures

3 ml of plasma, 1 ml of an aqueous solution of 1,1′-diethyl-4,4′-dipyridyl (1 μg ion per ml for FID and 0.1 μg ion per ml for NFID) and 1 ml of a 25% aqueous solution of trichloroacetic acid were thoroughly mixed and centrifuged (2500 rpm). The protein precipitate was resuspended in 5 ml of a 5% aqueous solution of trichloroacetic acid, mixed and centrifuged. The combined supernatant was placed in a 50-ml tube, adjusted to pH 10 with 1 ml of 5 *N* NaOH and 150 mg of $NaBH_4$ added. The mixture was left at room temperature with intermittent shaking for a period of 60–90 min. (The reaction time to produce approximately 90% conversion to II was checked for individual batches of commercial reagent). Sodium acetate (8 g) was added to the reduction mixture which was then extracted twice with 9 ml of ether. The ether layers were back-extracted in turn into 0.5 ml 1 *N* HCl contained in a 10-ml tapered tube. The ether layers were discarded and the acid transferred to a 3-ml Reacti-vial (Pierce, Rockford, Ill., U.S.A.) and traces of ether were removed under a stream of nitrogen.

For FID analysis, the solution was adjusted to alkaline pH with 0.15 ml of 5 *N* NaOH and thoroughly mixed with 30 μl of dichloromethane. After centrifuging, a 3-μl aliquot of the organic layer was used for GC. The chromatographic conditions were: oven 180°, injection block 210°, detector 250°, and nitrogen flow-rate 20 ml/min. Retention data are noted in Table I. Quantification was based upon peak height ratio of II to the internal standard with interpolation of unknowns on a standard curve for paraquat ion in plasma in the range 0.1–1.0 μg/ml. (See Fig. 2.)

For analysis by NFID, dichloromethane could not be used as a solvent. Following extraction into 0.5 ml of 1 *N* HCl and readjustment to basic pH, the reduction products were recovered into ether (2 × 1 ml). The ether was evaporated under nitrogen and the residue reconstituted in 20 μl of methanol. A 2-μl aliquot was used for GC. The chromatographic conditions were: oven 200° and detector 400°; helium (carrier gas) flow-rate 60 ml/min, hydrogen 28 ml/min and air 180 ml/min. Adjustment of the crystal position for maximum selectivity in response to nitrogen was carried out daily prior to paraquat analysis and was most conveniently effected by injecting 10 ng of diphenylamine and 5 μg of *n*-C_{20} alkane in 1 μl of hexane. The crystal was adjusted until peaks of equal height were obtained. The calibration range for paraquat ion in plasma was 0.025–0.2 μg/ml.

TABLE I

GAS CHROMATOGRAPHIC RETENTION DATA FOR SODIUM BOROHYDRIDE REDUCTION PRODUCTS OF DIPYRIDYL DERIVATIVES

Conditions: 5 × 2 mm I.D. glass column of 0.75% Carbowax 20M 5% KOH on Chromosorb G (80–100 mesh) at 180°; Pye Model 104 instrument; detection, FID.

Dipyridyl derivative	*Reduction product*	*Retention time (min)*	*Relative retention time*
Paraquat (I)	diene (II)	3.55	1.00
	monoene	1.50	0.40
1,1′-Diethyl-4,4′-dipyridyl	diene	5.90	1.66
	monoene	2.50	0.70
Diquat (III)	diene	3.10	0.87

Aliquots of tissue (1–3 g) after homogenisation in 5 volumes of 10% trichloroacetic acid could be treated in a similar manner. For certain urine and bile samples, protein precipitation could be omitted and it was possible to proceed to reduction with sodium borohydride. Quantitative methods have been evaluated fully in terms of the extraction sequence and possible endogenous substance interference only for plasma.

RESULTS AND DISCUSSION

Sodium borohydride reduction

Sodium borohydride reduction of paraquat dichloride yielded an ether-soluble crystalline solid of molecular formula $C_{12}H_{20}N_2$ corresponding to a hexahydro derivative. The base peak in the mass spectrum at *m/e* 96, shifted 3 a.m.u. in the spectrum of the trideuteromethyl analogue and 14 a.m.u. in the diethyl analogue (Fig. 1) was attributed to cleavage of the 4,4′-bond and indicated a bis(dehydropiperidine) structure.

The course of reduction of paraquat was examined in aqueous solution with 10 mg of the dichloride salt and a substantial excess of sodium borohydride (20 mg). [^{14}C-Methyl] paraquat was used as a tracer and the products were examined by GC–MS. Reaction to ether extractable radioactivity was 30% at 1 min, 80% at 5 min and complete at 10 min. A by-product of higher volatility than the diene was formed and identified by GC–MS as a monoene. Retention data are discussed below. This material accounted for 7% of the reduction product at 5 min and 9% at 10 min based on integration of GC peak areas. Reduction and subsequent extraction prior to GC was conveniently standardised by incorporating 1,1′-diethyl-4,4′-dipyridyl (II) as the reference. Reduction followed an analogous course to that for paraquat, the major product being the diene.

The related herbicide diquat (III) combined with paraquat in the commercial preparation Weedol and frequently encountered in poisoning cases, was similarly reduced to a hexahydro product.

Extraction from biological fluid

Protein precipitation using trichloroacetic acid could be applied to plasma and to tissue homogenates and was adopted as the initial clean-up step in the recovery of paraquat from biological material. Yields at each stage in the extraction sequence were checked with [^{14}C-methyl] paraquat at a concentration of 1 μg per 3 ml of plasma. Separation from protein into the trichloroacetic acid supernatant could be made quantitatively. The rate of reduction in this medium following readjustment to basic pH was slower than in water. Reaction to ether extractable radioactivity was 60% after 40 min, 90% at 70 min and complete at 90 min. Typically, the reduction was allowed to proceed for 60–90 min. The subsequent stages of ether extraction and back-extraction into HCl resulted in quentitative recoveries of radioactivity. In the final partition between dichloromethane (0.03 ml) and the basic aqueous phase (0.65 ml), 60–65% of the radioactivity was concentrated in the organic layer. One tenth of the organic extract was used for FID analysis.

The reduction products were found to be markedly adsorbed on glass surfaces. Thus, for NFID assay, where concentration by solvent evaporation was substituted

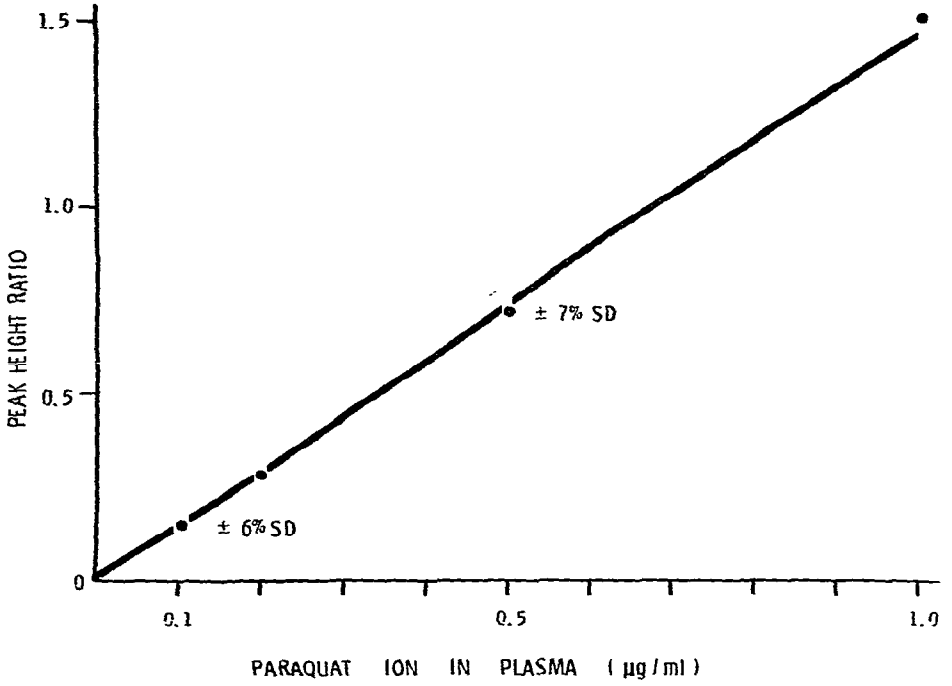


Fig. 2. Calibration and precision of measurement (as % relative standard deviation on the mean) for paraquat in plasma using FID. Response ratio of II to the internal reference is plotted against paraquat ion concentration.

for back-extraction into dichloromethane, care had to be taken to wash the walls of the tube with methanol in reconstitution of extracts for chromatography. Dispersal on glass surfaces during the removal of large volumes of ether was avoided where possible.

GC conditions for plasma assay

The reduction products of paraquat and its diethyl analogue could be chromatographed on SE-30, OV-1, OV-17, carbowax 20 M and on Carbowax coated on base-treated supports. The combination of 0.75% Carbowax 20M and 5% KOH on Chromosorb G provided good peak shape, and negligible memory effects or adsorptive loss at low concentration of sample. Retention data for dipyridyl reduction products are reported in Table I.

With FID, 5 ng of the diene II could be detected and with NFID the detection limit was approximately 0.1 ng. In FID analysis, calibration was linear in the range 0.1–1.0 μg/ml for paraquat ion based on the extraction of 3 ml of plasma (Fig. 2). The precision of measurement at 0.1 μg/ml was $\pm$ 6% (S.D., $n = 6$) and at 0.5 μg/ml was $\pm$ 7% (S.D., $n = 6$). The NFID procedure was used chiefly in the range 0.025–0.2 μg/ml. Fig. 3 shows a chromatogram obtained using NFID from plasma containing 0.025 μg/ml of paraquat and 0.1 μg/ml of the internal standard. Control extracts showed no interference from endogenous substances.

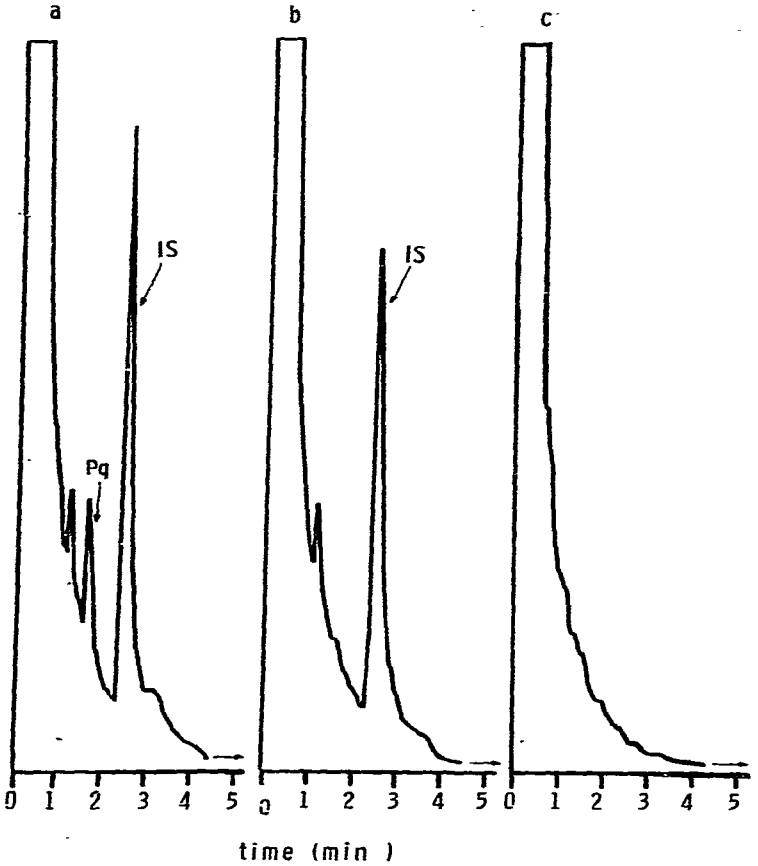
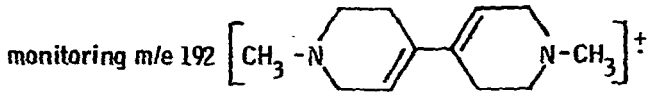


Fig. 3. NFID analysis after sodium borohydride reduction of plasma extracts: (a) 0.025 μg/ml of paraquat ion (Pq) and 0.1 μg/ml of 1,1′-diethyl-4,4′-dipyridyl (internal standard, IS), (b) internal standard only and (c) control plasma.

GC–MS analysis

Enhancement in selectivity and sensitivity was obtainable using SIM mass spectrometry as the GC detection technique. Fig. 4 illustrates the detection of paraquat at the molecular ion of the reduction product, in the plasma of a patient on the 10th day after ingestion of an unspecified quantity of weed killer. Based, in this example, on external standardisation, the level was estimated to be between 5 and 10 ng/ml. Quantitative SIM could be achieved using the diethyl analogue as the internal standard or by stable isotope dilution employing paraquat-d_6. However, the principal use of GS–MS has been in the positive identification of paraquat at trace levels in forensic analysis. Fig. 5 shows the determination of paraquat in the *post-mortem* bile of a murder victim, monitoring the response ratio at *m/e* 192–190. Advantage was taken of a characteristic effect of source temperature on M—2/M ratio which ranged from 0.12 at 205° to 0.30 at 280°. The identity of retention time and of response ratio under two sets of operating conditions with the values for the authentic substance provided convincing evidence for the presence of paraquat in the victim. In this case,



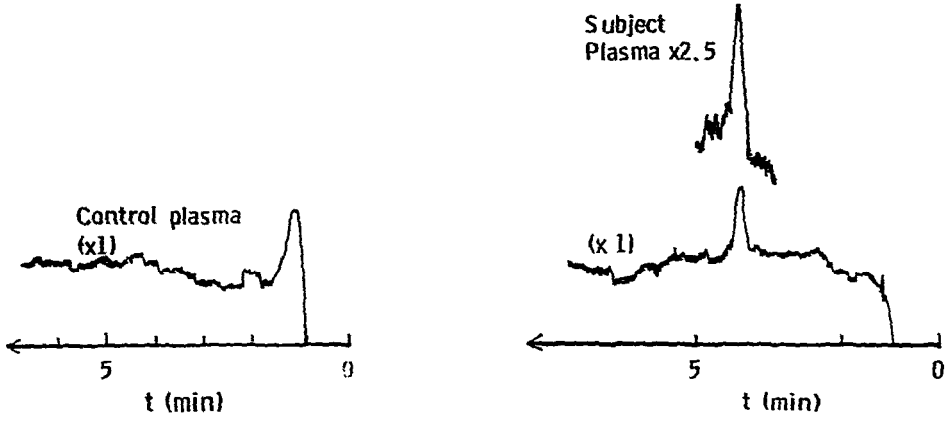


Fig. 4. Selected ion monitoring detection at the molecular ion (*m/e* 192) of II in extracts of plasma 10 days after ingestion of paraquat in man.

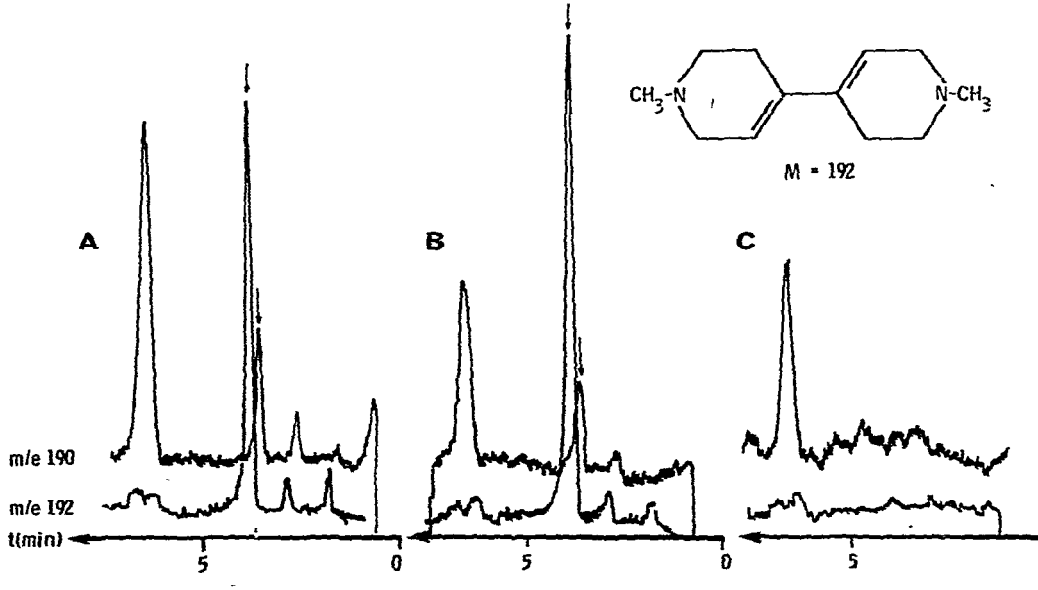


Fig. 5. The identification of paraquat as its reduction product II in *post-mortem* bile. Arrowed points indicate channel offset and correspond to the elution time of authentic II. M/M. − 2 (*m/e* 192/190) ratios at source temperatures of (A) 280° and and (B) 205° were consistent with the values for the authentic substance, II; C, control bile extract.

poisoning had not been suspected until some 8 days after admission to hospital. At death on the 12th day the residue in the body was close to the limits of determination by conventional techniques.

CONCLUSIONS

An accurate and sensitive method has been developed to measure paraquat in plasma. With this technique, we have shown that excretion of paraquat in urine is not a reliable guide to the severity of intoxication[14,15]. Paraquat can cause severe renal failure such that even in the presence of high systemic concentrations of the chemical only small amounts are excreted in the urine. Further, we have found that patients with plasma concentrations in excess of 0.25 μg/ml from 12 to 68 h after ingestion, with associated renal failure, usually do not survive. Our views on the pharmacokinetics of paraquat and the treatment of paraquat poisoning have now been presented in detail[15].

NOTE ADDED IN PROOF

The analytical procedures described have now been further developed with enhanced speed of analysis by Dr. G. Steele, ICI Central Toxicology Laboratories, Great Britain and by Professor Maes, University Toxicological Centre, Vondellaan 14, Utrecht, The Netherlands. (Personal communications to the authors.)

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