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# THE ANALYSIS OF PARAQUAT IN URINE BY HIGH-SPEED LIQUID CHROMATOGRAPHY

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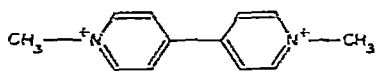
## SUMMARY

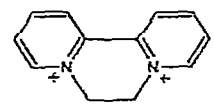
The paraquat content of urine can be directly determined by high-speed liquid chromatography using ultraviolet spectrophotometric detection. The method separates paraquat (and diquat) from the ultraviolet-absorbing components of urine and no extraction or pre-treatment of the sample is required prior to analysis. Concentrations down to 100 μg/l of paraquat in urine were determined. Quantitative results are in good agreement with those obtained by a colorimetric method. Diquat does not interfere with the analysis of paraquat, and it would also be possible to analyse diquat in paraquat-containing urine.

## INTRODUCTION

Paraquat is a bipyridilium herbicide currently marketed alone or in combination with the related herbicide diquat. In recent years there have been many cases of poisoning following ingestion of paraquat, and deaths have been reported. As there is no specific antidote for paraquat, treatment of paraquat poisoning involves eliminating the herbicide from the body as quickly as possible[1,2]. A fast analytical method for the detection and analysis of paraquat in body fluids is therefore desirable.



Paraquat



Diquat

A spot test for paraquat in urine sensitive to *ca.* 1 mg/l involves reduction of

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the paraquat to an intensely blue radical using alkaline dithionite[3]. For quantitative analysis[3–6], the paraquat (or diquat) is separated from the urine by cation-exchange chromatography and the reduced herbicide measured spectrophotometrically. Such ion exchange–colorimetric procedures are lengthy and in practice are not really satisfactory at paraquat concentrations below 500 μg/l.

The high-speed liquid chromatography (HSLC) method described in this paper has been used to analyze paraquat concentrations of 100–2000 μg/l in urine and has the advantage of requiring no derivative formation or extraction procedures.

## EXPERIMENTAL

### *Apparatus*

The liquid chromatograph was constructed in the laboratory, the main features being a reciprocating pump DMP 1515 (Orlita, Giessen, G.F.R.) and a CE 212 variable-wavelength monitor (Cecil, Cambridge, Great Britain) fitted with an 8 μl flow-cell. Columns (12.5 cm × 5 mm I.D.) of internally polished stainless steel were terminated by 10-μm PTFE frits. Column fittings were made according to designs described elsewhere[7].

### *Column-packing material*

The column-packing material was prepared by chemically bonding γ-aminopropyltriethoxysilane (Pierce, Rockford, Ill., U.S.A.) to the surface of spherical 30-μm alumina (Spherisorb A20Y; Phase Separations, Queensferry, Great Britain) via stable monomolecular Si–O–Al bonds, by treating the silane and the freshly dried support material with an organic solvent at an elevated temperature. Further details of the preparation and properties of a range of chemically bonded supports have been published separately[8]. Three batches of material prepared as described above showed similar chromatographic properties. Microanalysis figures for two batches are given in Table I; batch No. 246 was used throughout this study.

TABLE I

MICROANALYSIS RESULTS FOR THE COLUMN-PACKING MATERIAL

| *Batch* | *Analysis (%)* | | |
|---|---|---|---|
| | *C* | *H* | *N* |
| WLCU No. 241 | 2.42 | 0.54 | 0.49 |
| WLCU No. 246 | 2.65 | 0.68 | 0.60 |

### *General chromatographic technique*

Solvents were degassed by heating under reflux. A home-made pulse-damper connected to the high-pressure line comprised a 500 × 5 mm I.D. stainless-steel tube which was sealed at one end and connected at the other end to a Hone valve. Baseline noise caused by the stroke of the reciprocating pump was effectively damped by air trapped in the tube, even at the highest detector sensitivities [0.01 absorbance units full scale (a.u.f.s.)] used in this study.

Columns were packed by the rotate, bounce and tap method[9] and were oper-

ated at ambient temperature. The column and connecting tubing from the column to the flow-cell were lagged in order to prevent thermal drift of the baseline at high sensitivity due to draughts. The flow-rate was continuously monitored, and corrections were made for the effects of any small variation in flow-rate on the peak area on the basis that the peak area for a given quantity of solute is inversely proportional to the flow-rate. Such variations were normally less than $\pm 5\%$ over an 8-h period.

Small sample volumes ($< 5\ \mu l$) were injected without stopping the flow of mobile phase; for larger volumes (typically, 50 $\mu$l), the flow was stopped by means of a Whitey valve prior to injection. In order to avoid contamination, syringes were thoroughly washed with water and acetone and dried after each injection. Prior to each injection, the samples were mixed by inversion and the syringe thoroughly flushed with the sample to be injected. Injection of a blank sample after an injection of paraquat showed that all the paraquat had been removed from the syringe.

Peak areas were measured by constructing triangles about the peak and the baseline and measuring the area. For samples of urine containing paraquat, the sloping baseline was estimated as shown in Fig. 1.

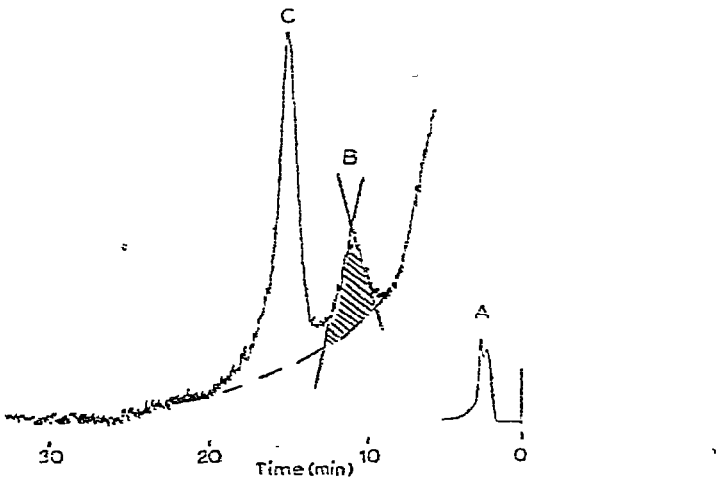


Fig. 1. Estimation of peak area for urine containing paraquat (sample 2, Table II, containing 800 $\mu$g/l of paraquat). Column (12.5 cm × 5 mm I.D.): stainless steel, packed with $\gamma$-aminopropyltriethoxysilane bonded to Spherisorb A20Y; temperature, ambient; pressure, 135 p.s.i. Mobile phase: buffer (0.01 *M* $KH_2PO_4$ + $H_3PO_4$ to pH 2.45)–methanol (11:14, v/v); flow-rate, 0.66 ml/min. Detector: Cecil CE 212 UV photometer operating at 258 nm. Injection by stopped flow; sample volume, 50 $\mu$l of neat urine containing paraquat. Peaks: A (sensitivity 2.0 a.u.f.s.) = endogenous urine components; B (0.01 a.u.f.s.) = paraquat; C (0.01 a.u.f.s.) = endogenous urine component.

*Preparation of standards and solvents*

Standard solutions of paraquat were prepared from paraquat dichloride (I.C.I., Macclesfield, Great Britain) which had been heated at 120° for 24 h in a vacuum-oven and then allowed to cool in a desiccator containing phosphorus pentoxide. The samples were weighed on a Cahn electrobalance, and dissolved in doubly distilled water. Methanol (AnalaR grade) was used as supplied (Fisons, Loughborough, Great Britain). The buffer solution was made by dissolving potassium dihydrogen-

phosphate (1.36 g, 0.01 *M*) in 1 l of doubly distilled water and adjusting the pH of the solution to a value of 2.45 by addition of *ca.* 1.1 g/l of phosphoric acid. Since the retention time of the paraquat peak was fairly sensitive to the buffer:methanol ratio in the mobile phase, *ca.* 2.5 l of mobile phase were prepared each time. A typical mobile phase composition for the analysis of paraquat in urine was methanol–buffer (14:11, v/v).

*Ion-exchange–colorimetric method*

The method used was based on published procedures[3,4]. Recoveries were 90% at 500 μg/l with a precision of 5.7%, and 86% at 2000 μg/l with a precision of 6.5%. Recoveries were calculated from 3 separate determinations on separate columns; precisions were calculated from the results of 5 replicate determinations on separate columns.

## RESULTS AND DISCUSSION

*Chromatographic phase and conditions*

Chromatographic materials formed by bonding organosilyl groups to the surface of silica via thermally and hydrolytically stable Si–O–Si bonds or direct Si–C bonds are now well established[10–19], and such materials have been used in many novel applications[20]. This is the first instance in which a silane has been bonded to the surface of alumina for the purposes of HSLC. The phase was stable to prolonged treatment with methanol–buffer mixtures (at an apparent pH of 3.3), and its performance was unaffected by repeated injections of neat urine, including samples which had been concentrated to thirty times their original urine (and paraquat) concentration.

The mechanism of retention of the paraquat (and diquat) by the support is not well understood at present. The use of buffer solutions of pH $> 4$ caused paraquat and diquat to be eluted as unretained peaks. The buffer used in this study (pH 2.45), when used as the mobile phase without additional methanol, caused the paraquat to elute as the unretained peak, with diquat slightly retained. At this pH, the aminopropyl-bonded phase would be in the cationic form, *viz.* $(CH_2)_3NH_3^+X^-$. Addition of increasing proportions of methanol caused the paraquat and diquat to be increasingly separated and retained. The methanol–buffer mixture finally chosen for the determination of paraquat in urine was the one which gave the best separation of the paraquat from the endogenous ultraviolet (UV)-absorbing components of urine. The mechanism of retention and separation of paraquat and diquat on the anion-exchange material in acid solution is clearly not one of ion exchange. There is probably a streaming of the mobile phase into two layers, with the bonded organic coating on the particle surface extracting the methanol from the mobile phase to form a methanol-rich layer on the particle surface, into which paraquat and diquat ion-pairs could be partitioned.

The paraquat and diquat peaks themselves were fairly broad, but probably not more than expected for doubly charged ions, and both peaks were fairly symmetrical.

*Analysis of paraquat in urine*

The UV spectra of paraquat and diquat in the methanol–buffer used as mobile

phase in this study were similar to those reported elsewhere[3]. In particular, paraquat had an absorbance maximum, $\lambda_{max.}$ at 258 nm ($\varepsilon_{max.}$ = 14,000), and diquat at 308 nm ($\varepsilon_{max.}$ = 17,500 l/mole·cm). Urine is strongly absorbing at *ca.* 258 nm, but has negligible absorbance at 308 nm. Thus interference from UV-absorbing components of the urine is much more severe in the estimation of paraquat than of diquat in urine. The present method relies on chromatographic separation of the paraquat from other much more highly absorbing endogenous compounds in the urine (Fig. 1). After separation, the paraquat peak is measured at 258 nm and quantitated by comparison of the peak area against those of standard solutions of paraquat. As all except one of the UV-absorbing components (at 258 nm) of urine elute with or soon after the solvent front, interference from the relatively large amount of these compounds can therefore be largely eliminated. The paraquat elutes on the sloping baseline of the endogenous components, prior to the most strongly retained endogenous component. The endogenous peaks were eluted at low detector sensitivity and the sensitivity was increased to 0.01 a.u.f.s. before the paraquat eluted. The capacity ratio of the paraquat peak was relatively sensitive to the composition of the mobile phase, and this was used to advantage to arrange the elution of paraquat to occur just between the final endogenous peaks.

*Paraquat standards*

Calibration graphs of peak area *versus* the amount of paraquat injected were constructed from duplicate injections of three or four standard solutions of paraquat, whose concentration range embraced the values of the clinical samples to be analyzed. A typical graph is shown in Fig. 2. Calibration graphs obtained with paraquat in water and urine showed no significant difference and standard solutions were thus made up in water for convenience. In order to even out any variations in the operating conditions, clinical samples and standard solutions were injected consecutively, and duplicate injections were made after an interval of several injections. The samples and standard solutions were injected in the same volume of water and urine respectively.

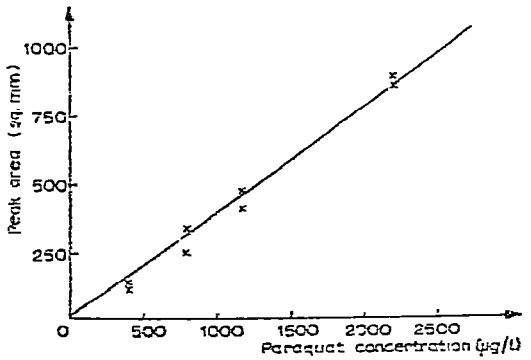


Fig. 2. Calibration graph of peak area *versus* amount of paraquat injected from chromatograms of standard solutions of paraquat (50 µl injections).

*Clinical samples*

The urine samples studied were obtained from two patients who had ingested paraquat. The first patient (samples 1–3, Table II) was a 4-year old boy and the

TABLE II

DETERMINATION OF PARAQUAT CONCENTRATIONS IN URINE BY THE HSLC AND ION-EXCHANGE–COLORIMETRIC METHODS

| *Sample** | *Paraquat concentration (μg/l)* | |
|---|---|---|
| | *HSLC*** | *Ion exchange–colorimetry**** |
| 1 | 21 800 | 22 000 |
| 2 | 800 | 900 |
| 3 | 100 | trace amount |
| 4 | 2650 | 3000 |
| 5 | 1600 | 1700 |
| 6 | 800, 900 | 1250 |
| 7 | 950, 1000 | 1550 |
| 8 | 600 | 1150 |
| 9 | 650 | 1150 |
| 10 | 200 | 300 |

* For origin of samples, see text.

** The values quoted for sample 1 and for samples 2–10 are the averages of triplicate and duplicate analyses respectively. Sample 1 was analyzed by injecting 2 μl of untreated urine, samples 2–10 by injecting 50 μl of untreated urine.

*** The values are the result of a single analysis.

second patient (samples 4–10, Table II) a 47-year old man, both of whom recovered.

Table II lists the values of paraquat concentration obtained by HSLC and by the colorimetric method. The results are arranged in serial order of collection. The values vary from 100 to 22,000 μg/l. For the determination of paraquat in the dilute samples (*i.e.* 2–10), a convenient volume for injection at the maximum instrument sensitivity of 0.01 a.u.f.s. was 50 μl, which in every case gave paraquat peaks

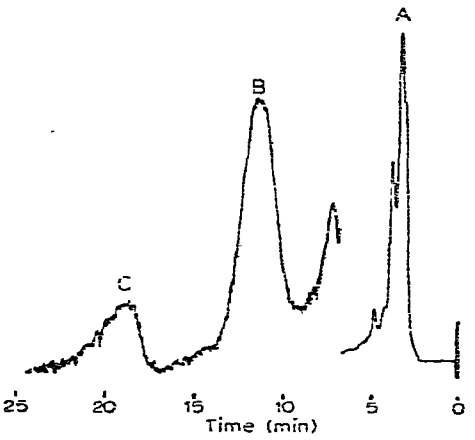


Fig. 3. Determination of high concentration of paraquat in urine (sample 1, Table II, containing 21,800 μg/l of paraquat). Column pressure, 125 p.s.i. Mobile phase: buffer (See Fig. 1)–methanol (23:27, v/v); flow-rate, 0.5 ml/min. Direct injection; sample volume, 2 μl of neat urine containing paraquat. Peaks: A (sensitivity 0.5 a.u.f.s.) = endogenous urine components; B (0.01 a.u.f.s.) = paraquat; C (0.01 a.u.f.s.) = endogenous urine component. Other conditions as in Fig. 1.

whose areas were readily measured and quantitated. For the more concentrated sample (1), direct injection of a 2-$\mu$l volume gave a readily measurable paraquat peak at maximum sensitivity (Fig. 3). The initial sample volume is relatively unimportant (the elution volume of the paraquat peak is *ca.* 2.5 ml), because of the dilution effect on the paraquat when the sample is injected onto the column. Thus lower concentrations of paraquat can probably be measured simply by injecting larger volumes onto the column, or alternatively by injection of the same volume of a concentrated urine sample. In the course of this study, chromatograms of urine samples (containing paraquat) which had been concentrated up to thirty times their original urine concentration were obtained but not quantitated. The effect of continued injection of urine samples (*ca.* 70 × 50 $\mu$l at normal concentration, and 45 × 3 $\mu$l samples including 3, 2 and 4 which had been concentrated 30, 15 and 10 times, respectively) on the efficiency of the column-packing material appeared to be minimal, and the column used throughout this study showed no deterioration in performance. No guard column was necessary, and the only precaution taken was to flush out the acid buffer from the column with distilled water before leaving the column overnight. The paraquat was quantitated by duplicate or triplicate injection of a sample and averaging the peak areas obtained. The paraquat concentration was then read from the calibration graph.

*Reproducibility and recovery*

The reproducibility of the method was determined by measuring the paraquat peak areas of ten consecutive injections of sample 7 containing *ca.* 1900 $\mu$g/l of paraquat. The coefficient of variation was 6.6%. The recoveries of two samples of paraquat added to sample 9 were quantitative within the limits of the reproducibility of the method, and thus no recovery correction was made to the values of paraquat concentration estimated from the calibration graphs.

There is no clear explanation for the consistently higher values of the paraquat concentration obtained by the ion-exchange–colorimetric method, but difficulty in obtaining true blank values and variations in the degree of recovery of paraquat from the ion-exchange resin may be partial explanations. Interference by diquat does not cause this discrepancy because although the specific absorbance is very low at 605 nm but moderately high at 395 nm, measurements at these wavelengths separately produced identical results for the concentration of paraquat.

There is no doubt that HSLC is superior for the assay of low concentrations of paraquat, direct application of urine being the greatest advantage. At high concentrations, the specific colour reactions can be used without resorting to ion-exchange procedures, and some of the disadvantages of the colorimetric method are eliminated.

*Paraquat in gastric aspirate*

A sample of gastric aspirate containing a high concentration of paraquat (140 mg/l, colorimetric determination) was examined briefly by this method. The gastric aspirate showed considerably fewer interfering peaks than did urine (Fig. 4). Variation in the capacity ratio of the paraquat peak caused by adjusting the proportions of methanol and buffer in the mobile phase did not lead to the appearance of any shoulders on the paraquat peak, and hence it was assumed that the peak shown represents pure paraquat.

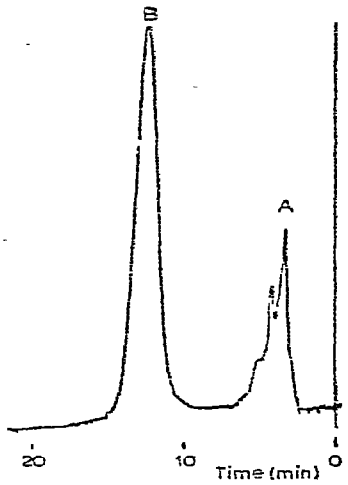


Fig. 4. Determination of paraquat in gastric aspirate (containing 140 mg/l of paraquat). Column pressure, 120 p.s.i. Mobile phase: as in Fig. 3; flow-rate, 0.47 ml/min. Direct injection; sample volume, 3 $\mu$l of neat gastric aspirate containing paraquat. Instrument sensitivity, 0.02 a.u.f.s. throughout. Peaks: A = endogenous components of gastric aspirate; B = paraquat.

*Diquat in urine*

Diquat can be detected in urine, and does not interfere with the determination of paraquat in urine. Diquat has a very low absorbance at 258 nm, the wavelength used to detect paraquat, and, under the chromatographic conditions used to analyse paraquat, diquat is much more strongly retained by the column. However, diquat is readily detected in the presence of paraquat by changing the wavelength to 310 nm after the elution of paraquat and the endogenous components (Fig. 5). Diquat alone in urine is readily detected, the chromatogram being run at 310 nm with little interference from the endogenous peaks (Fig. 6).

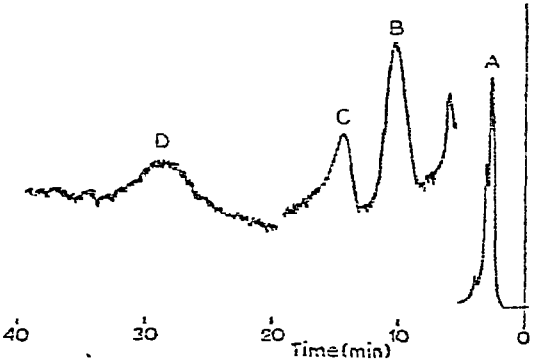


Fig. 5. Determination of diquat in the presence of paraquat in urine. Column pressure, 180 p.s.i. Mobile phase: buffer (see Fig. 1)–methanol (53:47, v/v); flow-rate, 0.59 ml/min. Direct injection; sample volume, 5 $\mu$l of a synthetic mixture containing 1.5 $\mu$l of sample 1 (Table II, 21,800 $\mu$g/l of paraquat) and 70 ng of diquat in 3.5 $\mu$l of water. Peaks: A (sensitivity 0.5 a.u.f.s.; wavelength 258 nm) = endogenous urine components; B (0.01 a.u.f.s.; 258 nm) = paraquat; C (0.01 a.u.f.s.; 258 nm) = endogenous urine component; D (0.01 a.u.f.s.; 310 nm) = diquat. Other conditions as in Fig. 1.

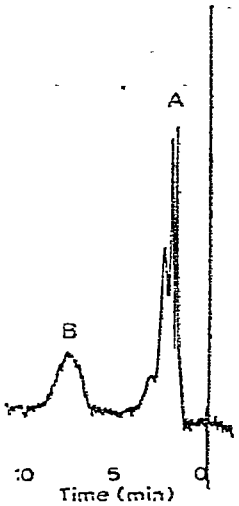


Fig. 6. Determination of diquat in urine. Column pressure, 230 p.s.i. Mobile phase: buffer (see Fig. 1)–methanol (3:2 v/v); flow-rate, 0.9 ml/min. Direct injection; sample volume, 2.5 $\mu$l of a synthetic mixture consisting of 1 $\mu$l of neat urine and 30 ng of diquat in 1.5 $\mu$l of water. Instrument sensitivity, 0.01 a.u.f.s. throughout. Wavelength, 310 nm throughout. Peaks: A = endogenous urine components; B = diquat. Other conditions as in Fig. 1.

*Interfering substances*

The method described is based on the assumption that no interfering substances elute with the paraquat peak. This seems reasonable since the paraquat peak, which is sensitive to changes in the composition of the mobile phase, can be shifted to merge with either the preceding or succeeding endogenous peaks by suitable changes in the composition of the mobile phase. For instance, 5% increase in the methanol concentration causes the paraquat to be more strongly retained and to overlap with peak C (Fig. 1). Correspondingly, a similar decrease in the methanol concentration causes the paraquat peak to be less strongly retained and to merge with the tail of the large endogenous peaks. The behaviour of paraquat (and diquat) in this respect seems characteristic and it is unlikely that there are other components present which shift by the same amount under the same treatment.

In cases of paraquat poisoning, where other substances are present which mask the region of the chromatogram where the paraquat normally elutes, the paraquat could be retained and eluted after the longest-retained urine peak (C, Fig. 1) by an increase in the methanol concentration. This would, however, be at the expense of chromatographic efficiency, and the width of the paraquat peak would increase with increasing time of retention. The minimum detectable concentration of paraquat would then be increased.

*Notes*

A variable-wavelength spectrometer was used in this study. Commercial chromatographs with UV detection at 254 and 280 nm would be suitable for the determination of paraquat by this method, but not for the determination of diquat where detection in the 310-nm region is desirable. Gradient elution could be employed to

speed up the separations described, and the accuracy of the method would be improved by the use of electronic integration.

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