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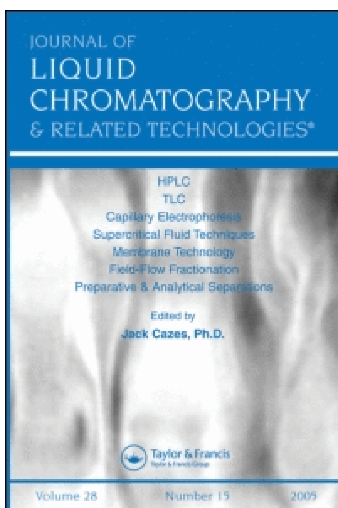


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# LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF PARAQUAT AND DIQUAT IN PLASMA, URINE AND VITREOUS HUMOUR

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## ABSTRACT

An HPLC method for the simultaneous determination of paraquat and diquat in aqueous solutions and biological fluids (plasma, urine and vitreous humour) was developed. This method is based on the initial ion-pair solvent extraction of both herbicides from plasma or urine. Vitreous humour samples required a protein precipitation and concentration process. Relatively small sample volumes (1 mL of plasma or urine, and 100 µL of vitreous humour) were enough to determine paraquat and diquat by the proposed technique. Chromatography was carried out using a LiChrospher® 100 RP-18 (5 µm) column for aqueous solutions and plasma and urine extracts, or a Nova-Pak $C_{18}$ (3.9x150 mm) column for vitreous humour extracts. Two ultraviolet wavelengths were selected, 254 nm for paraquat and 310 nm for diquat. The calibration curves were linear in the concentration ranges 0.42-8.4 µg/mL for aqueous solutions, 0.1-2 µg/mL for plasma, 0.1-3 µg/mL for urine and 0.5-5 µg/mL for vitreous humour.



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## INTRODUCTION

Weeds have been an important trouble in agriculture since the far-distant past. Some attempts for their control have been assumed through the ages. Nevertheless, the use of chemicals caused a real revolution in the weed control after World War II.

Bipyridylium herbicides (paraquat and diquat) are widely used in agriculture. Paraquat, prototype of that group, was first commercialized in 1962. The safety of paraquat in its correct use is well known. However, serious poisoning and high mortality have been associated with accidental or suicidal ingestion of paraquat. Although various measures to prevent paraquat poisoning (such as the addition of emetics, dyes, odorants and bitter substances) have been introduced, high lethality of paraquat hasn't been reduced. Since diquat toxicity is lower than paraquat toxicity, it has been considered advisable to reduce paraquat content of commercial product and to replace it with diquat.

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Determination of paraquat (PQ) and diquat (DQ) is required to know if these compounds are involved in a poisoning, to assess the severity of the intoxication,[1-5] and to monitor the terapy.

Spectrophotometric determination of PQ after reduction with dithionite is probably the most used technique. Derivative spectroscopy methods (6-8) have been recently reported for the determination of PQ and DQ in biological fluids. The use of derivative mode enhances the sensitivity and specificity of spectroscopy, as interference is eliminated. Other techniques, such as RIA,[9-12] PFIA,[13] ELISA,[14-16] GC,[17-19] TLC-FID[20] or capillary electrophoresis,[21] have been proposed. RIA and PFIA are very sensitive techniques and require small sample volumes, but cannot be commonly used.

Gill et al.[22] described in 1983 a method for the determination of both PQ and DQ in urine. The reported lower limit of detection was approximately 1 μg/mL for both herbicides. That limit required to be improved.

Querée et al.[23] reported in 1985 an HPLC method for the determination of PQ in liver and haemolysed blood which was based on the ion-pair solvent extraction of PQ with sodium dodecyl sulphate prior to ion-pair reverse phase chromatography. The reported limit of detection for haemolysed blood was 0.05 μg/mL.

Nakagiri et al.[24] developed in 1989 a new system in which an automated pretreatment apparatus was connected to ion-exchange HPLC. Measurement of PQ and DQ was automatically carried out after injecting a microsample of serum or urine into an injection port.

An ion-pair, reverse phase HPLC method with ultraviolet (UV) detection was later developed by Corasaniti et al.[25] to measure PQ concentrations in brain.

Ito et al.[26] have recently proposed a liquid chromatographic method for simultaneous determination of PQ and DQ in human tissues. This method was based on the extraction of PQ and DQ from the sample using a Sep-Pak $C_{18}$ cartridge. L-tyrosine was used as the internal standard.

Chromatography was carried out using an octadecyl silica column with a mobile phase of potassium bromide in methanol solution. Two UV wavelengths were selected, 256 nm for PQ as well as the internal standard, and 310 nm for DQ. The lower limit of detection was 0.05 μg/g.

Other authors[27,28] developed liquid chromatographic methods for determination of PQ and DQ in crops.

Gill's HPLC method for the quantification of PQ in urine has been applied to serum by Croes et al.[29] Sample preparation consisted of ion-pair extraction on disposable cartridges of end-capped octadecyl silica. PQ was determined by HPLC using 1,1'-diethyl-4,4'-dipyridyl dichloride as an internal standard.

A reverse phase ion-pair high performance liquid chromatographic system with UV detection is presented. This method separates PQ and DQ in under 8 min.

A rapid procedure for the previous extraction of the herbicides from plasma and urine, has been tuned up. The extraction method is a modified version of one reported by Querée et al.[23] Pretreatment of vitreous humour samples consisted of a simple protein precipitation and sample concentration process.

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## MATERIAL

### Reagents

Chemicals and HPLC-grade solvents were obtained from Merck. So, methanol, orthophosphoric acid, sulphuric acid, sodium dihydrogenphosphate, n-hexane, acetonitrile, diethylamine, methylisobutylketone (MIBK), isobutanol and sodium dodecyl sulphate were used. Heptanesulphonic acid sodium salt and octane sulphonic acid were supplied by Sigma, and sodium carbonate by Panreac. All chemicals used were of analytical grade.

### Standards

Paraquat dichloride was obtained from Sugelabor (Barcelona). Diquat dibromide was purchased from ICI.

### Apparatus

Chromatograms were obtained from a high-performance liquid chromatographic system consisting of a manual injector, LiChrospher® 100RP-18 (5 μm) column in LiChroCart® 125-4 (Merck) preceded by a 4x4 mm guard column ($C_{18}$ reversed-phase, particle size 5 μm), or 3.9x150 mm Waters Nova-Pak $C_{18}$ column with Waters Guard-Pak™ precolumn for vitreous humour samples, two Waters Model 501 pumps, a Waters Model 490 programmable multiwavelength UV detector, and a Waters system interface module. That chromatographic system was interfaced to a NEC PowerMate SX/16 microcomputer running MAXIMA&BASELINE software.

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## METHODS

### Solvents and Reagents Preparation

The MIBK was washed with 100 mL sodium carbonate (100 g/L) per liter of MIBK and then with distilled water. The extractant was prepared by mixing equal volumes of water-saturated isobutanol and MIBK, into which enough sodium n-dodecylsulphate was dissolved to obtain a final concentration of 25 g/L (23). 1M aqueous sulphuric acid was used as the aqueous phase of the plasma and urine extraction procedure. 0.5 and 2M $H_2SO_4$ were also tested.

### Preparation of Paraquat and Diquat Solutions

PQ dichloride was dried to constant weight at 100°C overnight and stored in a dessicator prior to use. Two stock solutions containing 50 mg/L and 100 mg/L, were then prepared in redistilled water.

Standards of DQ dibromide containing 50 mg/L and 100 mg/L, were also made similarly in redistilled water.

The above stock solutions were used to make working solutions containing 0.42-8.4 μg/mL PQ and/or DQ in mobile phase. The concentration range

**Table 1**

**Working Solutions**

| Set | Matrix | Concentrations (μg/mL) PQ | DQ |
|---|---|---|---|
| **PQ** | Aqueous | 0.42-2.1-4.2-6.3 and 8.4 | ----------- |
| | Plasma | 0.1-0.5-1.0-1.5 and 2.0 | ----------- |
| | Urine | 0.1-0.5-1.0-2.0 and 3.0 | ----------- |
| | Vitreous Humour | 0.5-1.0-2.0-3.0 and 5.0 | ----------- |
| **DQ** | Aqueous | ----------- | 0.42-2.1-4.2-6.3 and 8.4 |
| | Plasma | ----------- | 0.1-0.5-1.0-1.5 and 2.0 |
| | Urine | ----------- | 0.1-0.5-1.0-2.0 and 3.0 |
| | Vitreous Humour | ----------- | 0.5-1.0-2.0-3.0 and 5.0 |
| **PQ + DQ** | Aqueous | 0.42-2.1-4.2-6.3 and 8.4 | 0.42-2.1-4.2-6.3 and 8.4 |
| | Plasma | 0.1-0.5-1.0-1.5 and 2.0 | 0.1-0.5-1.0-1.5 and 2.0 |
| | Urine | 0.1-0.5-1.0-2.0 and 3.0 | 0.1-0.5-1.0-2.0 and 3.0 |
| | Vitreous Humour | 0.5-1.0-2.0-3.0 and 5.0 | 0.5-1.0-2.0-3.0 and 5.0 |

studied in plasma was 0.1-2 μg/mL for both compounds. Although the mentioned ranges for aqueous and plasma solutions are apparently different, the net amounts of each compound from aqueous solutions injected onto the HPLC, are the same as those from plasma solutions if the recovery obtained with the extraction procedure applied was 100%.

To calculate the recovery of the extraction method, we must consider that not only 4.2 mL of the 5 mL of extraction solvent used are transferred to a clean tube, but also 25 μL of the 200 μL $H_2SO_4$ added at the end of the process are injected onto the HPLC.

The concentration ranges selected for urine and vitreous humour samples were 0.1-3 μg/mL and 0.5-5 μg/mL, respectively.

We assayed three types of solutions, namely: a) a set containing PQ, b) another set containing DQ, and c) mixtures of the two pesticides, as can be seen in table 1. All these solutions were prepared in triplicate.

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### Extraction Procedure

The extraction procedure used was based on one reported by Querée et al.[23] Nevertheless, our liquid-liquid extraction method is shorter and easier to perform than the method of Querée.

A volume of 1 mL of biological fluid (plasma or urine) containing a given concentration of one or the two herbicides was mixed gently with 1 mL of redistilled water and 5 mL of extractant on a roller mixer and then centrifuged for 10-15 min, after which 4,2 mL of the organic layer were extracted and added 200 μL of 1M $H_2SO_4$. The mixture was then shaken vigorously for 5 min and centrifuged for 2 min, after which the acid extract was recovered and washed twice with 2 mL of n-hexane. 25 μL of the washed extract were injected onto the HPLC.

### Preparation of Vitreous Humour Samples

100 μL of vitreous humour and 100 μL of acetonitrile were mixed and shaken. The mixture was then centrifuged and 170 μL of the upper layer were recovered and concentrated to dryness into the speed-vac. The residues were dissolved in 28 μL mobile phase, 25 μL of which were injected onto the column.

### HPLC Method

The analytical columns used were those previously reported. Guard columns were also used. After different mobile phases were assayed, one proposed by Querée et al.[23] was selected to be applied to our chromatographic study. This one consisted of 25% aqueous methanol containing 10mM octane sulphonic acid and 13.4 mL/L o-phosphoric acid. Diethylamine was used to adjust the pH of the mobile phase to 3. The flow rate was set at 1 mL/min. The injected volume was 25 μL. We examined the suitability of different wavelengths to detect PQ and DQ. In spite of the first tests with aqueous solutions of mixtures of PQ and DQ confirmed that both compounds can be detected at 290 nm, we selected two detection wavelengths, 254 nm for PQ and 310 nm for DQ. Detector AUFS was set as referenced below:

| MATRIX | 254 nm | 310 nm |
|---|---|---|
| Aqueous | 0.06 aufs | 0.03 aufs |
| Plasma | 0.03 aufs | 0.03 aufs |
| Urine | 0.03 aufs | 0.03 aufs |

The separation of PQ and DQ was complete within 8 min.

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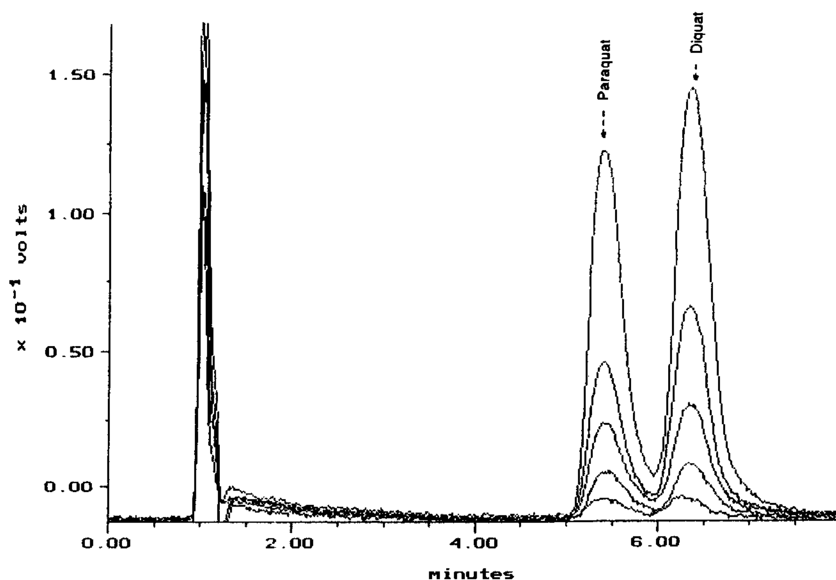


**Figure 1.** Chromatograms of mixtures of PQ and DQ in mobile phase at 290 nm.

## RESULTS

Figure 1 shows the chromatograms of mixtures of PQ and DQ (concentration range 0.4-8 μg/mL for both compounds) obtained at 290 nm.

There is a linear relationship between the peak areas of each one of the two compounds and the amounts of these in the injection volume. Table 2 lists the results of the regression analysis performed.

As can be seen in figures 2, 3, 4 and 5, the chromatograms obtained under the selected conditions, at 254 and 310 nm (for the detection of PQ and DQ, respectively), show two peaks corresponding to as many compounds with retention times of 4.258 ± 0.4258 min (PQ, 254 nm) and 5.183 ± 0.5183 min (DQ, 310 nm).

The peak areas of PQ and DQ were found to be linearly related to the drug concentrations. Table 3 summarizes the results of the regression analysis performed.

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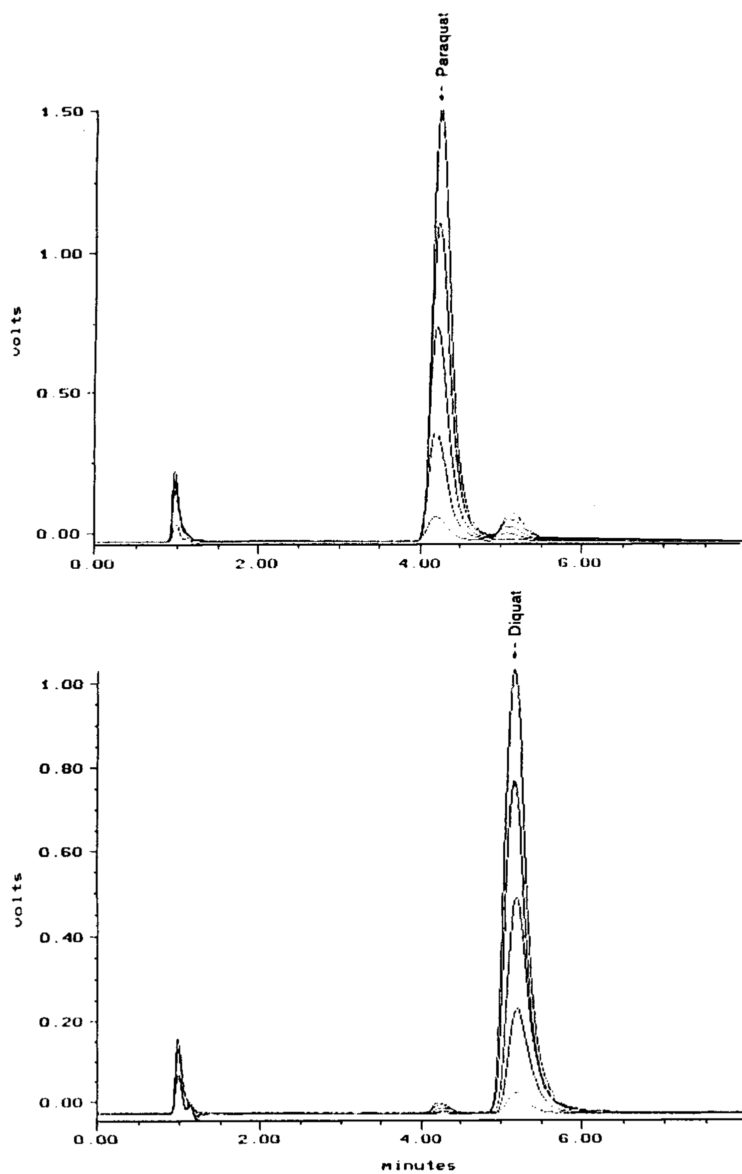


**Figure 2.** Chromatograms of mixtures of PQ and DQ in mobile phase at 254 nm (at the top) and 310 nm (at the bottom).

**Table 2**

**Equations of the Calibration Curves Obtained for Paraquat and Duquat in mobile phase at 290 nm (n = 3)**

| Compound | Solution | Intercept | Slope | Correlation Coefficient |
|---|---|---|---|---|
| PQ | PQ (0.4-8 μg/mL) + DQ (0.4-8 μg/mL) | 3.302E-02 | 1.458E-06 | 0.9975 |
| DQ | PQ (0.4-8μg/mL) + DQ (0.4-8 μg/mL) | 4.111E-02 | 1.22E-06 | 0.9991 |

y = ax + b
x = peak area
y = concentration ˙ injection volume

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The mean recoveries of PQ from plasma samples, with or without DQ, were 36% and 38%, respectively. The recoveries of PQ from urine were 23% and 16%, respectively.

The average recoveries obtained for DQ in plasma were 34.5% and 53.5%, with or without PQ, respectively. The mean values in urine were 40% and 35%, respectively.

In spite of the above mentioned recoveries, a good sensitivity for both herbicides was achieved. The quantification limits were 0.1 μg of PQ or DQ per millilitre of plasma or urine, and 0.5 μg of PQ or DQ per millilitre of vitreous humour.

The reproducibility of our method was determined by analysing every solution shown in table 1 in triplicate. The average coefficients of variation for PQ in mobile phase, plasma, urine and vitreous humour were 5, 11, 8 and 5, respectively. The mean coefficients of DQ in these types of matrix were 2, 12, 6 and 5, respectively. These values can be considered acceptable.

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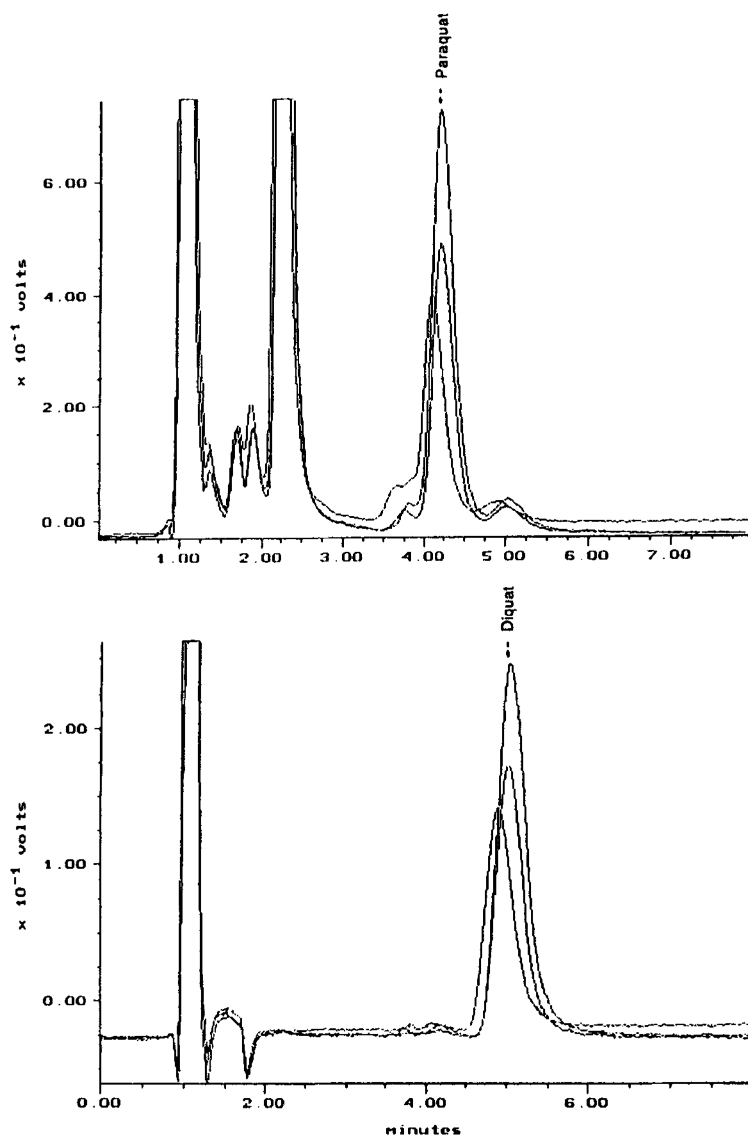


**Figure 3.** Chromatograms of mixtures of PQ and DQ in plasma at 254 nm (at the top) and 310 nm (at the bottom).

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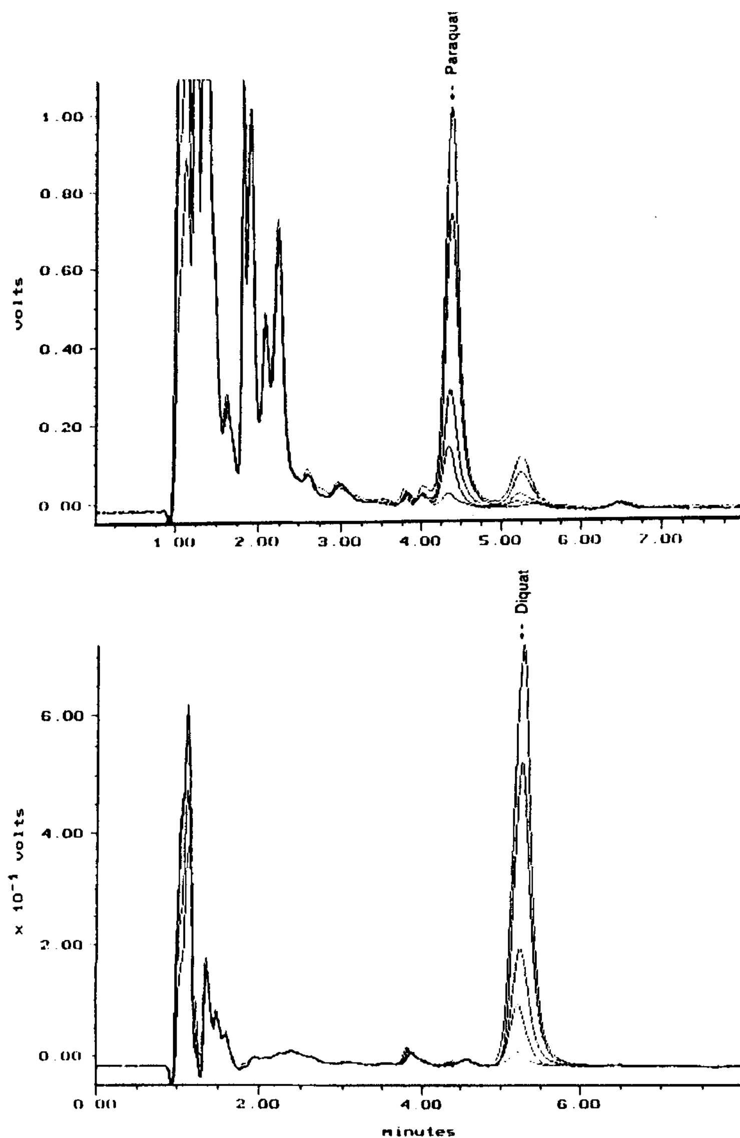


**Figure 4.** Chromatograms of mixtures of PQ and DQ in urine at 254 nm (at the top) and 310 nm (at the bottom).

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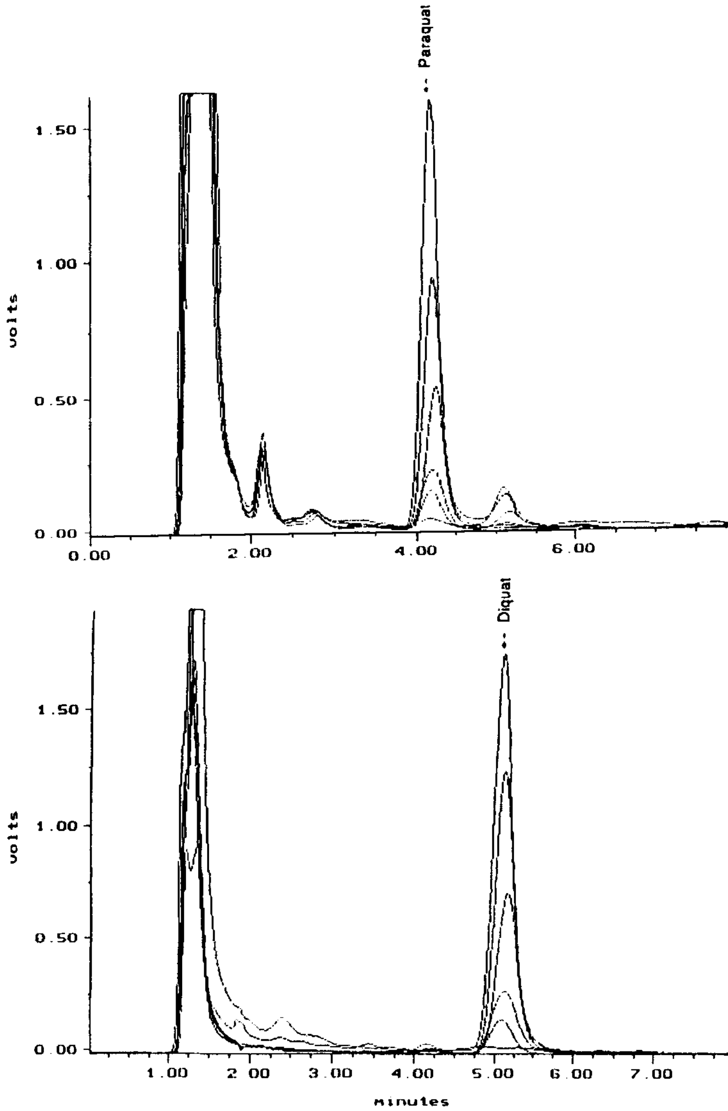


**Figure 5.** Chromatograms of mixtues of PQ and DQ in vitreous humour at 254 nm (at the top) and 310 nm (at the bottom)

**Table 3**

**Equations of Calibration Curves Obtained for Paraquat and Diquat at 254 and 310 nm, respectively (n = 3)**

| Compound | Matrix | Solution | Intercept | Slope | Correlation Coefficient |
|---|---|---|---|---|---|
| **PQ** | Aqueous | PQ alone | -6.060E+05 | 23.9336E+06 | 0.9886 |
| | | PQ + DQ | -2.203E+05 | 24.6361E+06 | 0.9994 |
| | Plasma | PQ alone | -5.454E+04 | 8.2598E+06 | 0.9898 |
| | | PQ +DQ | -1.254E+04 | 7.9721E+06 | 0.9810 |
| | Urine | PQ alone | 1.566E+04 | 4.3864E+06 | 0.9933 |
| | | PQ + DQ | -3.375E+05 | 4.4409E+06 | 0.9875 |
| | Vitreous Humour | PQ alone | 3.188E+05 | 4.7392E+06 | 0.9911 |
| | | PQ + DQ | -2.045E+05 | 4.5026E+06 | 0.9872 |
| **DQ** | Aqueous | DQ alone | -0.395E+04 | 10.4855E+06 | 0.9996 |
| | | DQ + PQ | -11.499E+04 | 10.5110E+06 | 0.9997 |
| | Plasma | DQ alone | -8.922E+04 | 3.8335E+06 | 0.9847 |
| | | DQ + PQ | 3.325E+05 | 3.6115E+06 | 0.9700 |
| | Urine | DQ alone | 1.248E+05 | 3.9853E+06 | 0.9948 |
| | | DQ + PQ | -2.039E+05 | 4.229E+06 | 0.9922 |
| | Vitreous Humour | DQ alone | -4.143E+04 | 6.9661E+06 | 0.9984 |
| | | DQ + PQ | 1.087E+05 | 5.9756E+-06 | 0.9958 |

$y = ax + b$
$x$ = concentration (µg/mL)
$y$ = peak area

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## DISCUSSION

The proposed HPLC method separates PQ and DQ. Quantification of low levels of these compounds, with an important prognostic value, can be made by this method.

Despite the good results obtained with the first tests at 290 nm, we chose two wavelengths, 254 and 310 nm, to detect PQ and DQ, respectively. PQ and DQ peaks were successively recorded at 290 nm (figure 1). There was nearly 1 min between both peaks, but the tail of PQ peak overlapped the initial plot of DQ peak.

The absorptivity of PQ at 254 nm and the one of DQ at 310 nm were stronger than those at 290 nm. Thus the presence of PQ and DQ can be determined by selecting the appropriate detector wavelength, 254 nm for PQ

and 310 nm for DQ. This procedure enhanced the sensitivity of the method for both herbicides. Moreover, PQ absorptivity at 310 nm and DQ absorptivity at 254 nm were very low. So, the interference observed at 290 nm was extremely reduced.

The liquid-liquid ion-pair extraction method developed offers the advantage that is more expeditious and easier to perform than the original extraction procedure of Querée. In the case of vitreous humour samples, the features of this biological medium allow to apply a rapid and simple procedure consisting of protein denaturalization and precipitation following by a concentration process. This sample pretreatment is applied on a little volume of that one (100 µL), which is a very important characteristic to study fatal real cases of poisoning.

The quantification limits achieved (0.1 µg/mL of PQ or DQ in plasma or urine, and 0.5 µg/mL of PQ or DQ in vitreous humour) happened to be adequate at the sight of the results obtained in our laboratory, where cases of poisoning caused by PQ and DQ or PQ alone were studied. The developed chromatographic method has been also used by our group for determination of PQ and/or DQ concentrations in a toxicokinetic study of these compounds made in rabbits and not yet published.

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