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Simultaneous determination of paraquat and diquat in human tissues by high-performance liquid chromatography

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ABSTRACT

A simple, sensitive, reliable, and economical method for simultaneous determination of paraquat dichloride and diquat dibromide in human biological materials has been developed, using high-performance liquid chromatography. The drugs were extracted from the sample with a Sep-Pak C_{18} cartridge and applied to a chromatograph with the internal standard, L-tyrosine. Paraquat and diquat were clearly separated on the octadecylsilica column with a mobile phase of 0.5% potassium bromide in 5% methanol solution, containing triethylamine (1 ml/l). The pH of the mobile phase was adjusted to 3–4 with 1.3 *M* phosphoric acid. Two ultraviolet wavelengths were selected, 256 nm for paraquat as well as the internal standard, and 310 nm for diquat. The calibration curves were linear in the concentration range 0.1–10 μg/g, and the lower limit of detection was 0.05 μg/g. We used this method to examine the concentrations of paraquat and diquat in tissues of an individual at autopsy.

INTRODUCTION

Cases of poisoning attributed to the mixture of paraquat dichloride (PQ) and diquat dibromide (DQ), N-alkyldipyridium salt herbicides, have increased in Japan since 1986, a time when the drug was marketed to reduce the toxicity of PQ. The simultaneous determination of both PQ and DQ is necessary in order to obtain proof of poisoning.

Methods for the simultaneous analysis of PQ and DQ involve reaction and derivatization of the drug for analysis by gas chromatography [1] or high-performance liquid chromatography (HPLC) [2]. Nakagiri *et al.* [3] used the system to analyse PQ and DQ in biological fluids. Tomita *et al.* [4] reported the simultaneous analysis of PQ and DQ in serum using capillary electrophoresis. Gill *et al.* [5] reported that PQ and DQ could be separated by adding the organic ion-pair reagent, sodium heptanesulphonate, to the HPLC mobile phase. Their method, however, requires use of the expensive organic ion-pair reagent, which makes it unsuitable for routine work, and it was applied only to urine testing. Forensic examinations require that solid materials be tested.

We have devised a more reliable and simple

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analytical method using an inorganic ion-pair reagent with which solid tissues can be examined.

EXPERIMENTAL

Reagents

PQ and DQ were provided by ICI Japan (Tokyo, Japan) and Sep-Pak C_{18} cartridges (360 mg sorbent) were purchased from Waters Assoc. (Milford, MA, USA). Methanol was of analytical-reagent grade and was purified by distillation. Other chemicals used were of analytical-reagent grade.

Biological samples

Human tissue samples obtained at the time of autopsy were stored at −20°C until analysis. Outdated human whole blood obtained from a blood bank was used as control samples.

Standard solution of paraquat and diquat

PQ (10 mg) was dissolved in distilled water to give a concentration of 1 μg/μl, and was then further diluted to concentrations of 100 and 10 ng/μl. A standard solution of DQ was prepared in the same manner.

L-Tyrosine (10 mg), purchased from Ishizu Seiyaku (Osaka, Japan), was selected as an internal standard (I.S.), and was dissolved in 100 ml of the HPLC mobile phase to make a concentration of 100 μg/ml, and this solution was used as a standard I.S. solution.

Extraction procedure

The extraction procedure reported by Tsunoda [6] was modified so that solid tissue samples could be tested. Tissue samples of 0.1–1.0 g were weighed and homogenized in 3 ml of 3% perchloric acid in a 30-ml centrifuge tube. The mixture was shaken for 5 min and centrifuged at 2000 *g* for 10 min. The supernatant was transferred to another test-tube. A 1-ml volume of 3% perchloric acid was added to the remaining precipitate, and the mixture was shaken, then centrifuged. This re-extraction procedure was repeated once more. After the supernatants obtained from the extractions were combined, the pH was adjusted to *ca.* 11 by adding 5 ml of 20% sodium carbonate solution. The preparation was applied to a pre-activated Sep-Pak C_{18} column. After the cartridge had been washed successively with 20 ml each of methanol, distilled water and methanol, the drugs were eluted with 7 ml of 0.1 *M* hydrochloric acid. The eluates were evaporated to dryness *in vacuo* at 60°C. The residue was dissolved in 500 μl of mobile phase containing the I.S., and applied to the HPLC column. The extraction procedure is summarized in Fig. 1.

```
Sample (0.1-1.0 g)
    |     1) Add 3 % perchloric acid (3 ml)
    |     2) Homogenize
    |     3) Shake(2 min) and centrifuge(2000g,10 min)
    |     4) Extract twice with 3% perchloric acid (1 ml)
    |
Supernatant
    |     1) Add 20 % sodium carbonate (5 ml)
    |     2) Apply to Sep-Pak C18 cartrige
    |     3) Wash with distilled water, methanol and
    |        distilled water (20ml each)
    |     4) Elute with 0.1 M hydrochloric acid (7 ml)
    |
Eluate
    |     1) Evaporate in vacuo
    |     2) Dissolve in mobile phase (500 µl)
    |        containing I.S. (L-tyrosine 100 µg/ml)
    |     3) Pass through a 0.45 µm filter
    |
Filtrate
          1) Apply onto HPLC (50 - 100 µl)
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Fig. 1. Extraction procedure for PQ and DQ.

Preparation of calibration graphs

Whole blood samples were prepared to contain PQ and DQ at the concentrations of 0.25, 0.5, 1.0, 2.5, 5.0 and 10 μg/g each. These samples were extracted as described above. Calibration graphs were obtained by plotting the peak-area ratio of PQ or DQ to the I.S. *versus* the amounts of PQ or DQ.

HPLC conditions

The apparatus was a Model M-600 high-performance liquid chromatograph (Waters Assoc.) with a 490E UV detector. The column was a 300 mm × 3.9 mm I.D. stainless-steel tube packed with 10 μm particle size octadecylsilica (μBondapak C_{18}, Waters). Two UV wavelengths were selected, 256 nm for PQ and the I.S., and 310 nm for DQ.

The mobile phase was 0.5% potassium bromide solution in 5% methanol, containing triethylamine (1 ml/l) and adjusted to pH 3–4 with 1.3 *M* phosphoric acid to protect the column. The column temperature was maintained at 30°C and the flow-rate was set to 0.8 ml/min.

RESULTS AND DISCUSSION

Extraction procedure

The interfering peaks derived from tissue samples were effectively removed by increasing the volume of washing solvent, methanol and distilled water, through the Sep-Pak C_{18} cartridge, from 5 to 20 ml. The problem of the instability of DQ under alkaline conditions with the pH over 13 [7], yet a good recovery of PQ with the pH over 10 [6], was overcome by adding sodium carbonate to adjust the pH to *ca.* 11, after deproteinization with perchloric acid.

HPLC conditions

Other workers reported that PQ and DQ could be separated by HPLC with an eluent containing an organic ion-pair salt, sodium heptanesulphonate [5]. Corasaniti *et al.* [8] used the same reagent for the analysis of PQ in rat brain. When we used this salt with our ODS column, μBondapak C_{18}, separation of both substances was unsatisfactory. To replace the sodium heptanesulphonate, several inorganic salts were examined including potassium bromide and calcium bromide. Potassium bromide proved to be the most favorable ion-pair salt for separating PQ and DQ, and the sensitivity was high and cost low. This inexpensive reagent makes feasible routine laboratory examinations. The peak shape was improved by adding diethylamine to the eluent to mask the residual silanol groups on the silica matrix.

L-Tyrosine present in the human tissues could be completely removed by washing the Sep-Pak C_{18} column with methanol and distilled water, with no effect on the peak of the I.S.

Determination of paraquat and diquat by HPLC

Fig. 2 shows chromatograms obtained from a plasma sample containing PQ and DQ (2 μg/g

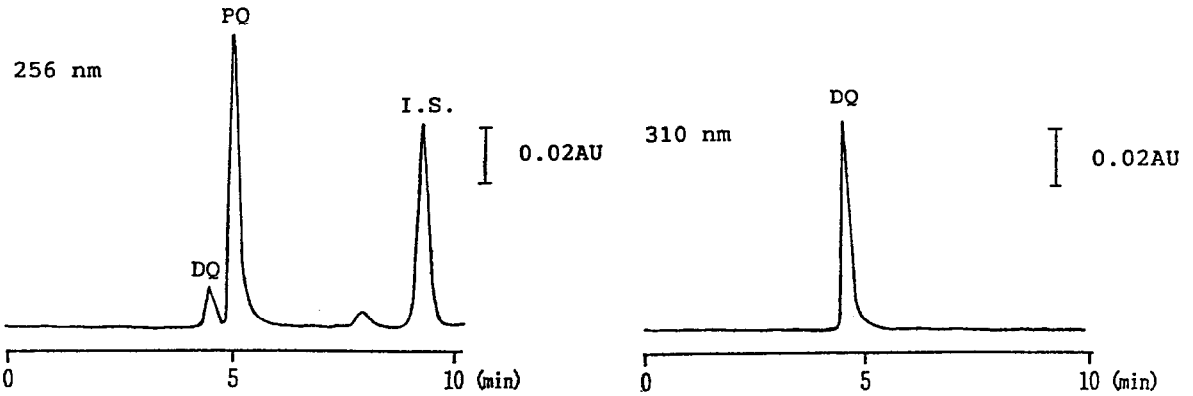

Fig. 2. Chromatograms of an extract from blood containing 2 μg/g each of PQ, DQ and the I.S., with detection of 256 nm and 310 nm.

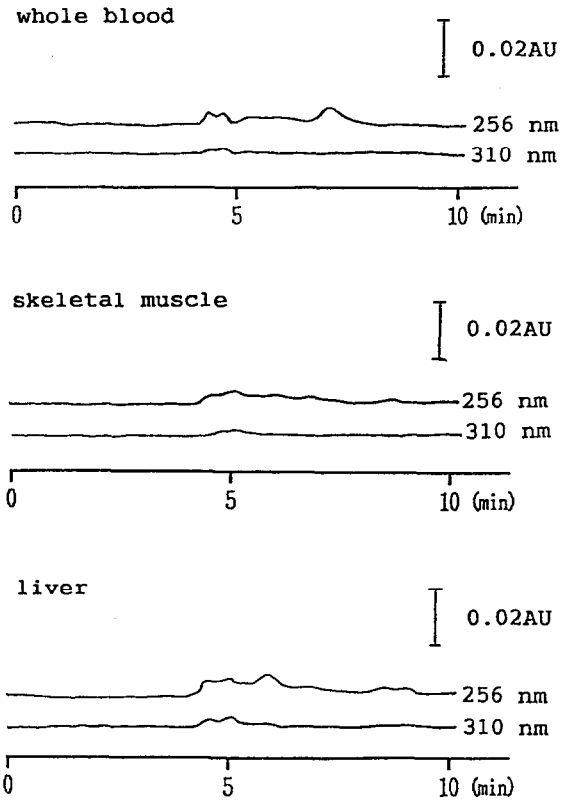

Fig. 3. Chromatograms of an extract from drug-free whole blood, skeletal muscle and liver, with detection at 256 nm and 310 nm.

each). PQ, DQ and the I.S. were clearly separated, with retention times of 5.4, 4.7 and 9.2 min, respectively.

Chromatograms of the blank blood, skeletal muscle and liver are shown in Fig. 3: few or no interfering peaks appeared.

The calibration graphs for PQ and DQ were linear in the concentration range from 0.1 μg/g to at least 10.0 μg/g, with correlation coefficients of 0.999 and 0.998, respectively. The limit of detection was 0.05 μg/g for each drug. The absolute recoveries were 89–95% for PQ, and 77–80% for DQ, at the concentrations of 0.25 μg/g and 5 μg/g, respectively.

Within-day and between-day precisions were obtained using two different concentrations (0.5 and 5 μg/g) by adding PQ and DQ to blank blood. The coefficients of variation (C.V.) for these compounds ranged from 3.5 to 6.7% for the within-day and from 5.4 to 10.3% for the between-day precision (see Table I).

TABLE I

PRECISION DATA FOR PQ AND DQ IN WHOLE BLOOD

Drug	Within-day (n = 5)		Between-day (n = 5)	
	Concentration (mean ± S.D.) (μg/g)	C.V. (%)	Concentration (mean ± S.D.) (μg/g)	C.V. (%)
Paraquat	0.53 ± 0.03	5.2	0.50 ± 0.04	7.1
	5.14 ± 0.18	3.5	5.13 ± 0.28	5.4
Diquat	0.49 ± 0.03	6.7	0.50 ± 0.05	10.3
	4.89 ± 0.20	4.0	4.92 ± 0.30	6.1

Practical application

A 30-year-old man who drank 100 ml of herbicide solution containing PQ and DQ was sent to a hospital. After dialytic treatment for 12 h, the tube of the dialyser was cut, and the patient died 6 h later.

A legal autopsy was done followed by a toxicological examination. Using our method, high concentrations of PQ and DQ were identified in the blood, urine, liver, kidney and lung, as shown in Table II, even after 12 h of dialytic treatment. These values are lethal, according to reported data [9,10].

The official cause of death of the patient was poisoning with PQ and DQ. Interruption of the dialysation was excluded from the investigation.

TABLE II

CONCENTRATIONS OF PQ AND DQ IN BLOOD, URINE AND VISCERAL TISSUES

Sample	Concentration (μg/g)	
	PQ	DQ
Blood	2.01	1.96
Urine	5.12	4.29
Liver	10.1	3.32
Kidney	52.1	42.8
Lung	18.0	3.39

CONCLUSION

A simple, sensitive, reliable, and economical HPLC method was developed for the simultaneous determination of PQ and DQ. This technique makes feasible the rapid and routine analysis of biological solid materials, including human body tissues.

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