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Note

Rapid quantification of paraquat and diquat in serum and urine using high-performance liquid chromatography with automated sample pretreatment

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Many methods have been employed for the quantification of paraquat and diquat, bipyridylium herbicides, including colorimetry with an alkaline dithionite reaction[1,2], gas–liquid chromatography[3,4], thin-layer chromatography[5,6] and high-performance liquid chromatography (HPLC)[7–9]. Each of these methods, however, requires pretreatment to extract paraquat or diquat from a serum or urine sample before measuring it.

In the present study, we developed a new system in which an automated pretreatment apparatus is connected to ion-exchange HPLC. The automated pretreatment, using a column switching method, made measurement of paraquat and diquat with HPLC easier and faster. After injecting a microsample of serum or urine, pretreatment and measurement were carried out automatically.

METHODS

Standard reagents

Paraquat dichloride and diquat dibromide standards (Wako Junyaku, Japan) for the test of drug residuals were used. All other agents were of special reagent grade.

Apparatus

The following pieces of analytical apparatus were used: a pump (CCPM, Tosoh, Yamaguchi, Japan), an automatic pretreatment apparatus (PT-8000, Tosoh), an ultraviolet detector (UV-8000, Tosoh) and a data analyzer (Chromatocorder 12, Tosoh). Columns for gel filtration chromatography (TSK precolumn PW, 3.5 cm × 4.6 mm I.D., Tosoh) and for ion-exchange chromatography (TSK gel SP-2 SW, 25 cm × 4.6 mm I.D., Tosoh) were used as the preparation column and the analytical column, respectively. The chromatographic conditions were as follows: column temperature, room temperature; mobile phase for preparation, 0.1 *M* sodium perchlorate solution containing 5 m*M* sodium dihydrogenphospate adjusted to pH 3 with phosphoric acid; mobile phase for analysis, 0.2 *M* sodium dihydrogenphosphate solution adjusted to pH 3 with phosphoric acid, and acetonitrile–0.2 *M* sodium dihydrogen-

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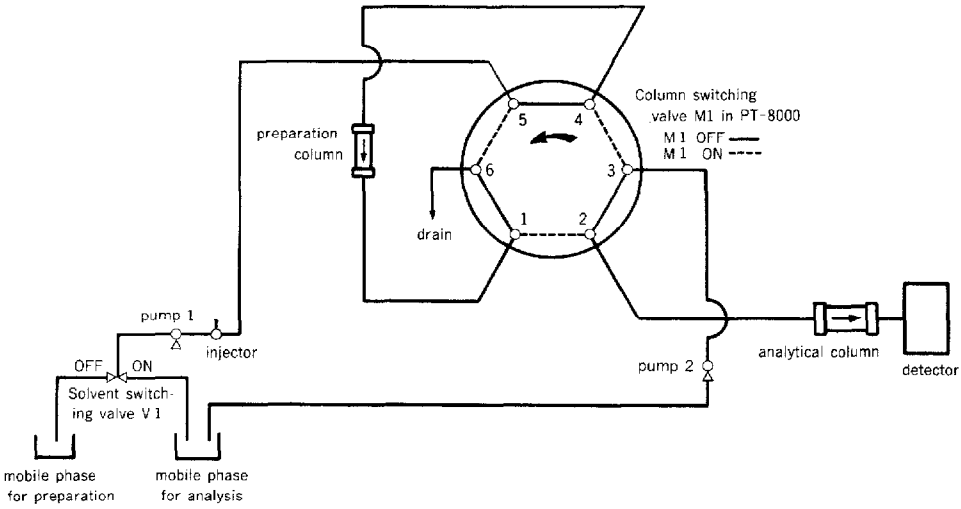

Fig. 1. Flow diagram of HPLC system with automatic sample preparation.

phosphate solution pH 3 (20:80, v/v); flow-rate, both 1.0 ml/min; detection wavelength, 290 nm.

Operation

A schematic drawing of the system detailing each component for analysis is shown in Fig. 1. A 50-μl sample was injected into the preparation column under a flow of the mobile phase for preparation (the solvent switching valve is off). Intensely hydrophilic components in the serum or urine are not retained in the preparation column, but are eluted and eliminated. Paraquat and diquat are retained in the column. The flow route was subsequently changed to that indicated by the dotted line by turning on the column switching valve in the automatic pretreatment apparatus as shown in Fig. 1. The mobile phase for analysis was directed to the analytical column by pump 2 and then redirected to the preparation column. Paraquat and diquat retained in the preparation column were extracted and transferred to the analytical column with the mobile phase for analysis. Then, with the solvent switching valve on

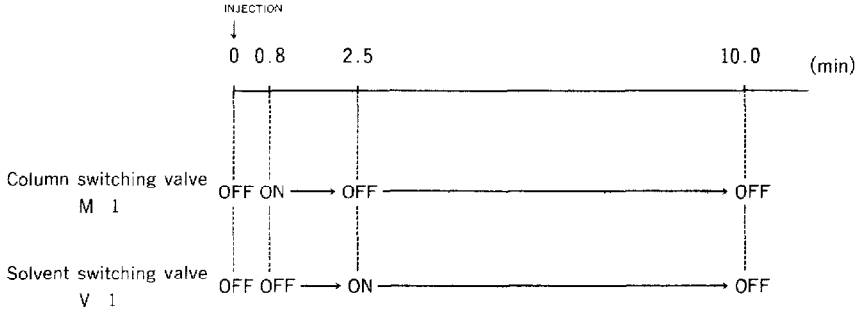

Fig. 2. Time program.

and the column switching valve turned back after 2.5 min of injection, the preparation column was washed with the mobile phase for analysis. When the solvent switching valve was again turned off after 10 min of injection, this column was recharged with the mobile phase for preparation.

In this manner, washing and recharging take place while the analysis is progressing. These procedures are performed by a program for turning the column switching valve and solvent switching valve in the automatic pretreatment apparatus off and on as shown in Fig. 2.

We examined the accuracy of this system for analyzing paraquat or diquat in serum or urine.

RESULTS AND DISCUSSION

Confirmation of deproteinization on the preparation column

Deproteinization on the preparation column was confirmed by using a serum sample containing 5 μg/ml each of paraquat and diquat (protein content 7.4 g/dl with albumin content 3.8 g/dl). Since highly hydrophilic protein is not retained in the

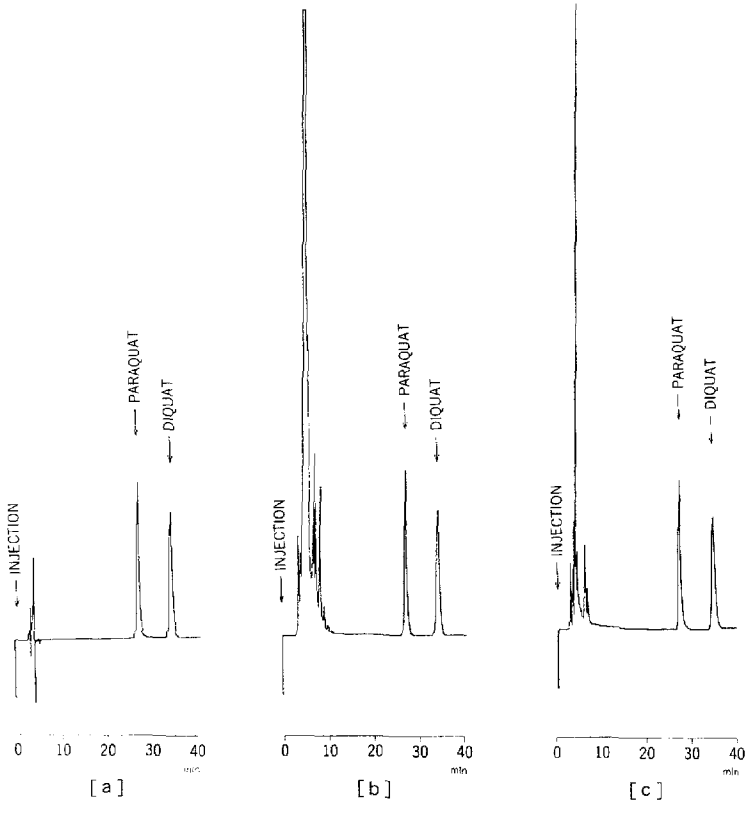

Fig. 3. Chromatograms of paraquat and diquat. (a) Standard paraquat (5 μg/ml) and diquat (5 μg/ml); (b) paraquat (5 μg/ml) and diquat (5 μg/ml) in serum; (c) paraquat (5 μg/ml) and diquat (5 μg/ml) in urine.

preparation column but is eluted and eliminated, we measured the amount of protein eluted using the method of Lowry *et al.*[10] with Folin-Ciocalteu reagent (Wako Junyaku). About 73% of the protein was detected in the waste fluid obtained after 10 min. The fractions containing paraquat and diquat, however, included no protein. These findings confirmed the satisfactory separation of paraquat and diquat from the protein of the serum sample.

Linearity of correlation between known and measured concentrations of paraquat or diquat

Paraquat dichloride and diquat dibromide were dissolved in 0.2 *M* NaH_2PO_4 in water to prepare 0.1–50 μg/ml standard solutions. Each calibration graph showed a good linear relationship between concentrations in standard solutions and those calculated from peak areas.

The relationships between the theoretical value, *i.e.* x (μg/ml), and the observed value, *i.e.* y (μg/ml), were: $y=0.983x + 0.011$, $r=0.999$ for paraquat; $y=0.938x + 0.009$, $r=0.999$ for diquat in the range of 0.1–1 μg/ml ($n=4$); $y=0.984x + 0.222$,

TABLE I

RECOVERY OF PARAQUAT AND DIQUAT BY THE AUTOMATIC SAMPLE PREPARATION METHOD

	Standard in water (μg/ml)	*Found (μg/ml)*	*n*	*C.V. (%)*	
Paraquat	20	19.65[a] ± 0.14	5	0.71	
	10	10.10[a] ± 0.10	5	0.99	
	1	0.93[a] ± 0.01	5	1.07	
Diquat	20	19.66[a] ± 0.13	5	0.66	
	10	10.28[a] ± 0.16	5	1.56	
	1	0.98[a] ± 0.02	5	2.04	
	Standard in serum (μg/ml)	*Found (μg/ml)*	*n*	*C.V. (%)*	*Recovery (b/a) · 100 (%)*
Paraquat	20	20.02[b] ± 0.13	5	0.64	101.9
	10	10.10[b] ± 0.24	5	2.38	100.0
	1	0.96[b] ± 0.03	5	3.13	103.2
Diquat	20	19.79[b] ± 0.15	5	0.76	100.7
	10	9.94[b] ± 0.18	5	1.81	96.7
	1	0.97[b] ± 0.03	5	3.09	99.0
	Standard in urine (μg/ml)	*Found (μg/ml)*	*n*	*C.V. (%)*	*Recovery (c/a) · 100 (%)*
Paraquat	20	19.59[c] ± 0.13	5	0.66	99.7
	10	10.09[c] ± 0.07	5	0.69	99.9
	1	0.99[c] ± 0.03	5	3.03	106.5
Diquat	20	19.71[c] ± 0.29	5	1.47	100.3
	10	10.02[c] ± 0.19	5	1.90	97.5
	1	0.95[c] ± 0.02	5	2.11	96.9

$r = 0.999$ for paraquat, and $y = 0.996x + 0.297$, $r = 0.999$ for diquat in the range of 1–50 μg/ml ($n = 6$).

Recovery rate and simultaneous reproducibility of this system

Fig. 3 shows chromatograms of paraquat (5 μg/ml) and diquat (5 μg/ml) in the standard solution, serum and urine.

The recovery rate and simultaneous reproducibility of our system are given in Table I. Recovery rates of paraquat and diquat in pooled human serum or urine were obtained by dividing values measured in the pooled samples by those in the authentic standards, multiplied by 100. The analytical recoveries and the coefficients of variation (C.V.s) for the three different concentrations (1, 10 and 20 μg/ml) ranged from 96.7 to 106.5% and from 0.64 to 3.13%, respectively.

The recovery rate and reproducibility were excellent.

CONCLUSIONS

Samples obtained from living organisms generally include a wide range of substances from higher, *e.g.*, proteins to lower molecular weight, *e.g.*, amino acids. Direct injection of a serum sample into an analytical column hence results in a clogging condensation on the column, leading to an increase in intracolumnar pressure, fluctuation of the chromatogram baseline and changes in the retention time and tailing of the peak. We must therefore pretreat a serum sample to measure paraquat and diquat. The pretreatment procedure requires time, and, in addition, has been an obstacle to measuring paraquat and diquat automatically with HPLC. Because our system includes a pretreatment apparatus, measurement of paraquat or diquat is automatically carried out after injecting a microsample of serum or urine into an injection port. This can provide quick and important information for the physician.

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