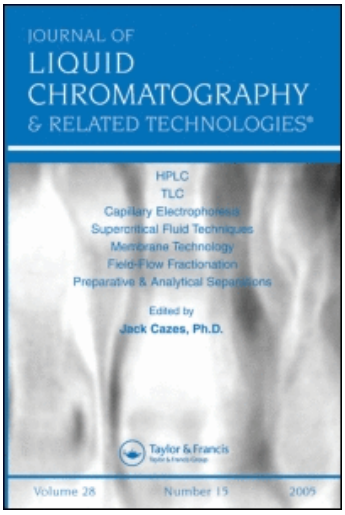

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A RAPID MONITORING METHOD OF PARAQUAT AND DIQUAT IN SERUM AND URINE USING ION-PAIRING BARE-SILICA STATIONARY PHASE HPLC FOLLOWING A SINGLE ACIDIFICATION STEP OF SAMPLE PRETREATMENT

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ABSTRACT

Intoxication by paraquat, an agricultral chemical, is frequently reported in Asian and eastern European countries because of its availablity by general population. Because paraquat is not significantly biotransformed in man, it requires an intensive monitoring of paraquat level during both stages of rescue and recuperation. It is critical to design an effective technique to eliminate the paraquat from the patient at the earlier stage of the therapy, and a rapid method is required for monitoring the paraquat concentration. Diquat is a less toxic analog of paraquat, and is often mixed with paraquat for agricultural usage. Recently, a high rate of fatal cases were reported on the mixture of diquat and paraquat among all paraquat incidences. In this study, a rapid HPLC method has been developed for monitoring paraquat and diquat in serum and urine samples.

The total analysis is less than 6 minutes. It only requires a minimal sample pretreatment, including acidification and centrifugation of sample at 9500 x g for 1 minute. This method utilizes ion-pairing HPLC with bare silica (150 mm X 4.6 mm) as stationary phase. After acidification with 2M phosphoric acid, the supernatant of samples can be injected directly into the HPLC system. The mobile phase was 25/75 acetonitrile/water (V/V); containing 10 mM 1- Heptanosulphonic Sodium Salt, 4 mM potassium phosphate, 10 mM potassium chloride, pH 3.0. The analysis was performed at room temperature with a flow rate of 1.0 mL/min. The optimum UV wavelength for paraquat and diquat were 257 nm and 310 nm, respectively; the corresponding detection limits were measured at 63 ng/mL (1.25 ng on column) for diaquat and 125 ng/mL (2.5 ng on column) for paraquat, respectively. For simultaneous monitoring of both compounds, a common wavelength of 290 nm can be used; with an injection volume of 20 μL, paraquat could be detected at 500 ng/mL(10 ng on column) and 125 ng/mL (2.5 ng on column) for diquat in both urine and serum. A lower detection limit at 290 nm can be achieved by using a larger injection volume. The CV% of retention data are less than 1.2% for both compounds. Interference studies were also conducted for common drugs and metabolites. Additional tests were conducted on drugs which are strongly retained on cation exchange methods: chloroquine, strychnine and nicotine. All these three compounds did not interfere with the method.

INTRODUCTION

Agriculture is a major industry in Asian and eastern European countries. Intoxication by agricultural chemicals is frequently reported in these regions because of the easy accessibility by general population. Among different agricultural chemicals, paraquat intoxication is a major concern because of its toxicity and its frequent use for a suicidal agent.

Paraquat (Gramoxone W, Weedol, methyl viologen) is a bis-quaternary ammonium compound that has been widely used since 1962 as a domestic and commercial herbicide.[1] The dichloride salt is supplied as a 5% powder for domestic use or a 10% to 30% aqueous concentrate for agricultural purposes. The compound is known to be absorbed via dermal contact, inhalation and ingestion.

Diquat is a less toxic analogue of paraquat (an ortho-bipyridyl analogue), and it is usually supplied as the dibromide in a 2% concentrate for spraying. Combinations of paraquat and diquat are also avaliable for agricultural usage.

There were many death since the introduction of this compound.[1,2] From 1964 to 1974, over 200 fatal cases were reported from ingestion of paraquat in all countries of use.[1] In recent years, higher incidence frequencies were reported. For example, in Japan alone,[3] 355 fatal cases of paraquat (PQ) and diquat (DQ) were reported in 1994 (93 PQ, 1 DQ, 261 mixture); 361 cases in 1993 (88 PQ, 5 DQ, 268 mixture).

Because paraquat is not significantly biotransformed in man, it requires intensive monitoring during both stages of rescue and recuperation. It is reported that patients with plasma concentrations in excess of 0.25 μg/mL from 12 to 68 hours after ingestion, with associated renal failure, usually do not survive.[1] Another report indicated that patients whose plasma concentrations do not exceed 2.0, 6.0, 0.3, 0.16, and 0.1 μg/mL at 4, 6, 10, 16, and 24 hours, respectively, are likely to survive.[4] Therefore, it is critical to design an effective technique to eliminate the paraquat from patient at the earlier stage of the therapy, and a rapid method is useful for monitoring the paraquat concentration at 0.1 μg/mL or above.

Early colorimetric methods[5,6] were developed with a detection limit of 5 μg/mL. Later, several modifications were able to improve detection limit to 1 μg/mL, including isolation by cation-exchange chromatography,[7,8] protein precipitation,[9] ion-pairing extraction,[10] or direct addition of a reagent to urine.[11] A thin layer chromatography (TLC) method was developed for detection of residue remaining on vegetation,[12] which also required extensive sample preparation.

There were also several gas chromatography (GC) methods initially developed for environmental and safety concerns.[13,14] Later, a GC method for biological fluid had a detection limit of 0.5 μg/mL for paraquat and 1.0 μg/mL for diaquat.[14] However, the sample pretreatment method (1 hour) was complex and impractical. Another GC method claimed a sensitivity of 0.025 μg/mL with nitrogen-selective detector,[1] and again the sample preparation procedure was long (>1.5 hours) and a large volume of patient sample was required (3 mL).

A GC/MS method for forensic tissue samples was able to detect paraquat at 10 ng/mL when operating at selected ion monitoring (SIM) mode.[1] Either thermospray[16,17] or particle beam techniques[18] were used for the interface of

LC/MS for the detection of diquat and paraquat in soil and water, with the detection limits of 5 ng/mL to 20 ng/mL in SIM mode. Sample volumes were from 10 grams of soil to 4 liters of water.

Many HPLC methods were developed for paraquat formulation analysis,[19] urinary analysis (1 mL urine),[20] direct injection (3 μL to 5 μL) of spiked urine, spiked water, and gastric aspirate,[21] column switching (40 minutes run time),[22] ion pairing reversed phase HPLC,[23] and post column reaction.[24]

Different HPLC techniques were used in these studies.[19-24] For example, reversed phase stationary phase (Silica base ODS C_{18}) with ion-pairing reagent at low pH (pH 2-3) is the most commonly reported method.[20,23] Other types of stationary phases were also used, such as ion-exchange resin,[19,22] γ-aminopropyltriethoxysilane bonded alumina,[21] and bare silica.[24] However, these methods all required tedious off-line extractionprocedure for removing interference materials from matrix, except Pryde's study.[21] A column switching technique was reported[22] for on-line extraction procedure by using gel filtration.

Other techniques such as capillary electrophoresis (CE), radioimmunoassay (RIA), and fluoresence-polarization immunoassay (TDx) were also reported.[23,25,26] For example, a CE method was reported on using acetic acid-sodium acetate (pH 4.0) with 100 mM sodium chloride as buffer and electrokinetic injection;[23] an RIA method was able to detect paraquat;[25] a TDx was reported to have a detection limit of 5 ng/mL of paraquat in serum.[26]

In this study, we developed a rapid direct injection method to meet the needs of clinical treatment situation. Both serum and urine samples can be analyzed directly folowing a single step of acidification, and the total analysis time is less than 6 minutes with a detection limit of 63 ng/mL. The method only requires a single pump isocratic elution and a single analytical cartridge. The separation is based on the ion-pairing chromatography with bare silica.

MATERIALS AND METHODS

Materials

Standards of paraquat (1,1'-Dimethyl-4,4'-bipyridinium dichloride) and diquat dibromide (6,7-Didropyridol[1,2-a:2'1'-c]pyrazidinium dibromide) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Chemical

Service Inc. (West Chester, PA, USA), respectively. 1- Heptanesulphonic Sodium Salt, 1-hydrate (HPLC grade) was from Eastman Kodak Company (Rochester, NY, USA). Drugs used for interference studies were obtained from Sigma, Alltech (State College, PA, USA), or their respective manufacturers.

A total of 18 compounds were tested, including diazepam, amphetamine, imipramine, morphine, hydrocodone, benzoylecgonine, methamphetamine, methadone, codeine, trimethoprim, pseudoephedrine, nortriptyline, propoxyphene, EDDP (methadone metabolite), dextromethorphan, strycinine, chloroquine, and nicotine.

HPLC

An HPLC system was consisted of a 1350 HPLC pump, a UV monitor (UV-1816 UV/Vis Detector), and an ALR 486 computer. The BDS software (BarSpec, Israel) was used for data collection. An automated sample injector equipped with 20 μL sample loop (Model AS-100 HPLC Automatic Sampling System) was used. All above mentioned HPLC modules and software were avaliable from Bio-Rad Laboratories (Hercules, California 94547, USA).

Separation conditions

A single bare silica cartridge (150 mm X 4.6 mm I.D.) was used (Bio-Rad Laboratories, part # 1957145). The mobile phase was 25/75 acetonitrile/water (V/V; containing 10 mM 1- Heptanosulphonic Sodium Salt, 1-hydrate, 4 mM KH_2PO_4, 10 mM KCl, pH 3.0 adjusted by H_3PO_4). The mobile phase was filtered by 0.45 μm Nylon 66 filter membrane. The flow rate was set at 1.0 mL/min. The analysis was performed at room temperature.

The optimum UV wavelength for paraquat and diquat were 257 nm and 310 nm, respectively. For simulataneous monitoring, a common wavelength of 290 nm can be used for these two compounds. The samples was kept at 4°C in the sample tray.

Sample Preparation (Acidification)

Minimal sample preparation is required. 50 μL of phosphoric acid (2 M) was added to 1 mL sample to acidify the sample. Then it was centrifuged at 9500 x g for 1 minute at room temperarture. After the centrifugation, 20 μL of the supernatant was injected to the system directly.

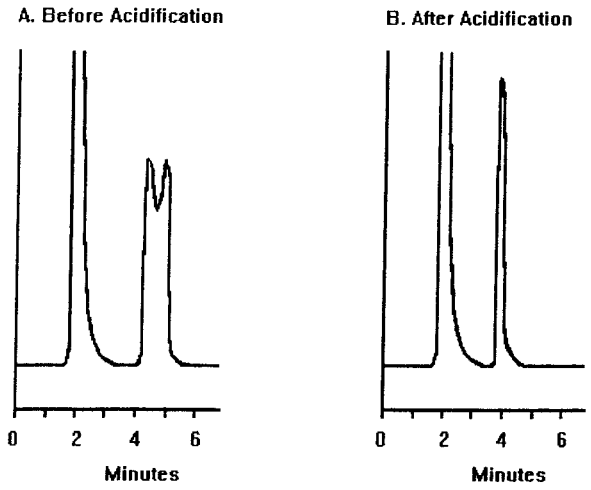

Figure 1. (A) A splitting peak of diquat was observed when urine sample was not acidified. (B) Splitting peak of diquat became a single peak after addition of 50 μL of 2 M phosphoric acid.

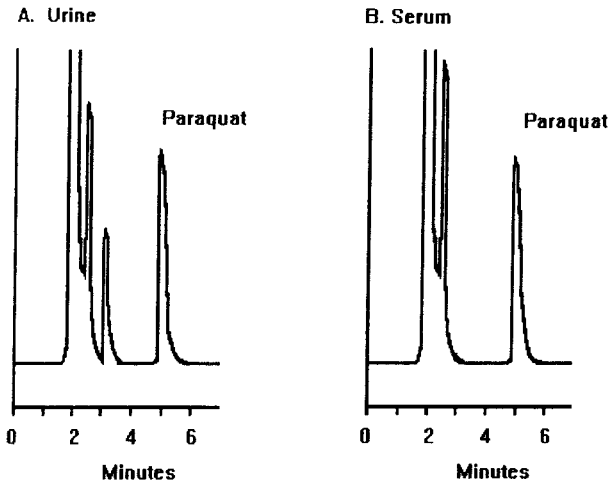

Figure 2. Chromatograms of paraquat in urine (A) and serum (B) with a detection at a UV wavelength of 257 nm.

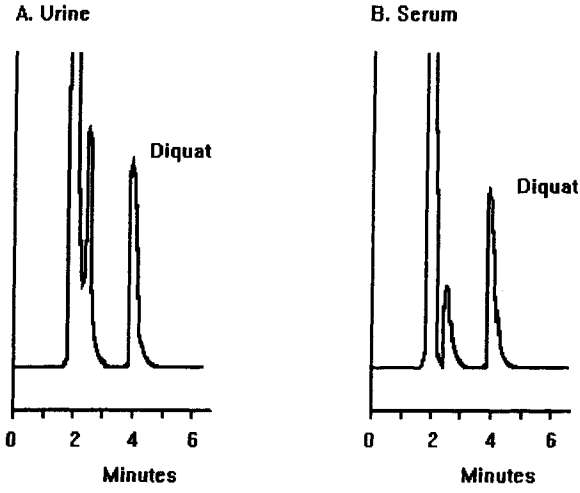

Figure 3. Chromatograms of diquat in urine (A) and serum (B) with a detection at a UV wavelength of 310 nm.

RESULTS AND DISCUSSION

Acidification of Sample

As shown in Figure 1-a, a splitting peak was observed when the spiked diquat in drug free urine was analyzed without any treatment. This might be due to the fact that there are components in the sample matrix which are adsorbed to the front end of the silica cartridge.[27] This problem was resolved by acidification of the sample matrix. As depicted in Figure 1-b, after an addition of 50 μL of 2 M phosphooric acid to 1 mL of sample, the split diquat peak became a single and sharp peak.

Analytical Separation

Figures 2-a and 2-b are chromatograms of paraquat standard at UV detection of 257 nm in urine and serum. Figures 3-a and 3-b are chromatograms of diquat at 310 nm in urine and serum.

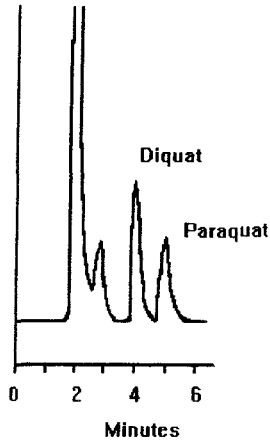

Figure 4. Chromatogram of mixture of diquat and paraquat at a UV wavelength of 290 nm in serum.

As shown in these four chromatograms, the endogenous components in both urine and serum matrices did not interfere with the detection of paraquat and diquat at either 257 nm or 310 nm. Figure 4 shows that a simultaneous monitoring can be conducted at a wavelength of 290 nm.

Linearity

A linearity study was conducted at the concentration range of 0.5 - 10 μg/mL (a total on-column amount of 10 - 200 ng). As shown in Table 1, a good linear coefficient is observed for paraquat and diquat in urine and serum samples. The peak height in serum was about 20% less than urine samples, and this was due to a slight broad peak for serum sample.

Reproducibility

As shown in Table 2, within-run study was performed by 10 consecutive runs at 5 μg/mL in both urine and serum. The CV% of retention data are less than 1.2% for both compounds. Paraquat showed a higher CV% of peak height in serum (8.18%).

Table 1

Linearity*

	Linearity	r=
Paraquat in Urine	Y = 7.6368X - 2.0199	0.99961
Paraquat in Serum	Y = 9.1716X + 0.4478	0.99971
Diquat in Urine	Y = 5.9876X - 0.9527	0.99989
Diquat in Serum	Y = 7.8980X - 0.5124	0.99999

*0.5 μg/mL to 10.0 μg/mL; = 5

Table 2

Within-Run Reproducibility*

Peak Height (Absorbance Unit)	PQ in Urine	PQ in Serum	DQ in Urine	DQ in Serum
Average	0.018	0.014	0.022	0.018
STDV	0.0003	0.0012	0.0003	0.0005
CV%	1.77	8.18	1.43	2.73
Retention Time (Minutes)				
Average	4.97	5.05	4.01	4.03
STDV	0.0350	0.0359	0.0467	0.0459
CV%	0.70	0171	1.16	1.14

* n = 10, 5μg/mL
Note: PQ: Paraquat, DQ: Diquat

Detection Limits

Clinical requirement

For paraquat, in most cases, blood concentrations greater than 2 μg/mL at 4 hours and 0.1 μg/mL at 24 hours are likely to be lethal, but individual

response is variable and some cases with much higher concentration have recovered. Therefore, the detection limit of paraquat must be at least 100 ng/mL or 2 ng on column.

Analytical detection

The detection limits were measured at 63 ng/mL (1.25 ng on column) and 125 ng/mL (2.50 ng/mL) for diaquat and paraquat, respectively. The detection wavelength for paraquat and diquat was 257 nm and 310 nm at 0.01 absorbance range, respectively. The detection limit was defined with a signal to noise ratio of 10. For a simultaneous monitoring of two compounds at 290 nm, paraquat could be detected at 10 ng (20 μL of 500 ng/mL) and 2.5 ng for diquat (20 μL of 125 ng/mL) in both urine and serum. A lower detection limit can be achieved by using a larger injection volume such as 50 μL.

Drug Interference Studies

The analytical cartridge of bare silica has been used for the separation of common drugs and metabolites with mobile phases at pH of 6.7[28] and 6.5[29] because of its weak cation exchange chromatographic behavior. In this study, with the adjustment of a low pH (3.0) and the addition of an ion pairing reagent to the mobile phase, all the common drugs and metabolites lost retention on the bare silica. They all eluted at the endogenous region (before 3 minutes) of sample matrices for this HPLC assay, and they did not cause any interference for the detection of paraquat and diquat. A total of 15 compounds were tested, including diazepam, amphetamine, imipramine, morphine, hydrocodone, benzoylecgonine, methamphetamine, methadone, codeine, trimethoprim, pseudoephedrine, nortriptyline, propoxyphene, EDDP (methadone metabolite), and dextromethorphan.

Additional tests were conducted on drugs which were strongly retained under the cation exchange chromatographic conditions;[28,29] chloroquine, strychnine, and nicotine. Again, all these three compounds eluted at the endogenous region (before 3 minutes) in this HPLC method, and they did not interfere with the detection of paraquat and diquat.

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