A SIMPLE AND SELECTIVE HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF PARAQUAT AND DIQUAT IN HUMAN URINE

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Abstract

A simple and selective HPLC method for the simultaneous determination of paraquat and diquat in human urine has been developed. After centrifugation with ultrafiltration device (Ultrafree-MC®), the samples were separated on a reversed-phase column and subsequently reduced to their radicals with alkaline sodium hydrosulfite solution. These radicals were monitored with a UV/VIS detector at 600 nm. This method permitted the reliable quantification of paraquat over linear ranges of 0.5 – 100 μg/ml for both of paraquat and diquat in human urine. This method was applied to determine the paraquat and diquat urine levels in a patient who had ingested herbicide (Preeglox L®) containing paraquat and diquat.

Introduction

Paraquat (1,1'-dimethyl-4,4'-dipyridyllium dichloride: PQ) and diquat (1,1'-ethylene-2,2'-dipyridyllium dibromide: DQ) are popular herbicides that are extensively used throughout the world [1]. A mixture of PQ and DQ has been used instead of PQ alone in Japan to reduce herbicide toxicity. The incidence of herbicide poisoning, often through suicidal intent rather than accidental ingestion, is still a major problem in many countries. Therefore, a rapid and sensitive technique for the simultaneous determination of PQ and DQ in the patients' serum is required. We recently developed a rapid and sensitive HPLC method for the simultaneous determination of PQ and DQ in human serum [2]. This method was based on a post-column reductive reaction with alkaline sodium hydrosulfite solution.

In general, the majority of PQ and DQ absorbed into the body is eliminated unmetabolized in the urine [3,4]. The PQ concentration in the urine obtained within the 24

h of ingestion can also be used to estimate prognosis. Scherrmann *et al* reported that urinary concentrations of less than 1μg/ml within 24 h of overdose survived[5]. Urinary concentrations in those who died were 10－10000 μg/ml; concentrations in those who died later of pulmonary fibrosis were 1－1000 μg/ml.

Several HPLC methods have been developed for the simultaneous determination of PQ and DQ in human urine. These methods require a liquid-liquid [5] or solid-phase [6] extraction procedure, and have not yet been applied to the determination of PQ and DQ in patient urine. Then, we applied our method to the analysis of the herbicides in urine and have improved the method to selectively quantify them.

Experimental

Chemicals and solutions

All chemicals and solvents were of analytical-reagent grade, unless otherwise noted. Distilled water, purified with Milli-Q system (Japan Millipore Ltd., Tokyo, Japan), was used for all aqueous solutions. PQ dichloride and DQ dibromide monohydrate were purchased from Wako Pure Chemical (Osaka, Japan). 1,1'-Diethyl-4,4'-bipyridyl dichloride (IS) was synthesized according to the procedure described by Calderbank and Yuen [8]. Sodium hydrosulfite was obtained from Wako Pure Chemical and was of Wako Practical grade. An alkaline sodium hydrosulfite solution was prepared by dissolving sodium hydrosulfite (50 mg) in 100 ml of 1 M sodium hydroxide. This solution could be used for more than two days when stored at 4 °C. PQ, DQ and IS solutions (100 μg/ml each) were prepared in water, and stored in a refrigerator at 4 °C, and then further diluted to appropriate concentrations with water before undergoing analysis. Urine and serum specimens were obtained from healthy volunteers in this laboratory.

HPLC apparatus and conditions

Chromatography was performed using L-7120 pump (Hitachi, Tokyo, Japan) equipped with a Rheodyne 7125 syringe loading sample injector valve (20 μl loop). PQ, DQ and IS were separated on a reversed-phase column, Capcell Pak C18 UG120 (150×4.6 mm i.d.; particle size, 5 μm; Shiseido Co., Tokyo, Japan), which was preceded by a Capcell Pak C18 UG120 guard column (10×4.0 mm i.d., 5 μm), by isocratic elution with a mixture of methanol and 200 mM phosphoric acid solution containing both 0.15 M diethyl amine and 15 mM sodium 1-heptane sulfonate (1:4, v/v) as eluent. The flow-rate of the mobile phase was set at 0.4 ml/min, and the column temperature was ambient (15-20 °C). The eluate from the HPLC column was mixed with the alkaline sodium hydrosulfite solution delivered by means of a Hitachi L-7120 pump using a polyether ether ketone (PEEK) T-type mixing device. The flow-rate of the alkaline sodium hydrosulfite solution was 0.4 ml/min. The mixture in the coil (1 m×0.5 mm i.d., PEEK tube) was warmed in an SB-9 (EYELA, Tokyo, Japan) water bath (20 °C) and it was detected at 600 nm with a Hitachi L-7405 UV/VIS detector. Peak areas were used

for quantification with a C-R6A Chromatopak (Shimadzu, Kyoto, Japan).

Sample preparation

A 200 μl aliquot of the urine was mixed with 20 μl of the IS solution (10 μg/ml). The solution was ultrafiltered at 1000*g* with Ultrafree-MC® (0.5 ml, 0.22 μm). Thereafter, the filtrate (25 μl) was injected into the chromatograph. Serum samples were treated according to the previous report [2]. The calibration graph was prepared as described in the above procedure, except that 20 μl of the IS solution was replaced with IS solutions, each containing 0.1−20 μg of both PQ and DQ. The net peak area ratios of the individual PQ, DQ and IS were plotted against the concentrations of the spiked PQ and DQ.

Case history

An 82-year old male was admitted to Fukuoka University Hospital at 9:40 a.m. (Dec., 17, 2001). He had taken Preeglox L® (containing 5 % PQ dichloride and 7 % DQ dibromide) and carbamate insecticide in a suicide attempt. After arrival, he underwent emergency medical treatment, including continuous hemodiafiltration and hemoperfusion, but unfortunately he died 3 days after admission. Blood and urine samples were collected at 0 (initial treatments), 2, 4, 6, 8 and 12 h. Each blood sample was allowed to stand for 30 min at room temperature (20-25 °C), and it was then centrifuged at 1000*g* for 10 min. Each blood and urine sample was frozen at −20 °C until analysis.

Results and Discussion

LC separation

PQ and DQ have been reported to be separable on a Capcell Pak C18 UG120 reversed-phase columns by elution with a methanol-200 mM phosphoric acid solution containing both 0.1 M diethyl amine and 12 mM sodium 1-heptane sulfonate. The best separation of PQ, DQ, and IS in urine samples was achieved on the reversed-phase column with methanol-200 mM phosphoric acid solution containing both 0.15 M diethyl amine and 15 mM sodium 1-heptane sulfonate (1:4, v/v) as an eluent. Unidentified peaks from urine appeared

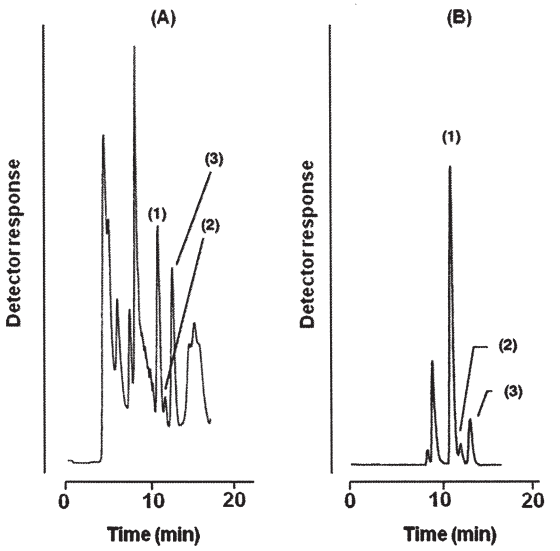

Figure 1. Chromatograms detected at 391 nm (A) and at 600 nm (B), which were obtained with urine spiked with PQ, DQ and IS (5 μg/ml). Peaks: 1, PQ; 2, DQ; 3, IS; others, endogenous compounds.

on the chromatogram around those of PQ, DQ, and IS when they were detected by UV at 391 nm (λ_1 abs of PQ radical: $\varepsilon = 4.5 \times 10^4$) (Fig. 1A). The detection at 600 nm (λ_2 abs PQ radical: $\varepsilon = 1.4 \times 10^4$) is suitable for PQ and IS, but the detection wavelength is far distant from the $\lambda_2$abs of DQ radical (430 nm). Figure 1B shows a typical chromatogram obtained with urine spiked with PQ, DQ, and IS. No interfering peaks were observed in the region of the retention times for those herbicides.

Post-column reduction conditions

The absorption spectra of the PQ, DQ, and IS radicals obtained by the reduction with alkaline sodium hydrosulfite solution showed maximum absorbances at 391 and 600 nm for PQ and IS, and at 378 and 430 nm for DQ (Fig. 2) [9, 10]. Post-column reductive conditions were examined using PEEK tube coil (1 m×0.5 mm i.d.). The reductive reaction of PQ, DQ and IS with sodium hydrosulfite occurred effectively under alkaline conditions at 20°C. Constant and maximum absorbance values were obtained when 0.05 % sodium hydrosulfite in 1 M sodium hydroxide was used for post-column reduction.

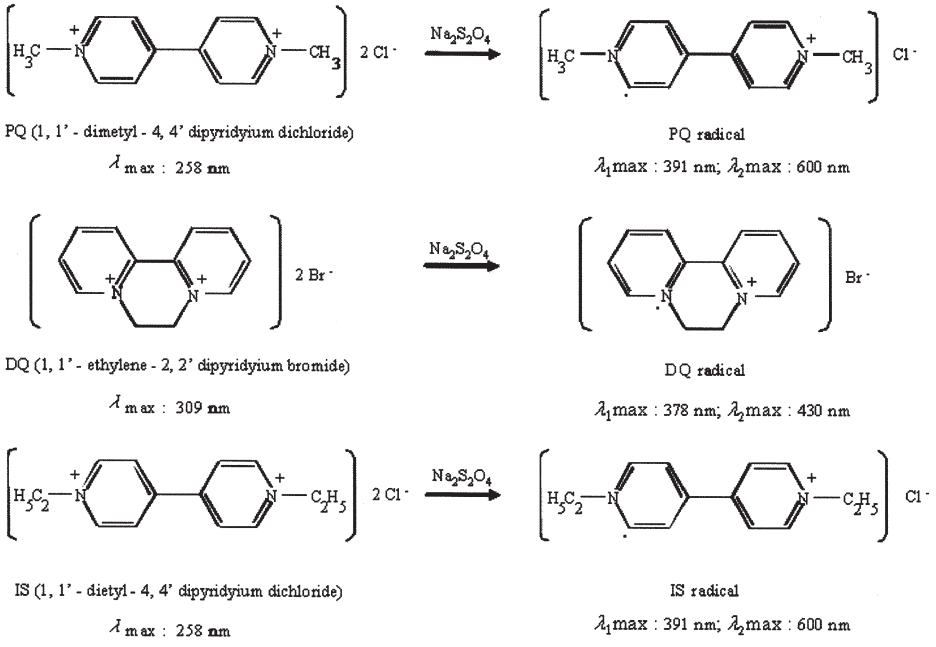

Figure 2. General scheme of the radicals from PQ, DQ, and IS by reduction with alkaline sodium hydrosulfite solution.

Quantitative analysis of PQ and DQ in human urine

Several HPLC methods have been reported to determine the levels of PQ and DQ in

human urine following clean-up procedures such as ion-pair liquid-liquid extraction [6] or solid-phase extraction [7]. In order to simplify the analysis procedure, we employed ultrafiltration device (Ultrafree-MC®) to remove proteins and crystalline uric acid which might damage an analytical column. The recovery (n=6) ranges of PQ, DQ and IS were 96.4—101, 80.5—86.5 and 96.7—101% in the concentration range of 1.0–10.0 μg/ml, respectively. Relative standard deviation percentages are between 0.9% and 8.0%.

A linear relationship was observed between the ratios of the peak areas of PQ and DQ to that of IS and the amounts of PQ and DQ added to urine over the range of 0.5 μg—100 μg/ml urine. A linear-regression analysis [the linear correlation coefficients (n = 7) in parentheses] of the curves for PQ and DQ showed that $y = 1.133x - 0.139$ ($r = 0.999$) and $y = 0.043x - 0.007$ ($r = 0.996$), where y and x are the peak-area ratio and the concentration (μg/ml in the urine) of each compound, respectively. These results demonstrate that this technique allows for the determination of the of PQ and DQ levels in urine over a wide range of concentrations.

Precision and accuracy

The within- and between-day precisions were examined by performing six separate analyses using urines spiked with PQ and DQ at low, medium and high concentrations. The results are listed in Table 1. These data indicate that the HPLC method is reliable both within the same day and on different days.

The detection limits (signal-to-noise ratio = 3) for PQ and DQ were 20 ng and 0.5 μg (76 pmol and 1.5 nmol, respectively) per ml urine. The sensitivity is approximately five times higher than those of the HPLC methods.[6, 7] The sensitivity is sufficient for the assessment of the prognostic significance of the urine-PQ concentrations in poisoned patients.

Table 1. Within- and between-day precisions of HPLC method for determining PQ and DQ in human urine.

Concentration (μg/ml)	Coefficient variance (n=6)			
	Within-day, %		Between-day, %	
	PQ	DQ	PQ	DQ
1	3.02	5.72	1.62	5.3
5	1.34	5.15	1.01	10.9
10	1.36	5.33	2.33	12.6

Validation

To ascertain the usefulness of this method, we applied it to simultaneously determine the PQ and DQ levels in a patient who had ingested Preeglox L® containing paraquot and diquat. Figure 3 shows the PQ and DQ level changes from 0 h (initial treatment) to 12 h in serum and urine. The time at which the patient had ingested the herbicide was unknown, but it appeared that more than 5 h had elapsed prior to the start of emergency medical treatment. Proudfoot *et al* reported that patients whose serum concentrations do not exceed 2.0, 0.6, 0.3, 0.16 and 0.1 μg/ml at 4, 6, 10, 16, and 24 h after ingestion respectively are likely to survive[11]. On the other hand, Scherrmann *et al* reported that patients with urine PQ concentrations of less than 1μg/ml within 24-h of overdose survived, but patients with urine PQ concentrations of more than 1μg/ml had a high probability of death.[5] Judging from the these findings, it was therefore unlikely that the patient would survive.

The present method employing UV/VIS-detection at 600 nm significantly improved the selectivity of this protocol. The total analysis time of the method was *ca.* 20 min, which was twice or more times as rapid as those of HPLC methods.[6, 7] This method was successfully performed to determine the PQ and DQ levels in urine obtained from a PQ-poisoned patient. Therefore, the method should be useful in both assessing the severity as well as in predicting the outcome of PQ poisoning.

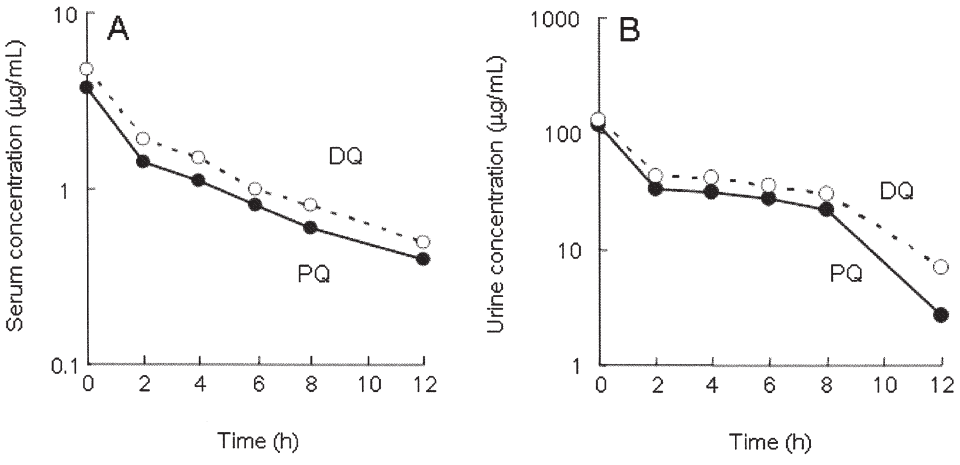

Figure 3. Time courses of serum (A) and urine (B) concentrations of PQ and DQ after the patient had been brought to the hospital.

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