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An improved approach for extraction and high-performance liquid chromatography analysis of paraquat in human plasma

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ABSTRACT

A simple, sensitive, reliable and economical HPLC method for quantifying paraquat concentration in human plasma has been developed, using diethyl paraquat as an internal standard. The drugs were extracted from the sample and separated on Xtimate C18 column with a mobile phase of 15% acetonitrile in 0.1 M orthophosphoric acid containing SDS (150 mg/l). The pH of the mobile phase was adjusted to 3 with triethylamine and the detection wavelength was 256 nm for both paraquat and the internal standard. The average extraction recoveries were 91.9%. Good linearity (R^2 = 0.9984) was observed throughout the range of 0.02–10 μg/ml in 0.5 ml plasma. The overall accuracy of this method was 97.6–107.3% and the lower limit of detection was 0.01 μg/ml. The intra- and inter-day variations were lower than 3.65% and 2.64%, respectively. We used this method to examine the paraquat concentrations of 53 patients with acute paraquat intoxication of whom 26 (49.1%) survived. In conclusion, this method was suitable for quantification of paraquat plasma concentration in toxicological samples. It was helpful in both assessing the severity of intoxication and predicting the outcome of paraquat poisoning.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium chloride, PQ) is widely used as a nonselective herbicide in the countryside. Although it has been proved safe in use, there have been numerous cases of PQ poisoning due to accidental or intentional swallowing of the commercial product [1,2]. The mechanism of PQ intoxication is associated with accumulation of reactive oxygen species (ROS) and toxic free radicals in various organs, especially in the lungs. This results in oxidative injuries and eventually leads to irreversible pulmonary fibrosis and injuries of other vital organs. PQ intoxication by ingestion is often fatal, the clinical outcome is closely associated with the amount consumed. Death occurs within few days and is usually a consequence of multi-organ failure (MOF) when an extremely large amount of PQ is ingested. Patients with lower doses who eventually die within several weeks or a longer timer are due to respiratory failure as a consequence of progressive and irreversible pulmonary fibrosis [3,4].

Current treatment of PQ poisoning mainly includes diminishing absorption of the remaining PQ in the gastrointestinal tract, removal of blood PQ by extracorporeal therapies such as hemoperfusion, administration of antioxidants and the maintenance of vital functions. However, these measures remain ineffective in improving patients' prognosis. According to many studies, the plasma PQ level is a key predictor of the clinical outcome [5,6]. If the plasma PQ level is above 2.0, 0.6, 0.3 or 0.1 μg/ml at 4, 6, 10 or 24 h after ingestion respectively, the survival chance of a patient is low. The fast and accurate establishment of the intoxication level is helpful for the physicians not only to choose proper treatment but also to evaluate the effects of some new therapeutic measures on clinical outcomes [6–10].

Various methods have been applied to the analysis of PQ in plasma, which include gas-chromatography (GC) [11], GC/mass-spectrometry (MS) [12], high-performance-liquid-chromatography (HPLC) [13–17], HPLC/MS [18], capillary electrophoresis (CE) [19,20]. However, some of these methods require special equipment or demand frequent and complicated steps. In our study, a simple, rapid and inexpensive procedure utilizing HPLC with ultraviolet (UV) detection was established to determine PQ plasma level in 53 patients with acute PQ intoxication.

2. Materials and methods

2.1. Chemicals and reagents

PQ and diethyl PQ as internal standard (IS) were purchased from Sigma–Aldrich Shanghai Trading Co. Ltd. (Shanghai, China), HPLC-

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grade acetonitrile, triethylamine and methylene chloride were purchased from TEDIA Shanghai Weston Trading Co. Ltd. (Shanghai, China), sodium dodecyl sulfate(SDS) from Shanghai Biological technology Co. Ltd. (Shanghai, China). Deionized water was produced by a Milli-Q Reagent Water System (Millipore China Co. Ltd., Shanghai, China). Drug-free health human plasma was obtained from department of laboratory medicine of West China Hospital of Sichuan University.

2.2. Apparatus and chromatographic conditions

A high performance liquid chromatographic (HPLC) system (Shimadzu, Japan) consisting of a LC-20AD pump, a SPD-20A ultraviolet detector, a SIL-20AC automatic sampler, a CTO-20A oven and a LC solution control software was used. The sample separation was achieved on a Xtimate C18 column (150 mm × 4.6 mm, 5 μm particle size) from Shimadzu Corporation (Japan). PQ and IS were eluted under 35 °C at a flow-rate of 1.0 ml/min and monitored by UV absorption at 256 nm. The mobile phase consisted of 0.1 M orthophosphoric acid with 15% (v/v) acetonitrile and 0.15 mg/ml SDS. The pH was adjusted to 3.0 with triethylamine.

2.3. Sample preparation

Stock standard solutions (200 μg/ml) of PQ and IS were prepared in deionized water and stored at 4 °C till use. The plasma samples (0.5 ml/sample) were spiked with 10ul of known amounts of IS to yield a final concentration of 10 μg/ml. Samples were vortex-mixed after adding 1 ml acetonitrile and then centrifuged for 5 min at 15,000 × *g* in an Eppendorf 5810R Centrifuge (Eppendorfchina Ltd., Shanghai, China), Supernatants were transferred into a round bottom glass tube containing 3 ml methylene chloride, vortex-mixed for 5 min and centrifuged for 5 min at 734 × *g*. Finally, 100 μl upper aqueous phase was injected to HPLC analysis.

2.4. Extraction recovery

Replicate ($n = 3$) plasma samples (0.5 ml/sample) were spiked with known amounts of PQ to yield final concentrations of 0.2, 1.0 and 5.0 μg/ml, the spiked plasma were treated and analyzed as described above. The extraction recoveries were calculated by comparing the chromatographic peak areas obtained from the extracts of the spiked human plasma with those obtained by direct HPLC injection of non-extracted compounds dissolved in the mobile phase. The efficiencies were determined at three different concentrations of PQ.

2.5. Calibration curves

A calibration curve was generated to confirm the linear relationship between the peak area ratio and the concentration of PQ in the test samples. PQ was added to plasma to yield final concentrations of 0.020, 0.039, 0.078, 0.156, 0.312, 0.625, 1.250, 2.500, 5.000 and 10.000 μg/ml in plasma. The spiked plasma and IS were extracted and analyzed as sample preparation above. Standard curves were performed weight linear regression ($1/C$) by plotting the peak area ratio of PQ/IS against the drug concentrations tested.

2.6. Intra-assay and inter-assay accuracy and precision

The intra- and inter-day precision, recovery and accuracy of the method were assessed by performing duplicate determinations of PQ in human plasma. PQ was added to drug-free plasma at concentrations of 0.04, 0.2, 1.0 and 5.0 μg/ml in plasma. The PQ plasmas concentration were determined as described above. The ratio of the measured concentration over the known concentration of PQ was used as the accuracy of the analytical method.

The intra-day precision was determined by analyzing a spiked sample at four PQ concentrations in sextuplicate on the same day. The same procedure was repeated on 6 days to determine the inter-day precision. The precision was given by the intra- and inter-day relative standard deviations.

2.7. Stability testing

The stability of PQ in plasma was assessed at four different concentrations (0.04, 0.2,1.0 and 5.0 μg/ml) at room temperature for 6 h and stored of −20°C for 26 days. Sample stability was accessed after three cycles of freezing (−20 °C)/thawing (room temperature) and extraction as described above. Freshly prepared standard curves were used for quantification of the samples.

2.8. Patients selection and samples collection

Fifty-three patients with acute PQ intoxication by accidental or suicidal ingestion, admitted to the emergency department, West China Hospital of Sichuan University, Chengdu, China, from June 3, 2010 to November 11, 2010, were studied. Blood samples for PQ concentration test were collected in heparinized tubes on admission. After centrifugation, plasma samples were stored at −20°C until analysis.

3. Results and discussions

3.1. Chromatographic separations

PQ can be separated by HPLC using aqueous mobile phases containing methanol, acetonitrile or ion pairing agents [21–23]. Several combinations of acetonitrile and 0.1 M (pH 3.0) orthophosphoric acid buffer were evaluated as possible mobile phases. We found that a combination of 0.1 M orthophosphoric acid (pH 3.0) and acetonitrile in a ratio of 85:15 (v/v) was the most suitable for separating PQ and IS from potentially interfering compounds. Typical chromatograms are shown in Fig. 1, with standard of PQ and IS (Fig. 1A) as well as blank human plasma samples (Fig. 1B), and Patient plasma samples were supplemented with IS to a final concentration of 0.2 μg/ml (Fig. 1C). The retention time of PQ and IS was respectively, 19.0 and 30.8 min. Interference of endogenous plasma compounds was absent confirming the specificity of the described method. Therefore, the specificity of the described method was good.

3.2. Quantitative analysis of PQ in human plasma

Several analytical methods have been reported to determine the concentration of PQ in plasma or urine, following various cleanup procedures such as ion-pair liquid–liquid extraction and solid-phase extraction. In order to shorten the time required for analyzing PQ in plasma, we studied a new sample preparation method which combined protein precipitation with organic solvent backwashing. Protein precipitation method usually uses acetonitrile, methanol, perchloric acid and trichloroacetic acid and backwashing uses methylene chloride, methenyl trichloride and hexane. The use of acetonitrile and methylene chloride was found to be the most effective for the removal of interfering compounds. The average recovery of PQ (91.9%) from plasma in the concentration range of 0.2–5.0 μg/ml as described above was satisfactory. The lower detection limit of our method was 0.01 μg/ml. The sensitivity is approximately two times higher than those of most HPLC methods [20–22] and the sensitivity was sufficient for the assessment of the

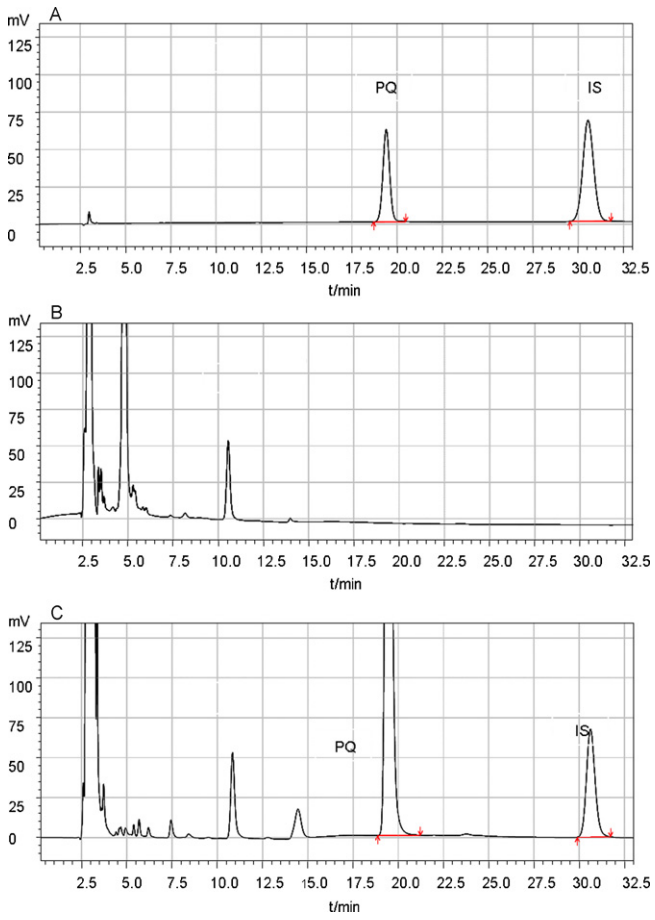

Fig. 1. HPLC chromatograms of PQ and IS: (A) standard of PQ and IS added; (B) blank human plasma; (C) plasma of a patient of a PQ poisoning with 0.2 μg/ml of IS added. The used HPLC conditions are described in Materials and methods. PQ: paraquat; I.S: internal standard.

prognostic significance of the plasma – PQ concentrations in poisoned patients. Paixao et al. [13] had reported a similar method, but our LOD was approximately 10× lower than theirs. The difference observed could be explained by the different sample volume and the different solvent used for sample preparation. First, in our method 0.5 ml plasma were added while Paixao et al. only added 0.2 ml, so much more PQ was concentrated for detection. Second, we used both acetonitrile and methylene chloride for sample preparation, the protein removal was more completely and the interference was still lesser. Third, 100 μl of the extracts obtained from the blood samples in our method were analyzed, and this is one more times than theirs (50 μl), These made our method more sensitive.

The calibration curve obtained from extraction of plasma containing known amounts of PQ was linear over the range from 0.02 μg/ml to 10.0 μg/ml and could be extrapolated to at least

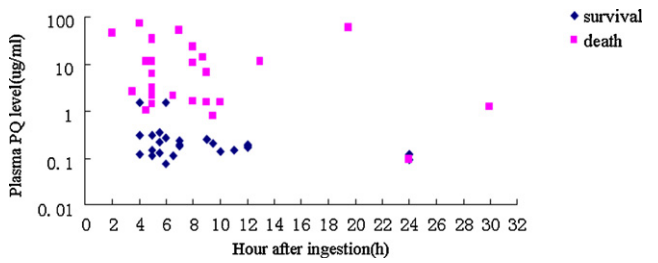

Fig. 2. Plasma paraquat concentrations related to time of ingestion of 53 patients ($n = 53$).

80.0 μg/ml (data not shown). The weighed regression equation ($1/C$) was $Y = 0.1841X - 0.007597$ ($R^2 = 0.9984$), in which Y was peak area ratio of PQ/IS and X was the concentration (μg/ml in plasma) of PQ. These results demonstrated that this technique allowed the determination of the level of PQ in plasma over a wide range of concentrations.

3.3. Accuracy, sensitivity and stability of the detection method

The intra- and inter-day precision and accuracy of the method were assessed by performing duplicate determinations of PQ in human plasma (Table 1). The intra-day and inter-day precision was less than 3.65% and 2.64%, respectively, for all the examined concentrations. The overall accuracy of the method, determined at four PQ plasma concentrations, was 97.6–107.3%. These results suggested that the procedures described above were satisfying with respect to both accuracy and precision.

The stability of PQ in plasma was assessed. The results obtained indicated that coefficients of variance (RSD) at room temperature, frozen/thawed at room temperature then refrozen at −20 °C and stored at −20 °C were less than 3.63%, 5.50% and 2.18%, respectively. Therefore, in human plasma, PQ was extremely stable for at least 26 days at −20 °C, for 6 h at room temperature and for three freeze/thaw cycles.

3.4. Clinical application

Human PQ intoxication was usually assessed by an estimated oral dose of PQ or by urinary or plasma concentrations of PQ [5,24]. In our study, PQ plasma levels of 53 cases were determined (22 men and 31 women, mean age was 30 years), which ranged from 0.094 μg/ml to 70.22 μg/ml. Twenty-six patients (49.1%) survived (Fig. 2). The upper limit of plasma PQ concentration in survivors was 0.34 μg/ml at 5 h. All patients with plasma PQ level above 3.08 μg/ml died. The minimum PQ concentration of the deaths was 0.094 μg/ml at 24 h. This showed that plasma PQ concentrations may be the most useful index in assessing the severity of intoxication and predicting the outcome of PQ poisoning.

Table 1
Intra- and inter-day precision, and accuracy of the determination of paraquat in human plasma.

Amount added (ng/ml)	Intra-day[a]			Inter-day[b]		
	Amount detected (ng/ml)	Precision (%)	Accuracy (%)	Amount detected (ng/ml)	Precision (%)	Accuracy (%)
40	42.6 ± 0.4[c]	0.99	106.5 ± 1.1	42.9 ± 0.4	0.85	107.3 ± 0.9
200	195.2 ± 5.2	2.66	97.6 ± 2.6	195.2 ± 3.7	1.87	97.6 ± 1.8
1000	961.6 ± 35.1	3.65	96.2 ± 3.5	995.2 ± 26.3	2.64	99.5 ± 2.6
5000	4954.4 ± 57.6	1.16	99.1 ± 1.2	4932.4 ± 80.0	1.62	98.7 ± 1.6

[a] Intra-day precisions were calculated from measurements of six spiked samples on the same day.
[b] Spiked human plasma were kept at −20 °C and analyzed on 6 separate days, with one sample each day.
[c] Values were shown as means ± SD.

4. Conclusions

This was a simple, sensitive, reliable and economical HPLC method for quantifying PQ levels in human plasma. This method was successfully performed to determine the PQ levels in plasma obtained from patients with PQ intoxication. Therefore, the method was suitable for not only quantification of PQ in toxicological samples but also the prognostic assessment of patients with PQ poisoning.

Competing interests

The authors declare that they have no competing interests.

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References

[1] R.D. Fairshter, A.F. Wilson, Am. J. Med. 59 (1975) 751.
[2] S. Okonek, Med. Welt. 27 (1976) 1401.
[3] L.L. Smith, Phil. Trans. R. Soc. Lond. B 311 (1985) 647.
[4] L.L. Smith, Hum. Toxicol. 6 (1987) 31.
[5] L. Senarathna, M. Eddleston, M.F. Wilks, B.H. Woollen, J.A. Tomenson, D.M. Roberts, N.A. Buckley, Q. J. Med. 102 (2009) 251.
[6] H.W. Gil, M.S. Kang, J.O. Yang, E.Y. Lee, S.Y. Hong, Clin. Toxicol. (Phila.) 46 (2008) 515.
[7] R.J. Dinis-Oliveira, H. Pontes, M.L. Bastos, Toxicology 255 (2009) 187.
[8] K. Tabei, Y. Asano, S. Hosoda, Artif. Organs 6 (1982) 37.
[9] S.Y. Hong, J.O. Yang, E.Y. Lee, S.H. Kim, Toxicol. Ind. Health 19 (2003) 17.
[10] R. Castro, C. Prata, L. Oliveira, Acta Med. Port. 18 (2005) 423.
[11] S. Kawase, S. Kanno, S. Ukai, J. Chromatogr. 283 (1984) 231.
[12] N. Tsunoda, O. Ootsuru, Eisei Kagaku. 35 (1989) 73.
[13] P. Paixao, P. Costa, T. Bugalho, C. Fidalgo, L.M. Pereira, J. Chromatogr. B 775 (2002) 109.
[14] I. Mika, H. Yasushi, F. Manami, O. Akira, K. Shinichiro, H. Yasuo, Y. Toshiharu, Biol. Pharm. Bull. 28 (2005) 725.
[15] R. Gill, S.C. Qua, A.C. Moffat, J. Chromatogr. 255 (1983) 483.
[16] T. Itagaki, S.J. Lai, S.R. Binder, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 3339.
[17] M.R. Brunetto, A.R. Morales, M. Gallignani, J.L. Burguera, M. Burguera, Talanta 59 (2003) 913.
[18] X.-P. Lee, T. Kumazawa, M. Fujishiro, C. Hasegawa, T. Arinobu, H. Seno, A. Ishii, K. Sato, J. Mass Spectrom. 39 (2004) 1147.
[19] O. Nïuñez, E. Moyano, L. Puignou, M.T. Galceran, J. Chromatogr. A 912 (2001) 353.
[20] O. Nïuñez, E Moyano, M.T. Galceran, J. Chromatogr. A 946 (2002) 275.
[21] S. Hara, N. Sasaki, D. Takase, S. Shiotsuka, K. Ogata, K. Futagami, K. Tamura, Anal. Sci. 23 (2007) 523.
[22] S. Ito, T. Nagata, K. Kudo, K. Kimura, T. Imamura, J. Chromatogr. 617 (1993) 119.
[23] I.S. Sellero, A. Cruz, P. Fernandez, M. Lopezrivadulla, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2009.
[24] A.T. Proudfoot, M.S. Stewart, T. Levitt, B. Widdop, Lancet II (1979) 330.