Original Article

Serum paraquat concentration detected by spectrophotometry in patients with paraquat poisoning

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BACKGROUND: Paraquat (PQ) is a world-wide used herbicide and also a type of common poison for suicide and accidental poisoning. Numerous studies have proved that the concentration of serum PQ plays an important role in prognosis. Spectrophotometry, including common spectrophotometry and second-derivative spectrophotometry, is commonly used for PQ detection in primary hospitals. So far, lack of systematic research on the reliability of the method and the correlation between clinical features of patients with PQ poisoning and the test results has restricted the clinical use of spectrophotometry. This study aimed to evaluate the reliability and value of spectrophotometry in detecting the concentration of serum PQ.

METHODS: The wavelengths for detecting the concentration of serum PQ by common and second-derivative spectrophotometry were determined. Second-derivative spectrophotometry was applied to detect the concentration of serum PQ. The linear range and precision for detection of PQ concentration by this method were confirmed. The concentration of serum PQ shown by second-derivative spectrophotometry and HPLC were compared in 8 patients with PQ poisoning. Altogether 21 patients with acute poisoning 4 hours after PQ ingestion treated in the period of October 2008 to September 2010 were retrospectively reviewed. The patients were divided into higher and lower than 1.8 µg/mL groups based on their concentrations of serum PQ measured by second-derivative spectrophotometry on admission. The severity of clinical manifestations between the two groups were analyzed with Student's *t* test or Fisher's exact test.

RESULTS: The absorption peak of 257 nm could not be found when common spectrophotometry was used to detect the PQ concentration in serum. The calibration curve in the 0.4–8.0 µg/mL range for PQ concentration shown by second-derivative spectrophotometry obeyed Beer's law with $r=0.996$. The average recovery rates of PQ were within a range of 95.0% to 99.5%, relative standard deviation (RSD) was within 1.35% to 5.41% ($n=6$), and the lower detection limit was 0.05 µg/mL. The PQ concentrations in serum of 8 patients with PQ poisoning shown by second-derivative spectrophotometry were consistent with the quantitative determinations by HPLC ($r=0.995$, $P<0.0001$). The survival rate was 22.2% in patients whose PQ concentration in serum was more than 1.8 µg/mL, and the incidences of acidosis, oliguria and pneumomediastinum in these patients were 55.6%, 55.6% and 77.8%, respectively. These clinical manifestations were different significantly from those of the patients whose PQ concentration in serum was less than 1.8 µg/mL ($P<0.05$).

CONCLUSIONS: For common spectrophotometry, the wavelength at 257 nm was not suitable for detecting serum PQ as no absorbance was shown. Second-derivative spectrophotometry was reliable for detecting serum paraquat concentration. Serum PQ concentration detected by second-derivative spectrophotometry could be used to predict the severity of clinical manifestations of patients with PQ poisoning, and PQ content higher than 1.8 µg/mL 4 hours after ingestion could be an important predictive factor for poor prognosis.

KEY WORDS: Spectrophotometry; Derivative spectrophotometry; Paraquat; Poisoning; Serum; Concentration

World J Emerg Med 2011;2(3):179-184

DOI: 10.5847/wjem.j.1920-8642.2011.03.004

INTRODUCTION

Paraquat (PQ) is a world-wide used herbicide,^[1] and also a common poison for suicide and accidental poisoning. Since its introduction to agriculture in 1962, thousands of people died from suicide or accidental poisoning of PQ.^[2, 3] This mortality rate can be as high as 60%-80% due to the lack of antidote.^[4] As it is difficult to accurately evaluate the severity of poisoning, clinicians can only estimate patient's condition by appropriate oral dosage offered by patient himself or his relatives.

Studies have proved that the concentration of PQ in serum plays an important role in prognosis.^[1, 5-7] For the measurement of the concentration of PQ, there are different ways including HPLC/MS,^[8-10] GC/MS,^[11] capillary zone electrophoresis,^[12] radioimmunoassay,^[13] ELISA,^[14] and time-resolved fluoroimmunoassay.^[15] However, expensive equipments as well as complicated procedures prevent their use in primary hospitals. Spectrophotometry including common spectrophotometry^[16, 17] and derivative spectrophotometry is commonly used to detect PQ in primary hospitals.^[18, 19] So far, inadequate evaluation of the reliability of the two methods and the correlation between the clinical features of patients with PQ poisoning and the results of these methods restrict the use of spectrophotometry. While evaluating the reliability of spectrophotometry to test PQ concentration in serum, we determined its clinical significance in treatment of PQ poisoning.

METHODS

Instruments and reagents

An UV-3010 photometer spectrophotometer (HITACHI, Japan) and a 5418 Style Desk Centrifuge (Eppendorf, Germany) were used.

Standard PQ samples were purchased from Sigma Company (Fluka, Cat#36541, USA). Trichloroacetic acid (TCA), sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_4$) and sodium hydroxide (NaOH) were from Sinopharm Chemical Reagent Co. Ltd. (AR, Shanghai, China). Sera collected from healthy people were provided by the Department of Clinical Laboratory in Tenth People's Hospital of Tongji University, Shanghai, China.

Serum sample and its maintenance

Altogether 22 PQ poisoned patients were treated at Tenth People's Hospital of Tongji University by medication. Among them 16 were male and 6 were female, with an average age of 31.2 ± 12.8 years. Twenty-

one patients were transferred to our hospital 4 hours after poisoning except one who came to the hospital 2 hours after poisoning. Serum samples of all patients were divided into two samples after collection and centrifugation. One sample was free from light in a -80°C freezer, and the other was sent to the Shanghai Pesticide Research Institute for PQ identification by GC/MS and PQ quantification by HPLC.

Preparation of standard PQ solution and serum sample

PQ stock solution was prepared by dissolving 100 mg PQ with 20 mL distilled water to a final concentration of 5 mg/mL, then it was sealed and kept in dark. Water standard solution was 10 $\mu\text{g/mL}$, and serum standard solutions were 50, 10, 8, 4, 2, 1, 0.8, 0.4, 0.2, 0.1 and 0.05 $\mu\text{g/mL}$ prepared from serum of healthy people.

Sample analysis

Common spectrophotometry method

Water standard solution with 10 $\mu\text{g/mL}$ PQ was scanned with a continuous wavelength (CW) between 200 to 300 nm, with pure distilled water as blank reference. Fresh serum or serum standard solutions with 10, 50 $\mu\text{g/mL}$ PQ were well mixed with 20% TCA at a volume ratio of 6:1. The mixture was centrifuged at $13000 \times g$ for 5 minutes. Then the supernatants were collected against CW scan from 200 to 300 nm.^[16]

Second derivative spectrophotometry

After the pretreatment of serum sample with the aforementioned method, the supernatant was moved to a new Eppendorf tube. The samples were sealed and shielded from light for further analysis. Before testing, 400 μL sample was gently and thoroughly mixed with 100 μL of mixture made with an equal volume of 10% $\text{Na}_2\text{S}_2\text{O}_4$ and 5 mol NaOH. The same method was applied to the reference serum. The data of a zero-order spectrum with a sampling interval of 0.5 nm were obtained by scanning from 300 to 500 nm (wavelength space $\Delta\lambda = 0.5$ nm) and recorded. Then the second derivative can be calculated as follows: $\Delta^2\text{Abs}/(\Delta\lambda)^2$ (derivative wavelength space $\Delta\lambda = 4$ nm).^[1]

Evaluation of second derivative spectrophotometry

Determination of standard curve and linear range

To set up a standard curve and linear range for

second derivative spectrophotometry, we selected serum standard with PQ at the concentrations of 10, 8, 4, 2, 0.8, 0.4, 0.2, 0.1, 0.05 $\mu\text{g/mL}$. Samples were treated and second derivative spectrophotometry was carried out as described above. Five replicates were achieved for each concentration. Regression analysis was made between the mean increment (y value) of the detective wavelength and the concentration (x value).

Determination of recovery

We determined the recovery using an additive recovery method. The supernatant collected from 5 serum samples (A, B, C, D, and E) containing PQ solution was mixed with PQ standard solution to get a theoretical concentration of 0.8, 2, 4, 6, 8 $\mu\text{g/mL}$, respectively. With the second derivative spectrophotometry mentioned, samples at each concentration was measured for 6 times and an average value was obtained. The recovery and relative standard deviation (RSD) was calculated.

Precision test

As described, the supernatant was collected from serum samples with PQ concentrations at 0.4, 0.8, 1, 2, 4 $\mu\text{g/mL}$ and kept in dark for later use. Before testing, 4 volumes of samples were thoroughly mixed with 1 volume of mixture made with an equal volume of 10% $\text{Na}_2\text{S}_2\text{O}_4$ and 5 mol NaOH. This determination process was performed with each concentration solution 6 times a day, and continued for 6 days. Finally RSD was calculated.

Correlation analysis for the results of HPLC

Eight serum samples, of which PQ concentration had been determined by HPLC were taken out from a -80°C freezer and treated as described at room temperature. PQ concentration was determined with second derivative spectrophotometry. This determination was repeated three times for each sample and the mean value was calculated. Regression analysis was made between the mean value and concentration determined by HPLC.

Period of determination

Serum samples containing 1.0 $\mu\text{g/mL}$ PQ were purified to get rid of protein. Then the supernatant was divided evenly into 30 parts and transferred into new EP tubes preventing light for later use. Fifteen samples were mixed with $\text{Na}_2\text{S}_2\text{O}_4$ and NaOH solution respectively and the PQ concentration was determined

by second-derivative spectrophotometry at 10, 20, 30 minutes, respectively ($n=5$). The PQ quantification for the other 15 samples was done by HPLC at the same time points.

Serum PQ concentration determination in patients with PQ poisoning

Twenty-one serum samples were taken from patients who were admitted to Tenth People's Hospital of Tongji University 4 hours after ingestion of PQ and the serum PQ concentration was determined with second derivative spectrophotometry. The patients were divided into two groups: one with serum PQ higher than 1.8 $\mu\text{g/mL}$ and the other lower than 1.8 $\mu\text{g/mL}$. Clinical features of the patients in the two groups were analyzed.

Statistical analysis

Quantification data were verified by Student's t test with two independent samples, and enumeration data were verified by Fisher's exact test. The results of second derivative spectrophotometry and HPLC were subjected to linear regression and linear correlation analysis. The software SPSS 13.0 was used for statistical analysis. $P<0.05$ was considered statistically significant.

RESULTS

Verification for detecting the wavelength by spectrophotometry

The absorption spectra of the PQ solution at a concentration of 10 $\mu\text{g/mL}$ showed maximum absorbance at 257 nm, while distilled water had negligible absorbance at this wavelength. However, repeated test verified that compared with serum of healthy people, the absorption spectra of the serum samples at a PQ concentration of 10 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ showed no absorbance at 257 nm (Figure 1, $n=5$).

It was reported that if the serum PQ is measured by second derivative spectrophotometry, a peak valley can be shown between 396 nm and 403 nm, and PQ concentration could be calculated by the increment between the valley and peak.^[1] Therefore, second derivative plot was drawn using serum containing 10 $\mu\text{g/mL}$ PQ and showed a peak valley at 396 nm and 403 nm. Then, the plot showed the correlation between the standard serum with 2, 4, 8 $\mu\text{g/mL}$ PQ and the height of the peak valley in detecting wavelength ranging from 396 nm to 403 nm by spectrophotometry (Figure 2). Hence, PQ can be determined by second

derivative spectrophotometry with wavelength at 396 nm and 403 nm, and the height/amplitude of the peak indicates PQ quantity.

Reliability evaluation of second derivative spectrophotometry

The linear regression equation for serum PQ concentration determined by second derivative spectrophotometry was as follows: $y=0.001x-0.000005$ ($r=0.996$, $R^2=0.991$). The linear range was: 0.4–8.0 $\mu\text{g/mL}$, and the detection limit was 0.05 $\mu\text{g/mL}$. RSD was 1.35%–5.41%. It varied from 3.58% to 7.42% within a day and from 1.96% to 7.34% between days (Tables 1 and 2).

Table 1. Recovery rate of serum PQ concentration by derivative spectrophotometry

Sample	Theoretical PQ concentration ($\mu\text{g/mL}$)	Actual PQ concentration ($\mu\text{g/mL}$)	Recovery (%)	RSD (%)
A	0.80	0.76	95.00	5.41
B	2.00	1.95	97.50	5.33
C	4.00	3.94	98.50	2.04
D	6.00	5.94	99.00	1.35
E	8.00	7.96	99.50	1.40

PQ: paraquat; RSD: relative standard deviation

Table 3. Derivative spectrometric and HPLC determinations of PQ concentrations

Patient's No.	PQ concentration ($\mu\text{g/mL}$)	
	Second-derivative spectrophotometry	HPLC
1	2.02	1.98
2	0.38	0.26
3	0.68	0.63
4	10.28	13.20
5	2.14	2.10
6	0.40	0.28
7	4.62	4.55
8	0.54	0.50

HPLC: high pressure liquid chromatography; correlation coefficient $r=0.995$, $P<0.0001$.

Figure 2. The second derivative plot of serum PQ concentration shown by spectrometry.

Correlation between derivative spectrometric and HPLC results

Data in Table 3 and Figure 3 indicate a significant correlation between the results shown by second derivative spectrophotometry and HPLC ($r=0.995$, $P<0.0001$).

Additionally, within 10 minutes, second derivative spectrophotometry showed the results consistent with those of HPLC. However, there was a significant difference at 20 and 30 minutes ($P<0.01$). The result shown by second derivative spectrophotometry was lower than the actual concentration; whereas HPLC showed a correct concentration (Figure 4).

Table 2. Precision of serum PQ concentration by derivative spectrophotometry

Paraquat concentration ($\mu\text{g/mL}$)	Intra-day		Inter-day	
	mean	RSD (%)	mean	RSD (%)
0.40	0.40	7.42	0.40	7.00
0.80	0.80	5.19	0.80	3.75
1.00	0.99	5.64	0.98	4.84
2.00	1.99	4.24	2.01	7.34
4.00	4.04	3.58	3.94	1.96

RSD: relative standard deviation.

Figure 1. Determination of detecting wavelength for serum PQ concentration by common spectrometry.

Figure 3. Linear regression analysis of PQ concentration by second derivative spectrophotometry and HPLC.

Figure 4. Effects of measuring time on PQ concentration determination between HPLC and second derivative spectrometry; * $P < 0.01$.

Table 4. Relationship between PQ concentration by derivative spectrophotometry and clinical manifestations in patients with PQ poisoning

Clinical manifestations	PQ concentration		P value
	>1.8 µg/mL (n=9)	<1.8 µg/mL (n=12)	
PQ ingestion amount (g)	9.6±7.4	2.3±1.3	0.019 ^a
Survivors	2 (22.2)	10 (83.3)	0.009
Acidosis ^b	5 (55.6)	0 (0)	0.006
Mediastinal emphysema	7 (77.8)	0 (7.7)	0.000
Oliguria ^c	5 (55.6)	1 (8.3)	0.046

Data were expressed as means ± SD or n (%). ^a: Except the data of PQ ingestion analyzed by Student's *t* test, other data were verified by Fisher's exact test. ^b: acidosis, pH < 7.35. ^c: oliguria, the urine output was less than 400 mL per day.

Relationship between serum PQ concentration and clinical manifestations

Compared with those with serum PQ concentration less than 1.8 µg/mL, the patients with serum PQ concentration more than 1.8 µg/mL ingested more PQ at beginning and had a possibility of acidosis, oliguria and mediastinal emphysema ($P < 0.05$) (Table 4).

DISCUSSION

Whether or not reagents such as Na₂S₂O₄, ascorbic acid, and sodium borohydride are used, common spectrophotometry can be classified as a derivative or non-derivative method for determining PQ concentration.^[16, 17] Yin et al^[20] published a systematic study on the deficiencies of derivative method including strict operation requirement and unstable blue compound, which severely affect the accuracy of the method. In the present study, therefore we selected non-derivative method instead of derivative method for determining PQ concentration.^[16] A clear absorption peak was observed at 257 nm when determined the

PQ concentration, compared with distilled water. This result is consistent with that reported by Kim.^[16] But we suggest that the serum from healthy people should be used as blank to determine the detection wavelength. When using serum from healthy people as blank, we could not find an absorption peak at 257 nm.

Second derivative spectrophotometry is superior than HPLC in determining serum PQ concentration in precision and correlation. Therefore, it is applicable in primary hospitals.

By adding Na₂S₂O₄ and NaOH, the results of second spectrophotometry matched very well with those of HPLC when the determination was finished within 10 minutes. But the concentration of PQ was lower than the actual concentration if the test time exceeded the limit. Possibly, PQ could undergo photolysis under alkaline conditions by ultraviolet light.^[11] Moreover PQ²⁺ can be reduced into PQ⁺ by Na₂S₂O₄ and PQ⁺ solution immediately turns to blue. However, the blue compound is unstable. It could easily be oxidized to transparent PQ²⁺ in the air. Therefore, to reduce the effect of photolysis, samples should be protected from light. CW Scan from 300 to 500 nm should be done within 10 minutes after adding of Na₂S₂O₄ and NaOH.

In the 21 patients with PQ poisoning who had their serum PQ concentration measured by second-derivative spectrophotometry on admission, the concentration of serum PQ as well as the incidences of pneumomediastinum, oliguria and acidosis, and vice versa were lower. The concentration of serum PQ shown by second-derivative spectrophotometry was consistent with the severity of clinical manifestations in patients with PQ poisoning. Second derivative spectrophotometry was proved to be reliable as a tool to determine the concentration of serum PQ. In addition, the serum concentration above 1.8 µg/mL 4 hours after PQ poisoning indicated more clinical manifestations and a high mortality. It was an important indicator for poor prognosis in patients whose serum concentration of PQ was more than 1.8 µg/mL.

In conclusion, the wavelength at 257 nm cannot be used to detect the serum concentration of PQ by common spectrophotometry. Second derivative spectrophotometry is more reliable for detecting the concentration of serum PQ. Moreover, second derivative spectrophotometry can be applied to evaluate the clinical severity of patients with PQ poisoning. The concentration of serum PQ above 1.8 µg/mL 4 hours after PQ ingestion can be considered an important criterion of prognosis.

ACKNOWLEDGEMENTS

This paper is proofread by Yun-fang Zhang, a staff of Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Chinese Academy of Science.

Funding: This study was supported by a grant from the National Natural Science Foundation of China (No. 30871202).

Ethical approval: The study was approved by the Ethics Committee of Tenth People's Hospital of Tongji University.

Conflicts of interest: The authors declare that there is no conflict of interest.

Contributors: Li CB proposed the study and wrote the paper. All authors contributed to the design and interpretation of the study and to further drafts.

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Received February 17, 2011

Accepted after revision June 6, 2011