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Gas chromatographic–mass spectrometric method for the determination of the herbicides paraquat and diquat in plasma and urine samples

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Abstract

In the present work, a method was developed and optimized aiming to determinate the herbicides paraquat (PQ) and diquat (DQ) in human plasma and urine samples. An initial procedure of chemical reduction of the analytes by adding $NaBH_4$ directly in the buffered samples (pH 8.0) was performed. This procedure was necessary to convert the quaternary ammonium substances into more volatile compounds for gas chromatographic analysis. The reduction compounds were extracted with C18 cartridges (solid-phase extraction). Ethyl paraquat (EPQ) was used as internal standard (IS). Gas chromatography–mass spectrometry (GC–MS) was used to identify and quantify the analytes in selected ion monitoring (SIM) mode. The limits of detection were 0.05 mg/l for both PQ and DQ. By using the weighted least squares linear regression ($1/x^{1/2}$ for plasma and $1/y$ for urine), the accuracy of the analytical method was improved at the lower end of the calibration curve (from 0.1 to 50 mg/l; $r > 0.98$). This method can be readily utilized as an important tool to confirm the suspicion of PQ and/or DQ poisoning and evaluate the extent of the intoxication.

Keywords: Paraquat; Diquat; Plasma and urine samples; SPE; GC–MS

1. Introduction

Paraquat (PQ) and diquat (DQ) are quaternary ammonium compounds widely used as non-selective contact herbicides. Commercial products are available in liquid and single (containing only PQ as active ingredient) or mixed forms (containing PQ and DQ). Occupational injuries are associated with irritative effects of PQ on the skin, nasal mucosa, and eyes of the exposed subjects [1]. However, most cases of serious intoxications are due to the deliberate ingestion of liquid formulations of these herbicides. Fatal cases of accidental, suicidal and homicidal poisonings have been reported worldwide [1–5].

When a liquid formulation of PQ is ingested, corrosive effects immediately occur in mouth, oral mucosa and throat with severe oesophageal and gastric lesions. Acute intoxications are also characterized by abdominal and substernal chest pain, bloody stools, dyspnea, anoxia, progressive fibrosis, coma, and death. While the pulmonary lesions are the most life-threatening effects in oral exposure, PQ induces multiorgan toxicity with necrotic damages to the liver, kidneys and heart [6]. Less substantial contact may result in delayed mortality from progressive pulmonary fibrosis and renal failure [7].

In acute poisonings, it is possible to infer the prognostic of survival based on the concentration of PQ in plasma. A number of reports on fatal intoxications indicated that plasma levels exceeding 2 mg/l are likely to be fatal in most cases but individual response is variable and some subjects with higher concentrations have recovered [3,8,9]. Concentrations of PQ in urine obtained within the 24 h of ingestion can also be used to estimate prognosis. Scherrmann et al. determined PQ urinary concentrations in 53 intoxicated patients. All patients with PQ concentrations of less than 1 mg/l within 24 h of overdose survived. Urinary concentrations in those who died were 10–10,000 mg/l; concentrations in those who died later of pulmonary fibrosis were 1–1000 mg/l [10].

For screening purposes of intoxication, colorimetric analysis in biological samples is performed by reducing PQ to its blue monocation radical with sodium dithionite under alkaline conditions [9,11,12]. If only DQ is present, a green color should appear [9]. For quantitative measurements, high-performance

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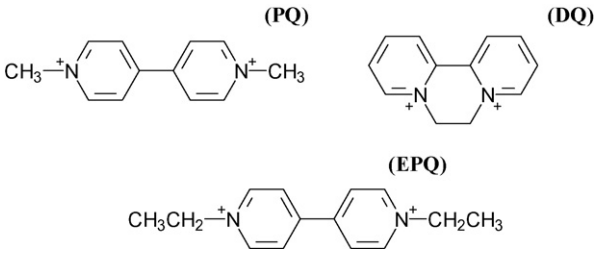

Fig. 1. Chemical structures of: paraquat (PQ) diquat (DQ) and ethyl paraquat (EPQ).

liquid chromatography (HPLC) or liquid chromatography coupled with mass spectrometry (LC–MS) are the main techniques used [4,8,13–16]. Other analytical techniques such as capillary electrophoresis and immunoassay were also proposed in methods to detect these herbicides in biological matrices [17,18].

However, to the knowledge of the authors, very few papers have reported the determination of PQ and DQ using gas chromatography–mass spectrometry (GC–MS) [19,20]. In the present work, after chemical reduction of the herbicides with $NaBH_4$ in more volatile compounds, solid-phase extraction (SPE) was used as sample preparation technique and the analytes were detected and quantified using GC–MS. Ethyl paraquat (EPQ) was used as internal standard (IS) for the chromatographic analysis. The chemical structures of the analytes (PQ and DQ) and the internal standard EPQ are shown in Fig. 1.

2. Experimental

2.1. Reagents and chemicals

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride hydrate), diquat dibromide monohydrate (1,1′-ethylene-2,2′-bipyridylium dibromide monohydrate) and ethyl paraquat dibromide (1,1′-diethyl-4,4′-bipyridinium dibromide) used as internal standards (IS), were purchased from Aldrich Chem. Co. (Milwaukee, WI, USA). Methanol (HPLC grade) and sodium borohydride were purchased from Merck (Darmstadt, Germany). Classic Sep-Pack® C18 cartridges (360 mg) were purchased from Waters (Bellefonte, PA, USA).

2.2. Synthesis of the reduction products of PQ, DQ and EPQ

The reduction products of the analytes were synthesized by the following procedure: sodium borohydride (300 mg) was added to a continuously stirred solution of PQ, DQ or EPQ (50 mg) in 5 ml of deionized water. After 10 min of reaction at 60 °C, the products were extracted with three aliquots of 10 ml of fresh distilled ether. The combined ether extracts were bubbled with HCl gas to produce dichloride salts of the reduction products. The excess of ether was removed by a gentle stream of nitrogen. Recrystallization and washing with solvents (acetone) were performed to purify the products. The substances were characterized by mass spectrometry (GC–MS).

2.3. Preparation of standard solutions

Two stock solutions of PQ and DQ were prepared separately by dissolving an appropriate amount of each compound in distilled water in order to achieve a concentration of 1 mg/ml. One solution was used to spike the calibration standards and the other one to prepare quality control (QC) samples. All stock solutions were stored at −4 °C. Working standard solutions of the compounds were prepared through serial dilution of the stock standard solutions with distilled water.

2.4. Instrumentation

GC–MS analyses for PQ and DQ were performed using a gas chromatograph model 6890 coupled with a mass selective detector (MSD) model 5972 (Hewlett Packard, Little Falls, DF, USA). Chromatographic separation was achieved on a HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d. film thickness 0.1 μm) using helium as carrier gas at 0.6 ml/min in a constant flow rate mode. MSD was operated by electronic impact (70 eV) in selected ion monitoring (SIM) mode. The injector port and interface temperature was 270 °C. The injector was operated in splitless mode. The oven temperature was maintained at 80 °C for 1 min; programmed at 10 °C/min to 200 °C and 20 °C/min to 270 °C with a hold at 270 °C for 4 min. The ions selected for each compound studied were: *m/z* 108, 135, 190 (DQ); *m/z* 134, 148, 192 (PQ) and *m/z* 148, 162, 220 (EPQ). The underline ions were used for quantification.

2.5. Sample extraction (plasma and urine)

An aliquot of 0.5 ml of plasma or urine, 1.5 ml of phosphate buffer (pH 8.0) and 20 μL of EPQ solution (100 μg/mL) were pipetted into a 15-ml plastic tube. Ten milligrams of a sodium borohydride ($NaBH_4$) were added to the solution. The reaction mixture was kept at for 10 min at 60 °C. For solid-phase extraction (SPE), the C18 cartridge was preconditioned with 2 ml of methanol and 2 ml of phosphate buffer (pH 8.0). The sample solution was transferred to the cartridge that was further washed with 2 ml of deionized water. Afterwards, the elution was performed with 2 ml of methanol and the eluate was evaporated at room temperature under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of methanol and 1 μl was injected into the GC–MS system.

2.6. Optimization of the conditions of chemical reduction reaction

The optimization study of the conditions for chemical reduction reaction was performed taking to consideration the influence of pH of the medium, time of reaction and the minimum quantity of sodium borohydride to efficiently reduce the analytes. Spiked plasma samples (0.5 ml) at a concentration of 50 mg/l of PQ and DQ were submitted to the method described in Section 2.4. The efficiency of reaction was evaluated by the average absolute area produced by the analytes in triplicate. The following parameters were studied: pH (7.0, 8.0, 9.0 and 10.0); quantity of $NaBH_4$

(10, 20, 30, 50, 100 and 150 mg) and time of reaction: (10, 20, 30, 40 and 60).

2.7. Validation of the method

The validation of the method was performed by establishing limits of detection (LOD), lower limits of quantification (LLOQ), recovery, linearity and intra- and inter-assay precision of the analytes. In order to obtain these validation data, two kinds of samples were prepared: calibration samples and quality control samples (QC), also used in routine analysis. These samples were independently prepared by spiking blank urine or plasma samples with appropriate volumes of PQ and DQ standard solutions. QC samples were prepared in the following concentrations of PQ and DQ: 0,3, 25 and 38 mg/l (QC1, QC2 and QC3).

2.7.1. Specificity

The specificity of the method was tested by analyzing six different blank plasma and urine samples obtained from healthy male and female volunteers to ensure the absence of endogenous compounds with the same retention time of PQ, DQ and EPQ.

2.7.2. Limit of detection (LOD) and lower limit of quantification (LLOQ)

LOD and LLOQ were determined by an empirical method that consists of analyzing a series of plasma and urine samples containing decreasing amounts of PQ and DQ [23]. The LOD was the lowest concentration that presented a CV that did not exceed 20% and the LLOQ the lowest concentration that presented a CV that did not exceed 15%. The LOD and LLOQ should still satisfy the predetermined acceptance criteria of qualification (retention time within 1% of standards and ion ratios within 20%).

2.7.3. Linearity

The study of linearity was performed by analyzing calibration plasma and urine samples in triplicate with the following concentrations for both PQ and DQ: 0.1, 1.0, 5.0, 10, 20 and 50 mg/l.

2.7.4. Recovery

The efficiency of the SPE method was evaluated through the recovery studies that were performed by preparing two sets of samples of each concentration. One of them (set A), consisting of three concentrations of the reduction PQ and DQ (0.3, 25 and 38 mg/l) was extracted using the method described in Section 2.4 without adding $NaBH_4$ (processed). The analyses were performed in five replicates for each concentration. The other one (set B), also consisted of five replicates of each concentration (0.3, 25 and 38 mg/l). However, the extract was spiked with standard solutions of reduced analytes before drying under nitrogen stream (unprocessed). To both sets (A and B), the reduced internal standard was added at concentration of 2 mg/l prior to the extraction of the matrix. The absolute recovery was evaluated by comparison of the mean response of extracted samples spiked before the extraction (processed) and the response of the extracted blank matrix to which analytes had been added at the same concentration just before the drying step (unprocessed). The unprocessed response represented 100% recovery.

2.7.5. Intra-day and inter-day precision

Precision, defined as the relative standard deviation or coefficient of variation (CV), was determined by intra- and inter-day repetitions. They were carried out by analyzing QC plasma and urine samples spiked with PQ and DQ in the concentration of 0.3, 25 and 38 mg/l (QC1, QC2 and QC3) for both analytes in three different days. The analyses were performed in five replicates for each day.

3. Results and discussion

Gas chromatographic analysis of quaternary ammonium compounds such as the herbicides PQ and DQ in biological samples is especially difficult due to their high polarity and low volatility. So, a previous conversion of these substances in thermally stable and volatile substances before GC injection is extremely necessary. In the present work, we evaluated the use of sodium borohydride to directly reduce PQ and DQ present in plasma and urine samples and make possible their analysis by GC–MS.

In order to obtain optimal conditions for the chemical reduction reaction in the sample medium, some parameters such as pH, time of reaction and quantity of sodium borohydride were evaluated. The temperature used for the reaction (60 °C) was the same used by Arys et al. [8]. Initially, it was known that alkaline medium is a favorable condition to occur the reduction reaction [8,19]. In our experiments, sample solution adjusted to pH 8.0 was found to be the optimal condition. A progressive decreasing in efficiency was observed in pH 9.0 and 10.0. This phenomenon can be associated with the chemical instability of PQ and DQ in higher values of pH [7,21]. The duration of the incubation has also a substantial influence in the reaction yield. The period of 10 min was found to be optimal since longer periods of reaction time (20, 30, 40 and 60 min) caused progressive loss of the products. No differences were observed in relation to the quantity of sodium borohydride used. Hence, the minimum quantity tested (10 mg) was found to be enough to reduce analytes efficiently in the highest concentration to be determined by the calibration curve (50 mg/l). In fact, when compared to the reduced standards, it was observed that the conversion rate was approximately 100% in the stated conditions (data not shown). Mass spectra in full scan mode of the reduction products of PQ, DQ and the internal standard EPQ are shown in Fig. 2.

In our first experiment of SPE using C18 cartridges containing 500 mg of stationary phase, it was observed that the sample solution often blocked their pores. On the other hand, the use of 360 mg cartridges allowed the use of plasma without pretreatment procedure of protein precipitation. Tests conducted with cartridges of 200 mg or mixed stationary phase (containing nonpolar C8 sorbent and a strong cation exchanger) did not provide good recoveries values for the analytes (less than 50%, data not shown).

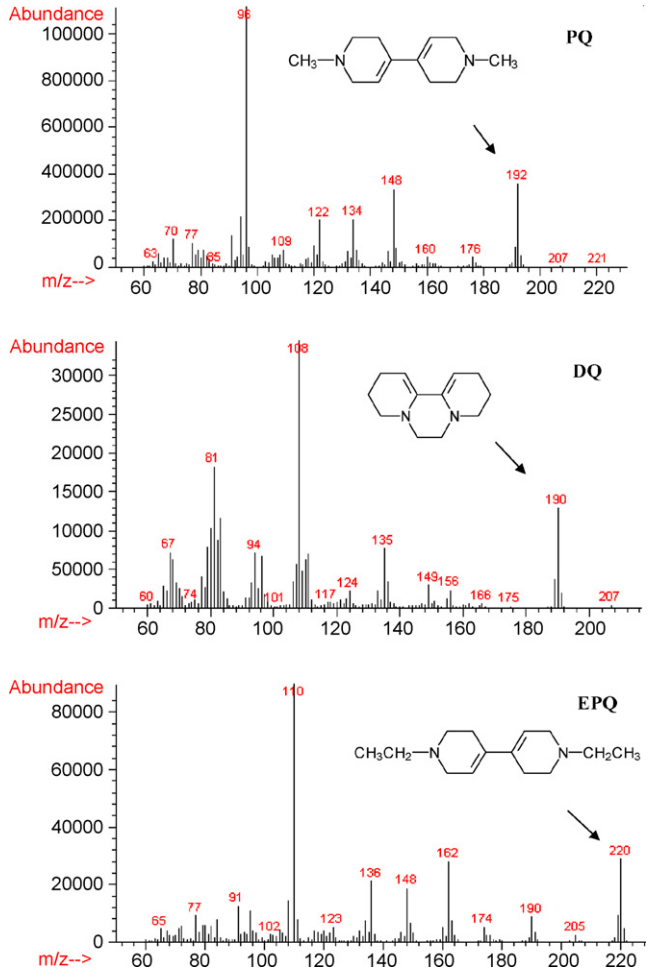

Fig. 2. Full scan mode mass spectra of the reduction products of paraquat (PQ), diquat (DQ) and ethyl paraquat (EPQ).

Previous works reported that PQ, DQ and even their reduction products adsorbs to glass surfaces [3,8]. So, during the entire sample preparation, silanized glassware and plastic test tubes were used.

The confidence parameters of the validated method (LOD, LLOQ, recovery, intra- and inter-assay precision) for the determination of PQ and DQ in plasma and urine samples are shown in Table 1.

In the calibration curve range (from 0.1 to 50.0 mg/l), the phenomenon of heteroscedasticity was present, probably due to the large range considered in the study of linearity (two orders of magnitude). In this case, larger deviations present at larger concentrations tend to influence the regression line more than smaller deviations associated with smaller concentrations [22]. Therefore, ordinary least square linear regression methods could result in large errors in the calculation of PQ and DQ concentrations, especially in low concentrations. By using weighted least squares linear regression, the sum of percentage of relative error (%RE) over the whole range indicated 'goodness of fit' in the evaluation of the effectiveness of the weighting factor used ($1/x^{1/2}$ for plasma and $1/y$ for urine). Other empirical weights such as $1/x^2$, $1/x$, $1/x^{1/2}$, and $1/y^2$ were also evaluated [22]. The weighted least squares linear regression equations and coefficients of correlation were: DQ: $y = 0.138x - 0.040$; $r = 0.981$ and PQ: $y = 0.200x + 0.201$; $r = 0.9811$ for plasma samples and DQ: $y = 0.109x + 0.017$; $r = 0.990$ and PQ: $y = 0.198x + 0.051$; $r = 0.997$ for urine samples, where y and x represent the relationship between the peak area ratio (compound/internal standard) and the corresponding calibration concentrations, respectively.

Table 1
Confidence parameters of the validated method for the determination of paraquat and diquat in plasma and urine samples

	Plasma		Urine	
	Paraquat	Diquat	Paraquat	Diquat
Mean recovery (%) ± SD				
QC1	68 ± 9	95 ± 12	83 ± 8	83 ± 12
QC2	64 ± 7	65 ± 10	86 ± 12	88 ± 7
QC3	98 ± 8	58 ± 9	88 ± 6	109 ± 9
LOD (mg/l)	0.05	0.05	0.05	0.05
LLOQ (mg/l)	0.1	0.1	0.1	0.1
Intra-day precision (CV%)				
QC1	9.3	10.1	9.8	11.6
QC2	14.1	6.3	9.2	10.5
QC3	11.0	8.0	4.6	13.1
Inter-day precision (CV%)				
QC1	6.0	11.0	14.9	11.7
QC2	16.8	7.2	7.3	5.7
QC3	12.6	17.2	5.2	8.9

LOD = Limit of detection; LLOQ = lower limit of quantification; QC1 = 0.3 mg/l; QC2 = 25 mg/l, QC3 = 38 mg/l.

For reliable quantitative purposes, an appropriate internal standard (IS) is necessary. The commercially available analogue of paraquat, ethyl paraquat, was evaluated. Ethyl paraquat behaves similarly to PQ and DQ during the entire course of sample preparation (including the reduction) and showed to be suitable for the GC–MS analysis. With the use of this IS, the precision varied slightly indicating that the reproducibility is acceptable over the studied concentration range (CV < 18%).

Fig. 3 shows chromatograms obtained with the practical use of this method to the analysis of urine samples (sample spiked with PQ and DQ at LLOQ levels, blank sample and sample deriving from an intoxication case). The analysis revealed the presence of PQ at a concentration of 32 mg/l in the urine from the intoxication case. Fig. 4 shows the chromatogram obtained with the analysis of a plasma sample deriving from another intoxicated patient. In this case, the analysis confirmed the presence of PQ at a concentration of 0.24 mg/l. The specificity of the method was demonstrated by analyzing six blank human plasma and urine samples. When the present method was used, no interference from the matrices was observed in the specific time detection windows of the compounds of interest.

The described method is time efficient and showed to be very practical. Over 20 plasma samples could be extracted, injected and analyzed by one analyst in approximately 8 h. This method can be readily utilized as an important tool to confirm the suspicion of paraquat and/or diquat poisoning and evaluate the extent of the intoxication in each case.

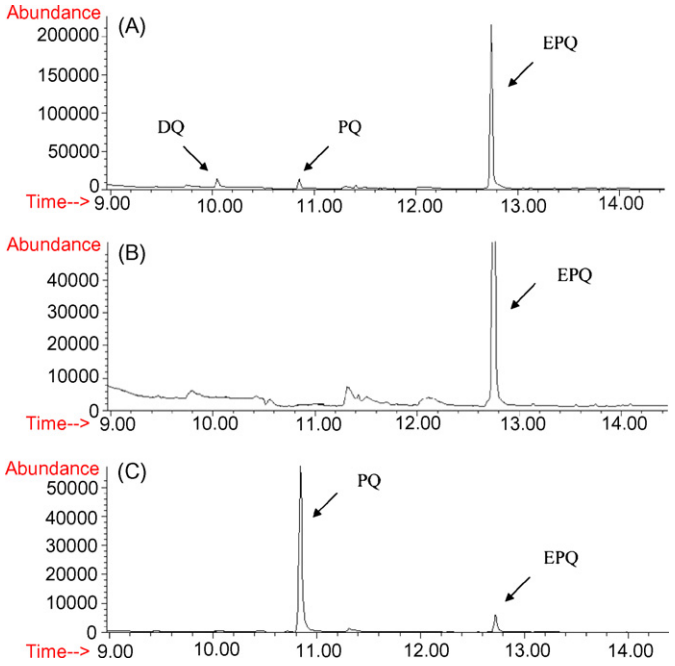

Fig. 3. (A) Chromatographic profile obtained with the SPE/GC–MS analysis of a spiked urine sample containing 0.1 mg/l (LLOQ) of both paraquat (PQ) and diquat (DQ); ethyl paraquat (EPQ). (B) Blank sample. (C) Urine sample deriving from an intoxicated patient. The analysis revealed the presence of PQ at a concentration of 32 mg/l.

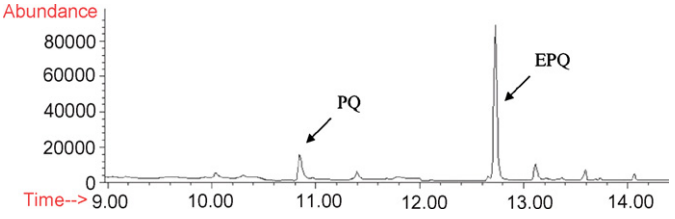

Fig. 4. Chromatographic profile obtained with the SPE/GC–MS analysis of a plasma sample deriving from an intoxicated patient. The analysis revealed the presence of paraquat (PQ) at a concentration of 0.3 mg/l. Ethyl paraquat (EPQ).

4. Conclusion

The conversion of quaternary ammonium compounds PQ and DQ into lipophilic and volatile derivatives with sodium borohydride was successfully applied to the gas chromatographic–mass spectrometric analysis of both plasma and urine samples. GC–MS was found to be specific, sensitive and selective enough for determining the concentrations to be expected in plasma and urine in cases of acute intoxication. Compared to the previous methods using gas chromatography [19,20], the present method showed to be simpler and faster since no deproteination procedure was necessary and the conditions of chemical reduction reaction of analytes were totally optimized.

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References

[1] S.M. Pond, in: L.R. Goldfrank, N.E. Flomenbaum, N.A. Lewin, R.S. Weisman, M.A. Howland, R.S. Hoffman (Eds.), Goldfrank's Toxicologic Emergencies, sixth ed., Appleton & Lange, Stamford, 1998, p. 1475.
[2] P.J. Taylor, P. Salm, P.I. Pillants, J. Anal. Toxicol. 25 (2001) 456.
[3] R. Castro, C. Prata, L. Oliveira, M.J. Carvalho, J. Santos, F. Carvalho, T. Morgado, Acta Med. Port. 18 (6) (2005) 423.
[4] M. Ito, Y. Hori, M. Fujisawa, A. Oda, S. Katsuyama, Y. Hirose, T. Yoshioka, Biol. Pharm. Bull. 28 (4) (2005) 725.
[5] A.R. González, J.F.N. González, M.L.M. Heras, C.M. Fernández, M.L.R. Errano, J.G. Pérez, An. Med. Int. 18 (2001) 208.
[6] D.J. Ecobichon, in: C.D. Klaassen (Ed.), Casarett & Doull's Toxicology: the Basic Science of Poisons, sixth ed., McGraw-Hill, New York, 2001, p. 763.
[7] A. Serra, F. Domingos, D.M. Prata, Acta Med. Port. 16 (2003) 25.
[8] K. Arys, V. Bocxlaer, K. Clauwaert, W. Lambert, M. Piette, C. Van Peteghem, A. De Leenheer, J. Anal. Toxicol. 24 (2000) 116.
[9] A.C. Moffat, M.D. Osselton, B. Widdop, Clarke's Analysis of Drugs and Posions, third ed., Pharmaceutical Press, London, 2004.
[10] J.M. Scherrmann, P. Houze, C. Bismuth, R. Bourdon, Hum. Toxicol. 6 (1987) 91.
[11] T. Kuo, D. Lin, R.H. Liu, F. Moriya, Y. Hashimoto, Forensic Sci. Int. 121 (2001) 134.
[12] C. Fuke, K. Ameno, S. Ameno, T. Kiriu, T. Shinohara, K. Sogo, I. Ijiri, J. Anal. Toxicol. 16 (1992) 214.
[13] M.M. Ariffin, R.A. Anderson, J. Chromatogr. B 842 (2006) 91.
[14] X.P. Lee, T. Kumazawa, M. Fujishiro, C. Hasegawa, T. Arinobu, H. Seno, A. Ishii, K. Sato, J. Mass. Spectrom. 39 (2004) 1147.
[15] P. Paixão, P. Costa, T. Bugalho, C. Fidalgo, L.M. Pereira, J. Chromatogr. B 775 (2002) 109.
[16] C. Fuke, T. Arao, Y. Morinaga, H. Takaesu, K. Ameno, T. Miyazaki, Legal Med. 4 (2002) 156.
[17] M.E. Koivunen, S.J. Gee, E.K. Park, K. Lee, M.B. Schenker, B.D. Hammock, Arch. Environ. Contam. Toxicol. 48 (2005) 184.
[18] E. Vinner, M. Stievenart, L. Humbert, D. Mathieu, M. Lhermitte, Biomed. Chromatogr. 15 (2001) 342.
[19] G.H. Draffan, D.L. Clare, D.L. Davies, G. Hawksworth, S. Murray, D.S. Davies, J. Chromatogr. 139 (1977) 311.
[20] S. Kawase, S. Kanno, S. Skai, J. Chromatogr. 283 (1984) 231.
[21] M. Ibáñez, Y. Picó, J. Mañes, J. Chromatogr. A 823 (1998) 137.
[22] A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, J. Chromatogr. B 774 (2002) 215.
[23] D.A. Armbruster, M.D. Tillman, L.M. Hubbs, Clin. Chem. 40 (1994) 1233.