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LC/MS/MS analysis of quaternary ammonium drugs and herbicides in whole blood☆

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Abstract

Quaternary ammonium drugs (atracurium, bretylium, edrophonium, ipratropium, mivacurium, neostigmine, pancuronium and rocuronium) and herbicides (difenzoquat, diquat and paraquat) in human whole blood were analysed by LC/MS/MS with positive electrospray ionisation (ESI), following extraction with Bond Elut® LRC-CBA cartridges. Internal standards were benzyldimethylphenylammonium chloride monohydrate and ethyl viologen for drug and herbicide analysis, respectively. Ion-pair chromatography used heptafluorobutyric acid (15 mM)–ammonium formate (20 mM) buffer adjusted to pH 3.30 with formic acid and a linear gradient from 5 to 90% methanol run over 18 min. Recoveries ranged from 79.7 to 105.1%, detection limits were between 3.6 and 20.4 ng/ml and the intra- and inter-day precisions were less than 18.6% at a concentration of 10 ng/ml. The method was applied to a case of accidental paraquat poisoning in which the concentration of paraquat in blood was 0.64 mg/l, which is within the range associated with fatal paraquat poisoning.
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1. Introduction

Quaternary ammonium (QA) compounds have been used as herbicides and anticholinergics drugs. Due to their toxicity, these compounds are found to be abused and encountered in many poisoning cases [1,2].

QA herbicides include two non-selective herbicides, namely paraquat and diquat and one highly selective herbicide, difenzoquat. For paraquat, an oral dose of only 1–2 g is fatal to most adults [3]. Paraquat is also believed to have deleterious effects on dopaminergic neurons and there is evidence suggesting that paraquat may be associated with the development of Parkinson's disease [4,5]. Diquat and difenzoquat poisoning are less common than paraquat poisoning, so that reports of human poisoning and animal experimental data for diquat and difenzoquat are less extensive than for paraquat.

QA drugs are normally used in adjunct to general anaesthesia to relax skeletal muscle. In the case of overdosing, it will cause prolonged muscle paralysis and changes in heart rate [6]. Some of these drugs have been known to be abused in equine sports [7].

Determination of QA compounds in various matrices by using gas-chromatography [8], liquid chromatography (LC) [9–11] and capillary electrophoresis [12] has been described. Ion-pairing liquid chromatography methods for determination of these compounds require additives to the mobile phase to improve the separation and resolution. Castro et al. [13] reported an LC/MS method for determination of QA herbicides by using volatile ion-pairing reagents; heptafluorobutyric acid (HFBA) has yielded satisfactory results.

In a forensic toxicology investigation, biological fluids such as whole blood, plasma or serum and urine are most commonly analysed. Screening of drugs in urine alone is not sufficient to support toxicological findings due to factors such as the amount of excretion and the time lapse after the intake of the drug. In cases like this, whole blood samples may be the sample of choice especially for deteriorated blood, when total separation of plasma and serum red cells is not possible [14]. In fatal cases,

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postmortem paraquat and diquat concentrations after 7 days of ingestion ranged from 0 to 0.44 mg/ml and 0–0.6 mg/ml, respectively [15]. In a suicidal case presented by Klys et al. [2], a post-mortem blood pancuronium level of 1.1 mg/ml was observed.

Reported data on the stability of QA drugs in post-mortem samples are limited. Farenc et al. reported that atracurium and its metabolite, laudanosine, were stable over 24 h in acidified plasma at room temperature [16]. Better stability of these compounds was displayed when stored at −30 °C. Pancuronium was found to be stable when stored at −20 °C in blood for up to seven months, however it degraded rapidly at 20 °C [17].

In the present work, we developed an efficient and simultaneous procedure for determining QA compounds in human whole blood by using weak cation exchange SPE and LC/MS/MS. The proposed method offers lower detection limits recovery and reproducibility when compared to previous methods [7,11] for most QA drugs in blood samples. For QA herbicides in blood analysis, this method displayed a slightly better repeatability when compared to the study done by Lee et al. [18], with additional data on difenzoquat.

2. Experimental

2.1. Materials

Paraquat dichloride, diquat dibromide and difenzoquat were obtained from Promochem (Middlesex, UK). Atracurium, bretylium besylate, edrophonium, ipratropium bromide and pancuronium were purchased from Sigma (St. Louis, MO, USA). Neostigmine bromide was from Roche (Hertfordshire, UK). Ethyl viologen and heptafluorobutyric acid were obtained from Aldrich (Milwaukee, WI, USA). Mivacurium chloride (Mivacron) was from Glaxo-Wellcome (Middlesex, UK). Rocuronium bromide (Esmeron) was from Ornagon (Oss, Holland). Benzyldimethylphenylammonium chloride monohydrate was obtained from Acros Organic (Geel, Belgium). Ammonium acetate and ammonium formate were purchased from Fluka (Buchs, Switzerland). Formic acid and methanol were of HPLC grade. Bond Elut® LRC-CBA cartridges were purchased from Varian (CA, USA).

2.2. Instrumentation

LC/MS/MS analyses of QA compounds were carried out using Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor HPLC system. Chromatographic separation was performed using an Atlantis® dC18 column 100 mm × 2.1 mm ID, particle size 5 μm (Waters, Milford, MA, USA).

2.3. Extraction procedure

Stock standard solutions (1 μg/ml) of QA compounds except for rocuronium bromide and mivacurium chloride were prepared by dissolving the dry chemical powder in methanol and stored at 4 °C in plastic bottles. Rocuronium bromide and mivacurium chloride were prepared by diluting the formulations to 1 μg/ml with methanol.

SPE with a weak cation exchanger (Bond Elut® LRC-CBA) has been used to extract QA compounds. The p*K*a of this cation exchanger is 4.8, thus adjustment of the eluent pH to below 2.8 facilitates the recovery of quaternary amines.

For QA herbicides, blood samples were prepared by diluting 1 ml of whole blood with 4 ml phosphate buffer (pH 6.0). Then the blood samples were vortex mixed and centrifuged for at 1121 × *g* for 10 min. Extraction cartridges were conditioned with 3 ml methanol followed by 3 ml phosphate buffer (pH 6.0). Each sample was loaded through a cartridge. It was then washed with 3 ml of phosphate buffer (pH 6.0) followed by 3 ml of methanol. The cartridge was eluted with 1 ml of 1.0 M HCl/methanol (70:30, v/v). The eluate was evaporated to dryness under nitrogen at 35 °C and the residue was dissolved in 1 ml of the initial HPLC mobile phase. The content was transferred to an autosampler vial for LC/MS/MS analysis.

Extractions for QA drugs were prepared in the same manner except for using ammonium acetate solution (pH 8.0) as a buffer. Except for atracurium, in the wash process, the cartridge was washed with 2 times of 3 ml buffer and no methanol was used since it will elute atracurium.

2.4. LC/MS/MS analysis

LC was carried out using HFBA (15 mM)–ammonium formate buffer (20 mM) adjusted to pH 3.30 by formic acid as solvent A, and 100% methanol as solvent B. The elution program consisted of a linear gradient from 5 to 90% of solvent B within 18 min. Twenty microlitres of samples were injected onto the Atlantis® dC18 column at 30 °C, operated at a flow rate of 0.2 ml/min.

Ionisation of analytes was performed using electrospray ionization (ESI) in the positive mode. A capillary temperature of 275 °C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 15 and 5 arbitrary LCQ units, respectively. QA compounds were analysed with MS parameters selected in Table 1 using product-ion scan MS/MS. Chromatograms of quantitation ions for all QA compounds are shown in Fig. 1.

2.5. Matrix effect study

This study was conducted to assess the interference caused by blood matrix during extraction. Five replicates of 1 ml blank blood were spiked with 50 ng of QA compounds and another five of standard line were prepared by spiking with the same concentration of QA compounds in 4 ml of loading buffer. All of the samples were vortexed, centrifuged and extracted using the described SPE procedure. One hundred nanograms of internal standard was added after the extraction. The percentage of matrix effect was calculated according to Eq. (1), where *a* and *b* are the peak area ratios of the analyte to internal standard in neat solution and blood extract, respectively.

$$\text{Percentage of matrix effect} = \frac{b}{a} \times 100\% \quad (1)$$

Table 1
MS parameters for the QA compounds by using LC–MS–MS in the positive ESI mode

QA compound	Precursor ion (m/z)	Product ions (m/z)	Relative Intensity	Collision energy (eV)
Atracurium	358	206.1	100.0	30
		207.1	8.40	
		327	5.30	
Bretylium	242	169.0	100.0	32
		71.9	20.2	
		224.9	17.8	
Diquat	183	157.1	100.0	39
		164.8	47.8	
		149.8	10.3	
Difenzoquat	249	208.1	100.0	47
		193.2	87.5	
		131.2	38.34	
Edrophonium	166	138.0	100.0	34
		137.0	32.3	
		148.8	12.7	
Ipratropium	332	166.1	100.0	35
		290.1	8.9	
		167.7	7.6	
Mivacurium	514	357.1	100.0	33
		428.2	68.3	
		342.3	44.5	
Neostigmine	223	208.0	100.0	40
		177.0	63.9	
		209.0	118	
Pancuronium	286	236.6	100.0	24
		100.0	9.3	
		206.8	12.3	
Paraquat	185	171.1	100.0	35
		166.8	52.3	
		166.0	12.3	
Rocuronium	529	487.2	100.0	32
		488.2	25.5	
		358.2	3.55	
Benzyldimethylphenyl-ammonium (Internal standard for drugs)	212	120.0	100.0	30
		121.1	40.2	
		134.0	37.4	
Ethyl Viologen (Internal standard for herbicides)	213	185.1	100.0	37
		195.0	86.5	
		194.2	3.2	

A value of <100% indicates analyte ion supression whereas >100% indicates analyte ion enrichment due to the matrix interference.

2.6. Recovery study

QA compounds each at 50 and 100 ng/ml were spiked in 1 ml of a drug free human blood sample ($n = 6$) were processed using the SPE procedure. 100 ng of internal standard (ethyl viologen for herbicides and benzyldimethylphenylammonium chloride monohydrate for drugs) was then added after the SPE. The recovery was determined by comparing peak area ratios obtained from QA ions of the compounds to that of the internal standard versus the peak area ratios of the same concentration of pure standards and internal standard spiked in extracted blood blank matrix.

2.7. Linearity and determination of the LOD and LOQ

The regression equations for QA compounds extracted from human whole blood were subjected to linear regression analysis of peak area ratios to internal standard (100 ng) against the spiked analytes (5, 10, 25, 50, 100 and 200 ng/ml). The LODs were calculated using Eqs. (2) and (3), where Y_B is the intercept, S_B is the standard error of the regression line and m is the gradient [19].

$$Y_{LOD} = Y_B + 3S_B \quad (2)$$

$$LOD = \frac{Y_{LOD} - Y_B}{m} \quad (3)$$

LOQ was calculated using the same method except that 10 times the standard error of the regression line was used (Eqs. (4) and (5)).

$$Y_{LOQ} = Y_B + 10S_B \quad (4)$$

$$LOQ = \frac{Y_{LOQ} - Y_B}{m} \quad (5)$$

2.8. Intra- and inter-day precisions

The intra- and inter-day precisions were determined by analysing 10, 50 and 100 ng/ml extracted spiked analytes in human whole blood six times in the same day and repeating this process on 6 separate days.

3. Results and discussion

3.1. Optimisation of the mobile phase

Initially, a mobile phase consisting of 15 mM HFBA (solvent A) and 100% methanol (solvent B) were used to separate QA compounds. HFBA was used as ion-pairing reagent to improve the separation and retention of QA herbicides [18]. Methanol was recommended over acetonitrile as an organic modifier to avoid a white film deposit on the ESI source [13].

However, when using this mobile phase, peaks obtained from paraquat and diquat separations were very poor and the detections were less sensitive. Therefore, experiments were carried out by adding 20 mM ammonium formate or 20 mM ammonium acetate to solvent A and adjusted to pH 3.30 with formic acid. Ammonium acetate and formic acid were recommended to improve the ionisation in the MS [20]. However, the ammonium formate mobile phase was found to be more sensitive and therefore was chosen for the determination and confirmation of QA compounds in LC/MS/MS.

3.2. Matrix effect

From the matrix effect results (Table 2), blood matrix components are found to cause interference with solid-phase extraction of the analytes especially for edrophonium and paraquat. Therefore, all standard solutions were prepared in whole blood matrix to mimic the actual sample conditions.

3.3. Quality parameters

The regression analysis between peak area ratios of QA compounds over the internal standard showed a good linearity in the

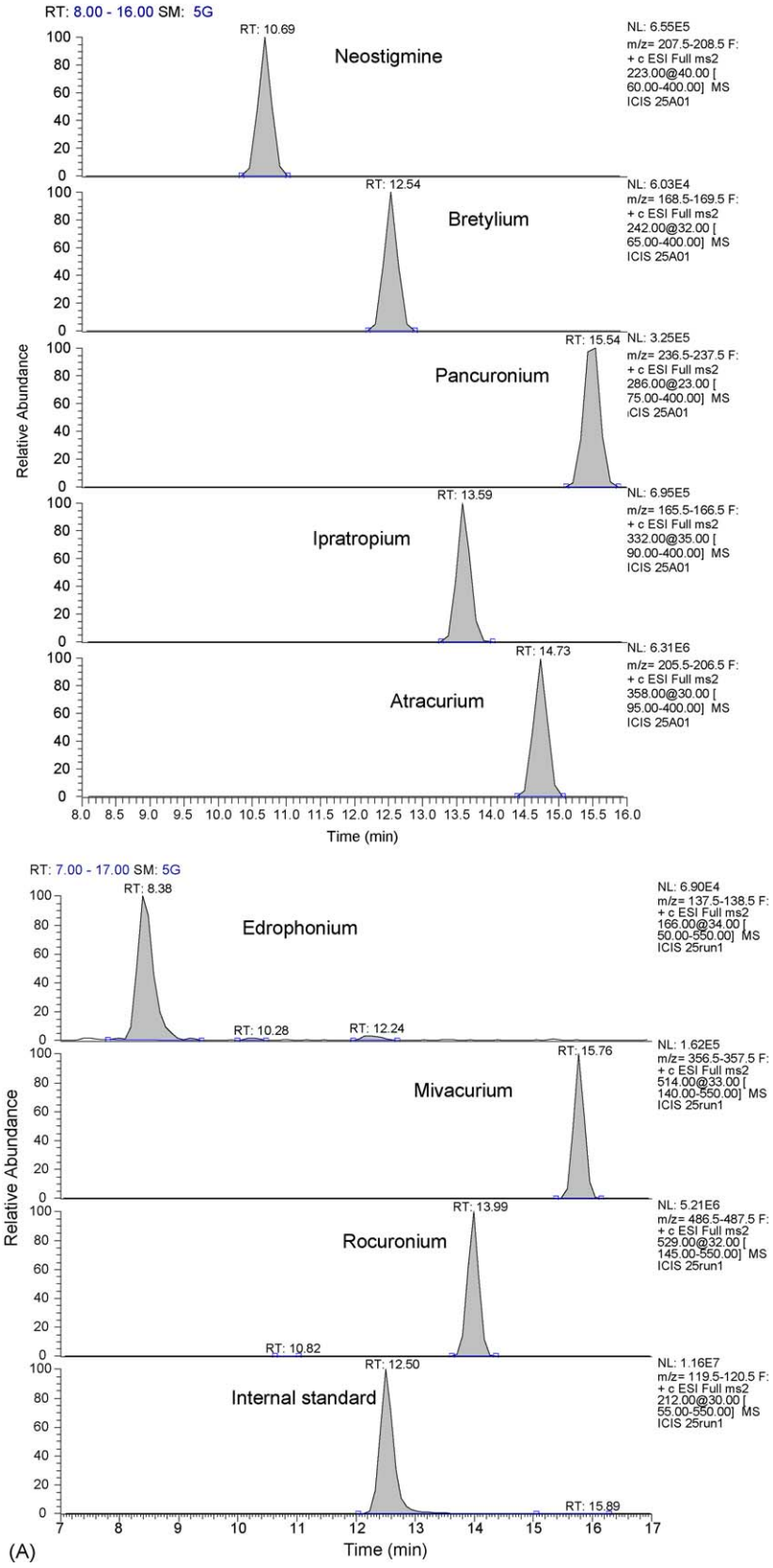

Fig. 1. (A) Chromatograms of the quantitation ions of QA drugs in blank blood spiked at a concentration of 25 ng/ml. (B) Chromatograms of the quantitation ions of QA herbicides in blank blood spiked at a concentration of 50 ng/ml.

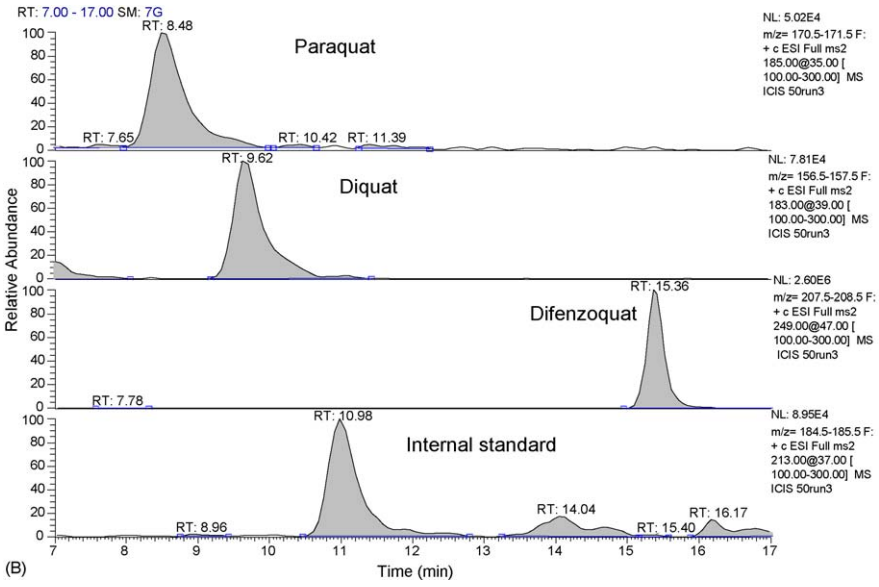

Fig. 1. (*Continued*).

Table 2
Blood matrix effect on sample extraction

QA compound	% Matrix effect
Atracurium	105.9
Bretylium	91.1
Diquat	101.2
Difenzoquat	84.2
Edrophonium	65.0
Ipratropium	78.8
Mivacurium	105.9
Neostigmine	85.5
Pancuronium	112.7
Paraquat	144.7
Rocuronium	86.6

range of 5–200 ng/ml. The correlation coefficients (r) for calibration curves were greater than 0.99. LODs and LOQs for all QA compounds are shown in Table 3.

Recoveries of QA compounds in human whole blood samples are presented in Table 4. The recoveries of all quaternary ammonium compounds ranged from 79.7 to 105.1%. The average recoveries and 100% value (null hypothesis) were not significantly different according to the calculated t-values, which were lower than the tabulated t-value of 2.5706 for $P = 0.05$.

Intra- and inter-day precisions results show that good precision can be obtained with the method described above. Relative standard deviations % (R.S.D.s) from intra- and inter-day precision ranging from 0.7 to 18.6% are shown in Table 5. At the LOQ level, 20% for precision and accuracy is acceptable [21].

4. Application in real sample

A 66-year-old man died 4 days after suffering from poisoning despite active therapy. A few days before he died, the deceased accidentally brought home three mineral water bottles containing Dextreone weedkiller from his workplace and placed them in his grocery bag. A friend, who helped him with his groceries, put the bottles in the fridge, believing them to be blackcurrant juice. Post mortem examination revealed jaundice, bilateral pleural

Table 3
Calibration, LOD and LOQ for QA compounds in human whole blood obtained using the proposed method

QA compound	r^2	b	a	LOD (ng/ml)	LOQ (ng/ml)
Atracurium	0.9994	0.0385	−0.0691	5.8	19.3
Bretylium	0.9997	0.0020	−0.0004	4.1	13.7
Diquat	0.9985	0.0038	0.0092	5.0	16.7
Difenzoquat	0.9997	0.0186	−0.0012	4.1	13.7
Edrophonium	0.9989	0.0003	0.0006	10.8	35.9
Ipratropium	0.9994	0.0115	−0.0148	5.9	19.5
Mivacurium	0.9927	0.0008	−0.0063	20.4	68.0
Neostigmine	0.9998	0.0075	−0.0059	3.6	12.1
Pancuronium	0.9981	0.0079	−0.0325	10.4	34.5
Paraquat	0.9984	0.0046	−0.0140	11.1	36.9
Rocuronium	0.9996	0.0180	−0.0264	4.9	16.2

Typical equation is $y = bx + a$, where x is the analyte concentration and y is the peak area ratio of analyte and internal standard. The calibration curves were determined over the range 5–200 ng/ml.

Table 4
Recoveries for QA compounds in human whole blood obtained from the proposed method

QA compound	50 ng/ml		100 ng/ml	
	Mean recovery (%)	R.S.D. of recovery (%)	Mean recovery (%)	R.S.D. of recovery (%)
Atracurium	105.1	3.8	94.3	3.1
Bretylium	79.7	17.5	90.7	15.7
Diquat	99.7	10.5	98.3	2.5
Difenzoquat	99.9	9.4	100.4	5.9
Edrophonium	95.2	3.1	90.2	6.6
Ipratropium	103.1	4.9	96.1	6.5
Mivacurium	93.1	8.1	92	3.8
Neostigmine	101.5	5.8	90.6	4.4
Pancuronium	94.3	7.6	96.5	2.1
Paraquat	84.9	2.8	89.7	5.7
Rocuronium	102.4	4.7	97.2	6.6

effusions, marked pulmonary oedema and mottling of the kidney indicative of death as the result of multiorgan failure. In addition there was an ulcerative oesophagitis and microscopic examination of the lungs revealed changes in keeping with ingestion of paraquat and its toxic effects. A blood sample was taken and the proposed methodology was applied to screening and confirmation of the paraquat level.

Quantification of paraquat was performed using a duplicate set of calibrators containing paraquat each at levels of 0, 10, 25, 50, 100, 200 and 300 ng/ml containing 200 ng/ml ethyl viologen as an internal standard. Fig. 2 shows the extracted ion chromatogram and product ion spectrum of paraquat standard. Both the retention time and the product ion spectrum of paraquat in the sample matched well with those of the authentic standard. This positive identification reveals the concentration of paraquat at 0.64 mg/l.

Table 5
Intra- and inter-day precision for QA compounds in human whole blood samples obtained using the proposed method

QA compound	Amount added (ng/ml)	Intra-day		Inter-day	
		Amount detected (ng/ml)	R.S.D. (%)	Amount detected (ng/ml)	R.S.D. (%)
Atracurium	10	10.4 ± 1.6	15.4	8.4 ± 1.5	17.5
	50	49.2 ± 3.6	7.2	47.6 ± 4.1	8.5
	100	96.3 ± 10.9	11.3	96.3 ± 7.2	7.5
Bretylium	10	10.4 ± 1.4	13.2	9.7 ± 1.3	13.8
	50	50.0 ± 4.6	9.1	47.7 ± 4.1	4.7
	100	100.0 ± 8.5	8.5	98.9 ± 6.0	6.1
Diquat	10	8.7 ± 1.6	17.9	10.7 ± 0.9	11.9
	50	46.0 ± 8.2	17.8	47.0 ± 0.3	0.7
	100	91.5 ± 5.5	6.0	96.0 ± 1.5	1.6
Difenzoquat	10	7.6 ± 1.1	14.3	10.7 ± 1.8	7.8
	50	47.1 ± 1.84	3.9	50.9 ± 2.4	4.8
	100	98.6 ± 3.1	3.1	102.2 ± 3.2	3.1
Edrophonium	10	10.1 ± 0.9	8.8	9.5 ± 1.4	14.2
	50	44.4 ± 4.8	10.7	51.1 ± 2.2	4.7
	100	94.2 ± 4.3	4.6	98.1 ± 5.3	5.4
Ipratropium	10	8.9 ± 0.7	8.2	9.4 ± 1.8	18.6
	50	47.7 ± 3.4	7.1	41.6 ± 2.9	6.9
	100	98.9 ± 7.1	7.2	98.2 ± 7.3	7.5
Mivacurium	10	10.1 ± 1.9	18.6	9.3 ± 1.7	18.4
	50	49.1 ± 4.4	9.0	48.6 ± 6.9	14.1
	100	101.8 ± 13.0	12.7	91.8 ± 11.7	12.8
Neostigmine	10	10.2 ± 1.3	12.8	9.7 ± 0.5	5.0
	50	49.9 ± 3.2	6.4	48.8 ± 2.8	5.7
	100	95.6 ± 5.6	5.9	96.6 ± 3.5	3.6
Pancuronium	10	8.5 ± 0.6	6.7	9.0 ± 1.3	14.3
	50	47.3 ± 0.94	2.0	49.0 ± 3.0	6.1
	100	94.5 ± 2.6	2.8	95.7 ± 7.5	7.8
Paraquat	10	10.1 ± 1.0	9.4	9.8 ± 1.5	15.2
	50	48.5 ± 4.8	9.8	49.9 ± 2.3	4.6
	100	103.6 ± 9.1	8.7	102.6 ± 3.3	3.2
Rocuronium	10	9.8 ± 1.3	13.4	10.0 ± .5	4.8
	50	49.7 ± 2.2	4.4	41.5 ± 2.8	6.7
	100	99.1 ± 3.5	3.6	99.5 ± 7.4	7.4

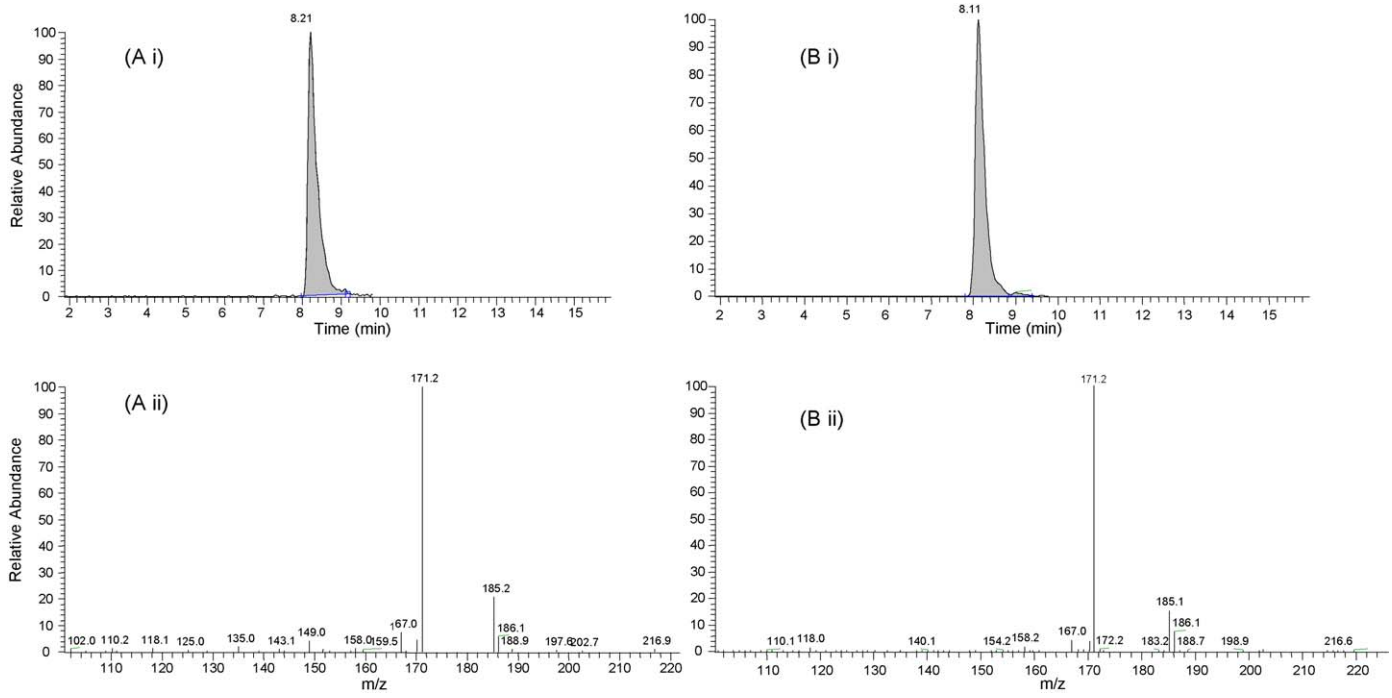

Fig. 2. (A) (i) extracted ion chromatogram of *m/z* 171 and (ii) product ion scan of paraquat *m/z* 185 $[M-H]^+$ for the poisoning sample; (B) (i) ion chromatogram of *m/z* 171 and (ii) product ion scan of paraquat *m/z* 185 $[M-H]^+$ of 300 ng/ml paraquat standard.

5. Conclusion

The proposed solid-phase extraction procedure and LC/MS/MS method provided an accurate assay for the determination of quaternary ammonium drugs and herbicides in human whole blood. Recovery and precision studies successfully quantified QA compounds in spiked human whole blood. The procedure has also been successfully applied to a fatal death case involving paraquat poisoning.

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