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Quantitative Analysis of the Aminosteroidal Muscle Relaxant Vecuronium in Rat Liver and Kidneys using Ion-pairing and Liquid Chromatography-Tandem Mass Spectrometry

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Abstract : *Muscle relaxants are occasionally encountered in forensic toxicological cases for identification and quantitation. Due to unavailability of human samples, the analysis of the muscle relaxant vecuronium was done using post-mortem rat visceral samples. Rats were injected with bolus overdose of the drug and the post mortem samples were subject to ion-pair extraction with metanil yellow dye. Method for identification of the drug by LC-MS-MS was established first with secondary standard aqueous vecuronium solutions and then applied on the questioned samples. Quantitation was done to find the quantity of the drug in liver and kidney samples.*

Keywords : Aminosteroid, Muscle relaxant Vecuronium, Liquid Chromatography-Tandem Mass Spectrometry.

Introduction

Muscle relaxants, better known as neuro-muscular-blocking drugs, are a class of drugs that block neuromuscular transmission at neuromuscular junction which causes paralysis of the affected skeletal muscles. Of the two classes, non-depolarizing neuromuscular agents work without depolarizing the motor end plate. They act as reversible competitive inhibitors and lead to a decrease in binding of acetylcholine at the receptors. The quaternary ammonium muscle relaxants belong to this class (Barash, *et al.,* 2009).

In clinical practice (Stuart and Cullen, 1959), neuromuscular blocking drugs (Raghavendra, 2002) are used in adjunction to anesthetic agents to cause relaxation of skeletal muscles for two main reasons (1) to permit incubation of the trachea by paralyzing the vocal chords, and (2) to inhibit spontaneous ventilation, laryngospasm, coughing, bucking, etc during surgery (Eckenhoff, 1959). Mechanical ventilation is made available to the patient so as to maintain respiration as these drugs may also paralyze the diaphragm at the therapeutic dose required (Dripps, 1959). Despite taking precautions, neuromuscular blocking drugs have been reported to have inadvertently caused deaths of patients by accident (Newsletters, Nurse Advise, 2006). These drugs have also been reported to have been used for execution of criminals by lethal injection (Huihui, *et al.,* 2009), homicides (Agentur, 2008) (Johnstone, 2011) (The Japan Times Weekly, 2004) and suicides (Sheikh, 2001). Hence, these drugs are forensically important and their presence in human tissues and body fluids has been investigated by toxicologists.

Vecuronium is a muscle relaxant in the category of non–depolarizing neuromuscular blocking agents. Its structure is derived from an amino-steroid and it is a mono-quaternary ammonium drug. It is a single-isomer preparation (McKenzie, 2000) (Figure 1a).

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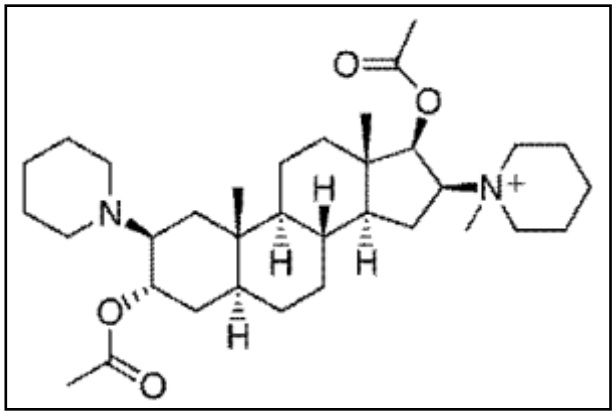

Fig. 1 (a) Vecuronium bromide

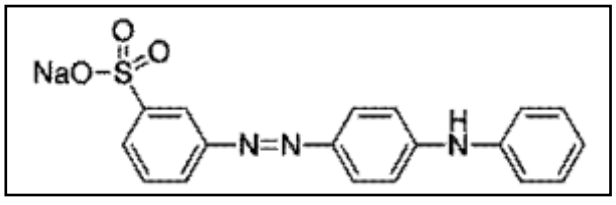

Fig.1 (b) Metanil Yellow

Vecuronium bromide acts as a paralyzing agent by inhibiting depolarisation as it blocks acetylcholine from binding to receptors on motor endplate. Its onset time is 1.5-2 min and duration of paralysis is 20-30 min. Vecuronium is known to be a potentially fatal drug that causes respiratory failure and in overdose, the effects are enhanced (Ohata, *et al.,* 2005) Cronnelly *et al.* (1983) have mentioned that in rats over 60% of vecuronium may be found unchanged in hepatic metabolism and the drug undergoes a non-renal elimination.

In this short communication, we aim to present simulated death case by intra-peritoneal injection of a non-depolarizing neuromuscular blocking drug vecuronium to rats and analysis of post mortem liver and kidneys by LC-MS-MS.

Materials and Methods

Experimental animal

Certified drug-free healthy female albino (Wistar sp.) rats were purchased and dissected with permission from Institutional Animal Ethical Committee, Department of Zoology, University of Allahabad.

Reagents and apparatus

All solvents were HPLC grade and purchased from different manufacturers. Chloroform from Qualigens; Dichloromethane, 1-Butanol, Glacial acetic acid, Hydrochloric acid from Rankem. Potassium iodide was also purchased from Rankem. Sodium chloride, Potassium dihydrogen phosphate, Potassium hydroxide and Metanil yellow dye were purchased from Merck. Injectable form of Vecuronium bromide-NEOVEC was purchased from Apollo Pharmacy and was used as a secondary standard in the study.

Glass bottles were used for preservation of viscera and polypropylene centrifugation tubes with screw caps were used for extraction purposes. All glassware were washed and cleaned with chromic acid solution before and after use. All polypropylene apparatus used were discarded after use.

Drug for injection

Vecuronium bromide injection was prepared by reconstituting 4 mg of the drug powder in 2 ml of sterile water supplied with the pack (Table 1).

Standards and control

0.015% solution of metanil yellow dye was prepared in water and was used as standard aqueous dye solution. The stock solutions were prepared in 20 mM phosphate buffer (pH

Table 1 Drug details of Rat.

1.	Room temperature	20 ± 1°C
2.	Weight of vecuronium bromide powder (NEOVEC) taken	4 mg
3.	Volume of sterile water taken to reconstitute the drug to make drug solution	2 ml
4.	Volume of drug solution administered to one rat by intraperitoneal injection	1 ml
5.	Drug administered to R1, R2, R3 and R4	2000 µg

4.8) and stored at -20°C in the dark in polypropylene vials. They were used as secondary standards.

For calibrating the instrument and determining the linearity of the method, 1ppm stock solution was prepared and diluted to concentrations 200, 150, 105, 52.5, 26.25, 10.11, 5.06 and 2.53 ppb to prepare secondary standard drug solutions.

Liver and kidneys of a chloroform-anesthetized and dissected (without injecting the drug) certified drug-free rat were homogenized and taken as blank samples.

For determination of matrix interference, 40 ppm stock solution was prepared and diluted to concentrations 20, 10, 5, 2.5 and 0.5 ppb. These were divided into two batches: the first batch samples were used as unextracted aqueous drug samples and the second batch samples were spiked in 1 ml blank viscera each and extracted thereafter so as to prepare extracted spiked samples.

Injection and postmortem viscera collection

Five rats were taken and labelled R1, R2, R3, R4 and R5 respectively. R1 was weighed and anesthetized with chloroform vapours at time T_0. After T_1 min, the anaesthetized rat was administered overdose of vecuronium by intra-peritoneal injection (LD_{50} = 2.630 mg/kg) (Rinsho, 1986) of 1 ml each at concentration 2.0 mg/ml (Table 2).

The behavioural response of the rat was observed and death was confirmed by cessation of all muscular activities and stoppage of the heart beat after T_2 min. After T_3 min, the rat was dissected and hole liver and both kidneys were isolated and collected (Millo, *et al.,* 2008) after T_4 min in clean and sterilized glass bottles with air tight screw caps (Table 3).

Note:

*** Time is calculated for the duration between two consecutive activities.**

For example In R1, anaesthesia was given at 13:00 hrs, drug injection was given at 13:07 hrs, death occurred at 13:12 hrs, autopsy was performed at 13:27 hrs and viscera were collected at 13:34 hrs. Likewise it is calculated for R2, R3 and R4. Preservation was done in saturated sodium chloride solution at -20°C in dark till analysis. Likewise was done to R2, R3 and R4. R5 was anesthetized and dissected without injection of the drug for collecting liver and kidneys for preparing control samples.

Table 2 Weight of Rat.

Sl. No.	Rat ID	Wt. of rat (kg)	Wt. of drug injected (mg)
1.	R1	0.143	2.0
2.	R2	0.169	2.0
3.	R3	0.189	2.0
4.	R4	0.187	2.0

Table 3 Time details of Rat.

Sl. No.	Rat ID	Anesthesia given to rat at time T_0 min*	Drug injection given after time T_1 min*	Death of rat after time T_2 min*	Dissection performed after T_3 min*	Viscera of rat collected after T_4 min*
1.	R1	0	7.0	5.0	15.0	7.0
2.	R2	0	3.0	15.0	15.0	7.0
3.	R3	0	5.0	7.0	17.0	5.0
4.	R4	0	7.0	4.0	17.0	5.0
5.	R5 BLANK#	0	-	-	21.0	8.0

Ion-pair extraction

The method suggested by Vorce *et al.* (2008) was modified and metanil yellow (Fig. 1b) was selected as the dye for extraction (in place of potassium iodide) as it is highly soluble in water and insoluble in dichloromethane (Maiti and Sethi, 1979). Tissue homogenates (1 g) from rat liver and kidneys were prepared by the addition of 1 ml of phosphate buffer (pH=4.8). 1 ml of standard aqueous metanil yellow dye solution was added to each sample and vortex mixed for 30 s and allowed to equilibrate at room temperature for 15 min. 5 ml of dichloromethane was added to each sample. The samples were vortex mixed for 5 min and then rotor mixed for another 5 min. The contents of the tube were then centrifuged at 3000 rpm for 10 min. The lower organic layer volume was transferred to clean polypropylene tube. Rat samples from liver (R1-L, R2-L, R3-L and R4-L) and kidney (R1-K, R2-K, R3-K and R4-K) were made upto 100 ml and 10 ml with dichloromethane respectively. The organic layer containing drug-dye complex was dried under nitrogen at 45 °C in a nitrogen concentrator. The dried complex was reconstituted in 2 ml of 0.1% aqueous formic acid and acetonitrile (1:1). The solution was then vortex mixed for 5 min, sonicated for 5 min and centrifuges at 15000 rpm at 27 °C.

Five spiked samples at concentrations 20, 10, 5, 2.5 and 0.5 ppb were also extracted using the above procedure.

Reproducibility of the extraction method was demonstrated by taking liver and kidney samples of four rats of different weights-all injected with the same concentration of the drug. Injection, dissection, collection and preservation, extraction and analyses in all four cases were done under identical conditions and the results obtained were comparable. Dissection of rats was performed at temperature 20± 1°C and all other experiments at 25 ± 2°C.

Liquid Chromatography Tandem Mass Spectrometry(LC-MS-MS)

An Agilent 6410 Triple Quad LC-MS-MS which incorporates an atmospheric pressure ionization (API) ion source, was used for the quantitative analysis of VEC. The LC system consisted of a vacuum degasser, binary pump, autosampler, and thermostatted column compartment. 5µL injections were made onto an Agilent ZORBAX 5 micron Eclipse XDB-C8 column, 4.6mm x 50-mm that was held at 45 °C.

The mobile phase consisted of two solvents: (a) 0.3% aqueous formic acid (95%) (b) 0.3% formic acid in acetonitrile (5%) A mobile phase gradient was used with a flow rate of 0.400 ml/min. The composition was held for a total run time of 10.0 min. An electrospray interface was used with nitrogen drying gas (12.0 l/min at 350 °C.

Base peak in the mass spectrum of vecuronium was found at m/z = 557.3. The most intense ions of the mass spectrum were found at 100.0, 381.1, 356.2, 338.1, 398.2, 497.3 and 416.1 For quantitative analysis, the ions m/z 100.0, and 356.2 were monitored. Fig. 2 gives the chromatogram in MS-MS-MS mode.

The working conditions for the LC-MS-MS method were established with secondary

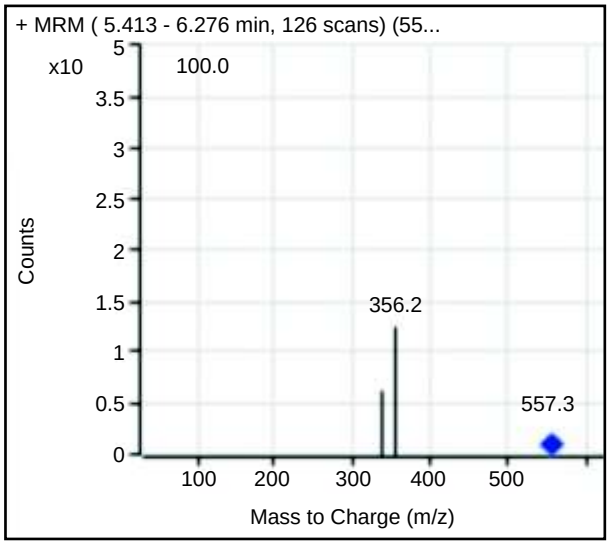

Fig. 2 Mass spectrum of vecuronium.

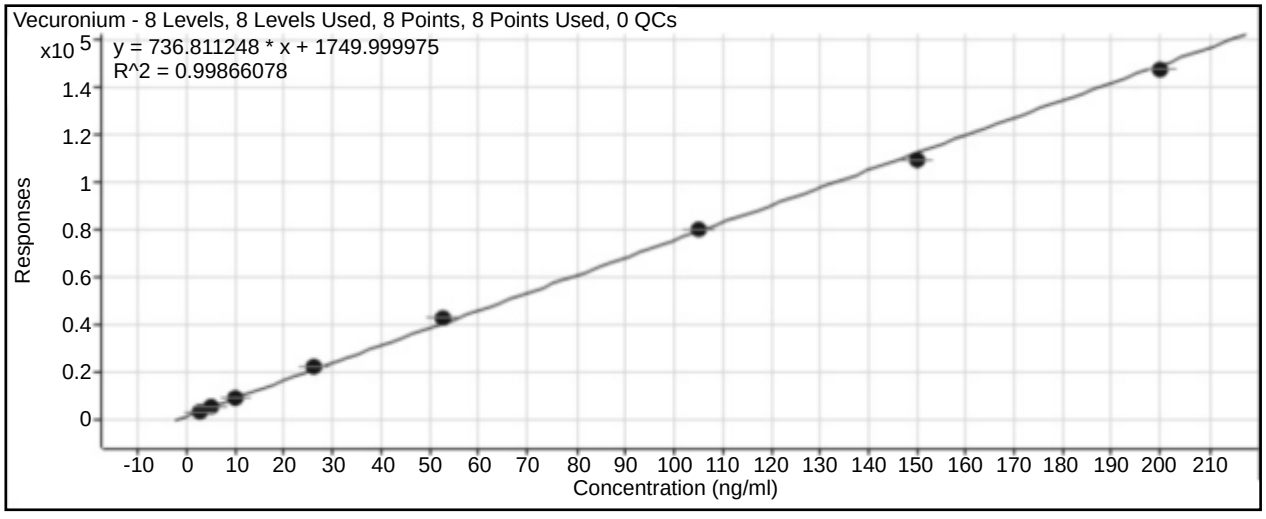

Fig. 3 Calibration Curve

Table 4 Result of LC-MS-MS Quantitative analysis of rat liver samples.

Sl. No.	Sample name	Wt. of sample (g)	Wt. of drug injected (mg)	Drug Conc. determined by LC-MS-MS (ppb)*	Wt. of drug determined in liver sample (mg) #	% of Drug found in rat liver sample
1.	R1-L	4.8920	2.0	7.0784	1.77	85%
2.	R2-L	6.5860	2.0	3.0077	0.75	37.5%
3.	R3-L	8.4640	2.0	6.4961	1.77	85%
4.	R4-L	7.3080	2.0	4.7837	1.20	60%

Table 5 Result of LC-MS-MS Quantitative analysis of rat kidney samples.

Sl. No.	Sample name	Wt. of sample (g)	Wt. of drug injected (mg)	Drug Conc. determined by LC-MS-MS (ppb)*	Wt. of drug determined in kidney sample (mg) #	% of drug found in rat kidney sample
1.	R1-K	0.9250	2.0	28.5908	0.0572	2.86 %
2.	R2-K	1.5100	2.0	39.5455	0.0791	4.00 %
3.	R3-K	1.5400	2.0	41.0153	0.0820	4.10 %
4.	R4-K	1.5380	2.0	34.2673	0.0685	3.43 %

* Actual concentration determined by LC-MS-MS analysis.

Weight of drug determined after considering the dilution factors, etc. and calculation.

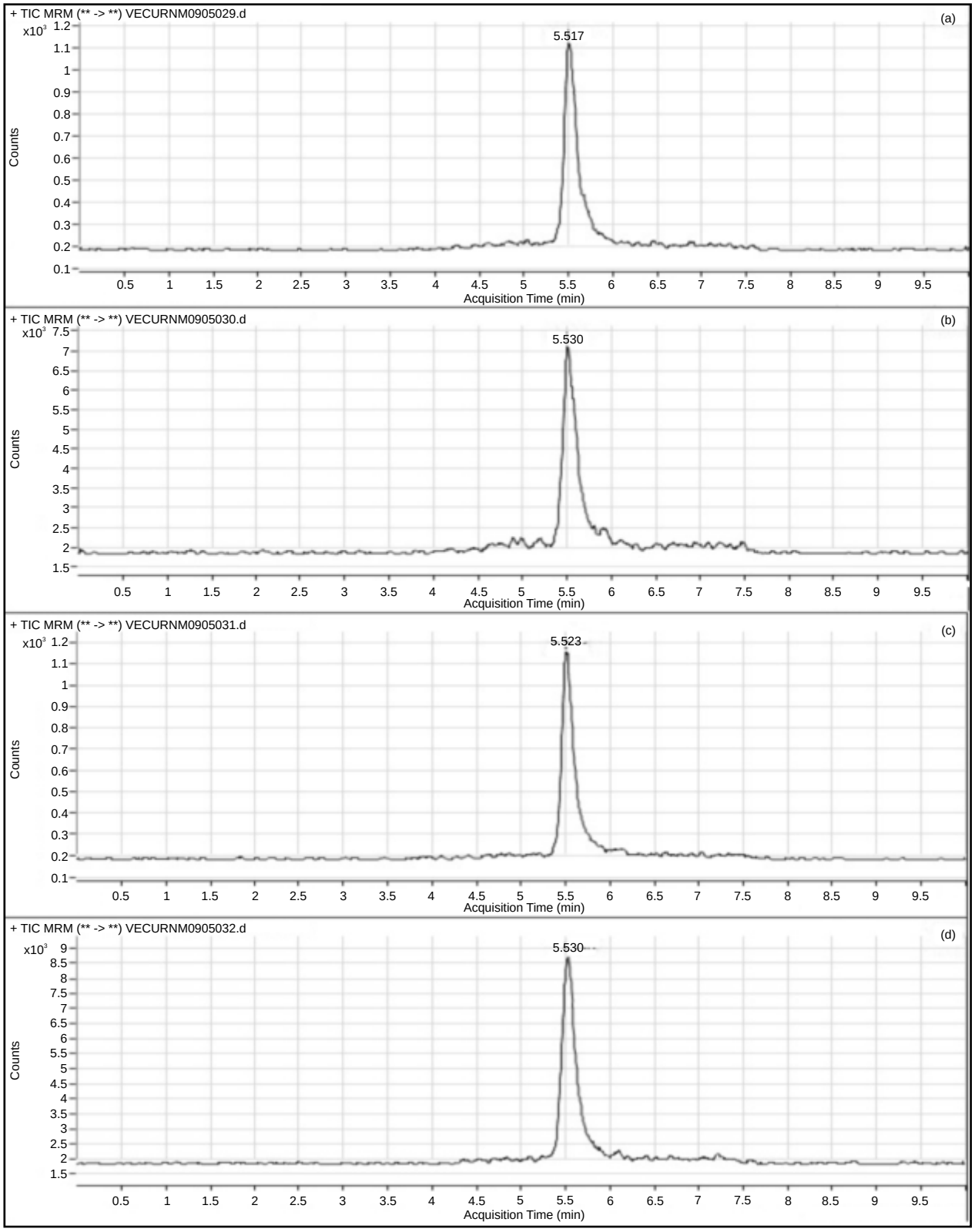

Fig. 4 Total ion chromatograms of rat liver samples (a) R1-L, (b) R2-L, (c) R3-L and (d) R4-L.

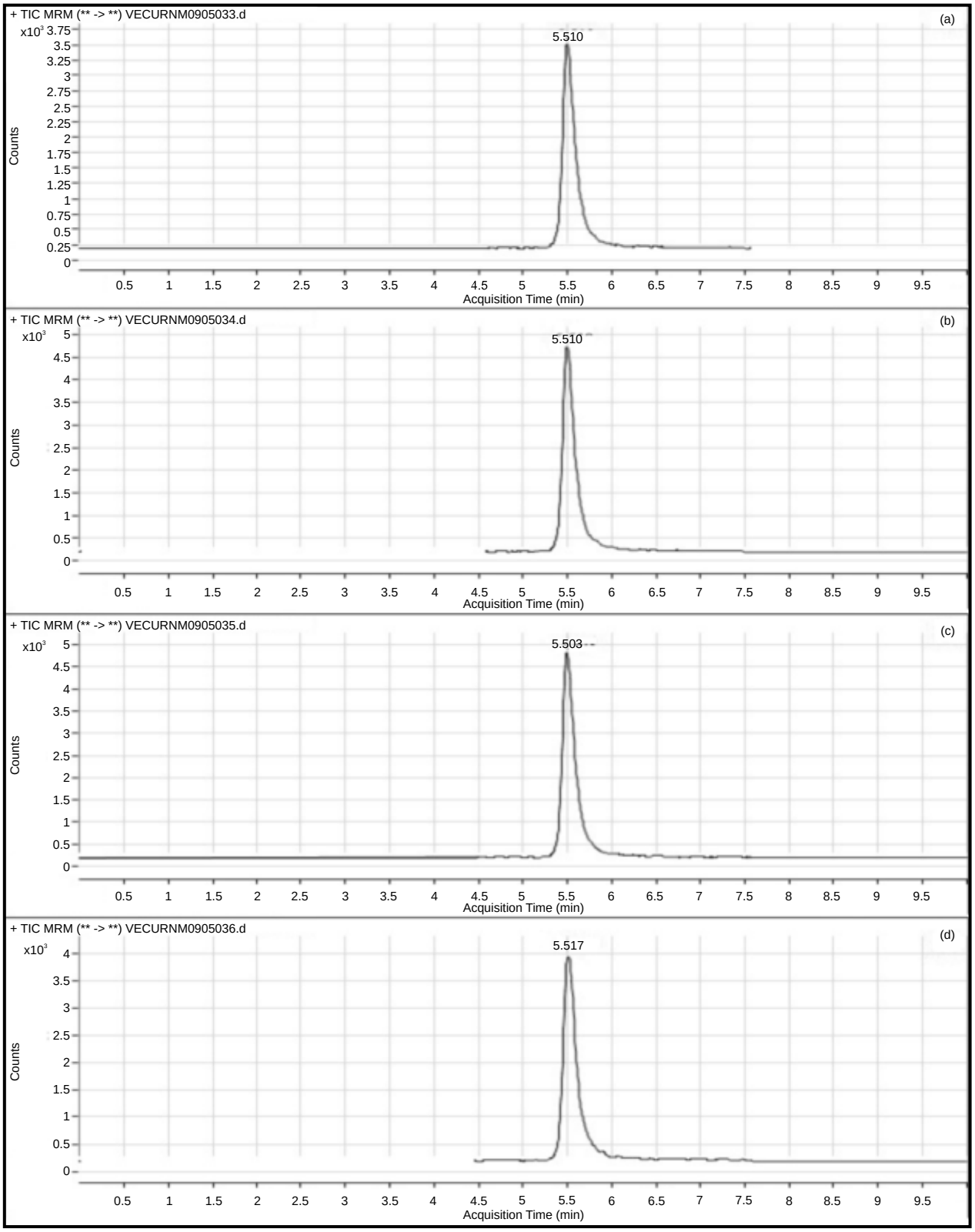

Fig. 5 Total ion chromatograms of rat kidney samples (a) R1-K, (b) R2-K, (c) R3-K & (d) R4-K.

Table 6 Quantitation data.

Sl. No.	Expected Conc (ppb)	Unextracted aqueous drug samples			Extracted spiked samples		
		Response	Final Conc (ppb)	Accuracy %	Response	Final Conc (ppb)	Accuracy %
1.	5.0000	12363	4.2470	84.94	1639 2	4.4376	88.75
2.	25.0000	47010	26.5784	106.31	5403 7	28.753 0	115 .01
3.	50.0000	90377	54.5305	109.06	8081 3	46.048 0	92.10
4.	100.0000	168252	104.7255	104.73	1759 82	107 .519 2	107 .52
5.	200.0000	300427	189.9186	94.96	30869 8	193 .242 2	96.62

standard aqueous drug solutions and then applied to the spiked samples. Injection volume was 5 µl and retention time for vecuronium was found to be 5.5 min under the conditions of analysis.

Calibration curves were obtained by plotting the peak-area ratio for the m/z 100.0 for vecuronium. Eight secondary standard drug solutions at concentrations 200, 150, 105, 52.5, 26.25, 10.11, 5.06 and 2.53 ppb were injected for calibration of the instrument and determination of linearity range prior to extraction of questioned samples. The regression equations was y = 736.811248 + 1749.999975. Coefficient of correlation was greater than R^2 = 0.998. Figure 3 gives the calibration curve.

Extracted rat samples from liver (R1-L, R2-L, R3-L and R4-L) and kidneys (R1-K, R2-K, R3-K and R4-K) were injected into the instrument for quantitative analysis. Figure 4 and 5 give the Total ion chromatograms of rat liver and kidney samples respectively. Blank samples were run before and after the questioned samples.

Matrix interference was determined by comparing the response of the extracted spiked samples (blank samples spiked at concentrations 200, 100, 50, 25 and 5 ppb) with those of unextracted aqueous drug samples at the same concentration. Tables 6 gives the quantitation data.

Results and Discussion

Four rats R1, R2, R3 and R4 of different weights 143, 169, 189 and 187 g respectively were taken for study and were completely anesthetized with chloroform vapours after 7, 3, 5 and 7 min respectively. When injected with overdose of vecuronium, death occurred after 5, 15, 7 and 4 minutes respectively. Dissection was performed after an average time of 16 min and viscera collected after 6 min from the time of death.

The sample preparation procedure used was rapid and simple. Ion-pairing was done with metanil yellow dye which is an ideal compound for visualising the colour of the drug-dye complex getting transferred from aqueous to organic phase.

LC-MS-MS was used to quantify the drug extracted from rat liver and kidney samples. In the chromatogram, peaks with a m/z 557.3, corresponding to vecuronium at 5.5 min retention time was found in all samples (Fig. 4 and 5). Confirmation and quantitation was done by fragment ions m/z 100.0, and 356.2. The quantity of vecuronium found in rat liver samples R1-L, R2-L, R3-L and R4-L were 1.77, 0.75, 1.77 and 1.20 mg respectively, whereas, in kidney samples R1-K, R2-K, R3-K and R4-K were 0.0572, 0.0791, 0.0820 and 0.0685 mg respectively.

Quantity of the drug analyzed in post-mortem rat liver was between 37.5 to 85% (Table 4) of the bolus overdose and death may be attributed paralysis of the skeletal muscles including the diaphragm which may have lead to respiratory failure as there was no respiratory failure.

Quantity found in the kidneys was in the range 2.86 to 4.10 % (Table 5) which explains the non-renal elimination of the drug as mentioned in the literature.

Ion-pairing extraction and LC-MS-MS analysis of such muscle relaxants is easy, fast and reliable that may be applied in case of human post-mortem samples as well in forensic toxicological laboratories.

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