ORIGINAL ARTICLE

# Detection of Metabolites of Vecuronium Bromide in Visceral Samples Solves A Typical Death Mystery- (Toxicokinetics- Studies Using Gc-Ms Technique)

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**ABSTRACT**

**Introduction**: On Sudden death of a medical student (20 year old girl) of National Institute of Medical Science autopsy materials along with site of injection were sent to F.S.L. It was informed during investigation that the girl took tetanus vaccine before death. In crime scene investigation Forensic Team observed that it was a case of gross negligence of dispensing wrong injection**. Material:** Tests were performed on viscera material viz., liver, spleen, kidney, lungs, brain, skin and blood etc. Mystery of suspected death was solved when a new GC-MS application was designed to get the unknown drug and various fragments of extracted material were studied. **Method:** A new method was developed on gas-chromatography-mass spectrophotometer and TL.C using various solvent systems is explained. **Results:** Metabolite fragments of vecuronium bromide a muscle relaxant were surprisingly observed in Viscera material, Blood sample and Skin piece from Leftt cubital fossa from this young girl, whose death is questioned. **Conclusion:** Structural elucidations of fragments provide a new approach to toxicokinetics. The explanation of fragments obtained were structurally compared with other neuromuscular blocking groups like atracurium and succinyl choline The presence of bromide attached cholest-5-en-Br, hydroxylated cholest-5-en-ol as hydroxylated product, acetylated fragments as cholest-5-en-acetate and piperidone-2-one present in visceral samples indicates structural part obtained from vecuronium bromide drug. These metabolites studies makes the case studies highly informative. Beside this new method of extractions, TLC systems and colouring reagents are also explained.

**Keywords:** Chromatography, Mass spectrometry, Thin layer chromatography, Toxico-kinetics

## INTRODUCTION

Vecuronium bromide is a neuromuscular blocker of intermediate duration. It is non-depolarizing skeletal muscle relaxant used for endotracheal intubations and to provide muscle relaxation in general anesthesia for surgical procedures.[1] It was quite surprising to get fragments of neuromuscular blocker in a 20-year-old student named Himani Bhati of NIMS, Jaipur (Rajasthan) who was found unconscious in her hostel room and got died within 40-45 minutes during treatment as stated by the police. Her friends and medical staff made out different stories about her death but no results match with these stories. Materials sent to FSL were analyzed. It was also conveyed that, she had taken tetanus vaccine, however clues like empty injection ampoule and used syringe were not found in the hostel room. Attempts were made to find out the unknown drug. Metabolic fragments of vacuronium bromide drug were observed under GC-MS analysis that was well co-related with basic molecular structure of vacuronium & death mystery was solved.

The attempt of identification of an unknown drug is very significant and the established fact about this drug correlates with the findings. This drug must be administered via trained personnel in an equipped facility to monitor, assist, and control respiration. Skeletal muscle weakness, decreased respiratory reserve, low tidal volume, or apnea are the symptoms associated with this drug overdose. A peripheral nerve stimulator may be used to monitor recovery from blockade and to differentiate between prolonged neuromuscular blockade from other causes of diminished respiratory reserve. In management the neuromuscular blockade may be reversed by administration of a cholinesterase inhibitor such as neostigmine, pyridostigmine or edrophonium.[2]

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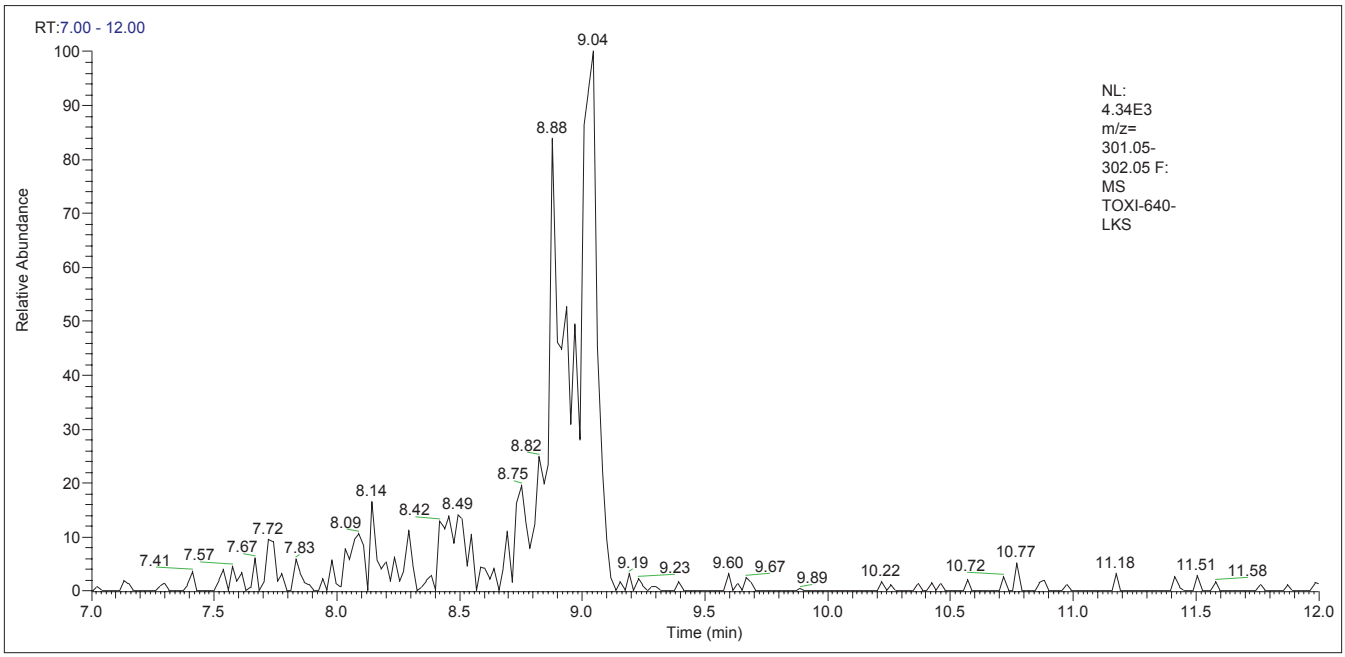
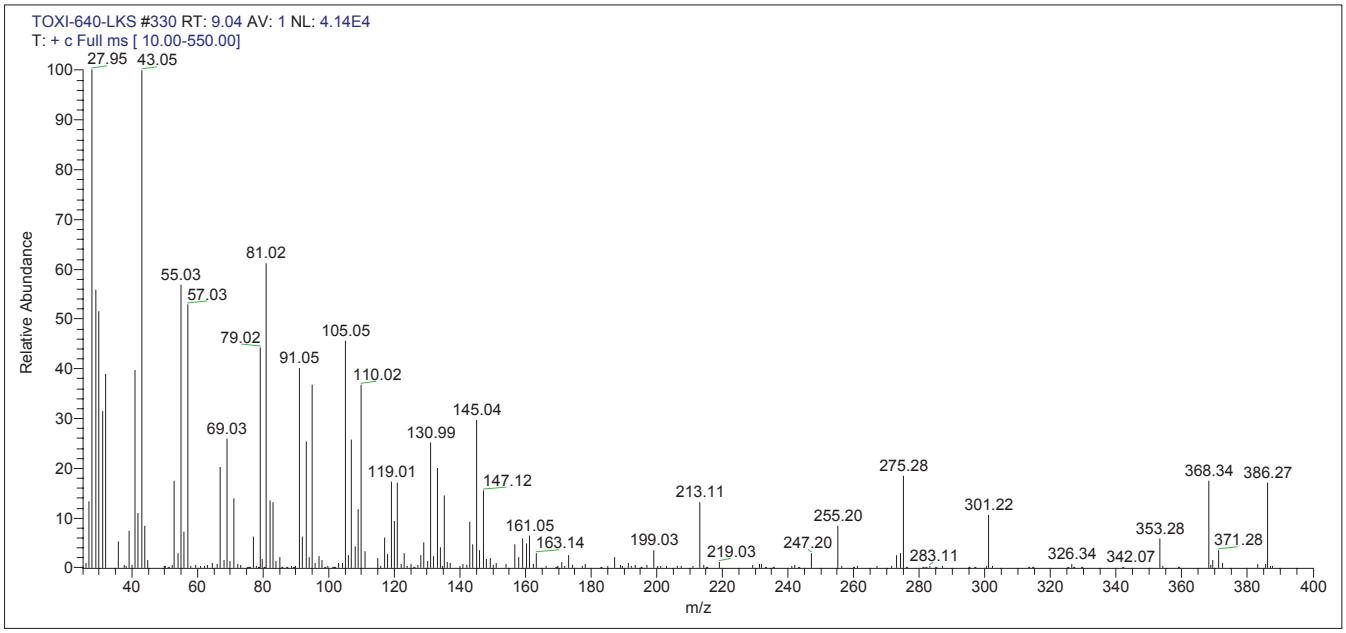



**Graph 1:** Gas-Chromatogram In Lsk




**Graph:**2 –MASS SPECTRUM IN LSK

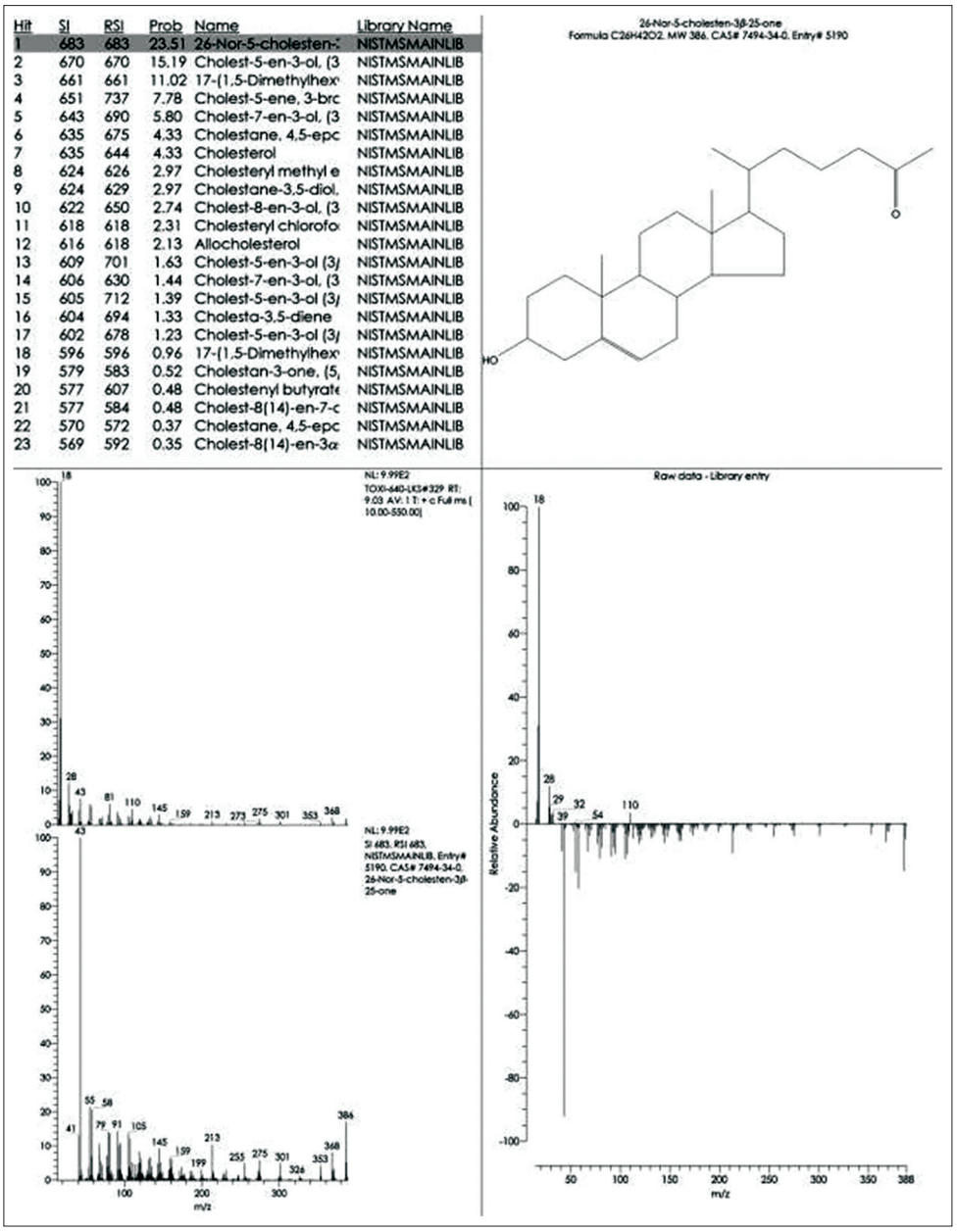
Vecuronium bromide can be used as part of a drug cocktail that prisons in the United States use as a means to put a condemned prisoner to death. Vecuronium bromide is used to paralyze the prisoner and stop his or her breathing, in conjunction with a sedative and potassium chloride to stop the prisoner's heart. Injections of vecuronium bromide without proper sedation have been known to cause excruciating pain to prisoners.[3] In 2001, Japanese nurse Daisuke Mori was reported to have murdered 10 patients using vecuronium bromide.[4] He was convicted of murder and was sentenced to life imprisonment.[5]

The method of detection by LC-MS-MS is widely explained by.[6]

The detection by mass using gas chromatography and T.L.C makes this work significant. The structural elucidation specifies differences with other similar group drugs works as neuromuscular blockers.

## MATERIAL AND METHOD

The viscera material is taken and digested using 25 gms ammonium sulphate and 0.5 N glacial acetic acid (50 ml) and

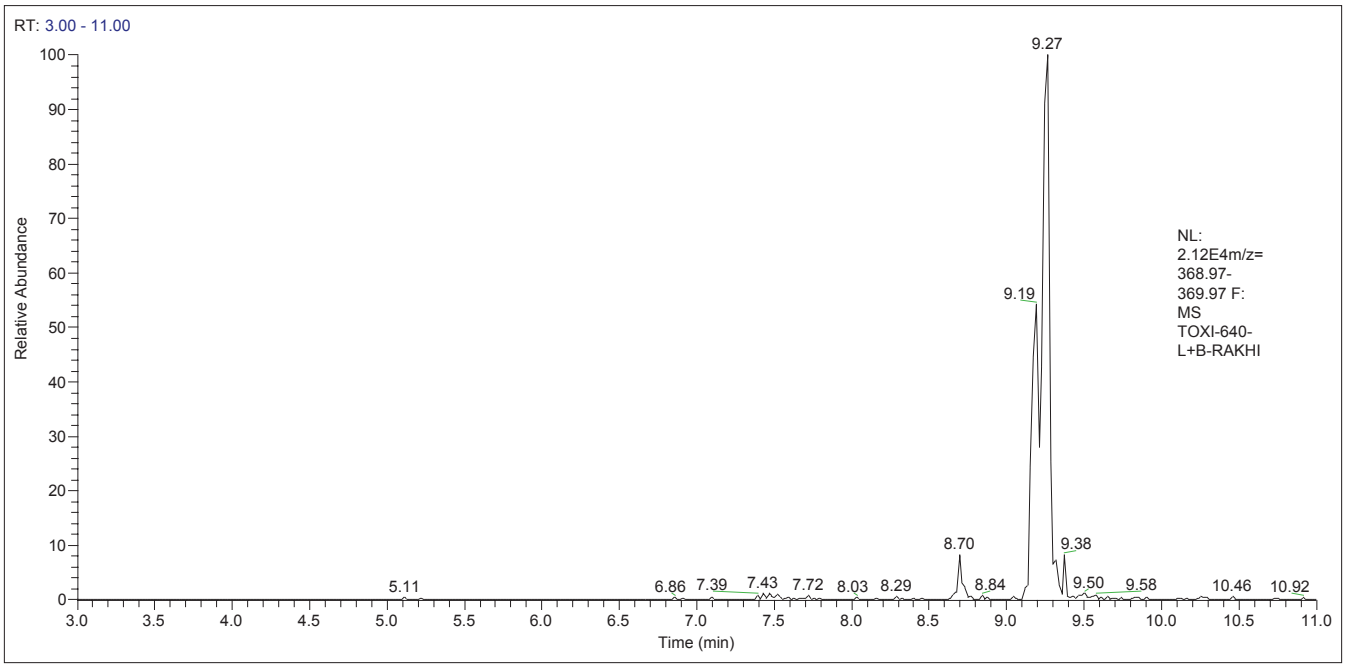



**Graph 3:** The gas chromatogram of Liver, Spleen, Kidney having peak at Rt- 9.04gave mass fragmentation for cholest-5-en-ol good probability

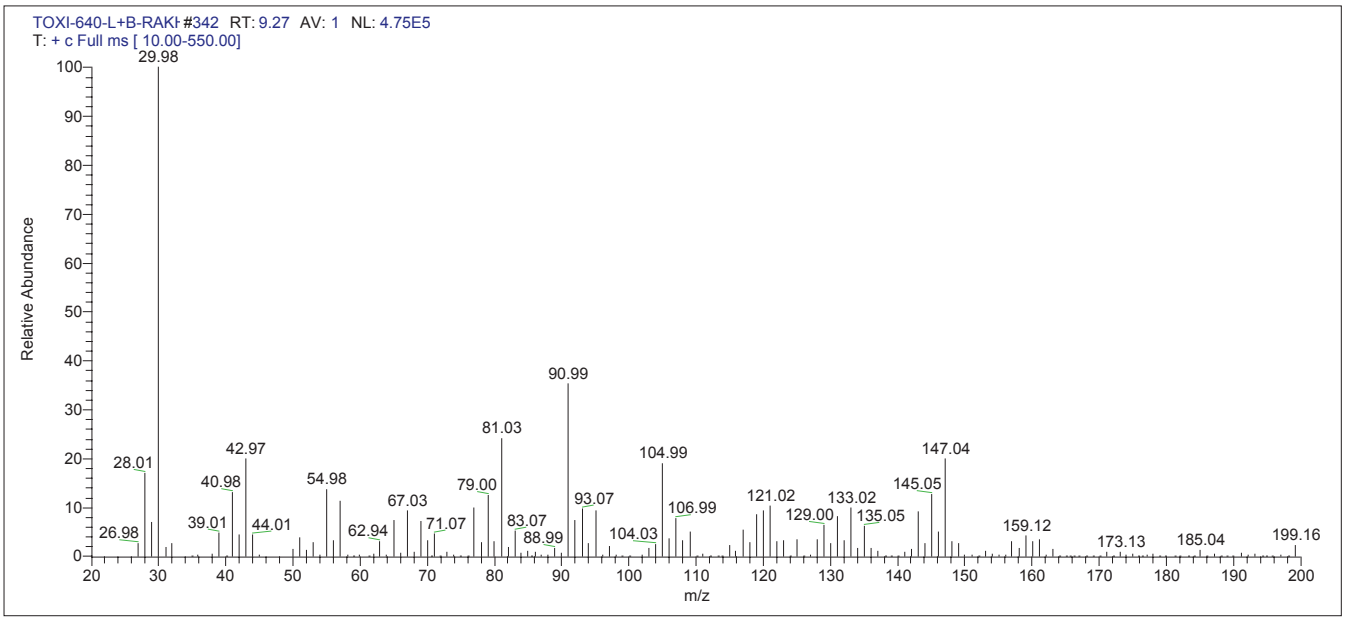
put on a boiling water bath for a period of three hours, after it was cooled at normal temperature Next day it was filtered using Whatmann filter paper -40 and taken in a separating funnel, Aqueous ammonia is added till it requires pH—9.5 and chloroform: ether is added in a ratio of 1:3, again the material in separating funnel is extracted to dissolve the required part for a period of 15 minutes. The chloroform layer is separated and tested for basic fractions. Then the residue made acidic with 0.1 MHCl and extracted using ether for 15 minutes. Ether layer was separated and tested for neutral fractions. Similarly the aqueous phase remained is again set at pH 9.5 by adding sodium carbonate extract with chloroform, filter the chloroform layer and checked for basic fractions. Similarly Viscera material was made acidic using sulphuric acid and extract with chloroform and chloroform layer is separated and tested for acidic fractions.

A) 1) Stomach & pieces of small intestine
   2) Pieces of Liver, spleen, Kidney
   3) Pieces of Lungs and Brain
   4) Skin Piece from Left Cubital Fossa
   5) Skin Piece from Right Cubical Fossa
   6) Blood Sample.
B) Standard chemicals, reagents & Glassware were used.
C) Instrument –Microwave Solvent Extraction Lab station System.

UV – VISIBLE Spectrophotometer by Perkin Elmer
GC – MS (Thermofinnigan).




**Graph 4:** Gas-Chromatogram In L+B




**Graph 5:** Mass Spectrum of cholest-5-en-3-bromo- compound in lungs and brain part

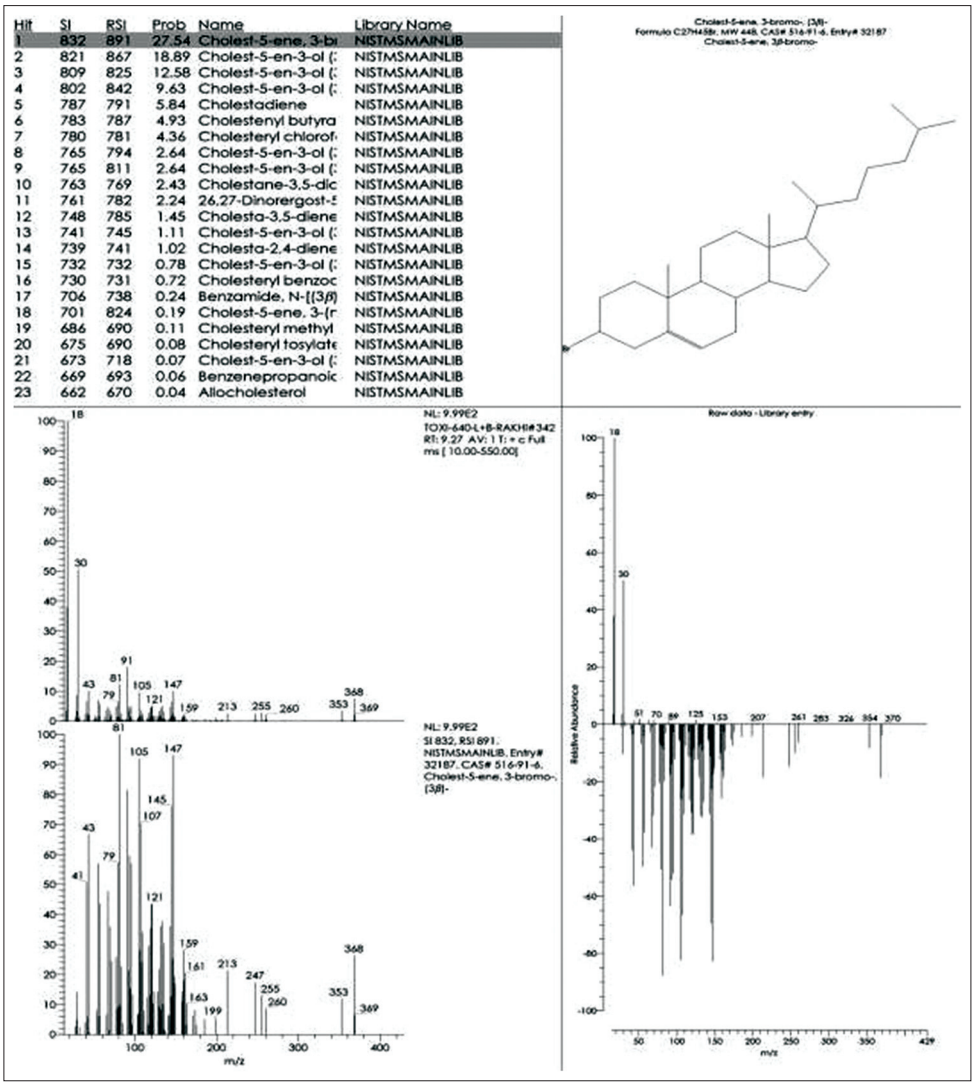
## Extraction Method and T.L.C

a) Strass Otto Extraction procedure was followed. Aqueous acidic filtrate of macerated tissue were filtered and made alkaline with ammonium hydroxide and extracted thrice with 100 ml of ether and chloroform in (3:1) ratio. Organic layer separated and dried.[7]

b) 20 gms tissue mixed with 30 ml of water and 20 ml of aluminum chloride reagent (10% w/v AlCl3 + 10% w/v citric acid in 2 NHCL), after protein coagulation, filtered it and add ammonia & saturated solution of sodium chloride, again add 5 gm KI in 5 ml of water. After continuous shaking vortexed with 10 ml of dichloromethane® organic layer separated and dried.[8]

c) Extracted samples from liver, spleen, kidney, lungs and brain and from Rt and Lt cubital fossa were dissolved in methanol along with control sample of Vecuronium bromide spotted on T.L.C plate, using four solvent system

1) (Chloroform: Acetone) system,
2) (Methanol: Ammonia),
3) (DMF + drops of ammonia),




**Graph 6:** The gas chromatogram of Lungs and Brain gives peak at Rt-9.27 the mass fragmentation pattern match with cholest-5-en-3 bromo compound in good probabilty

4) (Ammonium formate +formic acid + water + THF) gives good results in separation and running of samples.

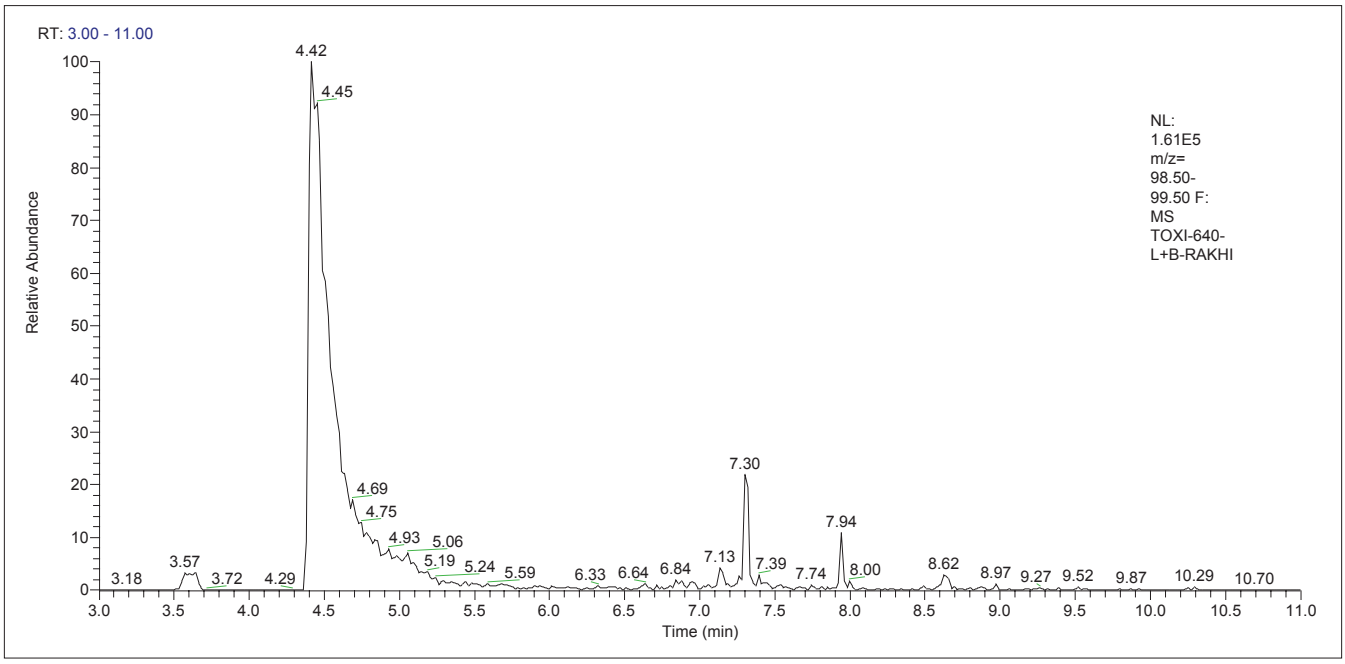
Vecuronium bromide and its metabolites give orange colour with Dragendorffs and purple brown with Iodoplatinate reagent on T.L.C plate applying the above systems.

## RESULTS

The results are shown in the respective Tables [Graphs 1-9].

Using methanol: ammonia in a ratio 8 ml: 10 drops, The material liver, spleen, kidney extracted in a mix gives a specific spot at Rf. 40, similarly Lungs and brain mix also gave us a spot at Rf-40. We find that extract of blood and samples of skin from left cubital fossa also gave a spot at Rf- 40. It indicates that spot is present in all these part, may resultive of drug metabolite or its moiety. Similarly the system chloroform: acetone and methanol was tried in (6:2:2) Several separated spots at Rf-20, 30, 75 in all organs mix indicates more degradation or metabolites formed in this process needs to tallied with standard sample of vecuronium drug. Similarly chloroform: acetone (8:2) and ammonium formate: formic acid: water and tetra hydrofuran (1:5:95:2) gives a single spot at Rf -20 as that of vecuronium drug. The results from the table itself explained that in blood, liver, spleen, kidney and lungs and brain give more separation spots than the standard sample and skin sample. Till now the inference can be taken out but for confirmation need of instrumental technique is must.

Reconstituted samples were subjected to GC-MS applications which confirm the presence of metabolite of vacuronium bromide.




**Graph 7:** Chromatogram In L+B

### Gc-Ms Method

Column used – EC™ - 5 alltech, Length – 30 meter, ID – 0.25 m meter, Film Thickness – 0.25 μm.

### Experimental Parameters

| GC - oven | | Mass detector spectrometer | ??? |
|---|---|---|---|
| Initial temp | 60°C | Ion source temp | 200°c |
| Final temp | 250°C | Mass-range | 10-650 |
| Oven run time | 9.33 min | Vaccum compensation | On |
| Injector temp | 250°C | Fragment mode | PI |
| Rise in temp | 20°C/min | Electron energy | 70ev |

## DISCUSSION

### Structure Elucidation & Inference

Vecuronium Bromide is chemically designated as 1-(3α, 17b-Diacetoxy- 2β-piperidino-5α-androstan-16β-yl)-1-methylpiperdinium bromide.

Vecuronium is amino-steroid NMB agent - The Steroid Nucleus provides a rigid bulky structural base. In vecuronium, A – ring Ach moiety is tertiary, without a methyl quaternizing group. Vecuronium is, therefore, D – ring Ach monoquaternary. It's A – ring Ach is a NOR (Nitrogen without radical) as in the trade name Norcuron. Although vecuronium bromide is often thought of as a muscle relaxant, it may be more accurate to classify it as a paralyzing agent.

Its NMB action can be attributable to its common cis D-ring Ach moiety. GC-MS Analysis performed & chromatographic peaks were observed, giving mass fragmentation for Cholest

**T.L.C Rf - values in solvent system**

| Material | MeOH: $NH_3$ (8 ml: 10 drops) | $CHCl_3$: acetone: MeoH (6:2:2) | $CHCl_3$: acetone (8:2) | $HCOONH_4$: HCOOH: $H_2O$: THF (1:5:95:2) |
|---|---|---|---|---|
| LSK | 40 | 20, 30, 75 | 25,47-55,60 | 20 |
| LB | 40 | 20, 30, 75 | 25, 75 | 20 |
| Blood | 40 | 20, 30, 75 | 25, 75 | 20 |
| Skin – Lt | 40 | 20, 75 | 25 | 20 |
| Vecuronium Bromide | 40 | 20 - - | 25, 75 | 20 |

LSK (Liver, Spleen and Kidney), L+B (Lungs & Brain)

– 5 – ene – 3 – ol (3β) i.e lanol at Rt = 9.03, prob = 52.08 in liver, spleen, kidney

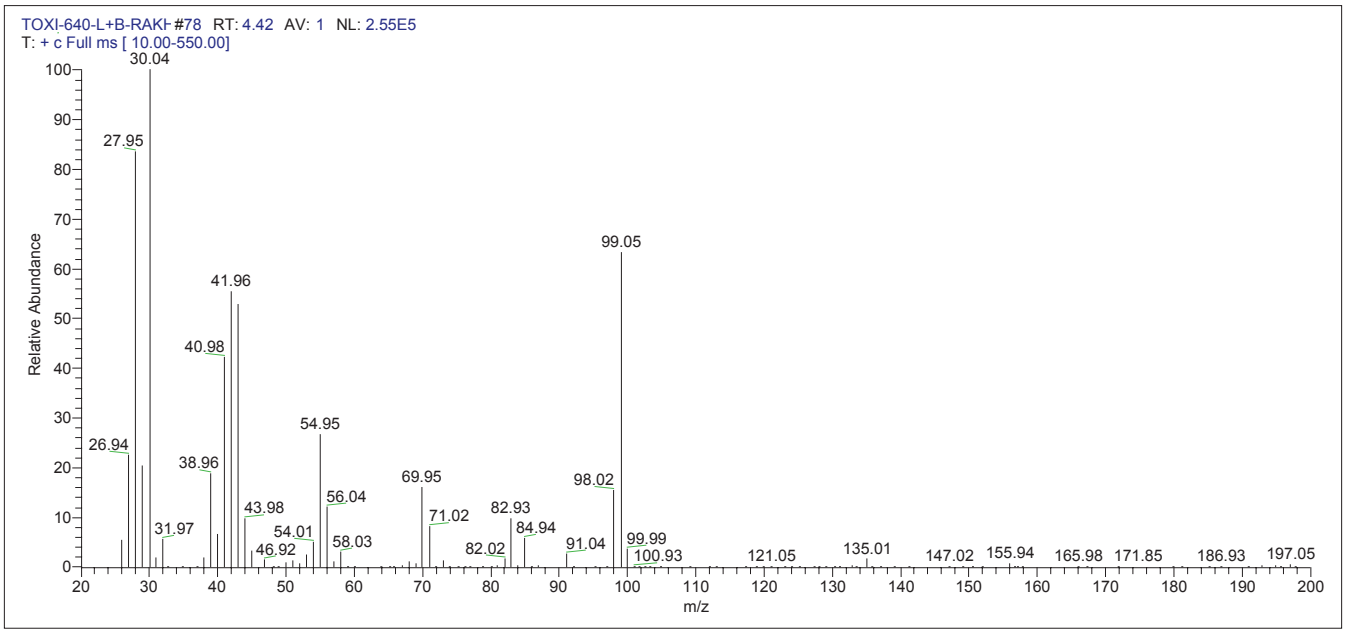
Cholest – 5 – ene – 3 – bromo- (3β) at Rf = 9.27, prob = 27.54 in lungs & brain

Cholestane – 3, 5, diol – diacetate (3β) – MW = 488, 2.43%, fragments were also observed.

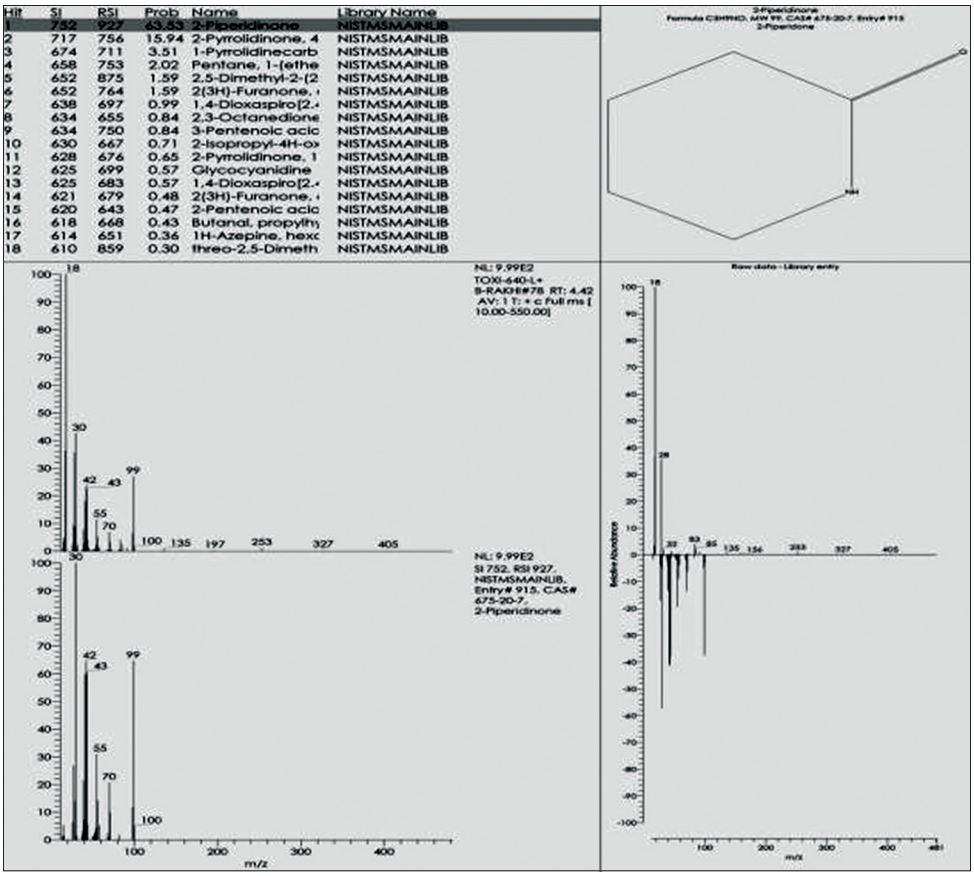
2-Piperidinone, Rt = 4.42, prob = 35.33 were observed.

Besides we have got fragmentation for 26-Nor-5-cholesten-3b-25-one and cholestane 3,5-diol, 5-acetate. The presence of basic molecular moiety i.e lanol and piperidinone concluded that these are main metabolic fragments of aminosteroid NMB especially of vecuronium.

Using LC-MS/MS on a Tandem Quadrupole/Time-of-Flight Instrument[9] clearly mentioned that Neuromuscular blocking agents such as succinylcholine, pancuronium,




**Graph 8:** Mass –Spectrum of lungs and Brain part for Rt-4.43 shows fragment 2-piperidone




**Graph 9:** The gas chromatogram of Lungs and Brain gives peak at Rt-4.43 the mass fragmentation pattern match with 2-piperidone compound in good probabilty

and tubocurarine, often used as paralytic agents during surgery, are occasionally suspected as paralytic poisoning agents involved in suspected homicide and suicide cases. In terms of toxicity and forensics, a clinically effective dose of one of these drugs can also be a lethal dose if respiratory assistance is not provided. Thus, neuromuscular blocking agents are potential murder weapons. They may be particularly attractive to a potential poisoner because

they are not routinely measured and, indeed, few forensic laboratories will even attempt to analyze these compounds. Several previous methods have been described for analyzing various neuromuscular blocking agents (and quaternary compounds in general) by mass spectrometric means.[10-24]

The neuromuscular blocking effects of equipotent doses of the three putative metabolites of vecuronium (3-hydroxy vecuronium, 17-hydroxy vecuronium and 3,17-dihydroxy vecuronium) were compared with those of an equipotent dose of vecuronium. following i. v. injection, in the cat.[25]

Rupp SM et al, (1987)[26] evaluated the effect of aging on the distribution, metabolism, and neuromuscular junction sensitivity to pancuronium and vecuronium, the authors determined the pharmacokinetics and pharmacodynamics of these drugs in 12 healthy elderly subjects.

GC-MS finding proves that cholest – 5 – ene – 3 – bromo, metabolite finds in Liver, Spleen, Kidney, lungs & Brain, which is the main moiety of vecuronium bromide. T.L.C provides us new method of separation, as it is water soluble drug; it is also observed that raising the alkalinity of developing system will increase the Rf value on TLC plate.

A newer muscle relaxant i.e Vecuronium is detected in mixture of 1) Lungs & Brain, 2) Liver, spleen, Kidney, 3) Blood and in 4) skin from Rt Cubital fossa.

Detection of Vecuronium bromide in Viscera, blood & skin is a new original attempt and provides us fruitful result about this case, and death mystery of NIMS girl solved.

## CONCLUSION

Study of metabolite and their structure elucidation concluded that Vecuronium bromide a muscle relaxant was injected to young girl resulted in her death and story of taking Titanus vaccine by herself due to some injury was framed. Designing GC-MS method for unknown drug solved a typical crime-scene of suspected drug. The drug bank data of Vecuronium is as available and according to it the accession number of Vecuronium Bromide is DB01339; This compound belongs to the steroid esters. These are compounds containing a steroid moeity which bears a carboxylic acid ester group Monoquaternary homolog of pancuronium. It is recorded that alternative parents of this drugs are Androgens and Derivatives; Piperidines; Tertiary Amines; Carboxylic Acid Esters; Enolates; Polyamines; Ethers and piperidine; tertiary amine; carboxylic acid ester; enolate; ether; carboxylic acid derivative; polyamine; amine; organonitrogen compound are its substitutes.

It is very clearly indicated that in forensics every case is of specific type and all neuromuscular blocking agent drugs having the basic similar structure are pancuronium, rocuronium and vecuronium while others neuromuscular blockers lacks this moiety like atracurium, succinyl choline etc. In every separation on mass spectrometer we got this moiety, cholest-ene-ol indicative of hydroxylated product. The presence of bromide attached with this structure again provides us a big reason for its presence. The piperidone fragment at every run is significantly approaching to the presence of this drug. The deacetylated molecule is widely accepted to form as metabolite again strengthen the results.

On these findings the medical staff was held responsible for negligence in treatment and question was raised against them for hiding the truth and terrorizing the students not to disclose the real event to anyone that will create defamation on institute name.

The detection by mass using gas chromatography and T.L.C makes this work significant. The structural elucidation specifies differences with other similar group drugs works as neuromuscular blockers.

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