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Case Report

Local anesthetic marcaine-bupivacaine hydrochloride and its metabolites in Forensic Samples: A case study

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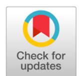

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ABSTRACT

The local anaesthetic bupivacaine and its metabolites such as Desbutyl bupivacaine, 3-hydroxybupivacaine, and 4-hydroxybupivacaine were extracted and found in the forensic sample from postmortem samples such as cerebrospinal fluid (CSF) and postmortem blood. To separate and detect the aforesaid substances in biological matrices, high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) and ATR-FTIR methods were used. When a couple of husband and wife who were riding on the side of road by a motor cycle which was abruptly collided by an unknown motor cycle. The couple were injured, especially in the left leg of the lady was severe pain due to bone fracture. Both were immediately admitted in nearby a Government Hospital for treatment. Anaesthetic was injected to the lady before starting the procedure of surgery. Within a few minutes she suffered pulmonary consequence and the mortality immediately occurred. Due to a therapeutic amount of bupivacaine was given, postmortem samples CSF and blood were received at forensic laboratory and extracted using a liquid-liquid extraction method (LLE). Certain chromatographic techniques and ATR-FTIR method were used to separate and detect bupivacaine and its metabolites in a forensic sample.

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1. Introduction

Treatments, mechanisms, and adverse reactions of anesthetics and analgesics is an important read for anyone working in pain management.[1] Bupivacaine is a long-acting local anaesthetic agent often known as Marcaine. Bupivacaine, unlike lignocaine or ropivacaine, causes irreversible circulatory collapse at doses much closer to the specified hazardous threshold.[2] For instance, Bupivacaine is four times as potent as lignocaine but it has sixteen times the cardio toxic potential. Cardio toxicity associated with Bupivacaine often proves difficult to treat.[3] Bupivacaine has been implicated in 76% of local anesthetic associated maternal deaths.[3] It remains to evaluate the efficacy of ropivacaine and Bupivacaine in infiltration analgesia. Bupivacaine hydrochloride (BPHC) Figure 1 is the hydrochloride of 1-butyl-N-(2,6-dimethylphenyl) piperidine-2-carboxamide. A white crystalline powder is a long-acting amide local anaesthetic and easily soluble in 95 % ethanol and distilled water and somewhat soluble in organic solvents such as chloroform or acetone. BPCH is used for intraoperative local anaesthetic, postoperative analgesia, and chronic pain management. In obstetrics, BPCH is commonly utilized. In lumbar epidural anaesthesia, the medication appears to be safe for both mother and foetus.

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Many adjuvants, such as opioids, dexamethasone and clonidine, have been administered with local anaesthetics aiming to increase the longevity of the block and reduce its toxic effects.[4] The local anaesthetic BPCH which is widely used to treat chronic and acute pain has been examined in numerous animal models.6.[5–10] Desbutylbupivacaine,3-hydroxybupivacaine, 4-hydroxybupivacaine and bupivacaine metabolites have been identified from researchers.[5–10]

Bupivacaine and its metabolites have been investigated in forensic evidence using a range of technologies, including high-performance liquid chromatography (HPLC) and gas chromatography (GC).[11–16] Extraction of the sections of interest on a TLC plate was followed by GC-MS analysis to identify bupivacaine metabolites. Desbutylbupivacaine and hydroxylated metabolites were identified. The GC-MS method paired with liquid-liquid extraction was used to analyse bupivacaine, 3-hydroxybupivacaine and desbutylbupivacaine.[17] Using nuclear magnetic resonance spectroscopy, three new metabolites such as N-butyl-pipecolyl-2-amide and two hydroxylated species were discovered (NMR). In general, GC-MS with selected ion monitoring (SIM) has been used to analyse bupivacaine and desbutylbupivacaine in foetal, maternal, and newborn plasma after administration as a caesarean section anaesthetic.[18] Bupivacaine and its metabolites, 3- and 4-hydroxybupivacaine, were recently determined using GC-MS with SIM[19]. Arvidsson and colleagues described an HPLC method for separating bupivacaine's 3- and 4-hydroxylated metabolites in human urine.[20] Though, the method was not compatible with mass spectrometric detection and structural elucidation of any of the sample components could not be performed.

Peak levels of BPCH in the blood are reached in 30 to 45 minutes after injection of BPHC for caudal, epidural, or peripheral nerve block in a man, followed by a drop to inconsequential levels over the next 3 to 6 hours.[21] One of the most important medical jobs has always been the management of pain. The International Association for the Study of Pain defines pain as an unpleasant sensory or emotional experience paired with the possibility of or actual tissue damage. Pain is a physiological symptom that serves as a warning to the body alerting it to injury or illness and is thus necessary for survival. It advises against bodily harm which is more crucial for preventing injuries and as a result for survival. Pain can be unpleasant for the patient due to acute injuries or it can change a person's life, lower their quality of life and have an effect on their family.[22]

Surgical patients experienced pain which is directly related to their treatment. Appropriate management of postoperative pain allowed wounds to heal faster and the patient to recover more quickly. Also, in child birth, the appropriate administration of analgesia reduces pain and makes delivery less stressful. Continuous analgesia with a combination of opioids and local anesthetics is becoming more prevalent in patients recovering after surgery. Even after severe and extensive abdominal or thoracic operations, it can be utilized safely and effectively.

From analytical approaches such as HPLC, GC-MS, LC-MS[23]) and by Capillary electrophoresis (CE)[24,25] have found their application in the analysis of bupivacaine in pharmaceutical formulations and/or biological fluids has benefited. The use of a single approach does not fulfil the objective of forensic analysis and an effective methodology is necessary because the samples encountered are polluted and in traces. Blood flow has a dangerous role in acute and chronic pathologies in peripheral nerves (Figure 2). Figure 2 shows spinal cord of human and epidural space. And also explains the collection of cerebrospinal fluid (CSF). The effects of local anesthetics and adjuvants on tissue perfusion and oxygenation are thought to be relevant factors in nerve injury following peripheral regional anaesthesia.[26] The relation between reduced tissue perfusion and subsequent tissue oxygenation is unknown due to local anesthetics.

2. Case Presentation

When a husband and wife were riding on a two-wheeler motor cycle after getting treatment for fever, an unknown two-wheeler crashed into their bike. Both of them were injured, especially the lady, who suffered extreme pain in her left leg due to a bone fracture. Hence, they were admitted to a nearby government hospital to get treatment. Due to a severe fracture, the lady was brought to surgery where an anaesthetic was injected prior to the operation. Within a few minutes, she had a pulmonary effect and subsequently died. A complaint was given by her husband, suspecting that his wife had been administered a heavy dose of anaesthesia so that she died. A postmortem was conducted after the case was filed. Then, the complete forensic profile was made using various analytical methodologies such as chemical tests, HPLC, FT-IR and GC-MS to confirm the presence of an anaesthetic such as bupivacaine.

Table 1: Thestudied mobile phases and its compositions for the separation

Mobile phases	Solvents
A	Methanol: Water (60:40)
	Acetonitrile: Water (80:20)
C	Methanol: Acetonitrile:Water (70:25:5)
D	pH 6.5 buffer: acetonitrile (50: 50)

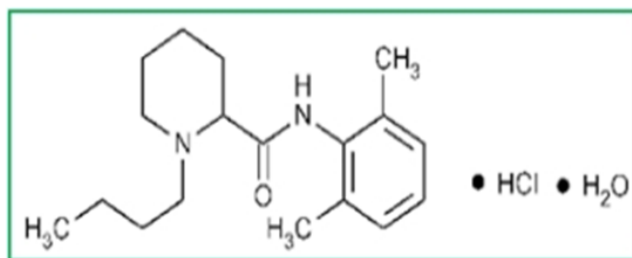

Figure 1: Chemical structure of bupivacaine hydrochloride (BPHC)

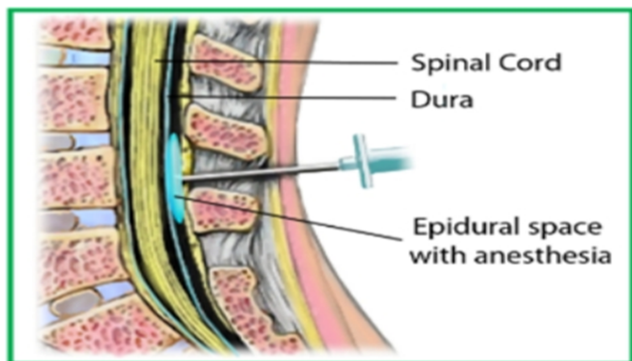

Figure 2: Epiduralneedle placement in spinal cord of humans

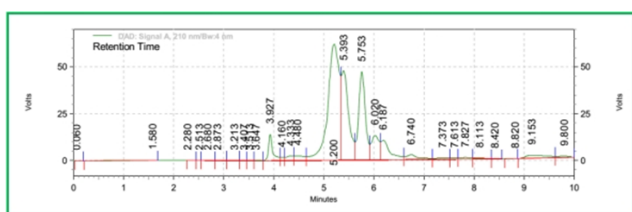

Figure 3: Chromatogram of the encountered forensic sample (BPHC) in mobile phase A at λ_{max}210nm

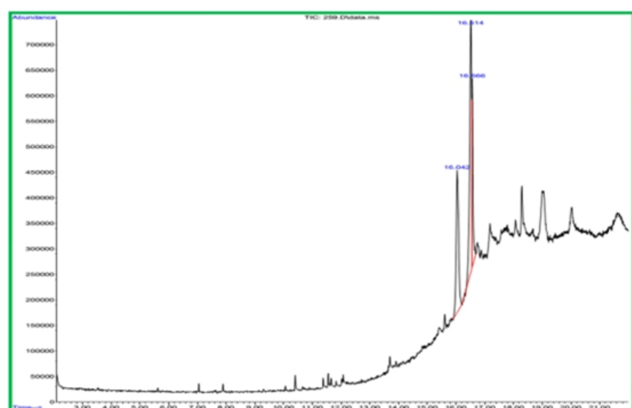

Figure 4: GC-MS Total ion chromatogram of BPHC in forensic sample

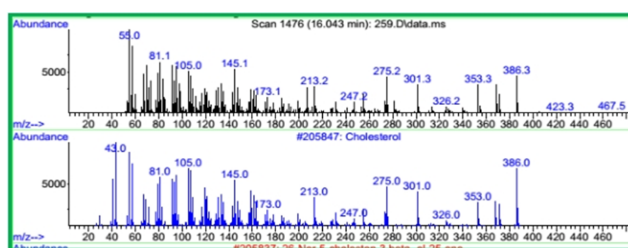

Figure 5: Mass spectral data of BPHC (forensic) sample when retention timeat 16.043 minute

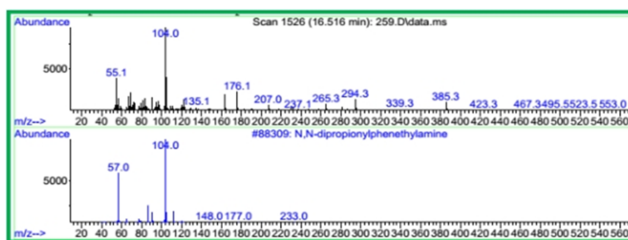

Figure 6: Mass spectral data of BPHC (forensic) sample when retention time at 16.516 minutes

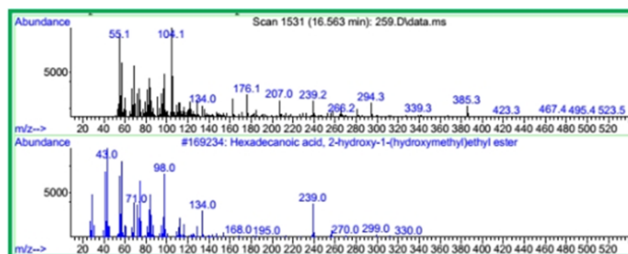

Figure 7: Mass spectral data of BPHC (forensic) sample retention time at 16.563 minutes

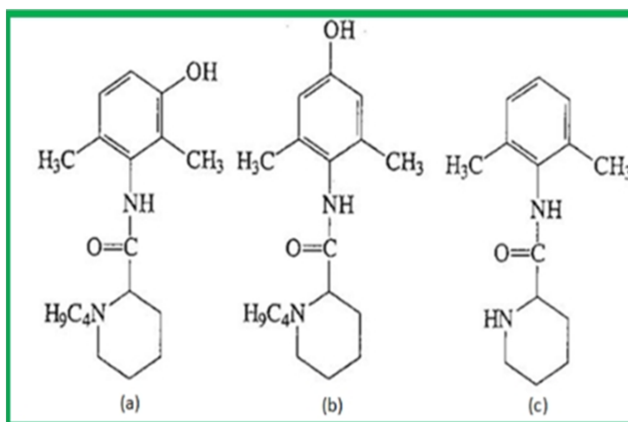

Figure 8: The structures of **(a)** 3'-hydroxybupivacaine **(b)** 4'-hydroxybupivacaine and **(c)** desbutyl bupivacaine (M=232.32).

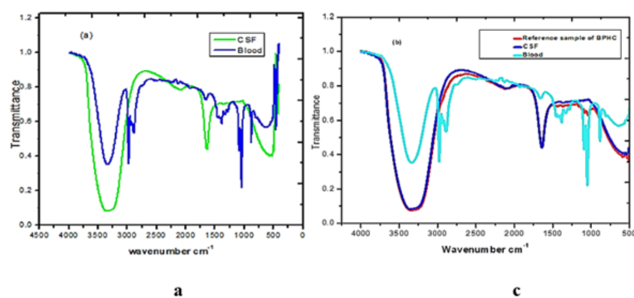

Figure 9: **(a)** ATR-FTIR Spectrum of blood, CSF **(b)** ATR-FTIR Spectrum of blood, CSF and Reference sample of BPHC

3. Materials and Methods

3.1. Chemicals, standards and reagents

Bupivacaine hydrochloride (BPHC) made by Systochem Laboratories Ltd, India and was obtained as a reference sample from Government Hospital. Water, acetonitrile and methanol were used in HPLC grade purity.

3.2. Mobile phase preparation

Mobile phase consists of pH 6.5 buffer and acetonitrile with (50: 50). Buffer was made by dissolving 6.8 g of potassium dihydrogen phosphate in 1000 mL Milli-Q water, which was precisely weighed. 5 mL triethylamine solution was added, the pH was adjusted to 6.5 with dilute orthophosphoric acid, and the solution was filtered through a 0.45 μm nylon membrane filter. At a flow rate 1.0 mL/min, the mobile phase was pumped from the solvent reservoir to the column. The column was maintained at 45°C and the volume of each injection was 10μL. Prior to injection of the solutions, column was equilibrated for at least 20 minutes with mobile phase flowing through the system. The eluents were monitored at 220 nm. Diluent: Milli-Q water: Methanol (50: 50).

3.3. Standard preparation

For the working standard, a 25.0 mg of BPHC was carefully weighed and put into a 100 mL clean dry volumetric flask. About 70 mL of diluent was added and it was sonicated for 15 minutes before being diluted with diluents to the appropriate volume.

3.4. Sample preparation

In a 50 mL clean dry volumetric flask, 5 mL of BPHC sample solution was obtained and brought up to volume 50 mL with diluent.

3.5. Extraction of biological fluids

The toxicological analysis was carried out systematically for unknown poisoning. The post-mortem samples of the deceased were examined for identification of different groups of poisons like gaseous, volatile and metallic poisons in addition to pesticides and drugs. Individual visceral samples such as the stomach and its contents are as the liver, kidney, small intestine and blood were examined for various poisons. 100 g of tissue samples were chopped into small pieces and divided into two equal parts. One fraction was processed to see if any drug was present. The tissue samples were acidified with 50 mL acetic acid and solid ammonium sulfate was added to it to obtain a saturated solution. This was kept on a water bath with thermostatic control at a temperature of 60 ºC for protein coagulation. The tissue samples were allowed to cool at ambient temperature before being filtered after complete coagulation. The aqueous layer was collected, and LLE was performed in two steps. The first stage involved extracting the aqueous filtrate three times with 50 mL chloroform and collecting the organic phases. Ammonia was added to the remaining aqueous component to make it alkaline (pH 9.0). The alkaline aqueous phase was extracted with 50 mL chloroform (3 times) and organic phases were collected. Both the organic layers were filtered through anhydrous sodium sulphate and evaporated to dryness. Methanol was added to the dried organic phases for analysis of drugs. The tissue samples were soaked overnight in a suitable amount of n-hexane. Anhydrous sodium sulphate was used to filter the samples. The organic layer was gathered in a dish and evaporated until it was completely dry. For drug analysis, the dried filtrate was reconstituted in chloroform.

To assist blood deproteination, one mL blood sample was mixed with two mL saturated sodium tungstate solution and one mL 1N H_2SO_4 and heated on a water bath at a regulated temperature of 60 °C. The deproteinized samples were filtered to collect the aqueous layer which separated into two halves and taken up for drug and other toxin extraction as stated.

In CSF, two mL CSF was mixed with five mL chloroform and filtered using Whatman filter paper. The extract was added with five mL methanol and sonicated for fifteen minutes. Finally, the sonicated sample was stored at temperature of 30 °C and used to detect drug in various analytical techniques.

3.6. High-Performance Liquid Chromatography Analysis

A Shimadzu LC-2030C, HPLC system was used to determine drug concentration (autosampler, pump with degassing unit Five Lines, and photo diode array detector) (Shimadzu, Japan). RP C18 column with a 5 μm packing as a stationary phase (Phenomenex, USA). At 254 nm, the

elution was performed at a flow rate of 5 mL/min with UV detection. A combination of acetonitrile with water (50:50) was used as the mobile phase. The flow rate was controlled at 1.0 mL/min. The effluent was monitored at 210 nm for 20 minutes with the drug retention time 6.0 min. Injection volume was 10 μL. The concentration of drug was determined from two standard curves in the range of 0.05–5 μg/mL (r^2=0.99992) and 1–50 (r^2=0.99999) μg/mL.

3.7. Gas Chromatography-Mass Spectrometry Analysis

Mass spectrometry (MS) is a strong analytical technique that can be utilized in a variety of applications including pharmaceutical studies, where high sensitivities are frequently required. The MS transfer line temperature was 310 °C and the source temperature was kept at 200 °C. Initial oven temperature was kept at 100 °C with a grasp time of 1 minute and was increased in linear ramp rate of 10 °C to a final temperature of 270 °C with a final hold time of 5 minutes and one μL was injected the sample. The mass spectrometer was operated in electron impact ionisation (EI) positive mode and full scan spectra in the mass range of 40-500 amu were acquired. Agilent DA Express data analysis software was used to collect and process the data and mass spectra libraries were tested for drug identification.

GC-MS instrument chromatographic analysis was carried out on an Agilent 7820A GC system equipped with 5977E mass selective detector (MSD) with triple axis detector (TAD). It augments signal-to-noise by merging resourceful ion collection and strengthening with the purging of neutral noise. DB-5 column width 0.25 μm, film thickness (30 m X 0.25 mm I.D. Germany) was used for separation. Splitless injection was used and the carrier gas helium was used at a flow rate of 1.2 mL/min. The data was acquired and processed by Agilent DA Express data analysis software and mass spectra libraries are screened for identification of drugs and poisons.

3.8. Attenuated total reflectance -fourier transform infrared spectroscopy analysis

Interactions of chemical bonding between structures can be studied using ATR-FTIR spectroscopy when samples are placed on platinum Diamond internal reflection elements (IREs). In this procedure, the IR radiation is first absorbed so as to penetrate the end of the IRE and reproduces down the length of the IRE. The IR radiation enters into the samples through the surface of the IRE with a short distance (~1 μm). The sample was placed on a diamond crystal mounted in attenuated total reflectance (ATR) cell (Perkin Elmer, Monza, Italy). Fourier transform infrared spectroscopy (FTIR) measurements were carried out with spectrophotometer (Perkin Elmer). The spectra were recorded at 4 cm^{-1} resolution, and 15 scans were collected over the wavenumber region 4,000−400 cm^{-1}.

ATR -FTIR is performed at room temperature in the range between 4000–400 cm^{-1} in Figure 9 (a) and (b) to detect any interaction between the drugs and the polymer matrix [27]. The fundamental vibrations of chemical bonds are due to the absorption of radiation, it also provides information related to the presence or absence of specific functional groups, as well as chemical structure of compounds. It takes place through ion exchange of F^- with -OH groups [28]. The spectra are collected as a result of 24 running scans at a resolution of 4 cm^{-1}.Software used in ATR-FTIR spectroscopy is “OPUS”. Water absorbs strongly in the mid-IR range [29] where the absorbance of components of samples also occur at 3600-3700 cm^{-1} . Thus, limiting the application of transmission and diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) [30] analyses to desiccated samples. However, ATR-FTIR has been successfully used to resolve analyzing processes occurring in aqueous conditions and at surface–liquid interfaces.

4. Results

The goal of preliminary test was to find the most acceptable and optimum settings for evaluating a universal and effective HPLC method. The C18 column was chosen because reverse phase columns are best for analyzing parameters like ideal mobile phase and optimum pH. The methanol and acetonitrile concentrations were adjusted depending on the asymmetry factor and peak area to produce a symmetric peak with short run duration. Table 1 lists the mobile phases and their compositions, which were used to separate and detect BPHC in this work (Figure 3). A series of systematic trials were conducted to optimize the chromatographic conditions in order to develop a sensitive, precise, and accurate RP-HPLC approach to detect BPHC in pharmaceutical formulations. This method uses pH 6.5 mobile phase buffers, with acetonitrile (50:50) proving to be the most suited.

The mobile phase A of 60:40 (v/v) methanol: water showed acceptable separation, well-resolved peaks, and excellent symmetrical peaks. The chromatogram of this system is presented in reference sample of BPHC, which was observed at a wavelength of max 210 nm. The retention time of BPHC was determined to be 4.10, 5.20, 5.30, and 6.02 minutes, respectively, using a strong baseline. In the obtained chromatogram for this system, the retention time 5.30 is extremely close to the standard chromatogram, which was seen at max 210 nm, as shown in Figure 3. The retention time of BPHC was determined to be 3.92, 5.20, 5.39, 5.75, 6.02, and 6.18 minutes, respectively, with a solid baseline. The retention times of 5.39 and 5.75 have been matched to the standard samples. As a result, the presence of BPHC was confirmed.

Figure 3 depicts the acquired chromatogram of the system, which was seen at a wavelength of 210 nm.The retention time of BPHC was determined to be 3.92, 5.20, 5.39, 5.75, 6.020, and 6.18 minutes, respectively, with a good baseline. The mobile phase A of 60:40 (v/v) methanol: water produced a nice, consistent separation. The BPHC retention duration of 5.39 and 5.75 minutes, respectively, showed a solid baseline. As a result, the HPLC chromatogram will have a good resolution, with the base line at 210 nm, which was the UV detector's optimal wavelength.

A forensic scientist's primary goal is to positively identify any banned material found in a forensic exhibit. GC-MS has been the preferred method for this investigation in most forensic laboratories. The GC-MS technology enables a rapid, semi-automated analysis of the sample and usually provides enough information to identify the substances in question. Figure 4 depicts the mass spectrum of the GC peak at 16.04, 16.51, and 16.56 minutes on a typical gas chromatograph of BPHC in chloroform solution. Figure 5 depicts strong signals at the mass to charge ratios (m/z) 55, 81, 105, 145, 173, 213, 247, 275, 301, 326, 353 and 386 with retention durations of 16.04 minutes. In these fragments, the most relevant BPHC fragmentation ions are 55, 81, 105, 145, 275, and 326.Actually, the molecular mass of BPHC is 324.89. There was a proton gain, and the presence of BPHC in the sample could be proven. At 16.043 retention time, the ropivacaine mass to charge ratio (m/z) is 275, and the bupivacaine the mass to charge ratio (m/z) is 326 (M+H+=325+1=326).

When the retention time was set to 16.516 minutes(Figure 6), the significant signals at m/z 55,104,135,176,207,237,265,294,339and 385 were seen. For BPHC, the reaction monitoring transitions m/z 272, 124 were selected. Obviously, the presence of BPHC in the encountered sample at m/z =325 could be established. When the retention time was set to 16.563 minutes(Figure 7), the main signals at m/z 55,84,98,104,126,176,207,239,266,294,339 and 385 were visible. For bupivacaine, the reaction monitoring transitions m/z 267, 130 were used. It also revealed that desbutyl bupivacaine was present. The presence of BPHC in the encountered sample at m/z = 323.8 could clearly be confirmed. The presence of BPHC in the samples found was then confirmed. It was also able to get a chromatogram at m/z 232 [M-93] that revealed the presence of desbutyl bupivacaine. In these mass spectra, only the signal for ropivacaine at m/z = 274.2 was observed.

The reaction monitoring transitions m/z 289, 140 and m/z 275, 126 were chosen for BPHC, according to Francis Beaudry[31]. Additionally, post-acquisition extracted ion chromatograms at m/z 233.1648 5 ppm (M + 16) and m/z 305.2224 5 ppm (M + 16) confirmed the existence of desbutylbupivacaine, 3 and 4-hydroxylated bupivacaine metabolites. Figure 8 shows the structures of 3-hydroxybupivacaine (a), 4-hydroxybupivacaine (b), and desbutyl-bupivacaine (c).

ATR-FTIR spectra of blood and CSF was detected as phenol -OH group strong stretching in the range 3610-3670 cm^{-1}.-NH Str stretching was recorded as a maximum in the range 3350-3500 cm^{-1}. These evidences are compatible with the presence of BPHC traces in the blood and CSF. Furthermore, the amide groups of υ(CO) slightly shifted towards higher wave numbers, and its intensity slightly decrease with respect to the raw BPHC (Figure 9 a & b). Since these variations were noticeable among three samples. It can be assumed that stomach and CSF strongly interacts with BPHC by means of -CH_2 and -CH_3 bonds. Even if it was not possible to quantify the amount of BPHC in the three samples by the common analytic techniques, this method could be used. C=C aromatic at 1500 cm^{-1} and M-disubstituted at 860-900 cm^{-1}were noted.

5. Discussion

Quantification of BPHC is a common question posed to forensic scientists by law enforcement authorities. As a result, forensic professionals were able to answer the questions using the following ways. The LOD and LOQ for bupivacaine were discovered to be 20 and 65 mg/mL, respectively using the HPLC technique. The LOD and LOQ for bupivacaine were 15 and 48 mg/mL respectively, according to the GC-MS technique. Desbutylbupivacaine, 3-hydroxybupivacaine, and 4-hydroxybupivacaine have detection limits of 13, 8, and 5 mg/mL in GC/MS.[32] All four Bupivacaine(1) and its metabolites Desbutylbupivacaine(2), 3-hydroxybupivacaine(3), and 4-hydroxybupivacaine(4) chemicals have linear ranges ranging from 0.5 to 13.8 mg/mL. All three compounds had their migration duration and peak height reproducibilities and extraction efficiencies assessed. Desbutylbupivacaine and bupivacaine had 3.6 percent relative standard deviation (RSD) peak height reproducibilities, while 3-hydroxybupivacaine and 4-hydroxybupivacaine had 5.9 percent relative standard deviation peak height reproducibilities. All compounds of bupivacaine had 3.4 percent migration time reproducibilities (n = 4).

6. Conclusion

In this case study, chromatographic techniques including HPLC, GC-MS, and spectroscopic technique ATR-FTIR were used in the blood and CSF of postmortem samples to validate the presence of BPHC and its metabolites. These techniques could be used to quantify anaesthetic samples sent to forensic science laboratories for analysis on a regular basis. As a result, monitoring the ion at m/z 324 could be used to detect BPHC. Accordingly these processes a therapeutic amount of bupivacaine

given to the woman was confirmed. While comparing HPLC and GC-MS process, GC-MS technique easily used to detect BPHC and its metabolites. Also detection limits of Desbutylbupivacaine,3-hydroxybupivacaine, and 4-hydroxybupivacaine could be easily detected in GC-MS. ATR-FTIR process is very quick and easily accessible than HPLC, GC-MS using reference sample of BPHC. Hence young scientists and researchers may also use ATR-FTIR method along with chromatographic techniques for giving reliable reports.

Abbreviations

1. BPHC: Bupivacaine hydrochloride
2. CSF: Cerebrospinal fluid
3. EI: Electron impact ionization
4. GC-MS: Gas Chromatography-Mass spectrometry
5. HPLC: High Performance Liquid Chromatography
6. LLE: Liquid -Liquid Extraction
7. MSD: Mass selective detector
8. LOD: Limit of detection
9. LOQ: Limit of quantification

7. TAD: Triple axis detector

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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