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Simultaneous Determination of Lidocaine and Bupivacaine in Human Saliva Using Gas Chromatography-Mass Spectrometry

التحديد المتزامن لليدوكائين والبوبيفاكايين في لعاب الإنسان باستخدام جهاز كروماتوجرافيا الغاز - مطياف الكتلة

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Abstract

Long-acting local anaesthetics have demonstrated effectiveness in managing intraoperative and postoperative pain. However, substances like lidocaine and paraben preservatives can trigger allergic reactions and methemoglobinemia in susceptible individuals. While bupivacaine shares common side effects with other local anesthetics, its adverse effects are amplified due to the physicochemical properties of long-acting local anesthetics. Recently, there has been growing interest in utilizing saliva as a potential bio matrix for drug testing because the drug concentration in saliva directly reflects the free, non-protein-bound drug in plasma. This study involved extracting lidocaine and bupivacaine from saliva collected from healthy male patients using a liquid-liquid extraction method. Gas Chromatography-Mass Spectrometry (GC-MS) with ropivacaine as an internal standard was employed for simultaneous determination of both drugs. The results demonstrated high precision and accuracy, with a limit of detection (LOD) and limit of quantification (LOQ) of 3 ng/ml and 10 ng/ml for lidocaine, and 20 ng/ml and 62 ng/ml for bupivacaine, respectively. This non-invasive and user-friendly method provides highly accurate drug analysis results.

Keywords: Forensic sciences, Local anesthetics, Lidocaine, Bupivacaine, GC-MS.

المستخلص

أثبتت أدوية التخدير الموضعي طويلة المفعول فاعليتها في إدارة الألم أثناء العملية الجراحية وبعدها. ومع ذلك، فإن المواد مثل: الليدوكائين والمواد الحافظة مثل: البارابين يمكن أن تؤدي إلى تفاعلات حساسية وميثيموغلوبينية الدم لدى الأفراد المعرضين للإصابة. في حين أن بوبيفاكايين يشترك في الآثار الجانبية الشائعة مع أدوية التخدير الموضعي الأخرى، إلا أن آثاره الضارة تتضخم بسبب الخصائص الفيزيائية والكيميائية للتخدير الموضعي طويل المفعول. وفي الآونة الأخيرة، كان هناك اهتمام متزايد باستخدام اللعاب كمصفوفة حيوية محتملة لاختبار الأدوية؛ لأن تركيز الدواء في اللعاب يعكس بشكل مباشر الدواء الحر غير المرتبط بالبروتين في البلازما. وقد تضمنت هذه الدراسة استخلاص الليدوكائين والبوبيفاكايين من اللعاب الذي تم جمعه من المرضى الذكور الأصحاء باستخدام طريقة الاستخلاص السائل. وتم استخدام تحليل كروماتوغرافيا الغاز - مطياف الكتلة (GC-MS) مع الروبيفاكايين كمعيار داخلي للتحديد المتزامن لكلا العقارين. وقد أظهرت النتائج دقة عالية، مع حد كشف (LOD) وحد قياس كمي (LOQ) يبلغ 3 نانوغرام/مل و10 نانوغرام/مل لليدوكائين، و20 نانوغرام/مل و62 نانوغرام/مل للبوبيفاكايين، على التوالي. وتوفر هذه الطريقة غير القاسية وسهلة الاستخدام تحليلًا دقيقًا للغاية للأدوية.

الكلمات المفتاحية: علوم الأدلة الجنائية، التخدير الموضعي، ليدوكائين، بوبيفاكايين، جهاز GC-MS.

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1. Introduction

Long-acting local anaesthetics have demonstrated their effectiveness in managing both intraoperative and postoperative pain [1], making them invaluable for prolonged dental procedures and preventing severe pain after various surgeries. Despite their minimal side effects, these anaesthetics can be quite toxic for susceptible individuals, especially with repetitive use [2-6]. Lidocaine 2% viscous jelly, a blend of lidocaine, preservatives, and suspending agents in water, serves as a topical anesthetic for irritated or inflamed mucous membranes of the mouth and throat, and helps reduce gag reflex during X-rays and dental impressions [7]. Lidocaine is easily absorbed through mucous membranes, the gastrointestinal tract, and damaged skin [8], leading to a range of symptoms after toxic doses, including lightheadedness, confusion, and cardiovascular issues [9]. Lidocaine, along with the paraben preservatives, can trigger allergic reactions and methemoglobinemia in susceptible individuals [10]. Since the introduction of long-acting agents like bupivacaine in dental anesthesia in 1983, their usage has rapidly increased. Animal studies have highlighted the heightened systemic toxicity of bupivacaine compared to lidocaine, with severe reactions like central nervous system and cardiovascular issues, often resulting in hemodynamic instability, cardiovascular collapse, and even death [11]. Although many side effects of bupivacaine are common among local anesthetics, the physiochemical properties of long-acting variants intensify their adverse effects. Therefore, it is crucial for dental practitioners employing these long-acting anesthetics to be well-versed in their potential adverse reactions [12]. This awareness is especially important considering the significant increase in drug-related emergency room visits in the United States, which doubled from 2004 to 2009, reaching 4.6 million cases [13].

In India, although there are no specific statistics regarding the number of patients reporting oral irritation after the dental administration of bupivacaine and topical lidocaine, there is a pressing need to develop a swift method for identifying and quantifying these drugs in saliva, especially in medico-legal cases, to ensure appropriate medical intervention. Instances of local anesthetic abuse and toxicity have been documented by the national anti-doping agency and criminalistics laboratories in India [14]. Saliva has recently garnered significant interest as a potential biomatrix for drug testing [15-18]. Unlike urine testing, where common methods of sample adulteration can be easily applied, saliva analysis offers distinct advantages [15]. Research has indicated a direct correlation between drug concentration in saliva and the free, non-protein-bound drug in plasma. Despite the knowledge of drugs being present in saliva for some time, fewer samples are tested compared to urine, mainly due to the limited sample size and the brief detection window, often less than 4 hours, coupled with relatively low drug concentrations. The presence of parent drugs in saliva occurs almost immediately after administration, distinguishing it from urine and perspiration. Interpreting drug concentration in saliva can be intricate due to various factors. Saliva's pH levels can affect the concentrations of certain basic drugs. Therefore, controlled collection conditions are essential to accurately correlate these levels with blood drug concentrations. Given the complexity of the sample matrix and the low concentrations of drugs and metabolites, sample preparation is crucial for preconcentration of analytes and matrix cleanup [19]. Methods such as liquid-liquid extraction and solid phase extraction are commonly employed for sample preparation before analysis. In oral fluid, the predominant species is the parent drug, and there is generally a correlation between oral fluid con-

centration and blood/plasma concentrations. The relatively low drug concentrations in saliva and the limited sample volume available for analysis necessitate the use of micro-extraction techniques, making them both attractive and necessary for this matrix [20-22].

Researchers have employed various analytical techniques like gas chromatography [23-27], liquid chromatography [28], and capillary electrophoresis [29] to analyze anesthetics. Some studies have attempted to determine the local anesthetic-free fraction in serum or plasma using ultrafiltration and micro-dialysis. In other cases, the free fraction of anesthetics was estimated by analyzing correlations between their protein binding degree, pH levels, temperature, and solute concentrations. The method devised in this study utilized liquid-liquid extraction to isolate lidocaine and bupivacaine from saliva samples obtained from healthy male patients. The outcomes demonstrated remarkable precision and accuracy.

2. Experimental

2.1. Materials and methods

Neon Pharmaceuticals Pvt. Ltd (India) provided the standard lidocaine and bupivacaine, while Sun Pharmaceuticals Pvt Ltd (India) supplied ropivacaine, the chosen internal standard. All chemicals used were of analytical reagent grade unless specified otherwise. High-performance liquid chromatography (HPLC) grade solvents, including chloroform, hexane, and methanol, were employed. Saliva samples were collected from healthy male volunteers who were not under the influence of any other drugs.

2.2. Extraction procedure

Saliva extraction was carried out using liquid-liquid extraction. In each milliliter of saliva sample, 100 µl of internal standard solution (0.2 mg/mL in methanol) was added. The mixture was then washed with 5 ml of chloroform, and the resulting organic layer was discarded. The remaining aqueous phase was rendered alkaline by adding carbonate buffer (pH= 9.2) [30] and shaken with 5 ml of hexane for 5 minutes. After centrifugation at 3000 rpm, the organic layer was separated and dried under a nitrogen stream. The dried extract was reconstituted with 100 µl of methanol, vortexed for 30 seconds, and 2 µl of the solution was injected into GC-MS for analysis. The samples were stored at -20°C until further analysis and were thawed prior to analysis. Blank saliva samples were tested to confirm the absence of any drugs or pharmaceutical additives.

2.3. Gas Chromatography-Mass Spectrometry

The analysis was performed using a Perkin Elmer Clarus 600 Gas Chromatograph coupled with a Clarus 600 S Mass Spectrometer and turbomass 5.3.0 software. Samples (2 µl) were injected in splitless mode. The analytes were separated on a ZB-5MS column (15m x 0.25mm x 0.1µm, Phenomenex) using helium as the carrier gas. The GC injection port and interface transfer line were maintained at 210°C and 310°C, respectively. The oven temperature was initially set at 90°C for 1 minute, then ramped up to 290°C at 35°C/min, and finally increased to 310°C at 10°C/min, where it was held for 3 minutes. The injection port temperature was kept at 260°C. The mass spectrometer operated in positive electron ionization (EI) mode with an electron energy of 70 eV. The source temperature was 150°C, and the mass scan was conducted in EI mode between 35-380 a.m.u. A solvent delay of 1.5 minutes was set for each analysis. Quantitation of lidocaine, bupivacaine, and Ropivacaine (IS) was done using m/z values 86, 140, and 126, respectively.

2.4. Preparation of working standard and quality control solutions

Methanol was used to prepare stock solutions of bupivacaine and lidocaine, resulting in a final concentration of 100 μg/ml. Calibration curve standard solutions were derived from the stock solution at concentrations of 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/ml. Methanol-based standards at concentrations of 2.5, 7.5, 75, and 750 ng/ml were prepared from the stock solution to evaluate precision and accuracy. All stock and standard solutions were refrigerated (2 to 8°C) until the day of use.

2.5. Method validation

The methods underwent validation to assess linearity, recovery, accuracy, and precision. Calibration curves were constructed through linear regression analysis, correlating the peak area of lidocaine and bupivacaine with their respective concentrations, employing a weight of 1/x^2. Precision (% relative standard deviation, R.S.D.) and accuracy (% error) were determined for four quality control (QC) samples. Intra-day accuracy and precision were evaluated by analyzing six replicates of each QC point. This process was repeated thrice across 3 days to establish inter-day accuracy and precision. Relative recoveries from saliva were computed for spiked samples ranging from 1 to 20 μg/mL (n=3) by dividing the peak area of the drugs by the peak area of an equivalent concentration of lidocaine and bupivacaine in deionized water.

2.5.1. Matrix effect and Carry over

The matrix effect was determined by comparing the signal of spiked compounds at concentrations of 0.2 μg/ml (LQC), 1 μg/ml (MQC), and 15 μg/ml (HQC) for lidocaine, and 0.5 μg/ml (LQC), 10 μg/ml (MQC), and 20 μg/ml (HQC) for bupivacaine in a methanolic solution of the drug with the signal obtained from drug spiked saliva extracts at the corresponding concentrations.

Furthermore, to investigate potential carryover, a sample devoid of drugs was injected immediately after analyzing a sample containing lidocaine and bupivacaine at concentrations of 18 μg/ml and 22 μg/ml, respectively.

3. Result and discussion

3.1. Chromatographic parameters

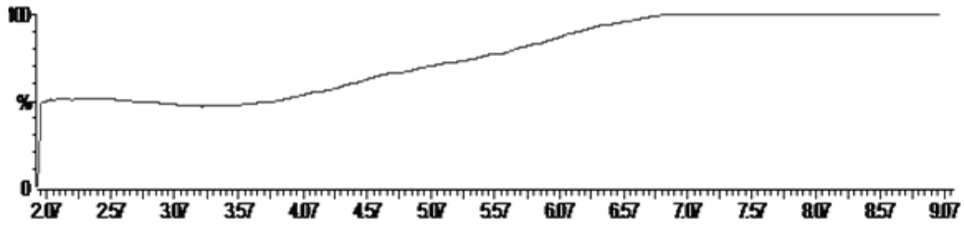
Figure1 displays chromatograms of a blank saliva sample after extraction Figure1 (a), saliva spiked with 1mg/ml of bupivacaine and lidocaine Figure1 (b), and the extracted saliva from a dental ulcer patient taken 15 minutes after intravenous injection of 1 mg/kg of bupivacaine and a topical application of 50mg of lidocaine Figure1 (c). For quantifying lidocaine and bupivacaine, the base peak (m/z) values of 86 and 140 were selected, as shown in figure 2 and 3. Table 1 outlines the chromatographic parameters for bupivacaine and lidocaine, including retention time, relative retention time, number of plates, width, and symmetric factors. Lidocaine eluted at 4.01 minutes, well before the internal standard and other drugs in the sample. Bupivacaine's retention time was 5.34 minutes, while ropivacaine eluted at 5.06 minutes.

3.2. Method validation

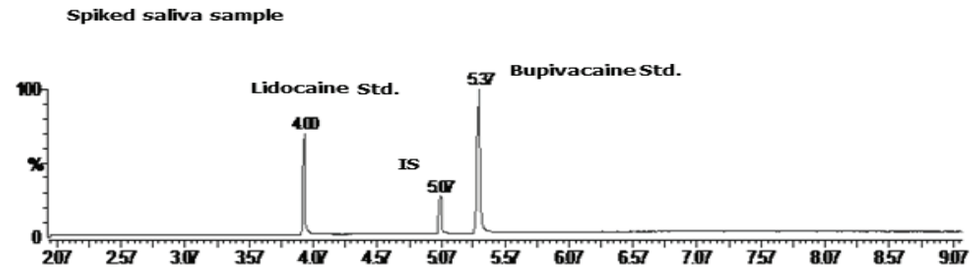
The method was validated according to the society of forensic toxicology guidelines for validation of analytical procedures.

3.2.1. Linearity

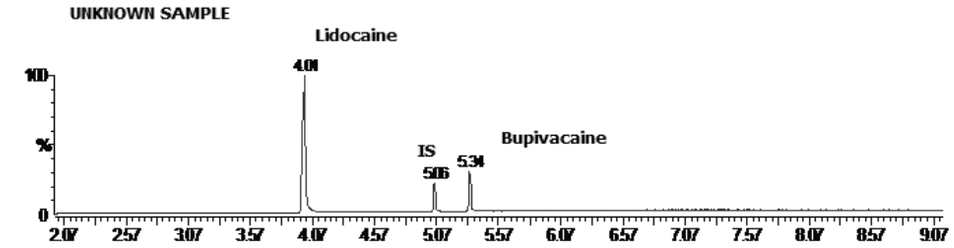
Linearity between the drug amounts and peak area in the mass chromatogram was established in EI mode. The minimum detectable concentration and the lowest quantifiable level for lidocaine and bupivacaine were determined, see Table 2. Lidocaine's LOD and LOQ were found to be 3 ng/ml and 10 ng/ml, respectively, while bupivacaine's values were 20 ng/ml (LOD) and 62 ng/ml (LOQ). These concen-



(a). Total ion chromatogram (TIC) obtained for blank saliva sample.



(b). Total ion chromatogram (TIC) obtained for spike saliva sample



(c). Total ion chromatogram (TIC) obtained for real saliva sample

Figure 1- *Showing the chromatogram obtained for saliva samples.*

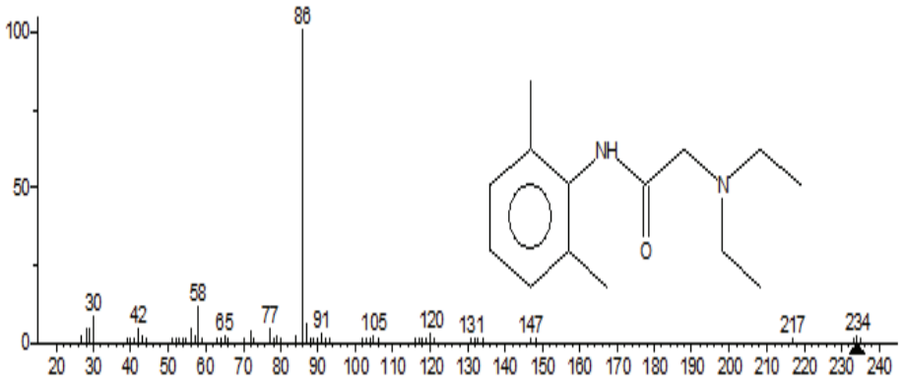
trations demonstrated precision and accuracy with a relative standard deviation of less than 20%. The standard curves for lidocaine and bupivacaine were linear within the concentration range of 1-20 μg/mL, based on three different concentrations, each injected five times. The standard curve equations were as follows: Y=50373X + 90484 (lidocaine, R2=0.9978) and Y= 51473X – 16262 (bupivacaine, R2=0.9985).

3.2.2. Precision

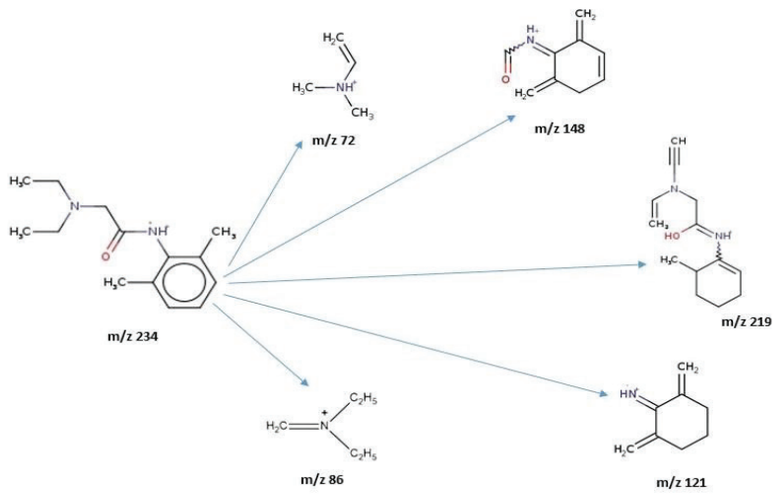
Intraday precision was assessed by analyzing six distinct preparations at concentrations of 3, 8, and 16 μg/mL for lidocaine and 6, 11, and 18 μg/mL for bupivacaine within the same day. To evaluate interday precision, the assays were compared over three consecutive days. The results, detailed in Table 3, indicate the method's favorable precision.

3.2.3. Accuracy

Accuracy was verified by calculating the recovery percentage of lidocaine and bupivacaine solutions at three concentration levels (50%, 100%, and 150% of the specified drug levels) for diluted samples



(a). Lidocaine Mass spectrum.

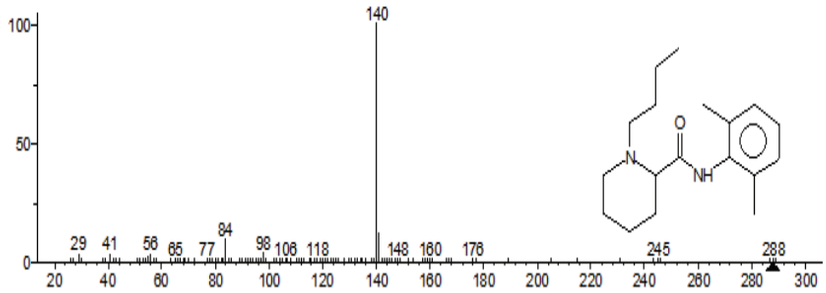


(b). Lidocaine plausible fragmentation.

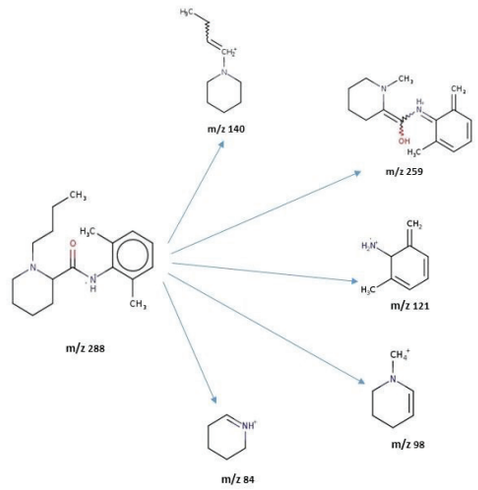
Figure 2- *Lidocaine mass spectrum and plausible fragmentation.*

Table 1- *Chromatographic parameters for lidocaine, bupivacaine and ropivacaine (I.S).*

Parameters	Lidocaine	Bupivacaine	Ropivacaine
Retention time (min)	4.01	5.34	5.06
Relative retention time	0.3	0.56	0.7
Width (min)	0.027	0.034	0.065
Number of plates	248 350	188 970	89 160
Symmetric factor	0.89	0.9	0.96



(a). Bupivacaine Mass spectrum.



(b). Bupivacaine plausible fragmentation.

Figure 3- *Bupivacaine mass spectrum and plausible fragmentation.*

Table 2- *Various parameters obtained for method validation of lidocaine and bupivacaine.*

Parameters	Lidocaine	Bupivacaine
LOD	3 ng/ml	20ng/ml
LOQ	10ng/ml	62ng/ml
Linearity	0.15 μg-18 μg/ml	0.4μg-22 μg/ml
Slope	50373	51473
Intercept	90484	-16262
R^2	0.9978	0.9985
Regression equation	Y=50373X + 90484	Y= 51473X - 16262

Table 3- *Showing intraday and interday reproducibility data.*

Compound	Amount added (μg/ml)	Intraday reproducibility		Interday reproducibility	
		Average amount found (μg/ml)	+SD (n=6)	Average amount found (μg/ml)	+SD (n=3)
Lidocaine	3	2.32	0.67	2.51	1.16
	8	7.88	1.32	7.72	1.82
	16	14.44	0.54	14.92	0.94
Bupivacaine	6	5.73	1.21	5.64	1.03
	11	9.28	0.37	9.53	1.51
	18	15.52	0.89	15.54	1.72

Table 4- *Data showing the recovery of lidocaine and bupivacaine in saliva (spiked and real sample).*

Compound	Amount added (μg/mL)	Amount found (μg/mL)	Recovery %	RSD [%] (n=3)
Saliva sample (spiked with lidocaine)	3	2.54	84.5	2.34
	8	7.32	91.2	1.54
	16	14.15	88.4	2.21
Saliva sample (spiked with bupivacaine)	6	5.38	89.6	3.12
	11	9.42	85.7	4.22
	18	15.75	87.5	1.56
Patient saliva (lidocaine)	-	8.38	-	1.92
Patient saliva (bupivacaine)	-	4.40	-	1.18

(three replicates for each concentration). To assess accuracy, the determined concentrations were compared. The recovery results are presented in Table 4.

3.2.4. Matrix effect and carry over

The matrix effect had a minimal impact on the signal, remaining below 16% for three different concentrations (LQC, MQC, HQC). Moreover, carryover in samples injected after high-concentration samples was negligible, being less than 1%.

4. Conclusion

The method put forward here is straightforward, precise, consistent, and rapid, enabling the analysis of Lidocaine and bupivacaine in saliva samples. This approach holds potential for routine use in scenarios involving accidental lidocaine and bupivacaine poisoning, drug abuse, and doping incidents. The study results demonstrate that employing Liquid-Liquid Extraction (LLE) followed by Electron Ionization (EI) detection in Gas Chromatography-Mass Spectrometry (GC-MS) is an efficient analytical technique for

determining the concentrations of lidocaine and bupivacaine in saliva samples. Significantly, this method's sensitivity arises from the choice of matrix, allowing the retrieval of the parent drug without undergoing metabolization. Remarkably, no previous method has been identified for simultaneous extraction and quantification of local anesthetics from saliva. Due to its brief runtime, this method is suited for routine applications in drug testing, forensic investigations, and clinical research laboratories.

Conflict of interest

The authors declare no conflicts of interest.

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