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RESEARCH ARTICLE

A Capillary Electrophoretic Method for the Analysis of Bupivacaine and Its Metabolites

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Abstract

The article studies a capillary electrophoretic (CE) method for the analysis of urinary extracts of the local anesthetic, bupivacaine, and its three main metabolites, desbutylbupi vacaine, 3'-hydroxybupivacaine, and 4'-hydroxybupivacaine, in rat urine. After collection of blank urine, the rats were given a 20 mg/kg intramuscular injection of bupivacaine, and urine was collected for 12 h after dosing. CE analyses were performed using the CAPEL®-205 capillary electrophoresis systems. The data was collected using the Elforan® specialized software. The use of methanol to reduce peak tailing was investigated at different concentrations, but 20% and 30% v/v were proved to be the most optimal at the preparatory stage of the experiment. The resolution of 3'-hydroxybupivacaine and 4'-hydroxybupivacaine was 1.09, 0.98, 0.89 and 0.89 at 15, 40, 70 and 110 s, respectively. The initial resolution (Rs) of desbutylbupi vacaine was achieved with all studied injection periods as Rs = 1.09, 0.97, 0.96 and 0.96 at 15, 40, 70 and 110 s, respectively. Separation efficiencies for 3'- and 4'-hydroxybupivacaine were312×10^3, 257×10^3, 196×10^3 *u* 169×10^3µlat injection times of15, 40, 70 and 110 s, respectively. The results showed that the mass of bupivacaine, desbutylbupi vacaine, and 3'- and 4'-hydroxybupivacaine significantly recovered in the rat urine after the dose was administered. The recoveries as a percent of the dose were 0.04, 0.80, 0.15 and 0.05% for desbutylbupi vacaine, bupivacaine, 3'-hydroxybupivacaine, and 4'-hydroxybupivacaine, respectively. Separation of bupivacaine and its metabolites was achieved in 15 min. A particular advantage of this approach over published HPLC methods is that separation of the two hydroxy positional isomers of bupivacaine is possible. A number of unknown peaks were also observed in the electropherograms from the rats dosed with bupivacaine. These did not correspond to any peaks appearing in the blank urine samples. Characterization of these unknown peaks may prove useful for the further understanding of bupivacaine metabolism.

Keywords: *Bupivacaine, Capillary electrophoresis, Desbutylbupivacaine, Hydroxybupivacaine, metabolites.*

Introduction

Bupivacaine is one of the most commonly used local anesthetics, particularly in obstetric anesthesia. Bupivacaine has a long-acting anesthetic effect after subcutaneous or intravenous injection, compering to other local anesthetics such as procaine and lidocaine.

However, the toxic effect is greater for the cardiovascular and central nervous systems [1], thus the patient may suffer from heart failure. Since the effects and associated toxicity are directly related to the concentration of local anesthetic in the systemic circulation, it is very important to determine the concentration of bupivacaine in plasma and other biological fluids. Capillary electrophoresis (CE) is known for its high efficiency, high resolution and ultra-small sample volume, and therefore is an alternative to HPLC.

It has been proven to be anexcellent separation technique for analytical and bioanalytical chemistry [2]. Optical detection is the most used in commercial CE, particularly UV absorbance and fluorescence detection.

However, the detection sensitivity of UV is relatively low due to the short optical path length. Fluorescence detection, namely laser-induced fluorescence, is highly sensitive, but quite expensive. Among other sensitive detection methods, electrochemical detection (ED) is limited by the poor stability, and mass spectrometry (MS) is expensive. However, no reported method in the literature clearly describes an analytical procedure for the simultaneous separation and analysis of bupivacaine and its desbutyl, 3'-hydroxy and 4'-hydroxy metabolites in a biological matrix.

Therefore, it is important for CE to develop a highly selective, sensitive, stable and economical detector, which makes the study relevant. The study describes the capillary electrophoretic (CE) and solid-phase extraction (SPE) conditions necessary for the simultaneous analysis of bupivacaine and the aforementioned metabolites in urine from rats administered a therapeutic dose of bupivacaine. The results showed that this method is simple, fast, selective and sensitive for the determination of bupivacaine in urine. The development of a CE-MS method employing electrospray ionization is currently underway in our laboratory for the identification of further metabolites of bupivacaine.

Literature Review

Capillary electrophoresis (CE) has long been used in the analysis of pharmaceuticals and their metabolites to a wide range of biological environments. The separation in CE is based on the charge-to-size characteristics of the analyte of interest rather than partitioning as in liquid chromatography (LC) [3]. Moreover, the separation efficiency of CE is frequently much higher than for LC.

For these reasons, separations not possible by LC may be possible by CE. Bupivacaine is one of the most commonly used local anesthetics, particularly in obstetric anesthesia, but careless and excessive use of bupivacaine and isoflupredone might lead to direct or indirect continuous contamination of food commodities [4].

Indirectly, bupivacaine can be released into the environment through patient excreta and hospital waste management. Thus, trace concentrations can be released into surface, ground and drinking waterthatlead to pollution of water and it is potential toxicity to human health. The infected water can also be used for agricultural irrigation and animal production, causing contamination of both plants and animals such as pigs, cattle and chicken through residues in muscle tissue, milk and eggs, respectively. The determination of the drug residues in animal foods is an integral part of food safety, due to their differences in functions, chemical structure, and physicochemical properties [5]. Since the effects and associated toxicity are directly related to the concentration of local anesthetic in the systemic circulation, it is very important to determine the concentration of bupivacaine in plasma and other biological fluids.

The development of analytical methods permitting simultaneous determination of bupivacaine and its metabolites is important in understanding its fate and distribution in tissues and biological fluids after therapeutic administration. The analysis of bupivacaine and its metabolites in biological tissues and fluids has been performed by thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC).

The earliest approaches to the analysis of bupivacaine and its metabolites made use of TLC and GC methods. The detection of bupivacaine, 3'-hydroxybupivacaine, and desbutylbupi vacaine in rat urine by GC-MS after liquid-liquid extraction has been previously reported [6].

Using this approach in conjunction with NMR analysis, three further metabolites, *N*-butyl-pipecolyl-2-amide and two mono-hydroxylated bupivacaine metabolites were identified in the urine samples. The GC-MS method incorporating selected ion monitoring was also used to determine bupivacaine and desbutylbupi vacaine in maternal, fetal and analysis neonatal plasma following its administration as an epidural anesthesia for Caesarean section [7]. The majority of analytical methods in recent years for the simultaneous analysis of bupivacaine and its metabolites in biological matrices have made use of HPLC in conjunction with liquid-liquid extraction [8], phase extraction (SPE) [9] or column switching schemes [10].

The analysis of bupivacaine, desbutylbupi vacaine and 4'-hydroxybupivacaine in human

serum, plasma and urine using liquid-liquid extraction and HPLC, has been reported by several authors [8, 11]. Using the chromatographic conditions described, excellent separation of all three compounds was possible. However, no separation or quantitation of 3'-hydroxybupivacaine was reported.

The use of capillary electrophoresis for the separation and quantitation of bupivacaine enantiomers in human plasma [12] and therapeutic monitoring of bupivacaine in drain fluid collected from patients after pulmonary surgery [13] has been reported. However, no reported method in the literature clearly describes an analytical procedure for the simultaneous separation and analysis of bupivacaine and its desbutyl, 3'-hydroxy and 4'-hydroxy metabolites in a biological matrix.

The study describes the capillary electrophoretic (CE) and solid-phase extraction (SPE) conditions necessary for the simultaneous analysis of bupivacaine and the aforementioned metabolites in urine from rats administered a therapeutic dose of bupivacaine. The results showed that this method is simple, fast, selective and sensitive for the determination of bupivacaine in urine.

Problem Statement

The problem of choosing a method for studying the properties of pharmacological drugs remains quite relevant, especially for anesthetics drugs used in medical practice during surgery. The widespread use of bupivacaine as local anesthetics is known, but no reported method in the literature clearly describes an analytical procedure for the simultaneous separation and analysis of bupivacaine and its desbutyl, 3'-hydroxy and 4'-hydroxy metabolites in a biological matrix.

In this regard, it is important for CE to develop a highly selective, sensitive, stable and economical detector, which makes the study relevant. Therefore, the aim of our study was to describe the capillary electrophoretic (CE) and solid-phase extraction (SPE) conditions necessary for the simultaneous analysis of bupivacaine and the aforementioned metabolites in urine from rats administered a therapeutic dose of bupivacaine. To achieve goal of the study, the following tasks were set up:

- Analyze the results of using different methods for studying the properties of bupivacaine and its metabolites;
- Determine the most effective method for obtaining high-quality results, analysis conditions, necessary reagents and available equipment;
- Find the most optimal order of migration of solutions of bupivacaine metabolites during capillary electrophoresis;
- Investigate the injection time and the resolution efficiency of bupivacaine and its metabolites (desbutyl, 3'-hydroxy and 4'-hydroxy);
- Determine the performance of extracted bupivacaine and its three metabolites in rat urine within 12 hours after the administration of a therapeutic dose of bupivacaine.

Methods and Materials

Materials and Reagents

Bupivacaine hydrochloride and prilocaine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Chloroform, used in the extraction method, was 99.9% A.C.S. grade. HPLC grade methanol was used in both the extraction method and as part of the CE buffer.

All other chemicals were reagent grade or better and were used as received.All water was purified by a double distilling and deionizing. Two buffers were used in this work. The CE buffer consisted of 30% v/v methanol and 70% v/v 214 mM ammonium acetate, pH 5.0. The extraction buffer consisted of 300 mM sodium bicarbonate, pH 10. All solutions were filtered through a 0.22 pm nylon filter prior to use.

Laboratory Rat Urine Collection

Female rats weighing 300-350g were hold in a metabolism cage (Zoon Lab). Urine was collected over a 12 h period in collection tubes submerged in dry ice. During the collection period, the animals were provided with food and water. After collection of blank urine, the rats were given a 20 mg/kg intramuscular injection of bupivacaine, and urine was collected for 12 h after dosing.

From rat 1, 11.2 mL urine was collected, while 8.0 mL was collected from rat 2. All urine samples were frozen until use, when they were thawed, extracted, and immediately analyzed by CE.

CE apparatus

CE analyses were performed using the CAPEL®-205 capillary electrophoresis system (Lumex-Marketing LLC, St. Petersburg) in accordance with the requirements of Technical Regulations CU TR 004/2011 on the safety of low-voltage equipment in the framework of the Customs Union and TR CU 020/2011 "Electromagnetic compatibility of technical means", and the Directive 2014/30/EU of the European Parliament on the harmonisation of the laws of the Member States relating to electromagnetic compatibility [14].

The data was collected using the Elforan® specialized software. A window was made in the capillary by placing a drop of concentrated sulfuric acid on the capillary, heating the droplet, wiping the exposed area with a tissue, and finally cleaning with methanol and water. All washings, sample applications, and sample elutions were performed at a flow rate of approximately 1 mL/min. The chloroform extract was evaporated to dryness and reconstituted in 0.200 mL of 0.1% formic acid, 20% v/v methanol. Reconstitution of the sample was aided by vortexing for 1 min, after which the sample was electro kinetically injected into the CE capillary.

Preparation of Stock and Standard Solutions

A stock solution of 1.5 mM desbutylbupivacaine, bupivacaine, 3'-hydroxybupivacaine, and 4'-hydroxybupivacaine and a stock solution of 1.0 mM prilocaine were prepared in water and stored at 4°C. Standards were prepared daily by serial dilutions of the two stock solutions into 20% v/v methanol and 0.1% v/v formic acid.

Statistical Reliability of Results

Statistical processing of data obtained on quantitative determination of local anesthetics of the studied concentrations displays positive repeatability and reproducibility of the results within the recommended analytical area. The coefficient of variation does not exceed 5%, and the mean error of the result is 1.42%.

Results

The CE separation of desbutylbupivacaine, 3'-hydroxybupivacaine, 4'-hydroxybupivacaine, bupivacaine, and prilocaine was investigated at several run buffer values. The experimental run buffers for the pH 4.5, pH 5.0, and pH 6.0 separations consisted of 70% v/v 214 mM ammonium acetate and 30% v/v methanol, while run buffer for the pH 7.0 separation consisted of 70 % v/v 171 mM potassium phosphate and 30% v/v methanol.

The order of migration is desbutylbupi vacaine (1), bupivacaine (2), and 3'-hydroxybupi vacaine (3) and 4' hydroxybupi vacaine (4), in all electropherograms. At pH 4.5, baseline resolution was achieved for all compounds with an analysis time of 15 min. With an increasing the pH of the buffer to 5.0 permitted baseline separation of all compounds in a reduced separation time of 14 min (Fig. 1).

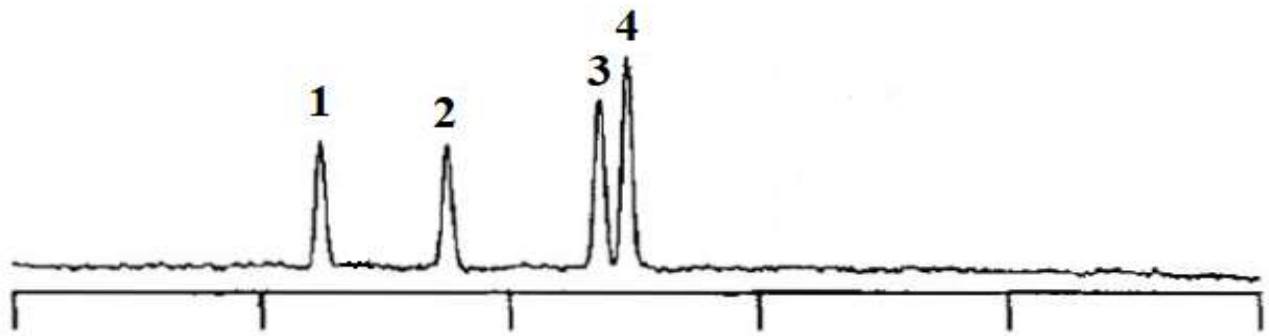

Figure 1: Electropherograms of separation of desbutylbupivacaine (1), bupivacaine (2), and 3'-hydroxybupivacaine (3) and 4 'hydroxybupivacaine (4) by capillary electrophoresis (CE) performed at pH 5.0

With the use of a pH 6.0 buffer, the analysis time was further improved to 1 min. This co migration of hydroxyl bupivacaine isomers was more pronounced with the use of a pH 7.0 run buffers. As the purpose of this pH optimization study was to define a set of CE run buffer conditions permitting baseline resolution of all compounds in as short an analysis time as possible, a run buffer pH of 5.0 was chosen for all future work.

It was noted that significant peak tailing occurred when aqueous buffers were employed. The use of methanol to reduce peak tailing was investigated at different concentrations, but 20% and 30% v/v were proved to be the most optimal at the preparatory stage of the experiment. With the use of 20% v/v methanol in the run buffer, peak tailing was reduced but not eliminated. Methanol at 30% v/v was found to eliminate peak tailing; thus, this concentration of methanol was used in the run buffer for all further analyses.

In the initial stages of this investigation, vacuum, hydrodynamic, and electrokinetic injection schemes were investigated. As the injection matrix is acidic, bupivacaine, its metabolites and prilocaine all exist in their ionized forms and are amenable to electrokinetic injection. The selection of electrokinetic injection for the final method was based on the better limits of detection associated with this injection technique compared to vacuum or hydrodynamic injection. While hydrodynamic injection was slightly more reproducible than electrokinetic injection, 1.6% *versus* 2.5% (n =3), electrokinetic injection provided more than a 12-fold improvement in detection limit. The effect of the injection time on the sensitivity and resolution of the CE method was also studied.It can be seen that the analyte response increases with increasing injection time; however, the resolution of 3'-hydroxybupivacaine and 4'-hydroxybupivacaine decreases as the injection time is increased.

The resolution of 3'-hydroxybupivacaine and 4'-hydroxybupivacaine was 1.09, 0.98, 0.89 and 0.89 at 15, 40, 70 and 110 s, respectively. The initial resolution (Rs) of desbutylbupi vacaine was achieved with all studied injection periods as Rs = 1.09, 0.97, 0.96 and 0.96 at 15, 40, 70 and 110 s, respectively. Separation efficiencies for 3'- and 4'-hydroxybupivacaine were*312×10³, 257×10³, 196×10³ u 169×10³*µlat injection times of 15, 40, 70 and 110 s, respectively. Although the use of longer injection times would result in improved limits of detection for this CE method, an injection time of 40 s was chosen as it permitted good resolution of all compounds, particularly that of the hydroxybupi vacaine isomers.

The detection limit using this 40 s injection time proved sufficient, when used in conjunction with SPE, for the analysis of bupivacaine and its desbutyl and hydroxy metabolites in urine. To demonstrate the utility of this method, the CE method was applied to the analysis of bupivacaine in the urine of two female rats previously administered a 20 mg/kg intramuscular dose of bupivacaine (Fig. 2).

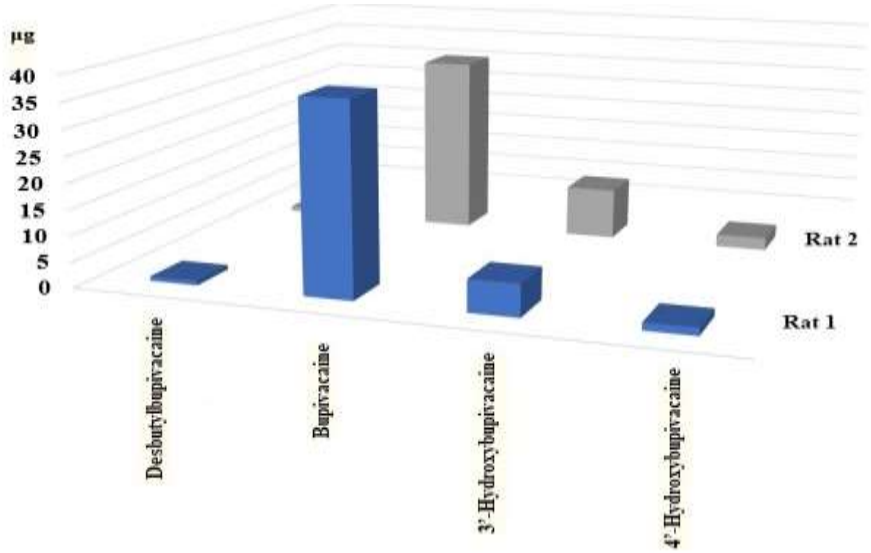

Figure 2: Recoveries of bupivacaine and its metabolites in rat urine 12h after dosing with 20 mg / kg bupivacaine

The results showed that the mass of bupivacaine, desbutylbupivacaine, and 3'- and 4' hydroxybupivacaine recovered, in addition to their recovery as a percentage of the administered dose. The recoveries as a percent of the dose were 0.04, 0.80, 0.15 and 0.05% for desbutylbupivacaine, bupivacaine, 3'-hydroxybupivacaine, and 4'-hydroxybupivacaine, respectively.

Discussion

Bupivacaine is a potent local anesthetic with unique characteristics from the amide group of local anesthetics, first discovered in 1957. Local anesthetics are used in regional anesthesia, epidural anesthesia, spinal anesthesia, and local infiltration [15]. It is known that bupivacaine infiltration in the preoperative period significantly reduce postoperative pain in accordance with placebo at the second and sixth hour after surgery.

Preoperative injection of bupivacaine is useful for controlling pain in the postoperative periodfor up to 24 hours [16]. Local anesthetics generally block the generation of an action potential in nerve cells by increasing the threshold for electrical excitation. However, the study of bupivacaine and its metabolitesproperties remains relevant, since the amount of bupivacaine used varies greatly.

Thus, it was shown that the urination time and restoration of the patient's motor functions are statistically comparable when using bupivacaine in a sufficiently high dose of 12.5 mg [17]. Therefore, "selective spinal anesthesia" or a combination of adjuvants technique to speed up the resolution of the unit for outpatient surgery is required. In the current study, less overall recovery of bupivacaine and its metabolites was achieved than in these studies [18, 19].

This lower recovery is likely due to the fact that urine was only collected for a 12 h period as opposed to a 24 h period in the previous studies.It has been shown that bupivacaine, lidocaine and azaleptin can be determined in biological fluids in the presence of coextracted substances using electrophoretic spectra and quantitative indicators [20]. The influence of several parameters, such as the buffer composition, pH, the ratio of the concentration of substances on the separation of the analyte is well known [21], but the nature of this effect remains poorly studied. The use of methanol to reduce peak tailing was investigated at different concentrations, but 20% and 30% v/v were proved to be the most optimal at the preparatory stage of the experiment. The addition of methanol to the working buffer decreases the peak concentration for some compounds [22]. Higher concentrations of methanol were not investigated because evaporation of the organic portion of the run buffer at higher organic concentrations can lead to irreproducible peak heights and migration times [23].

The effect of the injection time on the sensitivity and resolution of the CE method has also been studied. It can be seen that the analyte response increases with increasing injection time; however, the resolution of 3'-hydroxybupi vacaine and 4'-hydroxybupivacaine decreases as the injection time is increased. Lower resolution of the two hydroxy metabolites using longer injection times is due to the injection of a longer analyte band.

A longer analyte band leads to decreased resolution because the effective length of capillary available for separation of analytes is decreased. However, according to the aim of our study, namely the development of a method necessary for the simultaneous analysis of bupivacaine and the aforementioned metabolites in urine, it is clear that the CE can be used in combination with SPE.

The method offers superior resolution of the 3'- and 4'-hydroxy metabolites than can be achieved using reverse-phase LC methods [24, 25]. When compared to the use of radiochemical detection in earlier studies [26, 27] or the analysis of the hydroxy isomers, the CE method represents a more convenient and rapid approach for their determination in urine.

In addition, the CE method can be interfaced directly to a mass spectrometer (MS) to provide further identification of unknown peaks in the electropherograms of the urine extract, as shown in Fig. 1. However, equipment problems related to the reliability of automatic sampling and capillary contamination should be resolved before adopting these methods as routine methodologies in the laboratory for analysis of compounds.

He development of a CE-MS method [28, 29] employing electrospray ionization is currently underway in our laboratory for the identification of further metabolites of bupivacaine.

Conclusions

The study demonstrated an analytical method for the determination of the local anesthetic, bupivacaine, and its metabolites in rat urine using SPE and CE. The excellent resolution of the CE method makes this a viable approach for the *in vivo* analysis of bupivacaine and its metabolites.The order of migration is desbutylbupivacaine (1), bupivacaine (2), and 3'-hydroxybupivacaine (3) and 4' hydroxybupivacaine (4), in all electropherograms.

At pH 4.5, baseline resolution was achieved for all compounds with an analysis time of 15 min. With an increasing the pH of the buffer to 5.0 permitted baseline separation of all compounds in a reduced separation time of 14 min. It was noted that significant peak tailing occurred when aqueous buffers were employed.

The use of methanol to reduce peak tailing was investigated at different concentrations, but 20% and 30% v/v were proved to be the most optimal at the preparatory stage of the experiment. The resolution of 3'-hydroxybupivacaine and 4'-hydroxybupivacaine was 1.09, 0.98, 0.89 and 0.89 at 15, 40, 70 and 110 s, respectively. The initial resolution (Rs) of desbutylbupi vacaine was achieved with all studied injection periods as Rs = 1.09, 0.97, 0.96 and 0.96 at 15, 40, 70 and 110 s, respectively. Separation efficiencies for 3'- and 4'-hydroxybupivacaine were312×10^3, 257×10^3, 196×10^3 u 169×10^3µlat injection times of 15, 40, 70 and 110 s, respectively. The results showed that the mass of bupivacaine, desbutylbupi vacaine, and 3'- and 4'-hydroxybupivacaine significantly recoveredin the rat urine after the dose was administered. The recoveries as a percent of the dose were 0.04, 0.80, 0.15 and 0.05% for desbutylbupi vacaine, bupivacaine, 3'-hydroxybupivacaine, and 4'-hydroxybupivacaine, respectively.

A particular advantage of this approach over published HPLC methods is that separation of the two hydroxy positional isomers of bupivacaine is possible. A number of unknown peaks were also observed in the electropherograms from the rats dosed with bupivacaine. These did not correspond to any peaks appearing in the blank urine samples. Characterization of these unknown peaks may prove useful for the further understanding of bupivacaine metabolism. Qualitative determination of these unknowns by CE-MS is currently being carried out in our laboratories.

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