







RESEARCH ARTICLE

Synthesis and Characterization of 3-Hydroxybupivacaine and Deuterated 3-Hydroxybupivacaine for Use in Equine Medication Regulation

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ABSTRACT

Bupivacaine is a local anesthetic widely used in equine and human medicine. Use of bupivacaine in performance horses is regulated because its ability to block pain means that it can be misused for advantage in performance horses. In racing regulation, bupivacaine is classified by the Association of Racing Commissioners International (ARCI) as a Class 2 Penalty Class A Foreign substance, the detection of which can lead to significant penalties. In horses, bupivacaine is metabolized by Phase-I hydroxylation to yield 3-hydroxybupivacaine, which is then glucuronidated to yield the Phase-II metabolite bupivacaine-3-hydroxyglucuronide, which is excreted at relatively high concentrations in equine urine. Standard regulatory procedure during urinalysis is to perform an enzymatic hydrolysis, thereby enabling subsequent detection of 3-hydroxybupivacaine, the primary analyte used for bupivacaine regulation in urine samples from competition horses. We now report on the synthesis of 3-hydroxybupivacaine and deuterated 3-hydroxybupivacaine from piperidine-2-carboxylic acid in six successive steps with moderate yield. The compounds were characterized by ¹H and ¹³C NMR and their purity ascertained by HPLC-MS. The deuterated bupivacaine and 3-hydroxybupivacaine were further confirmed by HRMS. The synthesis of these compounds provides certified reference standards and stable isotope-labeled internal standards for drug testing in competitive equine sports including horse racing.

1 | Introduction

Current analytical procedures regarding the quantitative analysis of chemical and biological drugs and their metabolites recommends the use of reference standards for the substance and stable isotopic-labeled analyte as the internal standard for accurate quantitative analysis of drugs and their metabolites [1]. This technique is now the standard in mass spectrometry for quantitative forensic, metabolic, therapeutic, and diagnostic studies [2–6]. Advantages resulting from using stable isotope-labeled

analyte as an internal standard include preserving the similarities in the physicochemical properties (excluding mass) of the targeted compound and the labeled standard. When subjected to the analyte matrix, the labeled internal standard shows similar responses to matrix effects but remains distinguishable from the forensic analyte based on mass differences. Other advantages include high reproducibility, specificity, and quantitative precision of the analytical method because it overcomes the challenges associated with calibration and sample preparation matrix effects (such as intermolecular interactions and changes

in solubility and pH) that occur with analysis of drugs and drug metabolites in biological matrices [7].

The high sensitivity of current drug testing in sporting events has increased the requirement for reference standards and stable isotopic-labeled internal standards for quantitative and qualitative analysis of equine drug metabolites [8–10]. In regulated equine events, liquid chromatography-mass spectrometry is regularly employed for drug testing analysis, and the mass differences between the analyte and internal deuterated standard provides qualitative and quantitative analysis of the substance agent of interest [11–13]. Thus, this work aims to expand the availability and use of deuterated internal standards in equine medication regulation of local anesthetics that have been reported to be detected in equine samples.

Local anesthetics are a class of medications widely used to relieve/block local pain in human and veterinary medicine [14, 15]. Despite the long clinical use of these medications, their precise mode of action and active metabolites in the body are incompletely characterized [2]. Bupivacaine [(*RS*)-1-butyl-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide, C₁₈H₂₈N₂O, molar mass 288.435 g/mol] (Figure 1) is a potent local anesthetic that is on the World Health Organization's (WHO) list of essential medicines and is widely used in human and veterinary medicine. Bupivacaine is primarily used to produce local blockade of pain perception, for example, during minor surgery [16]. In equine medicine, however, it is routinely used in lameness diagnosis, where the local nature of the anesthetic response is used to block local lameness-producing lesions, to confirm or deny a specific location for the lameness-producing lesion(s). These capabilities also mean that local anesthetics such as bupivacaine can be used to block lameness in horses about to race or perform. Thus, use of these medications is considered inappropriate during or close to essentially all regulated equine events [17, 18].

Bupivacaine is therefore a strictly regulated substance in horse racing in the United States and is classified by the Association of Racing Commissioners International (ARCI) as a Class 2, Penalty Class A Foreign Substance [19]. As such, the detection of either bupivacaine itself or any of its Phase-I or Phase-II metabolites in a blood or urine sample can result in significant penalties for the connections of the horse. In post-bupivacaine administered urine samples, the principal urinary metabolite present is the Phase-II metabolite, 3-hydroxybupivacaine glucuronide, present at times in relatively high urinary concentrations. To use this urinary glucuronide metabolite in bupivacaine regulation, the urine sample is first subjected to enzymatic hydrolysis by beta-glucuronidase, which releases the Phase I 3-hydroxybupivacaine metabolite for recovery and analytical identification [20, 21]. This analytical procedure allows identification and quantitation of the Phase-I 3-hydroxybupivacaine metabolite by liquid chromatography/mass spectrometry (LC-MS) as we have previously described [16–18] and as depicted in Figure 1.

Given these regulatory realities 3-hydroxybupivacaine is often the critical urinary regulatory analyte for the regulation of bupivacaine in equine events worldwide [22–24]. There is therefore an ongoing regulatory need for a reference standard and a stable isotope internal standard for the Phase-I 3-hydroxybupivacaine metabolite of bupivacaine, and also for a corresponding deuterated internal standard for bupivacaine itself, the synthesis and characterization of each of which we now report. An additional consideration in structuring these synthetic procedures was that bupivacaine is chemically and pharmacologically similar to two other local anesthetics, ropivacaine and mepivacaine. The synthetic scheme presented for 3-hydroxybupivacaine and deuterated 3-hydroxybupivacaine was designed to also apply to the synthesis of regulatory and internal standards for the Phase I 3-hydroxy metabolites of ropivacaine and mepivacaine.

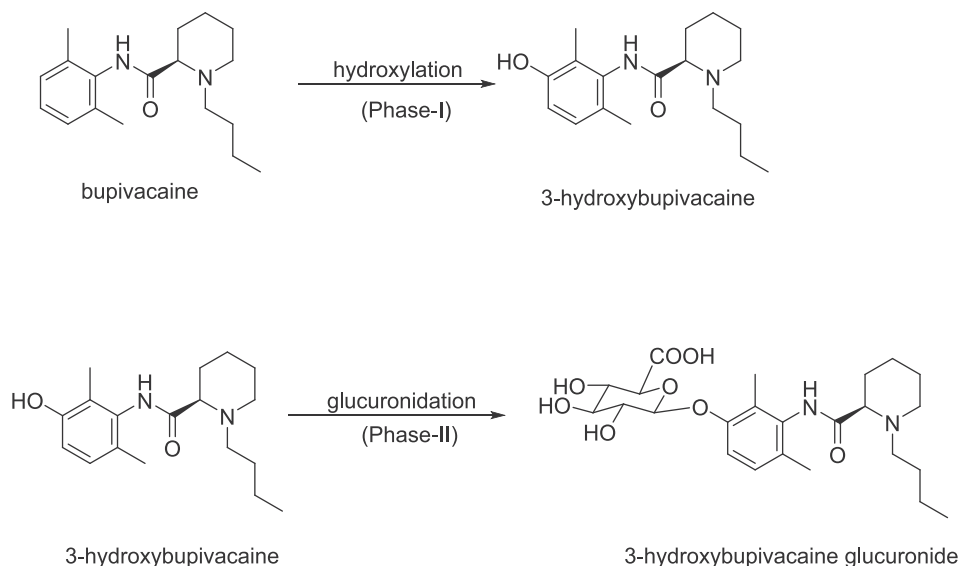


FIGURE 1 | Chemical structures of bupivacaine, 3-hydroxybupivacaine and 3-hydroxybupivacaine glucuronide, the Phase-II bupivacaine metabolite found at high concentrations in equine urine. Enzymatic hydrolysis of urine samples containing glucuronidated 3-hydroxybupivacaine is routinely performed to allow analytical recovery and identification of 3-hydroxybupivacaine, the Phase-I metabolite of bupivacaine and a widely used urinary regulatory analyte.

2 | Results and Discussion

Drug testing in racing horses is the longest established, broadest based, and most highly sensitive routine testing of any animal on earth, including humans [13–15]. Equine drug testing officially commenced in 1904 and has always tested for substances of concern at the highest available level of sensitivity [25, 26]. Testing started with saliva testing and then later moved to urine, and testing is now performed in urine, blood, and hair as required, with expanded testing for performance enhancing substances an ongoing process [13]. Scientifically correct testing creates a requirement for reference standards and stable isotope internal standards to allow detection and accurate quantification of therapeutic substances such as local anesthetics and or their equine metabolites in blood or urine samples from competition horses. Studies on the detection in plasma and urine of local anesthetics such as bupivacaine have focused on its Phase-I 3-hydroxybupivacaine metabolite as the regulatory analyte of choice [16]. In this work, we describe the synthesis of a reference standard for 3-hydroxybupivacaine and 3-hydroxybupivacaine- d_9 and also bupivacaine- d_9 for worldwide use as standards in the quantitative analysis of 3-hydroxybupivacaine, recovered as the principal regulatory analyte in the regulation of bupivacaine in equine forensic science.

The synthetic protocols described in this communication are also amenable to the synthesis of similar standards of parent substances or the 3-hydroxy metabolites of other local anesthetics such as 3-hydroxyropivacaine and 3-hydroxymepivacaine, chemically and pharmacologically related local anesthetics whose use in performance horses is also regulated by detection of their Phase-I 3-hydroxy metabolites recovered from plasma or urine samples.

Our synthesis of 3-hydroxybupivacaine and 3-hydroxybupivacaine- d_9 (7) was achieved in six successive steps (Figure 2). The first step involves the Boc-protection of the amine group of piperidine-2-carboxylic acid with di-*tert*-butyl dicarbonate in 1,4-dioxane affording 1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic acid (1) in 90% yield. This step was followed by

amide coupling with 2,6-dimethylaniline to yield *tert*-butyl 2-((2,6-dimethylphenyl)carbamoyl)piperidine-1-carboxylate and subsequent deprotection of the Boc group in trifluoroacetic acid/DCM to yield *N*-(2,6-dimethylphenyl) piperidine-2-carboxamide (2), with a 50% overall yield in these two steps. The *N*-(2,6-dimethylphenyl)piperidine-2-carboxamide generated was then alkylated with iodobutane- d_9 to yield bupivacaine- d_9 (4) at about 80% yield. Nitration in H_2SO_4/HNO_3 nitrating mixture afforded 3-nitrobupivacaine- d_9 (5) in about 86% yield, followed by reduction in Pd/C to give 3-aminobupivacaine- d_9 (6) in quantitative yield. The final step to make the desired compound involve diazotization of 3-amino derivative in 2-M H_2SO_4 using $NaNO_2$ and hydrolysis upon refluxing to afford 3-hydroxybupivacaine- d_9 (7) in 14% yield. This procedure can also be adapted for the synthesis of reference standards and internal standards for other structurally related local anesthetics, namely, mepivacaine and ropivacaine.

In the United States, lidocaine and mepivacaine are the only two local anesthetics currently approved by the ARCI for use in horses close to racing [16]. Lidocaine is regulated by means of a plasma threshold of 20 pg/mL of “total 3-hydroxylidocaine in plasma or serum” with a suggested dose of “200 milligrams of lidocaine as its hydrochloride salt administered subcutaneously” and a suggested “withdrawal time” of 72 h [6]. Mepivacaine is regulated by means of a urinary threshold of “10 nanograms of total hydroxymepivacaine per milliliter of urine or above level of detection of mepivacaine in plasma” with a suggested dose of a “single 0.07 milligrams per kilogram subcutaneous dose of mepivacaine” and a suggested “withdrawal time” of 72 h [19]. These regulatory criteria were made possible by the synthesis and commercial availability of the required certified reference standards and stable isotope internal standards by a University of Kentucky research program. For bupivacaine, however there are currently no ARCI plasma or urinary concentration thresholds, suggested dosage amounts, route of administration information, or suggested withdrawal times, which the availability of these 3-hydroxybupivacaine reference standards and deuterated internal standards addresses [18].

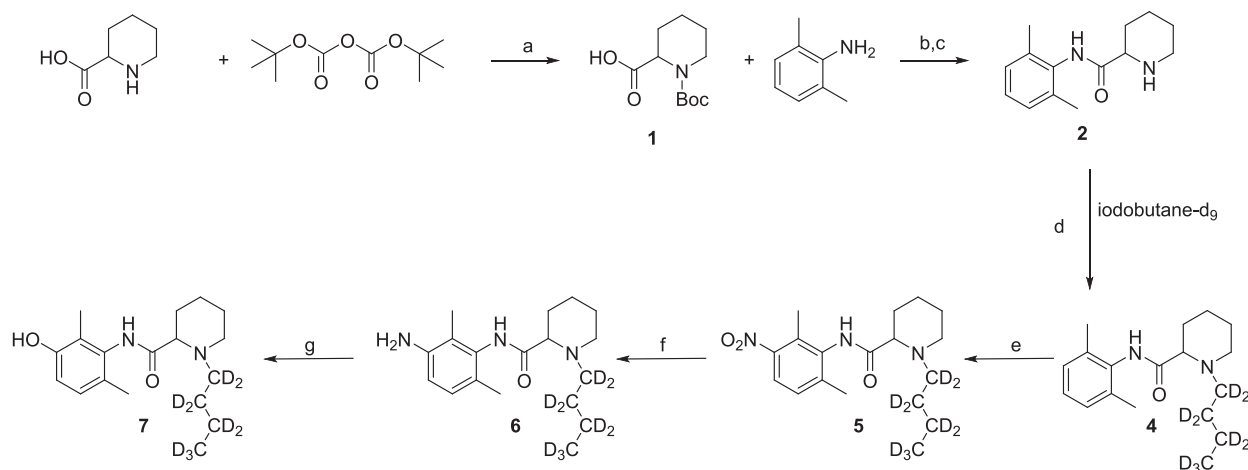


FIGURE 2 | General synthetic scheme for the synthesis of bupivacaine- d_9 and 3-hydroxybupivacaine- d_9 (a) 1,4-dioxane, NaOH, 0°C to RT, overnight. (b) EEDQ, Et_3N , $CHCl_3$, reflux, overnight, (c) TFA/DCM, 24 h, (d) K_2CO_3 , isopropanol, reflux, 8 h, (e) HNO_3/H_2SO_4 , 0°C to RT, overnight. (f) H_2 , Pd/C, ethanol, RT, 48 h (g) 2M H_2SO_4 , $NaNO_2$, 0°C to RT 30 min, reflux 30 min.

Bupivacaine is a highly effective local anesthetic, with a 0.25 mg/site perineural administration being the Highest No Effect Dose (HNED) for bupivacaine in horses. Administration of this 0.25 mg/site dose of bupivacaine to horses produced peak urinary concentrations of 3-hydroxybupivacaine recovered from urine of approximately 30 ng/mL. Given that the dose administered was an experimentally determined HNED, this value has been presented as a urinary concentration of recovered 3-hydroxybupivacaine that is unlikely to be associated with a pharmacological/local anesthetic effect in horses [16].

Figure 3 presents the history of bupivacaine detections/violations in US racing horses as per the ARCI. Bupivacaine detections commenced in 1983, shortly before the introduction of ELISA testing in equine forensic chemistry, with about 15 identifications in the 5 years between 1983 and 1988. In the 90s, there were about five total reported identifications, three in the years 2000 to 2010, and then one identification in 2016, a history suggesting good in place regulatory control of the use of bupivacaine in racing horses.

The synthesis and availability of these standards for 3-hydroxybupivacaine, deuterated 3-hydroxybupivacaine and bupivacaine will allow the performance of research on the forensically correct regulation of bupivacaine in competition horses. In particular, the 3-hydroxybupivacaine standard and its deuterated internal standard will allow accurate quantitation of plasma or urinary concentrations of 3-hydroxybupivacaine, the Phase-I metabolite (regulatory analyte) recovered post enzymatic hydrolysis [20, 21] of blood or urine samples containing 3-hydroxybupivacaine glucuronide, the major urinary bupivacaine metabolite detected in equine urine following bupivacaine administration.

In closing, this 3-hydroxybupivacaine standard and the corresponding deuterated internal standard will be made available to equine researchers, forensic scientists, and racing chemists worldwide through an in-place licensing agreement between Frontier BioPharm LLC and the University of Kentucky. Availability of these standards will allow the racing industry to establish regulatory guidelines for the appropriate clinical use of bupivacaine in performance horses related to competitive events. These guidelines will include plasma/urinary

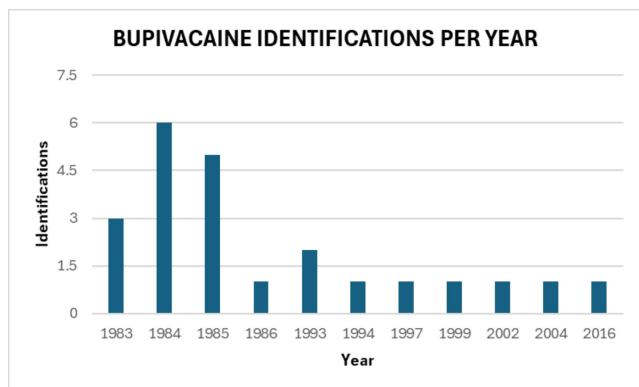


FIGURE 3 | Association of Racing Commissioners International (ARCI) reported bupivacaine violations from 1983 to 2016, showing a consistent decline in the number of bupivacaine regulatory violations in US racing.

concentration thresholds linked to suggested dosages and withdrawal time guidelines [22–24]. Such guidelines will enable equine veterinarians to more confidently use this highly effective local anesthetic in clinical situations where it is the most effective and appropriate treatment for specific clinical conditions in performance horses.

3 | Experimental

The overall scheme for the synthesis of 3-hydroxy bupivacaine and 3-hydroxybupivacaine- d_9 is as presented in Figure 2.

3.1 | General Information

All solvents used in this work were purchased from Greenfield Global (ACS grade) Shelbyville, KY, and were used without further purification. Piperidine-2-carboxylic acid was purchased from VWR (Radnor, Pennsylvania). 2,6-Dimethylaniline and 1-bromobutane were purchased from Sigma-Aldrich (St. Louis, Missouri), whereas EEDQ was purchased from Matrix Scientific (Columbia, South Carolina). Deuterated iodobutane was purchased from CDN Isotopes (Quebec, Canada). Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA), and the spectra were recorded on a Bruker Avance NEO 400-MHz spectrometer. The samples were calibrated for ^1H NMR (CDCl_3 $\delta = 7.26$ ppm), ^{13}C (^1H -decoupled) NMR (CDCl_3 $\delta = 77.16$). In addition to spectroscopic characterization, the purity of all compounds was assessed by RP-HPLC using an Agilent Technologies 1100 series HPLC instrument and an Agilent Phase Eclipse Plus C18 column (4.6 mm \times 100 mm; 3.5- μm particle size) and spectra data at 260 nm were reported. All compounds were found to be >95% pure. HRMS data were acquired by direct infusion of sample at a flow rate of 5 $\mu\text{L}/\text{min}$ on a Thermo Scientific Q Exactive Mass Spectrometer. Once the spectrum stabilized, data were collected for 1 min. All data were collected in positive ion mode, with a full scan range of 100 to 1000 m/z and a resolution of 140,000.

3.2 | Synthesis of 3-Hydroxybupivacaine- d_9

The synthesis of 3-hydroxybupivacaine- d_9 and its analogs were carried out using modified reported procedure [27, 28]. Compounds were characterized by ^1H NMR, ^{13}C NMR spectroscopy (Figures S1–S11); purity was assessed by HPLC-MS (Figures S12–S27) and found to be greater than 97%.

3.2.1 | Synthesis of 1-(*tert*-Butoxycarbonyl)piperidine-2-Carboxylic Acid (1)

To a 1-L flask containing dioxane/water (300 mL, 2:1 v/v) and piperidine-2-carboxylic acid (DL-pipecolic acid) (10.0 g, 77.4 mmol, 1.0 equiv.) at 0°C was added 1 M NaOH (200 mL) gradually till the solid dissolves. This was followed by the addition of di-*tert*-butyl dicarbonate (Boc_2O) (20.28 g, 92.92 mmol, 1.2 equiv.) in dioxane (100 mL), and the reaction was allowed to warm to room temperature. The reaction was stirred overnight at room temperature and concentrated on

vacuum, and the excess (Boc)₂O was removed by extracting with EtOAc (200 mL). The aqueous layer was then diluted with water followed by addition of 6M HCl until white precipitates formed. This acidified mixture containing the product was subsequently extracted with EtOAc (three times), washed with brine, dried with Na₂SO₄, and concentrated to give a colorless oil, which crystallizes to a white solid at room temperature. Yield: 16 g, 90 % ¹H NMR (400 MHz, chloroform-*d*) δ 9.39 (s, 1H), 4.85 (d, *J* = 67.1 Hz, 1H), 4.04–3.94 (m, 1H), 3.01–2.86 (m, 1H), 2.27–2.18 (m, 1H), 1.77–1.58 (m, 4H), 1.46 (s, 9H), 1.37–1.24 (m, 1H) (Figure S1). ¹³C NMR (101 MHz, CDCl₃) δ 178.05 (s), 155.96 (d, *J* = 61.6 Hz), 80.43 (s), 54.24 (d, *J* = 112.7 Hz), 41.68 (d, *J* = 104.9 Hz), 29.75–23.29 (m), 20.89 (s) (Figure S2).

3.2.2 | Synthesis of *N*-(2,6-Dimethylphenyl) piperidine-2-Carboxamide (2)

A mixture of **1** (4.0 g, 17.45 mmol, 1.0 equiv.), 2,6-dimethylaniline (2.5 mg, 20.9 mmol, 1.2 equiv.), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (5.178 g, 20.94 mmol, 1.2 equiv.), and triethylamine (4.4 mL, 31.41 mmol, 1.8 equiv.) in 200 mL of CHCl₃ was heated under reflux overnight. The resulting solution was evaporated, and ethyl acetate (100 mL) was added to redissolve the mixture. The resulting solution was washed successively with 100-mL solutions of 10% HCl, 0.1M NaOH, and H₂O. The mixture was dried over MgSO₄ and evaporated under reduced pressure to give a colorless oil. Deprotection was then carried out as follows: Anhydrous dichloromethane (200 mL) was added to the oil, and trifluoroacetic acid (20 mL)

was added dropwise under nitrogen at room temperature. The reaction was stirred overnight at room temperature, and the solvent was removed under vacuum to obtain the salt. Water (20 mL) was added, and the mixture was basified to pH 11 with 40% NaOH. The compound was then extracted using DCM (50 mL × 3). The combined organic layer was dried with MgSO₄ and concentrated under reduced pressure to give the product of **2** as a white powder. (Yield, 2.0 g, 50 % over two steps) ¹H NMR (400 MHz, chloroform-*d*) δ 8.25 (s, 1H), 7.10–7.05 (m, 3H), 3.42 (dd, *J* = 8, 4 Hz, 1H), 3.15–3.10 (m 1H), 2.82–2.72 (m, 1H), 2.22 (s, 6H), 2.12–2.02 (m, 1H), 1.88–1.80 (m, 2H), 1.67–1.60 (m, 2H), 1.53–1.45 (m, 2H) (Figure S3). ¹³C NMR (101 MHz, CDCl₃) δ 172.46, 135.26, 133.77, 128.32, 127.18, 60.83, 45.96, 30.51, 26.06, 24.14, 18.68 (Figure S4). ESI+ MS (Found) = 233.1 [M + 1]⁺ (calculated = 233.16) (Figure S21) Purity was determined to be > 97% by RP-HPLC: R_f = 3.5 min (Figure S13) using the following method: flow rate: 1 mL/min; λ = 260 nm; eluent A = DI water with 0.1% trifluoroacetic acid; eluent B = acetonitrile with 0.05% formic acid; solvent gradient: 0–4 min (100:0 H₂O:ACN), 6 min (0:100 H₂O:ACN), 10 min (100:0) 17 min until end of run (20 min) (50:50 H₂O:ACN).

3.2.3 | Synthesis of Bupivacaine-*d*₉ (4)

1-Iodobutane-*d*₉ (1.0 g, 5.23 mmol, 3.0 equiv.), K₂CO₃ (723 mg, 5.23 mmol, 3.0 equiv.) were added to a solution of *N*-(2,6-dimethylphenyl) piperidine-2-carboxamide **2** (405 mg, 1.74 mmol, 1.0 equiv.) in isopropyl alcohol (50 mL). Water (10 mL) was added, and the mixture was stirred at reflux

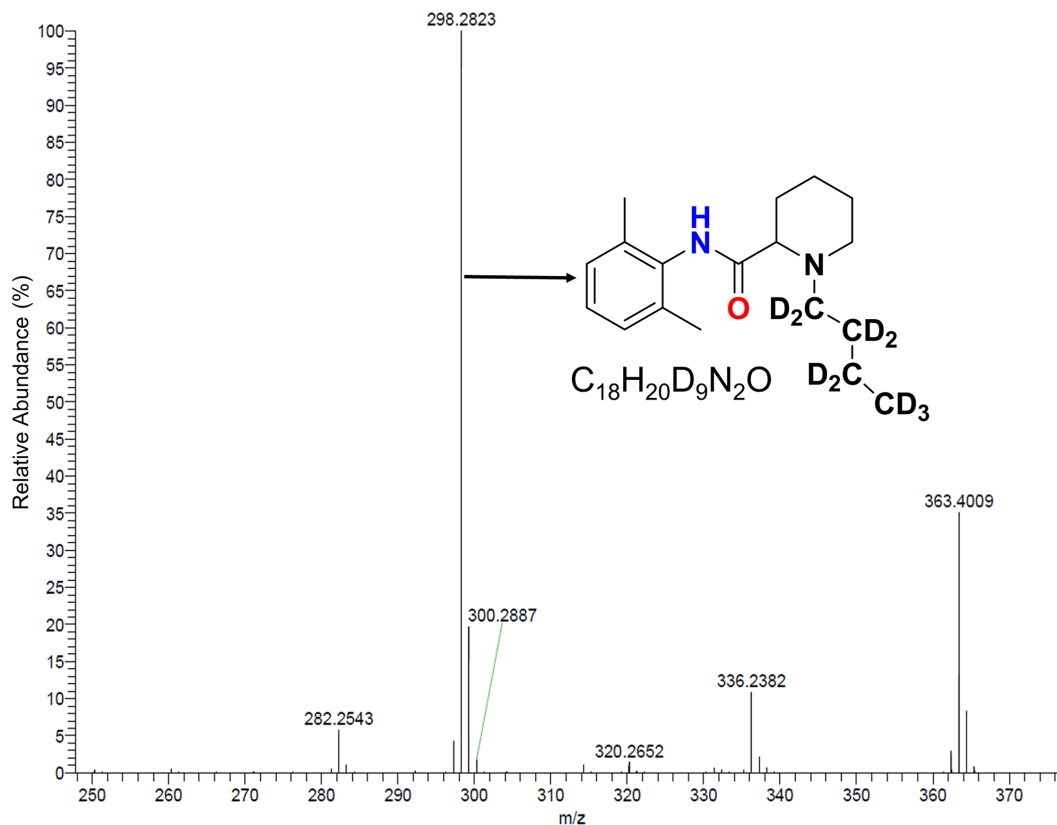


FIGURE 4 | High-resolution mass spectrum of bupivacaine-*d*₉. [M + 1]⁺ = 298.2823.

overnight. The solvents were evaporated, and the residue was treated with 50 mL of a toluene-water mixture (1:1 v/v) under gentle heating at 50°C. The layers were separated, and the organic layer was washed with warm water at 40°C (40 x 2 mL). The organic layer was concentrated and stored in the refrigerator overnight. Recrystallization of the crude product from toluene gave bupivacaine-*d*₉ as an off-white solid. Yield: 470 mg, 95% ¹H NMR (400 MHz, chloroform-*d*) δ 8.16 (s, 1H), 7.11–7.07 (m, 3H), 3.22–3.17 (m, 1H), 2.87 (dd, *J* = 8, 4 Hz, 1H), 2.25 (s, 6H), 2.14–2.01 (m, 2H), 1.82–1.67 (m, 3H), 1.59–1.46 (m, 1H), 1.39–1.28 (m, 1H) (Figure S5). ¹³C NMR (101 MHz, CDCl₃) δ 172.88, 135.17, 133.51, 128.16, 126.90, 68.45, 51.40, 30.66, 28.24, 24.79, 23.38, 18.56, 18.46, 18.17 (Figure S6). HRMS: Found [M + 1]⁺ = 298.2823, calculated [M + 1]⁺ = 298.2844 (Figure 4). Purity was determined to be > 97% by RP-HPLC: R_f = 2.48 min (Figure S18) using the following method: flow rate: 0.500 mL/min; λ = 260 nm; eluent A = DI water with 0.1% trifluoroacetic acid; eluent B = acetonitrile with 0.05% formic acid; solvent gradient: 0–17 min (85:15 H₂O:ACN), 17 min until end of run (20 min) (50:50 H₂O:ACN).

3.2.4 | Synthesis of 3-Nitrobupivacaine-*d*₉ (5)

In a 100-mL round bottom flask containing bupivacaine-*d*₉ **4** (500 mg, 1.74 mmol) and 85% H₂SO₄ (5 mL) at 0°C was added 70% nitric acid (133 μL) dropwise. The reaction was warmed up to room temperature and stirred overnight. The reaction was quenched by pouring the mixture into ice and 45 % NaOH solution added gradually until alkaline (pH = 11). Precipitate was dissolved with ethanol and insoluble salts filtered. The

filtrate was concentrated on rotary evaporator to give a dark oil. Yield: 500 mg, 87%. ¹H NMR (400 MHz, chloroform-*d*) δ 8.37 (s, 1H), 7.72 (d, *J* = 8 Hz, 1H), 7.19 (d, *J* = 8 Hz, 1H), 3.22–3.17 (m, 1H), 2.92 (dd, *J* = 8, 4.0 Hz, 1H), 2.38 (s, 3H), 2.30 (s, 3H), 2.13–2.03 (m, 2H), 1.77–1.67 (m, 3H), 1.54–1.42 (m, 1H), 1.42–1.32 (m, 1H) (Figure S7). ¹³C NMR (101 MHz, CDCl₃) δ 173.60, 149.56, 141.86, 135.95, 131.30, 128.77, 123.50, 68.50, 51.91, 30.64, 25.07, 23.83, 19.88, 15.37 (Figure S8). Purity was determined to be > 95% by RP-HPLC: R_f = 6.51 min (Figure S19) using the following method: flow rate: 0.500 mL/min; λ = 260 nm; eluent A = DI water with 0.1% trifluoroacetic acid; eluent B = acetonitrile with 0.05% formic acid; solvent gradient: 0–17 min (85:15 H₂O:ACN), 17 min until end of run (20 min) (50:50 H₂O:ACN). ESI + Found [M + 1]⁺ = 343.23, calculated [M + 1]⁺ = 343.26 (Figure S26).

3.2.5 | Synthesis of 3-Aminobupivacaine-*d*₉ (6)

3-Nitrobupivacaine-*d*₉ **5** (1.0 g, 3.0 mmol, 1.0 equiv.) was hydrogenated (using a hydrogen balloon) with Pd/C (300 mg, 2.82 mmol, 0.94 equiv., 10%) in EtOH (30 mL) at room temperature for 2 days. The mixture was filtered through celite and silica and the solvent evaporated in vacuo to afford a brown solid that was used in the next step without further purification. Yield: quantitative ¹H NMR (400 MHz, chloroform-*d*) δ 8.16 (s, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.57 (d, *J* = 8 Hz, 1H), 3.53 (s, 2H), 3.22–3.17 (m, 1H), 2.87 (dd, *J* = 8, 4 Hz, 1H), 2.20 (d, *J* = 4 Hz, 1H), 2.14 (s, 3H), 2.11–2.05 (m, 1H), 2.03 (s, 3H), 1.80–1.67 (m, 2H), 1.57–1.45 (m, 1H), 1.41–1.30 (m, 2H) (Figure S9). ¹³C NMR (101 MHz, CDCl₃) δ 173.73, 143.85,

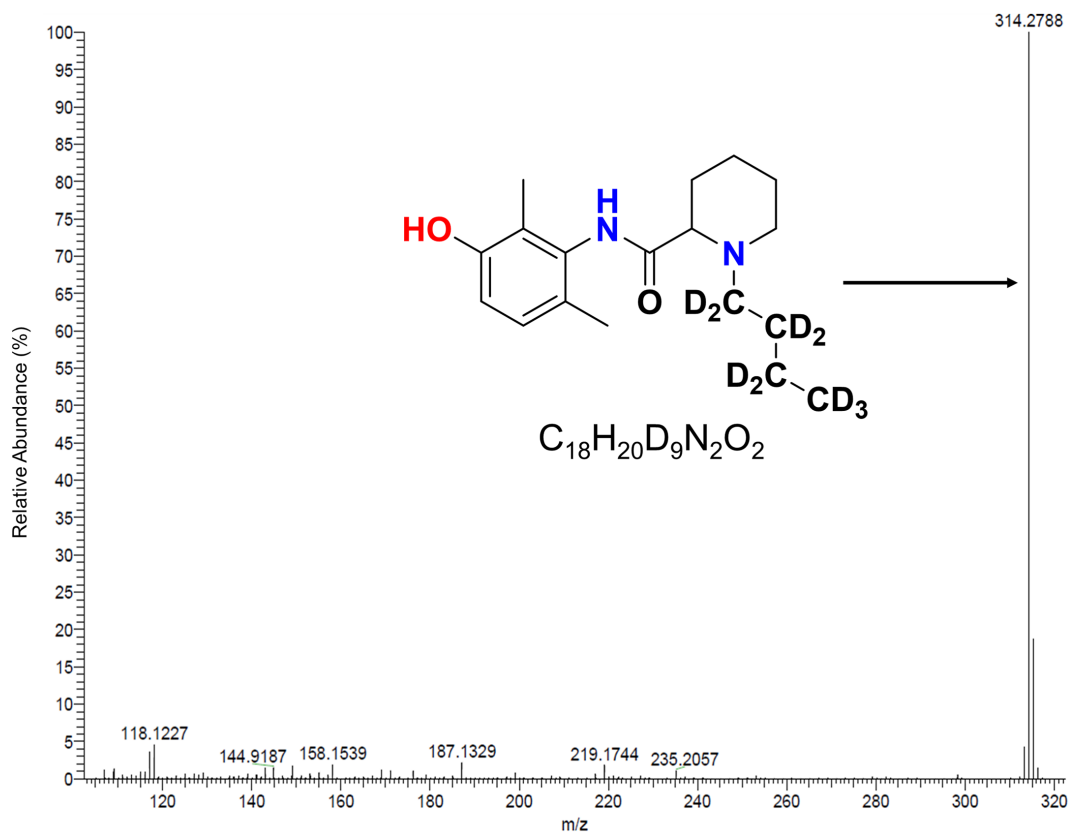


FIGURE 5 | High-resolution mass spectrum of 3-hydroxybupivacaine-*d*₉ [M + 1]⁺ = 314.2788.

134.26, 128.46, 125.33, 120.81, 114.80, 68.87, 52.02, 31.06, 25.25, 23.93, 18.67, 12.86 (Figure S10). Purity was determined to be > 95% by RP-HPLC: $R_f = 2.51$ min (Figure S20) using the following method: flow rate: 0.500 mL/min; $\lambda = 260$ nm; eluent A = DI water with 0.1% trifluoroacetic acid; eluent B = acetonitrile with 0.05% formic acid; solvent gradient: 0–17 min (85:15 H₂O:ACN), 17 min until end of run (20 min) (50:50 H₂O:ACN). ESI + Found $[M + 1]^+ = 313.29$, calculated $[M + 1]^+ = 313.29$ (Figure S27).

3.2.6 | Synthesis of 3-Hydroxybupivacaine-*d*₉ (7)

In a 50-mL round bottom flask was added 3-aminobupivacaine-*d*₉ **6** (700 mg, 2.24 mmol, 1 equiv.) and 2M H₂SO₄ (5 mL) was added at 0°C. The mixture was stirred and a solution of NaNO₂ (154.8 mg, 2.24 mmol, 1 equiv. in 2 mL of DI water) was added dropwise maintaining the temperature below 5°C. The reaction solution was stirred for 60 min at 0°C and stirred at reflux for 10 min. The solution was allowed to cool to room temperature and precipitated with NaOH to give a brown solid. The brown solid was chromatographed on silica using 10% MeOH/DCM and the product was recrystallized from acetone to give the pure compound. Yield: 100 mg, 14.2% ¹H NMR (500 MHz, chloroform-*d*) δ 8.20 (s, 1H), 6.79 (d, $J = 8$ Hz, 1H), 6.47 (d, $J = 8$ Hz, 1H), 6.13 (s, 1H), 3.22–3.17 (m, 1H), 2.89 (dd, $J = 8, 4$ Hz, 1H), 2.31–2.28 (m, 1H), 2.13 (s, 3H), 2.01 (s, 3H), 1.78–1.65 (m, 4H), 1.54–1.47 (m, 1H), 1.37–1.30 (m, 1H) (Figure S11). ¹³C NMR (126 MHz, chloroform-*d*) δ 174.04, 152.99, 133.85, 127.71, 126.67, 122.53, 114.60, 77.80, 77.37, 77.31, 77.11, 76.86, 76.61, 76.52, 68.48, 67.80, 67.23, 51.67, 30.79, 27.70, 24.93, 23.61, 18.29, 11.28 (Figure S12). HRMS: Found $[M + 1]^+ = 314.2788$, calculated $[M + 1]^+ = 314.2794$ (Figure 5). Purity was determined to be > 95% by RP-HPLC: $R_f = 2.3$ min using the following method: flow rate: 0.500 mL/min; $\lambda = 260$ nm; eluent A = DI water with 0.1% trifluoroacetic acid; eluent B = acetonitrile with 0.05% formic acid; solvent gradient: 0–17 min (85:15 H₂O:ACN), 17 min until end of run (20 min) (50:50 H₂O:ACN) (Figure S17).

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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