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A simple and sensitive LC–ESI-MS (ion trap) method for the determination of bupropion and its major metabolite, hydroxybupropion in rat plasma and brain microdialysates

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ABSTRACT

A specific and highly sensitive liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) method for the direct determination of bupropion (BUP) and its main metabolite hydroxybupropion (HBUP) in rat plasma and brain microdialysate has been developed and validated. The analysis was performed on a Bonus RP C18 (100 mm × 2.1 mm i.d., 3.5 μm particles) column using gradient elution with the mobile phase consisting of acetonitrile and ammonium formate buffer (10 mM, pH 4). Plasma samples were analyzed after a simple, one-step protein precipitation clean-up with trichloroacetic acid (TCA), however clean-up for microdialysis samples was not necessary, enabling direct injection of the samples into the LC–ESI-MS system. Signals of the compounds were monitored under the multiple reaction monitoring (MRM) mode of the LC–ESI-MS (ion trap) for quantification. The precursor to product ion transitions of *m/z* 240–184 and *m/z* 256–238 were used to measure BUP and HBUP, respectively. The method was validated in both plasma and microdialysate samples, and the obtained lower limit of quantification (LLOQ) was 1.5 ng mL^{-1} for BUP and HBUP in both matrices. The intra- and inter-day assay variability was less than 15% for both analytes. This LC–ESI-MS method provided simple sampling, rapid clean-up and short analysis time (<9 min), applicable to the routine therapeutic monitoring and pharmacokinetic studies of BUP and HBUP.

1. Introduction

Bupropion (BUP) (Fig. 1a) is an aminoketone derivative which is widely used as an antidepressant in the pharmacotherapy for smoking cessation. Although its exact mechanism in smoking cessation is not known, it is thought to be related to reduced reuptake of dopamine in the mesolimbic system and reduced reuptake of noradrenalin in the locus coeruleus. Also, nicotine is known to cause activation of the mesolimbic system, resulting in dopamine release in the nucleus accumbens similar to BUP's dopamine release effect [1].

BUP is metabolised to three active metabolites; hydroxybupropion, the major metabolite, threohydrobupropion and erythrohydrobupropion. Hydroxybupropion (HBUP), resulting from the hydroxylation of BUP catalyzed by cytochrome P450 enzyme CYP2B6, has 4–7-fold higher C_{max} and nearly 10-fold higher AUC compared to BUP in human plasma. Also, in *in vitro* studies the potency of HBUP was found to be comparable to that of BUP. The metabolites of threohydrobupropion and erythrohydrobupropion were evaluated as total concentration because of their lower concentration compared to HBUP and additionally the potency of these were one-fifth as potent as BUP [2–5].

The analysis of BUP in biological fluids [6–12] and in pharmaceuticals [13–15] has been performed with liquid chromatography [6–10,14], liquid chromatography–mass spectrometry [11,12], electrokinetic chromatography [13] and thin layer chromatography [15]. Also, several studies including chiral separations of BUP and HBUP enantiomers are described [16–18]. Using Cooper's method, BUP stability in human plasma has been reported [19]. In addition to published methods conducted on plasma of different species and on human urine [6,7,9–12], Suckow et al. investigated the pharmacokinetics of BUP and metabolites in plasma and brain homogenates of rats, mice and guinea pigs using Cooper's HPLC method [8]. The disposition of BUP and metabolites were investigated in different species of animals [20]. Reported HPLC methods, however, utilize ultraviolet detection and none analyze both BUP and HBUP in a single assay. Published single assay LC–MS methods do not include the determination of

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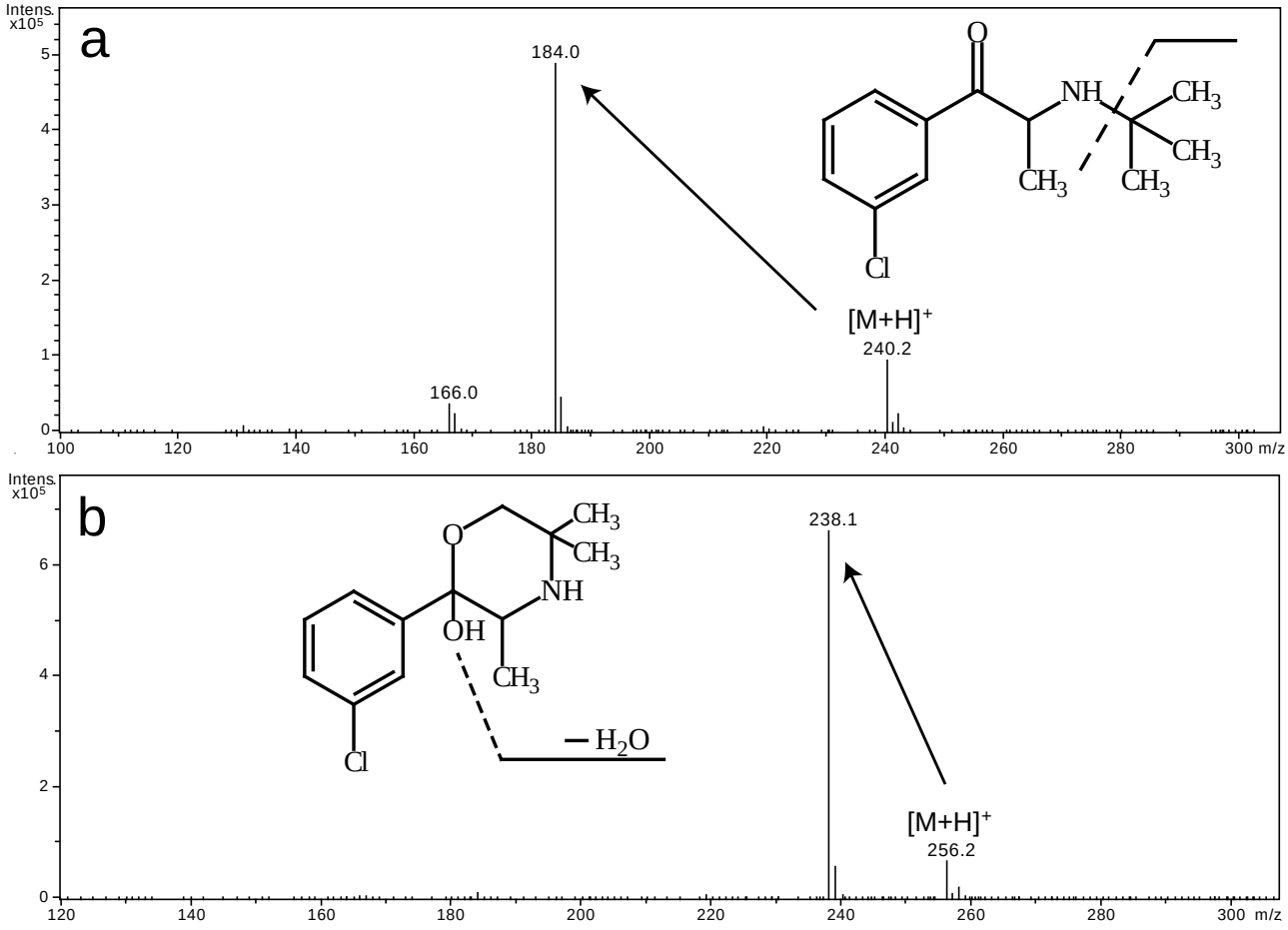

Fig. 1. Mass spectrums of (a) BUP and (b) HBUP obtained in positive-ion mode.

BUP and HBUP in rat brain microdialysate, as well as rat plasma [11,12].

Microdialysis is an *in vivo* sampling technique that involves the determination of unbound endogenous or exogenous compound concentrations by the insertion of a microdialysis probe into selected tissue or (body) fluid and continuous perfusion of a physiological solution. The mechanism is based on the passive diffusion of compounds down a concentration gradient, over a semipermeable membrane. The microdialysis technique provides several advantages for the *in vivo* sampling of drugs present in blood, brain or tissue [21–23]. Sampling can be continuously performed without any fluid loss; this process enables long-term sampling without any interference of the pharmacokinetic behavior of the drug. In contrast to other biological samples, microdialysis samples need no further clean-up because they are protein free and it is possible to directly inject the samples into the analytical system. Moreover, the number of subjects needed has been reduced using individual (freely moving) subjects. Since only the pharmacologically active free fraction of the drug is present in the dialysates, microdialysis is very important in establishing the pharmacokinetic and pharmacodynamic profile of the drug of interest, and microdialysis allows for sampling from specific tissues. This technique has been successfully used for the determination of neurotransmitter [24–28] and drug levels [22,23,29] in animal studies. In some of these reports, the effect of BUP on extracellular dopamine and norepinephrine concentrations was studied by *in vivo* microdialysis [25–28].

Microdialysis sampling has a few limitations, including small volume of the dialysates, low concentration of analytes which originates from the diluting effect of the dialysis procedure, and low probe recovery for the drug of interest [30]. To overcome these problems, the analytical method must be sensitive enough to determine the analyte at low concentrations. The LC–ESI-MS is a promising choice because of high selectivity and sensitivity with the capability of analyzing small sample volumes with low analyte concentrations present in the microdialysates.

The aim of this study is to develop a selective and sensitive LC–ESI-MS (ion trap) assay for the simultaneous determination of BUP and HBUP in rat plasma and brain microdialysates in a single assay, enabling to define the pharmacokinetic profile of BUP and HBUP.

2. Experimental

2.1. Chemicals and reagents

BUP and HBUP reference standards were kindly supplied by GlaxoSmithKline (Istanbul, Turkey). Acetonitrile, ammonium hydroxide, formic acid and ammonium formate were the products of Merck Co. (Darmstadt, Germany) of analytical reagent grade, and used with no further purification. Deionized water was obtained from a Millipore Ultrapure Water System (Molsheim, France). The dialysis perfusate used was Ringer's solution and consisted of 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$ and 125 mM NaCl [22] and all reagents used were of analytical reagent grade or better. The physiologic saline solution (SP) was 0.9% (w/v) sodium chloride.

2.2. Instrumentation and chromatographic conditions

Agilent 1100 series LC system (equipped with a binary pump, a degasser, an autosampler and a thermostatted column com-

partment) (Agilent Technologies, Waldbronn, Germany) and an Agilent MS Trap SL mass spectrometer (equipped with an electrospray ionization (ESI) source) were used for LC–MS trap analysis (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and peak processing were performed with LC–MSD Trap 4.2 Data Analysis Version 2.2 and LC–MSD Trap 4.2 Quant Analysis Version 1.5 Software, respectively (Bruker Daltonik GmbH, Bremen, Germany).

The compounds were separated on a Zorbax Bonus RP C18 (3.5 μm, 100 mm × 2.1 mm i.d.) analytical column. A gradient method consisting of solvent A: ammonium formate buffer (10 mM, pH 4) and solvent B: acetonitrile was used. The gradient elution was: 0 min, 5% B; 0–5 min, linear from 5% to 15% B; 5–10 min, linear from 15% to 70% B; 10–20 min, holding at 70% B for 10 min and then an immediate reduction to 5% B at 25 min; 25–30 min, initial conditions (5% B) for equilibration of the column. Efficient and symmetrical peaks were obtained at 40 °C temperature at a flow rate of 0.4 mL min^{-1} with a sample injection volume of 15 μL.

Ionization of the compounds was achieved by electrospray ionization (ESI) in positive ion mode. The capillary voltage was set at −4402 V for BUP and −4500 V for HBUP. Nitrogen was used as both nebulizer and dry gas. The common parameters of the ESI; nebulizer gas pressure, dry gas flow rate and dry gas temperature were set at 45 psi, 9 L min^{-1} and 350 °C, respectively. The ICC target and the accumulation time were also set at 60,000 and 100 ms, respectively.

Detection of the ions was performed in MRM mode monitoring the precursor to product ion transitions of *m*/*z* 240–184 and *m*/*z* 256–238 for BUP and HBUP, respectively (Fig. 1). Helium was used as a collision gas. Its collision amplitude and precursor ion width for isolation were set at 1 and 2, respectively for both BUP and HBUP.

Time segments (the range of 5–7 min for HBUP and 7–9 min for BUP) were determined for each analyte according to their retention times (Fig. 2). The MS signals of the compounds were individually optimized in these segments by the LC–MS software.

2.3. Preparation of stock solutions, calibration standards and quality control samples

Standard stock solutions of BUP and HBUP were prepared in 0.01 N HCl at a concentration of 1 mg mL^{-1}. These solutions were kept at −20 °C. Another stock solution at a concentration of 3.2 μg mL^{-1} including both BUP and HBUP was prepared in SP. Working standard solutions were prepared by spiking this solution into drug-free rat plasma and dialysate (Ringer's solution) to obtain BUP and HBUP concentrations in the range of 1.56–400 ng mL^{-1}.

The quality control (QC) samples were prepared in an analogous manner to the calibration standards to give concentrations of 1.56, 25 and 400 ng mL^{-1} for BUP and HBUP. All calibration standards and QC samples were stored at −20 °C until analysis.

Following the analysis of calibration standards and QC samples, the evaluation of data was performed using the GraphPad Prism v4.03 program.

2.4. Sample preparation

For plasma samples, a total volume of 0.2 mL of rat blood was collected and centrifuged at 3460 RCF for 15 min. 50 μL of clear supernatant was mixed with 30 μL of TCA (10%, w/v) to precipitate plasma proteins and the mixture was vortexed for 30 s. The vortexed solution was centrifuged at 3460 RCF for 15 min and 15 μL of the final clear supernatant was injected directly into the LC–MS system by the autosampler. For microdialysis samples, 30 μL of dialysate was collected and 15 μL was directly injected to the system for analysis.

2.5. Microdialysis procedure

Adult male Sprague–Dawley rats weighing 250–350 g were obtained from the Laboratory Animal Center at Osmangazi University. Rats were housed in a temperature-controlled laboratory and maintained under a 12 h light–dark cycle with free access to food and water at the beginning of the experiments. All animal experiments were performed in accordance with the principles of animal use and care approved by the ethical committee of the Medical Faculty of Osmangazi University (Approval File No. 163/2010). The rats were anesthetized with urethane (1.5 g kg^{-1}) by intra-peritoneal (i.p.) injection and remained anesthetized throughout the experimental period [24]. In surgery, the right femoral artery and the right femoral vein were cannulated with PE50 cannula, respectively for blood collection and fluid replacement (with 25 μL min^{-1} of SP during the blood sampling). Following the catheterization, a volume of 0.2 mL heparinized SP (containing 100 U mL^{-1} heparine) was administered *via* the right femoral vein to prevent coagulation. Six rats were used in the experiment and 10 mg kg^{-1} of BUP was given by i.p. injection. Following the dose, 0.2 mL of blood was collected every 30 min for 4 h and every 60 min for 6 h, so that blood sampling was continued for 10 h and 15 samples were taken. After each sampling, a volume of 0.2 mL SP was administered to sustain isotonic fluid balance.

After catheterization, the rats were mounted on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Two different microdialysis probes, CMA/12 (PAES): cut-off 100 kDa and 1 mm membrane length and CMA/12 (PC): cut-off 20 kDa and 2 mm membrane length, were used in the experiments. The probe was placed into the "nucleus accumbens" area of the brain. This area was selected because of BUP's dopamine related antidepressant and smoking cessation activity, and rich dopamine innervation of the nucleus accumbens [25–28]. The probe coordinates relative to the bregma were: anterior posterior 1.6 mm, lateral 1.75 mm, depth 8.0 mm from the top of the skull according to the Paxinos and Watson atlas [31]. Body temperature was maintained between 37.1 and 37.3 °C by a thermostatically controlled electric heating pad (CMA150). Before sample collection, the probe was conditioned and perfused with Ringer's solution at a flow rate of 1.0 μL min^{-1} for 1 h. Brain microdialysates were collected at the same flow rate, every 30 min at 4 °C using fraction collector and stored at −20 °C until LC–MS analysis. All microdialysis apparatus were obtained from CMA (Solna, Sweden). The brain microdialysate and the plasma samples were collected simultaneously after i.p. injection of BUP.

2.6. Microdialysis probe calibration

In microdialysis studies, the probe should be calibrated by determining the recovery of the probe to obtain an accurate quantification of the analyte. The relative recovery is defined as the ratio between the concentration of a substance in the dialysate and the concentration of the same substance in the solution outside the probe, usually expressed as a percentage [32].

In vitro recovery by dialysis (R_{dial}) was determined by immersing the microdialysis probe in Ringer's solution containing BUP and HBUP (C_s) at two concentrations (25 and 100 ng mL^{-1}) and perfusing with Ringer's solution ($C_{in} = 0$) at a flow rate of 1.0 μL min^{-1} using a CMA 102 microdialysis perfusion pump. Microdialysate samples were collected every 30 min and dialysate concentrations of the analytes (C_{out}) were determined to establish *in vitro* recovery. The *in vitro* recovery is estimated as follows: $R_{dial} = \frac{C_{in} - C_{out}}{C_{in} - C_s}$ or $R_{dial} = \frac{C_{out}}{C_s}$. Several reports indicate that *in vitro* recovery is insufficient in estimating *in vivo* recovery and the efficiency of microdialysis probes may be affected by *in vivo* conditions, such as the possible interaction of plasma or tissue components

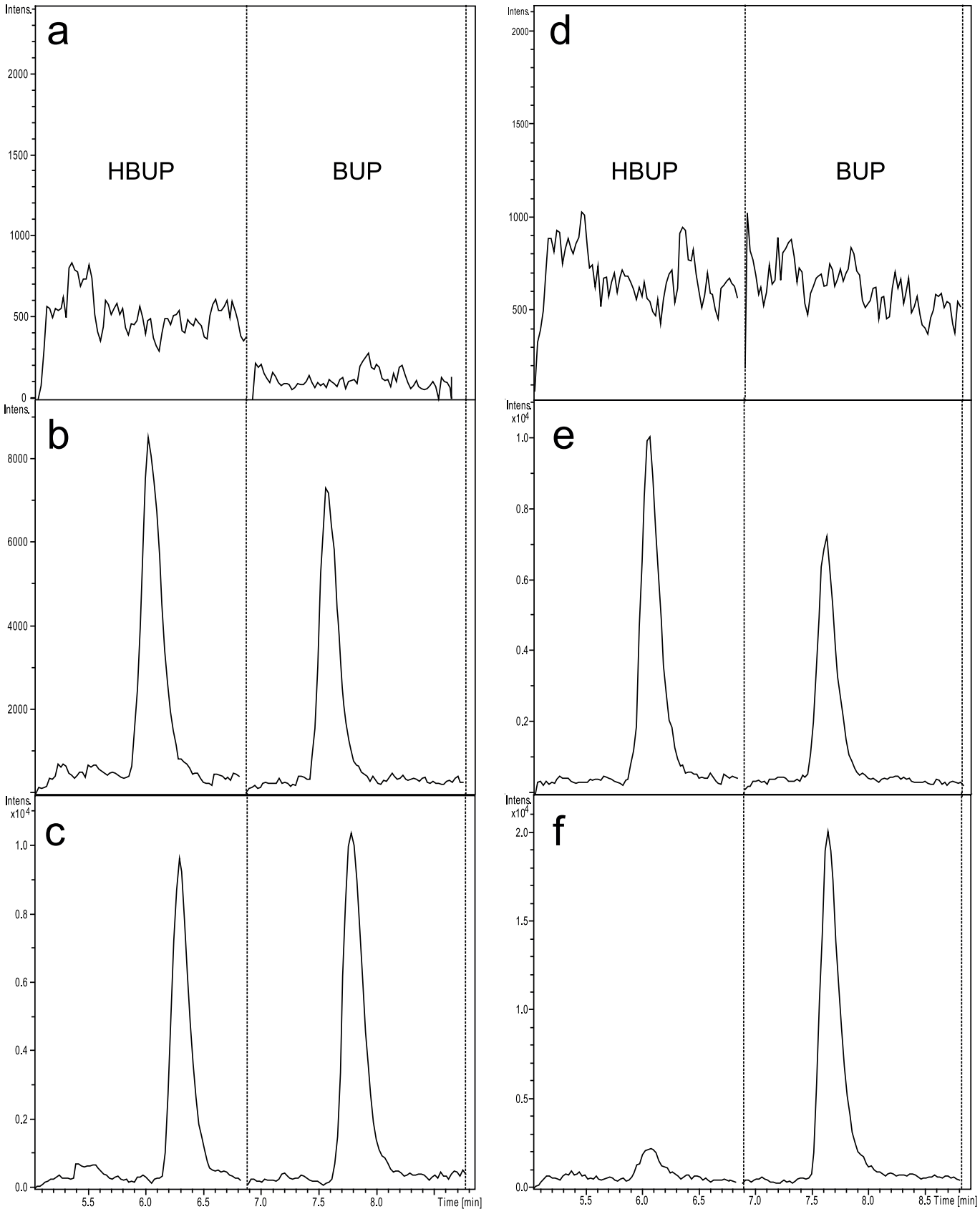

Fig. 2. Typical chromatograms of (a) blank rat plasma collected before drug administration; (b) rat plasma spiked with BUP and HBUP (25 ng mL^{-1}); (c) plasma sample collected at 420 min following BUP administration (10 mg kg^{-1}, i.p.); (d) blank microdialysate collected before drug administration; (e) Ringer's solution spiked with BUP and HBUP (25 ng mL^{-1}); (f) microdialysis sample collected at 165 min following BUP administration (10 mg kg^{-1}, i.p.). The vertical dashed lines are used to separate the time segments.

with the membrane materials. Thus, in a separate study *in vivo* recovery was determined using two blank rats. Following a series of regular surgical procedures, the microdialysis probe was implanted as described above and blank dialysate was perfused through the probe at a flow rate of 1.0 μL min^{-1} and stabilized for 1 h. The probe was then perfused with Ringer's solution containing BUP and HBUP (C_{in}) at two concentrations (25 and 100 ng mL^{-1}). Before perfusion, the rat was not treated with any drug ($C_s = 0$). Following analysis of dialysate (C_{out}), the *in vivo* recovery by retrodialysis was calculated as follows: $R_{retro} = (C_{in} - C_{out})/(C_{in} - C_s)$ or $R_{retro} = (C_{in} - C_{out})/C_{in}$ [21].

Microdialysate concentrations of the analytes (C_{out}) were converted to unbound concentrations (C_u) as follows: $C_u = C_{out}/R_{in\,vivo}$.

3. Results and discussion

3.1. Optimization of chromatographic conditions and extraction procedure

To optimize the proposed LC–MS method for the simultaneous determination of BUP and HBUP, the effects of several parameters, including the type of organic modifier, buffer, and the concentration and pH of the buffer were investigated. The effect of these parameters was evaluated and optimized based on the peak areas, retention times and resolution of the resulting signals for BUP and HBUP.

The composition and pH of the mobile phase have a great effect on separation selectivity and sensitivity of the LC methods. Acetonitrile was used as an organic modifier because of its low ionic suppression effect. It is volatile, and therefore compatible with MS detection. The compounds were ionized in the positive electrospray ionization (ESI^+) source of the mass-spectrometer enhanced by the acidic mobile phase. The mobile phase additives ammonium acetate and ammonium formate buffer were investigated at different pH values according to their buffer ranges. The ammonium acetate buffer was tested at pH 4.0, 5.0 and 5.7; the ammonium formate buffer was tested at pH 3.0, 4.0 and 4.7. The sensitivity was significantly increased with the use of an ammonium formate buffer compared to an ammonium acetate buffer with 2 fold higher peak areas of HBUP and 4–6-fold higher peak areas of BUP. After obtaining increased sensitivity and reproducibility with the ammonium formate buffer at pH 4, different buffer concentrations of 5, 10 and 15 mM were tested. Excessive buffer concentration use was avoided in order to minimize the suppression of the ESI process, to assure the safety of the column, and to minimize the cost of analysis. Considering the efficiency and run time of separation, 10 mM was chosen as an optimum buffer concentration. Although various acetonitrile to ammonium formate buffer (10 mM, pH 4) ratios were tested, complete separation of BUP and HBUP with appropriate analysis time could not be achieved under isocratic conditions. Hence, gradient elution conditions were optimized to achieve the best separation of BUP and HBUP in rat plasma and microdialysates.

All reported methods including the analysis of BUP in biological samples have required time-consuming and expensive extraction steps [6–9,11,12]. Thus, the development of a simple and one step protein precipitation procedure to extract the analytes from rat plasma was one of the goals of this study. The proposed plasma deproteination technique with 10% (w/v) TCA has been already used for BUP in our laboratory because of its great efficiency at low volume ratios without diluting the sample [10]. The ratio of 1:0.2 (plasma:TCA) is sufficient for deproteination, however the best results are obtained with the ratio of 1:0.6 (plasma:TCA) [33]. Protein precipitation with TCA removed all the potentially interfering endogenous compounds, as demonstrated by the absence of any significant signal in the chromatographic trace shown in Fig. 2a. The average recovery (±SE) was 82.11 ± 3.26% for BUP and 84.24 ± 4.60% for HBUP ($n = 3$) at three concentrations (1.56, 25 and 400 ng mL^{-1}) for two analytes, indicating that sample treatment did not cause a significant loss of the analytes. An additional benefit of this technique is that the column utilized in this study, which was used for 300 injections, displayed an increase in back-pressure of 30 bar (100–130 bar), indicating that this sample preparation method ensures sufficient column longevity.

3.2. Microdialysis conditions and probe recovery

The microdialysis experiments were initiated with a probe of CMA/12 (PAES), cut-off 100 kDa and 1 mm membrane length, because of the position of the nucleus accumbens. However, most samples contained HBUP levels below the LLOQ and were therefore inadmissible in the pharmacokinetic analysis.

Following the insufficient HBUP concentrations obtained with the first probe, research indicating that several factors which influence the relative recovery of the probe, such as the membrane (length, diameter, material), the diffusion coefficient of the analyte, the time factor, temperature, composition of the perfusion fluid, perfusion flow-rate and the properties of the substances, was taken into consideration for the selection of an appropriate probe [32]. Thus, the microdialysis experiments were continued using a probe of CMA/12 (PC), cut-off 20 kDa and 2 mm membrane length.

In vitro recoveries of these probes (average recovery for two concentrations and four samples for each concentration ± SE) were 26.92 ± 4.05% and 22.53 ± 4.55%, while *in vivo* recoveries of the probes (average recovery for two rats and four samples for each rat ± SE) were 39.52 ± 4.82% and 23.94 ± 3.51% for BUP and HBUP, respectively. Results indicated that the *in vitro* recovery was similar to the *in vivo* recovery for HBUP and lower than the *in vivo* recovery for BUP. The actual concentrations of BUP and HBUP in the rats' brains were corrected by the average recovery of 39.52% and 23.94%, respectively. No difference in recovery values for the same region was observed after the addition of BUP and HBUP with different concentrations to the perfusate, indicating that the recoveries from the microdialysis probes in the rats' brains are independent of the concentration for these experiments.

3.3. Evaluation of matrix effect

A drawback of the use of ESI is its sensitivity to matrix effects. Co-eluting matrix compounds, present in microdialysis and plasma samples, can cause ion suppression and sometimes even ionization enhancement which generate accuracy and precision problems and reduce sensitivity. Although microdialysates are protein-free aqueous solutions, they contain a large amount of salts and other small molecules. Ion suppression effects can be observed due to matrix endogenous components present in the plasma samples. These non-volatile components can cause ion suppression of the analytes of interest and can clog the MS source [32].

Matrix effect on the LC–ESI-MS sensitivity was evaluated with a post-column analyte infusion experiment [11]. Two standard solutions containing 32 ng mL^{-1} of BUP and HBUP in ultra-pure water were individually infused post-column *via* a "T" connector into the mobile phase flow (0.4 mL min^{-1}) at an infusion rate of 15 μL min^{-1} with the use of an infusion pump. Aliquots of 15 μL of blank plasma (resulting from the aforementioned protein precipitation process) and microdialysate were then injected into the LC column and MRM LC–ESI-MS chromatograms were acquired for each analyte.

Comparable electrospray ionization suppression regions were observed in related matrices, however, elution of the analytes were obtained in regions of no suppression as seen in Fig. 3.

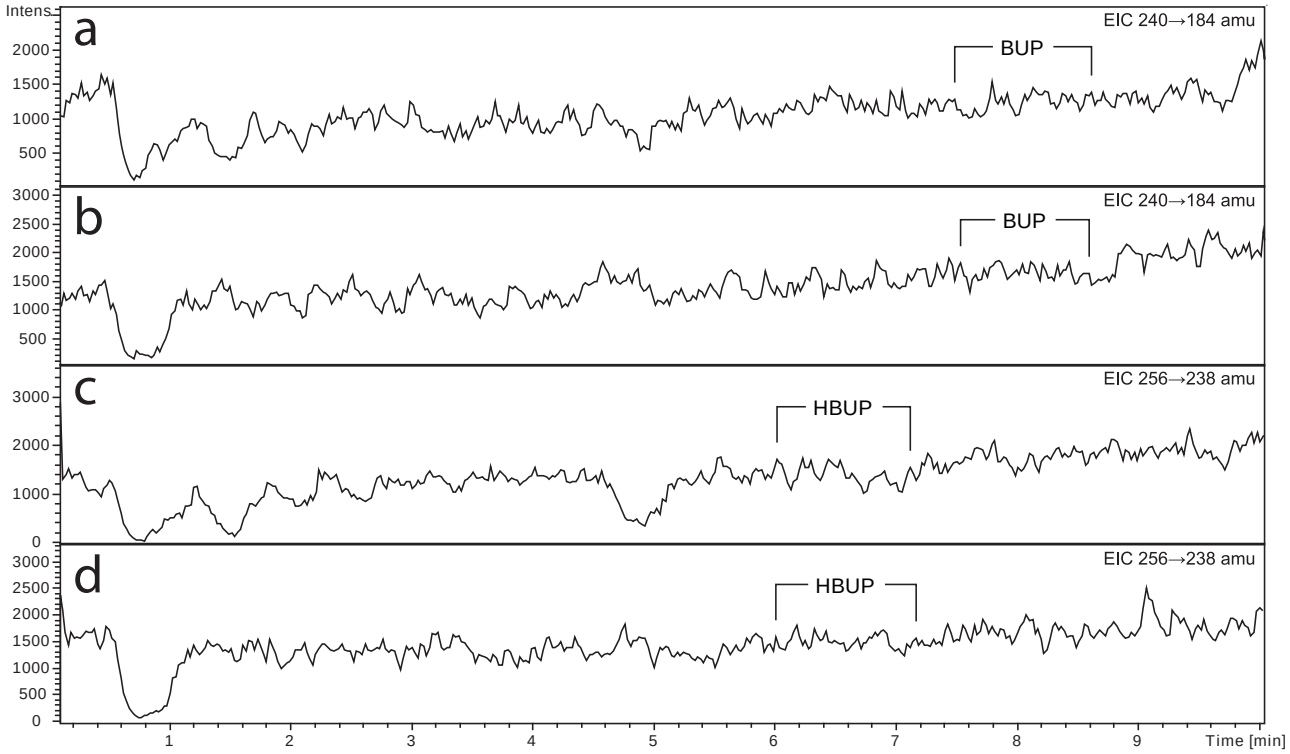

Fig. 3. Evaluation of the matrix effect. Representative post-column analyte infusion extracted ion chromatograms (EIC) for BUP (a) in rat plasma, (b) in rat microdialysate; and for HBUP (c) in rat plasma, (d) in rat microdialysate. Pointed regions show the retention times of the analytes.

3.4. Method validation

Validation of the proposed method was performed with respect to stability, specificity, linearity, lower limit of quantification (LLOQ), accuracy and precision in accordance with ICH and bioanalytical validation guidelines [34,35].

3.4.1. Linearity

Calibration curves were constructed by the external standard method and were established based on the peak area of analytes versus concentration using weighted ($w = 1/x^2$) linear regression analysis. The method was validated in rat plasma and microdialysates in the concentration range of 1.56–400 ng mL^{-1} for BUP and HBUP. Calibration curves prepared over this concentration range were linear with average determination coefficients greater than 0.99 for both parent drug and metabolite. Mean equations of the calibration curves in rat plasma for three days were $y = (3163 \pm 69.7)x + (286.3 \pm 282.7)$, $r^2 = 0.9958$ for BUP and $y = (4124 \pm 118.8)x + (61.2 \pm 481.6)$, $r^2 = 0.9946$ for HBUP ($n = 9$). Mean equations of the calibration curves in Ringer's solution for three days were $y = (3933 \pm 106.1)x - (162.6 \pm 430.1)$, $r^2 = 0.9897$ for BUP and $y = (5129 \pm 103.0)x + (464.5 \pm 417.7)$, $r^2 = 0.9966$ for HBUP, where y represents the peak area and x represents the concentration (ng mL^{-1}) of the analytes ($n = 9$).

The lower limit of quantification (LLOQ), defined as the lowest concentration of the calibration graph with acceptable accuracy (RSD and bias % less than 20%) [35], was found to be 1.56 ng mL^{-1} in rat plasma and microdialysates for each analyte, which is significantly lower than those of previously reported BUP assays [6–8,10,13–18].

3.4.2. Accuracy and precision

The intra- and inter-day assay precisions were evaluated using plasma and microdialysis samples of three different concentrations; after quantifying six replicates for each concentration on the same day (intra-day assay) and on six consecutive days (inter-day assay).

The overall precision in rat plasma, as defined by RSD, was ranged from 3.02% to 9.37% for BUP and from 2.45% to 8.69% for HBUP. Analytical accuracy, expressed as bias %, was varied from

Table 1
Precision and accuracy of BUP and HBUP in rat plasma.

Added concentration (ng mL^{-1})	Intra-day ($n = 6$)			Inter-day ($n = 6$)		
	Measured concentration (ng mL^{-1})	RSD	Bias %	Measured concentration (ng mL^{-1})	RSD	Bias %
BUP						
1.56	1.63	3.02	4.60	1.63	9.37	4.65
25	24.20	3.93	−3.19	24.24	8.70	−3.06
400	409.16	5.21	2.29	400.71	5.07	0.18
HBUP						
1.56	1.71	3.71	9.83	1.63	8.69	4.22
25	26.64	2.45	6.57	25.98	8.45	3.91
400	392.25	3.73	−1.94	396.92	4.72	−0.77

RSD: relative standard deviation. Bias %: [(found − added)/added] × 100.

Table 2
Precision and accuracy of BUP and HBUP in Ringer's solution.

Added concentration (ng mL^{-1})	Intra-day (n = 6)			Inter-day (n = 6)		
	Measured concentration (ng mL^{-1})	RSD	Bias %	Measured concentration (ng mL^{-1})	RSD	Bias %
BUP						
1.56	1.61	3.63	3.51	1.54	6.87	−1.08
25	25.89	2.74	3.57	25.27	6.75	1.06
400	373.88	3.67	−6.53	392.28	5.21	−1.93
HBUP						
1.56	1.48	6.81	−5.39	1.47	5.02	−6.03
25	24.19	3.49	−3.24	24.34	5.36	−2.64
400	352.48	2.04	−11.88	350.17	1.81	−12.46

RSD: relative standard deviation. Bias %: [(found − added)/added] × 100.

−3.19% to 4.65% for BUP and from −1.94% to 9.83% for HBUP as shown in Table 1. The precision of the analytes in Ringer's solution for microdialysis samples was also evaluated and RSD values were found in the ranges of 2.74–6.87% and 1.81–6.81%, whereas bias % values were varied from −6.53% to 3.57% and from −12.46% to −2.64% for BUP and HBUP, respectively (Table 2), indicating satisfactory results for accuracy and precision of the analytes in two matrices. As a result, lower values than the accepted criteria of ±15% were obtained [35].

3.4.3. Specificity

Specificity, described as the ability of a method to discriminate the analyte from all potential interfering substances, was confirmed by comparing the chromatograms of a blank rat plasma (Fig. 2a), rat plasma spiked with BUP and HBUP (Fig. 2b) and real plasma sample obtained from a rat that received BUP (Fig. 2c). Specificity of the method in microdialysis samples was evaluated in an analogous manner (Fig. 2d–f) with plasma samples.

The peaks of BUP and HBUP were well separated, no interfering peaks were observed, and no significant peaks were found at the retention times of the analytes in plasma and microdialysates. Specificity was found to be sufficient for accurately characterizing the pharmacokinetics of BUP and HBUP.

3.4.4. Stability

The stability of BUP and HBUP in rat plasma and microdialysates was evaluated under different storage conditions by spiking standard solutions into blank plasma and Ringer's solution at two concentrations (25 and 200 ng mL^{-1} for both BUP and HBUP). For short-term stability, spiked samples were kept at room temperature for 24 h. The long-term stability was assessed after storage of spiked samples in a freezer at −20 °C for 20 days. To test for freeze and thaw stability, the spiked samples were stored at −20 °C

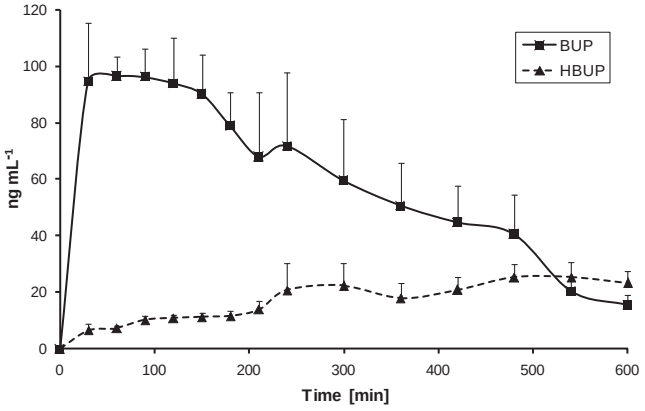

Fig. 4. Unbound plasma concentration–time profiles of BUP and HBUP after BUP administration (10 mg kg^{-1}, i.p.).

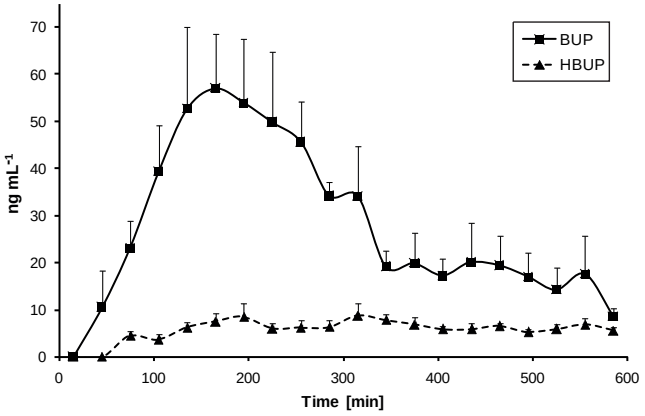

Fig. 5. Unbound brain (nucleus accumbens) concentration–time profiles of BUP and HBUP after BUP administration (10 mg kg^{-1}, i.p.).

Table 3
Stability of BUP and HBUP in rat plasma and Ringer's solution under different conditions (n = 3).

	Short-term stability (24 h, room temperature)		Long-term stability (20 days, −20 °C)		Freeze–thaw stability (3 cycles)	
	Remained (%)	RSD	Remained (%)	RSD	Remained (%)	RSD
Plasma (added (ng mL^{-1}))						
BUP						
25	92.54	3.09	98.14	4.21	88.65	2.51
200	99.11	1.80	106.81	2.98	95.56	2.14
HBUP						
25	100.76	9.41	93.57	1.99	91.76	1.93
200	101.08	3.77	103.41	6.10	99.10	0.98
Ringer's solution (added (ng mL^{-1}))						
BUP						
25	102.98	1.26	93.35	1.80	94.07	4.45
200	105.80	0.59	104.21	1.70	92.72	2.72
HBUP						
25	108.23	1.81	103.65	3.96	93.32	5.27
200	107.04	2.96	105.71	0.51	101.78	2.28

RSD: relative standard deviation. Remained % = (peak area of BUP or HBUP after the mentioned storage conditions/peak area of fresh BUP or HBUP) × 100.

Table 4
Pharmacokinetic results of BUP and HBUP for *in vivo* experiments.

	Plasma ($n=6$)		Nucleus accumbens ($n=6$)	
	BUP	HBUP	BUP	HBUP
AUC ± SE (ng min mL^{-1})	35172 ± 6400	10487 ± 1837.44	16479 ± 2715	3336 ± 423.59
C_{max} ± SE (ng mL^{-1})	96.43 ± 17.65	25.28 ± 9.18	57.07 ± 13.93	8.74 ± 2.63
T_{max} ± SE (min)	60 ± 25.11	540 ± 63.76	165 ± 23.89	315 ± 23.34
$T_{1/2}$ ± SE (min)	230.40 ± 55.94		362.17 ± 34.34	

AUC: area under the drug concentration–time curve; SE: standard error; C_{max}: maximum concentration; T_{max}: time to reach peak concentration; $T_{1/2}$: elimination half life.

for 24 h and thawed to room temperature three times and then analyzed. The stability results were evaluated by comparing peak areas of BUP and HBUP with those of freshly prepared samples. The results presented in Table 3 indicate that BUP and HBUP can be considered stable in rat plasma and Ringer's solution under the aforementioned conditions.

3.5. Method application

The concentration–time profiles for unbound BUP and HBUP in rat plasma and brain tissue (nucleus accumbens) after administration of 10 mg kg^{-1} of BUP to individual rats ($n=6$) are shown in Figs. 4 and 5.

The pharmacokinetic parameters are calculated using the GraphPad Prism v4.03 program and summarized in Table 4.

In the literature several reports indicate that BUP is metabolized differently in certain species of animals. HBUP is the major basic metabolite in mice, rats, dogs and rabbits and all species except the rat have higher concentrations of HBUP than of BUP. It has been suggested that the possibility of pharmacologically significant species differences in BUP metabolism mediates the absence of the anti-tetrabenazine activity of the drug in rats [8,20]. As shown in Figs. 4 and 5, the brain levels of HBUP are extremely low compared to its plasma levels, limiting HBUP accumulation into rats' brains. Such a conclusion supports the results of previous studies and also rouses interest in the further study of BUP with regard to its effects on smoking cessation.

4. Conclusion

Liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) has emerged as a powerful analytical technique for the determination of drugs and metabolites in biological fluids. By the use of the MRM mode the selection and quantification of compounds enable a reduction in the interference through the co-eluting substances and a considerable improvement in assay selectivity and sensitivity [36].

This is the first study on the simultaneous analysis of BUP and HBUP in both rat brain microdialysates and rat plasma. In addition, the new LC–ESI-MS method fulfills all the required analytical characteristics for this purpose. The LLOQ of the method (1.5 ng mL^{-1}) is significantly low, allowing the analysis of small volume of samples with low analyte concentrations. No time-consuming and expensive extraction steps are required, and the analysis can be performed directly on the microdialysis sample with no clean-up, and on the plasma sample after a simple and fast protein precipitation step. This assay can thus be applied for the analysis of BUP and HBUP in rat plasma and brain microdialysis samples collected during clinical and pharmacokinetic studies.

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