ELSEVIER

Journal of Chromatography B, 755 (2001) 379–382

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Extraction and quantification of epibatidine in plasma

M.A. Javors[a,b,*], J.J. Sanchez[a], T.S. King[c], A.R. Rohde[a], S.G. Wilson[e], C.M. Flores[b,d]

[a]*Department of Psychiatry, The University of Texas HSC, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA*
[b]*Department of Pharmacology, The University of Texas HSC, San Antonio, TX 78229, USA*
[c]*Department of Cellular and Structural Biology, The University of Texas HSC, San Antonio, TX 78229, USA*
[d]*Department of Endodontics, The University of Texas HSC, San Antonio, TX 78229, USA*
[e]*Department of Psychology and Program in Neuroscience, University of Illinois at Urbana-Champaign, 603 East Daniel Street, Champaign, IL 61820, USA*

Received 7 August 2000; received in revised form 26 January 2001; accepted 6 February 2001

Abstract

Epibatidine was extracted from human and mouse plasma into a hexane–isopropanol mixture and back-extracted into a phosphate buffer, pH 2.5, then identified by HPLC isocratically using a CN column and quantified with ultraviolet detection at a fixed wavelength of 214 nm. The percent recovery of epibatidine from spiked plasma samples was 83.6% and the percent extraction was linear between 10 and 1000 ng/ml. Desipramine was used as the internal standard. For spiked control samples containing 50 and 750 ng/ml, between-day precisions were 20.8 and 7.2% (RSD%), respectively; accuracy was 87.0 and 99.1%, respectively. The limit of detection was 2 ng/ml. Using this method, an intraperitoneal dose of 0.1 mg/kg of epibatidine produced mean levels of 7.3 and 37.1 ng/ml in pooled male and female plasma samples from C57BL/10 J mice, respectively. This is a simple and straightforward procedure by which plasma samples may be analyzed for epibatidine.

Keywords: Epibatidine

1. Introduction

Epibatidine, exo-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane, is a nicotine analog that was originally extracted from the skin of the Ecuadoran poison frog, *Epipedobates tricolor* [1]. Although this diet-derived alkaloid is not produced by captive-raised frogs, total synthesis of epibatidine has been achieved by several laboratories (for review, see [2]). Substantial interest in this compound derives from its profound antinociceptive properties, which are mediated via neuronal-type nicotinic acetylcholine receptors [3,4]. Indeed, epibatidine is currently recognized as the most potent neuronal nicotinic receptor agonist yet discovered. Tritiated epibatidine predominantly labels two sites with K_d values of approximately 15 and 500 p*M* in both mammalian central [5] and peripheral [6] nervous systems. At this time, however, very little is known regarding the in vivo distribution and metabolism of epibatidine in any species.

Recently, it was demonstrated that epibatidine exhibits profound pharmacogenetic variability related to its antinociceptive effects [7]. Thus, among 8

*Corresponding author. Tel.: +1-210-567-5532; fax: +1-210-567-3759.

E-mail address: javors@uthscsa.edu (M.A. Javors).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00120-7

different inbred mouse strains, there was as much as a 20-fold difference in antinociceptive potency between strains. Interestingly, this phenomenon was manifest in both the magnitude and duration of epibatidine-induced antinociception. However, these studies did not identify the source of this variability, leaving open the possibility that epibatidine might exhibit strain-dependent differences in its pharmacokinetics, pharmacodynamics, or both. In terms of the latter, investigations have been hampered by the lack of a reliable assay for the quantification of epibatidine in tissue. Although there are reports of studies in which HPLC techniques have been used to separate the chiral isomers of epibatidine and to separate epibatidine from other chemical analogues [8,9], a well-characterized analytic technique for the quantification of epibatidine in biological samples has not been published to our knowledge. The purpose of the present studies, therefore, was to develop a sensitive and reliable method for the chromatographic identification and quantification of epibatidine in plasma samples.

2. Methods

2.1. Chemicals and reagents

High purity-grade acetonitrile, isopropanol, hexane and methanol were purchased from Burdick and Jackson (Muskegon, MI, USA). Water used in the assay was purified with a Milli-Q Water System (Millipore, Bedford, MA, USA). Potassium phosphate and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Human plasma for the preparation of standards and calibrators was purchased from the University Hospital in San Antonio, TX, USA. (±)-Epibatidine and desipramine, the internal standard, were purchased from Sigma (St. Louis, MO, USA). (+)-Epibatidine and (−)-epibatidine were purchased from RBI (Natick, MA, USA).

2.2. Instrumentation

For the extraction procedure, shaking was performed with an Eberbach shaker (Eberbach, Ann Arbor, MI, USA) and centrifugation with a Beckman Model TJ-6 centrifuge equipped with a swinging bucket rotor (Fullerton, CA, USA). The HPLC system included a Beckman 110B HPLC pump, a Waters 717 sample injector, a Waters 2487 UV detector for HPLC, and a Spherisorb CN column (300×4.5 mm; 5 μm;) for the analysis of epibatidine.

2.3. Extraction and quantification of epibatidine

Aliquots (1 ml) of plasma for unknown, calibrator, and control samples were placed in polypropylene tubes with 20 μl of desipramine in water (stock, 10 μg/ml; final concentration in sample, 200 ng/ml) as the internal standard. Next, 100 μl of 5 *M* NaOH were added to the samples. The samples were vortexed, then 3 ml of a mixture containing 5% isopropanol in hexane were added to the samples followed by gentle shaking. Samples were then centrifuged at 3000 rpm (1200 *g*) for 10 min at 18°C in a Beckman TJ-6 centrifuge. Samples were placed in a methanol–dry ice bath, which froze the aqueous layer. The upper organic layer was transferred to another tube and mixed with 500 μl of a solution containing 12 m*M* monobasic potassium phosphate, pH 2.5, for back extraction of the drugs. The samples were shaken, centrifuged, and placed in the methanol–dry ice bath. After the aqueous layer was frozen, the upper organic layer was removed. The potassium phosphate fraction was placed in the hood for 45 min to allow any residual isopropanol–hexane to evaporate, then 100 μl of each of the samples were injected into the HPLC system.

The absorbance of epibatidine was quantified at a fixed wavelength of 214 nm, a time constant of 0.5 s, and a range setting of 0.005 AUFS on the UV detector. The mobile phase contained 70% acetonitrile, 13% methanol and 17% of a solution containing 10 m*M* monobasic potassium phosphate at pH 6.7. The flow-rate of the mobile phase was 2 ml/min. The peak areas of epibatidine and the internal standard desipramine were determined with Waters MAXIMA chromatography software, which subsequently calculated the ratio of peak areas. Epibatidine was quantified by comparing the peak area ratios of epibatidine:desipramine against the linear regression of calibrator sample ratios using a six-point standard curve (10, 50, 250, 500, 750 and

1000 ng/ml). Control plasma samples spiked with 50 and 750 ng/ml of epibatidine were used to calculate precision and accuracy of the test. The detection limit (sensitivity) of the assay was determined to be 2 ng/ml (signal-to-noise ratio of 5).

2.4. Measurement of epibatidine in plasma of C57BL/10J mice

Four male and four female Jackson Laboratory substrain mice were injected intraperitoneally with 0.1 mg/kg of epibatidine, then sacrificed 10 min later by decapitation. Trunk blood was collected immediately and plasma was prepared by centrifugation. The plasma samples were stored at −80°C until the day they were analyzed. On the day of the assay, the samples were thawed on ice, and then the male and female samples were pooled separately. Epibatidine was extracted from mouse plasma and quantified according to the methods described above.

3. Results and discussion

Epibatidine and the internal standard desipramine eluted from the CN column at 7.6 and 16.6 min, respectively. Chromatographic profiles of a control sample in buffer (Fig. 1a) and an extracted plasma sample (Fig. 1b) spiked with epibatidine and desipramine (internal standard) are shown. The extraction procedure did not produce any peaks that interfered with the elution of epibatidine or desipramine. The percent recovery of epibatidine was 83.6% (Fig. 2). Recovery was determined by comparing the slopes of the concentration–response curves for a series of six concentrations of epibatidine, extracted versus non-extracted.

(±)-Epibatidine, (+)-epibatidine, and (–)-epibatidine coeluted under the chromatographic conditions used in this study (data not shown). (+)-Epibatidine was used to obtain the results presented in this study.

The mean *r* value of the linear regressions for a total of nine extracted calibrator curves over 3 days was 0.992±.0087. This result indicates that the concentration–response calibration curves were linear over a concentration range of 10–1000 ng/ml. The mean and standard deviation of the slopes of nine extracted calibrator curves, run over 3 days, was 0.00038±0.0000289. The relative standard deviation of the slopes of the calibrator curves for between-day variability was 7.6% and for within-day variability was 4.4%, indicating an acceptable level of variability.

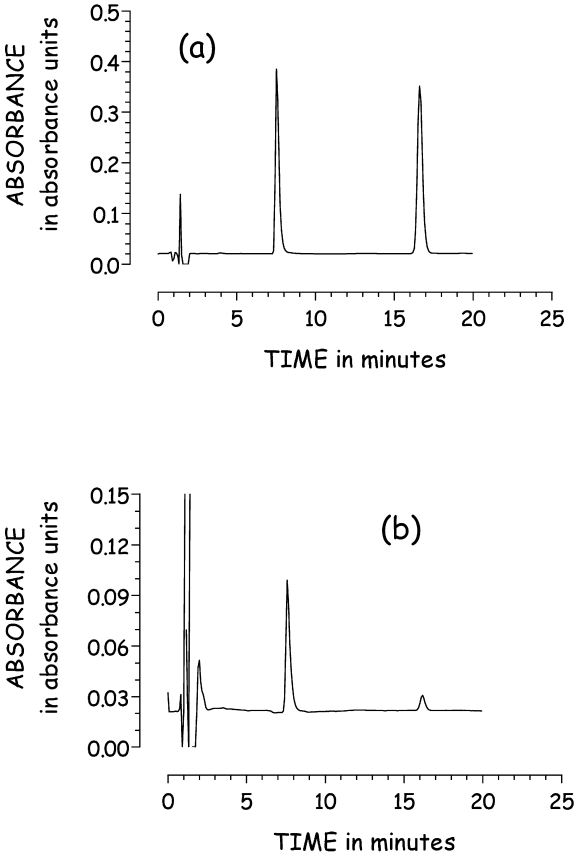

Fig. 1. Chromatographic profiles of epibatidine and the internal standard desipramine. Separation and detection of epibatidine and desipramine was achieved by HPLC using a CN column and a fixed ultraviolet wavelength of 214 nm. The details of the chromatographic procedure are described in the Methods. (a) Unextracted sample (100 μl injected) containing 10 μg/ml of epibatidine and 10 μg/ml of desipramine dissolved in water. (b) Extracted sample (100 μl injected) spiked with 1000 ng/ml epibatidine and 200 ng/ml desipramine.

The between-day precision for eight samples spiked with 50 ng/ml analyzed over 3 days was 20.8% (accuracy 87.0%) and for nine samples spiked with 750 ng/ml analyzed over 3 days was 7.2% (accuracy 99.1%).

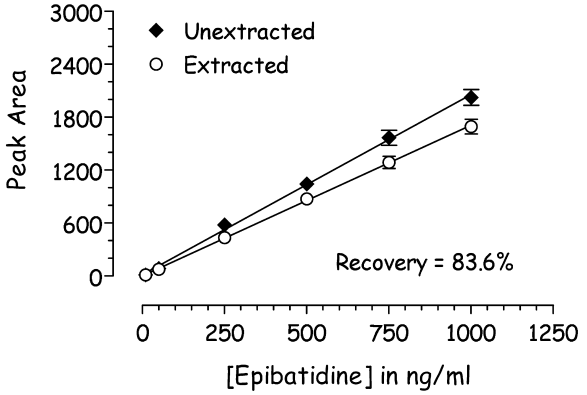

Fig. 2. Calibration curve of epibatidine extracted from spiked human plasma samples. Human plasma samples were spiked with epibatidine at concentrations of 10, 50, 250, 500, 750, and 1000 ng/ml. Epibatidine was extracted as described in Methods. Peak areas were quantified with MAXIMA chromatographic software by Waters. Recovery was determined by dividing the slope of the linear regression of the extracted calibrator curve (open circles) by the slope of the unextracted standard curve (filled diamonds).

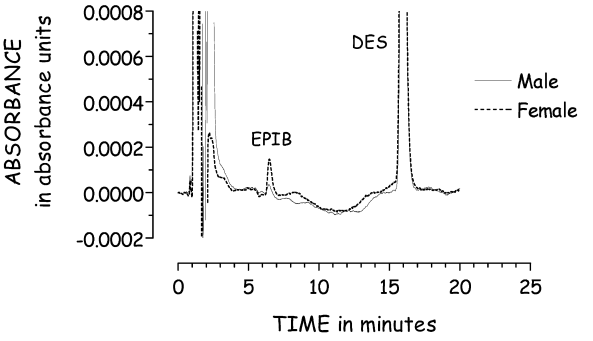

Fig. 3. Chromatographic tracings of epibatidine extracted from mouse plasma. Four male and four female C57BL/10J mice were injected with 0.1 mg/kg of epibatidine and sacrificed 10 min later as described in Methods. Blood was collected, then plasma was prepared and pooled for males and females for the quantification of epibatidine. Epibatidine was extracted from mouse plasma and quantified by the method described. The mean levels were determined to be 7.3 ng/ml for the males and 37.1 ng/ml for the females.

Epibatidine was quantified in pooled plasma samples of four male and four female C57BL/10J mice. Each animal was given a 0.1 mg/kg dose of epibatidine, then sacrificed 10 min later. Blood was collected and plasma prepared by centrifugation. The mean levels were 7.3 ng/ml for the males and 37.1 ng/ml for the females. This result suggests that metabolism or distribution of epibatidine may differ according to gender in C57/10J mice, and this discrepancy will be addressed in a future study. The chromatographic tracings for these samples are shown in Fig. 3.

These results indicate that epibatidine can be quantified in human and mouse plasma using a liquid–liquid extraction, followed by separation and quantification by HPLC using a CN column and ultraviolet detection at a fixed wavelength of 214 nm. The detection limit of the method is 2 ng/ml. The procedure for extraction and quantification of epibatidine in biological tissues described here should prove useful for future investigations aimed at elucidating relative pharmacokinetic and pharmacodynamic contributions to differences in the effects of this drug among genetically diverse groups of animals.

References

[1] T.F. Spande, H.M. Garraffo, M.W. Edwards, H.J.C. Yeh, L. Pannel, J.W. Daly, J. Am. Chem. Soc. 114 (1992) 3475.
[2] C. Broka, Med. Chem. Res. 4 (1994) 449.
[3] C. Qian, T. Li, T.Y. Shen, L. Libertine-Garahan, J. Eckman, T. Biftu, S. Ip, Eur. J. Pharmacol. 250 (1993) R13.
[4] B. Badio, J.W. Daly, Mol. Pharmacol. 45 (1994) 563.
[5] R.A. Houghtling, M.I. Davila-Garcia, K.J. Kellar, Mol. Pharmacol. 48 (1995) 280.
[6] C.M. Flores, R.M. DeCamp, S. Kilo, S.W. Rogers, K.M. Hargreaves, J. Neurosci. 16 (1996) 7892.
[7] C.M. Flores, S.G. Wilson, J.S. Mogil, Pharmacogenetics 9 (1999) 619.
[8] J.T. Patt, J.E. Spang, G. Westera, A. Buck, P.A. Schubiger, Nucl. Med. Biol. 26 (1999) 165.
[9] J.E. Spang, J.T. Patt, S. Bertrand, D. Bertrand, G. Westera, P.A. Schubiger, J. Recept. Signal Transduct. Res. 19 (1999) 521.