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Short Communication

Preparation of a fluorescent derivative of benzoylecgonine, and preliminary studies of its application to the analysis of urine

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ABSTRACT

A sensitive high-performance liquid chromatographic method using 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ) as a fluorescent labeling reagent is described for the determination of benzoylecgonine (BE) and ecgonine (EC). The Br-DMEQ derivatives of BE and EC were separated on a C_{18} column and detected at 455 nm with excitation at 370 nm. The detection limits of the proposed method were 18.7 fmol for BE and 12.5 pmol for EC at a signal-to-noise ratio of 3. Relative standard deviations of five replicate measurements were 1.94% (10 pmol) and 2.98% (50 pmol) for BE and 6.3% (250 pmol) and 5.62% (1.25 pmol) for EC. This method was applied to the determination of BE in human urine. BE was extracted from urine by solvent extraction with chloroform–isopropyl alcohol (9:1, v/v) solution. Levels of $2.5 \cdot 10^{-8}$ *M* BE in urine (25 pmol/ml) could be determined.

INTRODUCTION

Abuse of cocaine has caused serious social problems; for this reason a sensitive and selective method for the determination of cocaine and its metabolites has been desired in the fields of forensic, medical and pharmaceutical sciences [1–5]. Of the metabolites, benzoylecgonine (BE) is the main one [6,7] which could be detected in urine 48–72 h after intranasal administration of cocaine [8]. Recently, 3-bromo-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ) has been developed by one of the authors as a highly fluorescent labeling reagent for carboxylic acids and applied to the determination of fatty acids [9,10], phenylacetic acid [11], *p*- and *m*-hydroxyphenylacetic acids [12] and prostaglandins [13].

In this paper, a high-performance liquid chromatographic (HPLC) determination of BE and ecgonine (EC) was developed using Br-DMEQ as

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fluorescent label. The method was successfully applied to the assay of BE in human urine.

EXPERIMENTAL

Reagents and materials

Br-DMEQ was prepared by a procedure described previously [9]. BE and EC were prepared by published procedures [14,15]. 18-Crown-6 and tetra-*n*-butylammonium bromide were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile and other reagents were analytical-reagent grade and purchased from Wako (Osaka, Japan). Bond Elut C_8 cartridge was purchased from Varian (Harbor City, CA, USA). Deionized, glass-distilled water and distilled methanol were used for the preparation of the buffer solutions and the HPLC eluents.

Chromatography

A high-performance liquid chromatograph consisting of a Model LC6A pump (Shimadzu, Kyoto, Japan), a Model 7125 loop injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop for sample loading, an analytical column (150 mm × 4.6 mm I.D.) packed with TSK-gel ODS-80TM (particle size 5 μm; Tosoh, Tokyo, Japan), a guard column (15 mm × 3.2 mm I.D.) packed with TSK-guard gel ODS-80TM (Tosoh), a Model F1000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) and a Model 561 recorder (Hitachi) was used. The eluent used was methanol–phosphate–borate buffer (0.1 *M* KH_2PO_4–0.05 *M* $Na_2B_4O_7 \cdot 10H_2O$, pH 7.5) (60:40, v/v) containing 5 m*M* tetra-*n*-butylammonium bromide. The flow-rate was 0.8 ml/min. Separations were carried out at ambient temperature. Fluorescence of the DMEQ derivatives were monitored at 455 nm with excitation at 370 nm.

Derivatization of BE and EC with Br-DMEQ

To 500 μl of a mixture of BE and EC in acetonitrile were added 250 μl of 10 m*M* 18-crown-6 in acetonitrile and *ca.* 5 mg of K_2CO_3 (and *ca.* 2 g of Na_2SO_4 for the urine sample), and sonicated for 1 min. After adding 250 μl of 2 m*M* Br-DMEQ in acetonitrile, the mixture was heated at 80°C for 20 min. After cooling to room temperature, 20-μl aliquots of the mixture were applied in the HPLC system.

Extraction of BE from urine

To 1 ml of urine were added 10 μl of BE in acetonitrile. The pH was adjusted to *ca.* 9 with ammonia water. After 5 ml of chloroform–isopropyl alcohol (9:1, v/v) had been added, the mixture was mechanically shaken for 10 min and centrifuged at 1000 *g* for 10 min. The organic layer (4 ml) was evaporated, the residue was dissolved in 500 μl of acetonitrile, and the resulting solution was used for derivatization with Br-DMEQ.

RESULTS AND DISCUSSION

Fluorescence derivatization reaction

The fluorescence derivatization reactions of BE and EC with Br-DMEQ were examined. Fig. 1 shows the effect of the concentration of Br-DMEQ on peak height. At concentrations greater than 1 m*M*, maximum and constant peak heights for BE and almost the same results for

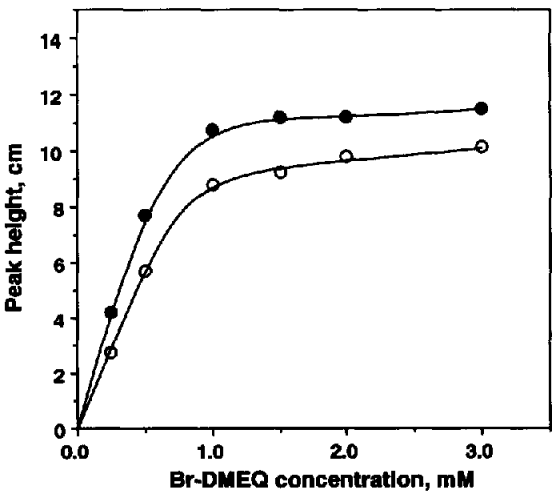

Fig. 1. Effect of the concentration of Br-DMEQ on peak heights of DMEQ-BE and BMEQ-EC. To 250 μl each of 4 μ*M* BE, 100 μ*M* EC and 10 m*M* 18-crown-6 in acetonitrile were added 5 mg of K_2CO_3 and the mixture was sonicated for 1 min. After the addition of 250 μl of Br-DMEQ in acetonitrile (0.25–3 m*M*), the mixture was heated for 20 min at 80°C, and applied in 20-μl aliquots to HPLC. (●) DMEQ-BE; (○) DMEQ-EC.

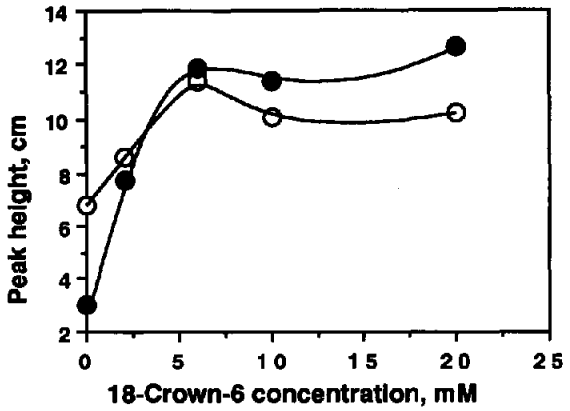

Fig. 2. Effect of the concentration of 18-crown-6 on peak heights of DMEQ-BE and DMEQ-EC. (●) BE, 4 μM; (○) EC, 100 μM; Br-DMEQ, 10 mM. Other derivatization conditions are as in Fig. 1.

EC were obtained. Thus 2 mM Br-DMEQ was used for the subsequent experiments.

18-Crown-6 and K_2CO_3 were used to promote the reaction. As shown in Fig. 2, peak heights were increased 3-fold for BE and 1.5-fold for EC above 4 mM 18-crown-6. The peak heights obtained in the absence of K_2CO_3 were very small and inconsistent compared to those obtained in the presence of 5 mg of K_2CO_3. The reaction yields were almost equal in the range 5–20 mg of K_2CO_3. However 1 min sonication of the mixture after adding K_2CO_3 gave high and reproducible peak heights. A saturated solution of K_2CO_3 in acetonitrile was also tried instead of powder. In this case, the reproducibility of the reaction yield was not so good. In this experiment, 10 mM 18-crown-6 and 5 mg of K_2CO_3 were used.

The time course of the reaction of Br-DMEQ with 4 μM BE and 100 μM EC at various temperatures was measured. The maximum peak heights for BE and EC were obtained after more than 10 min at 80°C. The yields at 60 and 25°C were lower than those at 80°C. Consequently 80°C and 20 min were chosen for the reaction temperature and time.

HPLC separations

A methanol–water system, which is generally used as an eluent for reversed-phase HPLC, was tested for its ability to separate BE-DMEQ and EC-DMEQ from reagent blank peaks. However, favorable separations could not be achieved by changing the methanol-to-water ratio. Thus 0.1 M phosphate–0.05 M borax buffers at three different pH values were examined instead of water. A methanol–0.1 M phosphate–0.05 M borax buffer (pH 7.5) (60:40, v/v) eluent gave relatively good separation, but the BE-DMEQ peak slightly overlapped the blank peak. To improve the separation, a cationic surfactant, tetra-*n*-butylammonium bromide, was used. The retention time of BE-DMEQ was affected by the concentration of tetra-*n*-butylammonium bromide (Fig. 3). In this experiment 5 mM tetra-*n*-butylammonium bromide, which gave a satisfactory separation, was used in the mobile phase. A representative chromatogram is shown in Fig. 4.

Calibration curves and detection limits

The calibration curves for BE and EC were linear over the ranges 0.1–50 pmol (r = 0.999) and 25–1250 pmol (r = 0.998), respectively. The detection limits were 18.7 fmol (BE) and 12.5 pmol (EC), at a signal-to-noise ratio of 3. Relative standard deviations for five replicate measure-

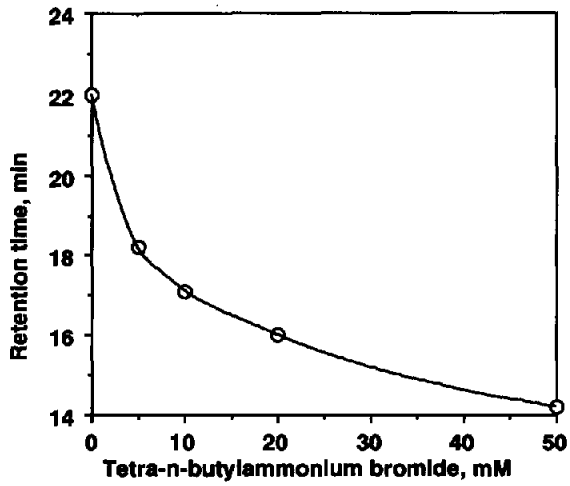

Fig. 3. Effect of tetra-*n*-butylammonium bromide on the retention time of DMEQ-BE. Eluent: methanol–0.1 M phosphate–0.05 M borax buffer (pH 7.5) (60:40, v/v) containing various concentrations of tetra-*n*-butylammonium bromide.

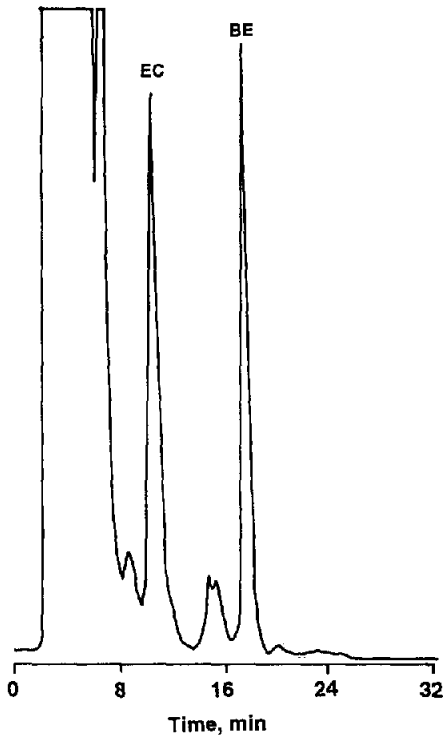

Fig. 4. Chromatogram of DMEQ-BE and DMEQ-EC. BE, 4 μM (20 pmol per injection); EC, 100 μM (500 pmol per injection). Other experimental conditions are as described in the text.

ments were 1.94% (10 pmol) and 2.98% (50 pmol) for BE and 6.3% (250 pmol) and 5.62% (1.25 nmol) for EC.

It is evident that the sensitivity of EC was very low compared to BE and was not good enough to determine EC in human urine [16]. This might be caused by the lower reactivity of EC towards Br-DMEQ. Consequently the proposed method was applied to the assay of BE in human urine.

Assay of BE in urine

Several methods to extract BE from urine have been reported. From the literature, solid-phase extraction using the Bond Elut C_8 cartridge has been examined in detail [17]. Although good recovery for the BE standard solution was obtained, no reproducible, high recovery for BE in urine could be attained. Thus solvent extractions using a chloroform–isopropyl alcohol system were adopted. After the pH of urine had been adjusted to *ca.* 9 with ammonia water, BE was extracted with three different chloroform–isopropyl alcohol systems: 9:1, 8:2 and 6:4 (v/v). As a reference, BE in 0.5 *M* ammonia buffer (pH 9) was used instead of urine sample. In the case of

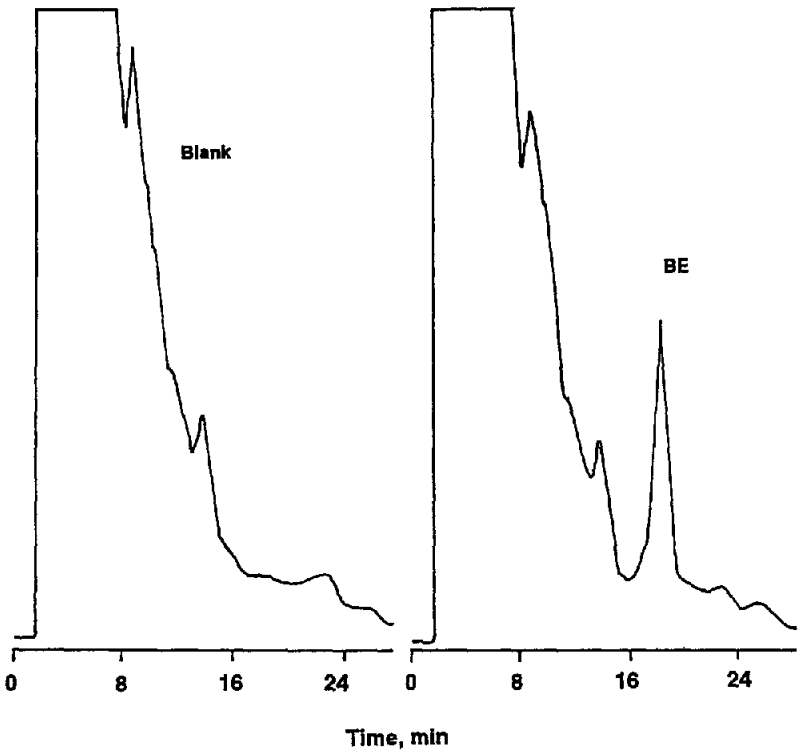

Fig. 5. Chromatogram of blank and BE-spiked urine. BE spiked, 2 nmol/ml; other experimental conditions are as described in the text.

the reference, the extraction ratio increased with increase in isopropyl alcohol content. On the other hand, for the urine samples, the background intensity (baseline level) was increased by high isopropyl alcohol content. In this experiment, a chloroform–isopropyl alcohol (9:1, v/v) system was chosen as extraction solvent. The means of the recoveries for seven measurements at 2 and 5 nmol/ml were 58 and 61%, respectively. The working curve for BE-spiked urine was linear over the range 0.125–5 nmol/ml (r = 0.985), and the detection limit was 25 pmol/ml at a signal-to-noise ratio of 3. The relative standard deviations for seven replicate measurements were 2.9% (2 nmol/ml) and 1.9% (5 nmol/ml). A representative chromatogram for the BE-spiked urine sample is shown in Fig. 5.

In conclusion, a highly sensitive HPLC method for the assay of BE in urine has been developed. The sensitivity is higher than those of other HPLC methods [1,2,17,18] and comparable to those of gas chromatography–mass spectrometry [19,20]. The proposed method should be useful for the identification of BE in forensic medicine and for biomedical or pharmaceutical studies of cocaine.

REFERENCES

1 B. K. Logan and D. T. Stafford, *J. Forensic Sci.*, 35 (1990) 1303.
2 C. E. Lau, F. Ma and J. L. Falk, *J. Chromatogr.*, 532 (1990) 95.
3 J. A. Fleming, R. Byck and P. G. Barash, *Anesthesiology*, 73 (1990) 518.
4 S. Dawling, E. G. Essex, N. Ward and B. Widdop, *Ann. Clin. Biochem.*, 27 (1990) 478.
5 J. J. Ambre, T. J. Connelly and T. I. Ruo, *J. Anal. Toxicol.*, 15 (1991) 17.
6 T. G. Vitti and R. L. Boni, in G. Barnett and C. N. Chiang (Editors), *Pharmacokinetics and Pharmacodynamics of Psychoactive Drugs*, Biomedical Publications, Foster City, CA, 1985, p. 427.
7 J. Y. Zhang and R. L. Foltz, *J. Anal. Toxicol.*, 14 (1990) 201.
8 J. J. Ambre, *J. Anal. Toxicol.*, 9 (1985) 241.
9 M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 346 (1985) 227.
10 M. Yamaguchi, R. Matsunaga, K. Fukuda, M. Nakamura and Y. Ohkura, *Anal. Biochem.*, 155 (1986) 256.
11 M. Yamaguchi and M. Nakamura, *Chem. Pharm. Bull.*, 35 (1987) 3740.
12 M. Yamaguchi, R. Matsunaga, K. Fukuda and M. Nakamura, *J. Chromatogr.*, 414 (1987) 275.
13 M. Yamaguchi, K. Fukuda, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 380 (1986) 257.
14 S. P. Findlay, *J. Am. Chem. Soc.*, 76 (1954) 2855.
15 M. R. Bell and S. Archer, *J. Am. Chem. Soc.*, 82 (1960) 4642.
16 A. R. Jeffcoat, M. P. Reyes, J. M. Hill, B. M. Sader and E. Cook, *Drug Metab. Dispos.*, 17 (1989) 153.
17 J. O. Svensson, *J. Anal. Toxicol.*, 10 (1986) 122.
18 L. R. Tebbett and Q. W. McCartney, *Forensic Sci. Int.*, 39 (1988) 287.
19 S. P. Jindal and P. Vestergaard, *J. Pharm. Sci.*, 67 (1978) 811.
20 D. M. Chinn, D. J. Crouch, M. A. Peat, B. S. Finkle and T. A. Jennison, *J. Anal. Toxicol.*, 4 (1980) 37.