ELSEVIER

Journal of Chromatography B, 658 (1994) 135–141

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Sensitive assay for cocaine and benzoylecgonine using solid-phase extraction and gas chromatography

Laszlo Virag[a,*], Subhash Jamdar[a], Conrad R. Chao[b,**], Hisayo O. Morishima[a]

[a]*Department of Anesthesiology, College of Physicians & Surgeons, Columbia University, 622 W. 168th Street, PH Box 9, New York, NY 10032, USA*

[b]*Department of Obstetrics and Gynecology, College of Physicians & Surgeons, Columbia University, 622 W. 168th Street, PH Box 9, New York, NY 10032, USA*

First received 2 August 1993; revised manuscript received 18 March 1994

Abstract

An improved method for the simultaneous determination of cocaine and benzoylecgonine by means of gas chromatography with nitrogen–phosphorus detection is described. Following a solid-phase extraction and derivatization of benzoylecgonine to its butyl ester, chromatography was performed using a capillary column. The *n*-propylester of benzoylecgonine served as the internal standard. The assay was linear from 4 to 2000 ng/ml for both cocaine and benzoylecgonine, with a good precision over this concentration range. This method may be particularly useful for small volume samples since it requires only 250 μl of plasma for analysis of both cocaine and benzoylecgonine.

1. Introduction

In research protocols involving measurements of cocaine and its metabolites it is often necessary to analyze small sample volumes such as in fetal or small animal samples. In the setting of pregnancy, another consideration is the relatively low concentration of these substances encountered in transplacental studies, where fetal drug concentration may be lower than that of the mother [1].

Although gas chromatography has been used previously to analyze plasma cocaine and benzoylecgonine (BE), its use for repeated blood sampling in smaller animals is not popular. Some investigators utilized radioactive-labeled cocaine in order to measure accurate concentration of the drug in small animals [2]. Most of the earlier methods require large amounts of plasma (1–2 ml) to analyze very low concentrations (*e.g.* 5 ng/ml) of these compounds [3–6]. An other disadvantage of these earlier procedures is that they rely on liquid–liquid extraction with large amounts of organic solvents. Often, the necessary phase separation is hindered by the formation of an emulsion, which results in the inconsistent recovery of the analytes and appearance of unknown peaks on the chromatograms. Pres-

* Corresponding author.

** Present address: Department of Obstetrics and Gynecology, Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, CA 90509, USA.

0378-4347/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(94)00198-E

ently, GC–MS is a popular method for analysis of low concentrations of cocaine and its metabolites [7–12], but the instrumentation is expensive and is not readily available in many laboratories.

Therefore, to fulfil the need to measure cocaine and BE at low concentrations in small sample volumes, we have developed a new gas chromatographic procedure for the simultaneous measurement of cocaine and BE in 250 μl of plasma.

2. Experimental

2.1. Materials

Methanol, methylene chloride, 1-butanol, 2-propanol and toluene (all HPLC grade), anhydrous sodium carbonate (certified ACS), ammonium hydroxide (reagent ACS), potassium hydroxide (certified ACS) and 0.1 *M* hydrochloric acid (certified) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Butyl acetate (HPLC grade) and potassium phosphate monobasic (reagent) were obtained from J.T. Baker (Phillipsburg, NJ, USA). *tert.*-Butyl alcohol (HPLC grade), N,N-dimethylformamide (anhydrous, 99%) and N,N-dimethylformamide di-*n*-propyl acetal (97%) were obtained from Aldrich (Milwaukee, WI, USA). Sulfuric acid 95.9% (analyical reagent) was obtained from Mallinckrodt (St. Louis, MO, USA), and oxalyl chloride (98% min.) from Eastman Kodak (Rochester, NY, USA). Cocaine and benzoylecgonine (BE) were obtained from the Research Triangle Institute (Research Triangle Park, NC, USA) through the National Institute on Drug Abuse (Rockville, MD, USA). Helium, hydrogen, nitrogen (each 99.9999%) and dry air (medical purity) were obtained from Matheson Scientific (Morris Plains, NJ, USA). Drug-free sheep plasma was purchased from Pocono Rabbit Farm and Laboratory (Canadensis, PA, USA). Bond Elut Certify extraction columns (3 ml; 130 mg solid-phase) were from Varian (Harbor City, CA, USA).

2.2. Methods

Gas chromatographic analysis was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Hewlett-Packard Ultra 2 fused-silica capillary column (12 m × 0.2 mm I.D.; 0.33 μm; cross-linked, 5% diphenyl- and 95% dimethylpolysiloxane stationary phase) and with a nitrogen-phosphorus detector. Injections (injection volume, 2 μl) were made by an automated injector in the splitless mode ("purge on" time, 0.5 min), using a deactivated borosilicate liner. Chromatograms were recorded on a Hewlett-Packard 3396A plotter–integrator.

The make-up (auxiliary) gas flow-rate was 30 ml/min; the hydrogen and air flow-rates were 4 ml/min and 90 ml/min, respectively, while the column head pressure was 135 kPa. The injection port and detector temperature were maintained at 250°C and 300°C, respectively. The oven temperature was held at 200°C for 2 min, then increased to 280°C at a rate of 30°C/min and held at this value until the end of the run. Total run time was 11 min.

Preparation of standards and other reagents

Stock solutions containing cocaine and BE, 1 mg/ml in methanol, were prepared and stored at −15°C. For calibration standards, working solutions of cocaine and BE were prepared at 0.1, 1 and 10 ng/μl, and stored under the same conditions. The propyl ester of BE served as the internal standard and was synthesized as described below. For extraction procedures, the following solutions were prepared: 0.1 *M* potassium phosphate buffer, pH 6.0; 2 *M* sodium carbonate; 0.5 *M* sulfuric acid; methylene chloride–2-propanol (4:1, v/v) with 2% ammonium hydroxide (fresh daily); toluene–*tert.*-butyl alcohol (9:1, v/v).

Synthesis of benzoylecgonine n-propyl ester

About 5 mg BE was treated with 100 μl of N,N-dimethylformamide and 100 μl of N,N-dimethylformamide di-*n*-propyl acetal. The mixture was refluxed for 30 s over an open flame and

allowed to cool at room temperature. The volatiles were removed by evaporation under a gentle stream of nitrogen at 50°C. Thereafter, the cooled mixture was treated with 1 ml of 0.5 *M* H_2SO_4, vortex-mixed and extracted with 5 ml of toluene–*tert.*-butyl alcohol (9:1, v/v). Following vortex-mixing and centrifugation (at 700 *g* for 12 min), the upper organic phase was removed by aspiration. The aqueous acid phase was neutralized with 2 ml of 2 *M* Na_2CO_3 and the benzoylecgonine propyl ester was extracted with 0.5 ml butyl acetate. The upper organic layer was then transferred into a clean tube and the solvent was evaporated under nitrogen at 50°C. The residue was reconstituted in 5 ml methanol and 2 μl of this solution was subjected to gas chromatography. An aliquot of this solution was diluted with methanol to a concentration of *ca.* 15 μg/ml to provide a peak of the same height as a calibration standard of 300 ng cocaine. Under the chromatographic conditions described above, the internal standard showed a single peak with a retention time of 4.7 min. The identity of the internal standard was confirmed by GC–MS, comparing the mass spectra (Fig. 1) of the laboratory synthesized compound and of the reference compound from Research Biochemicals International (Natick, MA, USA).

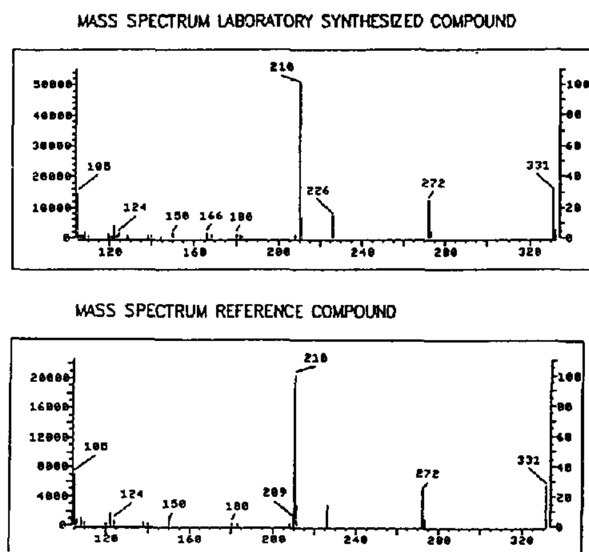

Fig. 1. GC–MS spectrum of the internal standard synthesized in our laboratory and of the reference compound.

Extraction and derivatization

Aliquots of 250 μl plasma samples were mixed with 250 μl drug-free plasma (see Results and discussion) in 10-ml glass centrifuge tubes; then, 20 μl of internal standard solution were pipetted in each and the tubes were briefly vortex-mixed. The samples were acidified with 1 ml of 0.1 *M* phosphate buffer, pH 6.0. The extraction procedure was similar to that described by the manufacturer for urine and hair samples [8]. Bond Elut Certify extraction columns were fitted in a Vac-Elut vacuum manifold. The extraction columns were activated by addition of 2 ml methanol and 2 ml of 0.1 *M* phosphate buffer, pH 6.0 (without allowing them to dry). Thereafter the samples were applied and slowly drawn through over a period of at least 2 min. The columns were sequentially rinsed with 3 ml deionized water and 3 ml 0.1 *M* HCl, and dried for 5–7 min under full vacuum and finally rinsed again with 6 ml methanol and dried for 1–2 min under full vacuum. The absorbed drugs and internal standard were eluted using 2 ml methylene chloride–2-propanol (4:1, v/v) containing 2% ammonium hydroxide. The extracts were transferred into 13 × 100 mm screw-cap glass culture tubes and evaporated under nitrogen at 50°C. Derivatization of BE to its butyl ester was performed as described by Jacob *et al.* [3]. To each standard and sample tube, 100 μl oxalyl chloride was added and the contents were briefly vortex-mixed. After 10 min, 1 ml of 1-butanol was added. The contents were mixed again and the capped (Teflon liner caps) tubes were heated for 20 min in a water bath at 90–95°C. Following derivatization, the samples were allowed to cool at room temperature and 1 ml of 2 *M* Na_2CO_3 was added. The samples were vortex-mixed briefly and then treated with 2 ml toluene–*tert.*-butyl alcohol (9:1, v/v), vortex-mixed for 4 min and centrifuged at 700 *g* for 10 min. The upper organic layer was transferred into a clean tube containing 0.5 ml 0.5 *M* H_2SO_4, and the tubes

were vortex-mixed and centrifuged as described above. The upper organic phase was discarded and the lower aqueous phase was mixed with 1 ml of 2 M Na_2CO_3 and 120 μl butyl acetate. The tubes were vortex-mixed and centrifuged and the butyl acetate extracts, containing the analytes were transferred to autosampler vials and subjected to gas chromatography.

Preparation of calibration curves

The calibration curves (Fig. 2) were constructed by spiking 0.5 ml of drug-free plasma with different amounts of cocaine and BE ranging from 1 to 500 ng, and 20 μl internal standard containing approximately 300 ng of BE propyl ester. Following addition of 1 ml potassium phosphate buffer, pH 6.0, the standards were subjected to extraction, derivatization and post-derivatization clean-up as described above. In every batch of samples analyzed, five standards (appropriate range) and plasma control (blank) were included.

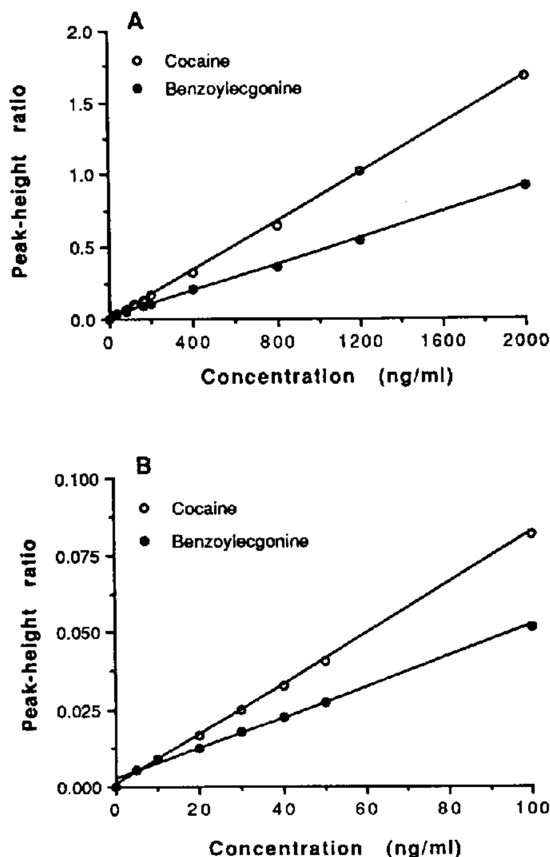

Fig. 2. Typical standard curves for cocaine and benzoylecgonine constructed using 0.5 ml of sheep plasma spiked with high (A) and low (B) concentrations of the analytes.

During preparation of calibration standards, it was observed that when the analytes dissolved in methanol were added to 250 μl of plasma, the system became turbid. To avoid this problem we increased the volume of plasma to 0.5 ml. Thus, calibration standards were prepared using 0.5 ml drug-free plasma, and 250 μl of drug-free plasma was added to the 250 μl actual samples in order to keep the extraction conditions identical for both standards and samples.

Quantitation of analytes was based on peak-height ratios of cocaine and BE to the internal standard. Standard curves (Fig. 2, A and B) were linear over the concentration range expected in our studies, *i.e.* from 4 ng/ml to 2000 ng/ml for both cocaine and BE. The equations for the standard curves, determined by linear regression from 10 different experiments, were $y = 0.00335x - 0.00133$, with $r^2 = 0.99967$, and $y = 0.00179x - 0.01450$, with $r^2 = 0.99921$, for cocaine and BE, respectively. For concentrations ranging from 4 to 100 ng/ml the equations were $y = 0.00323x + 0.00338$, with $r^2 = 0.99967$ and $y = 0.00199x + 0.01002$, with $r^2 = 0.99501$, respectively.

3. Results and discussion

We have developed an extremely sensitive GC procedure to quantitate low concentrations (4 ng/ml) of cocaine and benzoylecgonine. The procedure requires only 250 μl plasma and is based on the rapid solid-phase extraction procedure, which eliminated the problem of emulsion formation and produced very clean extracts.

The use of an acidic pH for the most part of the extraction–derivatization procedure was desirable, given the susceptibility of cocaine to basic hydrolysis [13]. We have also evaluated the extraction of the analytes under basic conditions (pH 9.5) using the non-polar Bond Elut C_8

Table 1
Day-to-day precision for spiked plasma samples

Analyte	Concentration (ng/ml)	Number of experiments	Concentration found (mean ± S.D.) (ng/ml)	Relative standard deviation (%)
Cocaine	5	6	6.49 ± 0.51	7.80
Benzoylecgonine	5	6	4.26 ± 0.38	8.92
Cocaine	10	10	11.92 ± 0.36	3.04
Benzoylecgonine	10	6	13.15 ± 0.98	7.46
Cocaine	50	10	49.21 ± 1.53	3.10
Benzoylecgonine	50	10	50.58 ± 2.90	5.74
Cocaine	300	10	302.99 ± 17.35	5.73
Benzoylecgonine	300	6	298.32 ± 19.81	6.64

columns (Varian), as described by Tebbett and McCartney [14]. However, our newly developed extraction procedure proved better and yielded cleaner extracts. This is due to the fact that the pre-elution column rinse with larger volumes of aqueous and organic solvents removed a major portion of both hydrophilic and hydrophobic contaminants (proteins and/or lipids).

To determine the overall extraction efficiency of the present procedure (including the derivatization and post-derivatization clean-up step), an aliquot of plasma spiked with different amounts of cocaine was subjected to the extraction–derivatization and 2 μl of the final extracts (from 120 μl) were analyzed by gas chromatography. Solutions containing the same amount of cocaine as those spiked in plasma were prepared and analyzed directly. The extraction efficiency, calculated on the basis of cocaine peak-heights before and after extraction, was found to be 70%.

The method is characterized by good precision as illustrated in Tables 1 and 2. In order to determine the precision of our chromatographic

Table 2
Within-day precision for spiked plasma samples

Analyte	Concentration (ng/ml)	Number of experiments	Concentration found (mean ± S.D.) (ng/ml)	Relative standard deviation (%)
Cocaine	5	6	5.87 ± 0.33	5.62
Benzoylecgonine	5	6	4.48 ± 0.31	6.92
Cocaine	20	10	20.63 ± 0.42	2.06
Benzoylecgonine	20	10	19.91 ± 1.35	6.79
Cocaine	100	6	97.92 ± 4.74	4.84
Benzoylecgonine	100	10	102.63 ± 6.88	6.71
Cocaine	200	6	195.23 ± 5.18	2.65
Benzoylecgonine	200	10	194.45 ± 13.33	6.86

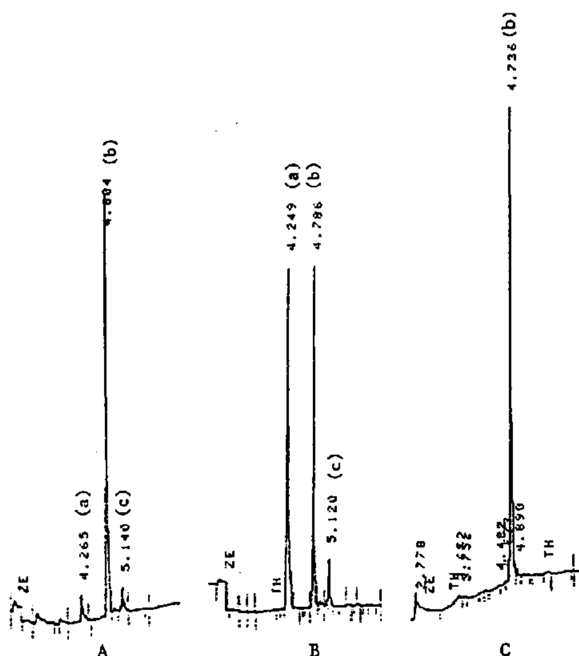

Fig. 3. Chromatogram of (A) extract of 0.5 ml sheep plasma spiked with 20 ng of cocaine (a), and 20 ng of BE (c); (b) is the internal standard peak; (B) 250 μl fetal lamb plasma drawn from the brachial artery during a fetal cocaine infusion at a rate of 1 mg/kg/min; (C) drug-free (blank) sheep plasma. Chart-speed 1 cm/min.

system, 2 μl of a methanol solution of cocaine (420 ng/ml) and internal standard were injected 10 times sequentially. The relative standard deviation for the peak-height ratios was 1.6%. Detection and quantitation limits were defined and determined according to the method described by Knoll [15] and were respectively ~1 ng/ml and 4 ng/ml for both cocaine and BE.

The method has a good selectivity as shown by the well resolved peaks of the chromatograms in Fig. 3.

4. Conclusion

The presented method for determination of cocaine and BE can be performed on as little as 250 μl of plasma with high sensitivity and linearity up to 2000 ng/ml. The method combines the advantages of simplicity, using only gas chromatography, and small sample size. This method may be especially useful for analyzing small sample volumes obtained from fetus and neonates as well as from small animals. We have already tested its usefulness in our laboratory while assaying *ca.* 300 samples collected during fetal and maternal cocaine infusion experiments in sheep.

Acknowledgments

We are grateful to the Research Technology Branch, National Institute on Drug Abuse, for providing the cocaine and benzoylecgonine standards; to Prof. Thomas B. Cooper (Department of Psychiatry) for his valuable suggestions, reviewing the manuscript and helping us with the GC–MS confirmation of the internal standard; to Dr. K.E. Jack and Ms. Khalida Khan for technical assistance. This study was supported in part by National Institute on Drug Abuse Grants ROI DA 07588 (CRC) and ROI DA 06648 (HOM).

References

[1] H.O. Morishima, C.R. Chao, M. Matsuo, Y. Abe and T.B. Cooper, *Anesthesiology*, 77 (1992) A1038.

[2] A.L. Misra, N.L. Vadlamani, R. Block, P.K. Nayak and S.J. Mule, *Res. Commun. Chem. Pathol. Pharmacol.*, 8 (1974) 55–63.

[3] P. Jacob III, B.A. Elias-Baker, R.T. Jones and N.L. Benowitz, *J. Chromatogr.*, 417 (1987) 277–286.

[4] B.H. Dvorchik, S.H. Miller and W.P. Graham, *J. Chromatogr.*, 135 (1977) 141–148.

[5] P.I. Jatlow and D.N. Bailey, *Clin. Chem.*, 21 (1975) 1918–1921.

[6] S. Koontz, D. Besemer, N. Mackey and R. Phillips, *J. Chromatogr.*, 85 (1973) 75–79.

[7] R.W. Taylor and S.D. Le, *J. Anal. Toxicol.*, 15 (1991) 276–278.

[8] M.R. Harkey, G.L. Henderson and C. Zhou, *J. Anal. Toxicol.*, 15 (1991) 260–265.

[9] D.S. Isenschmid, B.S. Levine and Y.H. Caplan, *J. Anal. Toxicol.*, 12 (1988) 242–245.

[10] S.J. Mule and G.A. Casella, *J. Anal. Toxicol.*, 12 (1988) 153–155.
[11] R.W. Taylor, N.C. Jain and M.P. George, *J. Anal. Toxicol.*, 11 (1987) 233–234.
[12] D.L. von Minden and N.A. D'Amato, *Anal. Chem.*, 49 (1977) 1974–1977.
[13] D.S. Isenschmid, B.S. Levine and Y.H. Caplan, *J. Anal. Toxicol.*, 13 (1989) 250–256.
[14] I. Tebbett and Q.W. McCartney, *Forensic Sci. Int.*, 39 (1988) 287–291.
[15] J.E. Knoll, *J. Chromatogr., Sci.*, 23 (1985) 422–425.