Determination of Esterase-Catalyzed Cocaine Metabolite Formation by High-Performance Liquid Chromatography with Ultraviolet Detection

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of cocaine metabolites produced in vitro by serum and liver esterases is described. Hydrolysis of cocaine at the methyl ester bond produces benzoylecgonine and methanol, whereas hydrolysis at the benzoyl ester bond produces ecgonine methyl ester and benzoic acid. This method quantitates benzoic acid (as a measure of ecgonine methyl ester formation) and benzoylecgonine, which can be determined simultaneously and with great sensitivity by HPLC and ultraviolet detection. Cocaine is found to be hydrolyzed to both ecgonine methyl ester and benzoylecgonine; hepatic microsomes exhibit the highest specific activity.

Introduction

Cocaine is a widely abused drug that is primarily metabolized by serum and liver esterases (1). Alterations in esterase activity have been shown to influence cocaine-mediated toxicities such as respiratory depression, cardiac arrest, and liver damage (2–4). The two most frequently quantitated esterase products of cocaine metabolism are benzoylecgonine, which is produced by hydrolysis of the methyl ester bond, and ecgonine methyl ester, which is produced by hydrolysis of the benzoyl ester bond (Figure 1). Numerous high-performance liquid chromatographic (HPLC) methods for the simultaneous determination of cocaine and benzoylecgonine have been reported (5,6), but ecgonine methyl ester has been routinely quantitated by gas chromatography with mass spectrometry or nitrogen–phosphorus detection (7,8) because it lacks an ultraviolet (UV)-absorbing chromophore. Although one published protocol described the simultaneous determination of cocaine, benzoylecgonine, and ecgonine methyl ester by HPLC, this method required both UV and electrochemical detection (9), which may not be readily available in many laboratories. In this article, we described a method for separating and quantitating cocaine and its esterase-mediated metabolites by HPLC with UV detection using benzoic acid formation as a measure of benzoyl ester bond hydrolysis. This method provides an extremely simple and highly efficient alternative to directly measuring ecgonine methyl ester levels and permits the rapid quantitation of cocaine metabolites formed in vitro from several biological sources.

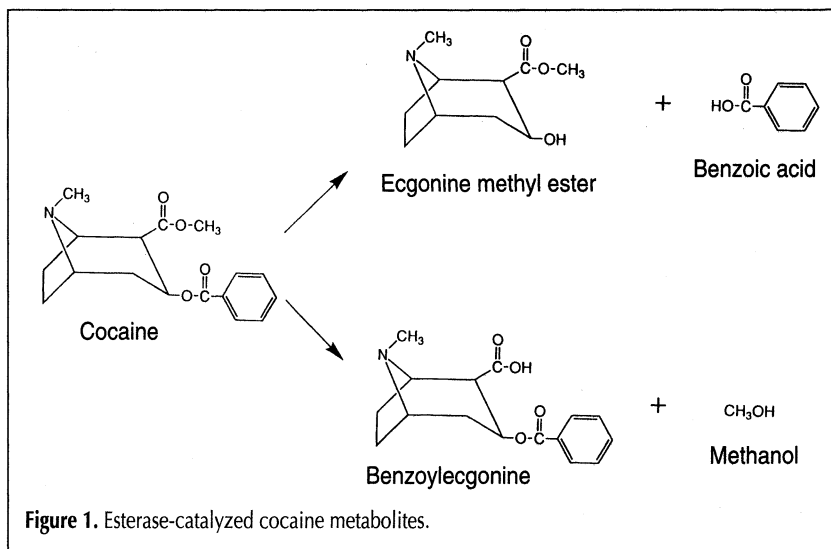

Figure 1. Esterase-catalyzed cocaine metabolites.

Experimental

Chemicals

Cocaine–HCl, tropacocaine–HCl, and benzoylecgonine were generously provided by the National Institute on Drug Abuse (Bethesda, MD). Benzoic acid was purchased from Sigma (St. Louis, MO). HPLC-grade acetonitrile, water, and phosphoric

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acid were purchased from Fisher Scientific (Springfield, NJ). All other chemicals were reagent grade.

Equipment

A Rainin HPLC system (Woburn, MA) consisting of a Rabbit HP/HPX solvent delivery system, control–interface module, and Dynamax UV-D II detector connected to a Macintosh computer via an RS-422 interface cable was used. Separations were performed at ambient temperature on an Alltech (Deerfield, IL) Alltima C_{18} column (250 × 4.6-mm i.d., 5-µm particle size). The flow rate was 1 mL/min, and the absorbance was monitored at 230 nm. The mobile phase consisted of a mixture (67:33, v/v) of acetonitrile and 0.05M monobasic potassium phosphate buffer acidified to pH 3 with 85% phosphoric acid.

Sample preparation

Serum, hepatic cytosol, and microsomes were obtained from male CF-1 mice (25–30 g) (Charles River, Wilmington, MA). Hepatic cytosol and microsomes were prepared from liver homogenates by differential centrifugation as described in the literature (10). To obtain serum, blood was drawn from the vena cava, allowed to clot on ice, and centrifuged at 2500*g* for 10 min. Protein concentrations of the samples were determined by the method of Lowry et al. (11) using bovine serum albumin as the standard.

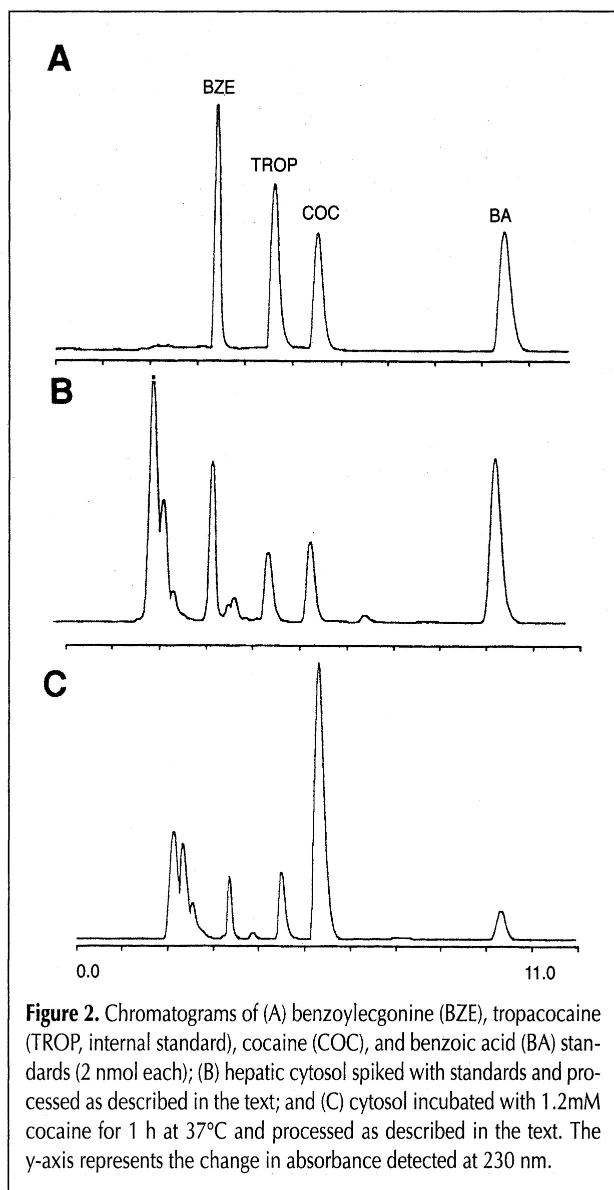

Figure 2. Chromatograms of (A) benzoylecgonine (BZE), tropacocaine (TROP, internal standard), cocaine (COC), and benzoic acid (BA) standards (2 nmol each); (B) hepatic cytosol spiked with standards and processed as described in the text; and (C) cytosol incubated with 1.2mM cocaine for 1 h at 37°C and processed as described in the text. The y-axis represents the change in absorbance detected at 230 nm.

In vitro incubation and extraction

Serum, hepatic cytosol, and microsomes were incubated with either cocaine (1.2mM) or benzoylecgonine (75µM) in methanol. Sodium fluoride (200mM) was added as noted (Figure 3) to inhibit esterase activity. The reaction was initiated by addition of substrate, incubated at 37°C for periods ranging from 0 to 150 min, and stopped by precipitation of proteins with 3 volumes of ethanol. Tropacocaine was added as the internal standard (25µM final concentration), and after centrifugation at 5000*g* for 5 min to pellet the proteins, a 200-µL aliquot of the supernatant was dried under nitrogen. The sample was reconstituted in 150 µL of mobile phase, filtered through a 0.45-µm nylon membrane, and then a 100-µL aliquot was injected onto the column. To determine the extraction efficiency, 50 nmol/mL of benzoylecgonine, tropacocaine, cocaine, and benzoic acid was added to blank tissue samples and processed as described above. The quantities recovered were compared with those obtained from standards dissolved directly in the mobile phase to determine the extraction efficiency of this procedure.

Calibration

Calibration standards were prepared by dissolving appropriate amounts of drugs in the mobile phase to achieve final concentrations of 0.05, 0.5, 1.0, 2.5, 10.0, 25.0, and 50.0 nmol per

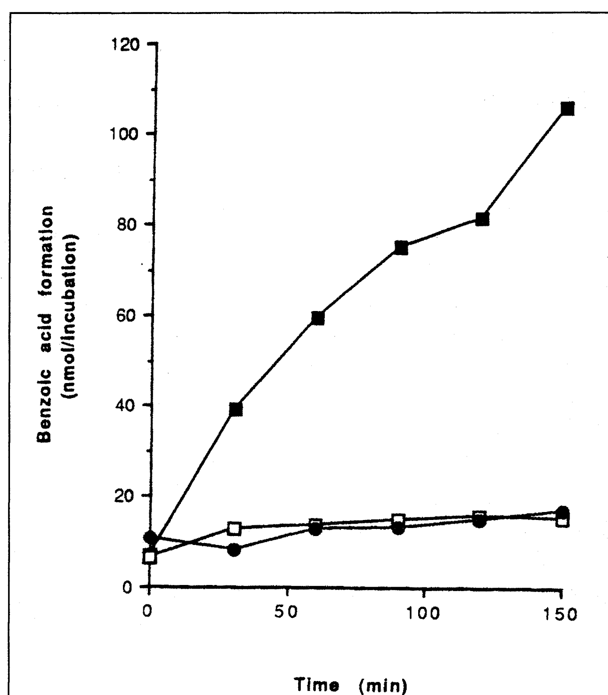

Figure 3. Time course of benzoic acid formation in hepatic cytosol incubated with 1.2mM cocaine (■), 1.2mM cocaine with 200mM sodium fluoride (□), or 75 µM benzoylecgonine (●).

100 μL of injection volume, and the linearity of peak area versus concentration was determined. Samples from in vitro incubations were prepared so that the concentrations of the drugs injected onto the column were within the linear range of the standard curve.

Results and Discussion

Benzoylecgonine, tropacocaine (internal standard), cocaine, and benzoic acid were separated with baseline resolution, exhibiting retention times of 3.4, 4.6, 5.5, and 9.5 min, respectively (Figure 2A). The calibration curves generated correlation coefficients (r) of 0.999 for all four compounds. The range of the standards was 0.05–50 nmol per 100 μL of injection volume. That is equivalent to 1.5–1500 nmol per milliliter of incubate, a range that includes the concentrations observed in the in vitro experiments. The limit of sensitivity for all four compounds was 0.3 nmol per milliliter of incubation mixture, and no intrinsic contaminating peaks in the serum, hepatic cytosol, or microsomes coeluted with any of the compounds of interest. The average extraction efficiencies for benzoylecgonine, tropacocaine, cocaine, and benzoic acid were 96, 82, 90, and 83%, respectively. Individual extraction efficiencies from serum, cytosol, and microsomes varied less than 5% for all of the drugs.

Incubation of cocaine with hepatic cytosol resulted in time-dependent benzoic acid formation (Figure 3). The addition of sodium fluoride, an esterase inhibitor, completely blocked benzoic acid formation. Similar findings were also observed with serum and hepatic microsomes (data not shown). Little or no benzoic acid was formed after incubation of benzoylecgonine, regardless of the tissue source with which it was incubated (Table I). The concentration of benzoylecgonine used in the incubations (75μM) reflects the approximate amount produced after the incubation of cocaine (1.2mM) with the various tissue samples for 1 h. The incubation of hepatic microsomes with cocaine resulted in benzoic acid (Table I) and benzoylecgonine formation (Table II) with the highest specific activity.

Table I. Specific Activity of Benzoic Acid Formation*

	Substrate	
Tissue sample	Cocaine	Benzoylecgonine
Serum	40 ± 5	0
Hepatic cytosol	51 ± 9	0
Hepatic microsomes	13800 ± 3201	0

* Tissue samples were incubated with cocaine (1.2 mM) or benzoylecgonine (75 μM) for 1 h at 37°C. Specific activities are given as pmol benzoic acid formed per milligram protein per minute. Values represent the average ± half the range of two determinations.

Table II. Specific Activity of Benzoylecgonine Formation*

Tissue sample	Protein concentration (mg/mL incubation)	Benzoylecgonine formed (nmol/mL incubation)	Specific activity† (pmol/mg/min)
None (buffer alone)	-	40 ± 5‡	-
Serum	41	116 ± 2	31 ± 1
Hepatic cytosol	18	37 ± 3	0
Hepatic microsomes	1	216 ± 2	3056 ± 235

* Tissue samples or buffer (0.1M potassium phosphate, pH 7.4) were incubated with cocaine (1.2mM) for 1 h at 37°C, and benzoylecgonine production was measured. The values represent the average ± half the range of two determinations.
† Specific activity was calculated from the total amount of benzoylecgonine formed in the incubation minus the amount of benzoylecgonine formed nonenzymatically.
‡ Represents the amount of benzoylecgonine formed nonenzymatically.

Conclusion

The method presented in this article was extremely simple and efficient and had the advantage that all compounds of interest could be quantitated by UV detection alone. In addition, the use of a double-endcapped C_{18} column eliminated the need for chemical modifiers in the mobile phase. In contrast, the use of a standard C_{18} column (Microsorb, Rainin Instruments) required the addition of 0.15mM tetrabutylammonium phosphate to the mobile phase to prevent the marked tailing of cocaine as well as benzoylecgonine (data not shown). Serum, hepatic cytosol, and hepatic microsomes were chosen as esterase sources for our studies because in vitro studies on cocaine metabolism have routinely utilized these tissues (1,7,12).

Hydrolysis of cocaine at the benzoyl ester bond produced benzoic acid and ecgonine methyl ester in a 1:1 molar ratio (Figure 1). Sodium fluoride inhibited benzoic acid formation, which indicated that it was indeed an esterase product. Little or no benzoic acid was produced when benzoylecgonine was incubated with esterases in any of the tissue sources examined. This indicated that benzoylecgonine was a poor substrate for these esterases, and thus benzoic acid formation could be associated specifically with cocaine hydrolysis. Because ecgonine methyl ester could be further hydrolyzed to ecgonine, especially after a prolonged incubation (13), quantitation of benzoic acid may, in fact, yield a more accurate measure of the rate of cocaine hydrolysis at the benzoyl ester bond.

Although our experiments were conducted with murine samples, Stewart et al. reported similar results using human samples (1). They also found little, if any, benzoic acid formation when benzoylecgonine was incubated with human serum or liver homogenates, and they confirmed that the levels of benzoic acid produced in incubations with cocaine correlated with the levels of ecgonine methyl ester. Their method, however, involved radiolabelled substrates and a thin-layer chromatographic separation, and thus lacked the ease and simplicity of the present method.

Acknowledgment

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