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Analysis of cocaine, benzoylecgonine, ecgonine methyl ester, ethylcocaine and norcocaine in human urine using HPLC with post-column ion-pair extraction and fluorescence detection*

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Abstract: The measurement of cocaine and its major metabolites has been achieved by an HPLC method that compensates for their different solubilities and detection properties. Although ecgonine methyl ester is a major metabolite it is generally not measured by HPLC because it is poorly detectable by UV, and its water solubility makes recovery from urine difficult. Using modified solid-phase extraction procedures recoveries of 85% for ecgonine methyl ester, 97% for cocaine, 106% for benzoylecgonine and 80% for ethylcocaine have been obtained from urine. Increased chromatographic retention and detection sensitivity has been obtained by formation of the *t*-butyldimethylsilyl derivative of ecgonine methyl ester which was found to be stable in the HPLC mobile phase for at least 1 week. Alkylation of norcocaine and benzoylecgonine has improved their detection sensitivity and also chromatographic resolution. All calibrations were linear over the range 200–1000 ng ml^{-1} in urine with correlation coefficients >0.99.

Keywords: *Cocaine; benzoylecgonine; ecgonine methyl ester; ethylcocaine; post-column high-performance liquid chromatography; silylation; alkylation.*

Introduction

The development of improved analytical procedures for drugs of abuse continues to be an active area of research because of the increasing availability of drugs world-wide. In this laboratory, sensitive and selective methods have been developed using pre-column derivatization [1], detection at 205 nm [2] and post-column ion-pair extraction [3] for drugs of abuse. Cocaine is widely abused for its CNS stimulant properties. In humans cocaine is metabolized and excreted in the urine mainly as benzoylecgonine (BE) and ecgonine methyl ester (EME). Typical values for cocaine excretion are BE 46%; EME 41%; cocaine 3% and the other metabolites about 10% [4]. In cases where cocaine is concurrently abused with ethanol an additional metabolite, ethylcocaine is produced [5]. Metabolism of cocaine by liver esterases forms EME and the resulting loss of the benzoyl group means that the metabolite has lost its important chromophore, has become more water soluble and is difficult to recover from urine using procedures suitable for cocaine or benzoylecgonine.

Numerous HPLC methods have been described for the measurement of cocaine and its metabolites [6, 7] but usually EME is not included, even in recent methods [8]. However, as a major metabolite of cocaine [9] EME is important in both drug metabolism studies and in forensic investigations since, with BE, it is an indication of when the cocaine was ingested. Examples of methods that measure EME include that by Miller and DeVane [10] who used an RP-HPLC procedure to measure C and BE by UV detection at 230 nm and also C and EME by electrochemical detection, with the two detectors arranged in series. A mobile phase of pH 8.8 was required to detect EME so that a polymeric column was required. Ethylcocaine was selected as internal standard, and the analytes measured in sheep plasma in the calibration range of 100–2400 ng ml^{-1}. GC with a nitrogen phosphorous detector has been used [11] to measure C, BE and EME in urine following SPE extraction and derivatization of BE and EME. Detection limits were 100 ng ml^{-1} for C and BE and 250 ng ml^{-1} for EME. Unfortunately, BE was derivatized to its ethyl ester, ethylcocaine and the percentage re-

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covery of EME was only 41%. GC–MS has been described [12] to measure C, BE, EME and EC in hair collected from cocaine users, and the presence of ethylcocaine found to be a particularly useful indication of cocaine use, since cocaine could conceivably contaminate the hair externally. In this work an HPLC method is reported for the simultaneous measurement of cocaine, benzoylecgonine, ecgonine methyl ester and ethylcocaine from urine by suitable modification of solid-phase extraction procedures, derivatizations to improve chromatography and extractions, followed by post-column ion-pair formation with fluorescence detection.

Experimental

Chemicals

Cocaine HCl was obtained from the dispensary of the School of Pharmacy and Pharmacology, University of Bath. Benzoylecgonine was synthesized following the procedure of Lampert and Stewart [6]. Ecgonine methyl ester was prepared from ecgonine following the procedure of Findlay [13]. Ecgonine was prepared by acid hydrolysis of cocaine. Ethylcocaine was prepared from ecgonine by treating it with iodoethane in the presence of potassium carbonate in acetone. Norcocaine was prepared following the procedure of Borne [14]. The structures of all the metabolites was confirmed by IR and ^{1}H NMR. The purity of the samples was confirmed by HPLC and for EME by GC. No extraneous peaks were observed.

Acetonitrile, methanol, ammonia, dichloroethane, dichloromethane, 9,10 dimethoxy anthracene sulphonate sodium salt (DAS) were HPLC grade and were obtained from Fisons (Loughborough, UK). Buffer components (analytical grade) were also obtained from Fisons. Iodoethane, iodobutane and iodohexane were synthesis grade (99% purity) and obtained from Aldrich Chemical Company (Gillingham, Dorset, UK). MTBSTFA (*N*-methyl-*N*{*tert*-butyldimethylsilyl}trifluoroacetamide) was obtained from Pierce Warriner Ltd (Cheshire, UK). Urine was Lyphocheck 'Screen Control Negative' from Bio-Rad Labs (Hemel Hempstead, UK). Water was distilled from an all glass still. Solid-phase extraction was carried out using a Vac Elut SP24 unit with Bond-Elut 'Certify' (300 mg) cartridges from Jones Chromatography (Hengoed, UK).

Instrumentation

Chromatography was performed using an SSI (State College, PA, USA) HPLC system consisting of a gradient system Model 402 with a microbore head that was modified to carry out gradients at low flow rates. Commercial low pressure gradient systems are designed to carry out gradient elution at flow rates of 1.0 ml min^{-1} or more with 4.6 mm i.d. columns. The volume of the mobile phase in the system, i.e. the gradient controller, the pump and the associated tubing before the column is about 5 ml and causes a delay in the time before the gradient reaches the column of about 5 min. If the same system is used at flow rates of 0.2–0.4 ml min^{-1}, as required for columns of 2.1 mm i.d., the delay time becomes 25–12.5 min. This delay also causes problems with the gradient pattern and so modifications are required to minimize this effect. This was achieved by using short 0.8 mm i.d. tubing to connect the pump to the gradient controller instead of the usual 2.0 mm i.d. tubing and using short narrow bore tubing (0.2 mm i.d.) between the column and the purge valve of the pump. These modifications reduced the delay time of 12.5 min to 4.0 min at 0.4 ml min^{-1}.

A Rheodyne 7125 model with a 50 μl loop in a model 505 oven and maintained at 40°C was connected to a 250 × 2.1 mm i.d. Supelco pKb 100 column, 5 μm particle size (Supelco, Bellefonte, PA, USA). The column was attached to the post-column ion-pair extraction detector as shown in Fig. 1, consisting of two model 350 pumps with either a Guardian pulse dampener or Model LP Lo-Pulse dampener (SSI). Additional pulse dampening was provided with 100 × 4.6 mm i.d. columns containing 10 μm Partisil silica (aqueous phase) and 10 μm CPS-Hypersil (organic phase). The addition of the aqueous reagent to the HPLC eluent and the segmentation of aqueous phase with organic phase was achieved using Tee-connectors, 1/16 × 0.015 inch (SSI). An Uptight precolumn, 20 × 2 mm i.d. (Anachem) packed with 75 μm glass Ballotini beads was used as mixing column. The extraction coil was 1.5 m × 0.8 mm i.d. s/s tubing. The phase separator, which has been described elsewhere [3] was connected to a Perkin–Elmer Model 204S Spectrofluorometer fitted with a 100 μl flow cell (Perkin–Elmer–Hitachi) and connected to a Servoger 120 chart recorder.

The mobile phase used an acetonitrile step

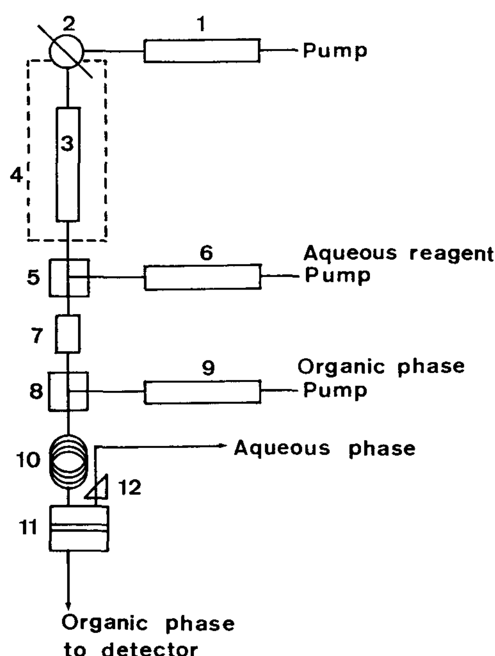

Figure 1
Schematic diagram of HPLC post-column system. 1,6 and 9 = in-line columns; 2 = injection valve; 3 = analytical column; 4 = oven; 5 and 8 = Tee connectors; 7 = mixing column; 10 = extraction coil; 11 = phase separator; and 12 = microneedle valves.

gradient from 11 to 30% v/v acetonitrile in 0.05 M phosphate buffer (pH 4.0) at 0.4 ml min^{-1} at 7.0 min after injection, and returned to 11% at 25 min. 9,10-dimethoxyanthracene sulphonate sodium salt (DAS, 34 mg l^{-1}) was added to the eluent flow post-column at a flow rate of 0.5 ml min^{-1}. Dichloroethane was used to segment and extract the drug–DAS ion-pairs at a flow rate of 0.6 ml min^{-1}. Fluorescence detection was achieved using an excitation wavelength of 383 nm and emission wavelength of 465 nm.

Extraction procedure

An appropriate amount of an acetonitrile stock solution of the various standards was pipetted into glass tubes, evaporated to dryness using nitrogen and 1.0 ml of Bio-Rad urine, prepared according to the manufacturer's instruction, was added to the dried residue. The sample was vortexed for 1 min and 4.0 ml of phosphate buffer (pH 6.0) added.

Bond-Elut 'Certify' LRC columns were inserted into a SP24 vacuum manifold and conditioned with 6.0 ml methanol and 6.0 ml phosphate buffer (pH 6.0). The columns were prevented from drying out at this stage by turning the vacuum off. The samples prepared above were poured into the prepared cartridges and gently pulled through. After this the cartridges were washed with 6.0 ml of water and air-dried for 5 min at 15 mm Hg. They were then washed with 9.0 ml of 0.1 N HCl followed by 7 ml methanol and 3 ml acetonitrile. Elution of the samples was performed with 6.0 ml of 1.5% NH_4OH in dichloromethane–isopropanol (80:20%, v/v). The samples were transferred to Reacti-vials (Pierce, Warrington, Cheshire, UK) and evaporated to dryness using a gentle stream of N_2 and derivatized.

Derivatization procedures

(a) The alkylation procedure was a modification of that described by Ortuno *et al.* [11] for the derivatization of benzoylecgonine and norcocaine. To the dried residue of standards, 180 μl of acetonitrile, 30 μl of iodobutane, 10.0 mg of K_2CO_3 and 20 μl of 0.20 mg ml^{-1} 18 Crown 6 ether in acetonitrile was added. The Reacti-vial was closed, vortexed for 30 s, and then heated at 85°C for 1 h. After the samples were allowed to cool they were evaporated to dryness under N_2.

(b) Silylation of EME. Acetonitrile (100 μl) and MTBSTFA (50 μl) were added to the dried residue from (a) and the vials heated at 60°C for 30 min. The samples were then allowed to cool and evaporated to dryness under a gentle stream of N_2. To the dried residue, internal standard (50 μl), mobile phase (350 μl) and 8.5% phosphoric acid (100 μl) were added and vortexed for 30 s. The sample was injected into the HPLC system. Peak height ratios were measured and plotted against concentration of standards. The recoveries of samples from urine were calculated by comparison with non-extracted standards.

Results and Discussion

Initial attempts with Bond-Elut 'Certify' cartridges (130 mg) following the manufacturer's instructions showed that recovery of EME from spiked urine was around 40%. Subsequent examination of the process showed that EME was being lost during the first washing procedure. To improve recoveries of EME the capacity of the cartridge was increased to 300 mg, and this raised recoveries to

70–80%. However this cartridge size also retained additional endogenous components from the urine. To clean the urine extract more thoroughly the volume of wash solvents and eluting solvents was also increased. Initially as recommended by the manufacturer methanol alone was used as the wash solvent but this lowered the recoveries of EME and ethylcocaine. Replacing methanol with acetonitrile gave a less clean blank chromatogram. A combination of 7.0 ml of methanol and 3.0 ml of acetonitrile was found to give the best compromise in terms of recoveries and clean blanks.

An additional complication was that some extra peaks were found to originate from the cartridge material itself and repeated washing of the cartridges with methanol or eluting solvents failed to remove them. The peaks were found to be proportional to the percentage of ammonia in the eluting solvent, as shown in Fig. 2, and is probably caused by ammonia attacking the silica-based packing material. Triethylamine had the same effect as

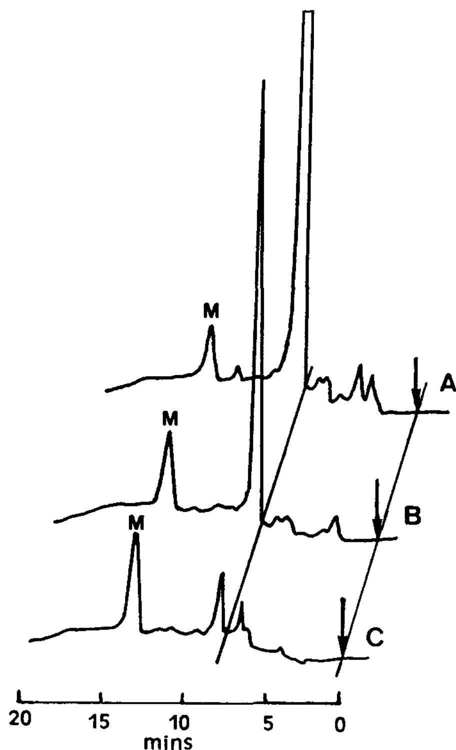

Figure 2
Effect of the percentage ammonia present in the dichloromethane–isopropyl alcohol elution step on Bond-Elut 'Certify' cartridges. No standards or urine was added to the cartridges. Methadone was added after the elution step as a marker. The cartridge residues were derivatized and alkylated as for the cocaine analysis. A = 2% ammonia; B = 1% ammonia; and C = 0.5% ammonia.

ammonia. Decreasing the percentage of ammonia in the eluting solvent also lowered the recoveries of BE, which is the most basic metabolite (pK_a value 11 compared to 8.5 for the other metabolites). As a compromise 1.5% (v/v) ammonia in the eluting solvent was the least that could be used for good recovery values.

In this application the derivatization procedures to increase the hydrophobicity of BE, NC and EME have two major benefits. Firstly their chromatographic retention times are increased, which moves them further away from the endogenous peaks extracted from urine, and secondly it improves the extraction of the ion-pair complexes into the organic phase and so increases sensitivity.

For benzoylecgonine and norcocaine, alkylation is appropriate but not the ethyl derivatives because cocaine is metabolized to ethylcocaine by those persons who also abuse alcohol. Initially the procedure of Ortuno *et al.* [11] was used with iodobutane replacing iodoethane. Derivatization was satisfactory and reproducible, but required 3 h at 60°C. Since crown ethers have been used as catalysts for alkyl halide derivatizations [15], 18 Crown 6 ether was selected in this study to speed up the reaction. Acetonitrile was used as the solvent instead of acetone so that the reaction temperature could be raised to 85°C and the vial caps remain secure. Acetonitrile is also more compatible with the silylation step.

18 Crown 6 ether is soluble in dichloroethane and produces a large peak near the solvent front, and so to minimize extraction into the post-column system a minimum amount of crown ether was used. Decreasing the amount of crown ether from 2.0 to 0.03 mg ml^{-1} did not make any difference to the derivatization yield or time required.

Silylation is widely used in GC to form trialkylsilyl ethers for compounds which contain hydroxyl groups. Most of the silylating agents are very sensitive to moisture and cannot be used for RP-HPLC. Tertiary butyldimethylsiloxyl groups (TBDMS) are 10^4 times more stable than tertiary methyl siloxyl groups [16, 17]. MTBSTFA was found to form a stable *t*-butyldimethlsilyl ecgonine methyl ester derivative which is resistant to hydrolysis for more than 1 week at room temperature. It is important to exclude any moisture during the derivatization procedure in order to obtain a successful, and reproducible derivatization.

The Supelco pKb 100 column is marketed for the chromatography of basic compounds and gave symmetrical narrow peaks for all the metabolites in this application. The column has been used for over a year without any major deterioration in its performance. A 2.1 mm i.d. column was preferred for this application in order to increase sensitivity and reduce solvent consumption and with care can be used under gradient conditions with little background noise.

Without derivatization, norcocaine appeared just after cocaine and these compounds were difficult to separate under gradient conditions. Changing the mobile phase pH or the organic modifier did not improve the situation. A step gradient was used to shorten the analysis time. Figure 3 shows a chromatogram of a mixture of derivatized standards. Norcocaine as its butyl derivative is well separated from cocaine and appears after ethylcocaine. Norcocaine was not used in spiked urine samples as there was some interferences from the cartridges at its retention time. Fortunately, norcocaine is a very minor metabolite in humans.

In the present application methadone has been used as an internal standard. However, methadone can also be present in the urine of some cocaine users as it is prescribed for the treatment of cocaine addicts. In that case hexylbenzoylecgonine can be used as an internal standard and the method can then also be used to measure methadone (Fig. 3).

The calibration curves for all the standards spiked into urine were linear from 200 to 1000 ng ml^{-1} with correlation coefficients >0.99 (Fig. 4). The equations of the calibrations with standard deviations shown in brackets were as follows: for cocaine, $y = 0.913 (\pm 0.124)x + 0.011 (\pm 0.081)$, $n = 4$, $r = 0.999$, limit of detection (LOD) = 0.059 μg ml^{-1}; for ethyl-cocaine, $y = 2.68 (\pm 0.712)x + 0.097 (\pm 0.395)$, $n = 4$, $r = 0.995$, LOD = 0.095 μg ml^{-1}; for benzoylecgonine, $y = 1.61 (\pm 0.165)x - 0.126 (\pm 0.114)$, $n = 4$, $r = 0.999$, LOD = 0.046 μg ml^{-1}; for ecgonine methyl ester, $y = 0.983 (\pm 0.156)x - 0.021 (\pm 0.123)$, $n = 4$, $r = 0.998$, LOD = 0.081 μg ml^{-1}. Within-day precision ($n = 4$) for cocaine was 0.78 ± 0.056, recovery 100%; for ethylcocaine 0.96 ± 0.047, recovery 80%; for benzoylecgonine 0.96 ± 0.045, recovery 91%; for ecgonine methyl ester 0.565 ± 0.037, recovery 98%. Much lower limits of detection are possible if the interfering peaks from the cartridges can be eliminated. The problem of interfering peaks was also observed from the

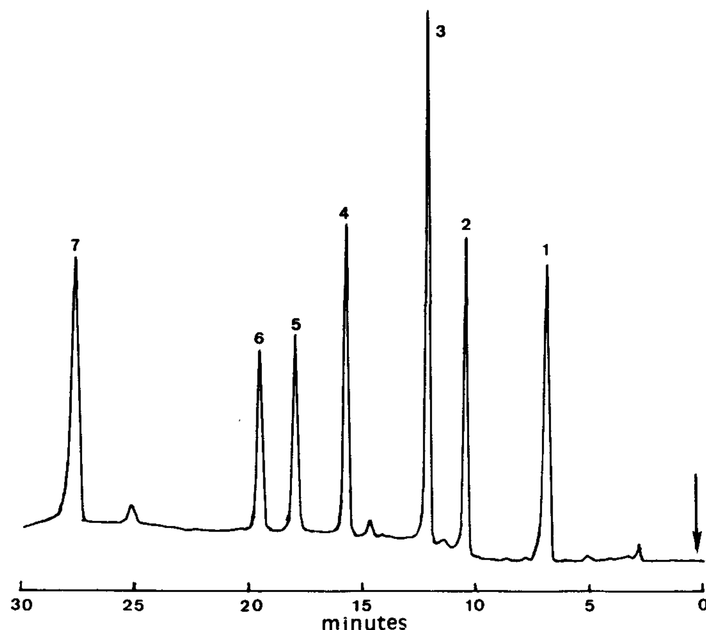

Figure 3
Chromatogram of standards derivatized at 65°C for 3 h [ref. 11] dissolved in mobile phase. 1 = cocaine 0.6 μg ml^{-1}; 2 = ethylcocaine 0.44 μg ml^{-1}; 3 = *N*-butylnorcocaine 0.4 μg ml^{-1}; 4 = butylbenzoylecgonine 0.5 μg ml^{-1}; 5 = methadone 0.3 μg ml^{-1}; 6 = *t*-butyldimethylsilyl ecgonine methyl ester 0.6 μg ml^{-1}; and 7 = hexylbenzoylecgonine 2 μg ml^{-1}. Chromatographic conditions as in Experimental.

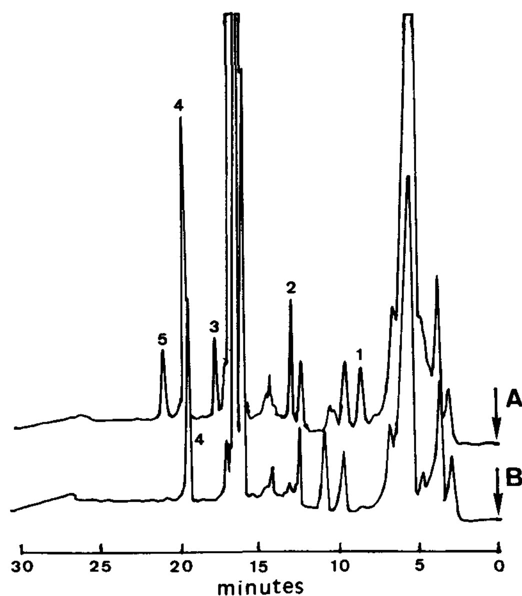

Figure 4
Chromatogram of standards in urine derivatized at 85°C for 1 h in the presence of 18 Crown 6 ether, as in Experimental. A = cocaine and metabolites in urine; B = urine with internal standard. 1 = cocaine 0.23 μg ml^{-1}; 2 = ethylcocaine 0.16 μg ml^{-1}; 3 = butylbenzoylecgonine 0.20 μg ml^{-1}; 4 = methadone 0.75 μg ml^{-1} (internal standard); 5 = *t*-butyldimethylsilyl ecgonine methyl ester 0.2 μg ml^{-1}. Chromatographic conditions as in Experimental.

cartridges of other manufacturers, namely J.T. Baker 'Narc 2' and World-wide Monitoring 'Clean Screen DAU'. This is because the cartridge materials and procedures for cocaine and metabolites recommended by these manufacturers are similar. If the interfering peaks are due to the reaction of ammonia on the silica of the packing material then a cartridge using a polymeric material would be a better alternative. This is currently under investigation.

Throughout the entire study the phase separator was operated at a separation efficiency of 0.8–0.9 without any problems and did not require any major adjustments apart from routine maintenance such as cleaning the needle valve outlet tubings with acetone and blowing air through them.

Conclusions

The method described permits the simultaneous measurement of cocaine, its major metabolites EME and BE, and also ethylcocaine in urine. The method could additionally measure methadone by using hexylbenzoylecgonine as internal standard. The recovery of EME from urine has been increased from typically 40% to at least 80% by the use of larger capacity cartridges. The silyl reagent MTBSTFA produced a stable *t*-butyldimethylsilyl-EME derivative for this LC application. Low pressure gradient systems can be used at low flow-rates, such as 0.4 ml min^{-1}, with 2 mm i.d. columns, provided that the internal volume of the gradient system is reduced.

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