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Radioimmunoassay of benzoylecgonine in samples of forensic interest

KATHERINE ROBINSON AND R. N. SMITH*

Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE1 7LP, UK.

A simple and economical radioimmunoassay for benzoylecgonine in blood or urine is described. Haemolysis, decomposition, common anticoagulants and sodium fluoride do not affect the results. A commercially available antiserum is used at a dilution of 1:600. The tracer is radioiodinated *p*-hydroxybenzoylecgonine. It is prepared by reacting *p*-acetoxybenzoic anhydride with methyl ecgonine followed by mild hydrolysis and radioiodination by the Iodo-gen method. The product is purified on a disposable silica cartridge and has a specific activity of about 30 TBq mmol^{-1}. Polyethylene glycol is used to separate the bound and free fractions in the assay. The range of the dose-response curve is 0–400 ng ml^{-1} benzoylecgonine. The assay is largely specific for benzoylecgonine. Cocaine, ecgonine, methylecgonine and cinnamoyl cocaine have slight or negligible cross-reactivities. The inter-assay coefficient of variation is 7·5% and the recovery of benzoylecgonine from 'spiked' blood is 103%. The 'cut-off' is 20 ng ml^{-1} benzoylecgonine for both blood and urine. Radioimmunoassay and high-performance liquid chromatography results agree well.

Cocaine is a local anaesthetic and potent sympathomimetic that is abused orally, intranasally, intravenously or by smoking. It is rapidly and extensively metabolized, less than 10% being excreted unchanged in the urine (Fish & Wilson 1969; Wilkinson et al 1980; Barnett et al 1981; Lindgren 1981). The principal breakdown products are benzoylecgonine, ecgonine and methylecgonine (Fish & Wilson 1969; Taylor et al 1976; Inaba et al 1978; Ambre et al 1982). Minor metabolites include norcocaine, *m*-hydroxybenzoylecgonine and ecgonidine methyl ester (Lowry et al 1979; Rafla & Epstein 1979). Cinnamoyl cocaine, which is an impurity in some illicit cocaine preparations, and cinnamoylecgonine have been identified in postmortem samples (Valentour et al 1978). Cocaethylene (ethyl cocaine) and ethylecgonine have been found in addition to cocaine and its metabolites in urines containing ethyl alcohol (Rafla & Epstein 1979).

The mechanism of cocaine breakdown in the body has been studied. Taylor et al (1976) incubated radiolabelled cocaine with human serum and found substantial conversion to benzoylecgonine, methylecgonine and ecgonine. Stewart et al (1977, 1979) demonstrated that the hydrolysis of cocaine to methylecgonine is mediated by plasma and liver esterases and concluded that benzoylecgonine is produced by non-enzymatic hydrolysis of cocaine under basic conditions. They suggested that the results of Taylor et al (1976) could be accounted for by non-enzymatic formation of benzoylecgonine under the experimental conditions used combined with enzymic hydrolysis of cocaine and benzoylecgonine by plasma cholinesterase to give methylecgonine and ecgonine respectively. The esterase activity is inhibited partially by fluoride and completely by diisopropylfluorophosphate or eserine (Stewart et al 1977). Non-enzymatic hydrolysis of cocaine in buffer occurs above pH 4 with benzoylecgonine as the sole product and so enhanced benzoylecgonine levels are to be expected if samples are extracted or stored under alkaline conditions (Fletcher & Hancock 1981; Das Gupta 1982). The decomposition of cocaine in a refrigerated blood sample has been investigated by Liu et al (1982) who found a 30% conversion of cocaine to benzoylecgonine in 36 days.

Johns et al (1977) measured benzoylecgonine levels in the blood of twelve subjects after a 200 mg intranasal dose of cocaine. They found about 30 ng ml^{-1} after 15 min and a peak of about 1 μg ml^{-1} after 4·5 h. The elimination half-life of benzoylecgonine from blood was 5·5 h. Much higher levels are found in urine, a peak of 75 μg ml^{-1} being observed after an intranasal dose of cocaine of 1·5 mg kg^{-1} (Hamilton et al 1977). Cocaine levels in blood and urine are considerably lower than benzoylecgonine levels (Van Dyke et al 1976; Miller et al 1977; Javaid et al 1978, 1983: Holmstedt et al 1979; Paly et al 1982) while norcocaine levels are lower still (Jindal et al 1978; Chinn et al 1980). Benzoylecgonine has therefore been the analyte of choice for the detection of cocaine abuse (Lindgren 1981).

Benzoylecgonine is both amphoteric and hydrophilic and so it is difficult to extract in good yield

* Correspondence.

from aqueous solutions. Radioimmunoassay (RIA) avoids this problem since no extraction is required and it has the additional advantages of high sensitivity, economy and the ease with which samples may be analysed in batches. Kaul et al (1976) developed an RIA for benzoylecgonine capable of detecting 5–10 ng benzoylecgonine in a 100 µl urine sample. An ecgonine-sheep γ-globulin conjugate was used to raise the antiserum and the radiolabelled antigens were an [^{125}I]benzoylecgonine derivative from Hoffman-La Roche and [^{14}C]cocaine. A commercially-available RIA, the benzoylecgonine Abuscreen (Roche Diagnostics, Hoffman-La Roche Inc, Nutley, New Jersey, U.S.A.) has been evaluated by Mulé et al (1977) and cross-reactivity data have been published (Budd 1981). Other immunoassays for benzoylecgonine that have been developed include a haemagglutination-inhibition technique (Decato et al 1977), a variant of which is commercially available (American Drug Research Institute Inc, P.O. Box 134, Park Forest, Illinois 60466, U.S.A.), and an Emit-dau enzyme immunoassay marketed by Syva (Van Dyke et al 1977).

This paper describes a simple and economical RIA for benzoylecgonine in blood or urine. The antiserum from an Emit-dau cocaine metabolite kit is used with ^{125}I-labelled *p*-hydroxybenzoylecgonine.

MATERIALS AND METHODS

Materials were purchased as follows: bovine γ-globulin (Cohn Fraction II), polyethylene glycol (average mol. wt 8000) and Norit A activated charcoal (untreated powder): Sigma London Chemical Co. Ltd, Fancy Road, Poole, Dorset, UK. Dicyclohexylcarbodiimide and *p*-hydroxybenzoic acid: Aldrich Chemical Co. Ltd, New Road, Gillingham, Dorset, UK. Iodo-gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril): Pierce and Warriner (UK) Ltd, Upper Northgate Street, Chester, Cheshire, UK. Na^{125}I (Code No. IMS. 30): Amersham International plc, White Lion Road, Amersham, Buckinghamshire, UK. Silica Sep-Pak cartridges: Waters Associates Ltd, Chester Road, Hartford, Northwich, Cheshire, UK. Methanol (hplc-grade): Fisons Scientific Apparatus, Bishop Meadow Road, Loughborough, Leicestershire, UK.

Other solvents were analytical grade and other chemicals were general-purpose grade.

Thin-layer chromatography (tlc)

The purity of the cocaine derivatives and the progress of reactions were monitored by tlc according to Jane et al (1981) with the exception that the tlc plates were not pre-treated with caustic soda (R_F values: cocaine 0·42, benzoylecgonine 0·25, methylecgonine 0·33, ecgonine 0·15, *p*-acetoxycocaine 0·40, *p*-hydroxybenzoylecgonine 0·24).

Mass spectrometry (ms)

Samples were analysed by ms using a VG Micromass 12-12F quadrupole mass spectrometer (VG Analytical, Altrincham, Cheshire, UK) in the electron impact mode using a direct insertion probe, a source temperature of 230 °C and an ionization energy of 70 eV.

Benzoylecgonine preparation

Cocaine hydrochloride, 1 g, was refluxed in 25 ml water. The pH of the solution was adjusted to 7·0 at intervals with 0·1 M sodium hydroxide. The reaction was monitored by tlc and was complete after 12 h. No ecgonine was formed. The hydrolysate was taken to dryness on a rotary evaporator and the residue was recrystallized from water. The white crystals of benzoylecgonine were dried at 60 °C in a vacuum oven. A substantial amount of benzoylecgonine remained in the mother liquor. The product, 172 mg, was pure by high-performance liquid chromatography (Jane et al 1981) and its infrared (ir) spectrum matched that of an authentic sample.

Ecgonine preparation

Ecgonine was prepared by acid hydrolysis of cocaine according to Bell & Archer (1960). Cocaine hydrochloride, 5 g, was refluxed for 15 h in 130 ml 0·7 M hydrochloric acid. The hydrolysate was extracted three times with diethyl ether to remove benzoic acid and taken to dryness on a rotary evaporator to yield 2·67 g ecgonine hydrochloride as a white solid that was pure by tlc.

Methylecgonine preparation

Methylecgonine was prepared by diazomethane methylation of ecgonine (Jane et al 1981). Ecgonine hydrochloride, 2 g, in 20 ml methanol was added slowly to diazomethane in 50 ml diethyl ether. The solvents were removed on a rotary evaporator then the residue was dissolved in a little methanol, treated with Norit A charcoal and filtered. The methanol was evaporated to yield 1·1 g methylecgonine as a pale yellow oil that was pure by tlc.

p-Acetoxybenzoic anhydride preparation

p-Hydroxybenzoic acid, 10 g, was dissolved in 40 ml acetic anhydride and 200 µl phosphoric acid were added. White crystals of *p*-acetoxybenzoic acid

separated out on standing at room temperature (20 °C) overnight. The crystals, 4·2 g, were separated by filtration and dried in a vacuum oven at 60 °C.

Dicyclohexylcarbodiimide (DCC), 1·14 g, in 50 ml diethyl ether was added slowly to 2 g *p*-acetoxybenzoic acid in 100 ml diethyl ether at room temperature. Dicyclohexylurea was precipitated on standing at 2 °C overnight and was removed by filtration. The ether solution was washed three times with dilute aqueous sodium bicarbonate to remove unreacted *p*-acetoxybenzoic acid then it was dried over anhydrous sodium sulphate. An aliquot was evaporated to yield a white solid that was examined by ir spectroscopy. A strong absorption band at 2122 cm^{-1} showed the presence of unreacted DCC and so an additional 750 mg *p*-acetoxybenzoic acid were added to the ether solution which was then left at 2 °C overnight. A portion of the product was isolated as before and found to contain no unreacted DCC. The remainder of the product was therefore isolated to yield 1·3 g of white solid. Comparison of the ir spectra of the product and *p*-acetoxybenzoic acid with those of benzoic anhydride and benzoic acid indicated that the product was *p*-acetoxybenzoic anhydride as expected. It was not characterized further.

p-Hydroxybenzoylecgonine preparation

The method was analogous to that used by Jane et al (1981) for the preparation of cinnamoyl cocaine. Methylecgonine, 250 mg, was refluxed in 20 ml benzene with 1 g *p*-acetoxybenzoic anhydride and 150 mg anhydrous sodium carbonate. The reaction was monitored by tlc and was virtually complete after 16 h. Insoluble material was removed by filtration and the benzene solution was extracted three times with 0·5 M hydrochloric acid. The acid extracts were combined, made alkaline with concentrated ammonium hydroxide and extracted three times with diethyl ether. The ether extracts were combined, dried over anhydrous sodium sulphate and evaporated to yield 200 mg of a colourless oil, presumably *p*-acetoxycocaine, which was pure by tlc but was not characterized further.

A portion of the *p*-acetoxycocaine was hydrolysed to *p*-hydroxybenzoylecgonine as described above for the preparation of benzoylecgonine. The product, 40 mg, was pure by tlc. Its identity was confirmed by ms of its methyl and ethyl derivatives. The methyl derivative was prepared by treatment with diazomethane and the ethyl derivative was prepared according to Greeley (1974a, b) using *NN*-dimethylacetamide, tetramethylammonium hydroxide and ethyl iodide. Molecular ions of *m/z* 333 and 361 were observed in the spectra showing that dialkylation had occurred in both cases. In addition, the spectrum of the dimethyl derivative contained intense ions of *m/z* 82 and 182 characteristic of the tropane moiety of cocaine, while that of the diethyl derivative contained the corresponding ions of *m/z* 82 and 196 characteristic of the tropane moiety of cocaethylene (Rafla et al 1979).

Radioiodination of p*-hydroxybenzoylecgonine*

The Iodo-gen method is used (Fraker & Speck 1978). *p*-Hydroxybenzoylecgonine, 80 ng, in 25 µl 10 mM Tris buffer, pH 7·8, and 18·5 MBq Na ^{125}I made up to 25 µl with water are put in a 1·5 ml polypropylene tube coated with 40 µg Iodo-gen by evaporating 100 µl Iodo-gen in chloroform under a stream of nitrogen. The mixture is allowed to stand for 10 min with occasional vortexing and is then transferred to a silica Sep-Pak cartridge. Unreacted radioiodide is eluted with 3 ml acetone followed by 2·5 ml methanol, then radioiodinated *p*-hydroxybenzoylecgonine is eluted with 7·5 ml methanol. The solvents are passed through the Sep-Pak cartridge at a moderate rate using a syringe pipette with a Luer fitting. The product is collected in a silanized glass vial and stored at −20 °C.

For use in an assay, an aliquot is diluted (approx. 1:200) in buffer to give 10 000 counts min^{-1} per 50 µl.

Buffer

0·067 M phosphate, pH 7·4, containing 0·2% bovine γ-globulin (Cohn Fraction II) and 0·1% sodium azide.

Antiserum

Antiserum from an Emit-dau cocaine metabolite kit (Syva UK, Syntex House, St. Ives Road, Maidenhead, Berkshire, U.K.) is diluted 1:10 with buffer, aliquotted and stored at −20 °C. For use in an assay, an aliquot is allowed to thaw and is then diluted with buffer so that 50–60% of the label is bound in the absence of unlabelled benzoylecgonine. The correct dilution is determined from an antiserum dilution curve and is approximately 1:600.

Benzoylecgonine standards

Benzoylecgonine standards in buffer ranging from 0–400 ng ml^{-1} are stored in silanized glass vials at 2 °C.

Polyethylene glycol (PEG) solution
23% w/v PEG (average mol. wt 8000) in buffer containing no γ-globulin.

Sample preparation
Blood or urine, 50 µl, is diluted with an equal volume of buffer. This is done to conserve the sample. Further dilution may be necessary to ensure that the result lies within the range of the standard curve.

Assay protocol
The protocol is identical to that used in a previously published method (Robinson & Smith 1983). Sample or standard, 25 µl, and 50 µl each of label and antiserum are pipetted into duplicate sets of polypropylene microcentrifuge tubes. The tubes are vortexed, incubated at room temperature for 1 h and then 400 µl of PEG solution are added. The tubes are vortexed thoroughly and allowed to stand for 5–10 min before centrifuging (2 min, 12 000*g*). The supernatants are aspirated and the precipitates are counted in a γ-counter. Two extra tubes containing only 50 µl radioiodinated *p*-hydroxybenzoylecgonine are also counted to measure the total activity per tube, and the results are plotted.

Hplc determination of benzoylecgonine in blood
(I. Jane, S. D. R. Humphrey, A. McKinnon, A. E. Pendlebury, unpublished)
Samples and standards are buffered to pH 9·2 and internal standard is added (200 µl aqueous amitriptyline hydrochloride, 10 ng µl^{-1}). The solutions (total volume 1·5 ml) are saturated with sodium chloride and extracted once with 8 ml chloroform–ethanol (4:1). The extracts are evaporated to dryness and taken up in 250 µl hplc eluant consisting of hplc-grade methanol-aqueous ammonium acetate (9:1) adjusted to pH 7 with glacial acetic acid. A valve injector is used to transfer 20 µl extract on to a 250 × 4·9 mm column of Partisil-5 silica (Whatman, Springfield Mill, Maidstone, Kent, U.K.) pretreated at 800 °C. The eluant flow-rate is 1·0 ml min^{-1} and the separated components are detected spectrophotometrically at 232 nm. Peak-height ratios of benzoylecgonine to internal standard are plotted against benzoylecgonine concentration. The calibration is linear to at least 8 µg ml^{-1} benzoylecgonine.

RESULTS AND DISCUSSION

The benzoylecgonine RIA described here is both simple and economical. The antiserum is commercially available and the radioiodinated *p*-hydroxybenzoylecgonine may be prepared in the laboratory. The running cost (excluding labour) is about 2·5p per tube which is considerably less than that of any commercially-available assay.

The structures of cocaine and its derivatives are given in Fig. 1. *p*-Hydroxybenzoylecgonine is relatively simple to prepare and it is easily radioiodinated by the Iodo-gen method as described. Initial attempts to isolate radioiodinated *p*-hydroxybenzoylecgonine by solvent extraction or ion exchange were unsuccessful due to the amphoteric nature of the compound. We therefore employed a novel procedure using an inexpensive Sep-Pak cartridge which is simple, effective and quick. Radioiodination and isolation of the product is completed within 15 min with negligible exposure of the operator to radiation. Preliminary experiments showed that elution from a Sep-Pak cartridge as described isolated 85% of the product with minimal contamination by extraneous activity (80–90% of the isolated product is bound by a 1:10 dilution of the antiserum). The specific activity of a typical radioiodinated *p*-hydroxybenzoylecgonine preparation is about 30 TBq mmol^{-1} (calculated from the activity in the product assuming 85% overall recovery). The structure of the radioiodinated *p*-hydroxybenzoylecgonine was not determined but, by analogy with other radioiodinated phenolic compounds (Seevers & Counsell 1982), there is little doubt that sub-

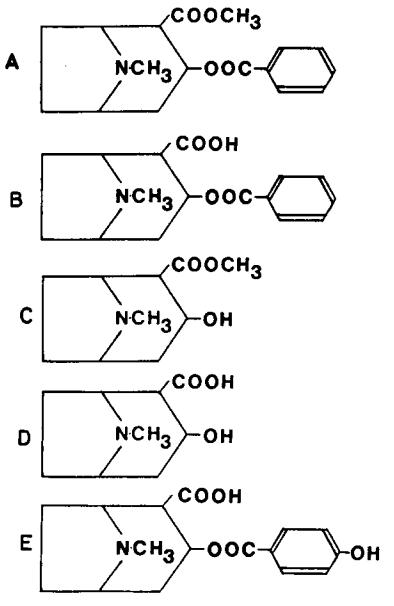

FIG. 1. Structures of cocaine and its derivatives. A, Cocaine. B, Benzoylecgonine. C, Methylecgonine. D, Ecgonine. E, *p*-Hydroxybenzoylecgonine.

stitution by ^{125}I occurs *ortho* to the phenolic hydroxyl group. Radioiodinated *p*-hydroxybenzoylecgonine is usable for at least one half-life of the isotope and successive preparations have proved equally effective.

The dose-response curve of the assay has a useful range of 0–400 ng ml^{-1} benzoylecgonine when the initial binding of label by the antiserum is 50–60% in the absence of unlabelled benzoylecgonine. A 50% displacement of the label is given by 50–55 ng ml^{-1} benzoylecgonine. Non-specific binding (3%) is low enough to be ignored.

Cross-reactivity data are given in Table 1. The assay cross-reacts primarily with benzoylecgonine and only slightly with related compounds. The lack of sensitivity to cocaine is no disadvantage in practice due to the ready conversion of cocaine to benzoylecgonine both in-vitro and in-vivo.

Table 1. Cross-reactivity data of benzoylecgonine RIA.

	Cross-reactivity*
Benzoylecgonine	100
Cocaine	2
Ecgonine	1
Methylecgonine	<0·25
Cinnamoyl cocaine	≪0·25**

* Weight of benzoylecgonine required to displace 50% of the label × 100/weight of compound required to displace 50% of the label.

** 5% displacement of label by 20 μg ml^{-1} cinnamoyl cocaine.

A 'cut-off' value of 20 ng ml^{-1} benzoylecgonine for both blood (n = 53) and urine (n = 56) was defined by determining the average response of blank samples plus three standard deviations. The samples were obtained from healthy subjects who had not ingested cocaine. The blood samples were haemolysed and varied in condition from fresh to putrid. Urine samples varied in age from several days to about a year. Heparin, ethylenediaminetetraacetic acid (dipotassium salt), potassium oxalate or sodium fluoride present in some of the samples had no significant effect on the results.

The inter-assay coefficient of variation was found to be 7·5% and the mean recovery from 'spiked' blood was 103% (n = 10; determined by assaying haemolysed blood 'spiked' with 100 ng ml^{-1} benzoylecgonine).

The assay has been used in forensic case-work for eight months and no problems have arisen. Most samples found to be positive for benzoylecgonine were from cases involving multiple drug abuse and, while the presence of benzoylecgonine was confirmed qualitatively by other means, quantitation was either not required or was not possible due to a lack of sample. In four cases, however, blood levels of 1·9, 3·1, 4·2 and 9·2 μg ml^{-1} benzoylecgonine were found by RIA while hplc gave levels of 1·7, 3·0, 4·0 and 11·0 μg ml^{-1} respectively. These results are insufficient to permit a statistical comparison of the two methods but they indicate that the RIA performs as well with 'real' samples as with 'spiked' blood.

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