Journal of Chromatography, 196 (1980) 337–341
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,886

Note

Qualitative screening procedure for the detection of benzoyl ecgonine in the urine of greyhounds

JOHN H. LEWIS*
Department of Pharmacology, University of Sydney, N.S.W. 2006 (Australia)
(First received March 3rd, 1980; revised manuscript received April 14th, 1980)

The illicit use of the stimulant drug cocaine as a doping agent in racing greyhounds has prompted a study of its metabolism, excretion and subsequent detection in urine.

Although the efficacy of orally administered cocaine on the performance of racing greyhounds has not been established, it was deemed necessary to include the drug in the routine screening system as used in the author's drug detection laboratory.

Cocaine has been shown to be extensively metabolished in the rat[1] and in man[2–6], the major metabolite being benzoyl ecgonine. There appears to be little published information as to the metabolic fate in the greyhound.

Preliminary experiments conducted on laboratory greyhounds revealed that following a single oral administration of cocaine hydrochloride (1.8 mg/kg), benzoyl ecgonine was the only metabolite detected in the urine. Although numerous methods have been published on the detection of benzoyl ecgonine in urine, none could be satisfactorily incorporated into an existing routine screening process where only a limited quantity of urine was available. Additionally, a method was required that would take up little extra analysis time, could be performed by technical personnel and utilised simple analytical procedures.

EXPERIMENTAL

Reagents and equipment

All reagents were analytical-reagent grade and were used without further purification.

Thin-layer chromatographic (TLC) plates and potasium iodide spray were prepared as described previously[7]. The TLC solvent was chloroform–methanol–ammonia–water (70:30:1:0.5).

The extraction tubes were 50-ml glass (Corex, U.S.A.) and the extractor was a Clements rotating type (30 rpm).

The methylating reagent was trimethylanilinium hydroxide (TMAH, Methelute®, Pierce, Rockford, IL, U.S.A.).

* Present address: Oliver Latham Laboratory, Psychiatric Centre, Cox's Road, North Ryde, NSW 2113, Australia.

0021-9673/80/0000–0000/$02.25

The internal marker for gas–liquid chromatography (GLC) was mefenamic acid (125 μg/ml in chloroform).

Procedure

Cocaine hydrochloride (1.8 mg/kg) contained in a gelatin capsule, was administered orally to three male greyhounds (weight range 28–42 kg). The animals were fasted overnight prior to administration. Control urines were collected immediately before dosing, followed by samples at 30 min, 1, 2, 4, 6 and 8 h after administration. The greyhounds were allowed water *ad libitum* during the experiments. Urine samples were frozen until analysed.

Extraction

Unchanged cocaine. A 15-ml volume of urine was adjusted to pH 3–4 with acetic acid and extracted for 10 min with 20 ml of chloroform. The organic phase was washed with 5 ml of saturated sodium bicarbonate solution, filtered through a filter paper (Whatman No. 1) into an evaporating beaker, and evaporated to dryness on a water bath at 40°C using a gentle stream of nitrogen. The dried extracts were reconstituted in 50 μl chloroform and spotted onto TLC plates along with cocaine standard. The plates were developed in the solvent methanol–ammonia (100:1.5), air dried and finally sprayed with potassium triiodide.

Benzoyl ecgonine. The remaining aqueous phase was adjusted to pH 9–10 by the addition of solid sodium carbonate. A 30-ml volume of chloroform–isopropanol (95:5) was added and the urine extracted for 15 min. After centrifugation, the organic phase was transferred to a 100-ml separating funnel. A second extraction was performed using 20 ml of solvent. The organic phases containing the benzoyl ecgonine were combined and then shaken for 2 min with 5 ml of 0.1 *M* sulphuric acid. The organic layer was then discarded.

The acid phase was transferred back into a 50-ml extraction tube, the pH adjusted to 9–10 as before and the sample extracted twice for 10 min with 15 ml of chloroform–isopropanol.

The solvent was dried through a filter paper (Whatman No. 1) into an evaporating beaker and gently evaporated to dryness on a waterbath at 40°C using a stream of nitrogen.

Analysis

TLC of urine samples. The urine extracts were reconstituted in 100 μl of chloroform–isopropanol and 5–10 μl spotted onto a TLC plate. Authentic benzoyl ecgonine was also spotted. The plate was developed to a distance of 15 cm. The developed plate was air dried and the spots due to benzoyl ecgonine were located by spraying with potassium triiodide. These spots appeared dark brown.

GLC. Following visualisation of the spots on TLC, confirmation of the presence of benzoyl ecgonine was achieved by its derivatisation to cocaine followed by GLC.

The column chosen was 3% OV-17 on Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.).

The conditions were as follows. Instrument: Bendix 2500 Series gas chromatograph fitted with flame-ionisation detectors; nitrogen flow-rate, 40 ml/min; air

flow-rate, 300 ml/min; hydrogen flow-rate, 40 ml/min; temperatures: injector, 245°C, detector, 245°C; oven, 210°C; recorder, Perkin Elmer Model 56, chart speed, 5 mm/min.

To the urine extracts remaining from TLC were added 50 μl of mefenamic acid (125 μg/ml in chloroform) and the samples allowed to evaporate. The extracts were then reconstituted in 50 μl trimethylanilinium hydroxide and 1 μl was injected onto the column.

RESULTS

TLC of urine extracts

Following the administration of cocaine hydrochloride to greyhounds no unchanged cocaine was detected in the urine.

In all cases benzoyl ecgonine was readily detected at time intervals between 1 and 8 h after ingestion. At 30 min a spot appeared that could not be satisfactorily distinguished from the control sample. Using the solvent system described, good separation of benzoyl ecgonine was achieved from naturally occurring pigments (Fig. 1). R_F value for benzoyl ecgonine = 0.23

Potassium triiodide solution was found to be the most sensitive spray reagent for benzoyl ecgonine, reacting to less than 1 μg of drug.

GLC of urine extracts

Using OV-17, good separation of both cocaine and the internal marker (methyl derivative of mefenamic acid) was achieved from the other constituents (Fig. 2).

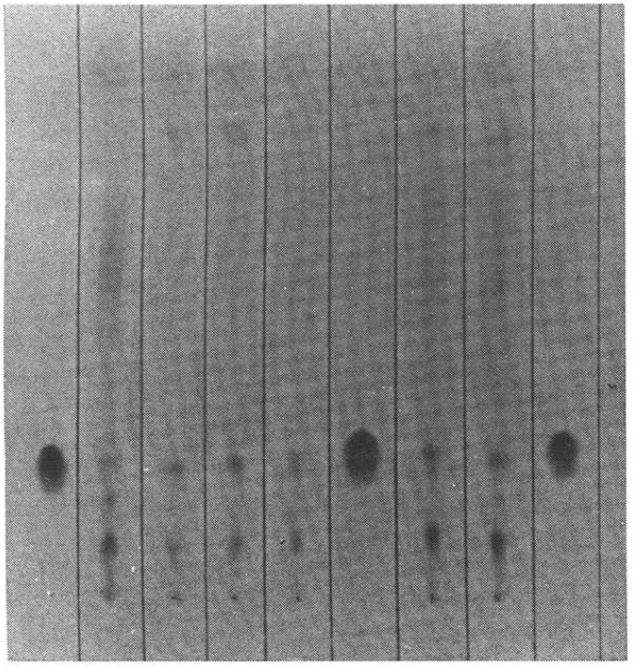

Fig. 1. TLC of urine extracts following the administration of cocaine. Left to right: benzoyl ecgonine (2 μg), 30 min, 1 h, 2 h, 4 h, benzoyl ecgonine (2 μg), 6 h, 8 h, benzoyl ecgonine (2 μg).

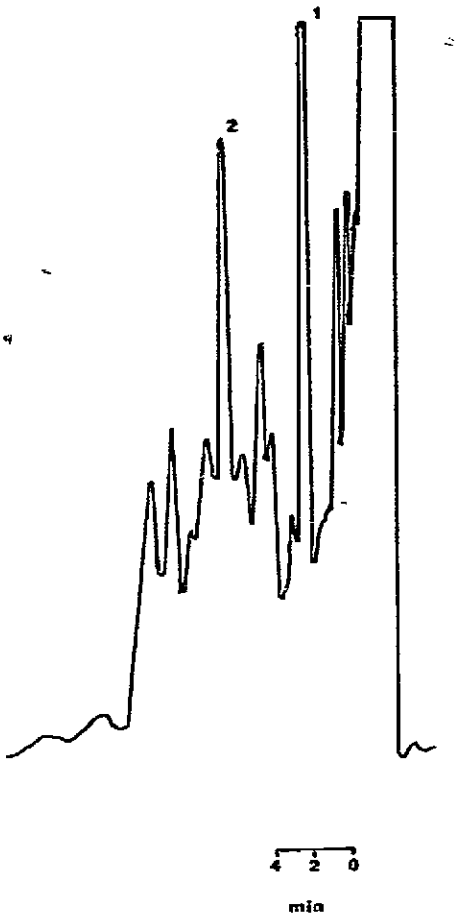

Fig. 2. GLC on OV-17 of urine extract at 210°C. 1 = Cocaine, 2 = mefenamic acid–methyl derivative.

Benzoyl ecgonine (as cocaine) was readily detected at all time intervals tested from 1 to 8 h. At 30 min a small peak appeared with the same relative retention time as cocaine. This peak did not occur in the control. Relative retention time (RRT) mefenamic acid derivative = 1.0; cocaine = 0.52.

DISCUSSION

A method has been devised for the routine screening of the major metabolite of cocaine (benzoyl ecgonine) in the urine of greyhounds. The method utilises simple procedures and is thus suitable for inclusion into a routine screening program operated by technical personnel. An additional advantage is that the benzoyl ecgonine may be isolated in the same fraction as other basic drugs, thus saving analysis time and reducing the specimen volume required.

Trimethylanilinium hydroxide[5] was found to be suitable as a methylating

reagent for greyhound urine extracts when used following identification by TLC. No false negatives or positives have been detected with the method.

No attempt was made in this study to quantitate the results. However, readily detectable benzoyl ecgonine was still being excreted 8 h after administration. Under these circumstances one could expect to detect any illicitly administered cocaine when given prior to racing. Although it appeared that benzoyl ecgonine was excreted in the urine 30 min after administration, the GLC results could not be confirmed by TLC.

ACKNOWLEDGEMENTS

The author is grateful to Ms. Susan Shadbolt for her technical assistance and to Dr. David Jackson for his advice and comments. Appreciation is expressed to the Australian Government Analytical Laboratories for a sample of benzoyl ecgonine.

REFERENCES

1 H. E. Hamilton, J. E. Wallace, E. L. Shimek Jr., Pam Land, S. C. Harris and J. G. Christenson, *J. Forensic Sci.*, 22 (1977) 697.
2 F. Fish and W. D. C. Wilson, *J. Pharm. Pharmacol.*, 21 (1969) 135S.
3 M. L. Bastos, D. Jukofsky and S. J. Mulé, *J. Chromatogr.*, 89 (1974) 335.
4 J. E. Wallace, H. E. Hamilton, H. Schwertner, D. E. King, J. L. McNay and K. Blum, *J. Chromatogr.*, 114 (1975) 433.
5 N. C. Jain, D. M. Chinn, R. D. Budd, T. S. Sneath and W. J. Leung, *28th Annual Meeting, American Academy of Forensic Sciences, Washington, DC, February 17–20th, 1976.*
6 N. N. Valanju, M. M. Baden, S. N. Valanju, D. Mulligan and S. K. Verma, *J. Chromatogr.*, 81 (1973) 170.
7 J. H. Lewis, *J. Chromatogr.*, 172 (1979) 295.