Journal of Chromatography, 114 (1975) 433–441

CHROM. 8416

THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF COCAINE AND BENZOYLECGONINE IN URINE

JACK E. WALLACE, HORACE E. HAMILTON, HARVEY SCHWERTNER and DAVID E. KING

Department of Pathology, The University of Texas Health Science Center, San Antonio, Texas (U.S.A.)

and

JOHN L. McNAY and KENNETH BLUM

Department of Pharmacology, The University of Texas Health Science Center, San Antonio, Texas (U.S.A.)

(Received May 6th, 1975)

SUMMARY

The sensitivity achieved by the described thin-layer chromatographic (TLC) method greatly exceeds that of previously published TLC methods for the determination of cocaine and its principal metabolite, benzoylecgonine, in urine. Sensitivity for cocaine and benzoylecgonine approaches 0.1 and 0.25 μg/ml, respectively, for a 5.0-ml specimen. A simple extraction with a mixed organic solvent provides the basic mechanism for isolating the drugs from biologic specimens. Cocaine and its metabolites are stable in sulfuric acid solutions but labile in aqueous media containing certain other inorganic and organic acids; therefore, an emphasis on the utilization of sulfuric acid solutions is employed throughout the procedure. An evaluation of sensitivities achieved for cocaine and benzoylecgonine by various detection reagents is presented. The technique is applicable to drug screening programs.

INTRODUCTION

The current trend toward illicit use of cocaine[1–6] has prompted considerable interest in the development of rapid, reliable and inexpensive screening methods for the detection of users and abusers of the drug. Urine analysis has proven to be the most effective means presently available for the routine detection of most drugs of abuse[7]. Thin-layer chromatography (TLC) provides the potential for rapid, inexpensive screening for a number of drugs and metabolites and is currently the most common screening method employed by drug abuse detection laboratories[8]. Sunshine stated in a recent comprehensive report on analytical toxicology that for laboratories plagued with a large work load there is no substitute for TLC as a screening analysis[9]. Consequently, a vast number of reports pertaining to the TLC detection of cocaine exists in the literature.

A review of the literature, however, reveals that the majority of these papers describe general screening methods for a large number of opiates and alkaline drugs, *i.e.*, cocaine is only one of a number of drugs considered. Further, many published reports offer little other than R_F values for a specific solvent system (one paper (ref. 10) presents R_F values for cocaine in seventeen solvent systems) and the inference that iodoplatinate spray reagent is the most applicable for the detection of cocaine and benzoylecgonine. Only a few of the TLC methods provide any type of sensitivity limits or acknowledge the existence of cocaine metabolites. The failure to consider metabolites constitutes a significant discrepancy since cocaine is rapidly and extensively metabolized *in vivo*. Valanju *et al.*[11] detected unchanged cocaine in the urine of only 15% of addict urine specimens containing cocaine metabolites, and Bastos and Hoffman[2] estimated that greater than 98% of ingested cocaine is excreted as the water-soluble metabolites benzoylecgonine and ecgonine.

It is apparent that, although a number of methods for the detection of cocaine have been described, an explicit need exists for a rapid and sensitive (submicrogram) screening technique that gives appropriate consideration to the chemical properties of cocaine and benzoylecgonine. The present report describes a TLC method that provides a sensitivity level of approximately 0.1 μg/ml for cocaine and 0.25 μg/ml for benzoylecgoine in the analysis of a 5-ml urine specimen. The procedure utilizes Dragendorff's reagent followed by an overspray with dilute sulfuric acid and exposure to iodine vapors. Data comparing the relative sensitivities achieved for cocaine and benzoylecgonine with the more common detection sprays are presented. Post-surgery urine specimens from patients receiving cocaine anesthetic have also been assayed.

EXPERIMENTAL

Materials

Extraction solvent. Twenty milliliters of ethanol (Commercial Solvents, Terre Haute, Ind., U.S.A.) were mixed with 80 ml of chloroform (No. 4440 or equivalent of Mallinckrodt, St. Louis, Mo., U.S.A.).

Chromatographic solvent. Chloroform (Mallinckrodt No. 4440), methanol (No. A-412; Fisher Scientific, Fair Lawn, N.J., U.S.A.) and concentrated ammonium hydroxide (Mallinckrodt No. 3256) were mixed in a ratio of 100:20:1. An alternate chromatographic solvent contained the same components at a ratio of 60:60:1.

TLC plates. The chromatographic plates used (Uniplate®), Silica Gel G, 250 μ, 5 cm × 20 cm and 20 cm × 20 cm, were obtained from Analtech, Newark, Del., U.S.A.

Chromatographic tanks. The glass tanks used were 9.0 cm × 25 cm at the base and 24 cm deep with a glass plate lid sealed with vacuum grease. A paper lining placed in the interior of the tank significantly reduced the migration time for chromatogram development.

Iodine. Iodine was used in the form of resublimed crystals (Fisher certified, A.C.S. reagent).

Dragendorff's spray reagent. (a) Two grams of bismuth subnitrate (No. Bx825, CB222, Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) were mixed with 25 ml of glacial acetic acid (A.C.S. grade, Fisher Scientific No. A-38) and 100 ml of distilled water; (b) 40 g of potassium iodide (Fisher Scientific No. P-140) were

dissolved in 100 ml of distilled water. Ten milliliters each of (a) and (b) were combined with 20 ml of glacial acetic acid and 100 ml of distilled water prior to utilization. The final spray reagent was stored in a light-protected bottle at 4°.

Sulfuric acid spray reagent. Six milliliters of concentrated sulfuric acid (Fisher Scientific No. A-298) were carefully dissolved in 30 ml of distilled water.

Procedure

Five milliliters of urine are pipetted into a 50-ml glass-stoppered centrifuge tube containing 25 ml of the chloroform–ethanol extracting solvent. The mixture is shaken for 10 min on a mechanical shaker and subsequently centrifuged for 5 min at 560–695 *g*. The organic solvent layer is transferred to a 40-ml conical centrifuge tube and carefully evaporated to almost dryness (volume *ca.* 50–100 μl) in a water-bath at 55° under a gentle stream of dry air. Evaporation of solvent to total dryness results in a decreased sensitivity. The chloroform–ethanol can be evaporated from several tubes simultaneously by utilization of an appropriate manifold to effectively divide and distribute the air stream. It has been convenient in our laboratory to evaporate with one manifold eighteen specimens simultaneously. Approximately 100 μl of chloroform is sprayed as a fine mist from a chromatosprayer into the conical tube containing the residual amount of solvent. Solid materials dried to the side of the tube are effectively washed to the bottom of the tube by this operation. After a 90-sec vortexing the mixed contents of the tube are streaked or spotted on a TLC plate. A 1–2 cm streak provides after migration in the solvent system a satisfactory combination of clarity and sensitivity.

The chromatograms are developed by the solvents previously described. The migration time required for the solvent front to reach 15–18 cm was routinely approximately 50–60 min with paper-lined tanks. Upon removal of the developed plates from the solvent a time interval of 15–20 min for air drying is permitted. The air-dried plates are sprayed first with Dragendorff's reagent (in a hood). After 1–2 min the plates are sprayed with the sulfuric acid solution. Subsequent additional spraying of the plates in the manner described generally establishes the original observed intensities for the areas containing cocaine and benzoylecgonine. After the spraying sequence additional sensitivity can be obtained by placing the plates for 60–90 sec in a developing tank containing iodine crystals. Covering the thin-layer chromatograms, after spraying, with clear glass plates allows the color associated with cocaine, benzoylecgonine and other extracted drugs to be stabilized for several weeks. If the covered plate is immediately stored in a freezer the resultant chromatogram can serve as a permanent stable record.

RESULTS AND DISCUSSION

Extraction

Unchanged cocaine may be effectively extracted from urine by simple organic solvents such as ethyl ether[7] or chloroform[12,13], or by mixed solvents such as chloroform–isopropanol[14–16]. The polar metabolite benzoylecgonine specifically requires mixed solvents[11,17] or salting-out techniques[13,18]. Cocaine extractions have been reported at both moderately alkaline conditions, pH 8–9 (refs. 11 and 14–16), and

at higher pH values, 10–11 (refs. 7 and 17). Benzoylecgonine extractions have similarly been reported at both slightly alkaline[11,13,18] and strongly alkaline[17] pH values.

Our studies demonstrate that 20% ethanol in chloroform (v/v) is an effective solvent for the extraction of both unchanged cocaine and benzoylecgonine. The recovery of benzoylecgonine from urine was enhanced with increasing amounts of ethanol up to approximately 20% (v/v) and with increasing solvent-to-sample ratios of up to approximately 5:1. The extraction efficiency for both compounds was relatively pH independent over the pH range 5.5–9.5 with cocaine recovery diminishing at pH > 10. Saturation of the urine with sodium sulfate or ammonium sulfate produces a slight enhancement of absolute recovery, but the concurrent increased extraction of interfering natural constituents negates the effectiveness of this technique. Our studies confirmed Mulé's observation that acid hydrolysis, required for sensitive opiate determinations, destroys both cocaine and its principal metabolites. Quantitative evaluation by gas–liquid chromatography[19] indicated that the extraction recoveries achieved with 20% ethanol in chloroform are 90–95% for cocaine and 65–70% for benzoylecgonine.

Chromatography

Numerous developing solvents have been described for the detection of cocaine; Noirfalise and Mees[20] report R_F values for cocaine in nine solvent systems and Comer and Comer[10] list data on seventeen solvent systems. Few methods have been described for the specific detection of benzoylecgonine and ecgonine, however, and those that have generally utilized the solvent systems of Davidow[21], Sunshine[22], or minor modifications of these systems.

A number of developing solvents were examined in our laboratory to determine specifically optimum chromatographic conditions for the separation and determination of benzoylecgonine since the metabolite rather than the unchanged drug predominates in *in vivo* specimens. Various combinations of methanol, ethanol, *n*-propanol or *n*-butanol with water and several acids were examined and rejected. It was observed that the presence of hydrochloric or acetic acid significantly diminished the sensitivity achieved; other acids tested and found to interfere with the analysis of benzoylecgonine were perchloric, citric, nitric, and boric acids. Sulfuric acid did not induce a decreased sensitivity. In an additional experiment residual acetic acid on a developed chromatogram was removed by air-drying; however, the sensitivity for benzoylecgonine was not increased, indicating that the decreased sensitivity for the cocaine derivatives due to acids is non-reversible. A developing solvent consisting of butanol–sulfuric acid (95:5) saturated with water was very effective in the separation of benzoylecgonine and cocaine from most drugs and provided for acceptable sensitivity. This solvent, however, required an extensive migration time, *viz.* 7–8 h in unlined tanks or 5–6 h in paper-lined tanks saturated with the developing solvent. The utilization of solvent systems containing methanol, chloroform and ammonium hydroxide, however, permitted effective separation of the two compounds from one another, from natural urine constituents, and from other major drugs of abuse, yet required only 50–60 min of developing time. Chromatograms developed with chloroform–methanol–ammonium hydroxide (100:20:1) were found to be extremely effective for the separation of benzoylecgonine from normal urine contaminants, and in effect gave the best separation and identification of benzoylecgonine. R_F values for

TABLE I

R_F VALUES OF SELECTED DRUGS

Solvent system: Chloroform–methanol–ammonium hydroxide (100:20:1).

Drug	*R_F value*
Benzoylecgonine	0.20
Cocaine	0.87
Morphine	0.43
Methadone	0.77
Amitriptyline	0.90
Antistine	0.66
Meperidine	0.87
Propoxyphene	0.93
Chlorpromazine	0.89
Atropine	0.33

several drugs in the proposed solvent system are given in Table I. Representative chromatograms of urines spiked with varying concentrations of cocaine and benzoylecgonine are shown in Fig. 1. Berry and Grove[14], Mulé[17] and Davidow *et al.*[21] have noted the difference between R_F values of drugs extracted from urine and those of non-extracted reference standards, and have attributed the differences in migration rates to the influence of co-extractable material. A co-extraction influence is observed in the separation of benzoylecgonine by the developing solvent described in this report, thus making extracted standards a recommended part of the technique.

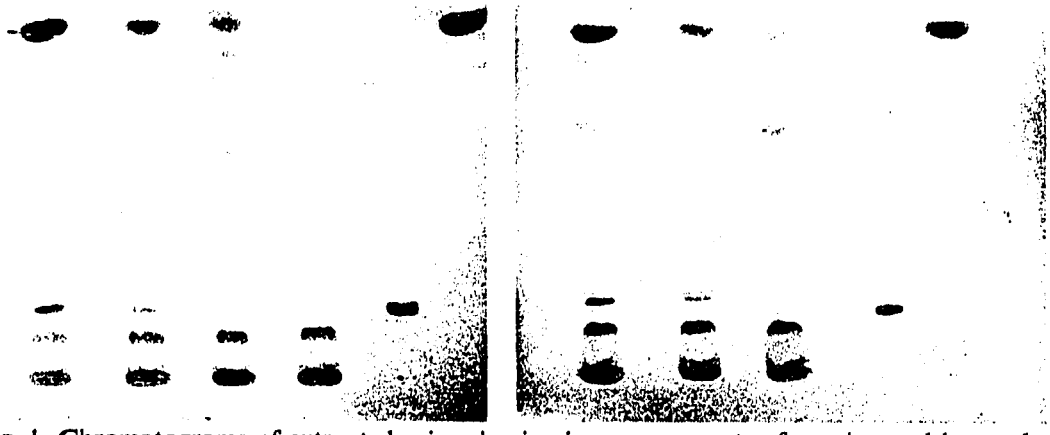

Fig. 1. Chromatograms of extracted urines having known amounts of cocaine and benzoylecgonine. Chromatograms contain, from left to right, extracts of urines containing 3, 1, 0.5 and 0 μg/ml of cocaine and benzoylecgonine, reference standards of benzoylecgonine and cocaine, urine extracts of 2, 0.75 and 0.25 μg/ml, and additional reference standards.

Detection

The vast majority of TLC methods for cocaine and those specific for the identification of cocaine metabolites utilize iodoplatinate (neutral or acidified) as the detecting agent alone or in combination with other reagents. Modifications described

for iodoplatinate detection of cocaine and its metabolites include a double spray of that reagent[18], an overspray of the initial iodoplatinate with dilute sulfuric acid[17] or the utilization of Dragendorff's reagent prior[11] or subsequent to iodoplatinate[20]. Gorodetzky[23] has presented quantitative data precisely defining the sensitivity achievable for cocaine in non-biologic media by iodoplatinate, but does not present data for benzoylecgonine. Although various investigators have described a variety of detection techniques for cocaine and its metabolites, they generally have not presented data substantiating their choice.

An evaluation of the various iodoplatinate detection techniques was performed in our laboratory with urine specimens to which were added differing amounts of cocaine and benzoylecgonine. All specimens were extracted and chromatographed by the procedure described in this report. Individuals spraying and interpreting the chromatograms were unaware of the concentrations employed in the study and were identified separately from the technicians who performed the extractions and the streaking of the chromatographic plates. Included in the study along with various applications of iodoplatinate reagent were Dragendorff's reagent followed by an overspray of dilute sulfuric acid, a system previously employed for the paper chromatographic detection of cocaine, benzoylecgonine, and ecgonine[24] and Dragendorff's reagent followed by sulfuric acid and subsequent exposure to iodine vapors. The results of the evaluation are presented in Table II. Dragendorff's reagent followed by a light spraying of 20% aqueous sulfuric acid and a subsequent brief exposure to iodine vapors provided the optimum sensitivity for the detection of both cocaine and benzoylecgonine. The sulfuric acid overspray provides an approximate twofold increase in sensitivity for cocaine and a five- to tenfold enhanced sensitivity of benzoylecgonine over that achieved with Dragendorff's reagent alone. A final 30- to 90-sec exposure to iodine vapors results in a slight but definite additional enhancement of sensitivity; a more prolonged iodine exposure produces a general darkening of the chromatogram background, resulting in decreased sensitivity. The darkening of plate background was more evident when plates other than Uniplate® were used. Iodo-

TABLE II

EVALUATION OF VARIOUS REAGENTS FOR THE DETECTION OF COCAINE AND BENZOYLECGONINE IN URINE*

Detection reagent(s)	*Cocaine (μg/ml)*			*Benzoylecgonine (μg/ml)*		
	0.5	*1.0*	*2.0*	*0.5*	*1.0*	*2.0*
Dragendorff's	100**	100	100	12	55	67
Dragendorff's + H_2SO_4 + I_2	100	100	100	83	100	100
Iodoplatinate, iodoplatinate + Dragendorff's, or Dragendroff's + iodoplatinate	13	75	92	0	0	62
Iodoplatinate + H_2SO_4	50	50	75	0	0	75

* 5-ml urine specimens were extracted and chromatographed by the method described in this report.

** The indicated values are the per cent of specimens of that concentration determined to be positive. The number of specimens examined per combination of reagents and concentration was ten to fifteen, except for iodoplatinate followed by sulfuric acid, for which only four determinations were made.

platinate, alone or in the combinations examined, provided a significantly lower sensitivity for both cocaine and benzoylecgonine. An overspray of dilute sulfuric acid over iodoplatinate resulted in an enhanced sensitivity for benzoylecgonine but a slight decrease in the observed sensitivity for cocaine. The sensitivity for cocaine and benzoylecgonine achieved by Dragendorff's reagent followed by spraying with sulfuric acid could also be obtained by modifying the spray reagent to contain sulfuric acid in a quantity equal to that of acetic acid. However, the modified reagent was unstable and gave a slightly darker background on the plate.

The sensitivity of the proposed method greatly exceeds that achieved for cocaine and benzoylecgonine by previously published TLC methods. The sensitivity for benzoylecgonine approaches 0.25 μg/ml for a 5-ml specimen; concentrations of 0.5 μg/ml are readily and reliably detected. Data in Table II demonstrate that a major factor responsible for the enhanced sensitivity is the choice of detecting agent. At a concentration of 1.0 μg/ml, benzoylecgonine was detected with 100% reliability (fourteen determinations) by Dragendorff's reagent followed by sulfuric acid as opposed to 0% detection (fifteen determinations) by iodoplatinate, alone or by various combinations utilizing that reagent. The sensitivity of the proposed method is also due in part to the improved extraction and chromatography techniques employed by the iodoplatinate reagent (Table II); it is apparent that iodoplatinate is not the detection agent of choice in the TLC analysis of cocaine and benzoylecgonine. This is evident by the detection limits reported by other investigators utilizing iodoplatinate. For example, Bastos *et al.*[18] and Valanju *et al.*[11] reported sensitivity limits of 3–5 μg/ml benzoylecgonine for 10- and 25-ml specimens, respectively. Some investigators[14] have criticized the application of Dragendorff's reagent to the analysis of drugs of abuse, for the colors obtained with this reagent are less discriminating than those obtained with iodoplatinate. The enhanced sensitivity achieved with the reagent when used in conjunction with sulfuric acid and iodine vapors, however, far exceeds any liability inherent in a lack of color variability in the detection process.

Urine specimens of eight patients receiving cocaine anesthesia for rhinoplastic or septoplastic surgery (250 mg cocaine hydrochloride applied topically to nasal mucosa) were examined by the method described. Approximate quantitation was based upon visual comparison of specimens with similarly extracted and chromatographed urines to which had been added known amounts of cocaine and benzoylecgonine. Unchanged cocaine was generally low (*ca.* 1 μg/ml) in the initial 8-h specimen and absent in the two subsequently collected 8-h specimens. In contrast, benzoylecgonine levels were typically highest (*ca.* 47 μg/ml) in the initial 8-h specimen, decreasing to approximately 26 and 12 μg/ml for the latter two specimens, respectively (Table III, Fig. 2). The specimens were also assayed for benzoylecgonine by the EMIT® system (enzyme multiplied immunoassay technique, Syva, Palo Alto, Calif., U.S.A.)[18]. The excretion patterns of benzoylecgonine (the EMIT system does not detect cocaine at concentrations encountered in physiologic specimens) detected by the TLC method were generally approximated by the EMIT system, although the levels estimated by the latter system were inherently non-precise. The lack of precision achieved by the EMIT system becomes a more pronounced limiting factor at concentrations greater than 10 μg/ml due to the exponential basis of the assay. A more detailed evaluation of the patient specimens will be presented in a report describing a gas chromatographic procedure for quantitatively determining cocaine and benzoylecgonine[19].

TABLE III

ESTIMATED* COCAINE AND BENZOYLECGONINE LEVELS IN THE URINE** OF PATIENTS RECEIVING COCAINE ANESTHESIA***

	Cocaine (μg/ml)		*Benzoylecgonine (μg/ml)*	
	Mean	*Range*	*Mean*	*Range*
Initial 8-h specimen	1	0–5	47	10–150
Second 8-h specimen	0	0–2	26	7– 80
Third 8-h specimen	0	0–1	12	0– 5

* Approximate quantitation based upon visual comparison of specimens with similarly extracted and chromatographed urine standards.

** Urines from eight patients were each collected in three 8-h intervals.

*** 250 mg cocaine hydrochloride applied topically to nasal mucosa.

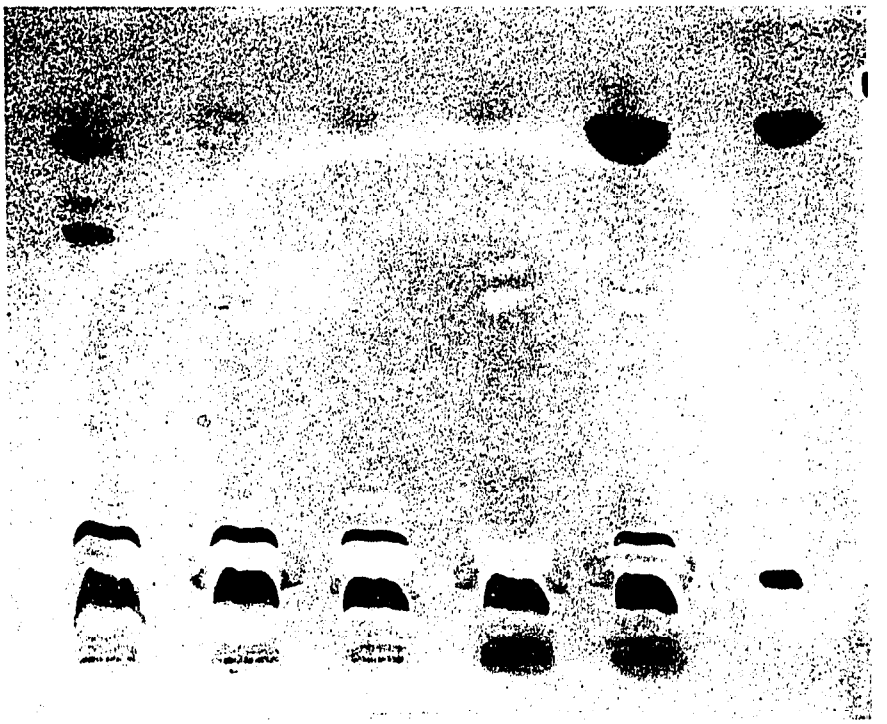

Fig. 2. Chromatogram of extracted urines of patient receiving cocaine anesthesia. Chromatogram contains, from left to right, extracts of the first, second and third 8-h collective specimens, urine blank, urine standard containing 5 μg/ml each of benzoylecgonine and cocaine, and a reference standard.

ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance of Diana Bason and Linda Goggin. Appreciation is also expressed to Donald L. Smith, John Alexander, M.D., and Kenneth Shanklin, M.D., USAF Wilford Hall Medical Center, for supplying post-surgery urine specimens of patients receiving cocaine anesthesia. This research

was supported by Grants R01 DA00594-01 and R01 DA00729-02 from the National Institute on Drug Abuse, NIH, Bethesda, Md., U.S.A.

REFERENCES

1 J. K. Brown, R. H. Schingler, M. G. Chaubal and M. H. Malone, *J. Chromatogr.*, 87 (1973) 211.
2 M. L. Bastos and D. B. Hoffman, *J. Chromatogr. Sci.*, 12 (1974) 269.
3 J. de Onis, Coca means Cocaine. It's also a way of life, *New York Times*, February 9, 1973.
4 G. Volsky, Illicit traffic of cocaine "growing by leaps and bounds" in Miami, *New York Times*, February 1, 1970.
5 H. M. Schmeck, Jr., Cocaine is re-emerging as a major problem, while marijuana remains popular, *New York Times*, November 15, 1972.
6 Cocaine found on the rise, *Washington Star-News*, April 10, 1974.
7 M. M. Baden, N. N. Valanju, S. K. Verma and S. N. Valanju, *Amer. J. Clin. Pathol.*, 57 (1972) 43.
8 B. S. Finkle, *Anal. Chem.*, 44 (9) (1972) 18A.
9 I. Sunshine, *Anal. Chem.*, 47 (2) (1975) 212A.
10 J. P. Comer and I. Comer, *J. Pharm. Sci.*, 56 (1967) 413.
11 N. N. Valanju, M. M. Baden, S. N. Valanju, D. Mulligan and S. K. Verma, *J. Chromatogr.*, 81 (1973) 170.
12 E. G. C. Clarke, *Isolation and Identification of Drugs*, Pharmaceutical Press, London, 1968, pp. 215, 267, 268.
13 S. Koontz, D. Besemer, N. Mackey and R. Phillips, *J. Chromatogr.*, 85 (1973) 75.
14 D. J. Berry and J. Grove, *J. Chromatogr.*, 61 (1971) 111.
15 G. A. Jansen and I. Bickers, *South. Med. J.*, 64 (1971) 1072.
16 V. P. Dole, A. Crowther, J. Johnson, M. Monsalvatge, B. Biller and S. S. Nelson, *N.Y. State J. Med.*, 72 (1972) 469.
17 S. J. Mulé, *J. Chromatogr.*, 55 (1971) 255.
18 M. L. Bastos, D. Jukofsky and S. J. Mulé, *J. Chromatogr.*, 89 (1974) 335.
19 J. E. Wallace and H. E. Hamilton, *Anal. Chem.*, submitted for publication.
20 A. Noirfalise and G. Mees, *J. Chromatogr.*, 31 (1967) 594.
21 B. Davidow, N. LiPetri, B. Quame, B. Fastich and J. Savitsky, *Amer. J. Clin. Pathol.*, 46 (1965) 58.
22 I. Sunshine, *Amer. J. Clin. Pathol.*, 40 (1963) 576.
23 C. W. Gorodetzky, *Toxicol. Appl. Pharmacol.*, 23 (1972) 511.
24 P. Majlát and I. Bayer, *J. Chromatogr.*, 20 (1965) 187.