Journal of Chromatography, 615 (1993) 365–368
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6804

Short Communication

Simplified method for simultaneous determination of diazepam and its metabolites in urine by thin-layer chromatography and direct densitometry

Raka Jain

Department of Psychiatry (Deaddiction), All India Institute of Medical Sciences, New Delhi 110 029 (India)

(First received July 9th, 1992; revised manuscript received February 16th, 1993)

ABSTRACT

A direct densitometric method for determination of diazepam and its metabolites in urine was developed. The proposed procedure involves acid hydrolysis of urine specimens, thereby converting diazepam and its metabolites into benzophenones [2-methylamino-5-chlorobenzophenone (MACB) and 2-amino-5-chlorobenzophenone (ACB)]. It is followed by extraction with chloroform–isopropanol (3:1, v/v). The two benzophenones were separated on thin-layer chromatography plates using hexane–diethyl ether–acetic acid (80:10:10) as a mobile phase. Quantitation of the MACB and ACB spots was achieved by direct ultraviolet densitometry. The limit of detection was 0.5 μg per ml of urine for both benzophenones. The proposed method is simple, rapid, reproducible and has been found to be effective for direct determination of diazepam and its metabolites in urine.

Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, is frequently prescribed as a tranquillizer for the treatment of anxiety and tension [1]. It is extensively used and abused [2–5]. Many patients form a dependence on benzodiazepine drugs. Therefore, benzodiazepines are encountered frequently in clinical or forensic toxicological analysis. Concentrations of benzodiazepines and their metabolites in urine are higher than those in plasma, so that it is preferable to use urine for their identification.

In the body, diazepam is rapidly and extensively metabolized to N-desmethyldiazepam, oxazepam and oxazepam glucuronide [5]. It is mainly excreted in the form of two glucuronides, very little unchanged diazepam being excreted. Acid hydrolysis converts both the glucuronides and free benzodiazepines into benzophenones, which are readily extractable [6]. Diazepam, 3-hydroxydiazepam and 3-hydroxydiazepam glucuronide are converted in 2-methylamino-5-chlorobenzophenone (MACB), and N-desmethyldiazepam, oxazepam and oxazepam glucuronide are converted to 2-amino-5-chlorobenzophenone (ACB).

Detection of these hydrolysis products (benzophenones) using thin-layer chromatography (TLC) [7,8], gas chromatography (GC) [9–11], high-performance liquid chromatography (HPLC) [12] and gas chromatography–mass spectrometry (GC–MS) [13,14] has been described previously.

The problem of diazepam abuse is apparently on the increase, and this situation has necessitated

0378-4347/93/$06.00

the search for a rapid method for quantitative analysis of diazepam in urine samples. The purpose of this communication is to report a simple, accurate, rapid and quantitative procedure for the determination of hydrolysis products of diazepam and its metabolites in urine by direct TLC–densitometry.

EXPERIMENTAL

Materials

2-Amino-5-chlorobenzophenone was obtained from SRL (Sico Research Labs., India) and 5-chloro-2-methylaminobenzophenone was a gift from the Forensic and Toxicoloy Laboratory, Institute of Legal Medicine (Padova, Italy). Benzophenones were also prepared from the parent benzodiazepines by acid hydrolysis for 1 h followed by extraction into diethyl ether [7]. Stock standard solutions (1 mg/ml) of each benzophenone were prepared in methanol. Silica gel-precoated 60F 254 TLC plates of 0.25 mm thickness were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

Apparatus

A Shimadzu CS-9000 dual-wavelength flying spot scanner was used with the following settings: scanning speed 5 mm/min with zig-zag scanning, swing width 10 mm, sample and reference beams (λ_S and λ_R) 250 nm and 300 nm, respectively, for both ACB and MACB, reflection mode, minimum area 1000, minimum width 3.00 mm, chromatogram ordinate scale (upper = 4.00 and lower = −1.00), chromatogram abscissa scale = ×0.5. The developed thin-layer plates were scanned in the direction parallel to the chromatogram flow. Ultraviolet (UV) absorption profiles of the thin-layer chromatogram and the integration curves of the peaks on the profiles were obtained simultaneously on the recorder.

Extraction

To 5 ml of urine was added 1 ml of concentrated hydrochloric acid. The solution was vortex-mixed for 5 min and autoclaved for 15 min at 120°C and 15 kPa. After cooling the tube in an ice water bath for 5 min, the pH was adjusted to 12 with 10 *M* sodium hydroxide and extracted with 2 × 8 ml of chloroform–isopropanol (3:1). The aqueous layer was discarded and the organic layer was evaporated to dryness in a 70°C water bath under a stream of nitrogen. After evaporation, the extracted residue was reconstituted with 20 μl of the methanol. The 10 μl of the residue and reference drug standards were spotted with 10-μl glass capillaries on silica gel TLC plates.

Thin-layer chromatography

TLC plates were developed by a freshly prepared mobile phase of hexane–diethyl ether–glacial acetic acid (80:10:10) at 25°C to a height of 8 cm from the point of application. After air-drying the MACB spots were visualized with short-wavelength UV light and the ACB spots with long-wavelength UV light and then subjected to densitometric analysis. The peak area of each spot was measured by densitometry.

RESULTS AND DISCUSSION

Analytical recovery

Known amounts of each benzophenone in methanol were added to drug-free urine to achieve the concentrations shown in Table I. Five samples of each concentration were processed by the procedure described above.

Precision and accuracy

Within-run precision was evaluated by analysing quadruplicate standard samples containing 2 μg/ml of each benzophenone. Between-run precision was studied by analysing the same standard of each benzophenone urine sample on five different days of one week. Urine sample extraction and TLC–densitometric analysis were performed as described above. The within-day precision (coefficient of variation, C.V.) was 4.62% for MACB and 2.10% for ACB. Between-day precision (C.V.) was 7.50 and 5.42% for MACB and ACB, respectively.

TABLE I

EXTRACTION EFFICIENCY OF BENZOPHENONES FROM URINE ($n = 5$)

Benzophenone	Added (μg)	Recovery (%)	
		Mean	S.D.
2-Amino-5-chlorobenzophenone (ACB)	20	86.94	3.14
	5	86.64	1.82
5-Chloro-2-methylaminobenzophenone (MACB)	20	90.2	1.20
	5	90.22	1.54

Quantitation

The calibration curves for benzophenones were prepared by analysing 5 ml of urine containing 50 μg of each benzophenone. The samples were processed as described above and each residue was dissolved in 500 μl of methanol. Various volumes, *i.e.*, 0.5, 2, 5, 10 and 20 μl, of residual extraction of each benzophenone were spotted on three TLC plates. After development the peak areas of benzophenone spots on the chromatograms were measured by densitometry. The mean value of each experimentally obtained datum was plotted for each concentration spotted. A straight-line calibration graph was obtained for each benzophenone based on peak-area measurements in the range 0.05–2.0 μg. Each point was taken as the average of two determinations. The correlation coefficient for MACB and ACB was 0.9983 and 0.9989, respectively.

The detection limit for both benzophenones

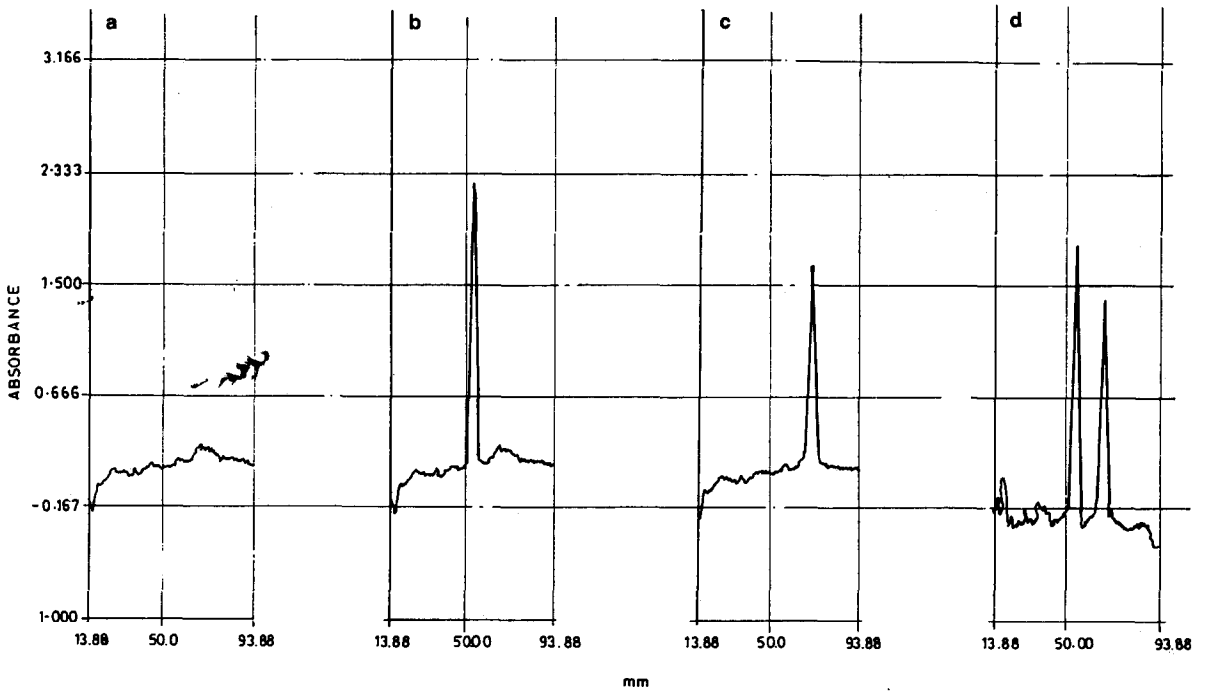

Fig. 1. Densitograms showing (a) an extract from a blank urine sample, (b) an extract from urine spiked with ACB (1 μg/ml), (c) an extract from urine spiked with MACB (1 μg/ml) and (d) an extract from urine of a diazepam-dependent patient.

was 0.5 μg/ml of urine. Chromatograms of control urine, spiked urine samples containing ACB and MACB, and urine from a diazepam-dependent patient are shown in Fig. 1.

In addition, neither benzophenone requires spray reagents for visualization. MACB is readily visualized as a fluorescent spot under short-wavelength UV light and ACB as a dark spot under long-wavelength UV light. Both spots appeared as yellow in visible light. The R_F value for MACB was 0.70 and for ACB was 0.55. For a sample to be considered positive for diazepam, both MACB and ACB must be present on the TLC plate [8].

Chlordiazepoxide and its metabolites yield an ACB spot but not an MACB spot on TLC. Thus possible interference from chlordiazepoxide is eliminated by the absence of an MACB spot. Similarly, camazepam, ketazepam, temazepam are also hydrolyzed to MACB but not ACB. Hence, these drugs will not interfere in the assay. However, note that a patient taking medazepam should give a positive result for diazepam and all its metabolites, as diazepam and its metabolites are metabolic products of medazepam. Medazepam, however, is not legal in India. Moreover, other benzodiazepines are metabolized to specific benzophenone derivatives, thus there is no possibility of interference from other benzodiazepines.

The method has been used to screen and quantitate 300 urine samples from diazepam abusers and has proved quite suitable for analysis of urine samples taken from abusers of diazepam. Hence, the method outlined above is simple, rapid, precise and most effective in detecting and quantitating metabolites of diazepam, especially the glucuronides.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. D. Mohan (Head of the Department of Psychiatry, Deaddiction) and Dr. R. Ray (Department of Psychiatry, Deaddiction), All India Institute of Medical Sciences, New Delhi, for their valuable advice. The technical assistance of Mr. S. D. Dhiman and Mr. N. P. Sharma is gratefully acknowledged.

REFERENCES

1 M. Linnoila, in E. Costa (Editor), *The Benzodiazepines — From Molecular Biology to Clinical Practice*, Raven Press, New York, 1983, p. 267.
2 J. C. Garriott, V. J. M. DiMaio, R. E. Zumwalt and C. S. Petty, *J. Forensic Sci.*, 22 (1977) 383.
3 T. A. Rejent and K. C. Wahl, *Clin. Chem.*, 22 (1976) 889.
4 J. Wiersum, *J. Am. Med. Assoc.*, 224 (1974) 79.
5 J. A. F. de Silva and C. V. Puglisi, *Anal. Chem.*, 42 (1970) 1725.
6 J. Bäumler and S. Rippstein, *Helv. Chim. Acta*, 44 (1961) 2208.
7 H. Schütz, *Benzodiazepines, II, A Handbook, Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature*, Springer, Heidelberg, 1989.
8 R. D. Budd and E. Walkin, *Clin. Toxicol.*, 16 (1980) 201.
9 M. Japp, K. Garthwaite, A. V. Geeson and M. D. Osselton, *J. Chromatogr.*, 439 (1988) 317.
10 H. Schütz and V. Westenberger, *Z. Rechtsmed.*, 82 (1978) 43.
11 H. Schütz and V. Westenberger, *J. Chromatogr.*, 169 (1979) 409.
12 K. Harzer and R. Barchet, *J. Chromatogr.*, 132 (1977) 83.
13 H. Maurer and K. Pfleger, *J. Chromatogr.*, 222 (1981) 409.
14 H. Maurer and K. Pfleger, *J. Chromatogr.*, 422 (1987) 85.