Journal of Chromatography, 299 (1984) 465–470
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,915

Note

Two-dimensional thin-layer chromatographic identification of twelve 1,4-benzodiazepines

M. BAKAVOLI*, V. NAVARATNAM and N. K. NAIR
National Drug Research Centre, University of Science Malaysia, Minden, Penang (Malaysia)
(Received May 15th, 1984)

Thin-layer chromatography (TLC) is widely used for the separation and identification of benzodiazepines or their hydrolysis products, *i.e.*, benzophenones. Most studies have focused on the separation of the parent benzodiazepine from its metabolites, impurities or related compounds, and only few papers deal with the separation of intact benzodiazepines for identification purposes. The most commonly used method to identify the benzodiazepines and their metabolites is by means of their aminobenzophenone hydrolysis products. However, it is not a specific method since different benzodiazepines can yield the same benzophenones, and some benzodiazepines do not form benzophenones when subjected to acidic hydrolysis. Comprehensive reviews of the determination of benzodiazepines and their metabolites have been published[1–4].

Among the procedures described for separation of intact benzodiazepines, two-dimensional and pH-gradient techniques seem to give the best separations[5–7]. More recently, a method involving the use of two or three chromatographic systems has been reported[8]. Although this was described as a very convenient method for identification purposes, it has the limitation of not being able to separate and consequently identify some mixtures of benzodiazepines. By using a modified version of this method in conjunction with the acidification of the unresolved benzodiazepines on TLC plates, satisfactory separation and identification can be achieved.

In this paper we describe such a method to separate and identify twelve benzodiazepines of interest in our national drug screening programme.

EXPERIMENTAL

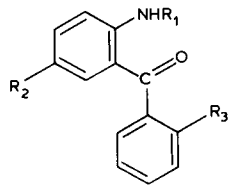
Sample

The benzophenones listed in Table I were prepared by acidification of the corresponding benzodiazepines as described by Roets and Hoogmartens[9]. The purity of the samples was established by gas chromatography using a 3% OV-17 column (6 ft. × 2 mm I.D.) with a flame ionization detector. The benzodiazepines given in Table II were obtained from Hoffmann-La Roche (Basle, Switzerland), except for I, II and V which were provided by Wyeth Laboratories (Taplow, Maidenhead, U.K.).

0021-9673/84/$03.00

TABLE I

BENZOPHENONES PREPARED



R_1	R_2	R_3	*Abbreviation*	*Systematic name*
H	NO_2	H	ANB	2-Amino-5-nitrobenzophenone
H	NO_2	Cl	ANCB	2-Amino-2′-chloro-5-nitrobenzophenone
CH_3	NO_2	F	MANFB	2′-Fluoro-2-methylamino-5-nitrobenzophenone
H	Cl	Cl	ADB	2-Amino-5,2′-dichlorobenzophenone
H	Cl	H	ACB	2-Amino-5-chlorobenzophenone
CH_3	Cl	H	MACB	5-Chloro-2-methylaminobenzophenone
C_3H_5	Cl	H	CPACB	5-Chloro-2-cyclopropylaminobenzophenone
$CH_2CH_2N(C_2H_5)_2$	Cl	F	DEACFB	5-Chloro-2-diethylaminoethylamino-2′-fluorobenzophenone

TABLE II

BENZODIAZEPINES AND THEIR RELATED BENZOPHENONES

Benzodiazepine	*Benzophenone**
Lorazepam (I)**	ADB (1)
Oxazepam (II)	ACB (2)
Desmethyldiazepam (III)	ACB (2)
Clonazepam (IV)**	ANCB (3)
Temazepam (V)	MACB (5)
Diazepam (VI)**	MACB (5)
Flunitrazepam (VII)**	MANFB (6)
Prazepam (VIII)**	CPACB (7)
Medazepam (IX)	MACB (5)
Chlorodiazepoxide (X)**	ACB (2)
Nitrazepam (XI)**	ANB (4)
Flurazepam (XII)**	DEACFB (8)

* For abbreviations, see Table I.
** Subject to acidic hydrolysis.

Stationary and mobile phases

Commercially coated silica gel Merck plates were used: 20 × 20 cm, silica gel 60, GF 254, thickness 0.2 mm, with alumina support. The mobile phases were chloroform–acetone (9:1) and dichloromethane–chloroform (1:1).

All solvents were of "reinst" grade.

Chromatographic procedure

Solutions of 1 mg/ml of each benzodiazepine in methanol and each benzo-

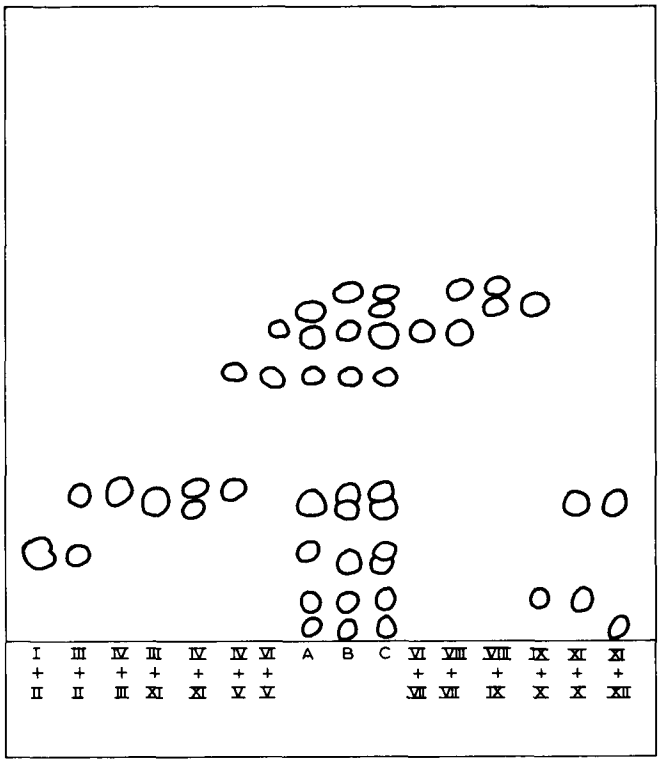


Fig. 1. TLC of mixtures of benzodiazepines (refer to Table II) run in chloroform–acetone (9:1, double development). A = I, III, V, VII, IX–XII; B = II, IV–VI, VIII, X–XII; C = I–XII.

phenone in chloroform were prepared. A 10-μl volume of each benzodiazepine (10 μg) was applied to a TLC plate on a line 3 cm from the bottom, with the first spot 1.5 cm from the side of the plate and the following spots at 1 cm intervals (Fig. 1). A mixture containing 10 μg of each benzodiazepine was spotted onto a second plate 3 cm from the side and base of the plate. Both plates were developed twice with chloroform–acetone (9:1) in pre-equilibrated chromatographic chambers. At room temperature the solvent front migrated 15 cm in *ca.* 25 min.

The second plate was air-dried and each unresolved spot treated with sulphuric acid in methanol (15%, v/v; 5 μl per component). The plate was placed between two glass plates (20 × 20 cm) and heated in an oven for 20 min at 110°C. After cooling to room temperature, the spots were treated with ammonia solution in methanol (25%, w/v; 10 μl per component). The plate was air-dried and heated for 10 min at 110°C. After cooling to room temperature, 2 μg of each benzophenone were applied in line with the developed spots. Finally, the plate was developed in the second direction with dichloromethane–chloroform (1:1) (Fig. 2), and examined under UV light at 254 and 366 nm.

Several mixtures of benzodiazepines on TLC plates were also treated with HCl in methanol (48%, v/v) and H_3PO_4 in methanol (12.5%, v/v).

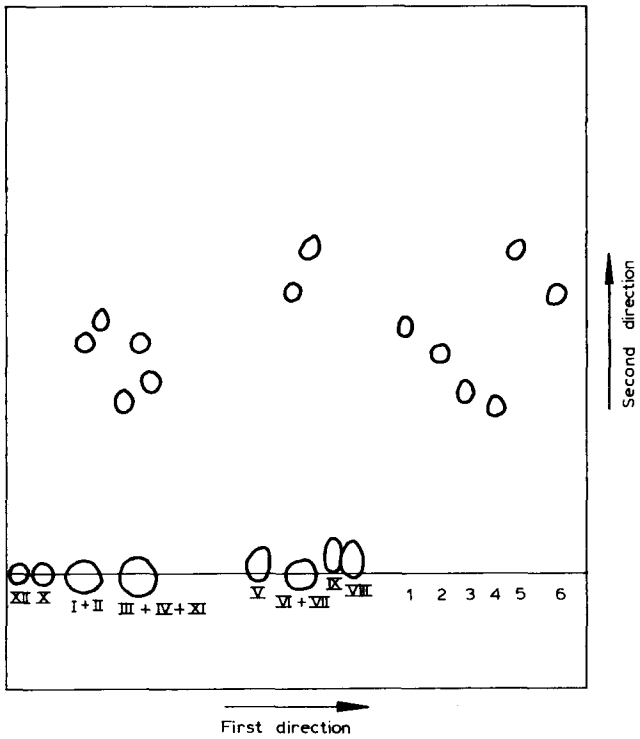


Fig. 2. TLC of a mixture of benzodiazepines (refer to Table II) run in chloroform–acetone (9:1, first direction) followed by on-plate acidic hydrolysis of the unresolved spots and subsequent development in dichloromethane–chloroform (1:1, second direction).

RESULTS AND DISCUSSION

The initial separation of the standard mixtures of benzodiazepines is shown in Fig. 1. Several mobile phases that had recently been reported as suitable for the separation of benzodiazepines were used[9–13], but the chloroform–acetone system was selected since it can separate most of the components of the mixture. A second development of the plate led to an even better separation. The R_F values are listed in Table III.

It is clear from Fig. 1 that the pairs I and II, VI and VII and the mixture III, IV and XI cannot be separated. However, IV and XI can be distinguished. Since these compounds produce different benzophenones on treatment with acid their separation and identification can be achieved through their hydrolysis products. Recently, several papers have reported the TLC of benzophenones in the identification of pure benzodiazepines or their metabolites in biological media[9,14,15]. Several of the mobile phases employed there were tested and dichloromethane–chloroform (1:1) was adopted as the solvent system of choice. The R_F values of the benzophenones derived from the hydrolysis of the unresolved parent 1,4-benzodiazepines (Fig. 1) are listed in Table III.

It is known that some benzodizepines when treated with acid produce several

TABLE III

R_F VALUES OF BENZODIAZEPINES AND BENZOPHENONES

Benzodiazepine	$R_F \times 100$	*Related benzophenone*	$R_F \times 100$
I	16	ADB	45.3
II	14	ACB	40.6
III	25.3	ACB	40.6
IV	26.3	ANCB	34.7
V	46.7	–	–
VI	54.7	MACB	49.3
VII	54.7	MANFB	56.7
VIII	62.7	–	–
IX	59.7	–	–
X	7.3	–	–
XI	24	ANB	30.7
XII	2	–	–

hydrolysis products[9,16–19]. Similarly, some of the benzodiazepines in this study undergo acidic hydrolysis fairly readily to produce not only the related benzophenones, but also other hydrolysis products. For example, compounds V, VI and VIII each produces at least two hydrolysis products, visible upon TLC as yellow spots. Therefore, complications could arise when a mixture of benzodiazepines are hydrolysed to their benzophenones for identification purposes. At first it appears that the separation and identification of such a complex mixture might be difficult. However, considering the fact that pure benzophenones have much higher R_F values than their impurities, their identification and that of the parent benzodiazepine can easily be achieved.

The hydrolysis of some benzodiazepines in aqueous HCl has been reported to give a higher yield of the hydrolysed products[20,21]. When aqueous HCl was used to hydrolyse the benzodiazepines on TLC plates, background interference made visualization of the spots difficult under UV light[9]. To reduce the effect of the background, the plate must be pre-dried at 150°C for 1 h. Further, the solution of HCl in methanol (48%, v/v) should be applied carefully to the plate in such a manner as to prevent spreading of the acid. Then by covering the spots and following the stated experimental procedure, normal detection is possible. Orthophosphoric acid (12%, v/v) was also used for hydrolysis of the benzodiazepines on the TLC plate and the results were satisfactory. However, sulphuric acid was found to be more suitable since it can easily be applied to the plate.

Fig. 2 shows that the twelve 1,4-benzodiazepines can be identified by initially separating them with chloroform–acetone (9:1) as the mobile phase. The unresolved benzodiazepine pairs I and II, VI and VII and the mixture III, IV and XI were then hydrolysed on the plate and the parent benzodiazepines were easily identified from the related benzophenones after chromatography in the second direction with the mobile phase dichloromethane–chloroform (1:1). To determine the detection limit of benzodiazepines via their related benzophenones, different amounts of benzodiazepines, ranging from 2 to 16 μg, were hydrolysed on the plate. Individual benzodiazepines are detectable down to 2 μg, but in a mixture the detection limit was 5 μg.

TABLE IV

TLC ANALYSIS OF SOME ILLICIT BENZODIAZEPINES

Sample	*Benzodiazepine detected*	$R_F \times 100$	*Related hydrolysis product* ($R_F \times 100$)
A	Medazepam	59	–
B	Clonazepam	27	35
C	Chlorodiazepoxide	8	–
D	Diazepam	56	51
E	Nitrazepam	25	31
F	Medazepam	59	–

The reliability of the TLC screening procedure was established by analysis of some illicit benzodiazepine tablets. The results of the analysis in Table IV were confirmed by gas chromatography using an OV-101 column (6 ft. × 2 mm I.D.). It is apparent that the TLC procedure described permits the separation and identification of some benzodiazepines for screening purposes.

ACKNOWLEDGEMENT

We thank the United Nations Fund for Drug Abuse Control for support of this work.

REFERENCES

1 D. M. Hailey, *J. Chromatogr.*, 98 (1974) 527.
2 J. M. Clifford and W. F. Smyth, *Analyst (London)*, 99 (1974) 241.
3 N. Y. Golovenko and W. G. Zinkovskii, *Khim-Farm. Zh.*, 12 (1978) 3.
4 J. A. F. de Silva, *Anal. Chem. Symp.*, 4 (1980) 349.
5 S. N. Tewari and S. K. Shukla, *J. Indian Acad. Forensic Sci.*, 17 (1980) 80.
6 S. N. Tewari and S. K. Shukla, *Pharmazie*, 32 (1977) 536.
7 E. Stahl and J. Müller, *J. Chromatogr.*, 209 (1981) 484.
8 I. Wouters, E. Roets and H. Hoogmartens, *J. Chromatogr.*, 179 (1978) 381.
9 E. Roets and J. Hoogmartens, *J. Chromatogr.*, 194 (1980) 262.
10 D. Owen, A. Pendlebury and A. C. Moffat, *J. Chromatogr.*, 161 (1978) 187.
11 I. Natar, L. Bajor, P. Marcu and L. Roman, *Clujul Med.*, 51 (1978) 351.
12 H. Shuetz and Z. Fresevias, *Anal. Chem.*, 294 (1979) 135.
13 J. M. Meola, G. T. Rosana and T. Swift, *Clin. Chem.*, 27 (1981) 1254.
14 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 154 (1978) 211.
15 H. Shuetz, S. Suphachearabhan and Z. Fresenius, *Anal. Chem.*, 314 (1983) 44.
16 G. Cille, J. Braun, D. Gravel and R. Plourde, *Can. J. Pharm. Sci.*, 8 (1973) 42.
17 M. E. Esteva, S. Lamdan and C. H. Gaozza, *Rev. Farm. (Buenos Aires)*, 118 (1976) 118.
18 J. Gasparic, J. Zimak, P. Sedmera, Z. Breberova and J. Volke, *Collect. Czech. Chem. Commun.*, 44 (1979) 2243.
19 J. T. M. Groenewegen and P. J. J. De Meijer, *Pharm. Weekbl.*, 115 (1980) 963.
20 A. Hayriye, S. Serpil and B. Nejla, *Instanbul Univ. Eczacilik Fak. Mecin.*, 16 (1980) 3.
21 J. Gasparic, J. Zinak, M. Machacek and J. Volke, *Cesk. Farm.*, 31 (1982) 55.