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Note

Thin-layer chromatography of the acid hydrolysis products of nineteen benzodiazepine derivatives

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Thin-layer chromatography (TLC) of the benzophenones obtained by acid hydrolysis of benzodiazepine derivatives is widely used for the identification of members of this group of psychopharmacological agents. As already stated previously, this method is not specific, as different benzodiazepines can give the same benzophenone, nor is it a general method[1]. Indeed, benzodiazepines such as triazolam, alprazolam and clobazam do not form benzophenones when they are treated in the usual way, and medazepam is stable towards hydrolysis. The advantage of TLC of the benzophenones rather than of the benzodiazepines themselves is that different metabolites of the same benzodiazepine can give the same benzophenone on hydrolysis, which makes this method more suitable for identification of these products in biological fluids. Work up to 1974 has already been reviewed[2,3]. TLC of benzophenones is also mentioned in several more recent papers as being useful for the identification of pure benzodiazepines or for the identification of benzodiazepines and metabolites in biological fluids[4–7].

In this paper we describe the separation of the nine benzophenones obtained by acid treatment of 19 benzodiazepines on the TLC plate.

EXPERIMENTAL

Samples

The origins and structures of the benzodiazepine derivatives I to XIX have been given previously[1]. Their names are listed in Table I, except for clobazam (VIII), triazolam (XVII) and alprazolam (XVIII), which were not hydrolysed into benzophenones. Table I also lists the corresponding benzophenones. Samples XXIV and XXV were kindly provided by Wyeth (Brussels, Belgium). All other benzophenones were obtained by hydrolysis of the first-mentioned corresponding benzodiazepine (Table I). For this purpose 25 mg of the benzodiazepine were dissolved in 10 ml of 4 *N* hydrochloric acid. The solution was kept in a boiling water-bath for 1 h, was then brought to pH 10 with 10 *N* sodium hydroxide solution and was finally extracted twice with 15 ml of chloroform. The combined organic layers were washed with water, dried over anhydrous sodium sulphate and evaporated to dryness. The residues were purified when necessary by column chromatography over silica gel 60 (0.040–0.063 mm)

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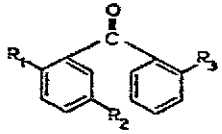
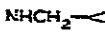
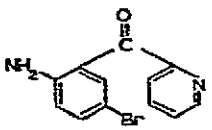
(E. Merck, Darmstadt, G.F.R.) with methylene chloride as the mobile phase, except for XX and XXI, where 5% (v/v) of acetone had to be added. All of the structures were confirmed by infrared spectroscopy and mass spectrometry.

TABLE I

BENZOPHENONES AND RELATED BENZODIAZEPINES



R_1	R_2	R_3	*Benzophenones*	*Benzodiazepines*
$NHCH_2CH_2N(C_2H_5)_2$	Cl	F	2-(Diethylaminoethylamino)-5-chloro-2'-fluorobenzophenone (XX)	Flurazepam (III)
NH_2	NO_2	H	2-Amino-5-nitrobenzophenone (XXII)	Nitrazepam (XIV)
NH_2	NO_2	Cl	2-Amino-5-nitro-2'-chlorobenzophenone (XXIII)	Clonazepam (XIII)
NH_2	Cl	H	2-Amino-5-chlorobenzophenone (XXIV)	Oxazepam (XVI) Chlordiazepoxide (XIX) Chlorazepate (X) Desmethyldiazepam (XI)
NH_2	Cl	Cl	2-Amino-2,5-dichlorobenzophenone (XXV)	Lorazepam (XV)
$NHCH_3$	NO_2	F	2-Methylamino-5-nitro-2'-fluorobenzophenone (XXVI)	Flunitrazepam (V)
$NHCH_3$	Cl	H	2-Methylamino-5-chlorobenzophenone (XXVII)	Camazepam (VI) Diazepam (IV) Temazepam (IX) Ketazolam (VII) Medazepam (II)
	Cl	H	2-Cyclopropylmethylamino-5-chlorobenzophenone (XXVIII)	Prazepam (I)
			2-Amino-5-bromobenzoylpyridine (XXI)	Bromazepam (XII)

Stationary and mobile phases

Generally DC-Fertigplatten Kieselgel 60 F 254 (Merck) were used, but other brands of ready-made silica gel plates were also tried, *viz.*, Stratochrom Si F 254 (Carlo Erba, Milan, Italy) and DC-Fertigplatten Si F (Riedel-de Haën, Hannover, G.F.R.). The plates were used without prior activation. Methylene chloride–chloroform (1:1) was generally used as the mobile phase (A). Both solvents were of "reinst" quality (Merck). Other mobile phases were also used, *viz.*: B, chloroform; C, benzene; D, benzene–chloroform (3:1); E, benzene–nitromethane (30:1); F, toluene–diethyl-

amine (4:1); G, benzene–methanol (96:6); and H, cyclohexane–acetone (9:1). All ratios are expressed in volumes.

Chromatographic procedure

The benzophenones were dissolved in chloroform (0.4%, w/w) and 0.5-μl (2-μg) amounts were spotted on the TLC plate. The benzodiazepines were dissolved in methanol (0.4%, w/v), except for X, which was dissolved in water and then diluted with methanol to a final concentration of 80% (v/v) of methanol. Amounts of 2.5 μl (10 μg) were spotted. Then 5 μl of 15% (v/v) sulphuric acid was placed over each spot, whereafter the TLC plate was covered with a glass plate and kept for 20 min in an oven at 120 °C. The plate was cooled to room temperature and each spot was covered with 10 μl of ammonia solution (25%, w/v). The spots were dried, first in a stream of air and then by heating at 120 °C for 5 min. Paper-lined chromatographic chambers were equilibrated with the mobile phase for at least 1 h. The plates were developed over a distance of 15 cm, dried in a stream of warm air and examined under a UV lamp with a maximum output a about 254 nm (Sylvania G 15 T8 A lamp) or at about 366 nm (Philips HPW 125-W lamp). The detection limit for benzophenones was less than 0.5 μg at the shorter wavelength; the sensitivity was lower at the higher wavelength. All experiments were carried out at temperatures between 20 and 25 °C.

Photography

The technique and materials have been mentioned previously[1]. Two Philips HPW 125-W lamps were used as the 366-nm light source.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of the pure benzophenones obtained with mobile phase A on a Merck plate. Comparable results were obtained with the other brands of plates. Table II lists the R_F values obtained with the different mobile phases. All except A and B have been mentioned in the literature as suitable for the separation of benzophenones[6–13]. R_F values from the literature are also given where available. No literature values are mentioned for mobile phase D since Lafargue *et al.* used aluminium oxide plates[11].

Mobile phase A separates the nine benzophenones but has the disadvantage that XX does not migrate. It must be noted that for this mobile phase the quality of the solvents is important, as the amount of ethanol, present as a stabiliser, can influence the separation. Mobile phase C is widely used for the separation of benzophenones. With this mobile phase an even better separation is obtained when no paper lining is used. The values thus obtained correspond best to those in the literature. The markedly higher values obtained by Hermans and Kamp[8] may be due to the use of home-made plates, which generally give higher R_F values. With this mobile phase lower R_F values are recorded and therefore it is less suitable for chromatography after hydrolysis on the TLC plate because several benzophenones could disappear amongst the lower background spots. For mobile phases E, F and G a reasonable agreement with the literature values is observed. The higher values for G can again be explained by the use of home-made plates by Gräfe and Schmeling[6]. For mobile phase H totally different values were obtained, probably owing to the use of a particular brand of

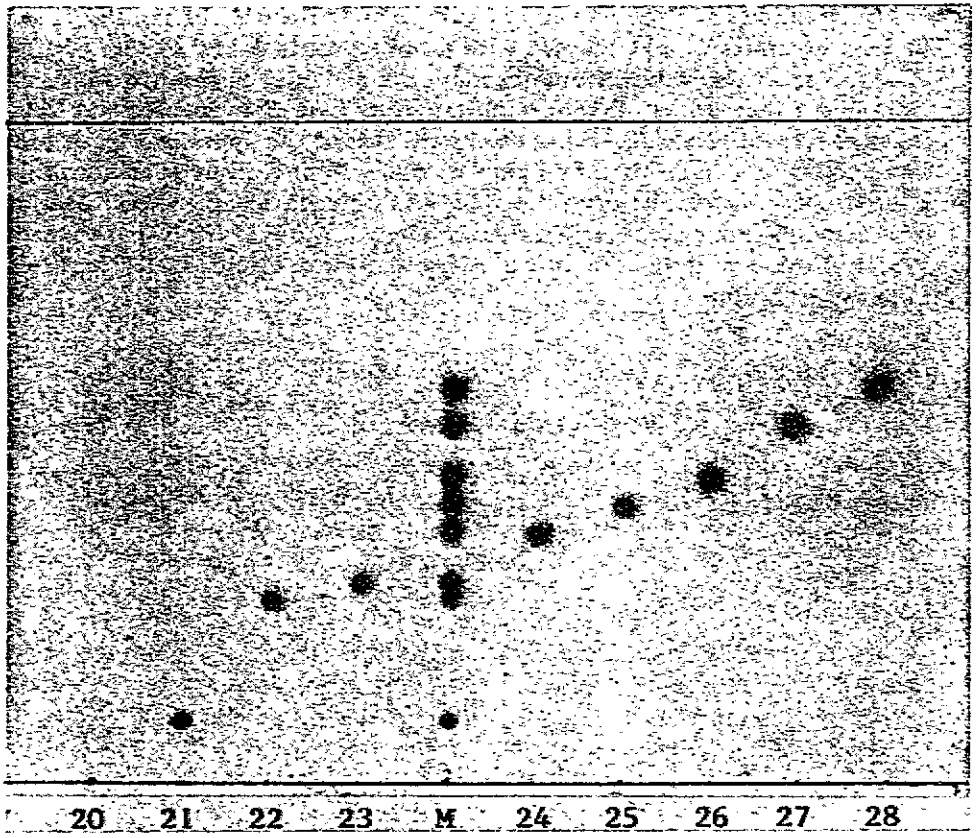


Fig. 1. Chromatogram of pure benzophenones obtained using methylene chloride–chloroform (1:1) (A) as the mobile phase. The arabic numbers of the spots correspond to the roman numbers listed in Table I.

TABLE II

R_F VALUES OF BENZOPHENONES ON MERCK PLATES

Benzo phenone	*Mobile phase*																	
	A	*B*	*C (with paper lining)*	*C (without paper lining)*				*D*	*E*			*F*		*G*		*H*	
				This work	*Ref. 8*	*Ref. 9*	*Ref. 10*		*This work*	*Ref. 12*		*This work*	*Ref. 13*	*This work*	*Ref. 6*	*This work*	*Ref. 7*
XX	0.00	0.01	0.00	0.00				0.00	0.00			0.64		0.10	0.17	0.03	
XXI	0.09	0.16	0.02	0.02				0.03	0.04			0.37		0.41		0.07	
XXII	0.29	0.31	0.10	0.15	0.20	0.17	0.20	0.14	0.15			0.27		0.43	0.47	0.05	
XXIII	0.32	0.32	0.11	0.19				0.15	0.17	0.13		0.25	0.27	0.42		0.04	
XXIV	0.38	0.43	0.18	0.30	0.39	0.34	0.32	0.24	0.26	0.20		0.44	0.47	0.54	0.61	0.15	0.43
XXV	0.43	0.45	0.24	0.39		0.46	0.41	0.28	0.31			0.40	0.42	0.54	0.61	0.11	
XXVI	0.47	0.52	0.21	0.35				0.29	0.29			0.54		0.58		0.10	
XXVII	0.55	0.58	0.36	0.56	0.73	0.69	0.56	0.42	0.43	0.37		0.63	0.63	0.63	0.73	0.34	0.73
XXVIII	0.61	0.62	0.45	0.69			0.70	0.50	0.52			0.66		0.66	0.75	0.42	

silica gel plates (Gelman ITLC Type SA) and possibly also to the use of an unsaturated chamber. The best separations are obtained with mobile phases A and C.

Figs. 2 and 3 show the same chromatogram obtained after *in situ* hydrolysis of the 19 benzodiazepine derivatives. The only difference is that 254- and 366-nm light sources were used, respectively. Observing the intensities of the spots in Fig. 3, one should not erroneously conclude that detection by black light is more sensitive: this effect is due only to a longer exposure time. The photographs clearly show that, in addition to the benzophenones, a considerable number of other hydrolysis products are formed. Most of these migrate very little and thus do not interfere. Their identities have not yet been investigated.

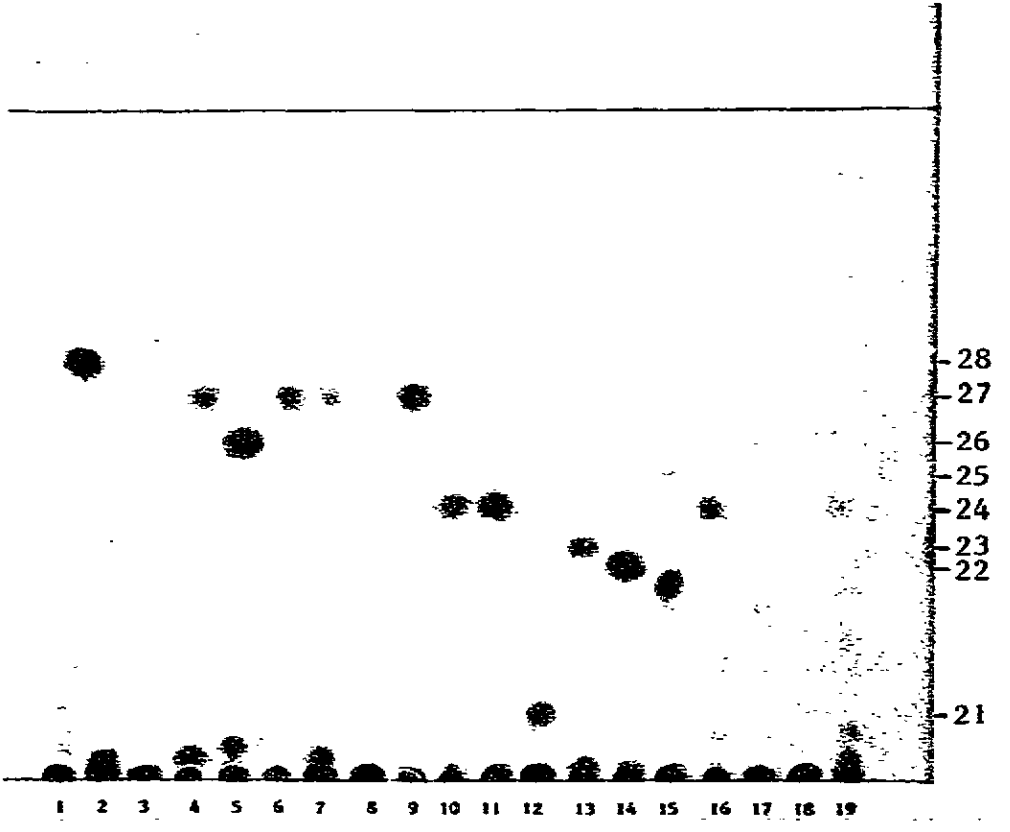


Fig. 2. Chromatogram obtained after hydrolysis of the benzodiazepines on the plate with methylene chloride–chloroform (1:1) (A) as the mobile phase. Detection with a 254-nm light source. The numbers of the spots correspond to those listed in Table I.

To obtain a good hydrolysis it is necessary to cover the spots, moistened with dilute sulphuric acid, with a glass plate; omitting this detail causes rapid evaporation of the water with partial or no hydrolysis as a consequence. This is probably the reason why Hermans and Kamp[8] did not succeed in hydrolysis on the plate. The importance of this detail was mentioned by Schillings *et al.*[14]. Schütz and Schütz[15]

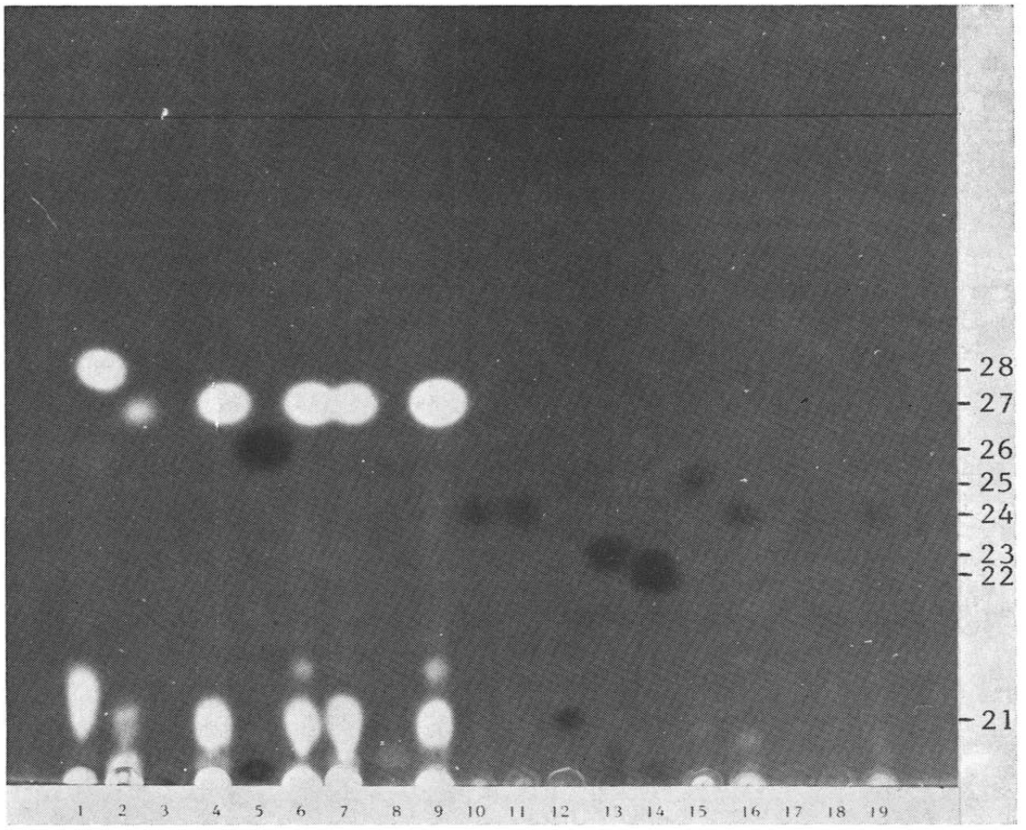
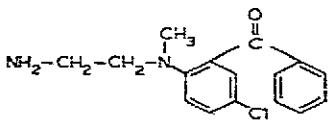


Fig. 3. Chromatogram obtained after hydrolysis of the benzodiazepines on the plate with methylene chloride–chloroform (1:1) (A) as the mobile phase. Detection with a 366-nm light source. The numbers of the spots correspond to those listed in Table I.

uses hydrochloric acid for the hydrolysis, but this acid is unsuitable when UV light is used for the detection: background effects prevent normal detection of the spots.

Medazepam (II) shows only a very weak spot of XXVII, which is best seen in Fig. 3. The acid hydrolysis of II is mentioned by some authors as being only very partial[16,17], others report it as not occurring[11,18], while some do not mention any problem in connection with this hydrolysis[9,10]. Although the hydrolysis on the plate of II into XXVII is only very partial, we obtained a better result when the hydrolysis was carried out as mentioned under *Samples*. The yield was about 10% of the theoretical value. One author even reports the formation of another benzophenone (XXIX) by hydrolysis of II[6].



XXIX

When the hydrolysis was carried out as stipulated by Gräfe and Schmeling[6], the benzodiazepine remained unchanged. Medazepam behaves chromatographically

in the same way as described for XXIX. In acidic conditions II shows up as a reddish spot on the plate, and when the plate is sprayed with an alkaline solution this colour disappears. Gräfe and Schmeling[6] noticed the same behaviour for their substance. We therefore conclude that the structure XXIX was erroneously assigned to the unchanged benzodiazepine.

Benzophenone XX does not migrate in system A and therefore III cannot be identified together with the other benzodiazepines. A second development of the plate in mobile phase F can solve this problem. Benzophenone XX is a tertiary aliphatic amine and therefore needs a base, stronger than ammonia, to be liberated from the corresponding salts. With regard to this, TLC of XX in mobile phase F, which contains diethylamine, is no problem. However, if another system were to be used it is recommended that the spot be treated with diethylamine prior to chromatography. Excess of base can easily be removed by a stream of hot air. This method, using a second development, is valid only when no other benzodiazepines are present. Indeed, the secondary hydrolysis products of several benzodiazepines migrate together with XX. The same has been observed for unreacted II.

Triazolam (XVII) and alprazolam (XVIII) do not hydrolyse on the plate. These benzodiazepines are also stable in hot 4 *N* hydrochloric acid and therefore cannot be identified by the benzophenone method. Clobazam (VIII) is not expected to form a benzophenone on hydrolysis as it is a 1,5-benzodiazepine. The benzodiazepines VIII, XVII and XVIII do not migrate with mobile phase A.

For lorazepam (XV), it can be seen in Fig. 2 that the spot of one of these secondary products, with R_F 0,29, is even more intense than the spot corresponding to the benzophenone itself (R_F 0.43). This substance is not detected by black light. As far as we know, secondary hydrolysis products were not described for XV. For oxazepam (XVI), which only differs from XV in that it has a supplementary chlorine substituent, several secondary products are mentioned in the literature[19–21], with different proposed structures. The hydrolysis was also carried out under different conditions, so that comparison is difficult. A first attempt to compare the reaction products obtained by the different hydrolysis procedures was carried out by TLC. Insufficient separation of the complex mixtures complicated the interpretation of the results and it was decided to try to solve the problem by high-performance liquid chromatography. The results will be reported later. From Fig. 2 it is already obvious that XV and XVI do not behave in a completely analogous way.

We believe that the TLC method presented here can be an aid in the identification of benzodiazepines and their derivatives.

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