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Note

Gas chromatographic determination of dibenzepine and its basic metabolites in biological material

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During the last 10 years there have been several reports of fatal poisonings by overdosage of dibenzepine[1–8]. Gas chromatographic procedures are available for the determination of this drug and its basic metabolites II–VI (see Fig. 2) in biological material based on the acetylation of the demethylated compounds[2,5–7,9,10]. These procedures are suitable for the measurement of the unchanged drug but, as demonstrated by the gas chromatograms published[9,10], poor sensitivity and specficity were achieved with acetylated IV, V and VI owing to their unsatisfactory gas chromatographic properties. For this reason, so far the pattern of all five demethylated metabolites has been determined only in the urine of patients who had received dibenzepine for a long period[9,10] and not in tissues or body fluids in cases of fatal intoxications.

In this investigation the metabolites of dibenzepine were determined after trifluoroacetylation, as the derivatives thus obtained exhibited excellent gas chromatographic properties. Further, a simple procedure is described for the separation of dibenzepine and its demethylated metabolites by ion-pair extraction. Thus it became possible to determine the total pattern of dibenzepine and its basic metabolites in tissues and body fluids.

MATERIALS AND METHODS

Amberlite XAD-2 resin was purified as described by Bradlow[11]. All solvents and reagents were of pro analisi grade and were used as supplied. Dibenzepine and the demethylated compounds were a gift from Wander GmbH (Frankfurt/Main, G.F.R.).

Tissue

Tissue (10 g) was homogenized in methanol (50 ml) and centrifuged (2200 *g*, 5 min). The precipitate was re-suspended in methanol, shaken for 10 min and centrifuged again. To complete the precipitation of protein the combined supernatants were stored overnight at 4°. After centrifugation the methanol was evaporated *in vacuo* and the aqueous residue (*ca.* 2 ml) was dissolved in 0.1 *M* hydrochloric acid (30 ml) and extracted twice with diethyl ether (20 ml). The aqueous phase was saturated with sodium chloride and treated with a further 20 ml of diethyl ether. The ethereal solutions were discarded and the dibenzepine was isolated by extracting the aqueous layer twice with chloroform (30 ml).

For isolation of the metabolites II–VI the aqueous phase was made alkaline with ammonia solution and extracted three times with chloroform (30 ml). Both chloroform solutions, one containing the dibenzepine and the other the metabolites, were washed twice with 3% ammonia solution (20 ml). The solvent was evaporated under a stream of nitrogen and residual water was removed with benzene–ethanol (1:1).

For gas chromatography the residue containing the dibenzepine was dissolved in an appropriate amount of acetone, while the demethylated metabolites were treated with trifluoroacetic anhydride (0.5 ml) at room temperature for 45 min. The reagent was removed under a stream of nitrogen and for gas chromatography the residue was dissolved in acetone.

The gas chromatographic conditions were as follows: Perkin-Elmer F22 gas chromatograph equipped with a nitrogen-sensitive detector; glass columns, 200 × 0.3 cm; stationary phase, 3% OV-17 on Chromosorb W AW DMCS (80–100 mesh); carrier gas, nitrogen at a flow-rate of 40 ml/min; column temperature, 240°; injection port temperature, 270°; and detection port temperature, 290°. The retention times of the trifluoroacetyl derivatives were 3.8 min (III), 5.0 min (VI), 5.8 min (IV), 8.8 min (V) and 10.6 min (II), and that of dibenzepine was 7.2 min.

Bile

Bile (3 ml) was diluted with 3% ammonia solution (30 ml) and transferred on to a column (I.D. 1.2 cm) containing 12 ml of a slurry of Amberlite XAD-2 (particle size 300–1000 μm) in water. The solution was slowly percolated through the column (*ca.* 12 drops/min). The column was washed with water (40 ml), which was discarded, and subsequently eluted with 100 ml of methanol. After evaporation of the organic solvent *in vacuo*, the aqueous residue was dissolved in 0.1 *M* hydrochloric acid and extracted as described above.

Blood

Blood (10 ml) was diluted with 10% ammonia solution (10 ml) and subjected to column chromatography on Amberlite XAD-2; the extraction and gas chromatography stages were as described for bile.

Urine

Urine (10 ml) was diluted with 0.5 *M* hydrochloric acid (10 ml). Extraction and gas chromatographic examination were carried out as described above.

Gas chromatography–Mass spectrometry

The mass spectra were recorded with a Hewlett-Packard 5992 A gas chromatograph–mass spectrometer at 70 eV.

RESULTS AND DISCUSSION

In the procedure described here dibenzepine was isolated from sodium chloride-saturated aqueous solutions by ion-pair extraction with chloroform. By this means a simple separation of dibenzepine and its basic metabolites was achieved, as under these conditions the demethylated compounds II–VI remain in the aqueous phase. This

finding corresponds well with the distribution coefficients described previously for dibenzepine and its metabolites II, III and IV in 2 *M* hydrochloric acid, chloroform and diethyl ether[2]. This separation procedure prevented interference in the gas chromatography from the large amount of the unchanged drug and its metabolites IV and V, which, especially after an acute poisoning, are formed to only a relatively small extent. Further ,the extracts finally obtained were of excellent purity so that a gas chromatographic examination could be carried out without further purification. Investigations with known amounts of all six compounds added to drug-free tissue and body fluid specimens yielded recoveries between 70 and 90%.

As described previously, only dibenzepine and not its metabolites can be subjected directly to gas chromatography[9,10]. For derivatization of the demethylated compounds so far only acetylation has been used[2,5–7,9,10], but this proved to be unsatisfactory with respect to the quality of the separations and the long retention times[9,10]. Therefore, the gas chromatographic properties of the derivatives obtained after silylation, acetylation and trifluoroacetylation were systematically investigated on different stationary phases. As demonstrated by the gas chromatogram in Fig. 1 optimal results were obtained by trifluoroacetylation and subsequent separation of the compounds on silicone OV-17. Compared with the acetylated substances, especially the trifluoroacetyl derivatives of metabolites IV and VI exhibited significantly improved gas chromatographic properties. This result is due to the fact that, as demonstrated by the corresponding mass spectra, the dealkylated nitrogen atom in the ring system reacts with trifluoroacetic anhydride but not with acetic anhydride.

Interestingly, the derivatives of the metabolites III, IV and VI ($R^3 = COCF_3$)

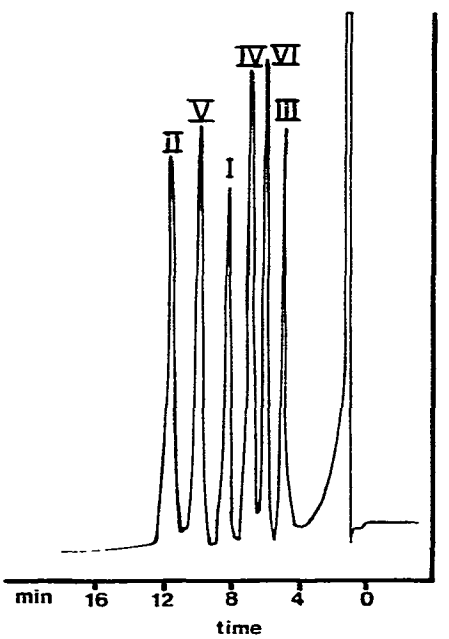
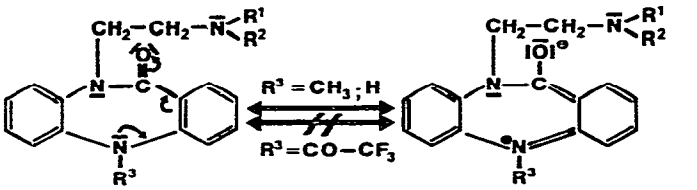


Fig. 1. Gas chromatogram of a mixture of dibenzepine and its metabolites II–VI after trifluoroacetylation. Silicone OV-17, 240°, N_2, 40 ml/min.

have shorter retention times than the methylated compounds ($R^3 = CH_3$). It is conceivable that the trifluoroacetyl substituent in the ring system reduces the electron mesomerism (Fig. 2), resulting in a decrease in the polarity and the retention time.

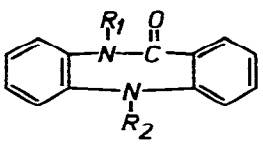
For elucidation of their structure, the trifluoroacetyl derivatives, which are described here for the first time, were subjected to gas chromatography–mass spectrometry. The structures and relative intensities of the ions obtained are given in Fig. 3.



	R^1	R^2	R^3
Dibenzepine I	CH_3	CH_3	CH_3
Metabolite II	H	CH_3	CH_3
Metabolite III	CH_3	CH_3	H
Metabolite IV	H	CH_3	H
Metabolite V	H	H	CH_3
Metabolite VI	H	H	H

Fig. 2. Structure and electron mesomerism of dibenzepine and its metabolites.

Structures



Fragment	R_1	R_2	m/e	Fragment	R_1	R_2	m/e
a	—— H ——		*209*	*g*	$-CH_2-$	$-COCF_3$	*319*
b	$-CH_2-$	$-CH_3$	*237*	*h*	$HN\overset{COCF_3}{\underset{CH_2CH_2}{}}$	O=C<	*376*
c	H	H	*210*	*i*	$-CH_2CH-$	O=C<	*263*
d	H	$-CH_3$	*224*	*j*	H	O=C<	*237*
e	$-CH_2\,CH-$	$-CH_3$	*250*	*k*	$-CH_2CH-$	$-COCF_3$	*332*
f	$-CH_2-$	H	*223*	*l*	H	$-COCF_3$	*306*

Intensities

Compound	Relative intensity of the fragment (%)												
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	M^+
II	*100*	*75*	*27*	*10*	*9*	*6*	–	–	–	–	–	–	*37*
III	–	–	–	–	–	–	–	–	–	*1,3*	–	–	*1,2*
IV	*3,5*	–	–	–	–	–	*100*	–	*73*	*25*	*43*	*14*	–
V	*100*	*83*	*28*	*12*	*4*	*14*	–	–	–	–	–	–	*55*
VI	*8*	–	–	–	–	–	*100*	*40*	*53*	*63*	*39*	*12*	*5,7*

Fig. 3. Structures and intensities of the fragments of the trifluoroacetyl derivatives.

Very similar spectra, especially with respect to the relative intensities, were obtained for those compounds which differ by only a methyl group in the side-chain nitrogen atom (II and V *versus* IV and VI). On the other hand, the relative intensities of the ions obtained by side-chain cleavage were influenced by the substituent on the ring nitrogen (II and V, $R^3 = CH_3$; IV and VI, $R^3 = COCF_3$). Compared with the mass spectra described previously for the acetylated compounds[10], the same ions resulting from the cleavage of the side-chain were observed but their relative intensities were different.

A completely different fragmentation pattern was obtained for the trifluoroacetyl derivative of metabolite III. Apart from the side-chain fragments $CH_2CH_2N(CH_3)_2$ (*m/e* 72; 100%) and $CH_2N(CH_3)_2$ (*m/e* 58; 100%), only one fragment containing the ring system was observed (j, 1.3%; Fig. 3).

The method presented here has been applied successfully to the determination of dibenzepine and its basic metabolites in blood, urine, bile and various tissues after fatal poisoning. A detection limit of *ca.* 300 ng/g has been achieved. The results of one typical investigation are given in Table I.

TABLE I

TISSUE CONCENTRATIONS OF DIBENZEPINE AND ITS METABOLITES AFTER FATAL POISONING

Compound	*Concentration (μg/g)*					
	Urine	*Liver*	*Brain*	*Kidney*	*Lung*	*Bile*
Dibenzepine	350	130	42	63	72	113
Metabolite II	180	120	8.3	28	30	80
Metabolite III	39	9	3.7	6.2	5.6	21
Metabolite IV	34	1	Trace*	3.2	1.9	36
Metabolite V	3	3.6	Trace	0.7	1.4	Trace
Metabolite VI	2	Trace	n.d.**	Trace	Trace	Trace

* Trace = concentration <0.3 μg/g.
** n.d. = not detectable.

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