Journal of Chromatography, 236 (1982) 157–168
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,370

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BENZOPHENONE DERIVATIVES FOR THE DETERMINATION OF BENZODIAZEPINES IN CLINICAL EMERGENCIES

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(First received June 22nd, 1981; revised manuscript received September 2nd, 1981)

SUMMARY

A screening procedure for fifteen 1,4-benzodiazepines in biological material (urine), suitable for clinical toxicology, is described. The benzodiazepines and/or their metabolites are hydrolysed to their benzophenones; after alkalinization, the benzophenones are extracted with chloroform. The extract is evaporated to dryness and the dried residue is dissolved in methanol and analysed by high-performance liquid chromatography on a reversed-phase column at 254 nm with methanol–water (1:1) as eluent.

INTRODUCTION

Benzodiazepines are widely used for their sedative and hypnotic activity and for anticonvulsant medication, and are responsible for considerable clinical demands in emergency toxicology. Although benzodiazepines are seldom responsible for fatal cases in toxicology, the danger is enhanced when they are taken in addition to other intoxicants such as alcohol, barbiturates or tricyclic antidepressants.

The analysis of benzodiazepines and their metabolites is often performed by gas–liquid chromatography (GLC)[1–12], mostly with electron-capture detection, high-performance liquid chromatography (HPLC)[13–18] and gas chromatography–mass spectrometry (GC–MS)[19–21]. These very specific and sensitive methods, focused on one or a few benzodiazepines, are very suitable for use in drug monitoring or when the intoxicant is known. In clinical toxicology, however, a less complex and less time-consuming procedure for screening a group of compounds can be very useful. From this point of view, the analysis of benzodiazepines as their hydrolysis products (benzophenones) is frequently used. The hydrolysis breaks down the benzodiazepines and their metabolites, both free and conjugated, to benzophenones, yielding an increased sensitivity. These benzophenones can be analysed by thin-layer chromatography[22–24], GLC[25] or GC–MS[26].

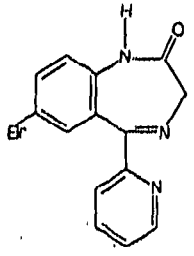
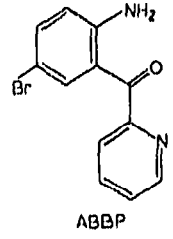
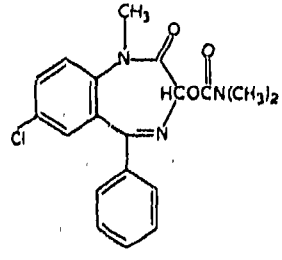
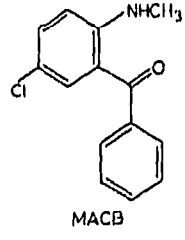
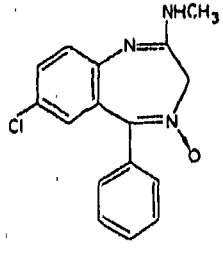
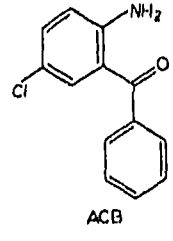
In the proposed method, benzodiazepines are analysed by the HPLC of their benzophenones, which are obtained by acid hydrolysis of biological samples. After

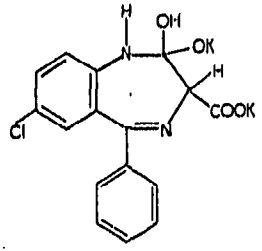
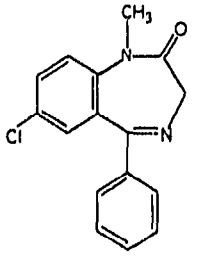
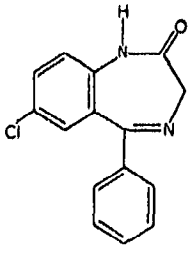
0021-9673/82/0000–0000/$02.75

TABLE I

BENZODIAZEPINES, THEIR METABOLITES AND THE RESPECTIVE BENZOPHENONES

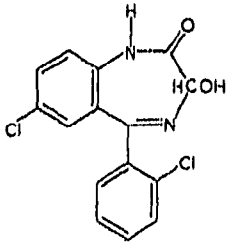
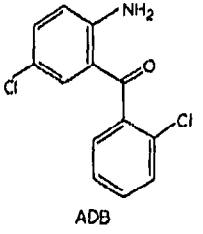
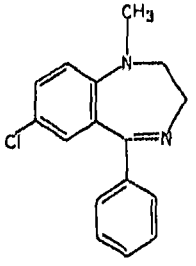
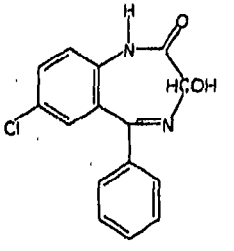
ABBP = 2-(2-Amino-5-bromobenzoyl)pyridine; MACB = 5-chloro-2-(methylamino)benzophenone; ACB = 2-amino-5-chlorobenzophenone; ADB = 2-amino-5-chloro-2′-chlorobenzophenone; ANB = 2-amino-5-nitrobenzophenone; DAB = 2,5-diaminobenzophenone; ANCB = 2-amino-2′-chloro-5-nitrobenzophenone; DACB = 2′-chloro-2,5-diaminobenzophenone; CACB = 5-chloro-2-(cyclopropylmethylamino)benzophenone; PACB = 5-chloro-2′-(propynylamino)benzophenone; ANFB = 2-amino-2′-fluoro-5-nitrobenzophenone; MANFB = 2′-fluoro-2-(methylamino)-5-nitrobenzophenone; MAAFB = 5-amino-2′-fluoro-2-(methylamino)benzophenone.

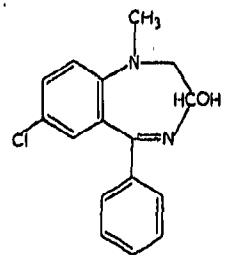
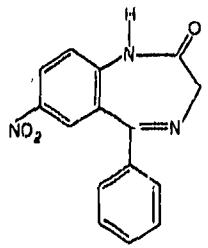
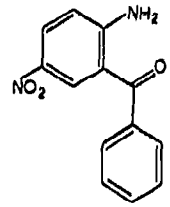
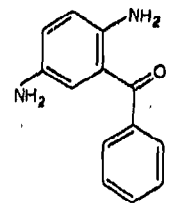
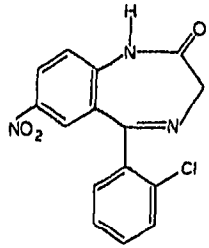
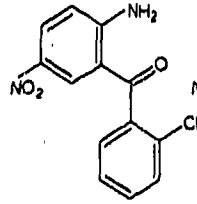
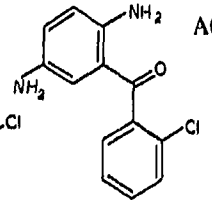
Name	*Formula*	*Urinary metabolites (free or conjugated)*	*Respective benzophenone(s)*	*Benzophenone(s) used*
Bromazepam (Lexotan)		3-Hydroxybromazepam 2-(2-Amino-5-bromobenzoyl)-pyridine 2-(2-Amino-5-bromo-3 hydroxy-benzoyl)pyridine	 ABBP	ABBP
Camazepam (Albego, Paxor)		N-Methyloxazepam	 MACB	MACB
Chlordiazepoxide (Librium)		Demethylchlordiazepoxide Demoxepam Nordiazepam	 ACB	ACB

Dipotassium chlorazepate (Belseren, Tranxène)		Nordiazepam Oxazepam	ACB	ACB
Diazepam (Valium)		Temazepam Nordiazepam Oxazepam	MACB, ACB	MACB, ACB
Demethyldiazepam (Madar)		Nordiazepam Oxazepam	ACB	ACB

(Continued on p. 160)

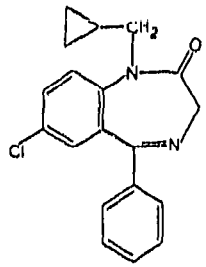
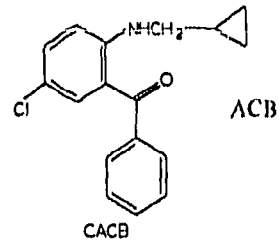
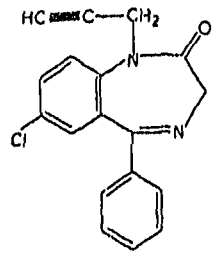
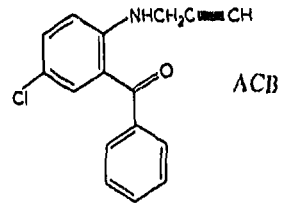
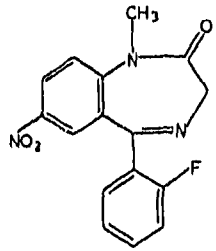
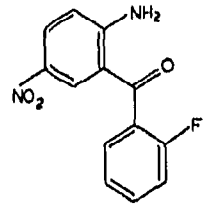
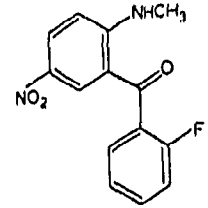
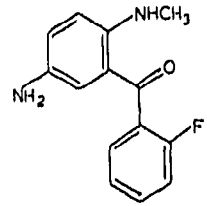
TABLE I *(continued)*

Name	*Formula*	*Urinary metabolites (free or conjugated)*	*Respective benzophenone(s)*	*Benzophenone(s) used*
Lorazepam (Temesta)		Almost not metabolized but excreted as glucuronide	 ADB	ADB
Medazepam (Nobrium)		Oxazepam (major metabolite) Diazepam Normedazepam Nordiazepam Dehydromedazepam	MACB, ACB	ACB, MACB
Oxazepam (Seresta)		Not metabolized but excreted as glucuronide	ACB	ACB

Temazepam (Levanxol)		Oxazepam Temazepam	MACB, ACB	ACB, MACB
Nitrazepam (Mogadon)		2-Amino-5-nitrobenzophenone 2-Amino-3-hydroxy-5-nitro-benzophenone 7-Aminonitrazepam 7-Acetamidonitrazepam	 ANB  DAB	ANB
Clonazepam (Rivotril)		7-Aminoclonazepam 7-Acetamidoclonazepam 3-Hydroxyclonazepam 7-Amino-3-hydroxyclonazepam	 ANCB  DACB	ACB

(Continued on p. 162)

TABLE I *(continued)*

Name	*Formula*	*Urinary metabolites (free or conjugated)*	*Respective benzophenone(s)*	*Benzophenone(s) used*
Prazepam (Lysanxia)		Nordiazepam Oxazepam	 ACB CACB	ACB
Pinazepam (Domar)		Nordiazepam Oxazepam (major metabolite) 3-Hydroxypinazepam	 ACB PACB	ACB
Flunitrazepam (Rohypnol)		Norflunitrazepam (major metabolite) Flunitrazepam 7-Aminoflunitrazepam 3-Hydroxyflunitrazepam	 ANFB  MANFB  MAAFB	ANFB, MANFB

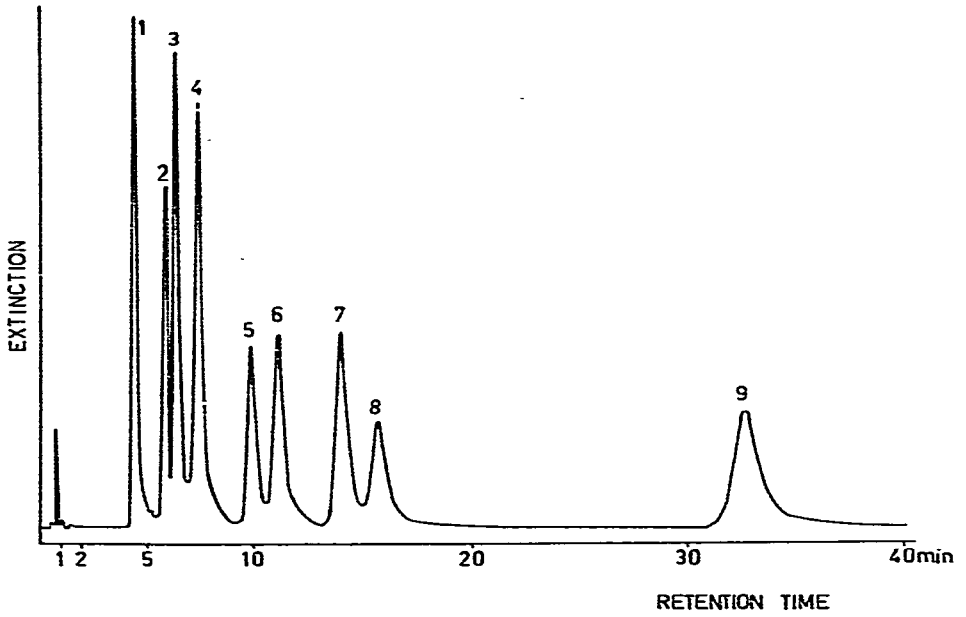


Fig. 1. Chromatogram of eight benzophenones and internal standard: 1 = ABBP; 2 = ANFB; 3 = ANB; 4 = ANCB; 5 = MANFB; 6 = camazepam; 7 = ACB; 8 = ADB; 9 = MACB.

alkalinization of the hydrolysate, the benzophenones are extracted with chloroform, the solution is evaporated to dryness, the residue is dissolved in methanol, an internal standard is added and HPLC is then performed, following a previously described method[27]. Identification and quantification of the benzophenones are performed by calculating relative retention times and relative peak heights. Hydrolysis and extraction yields were calculated for eight benzophenones derived from thirteen benzodiazepines.

EXPERIMENTAL

A survey of the benzodiazepines and their urinary metabolites[1,3,5,19,21,28–37], with their respective benzophenones, is given in Table I.

The eight benzophenones used (see Table I, last column) were analysed by HPLC. As described previously[27], the chromatogram permits the identification of methanolic solutions of the benzophenones (for the chromatogram of all of the benzophenones and the internal standard, see Fig. 1) from their relative retention times and peak heights, in the concentration range 0.1–1000 μg/ml.

The technique was applied to the analysis of urine.

Benzodiazepine and benzophenone standards

Benzodiazepine standards were obtained as follows: bromazepam, chlordiazepoxide, diazepam, medazepam, temazepam, desmethylflunitrazepam, flunitrazepam, nitrazepam and clonazepam from Roche (Brussels, Belgium); lorazepam and oxazepam from Wyeth (Münster, G.F.R.); camazepam from Sintesa (Milan, Italy);

dipotassium chlorazepate from Clin-Midy (Brussels, Belgium); and desmethyldiazepam from Willpharm (Brussels, Belgium).

Benzophenone standards were donated by Hoffmann-La Roche (Basle, Switzerland), except for ADB, which was synthesized[22].

A standard mixture of benzophenones (see Table I, last column), each at a concentration of 50 μg/ml in methanol, was prepared, and camazepam was added at the same concentration as an internal standard.

Hydrolysis and extraction

A 5-ml volume of sample (containing benzodiazepines and free or conjugated metabolites) was hydrolysed with concentrated hydrochloric acid for 15 min on a boiling water-bath. The hydrolysates were alkalinized to pH 12 with 30% sodium hydroxide solution. The samples were cooled and 10 ml of chloroform were added. After extraction for 5 min, the aqueous phase was aspirated. The organic layer was filtered through Whatman No. 1 phase-separating filters and evaporated to dryness. The residue was dissolved in 500 μl of methanol containing 25 μg of camazepam as an internal standard.

All reagents were of analytical-reagent grade from UCB.

Chromatographic conditions

The high-performance liquid chromatograph was a Hewlett-Packard Model 1081A with UV detection at 254 nm. The stationary phase was LiChrosorb Hibar (Merck, Darmstadt, G.F.R.) with a particle size of 7 μm, pre-packed in a 250 × 4 mm I.D. column. The mobile phase was methanol–water (1:1).

The column pressure was kept at 350 bar with a solvent flow-rate of 3.02 ml/min. The six-port valve loop injector was provided with a 10-μl loop. All work was carried out at ambient temperature.

RESULTS

Extraction of benzophenones

The percentage extractions of the benzophenones were determined by HPLC. Each benzophenone was extracted from water and blank urine, spiked with benzophenone (5 μg/ml) and the above extraction and HPLC procedure was carried out as described above. The results ($n = 15$, $\sigma = 0.05$) are given in Table II.

Hydrolysis yield of benzodiazepines to benzophenones

Knowing the percentage extraction, the hydrolysis yield to the benzophenone was determined for each benzodiazepine. Benzodiazepines were added to water and

TABLE II

EXTRACTION YIELDS (%) OF BENZOPHENONES FROM WATER AND URINE

Sample	*ABBP*	*MANFB*	*ANFB*	*MACB*	*ACB*	*ANB*	*ADB*	*ANCB*
Water	84	57	79	85	89	93	88	78
Urine	82	73	80	92	88	93	88	82

TABLE III

HYDROLYSIS YIELD OF BENZOPHENONES FROM BENZODIAZEPINES

Benzodiazepine	*Benzophenone*	*Hydrolysis yield (%)*	
		Water	*Urine*
Bromazepam	ABBP	42	45
Flunitrazepam	MANFB	81	78
Demethylflunitrazepam	ANFB	93	92
Diazepam	MACB	74	72
Temazepam	MACB	99	96
Camazepam	MACB	99	100
Medazepam	MACB	0	0
Oxazepam	ACB	83	78
Dipotassium chlorazepate	ACB	30	28
N-Demethyldiazepam	ACB	94	98
Chlordiazepoxide	ACB	60	72
Nitrazepam	ANB	93	96
Lorazepam	ADB	65	61
Clonazepam	ANCB	76	65

blank urine samples in the following concentrations: (mol.wt. benzodiazepine/mol.wt. benzophenone)·5 μg/ml. The above-described procedure was then performed. The results ($n = 15$, $\sigma = 0.06$) are given in Table III.

Using the hydrolysis and extraction yields in water, standard benzophenones for HPLC can be prepared from benzodiazepines and can be used in routine analysis.

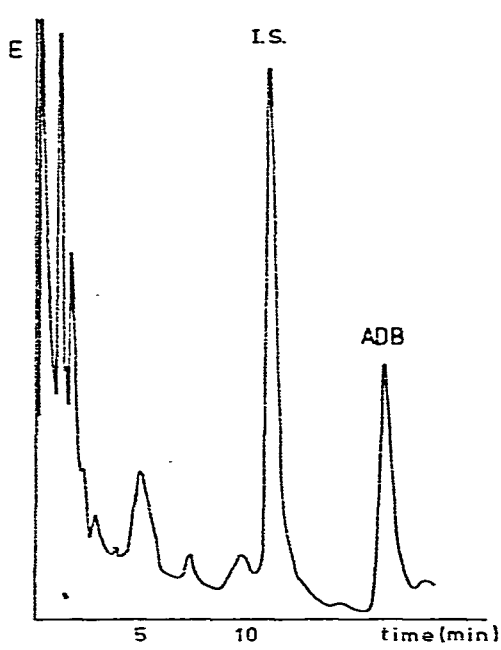
Analysis of benzodiazepines in urine

Fig. 2–6 are examples of chromatograms of benzophenones obtained on applying the described procedure to urine samples. Knowing the percentage extraction and hydrolysis yield, the amount of benzodiazepine equivalent to the amount of benzophenone can be calculated.

The sensitivity of the method, when the screening is performed as described above, is 0.01 μg/ml of benzophenone in urine. When a single dose of 1 mg of benzodiazepine (which is a low dose) is ingested, and assuming that 5% of the dose (minimal value, *i.e.*, for chlorazepate) is excreted in the urine, the benzophenone concentration will be 0.03 μg/ml in 24-h urine. If necessary, the sensitivity can be increased by dissolving the residue in less methanol or by increasing the sample size.

DISCUSSION

In practice, all of the 15 benzodiazepines and/or their metabolites (Table I) yield one or two of the eight benzophenones in acceptable amounts: bromazepam (ABBP), camazepam (MACB), chlordiazepoxide (ACB), dipotassium chlorazepate (ACB), demethyldiazepam (ACB), diazepam (ACB, MACB), lorazepam (ADB), oxazepam (ACB) and temazepam (ACB, MACB) are totally recovered as their benzophenones.



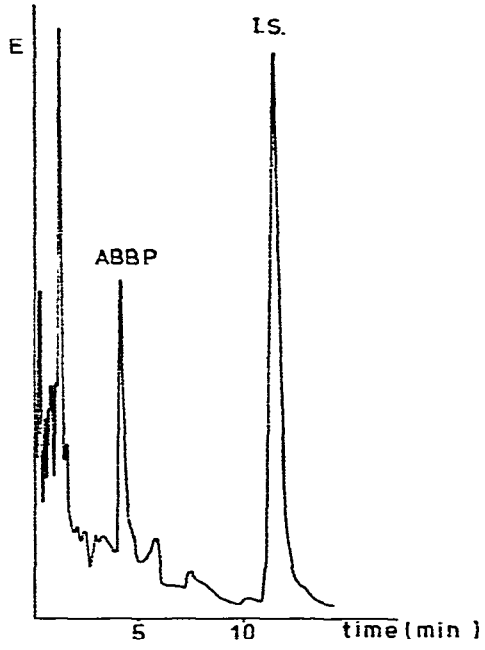
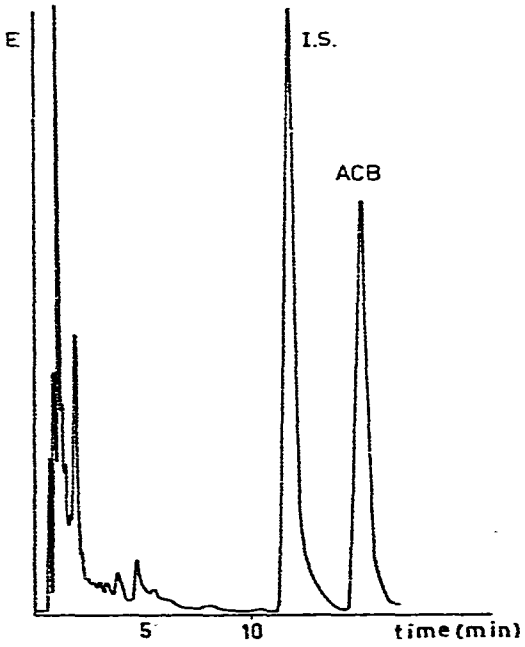


Fig. 2. Chromatogram of urine extract of ADB from a patient taking lorazepam (4 μg/ml of lorazepam in urine).

Fig. 3. Chromatogram of urine extract of ABBP from a patient taking bromazepam (1.2 μg/ml of bromazepam in urine).



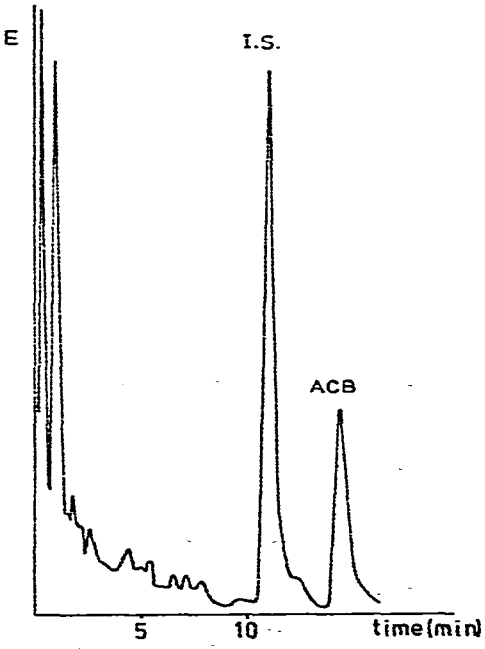


Fig. 4. Chromatogram of urine extract of ACB from a patient taking oxazepam (10 μg/ml of oxazepam in urine).

Fig. 5. Chromatogram of urine extract of ACB from a patient after ingestion of dipotassium chlorazepate (6.4 μg/ml of dipotassium chlorazepate in urine).

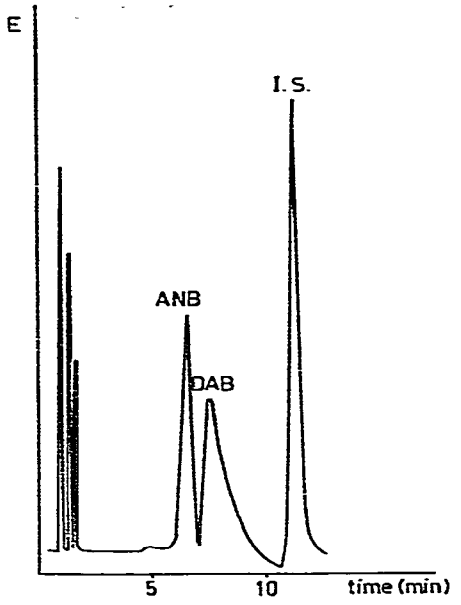


Fig. 6. Chromatogram of a urine extract of ANB and DAB after ingestion of nitrazepam.

Although medazepam cannot be hydrolysed, it is recovered in urine via its metabolization products, diazepam, nordiazepam and, as a major metabolite, oxazepam (MACB, ACB).

Flunitrazepam is quantitated via its own benzophenone MANFB and via ANFB, the benzophenone of the main metabolite.

Nitrazepam and its metabolites yield two benzophenones. ANB and DAB, from which only ANB is quantitated. DAB under these conditions gives too much tailing (see Fig. 6), and can be used only for qualitative purposes. Clonazepam is recovered as ANCB. The other benzophenone, DACB, does not chromatograph under these conditions. As pinazepam and prazepam are readily metabolized to their N-demethyl derivatives, these products can also be quantitated via the benzophenone ACB.

For the specific benzophenones ADB, ABBP, ANB, ANCB, MANFB and ANFB results of analysis in urine can be expressed in μg/ml of lorazepam, bromazepam, nitrazepam, clonazepam and flunitrazepam, respectively. When ACB and MACB are found the results are expressed in equivalents of diazepam; and when ACB is found, in equivalents of demethyldiazepam. If further identification and quantification of the unhydrolysed benzodiazepines and metabolites should be necessary, a supplementary procedure can follow the HPLC, *e.g.*, GLC with electron-capture detection[6]. The analysis time in routine application for total screening is 1 h, according to the retention time of the last eluting peak (MACB). As in some instances the amount of benzodiazepines might not be totally recovered (owing to non-detection of DACB, non-quantification of DAB or imperfect hydrolysis of metabolites), the method provides a semi-quantitative analysis of the benzodiazepines. As the benzodiazepines are analysed as their benzophenone derivatives, there is a loss of

specificity but a gain in sensitivity as benzodiazepines themselves and their metabolites, free or conjugated, yield the same benzophenone(s). This possible decrease in specificity can be offset against the possibility of screening all of these benzodiazepines simultaneously in a reasonable time.

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