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Note

Screening procedure for therapeutic benzodiazepines by high-performance liquid chromatography of their benzophenones

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Benzodiazepines can be analysed either as the compounds themselves and their metabolites, or as their hydrolysis products, *i.e.*, benzophenones.

The analysis of benzodiazepines and their metabolites is well known, but most studies have been focused on one or a few compounds. The most commonly used technique is gas–liquid chromatography, preferably with an electron-capture detector, as the molecules involved are electronegative[1–12]. High-performance liquid chromatography (HPLC) has been used for the same purpose[13–18]. These methods are specific and sensitive but, because of their complexity (extraction at specific pH, derivatization, variable chromatographic conditions), they are not easily applicable in screening tests.

On the other hand, benzodiazepines are often analysed as benzophenones after acid hydrolysis. With biological materials the sensitivity of detection is increased after hydrolysis as a benzodiazepine and its corresponding metabolites yield the same benzophenone(s). Separation of the benzophenones is performed by thin-layer chromatography (TLC) and they are detected under UV light or with Bratton–Marshall reagent if a primary amine is present[19–21]. Quantification is not possible, however.

HPLC is the method of choice for screening benzodiazepines as their benzophenones: the separation is more reliable than with TLC and the benzophenones are simultaneously quantified. The analysis consists simply of acid hydrolysis of the sample, followed by a single injection of the solution after addition of an internal standard. Identification and quantification are performed by calculating relative retention times and relative peak heights.

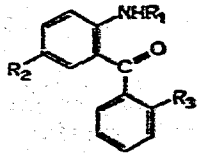
EXPERIMENTAL

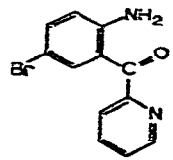
Materials

The apparatus consisted of a Hewlett-Packard liquid chromatograph with a built-in fixed-wavelength UV detector (254 nm). Hibar column pre-packed with LiChrosorb RP-8 of particle diameter of 7 μm was used. This was selected after examining RP-3, RP-8 and RP-18 columns; a particle diameter of 7 μm gave better results than 10 μm. The benzophenones listed in Table I were obtained from La Roche (Basle, Switzerland), except for ADB, which was synthesized[15]. Camazepam was provided by Sintesa (Milan, Italy).

TABLE I

BENZOPHENONES EXAMINED



R_1	R_2	R_3	*Abbreviation*	*Systematic name*
CH_3	Cl	H	MACB	5-Chloro-2-(methylamino)benzophenone
H	Cl	H	ACB	2-Amino-5-chlorobenzophenone
H	Cl	Cl	ADB	2-Amino-5-chloro-2′-chlorobenzophenone
CH_3	NO_2	F	MANFB	2′-Fluoro-2-(methylamino)-5-nitrobenzophenone
H	NO_2	F	ANFB	2-Amino-2′-fluoro-5-nitrobenzophenone
H	NO_2	H	ANB	2-Amino-5-nitrobenzophenone
NH_2	NO_2	Cl	ANCB	2-Amino-2′-chloro-5-nitrobenzophenone
			ABBP	2-(2-Amino-5-bromobenzoyl)pyridine

Chromatographic conditions

The mobile phase was methanol–water (1:1) and the column pressure was kept at 350 bar with a solvent flow-rate of 3.02 ml/min. A six-port valve loop injector with a 10-μl loop was used. The recorder speed was 38 cm/h. All work was carried out at ambient temperature.

Standard mixture

A standard mixture was prepared from the eight benzophenones listed in Table I (derived from the hydrolysis products of the thirteen 1,4-benzodiazepines

TABLE II

BENZODIAZEPINES AND THEIR BENZOPHENONE HYDROLYSIS PRODUCTS

Benzodiazepine	*Benzophenone**
Bromazepam (Lexotan)	ABBP
Camazepam (Albego, Paxor)	MACB, ACB
Chlordiazepoxide (Librium)	ACB
Di-K-Chlorazepate (Belseren, Tranxene)	ACB
Demethyldiazepam (Madar)	ACB
Diazepam (Valium)	MACB, ACB
Lorazepam (Temesta)	ADB
Medazepam (Nobrium)	MACB, ACB
Oxazepam (Seresta)	ACB
Temazepam (Levanxol)	MACB, ACB
Flunitrazepam (Rohypnol)	MANFB, ANFB
Nitrazepam (Mogadon)	ANB
Clonazepam (Rivotril)	ANCB

* For abbreviations, see Table I.

listed in Table II), each at a concentration of 50 μg/ml in methanol. Camazepam was added as the internal standard at the same concentration.

RESULTS

Fig. 1 shows a chromatogram, obtained under the above conditions, of the standard mixture containing the eight benzophenones and the internal standard. Retention times relative to camazepam (with their standard deviations) were calculated. The absolute retention time of camazepam is about 11 min 20 sec.

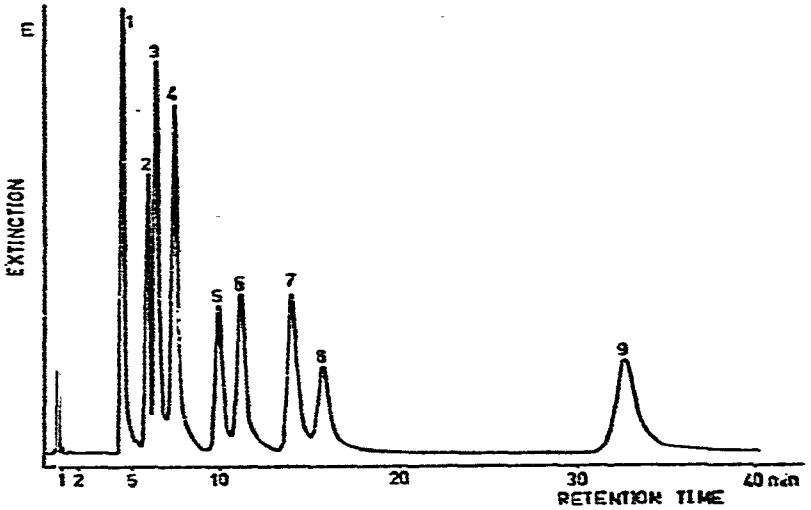


Fig. 1. Chromatogram of the standard mixture. Peaks: 1 = ABBP; 2 = ANFB; 3 = ANB; 4 = ANCB; 5 = MANFB; 6 = camazepam (internal standard); 7 = ACB; 8 = ADB; 9 = MACB.

The peak-height ratio of benzophenone to internal standard was used for quantification. Relative peak heights were calculated (with their standard deviations).

The results are given in Table III. For quantitative work a standard deviation of less than 2.5% for each of the benzophenones is obtained.

TABLE III

RELATIVE RETENTION TIMES (RR_t) AND RELATIVE PEAK HEIGHTS (R_h) WITH THEIR STANDARD DEVIATIONS (σ)

Compound	RR_t	σ_{RR_t} ($n = 31$)	R_h	σ_{R_h} ($n = 16$)
ABBP	0.399	0.004	2.725	0.032
ANFB	0.532	0.004	1.694	0.033
ANB	0.573	0.006	2.380	0.042
ANCB	0.668	0.006	2.088	0.044
MANFB	0.893	0.008	0.923	0.009
Camazepam	1		1	
ACB	1.265	0.009	0.975	0.024
ADB	1.421	0.010	0.533	0.012
MACB	2.958	0.027	0.566	0.012

Linearity of the detector response was tested for ABBP and camazepam (0.1–1000 μg/ml). The correlation coefficient of the linear regression between absorption and concentration was 0.994 for both products. Quantification over the whole concentration range was possible.

CONCLUSION

Eight benzophenones of thirteen 1,4-benzodiazepines could be separated and identified with the use of an internal standard. The products could be quantified by means of their relative peak heights with a standard deviation of less than 2.5%. Although the identification of a compound is the first requirement in toxicology, quantification is of particular interest for monitoring purposes, as the benzodiazepines are used not only as tranquillizers and hypnotics but also as anticonvulsants. The proposed screening method identifies and quantifies them simply and rapidly.

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