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ANALYSIS OF BENZODIAZEPINES

II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–FLUORESCENCE DETECTION AFTER MOLECULAR REARRANGEMENT TO ACRIDANONES

N. DE GIOVANNI* and M. CHIAROTTI

Istituto di Medicina Legale e delle Assicurazioni, Università Cattolica del Sacro Cuore, L go F. Vito 1, 00168 Rome (Italy)

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SUMMARY

A molecular rearrangement of benzophenones (hydrolysis products of 1,4-benzodiazepines) to 9-acridones has been studied. The compounds synthesized were analysed by high-performance liquid chromatography coupled with a fluorescence detector, because of their high luminescence. The method, which can detect sixteen benzodiazepines simultaneously, is highly sensitive and adequately specific. It is reliable for the analysis of these compounds in biological samples at therapeutic concentrations.

INTRODUCTION

The determination of benzodiazepines in biological fluids requires specific and sensitive techniques that can detect not only the parent drugs but also their metabolites, which are often pharmacologically active.

Numerous methods have been published based principally on gas chromatography with electron-capture detection (GC–ECD) [1–3], high-performance liquid chromatography (HPLC) [4,5] and immunochemical techniques employed as toxicological screening methods [6,7].

Fluorescence determination of some benzodiazepines has also been studied: these compounds require derivatization because of their low native fluorescence [8–10]. The high sensitivity of these methods is not generally matched by the specificity because of interferences due to endogenous and/or exogenous compounds; moreover, only a few molecules could be derivatized with each method.

In some cases [11–13] fluorescent compounds were formed after molecular

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rearrangement of benzophenones (easily formed from 1,4-benzodiazepines after acid hydrolysis) to acridone derivatives.

Weijers-Everhard et al. [14] used this rearrangement for flunitrazepam only, increasing the specificity of the method by using liquid chromatographic separation.

In a previous paper [15] we reported a study of different chromatographic techniques for the identification of benzodiazepines without derivatization, with very high specificity but at the expense of sensitivity when applied to biological specimens. The present paper describes a new toxicological screening technique with higher sensitivity for the quantitation of benzodiazepines at therapeutic concentrations in biological fluids. We re-worked the cyclization of benzophenones to acridones: our reaction conditions yielded the rearrangement of sixteen molecules. The proposed structure for the acridones was confirmed by published data [12,13], by chromatographic retention times, fluorescence spectroscopy and mass spectrometry. The subsequent analysis performed by HPLC with fluorimetric detection allowed the quantitation of the benzodiazepines with high sensitivity and adequate specificity in biological fluids.

EXPERIMENTAL

Standards and chemicals

Pure chemical standards of chlordiazepoxide, clonazepam, nordiazepam, diazepam, flunitrazepam, flurazepam, lorazepam, lormetazepam, nitrazepam, oxazepam, prazepam and temazepam were obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Camazepam, 2′-chloronordiazepam, clorazepate and pinazepam were obtained from commercial pharmaceutical products by methanolic extraction. 2-Amino-5-chlorobenzophenone, 2-methylamino-5-chlorobenzophenone, 2-amino-5-nitrobenzophenone, 2-amino-5,2′-dichlorobenzophenone and 2-aminobenzophenone were obtained from Fabbrica Italiana Sintetici (Alte di Montecchio Maggiore, Italy). Other benzophenones were obtained by acid hydrolysis of the parent 1,4-benzodiazepines according to Berry and Grove [16].

All chemicals and reagents were of analytical grade.

Apparatus

A Perkin-Elmer 3B liquid chromatograph equipped with an LS-2 filter fluorimeter was employed. The chromatographic separation was performed in the reversed-phase mode using a Perkin-Elmer 5-μm Bondapak C_8 column (12 cm long) (Waters Assoc., Milford, MA, U.S.A.) using acetonitrile–1 mM acetate buffer pH 4.0 (1:1) as the mobile phase. The fluorimetric detector was set at 260 nm (excitation) and 430 nm (emission).

Mass spectra were obtained with a Finnigan ion trap detector interfaced to a Carlo Erba Fractovap HRGC 5300 gas chromatograph. A 30-m glass capillary column with a 0.3-μm film of OV 101 stationary phase with a temperature programme set as follows was used: initial hold for 2 min at 60°C, then to 160°C at 40°C/min and to 260°C at 10°C/min, then hold at 260°C.

Extraction and derivatization

The 0.5–1 ml samples of biological fluids (urine or serum) were enzymatically hydrolysed with 100 μl of β-glucuronidase (100 000 Fishman units) for 5 h at 37°C [17]. Each sample was then pre-extracted with RP-18 Baker 10SPE octadecyl microcolumn (Baker, Deventer, Holland), prewashed with 2 ml of methanol and 2 ml of water. The retained benzodiazepines were eluted with 0.5 ml of methanol and dried to a residue, which was hydrolysed in 6 *M* hydrochloric acid at 100°C for 60 min. After diethyl ether extraction, the benzophenones were cyclized to 9-acridanones with 1 ml of 2 *M* sodium hydroxide and 20 mg of lead dioxide (as catalyst), or with 1 ml of dimethyl sulphoxide (DMSO) and 20 mg of lead dioxide. The reaction was performed at 120°C for 60 min when sodium hydroxide was used, or for 90 min with DMSO. The reaction mixture was extracted twice with ethyl acetate (after alkalinization when DMSO was used), and the organic layer was evaporated to dryness; the residue, reconstituted in 1 ml of acetonitrile, was then injected for HPLC analysis.

The internal standard (50 ng/ml 2-aminobenzophenone) was added immediately before the hydrolysis.

RESULTS AND DISCUSSION

The reactions by which 1,4-benzodiazepines are converted into acridanone derivatives are shown in Fig. 1. The first step is an acid hydrolysis that yields the corresponding benzophenones (ten different benzophenones are formed from the sixteen benzodiazepines considered); the second reaction is an internal nucleophilic aromatic substitution, in which the amino group is the nucleophile and the leaving group is represented by the R_2' group (halogen) or the hydrogen atom in the 6′-position.

The displacement of the leaving group is encouraged by the nucleophilic characteristics of the amino group (enhanced by the electron-withdrawing effect of the carbonyl group), by the presence of chlorine in the *para* position with respect to the amino group (activating mesomeric effect) and by the type of solvent and

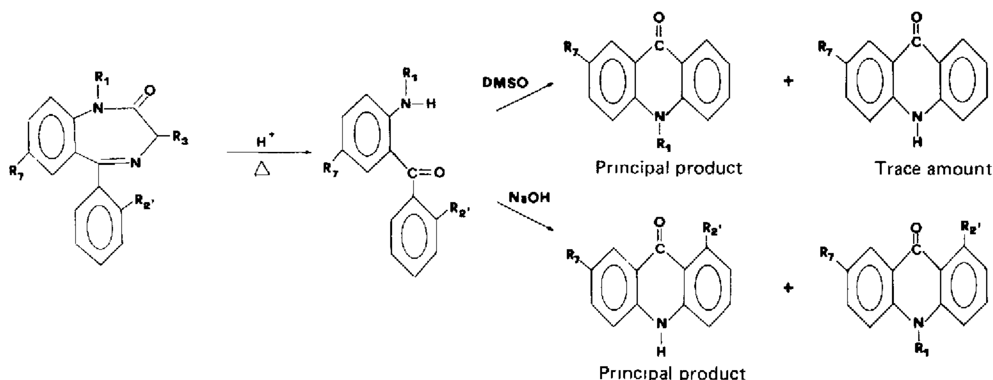


Fig. 1. Chemical reactions of 1,4-benzodiazepines to yield fluorescent acridanone derivatives.

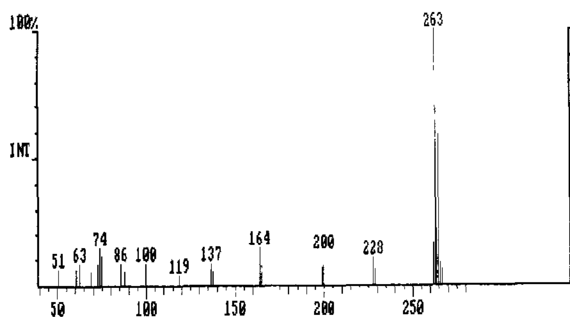
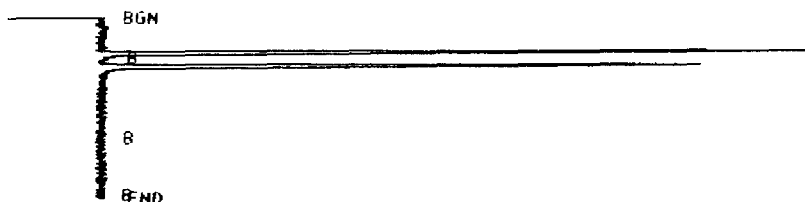


Fig. 2. Mass spectrum of the acridanone synthesized from lorazepam in sodium hydroxide (1,7-dichloro-9-acridanone).

INST 1 METH 1 FILE 12

RUN 1 DIAZEPAM ACRIDANONE 115.5 NG/ML PREP IN NaOH

SENSITIVITIES 1023 255



INST 1 METH 1 FILE 10

RUN 1 DIAZEPAM ACR 115.5 NG/ML PREP IN DMSO

SENSITIVITIES 1023 255

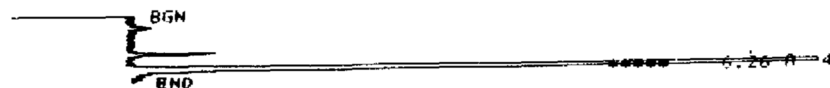
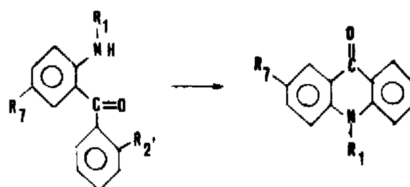


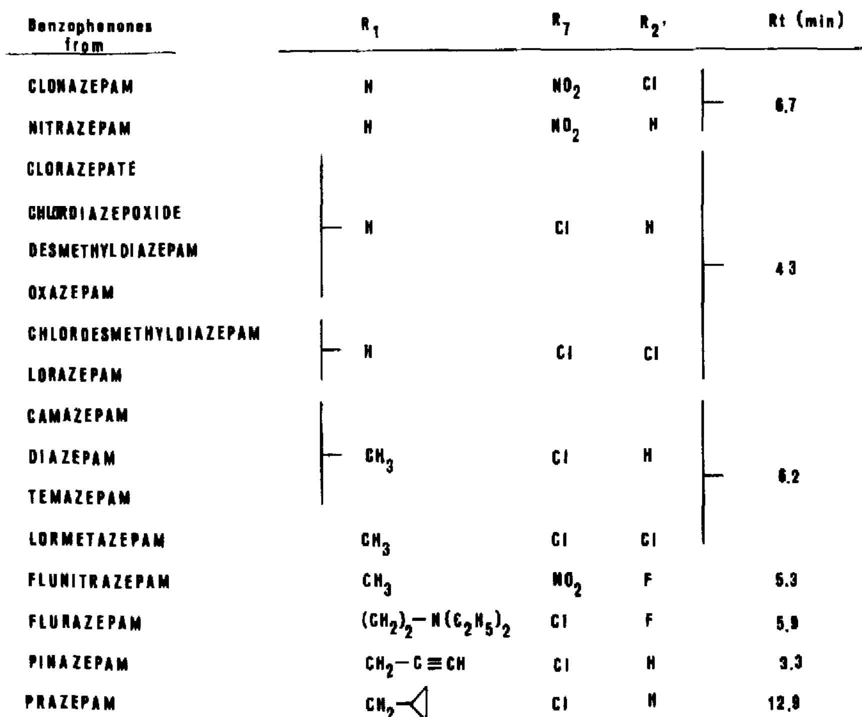
Fig. 3. Influence of the solvent on the cyclization: only one major product is formed when the benzophenone formed from diazepam is cyclized in DMSO (lower trace); the specificity decreases when sodium hydroxide is used because an N-dealkylation reaction also occurs (upper trace).

catalyst used [18]. In fact, when sodium hydroxide is used as solvent, the hydrogen is the preferred leaving group so the principal cyclization products still contain the halogen. DMSO, on the other hand, yields a principal product in which the halogen at R_2' is eliminated. Lorazepam benzophenone cyclizes with the loss of the hydrogen even when sodium hydroxide is used (the mass spectrum of this product is shown in Fig. 2), whereas DMSO encourages the substitution of the halogen to yield the same compound synthesized from oxazepam (2-chloro-9-acridanone).

Moreover, when the only leaving group present in the molecule is a hydrogen, the use of sodium hydroxide is necessary to achieve a suitable yield for analytical

DMSO





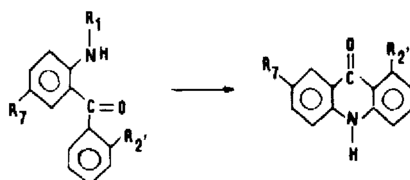
Benzophenones from	R_1	R_7	R_2'	Rt (min)
CLONAZEPAM	H	NO_2	Cl	6.7
NITRAZEPAM	H	NO_2	H	
CLORAZEPATE				
CHLORDIAZEPOXIDE	H	Cl	H	
DESMETHYLDIAZEPAM				4.3
OXAZEPAM				
CHLORODESMETHYLDIAZEPAM	H	Cl	Cl	
LORAZEPAM				
CAMAZEPAM				
DIAZEPAM	CH_3	Cl	H	6.2
TEMAZEPAM				
LORMETAZEPAM	CH_3	Cl	Cl	
FLUNITRAZEPAM	CH_3	NO_2	F	5.3
FLURAZEPAM	$(CH_2)_2-N(C_2H_5)_2$	Cl	F	5.9
PINAZEPAM	$CH_2-C \equiv CH$	Cl	H	3.3
PRAZEPAM	CH_2-cyclopropyl	Cl	H	12.9

Fig. 4. Acridanones synthesized from sixteen benzodiazepines using DMSO as cyclization solvent.

purposes: all benzodiazepines examined were able to react. In this case, however, a secondary reaction (N-dealkylation) could interfere, affecting the specificity (Fig. 1) and yielding a dealkylated by-product in larger amount, although the two products can be easily chromatographically resolved. Fig. 3 shows column liquid chromatograms of diazepam acridanone prepared in sodium hydroxide and DMSO: the latter solvent gives one major product, whereas sodium hydroxide gives a mixture.

The use of DMSO allows the cyclization of 2′-halobenzophenones and 7-nitrobenzophenones with high specificity (the N-dealkylation takes place in negligible yield). The formation of different acridones from all sixteen benzodiazepines examined, using the two solvents, are summarized in Figs. 4 and 5, respectively. As

Na OH



	R_1	R_7	$R_{2'}$	Rt (min)
CLONAZEPAM	H	NO_2	Cl	9.5
CLORAZEPATE				
CHLORDIAZEPOXIDE	H	Cl	H	
DESMETHYLDIAZEPAM				
OXAZEPAM				
CAMAZEPAM				4.3
DIAZEPAM	CH_3	Cl	H	
TEMAZEPAM				
PINAZEPAM	$CH_2-C\equiv CH$	Cl	H	
PRAZEPAM	CH_2–cyclopropyl	Cl	H	
CHLORDESMETHYLDIAZEPAM	H	Cl	Cl	
LORAZEPAM				7.1
LORMETAZEPAM	CH_3	Cl	Cl	
FLUNITRAZEPAM	CH_3	NO_2	F	8.2
FLURAZEPAM	$(CH_2)_2-N(C_2H_5)_2$	Cl	F	6.4
NITRAZEPAM	H	NO_2	H	6.7

Fig. 5. Acridanones synthesized from sixteen benzodiazepines using sodium hydroxide as cyclization solvent.

INST 1 METH 1 FILE 6

RUN 1

SENSITIVITIES 1000 200

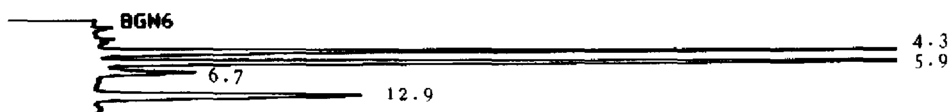


Fig 6. HPLC pattern of acridanones prepared from clonazepam, flurazepam, oxazepam and prazepam, when DMSO is used.

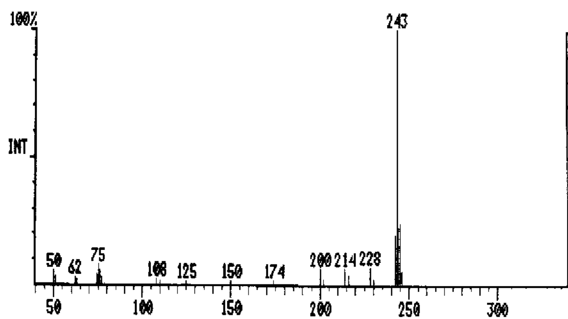


Fig. 7. Mass spectrum of 2-chloro-10-methyl-9-acridanone.

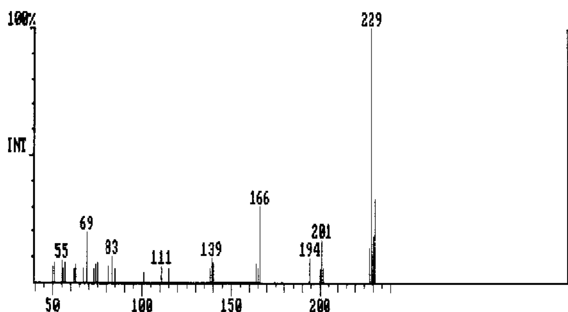


Fig. 8. Mass spectrum of 2-chloro-9-acridanone prepared from oxazepam.

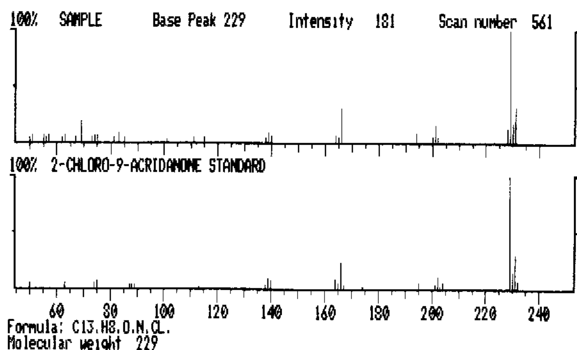


Fig. 9. Mass spectrum of 2-chloro-9-acridanone prepared from oxazepam, compared with a standard.

can be seen, the acid hydrolysis allows the formation of ten different benzophenones; these compounds give six different acridones when sodium hydroxide is used and seven with DMSO.

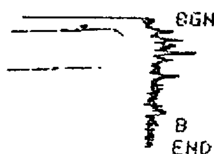
The retention times for the acridanones are different in the two solvents because the major products are significantly different. Fig. 6 shows the chromatographic pattern of the acridanones formed from clonazepam, flurazepam, oxazepam and prazepam, synthesized using DMSO. There is clearly the possibility of analysing them at the same time with complete separation.

Excitation/emission spectral characteristics, when compared with those reported in the literature [12,19], confirmed that the reaction products were mainly the acridones. Moreover, the mass spectrum of each compound was consistent

INST 1 METH 1 FILE 11

RUN 1 BLANK URINE EXTRACT N 5

SENSITIVITIES 1023 255



INST 1 METH 2 FILE 16

RUN 1 URINE SPIKED WITH OXAZEPAN

SENSITIVITIES 1023 255

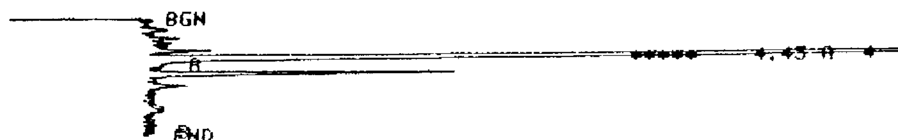


Fig. 10. HPLC patterns of blank urine (above) and blank urine spiked with oxazepam (below), determined as the acridanone derivative.

with the empirical formula for the corresponding acridanone. The reaction mixture obtained from diazepam benzophenone cyclized in sodium hydroxide, analysed by gas chromatography–mass spectrometry, showed several peaks, one of which indicated a mass spectrum corresponding to the benzophenone (2-methylamino-5-chlorobenzophenone), another one belonging to the dealkylated benzophenone (2-amino-5-chlorobenzophenone), one peak attributable to the alkylated acridanone that shows an m/z 243 fragment, corresponding to its molecular ion (Fig. 7) and the dealkylated one with an m/z 229 fragment (Fig. 8). In Fig. 9 the dealkylated acridanone mass spectrum is compared with a standard.

The reaction studied was then applied to biological material. The chromatographic pattern obtained from drug-free urine specimens spiked with therapeutic amounts of oxazepam is shown in Fig. 10. The upper part of the figure shows that a very clean extract is obtained under these conditions, allowing the quantitation of all the substances examined.

To obtain a clean extract, a pre-extraction step was necessary; its yield has been calculated to be higher than 95%, so it does not affect the final yield. The acid hydrolysis allowed a recovery of 70%; the yield of the cyclization step could not be calculated, owing to the lack of authentic acridanone standards. However, the recovery was sufficient to permit the determination of nanogram amounts of benzodiazepines.

Since the acridanones are intrinsically highly fluorescent, the present method permitted the detection of benzodiazepines at therapeutic concentrations. The detection limit (defined as twice the peak-to-peak value of the noise) was 2 ng/

ml of urine for oxazepam, chlordiazepoxide, clorazepate, nordiazepam, diazepam, lorazepam, pinazepam, prazepam, camazepam, temazepam and 2′,5-dichloronordiazepam, 50 ng/ml for clonazepam, flunitrazepam, flurazepam and lormetazepam and 500 ng/ml for nitrazepam.

The reproducibility is very high, with a coefficient of variation (C.V.) of less than 5%, and the calibration line is linear over the investigated concentration range (2–500 ng/ml) with a mean C.V. of 4.2%. For example, calibration curves for diazepam and oxazepam gave a correlation coefficient of 0.9994 and 0.9915, respectively.

The total chromatographic analysis allows complete separation of all acridanones synthesized in ca. 15 min thus ensuring adequate specificity (Fig. 6). We suggest the use of this method because of its very high sensitivity, which makes it preferable to other techniques that employ HPLC. Moreover, it requires less manipulation of biological samples than GC–ECD.

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