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CAPILLARY GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF SOME BENZODIAZEPINES AND THEIR UNCONJUGATED METABOLITES IN PLASMA

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SUMMARY

A gas chromatographic–mass spectrometric method for the identification and/or quantification of diazepam, clobazam, flunitrazepam, triazolam, midazolam, oxazepam and lorazepam and some of their desmethylated and hydroxylated metabolites in plasma is described. Benzodiazepines were extracted from plasma with butyl acetate at pH 9; the hydroxylated compounds were then silylated with N,O-bis(trimethylsilyltrifluoroacetamide). Analysis was performed using a compact mass-selective detector operating in the electron-impact mode. Depending on the concentration, identification was performed either by direct comparison of the observed mass spectra with reference spectra or by the relative intensities of the most intense and characteristic ions in the selected-ion monitoring (SIM) mode. Quantification was performed in the SIM mode using the most intense ion. The intra-assay precision and accuracy were better than 5–6%; linearity was satisfactory up to 1–2 μg/ml. The detection limit was 1–5 ng/ml for most of the benzodiazepines. This method can be easily used in clinical situations when a safe and rapid response is essential for patient treatment.

INTRODUCTION

Benzodiazepines (BZDs), an important class of pharmacologically active drugs, are frequently used in clinical practice as tranquillizers, sleep inducers, anti-epileptic and muscle-relaxant drugs [1,2]. Many clinical situations, such as pharmacokinetic studies, therapeutic monitoring, dosage regimens and identification of compounds in overdose cases (suicide attempts) require the development of an adequate methodology.

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Many quantification methods have been described. Various techniques have been used, e.g., thin-layer chromatography (TLC) [3,4], gas chromatography (GC) [5,6] and high-performance liquid chromatography (HPLC) [7,8]. Despite their selectivity, these methods, which identify unknown compounds only by their retention times, cannot solve unequivocally some identification problems. Therefore, combined gas chromatography–mass spectrometry (GC–MS), allowing the identification of a compound by its characteristic ion(s), is nowadays an essential tool [9,10].

The aim of this study was to develop a simple and reliable GC–MS method for the identification and/or quantification in plasma of some of the most commonly used BZDs, namely, diazepam, clobazam, flunitrazepam, triazolam, midazolam, oxazepam and lorazepam, and their main desmethylated or hydroxylated metabolites, N-desmethyldiazepam, N-desmethylclobazam, N-desmethylflunitrazepam and temazepam.

In most studies, BZDs were converted into benzophenones by acid hydrolysis before GC analysis [11,12], but in the last few years the chromatography of intact BZDs has been considered [5,6,13–16]. This leads to higher specificity (different BZDs could give the same benzophenone), improved reproducibility, easier manipulation and shorter analysis times. The method described here was based on a simple extraction of these drugs from plasma without acid hydrolysis. Further, this method was fully validated by classical linearity, reproducibility and sensitivity tests. This method could also be used for the quantification in plasma of some other BZDs, such as 4′-hydroxyclobazam, 4′-hydroxy-N-desmethylclobazam, α-hydroxymethyltriazolam, hydroxyethylflurazepam, α-hydroxymethylmidazolam, clonazepam and estazolam, but a full validation has not yet been carried out.

EXPERIMENTAL

Apparatus

Analysis was performed on an HP Series 5890 A gas chromatograph (Hewlett-Packard, Les Ulis, France) equipped with an injector operating in the splitless mode (with a 0.5-min splitless period) and set at 250°C. The carrier gas was helium N 55 with a column head pressure of 1.0 bar (septum purge 2–3 ml/min). The column was a fused-silica capillary column (12 m × 0.2 mm I.D.) coated with a cross-linked dimethylsilicone as stationary phase (0.33 μm film thickness) (Hewlett-Packard). New columns were conditioned before use by several slow temperature programmings (2°C/min) from 100 to 310°C and by heating at 310°C for 12 h. The oven temperature was monitored during analysis from 70 to 300°C at a step rate of 20°C/min.

The detection of the compounds was carried out with an HP Series 5970 B mass-selective detector (Hewlett-Packard) including a capillary interface heated at 280°C, an electron-impact ion source (electron energy 70 eV), a quadrupole mass filter and an electron multiplier detector.

The detection was performed either in the SCAN mode, in which total ionization is carried out by scanning of a continuous mass range (within a full mass

range of 10–800 a.m.u. allowed by the MS system), which gives the mass spectrum of the compound, or in the selected-ion monitoring (SIM) mode, in which the detector simultaneously monitors some specific ions characteristic of the compound. Data processing was carried out by an HP Series 59970 B computer.

Chemicals

Standard solutions of the different BZDs (kindly supplied by Hoffmann-LaRoche, Basle, Switzerland) were prepared by carefully weighing ca. 10 mg into a 10-ml volumetric flask, with successive dilutions in methanol.

Butyl acetate and toluene (Purex) and cyclohexane (Pestipur) were supplied by SDS (Peypin, France). Methanol (Uvasol) was from Merck (Darmstadt, F.R.G.).

Extraction and derivatization procedures

A 0.5-ml volume of plasma was added to a suitable volume of an appropriate internal standard solution (concentrated to ca. 30–50 μl in tapered 10-ml tubes) and extracted at pH 9 for 4 min with 0.4 ml of butyl acetate on a Vortex system. The internal standards are listed in Tables II and III. The tubes were then centrifuged for 20 min at 4500 *g*. The organic phase was transferred into 1.0-ml minivials (Pierce, Rockford, IL, U.S.A.) and evaporated to dryness under a stream of nitrogen.

For non-hydroxylated BZDs, the residue was dissolved prior to analysis in 50–200 μl of toluene, depending on the expected concentration. For hydroxylated BZDs, such an extraction was followed by silylation of the hydroxy group(s) with N,O-bis(trimethylsilyltrifluoroacetamide) (BSTFA). After evaporation of the organic phase, the residue was dissolved in 100 μl of cyclohexane; 20 μl of BSTFA were then added and the minivials heated at 85°C for 15 min. The reaction was stopped by placing the minivials in ice. Samples of 1–3 μl were used for GC–MS analysis.

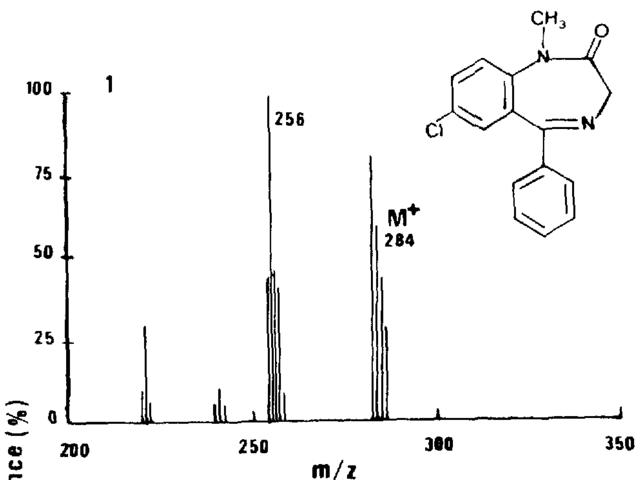
When a mixture of hydroxylated and N1-desalkyl BZDs had to be assayed simultaneously, i.e., in the same plasma sample, the extraction residue was dissolved in 100 μl of cyclohexane and this cyclohexane layer was first analysed for desalkylated BZDs (with the respective internal standard). The remaining ($\geqslant$97–98%) cyclohexane was then derivatized with BSTFA and analysed for hydroxylated BZDs (with their adequate internal standard).

RESULTS AND DISCUSSION

Mass spectra

The mass spectra obtained from unchanged non-hydroxylated BZDs and trimethylsilyl derivatives (TMS) of hydroxylated BZDs are shown in Fig. 1. Mass spectra were obtained in the mass range 50–500 a.m.u. but are shown only for the mass ranges 200–350 and 300–500 a.m.u. for non-hydroxylated and TMS derivatives of hydroxylated BZDs, respectively, as the most intense and characteristic ions used for quantification in the SIM mode were found in these ranges.

The molecular ions (M^+) and the most intense ions are listed in Table I. The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M+
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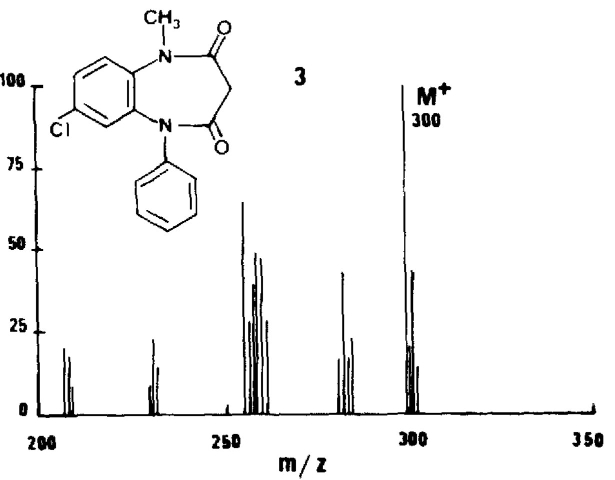
CH3
Cl
abundance (%)
m/z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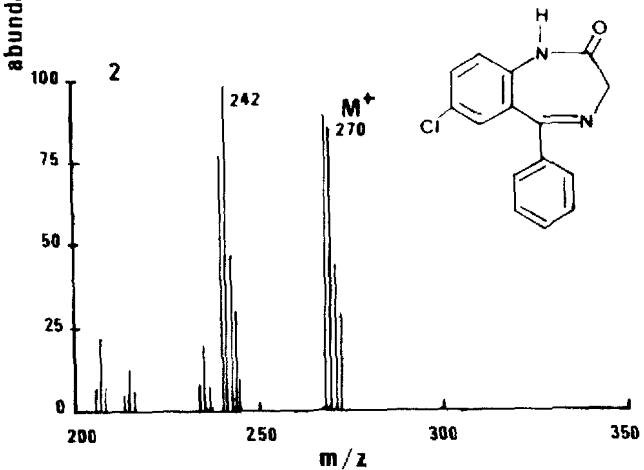
m/z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H
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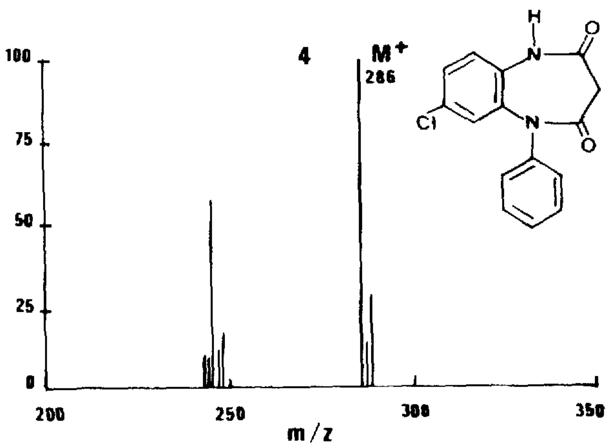
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H
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m/z

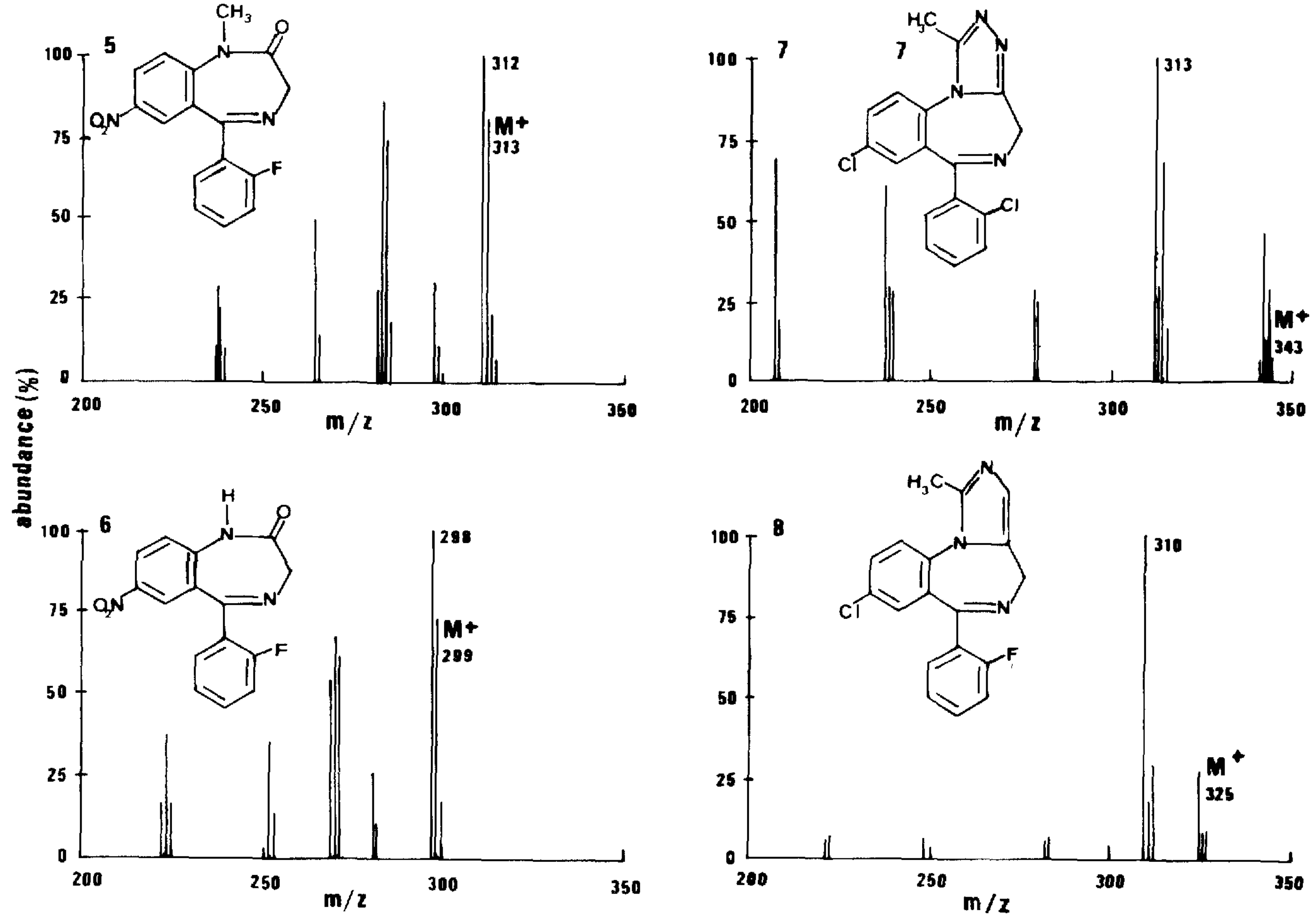

Fig 1.

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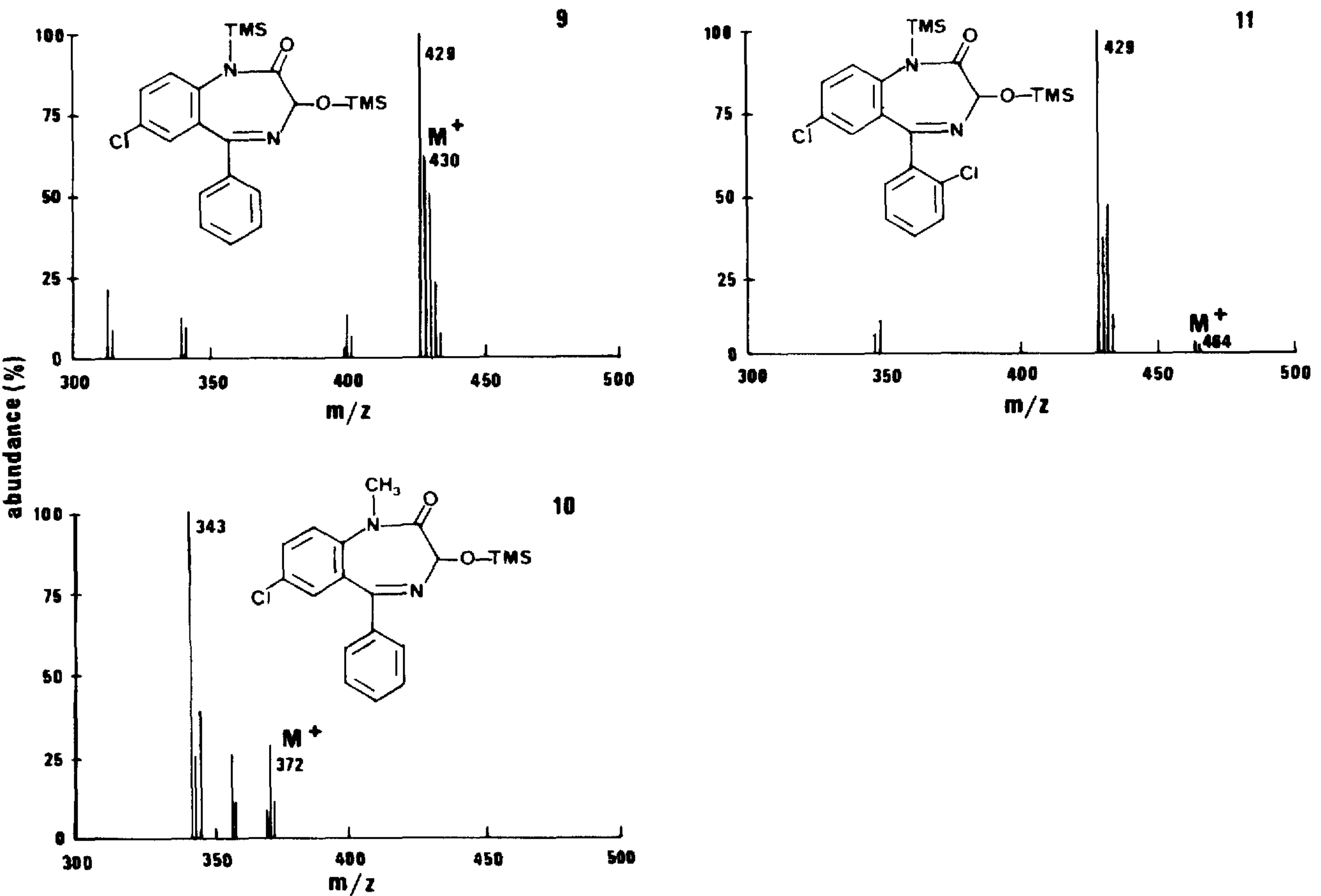


Fig. 1. Mass spectra of the investigated BZDS. See also Table I for mass spectrum numbers

TABLE I

MASS SPECTRA OF THE INVESTIGATED BZDs

m/z values and relative intensities (*I*) of the most intense and characteristic ions.

Compound	Mass spectrum No.	$(M)^+$		Most intense ion	
		m/z	*I* (%)[a]	*m/z*	Fragment
Diazepam	1	284	60	256	$(M-CO)^+$ or $(M-HCN-H)^+$
N-Desmethyldiazepam	2	270	91	242	$(M-CO)^+$ or $(M-HCN-H)^+$
Clobazam	3	300	100	300	$(M)^+$
N-Desmethylclobazam	4	286	100	286	$(M)^+$
Flunitrazepam	5	313	82	312	$(M-H)^+$
N-Desmethylflunitrazepam	6	299	74	298	$(M-H)^+$
Triazolam	7	343	15	313	$(M-2H-HCN)^+$
Midazolam	8	325	28	310	$(M-CH_3)^+$
Oxazepam	9	430	60	429	$(M-H)^+$ [b]
Temazepam	10	372	28	343	$(M-CHO)^+$ [c]
Lorazepam	11	464	1	429	$(M-Cl)^+$ [b]

[a] Abundance relative to the most intense ion taken as 100%
[b] $(M)^+$ corresponds to the bistrimethylsilyl (TMS) derivative
[c] $(M)^+$ corresponds to the monotrimethylsilyl derivative

most intense ions observed for thermally stable BZDs, e.g., diazepam, N-desmethyldiazepam, clobazam, N-desmethylclobazam and triazolam, confirmed the results in the literature [17]. For temazepam, oxazepam and lorazepam, the position of the TMS group depended on the structure of the compound. A double silylation has been reported for oxazepam and lorazepam, and their TMS derivatives have been shown to produce the same most intense fragment, *m/z* 429, corresponding to $(M-H)^+$ and $(M-Cl)^+$ ions, respectively [18]. Identical results were obtained here (Fig. 1). Temazepam, an N-methylated hydroxy-BZD, bore only one TMS group on the C_3 hydroxy; the most intense fragment was then $(M-CHO)^+$, *m/z* 343.

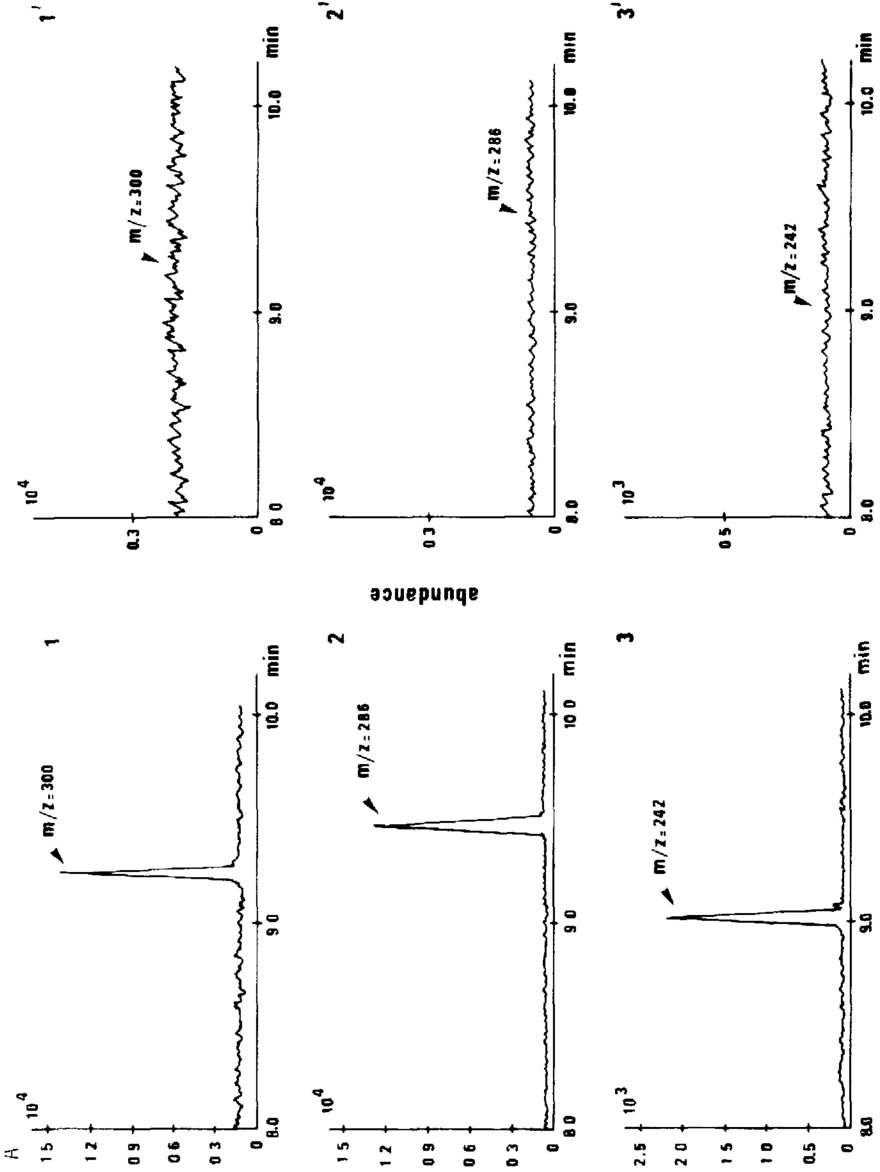
Chromatograms

Some typical chromatograms obtained in the SIM mode for control blank plasmas and for control plasmas spiked with clobazam and N-desmethylclobazam (internal standard N-desmethyldiazepam), and with temazepam and lorazepam (internal standard 3-hydroxyflunitrazepam), are presented in Fig. 2.

Intra- and inter-assay precision and accuracy

Results for the intra- and inter-assay precision (given by the relative standard deviation) and accuracy (defined by the difference between obtained and expected concentrations) are presented in Table II for all the investigated BZDs.

The intra- and inter-assay reproducibility were generally better than 95% for non-hydroxylated BZDs, but were sometimes lower for derivatized hydroxy BZDs. Indeed, a less satisfactory reproducibility of the chromatographic response was observed with the analysis of TMS derivatives. This was probably due to progressive column conditioning by the non-eliminated excess of BSTFA.


A
1
2
3
1'
2'
3'
m/z=300
m/z=286
m/z=242
abundance
min
10^4
10^3
8.0
9.0
10.0

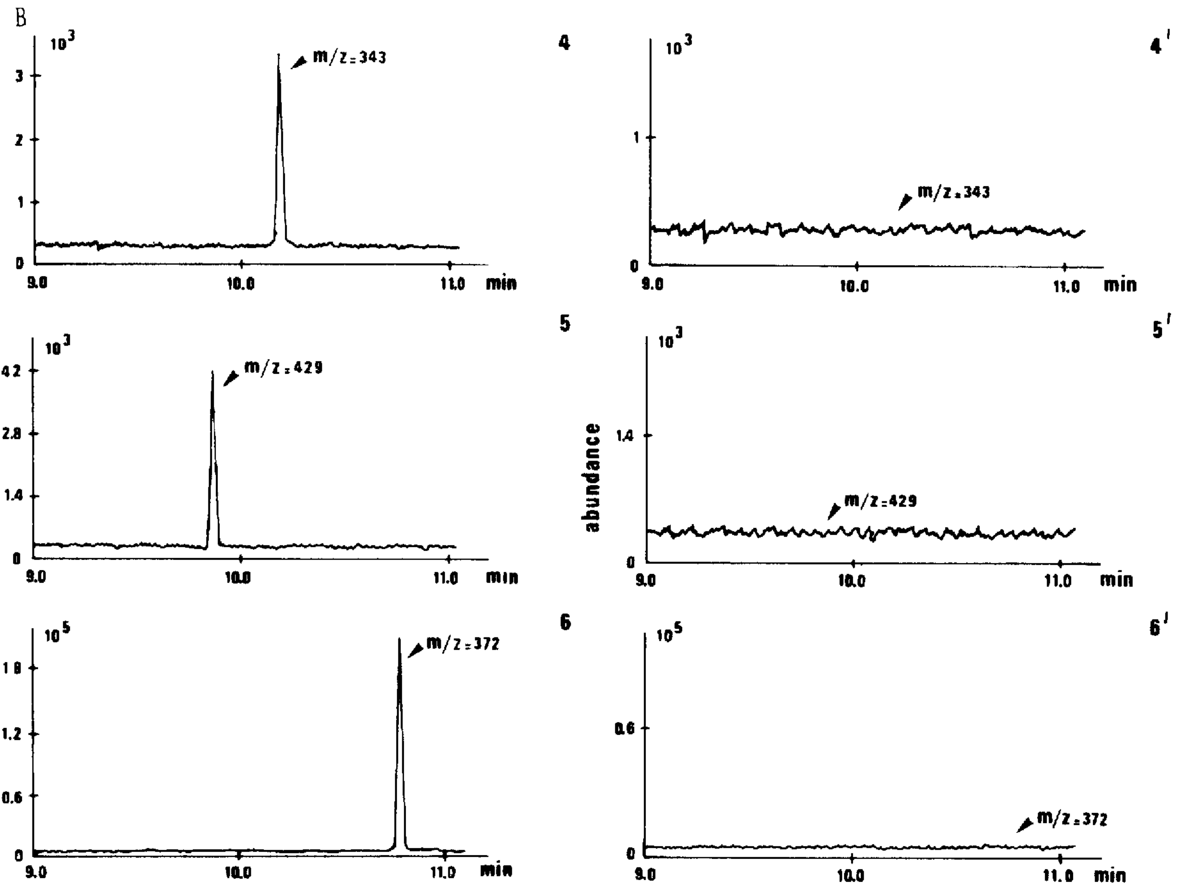


Fig. 2. Chromatograms obtained in the SIM mode. (A) After extraction of human control plasma spiked with (1) 0.316 μg/ml clobazam, (2) 0.218 μg/ml N-desmethylclobazam and (3) 0.024 μg/ml N-desmethyldiazepam (internal standard). (B) After extraction and derivatization of human control plasma spiked with (4) 0.595 μg/ml temazepam, (5) 0.535 μg/ml lorazepam and (6) 9.400 μg/ml 3-hydroxyflunitrazepam (internal standard). 1′–6′, respective ion chromatograms obtained from drug-free plasma samples.

TABLE II

INTRA- AND INTER-ASSAY PRECISION AND ACCURACY

Compound	Internal standard	n	Concentration added (μg/ml)	Concentration found (μg/ml)	CIM[a] (%)	Accuracy (%)
Intra-assay						
Diazepam	N-Desmethylclobazam	5	0.0195	0.0193	2.8	−1.0
		6	0.390	0.377	5.0	−3.4
N-Desmethyldiazepam	N-Desmethylclobazam	5	0.0488	0.0483	1.7	−1.0
		6	0.488	0.467	1.1	−4.3
Clobazam	N-Desmethyldiazepam	6	0.0220	0.0225	4.1	+2.3
		6	0.220	0.224	3.8	+1.8
N-Desmethylclobazam	N-Desmethyldiazepam	6	0.0420	0.0413	4.3	−1.6
		6	0.2100	0.2102	5.9	+0.1
Oxazepam	3-Hydroxyflunitrazepam	5	0.0250	0.0265	6.3	+5.6
		6	0.200	0.190	4.2	−5.2
		6	2.000	1.892	6.8	−5.7
Triazolam	Estazolam	6	0.320	0.337	4.7	+5.3
		6	1.600	1.560	2.2	−2.5
Inter-assay						
Diazepam	N-Desmethylclobazam	6	0.488	0.474	4.8	−2.9
		6	1.950	1.960	1.2	+0.7
Clobazam	N-Desmethyldiazepam	7	0.110	0.111	3.5	+0.9
		7	0.220	0.222	2.6	+0.9
Flunitrazepam	Diazepam	6	0.00520	0.00516	4.6	−0.8
		5	0.0208	0.0209	1.6	+0.5
Midazolam	Clobazam	5	0.050	0.048	4.2	−4.0
		6	0.200	0.202	2.5	+1.0
Temazepam	3-Hydroxyflunitrazepam	6	1.190	1.193	6.3	+0.3
		6	2.380	2.373	3.9	−0.3
Lorazepam	3-Hydroxyflunitrazepam	5	0.536	0.510	7.2	−4.9
		5	1.072	1.044	4.5	−2.6

[a]CIM = confidence interval of the mean (significance level 0.05).

TABLE III

LINEARITY TEST RESULTS FOR THE QUANTIFICATION OF THE INVESTIGATED BZDS IN PLASMA

Internal standard in parentheses.

Concentrations added (μg/ml)	Equation of the non-weighted linear regression line	Correlation coefficient
Diazepam (N-desmethylclobazam)		
0.005-0.01-0.015-0.02-0.03-0.04	$y = 2.92 \cdot 10^{-2}x - 0.004$	0.998
0.5-1.0-1.5-2.0-3.0-4.0	$y = 6.20 \cdot 10^{-4}x + 0.006$	0.999
N-Desmethyldiazepam (N-desmethylclobazam)		
0.024-0.048-0.072-0.096-0.144-0.192	$y = 8.64 \cdot 10^{-3}x - 0.023$	0.997
0.61-1.22-1.83-2.44-3.66-4.88	$y = 1.70 \cdot 10^{-4}x - 0.008$	0.998
Clobazam (N-desmethyldiazepam)		
0.0022-0.0055-0.011-0.022-0.044	$y = 1.60 \cdot 10^{-2}x + 0.0011$	0.999
0.055-0.11-0 17-0.22-0.33-0.44	$y = 2.21 \cdot 10^{-3}x - 0.002$	0.999
0.27-0.55-0.82-1.10-1.64-2.19	$y = 5.40 \cdot 10^{-4}x - 0.009$	0.999
N-Desmethylclobazam (N-desmethyldiazepam)		
0.010-0.026-0.052-0.10-0.16-0.21	$y = 4.37 \cdot 10^{-3}x - 0.004$	0.996
0.26-0.52-0.78-1.04-1.57-2.09	$y = 7.17 \cdot 10^{-4}x - 0.032$	0.997
Flunitrazepam (diazepam)		
0.0021-0.0052-0 010-0.016-0.021	$y = 2.39 \cdot 10^{-2}x - 0.005$	0.998
0.042-0.010-0.021-0.031-0.042	$y = 9.63 \cdot 10^{-3}x - 0.003$	0.998
N-Desmethylflunitrazepam (diazepam)		
0.0039-0.0099-0.020-0.030-0.039	$y = 8.97 \cdot 10^{-3}x - 0.007$	0.990
Triazolam (estazolam)		
0.5-1.0-1.5-2.0	$y = 1.42 \cdot 10^{-3}x - 0.009$	0.998
Midazolam (clobazam)		
0.05-0.10-0.15-0.20	$y = 1.13 \cdot 10^{-2}x - 0.022$	0 997
0.2-0.5-1.5-2.0	$y = 2.02 \cdot 10^{-3}x - 0.025$	0.994
Oxazepam (3-hydroxyflunitrazepam)		
0.025-0.050-0.075-0.10-0.15-0.20	$y = 2.03 \cdot 10^{-2}x + 0.004$	0.999
0.25-0.50-0.75-1.0-1.5-2.0	$y = 9.80 \cdot 10^{-4}x - 0.003$	0.999
0.5-1.0-1.5-2.0-3.0-4.0	$y = 9.20 \cdot 10^{-4}x - 0.042$	0.997
Temazepam (3-hydroxyflunitrazepam)		
0.03-0.06-0.09-0.12-0.18-0.24	$y = 1.04 \cdot 10^{-2}x + 0.004$	0.999
0.30-0.60-0.90-1.20-1.80-2.40	$y = 1.10 \cdot 10^{-3}x - 0.038$	0.998
Lorazepam (3-hydroxyflunitrazepam)		
0.022-0.054-0.082-0.108-0.216	$y = 6.02 \cdot 10^{-3}x + 0.007$	0.998
0.214-0.536-0.804-1.07-2.14	$y = 6.12 \cdot 10^{-4}x - 0.005$	0.999

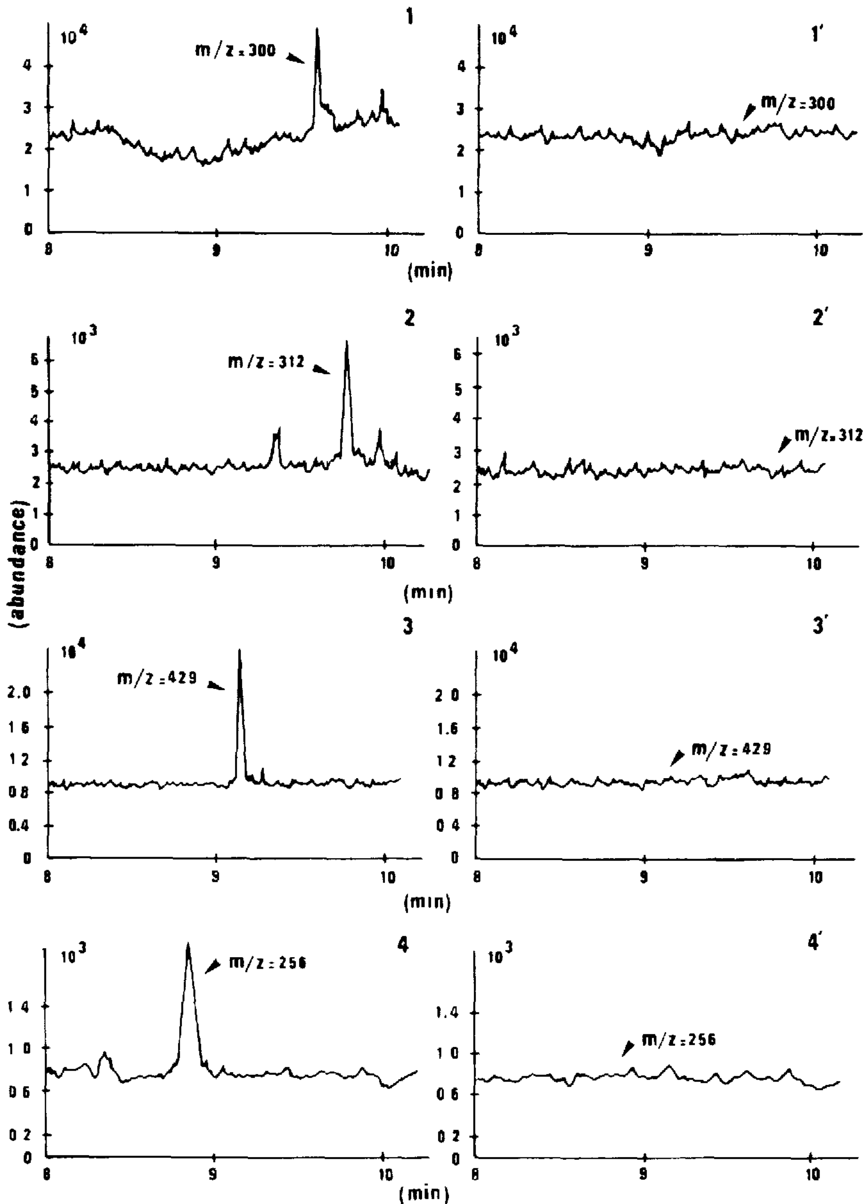


Fig 3 Chromatograms obtained in the SIM mode for human control plasma spiked with (1) 2.5 ng/ml clobazam, (2) 1.0 ng/ml flunitrazepam, (3) 5.0 ng/ml oxazepam and (4) 5.0 ng/ml diazepam. 1′–4′, respective ion chromatograms obtained from drug-free plasma samples.

Linearity

Some linearity test results are presented in Table III. In the concentration range checked, i.e., 0.002–4.8 μg/ml, the correlation coefficients of the linear regression curves were better than 0.990–0.995 and the intercepts did not differ significantly from zero.

Limit of detection

The limit of detection, defined by a signal-to-noise ratio of about 5, was in the approximate range 1–5 ng/ml for non-hydroxylated BZDs (diazepam, clobazam, flunitrazepam and their N-desmethylmetabolites) and 5 ng/ml for hydroxylated BZDs (oxazepam, temazepam, lorazepam). Fig. 3 shows some chromatograms obtained from control plasma samples spiked with BZDs at concentrations close to their respective limits of detection. These limits of detection were similar to those obtained by GC with electron-capture detection (ECD), which is the most sensitive method for the determination of BZDs.

Paradoxically, a detection limit of about 50–100 ng/ml was observed for triazolam, whereas by GC–ECD under the same chromatographic conditions the limit of detection was below 1 ng/ml for this BZD, but with solid injection instead of splitless injection [13]. For midazolam, the detection limit was about 20 ng/ml.

Extraction efficiency

The extraction recovery of the BZDs from human plasma was in the range 75–80%, regardless of the compound or its concentration.

Derivatization step

To quantify hydroxy BZDs in their underivatized form in plasma, some preliminary tests were performed, in particular with oxazepam, by using a direct butyl acetate extraction without silylation. However, these tests did not give satisfactory results on repeated injections of the same extract, because of an expected adsorption of this BZD by its free hydroxy group on the active sites of the column. Therefore, it was necessary to stabilize the hydroxy group by silylation.

Several parameters were tested for optimizing the derivatization reaction, in particular the nature of the derivatization solvent (acetonitrile, cyclohexane), the reaction temperature (40–120°C), the heating time (0.25–2 h) and the vol-

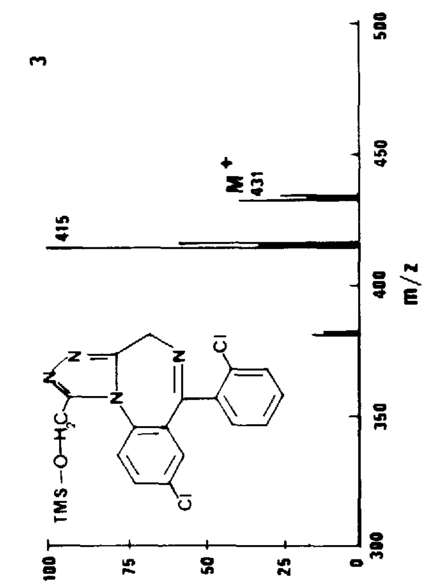
TABLE IV

MASS SPECTRA OF SOME OTHER BZDs AND HYDROXY METABOLITES OBTAINED UNDER THE SAME CONDITIONS

m/z values and relative intensities (*I*) of the most intense and characteristic ions.

Compound	Mass spectrum No.	$(M)^+$		Most intense ion	
		m/z	*I* (%)[a]	*m/z*	Fragment
4′-Hydroxyclobazam	1	388	100	388	$(M)^{+}$ [b]
4′-Hydroxy-N-desmethylclobazam	2	446	100	446	$(M)^{+}$ [c]
α-Hydroxymethyltriazolam	3	431	38	415	$(M-H-CH_3)^{+}$ [b]
Hydroxyethylflurazepam	4	404	4	288	$[M-(CH_2)_2-O-TMS]^{+}$ [b]
α-Hydroxymethylmidazolam	5	413	13	310	$(M-CH_2-O-TMS)^{+}$ [b]
Clonazepam	6	315	69	280	$(M-Cl)^+$
Estazolam	7	294	70	259	$(M-Cl)^+$

[a]Abundance relative to the most intense ion taken as 100%.
[b]$(M)^+$ corresponds to the monotrimethylsilyl (TMS) derivative.
[c]$(M)^+$ corresponds to the bistrimethylsilyl derivative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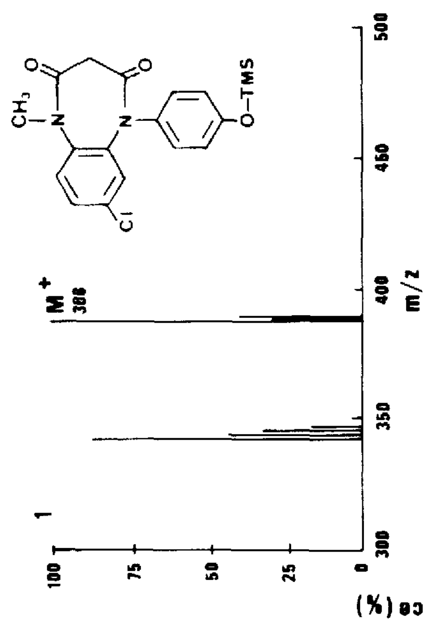
abundance (%)
m/z

3
415
m/z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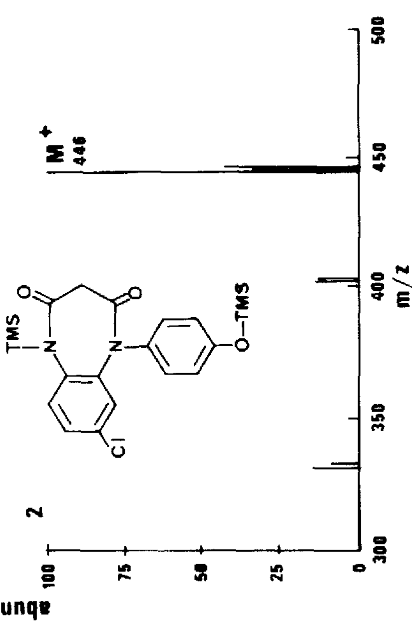
m/z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m/z

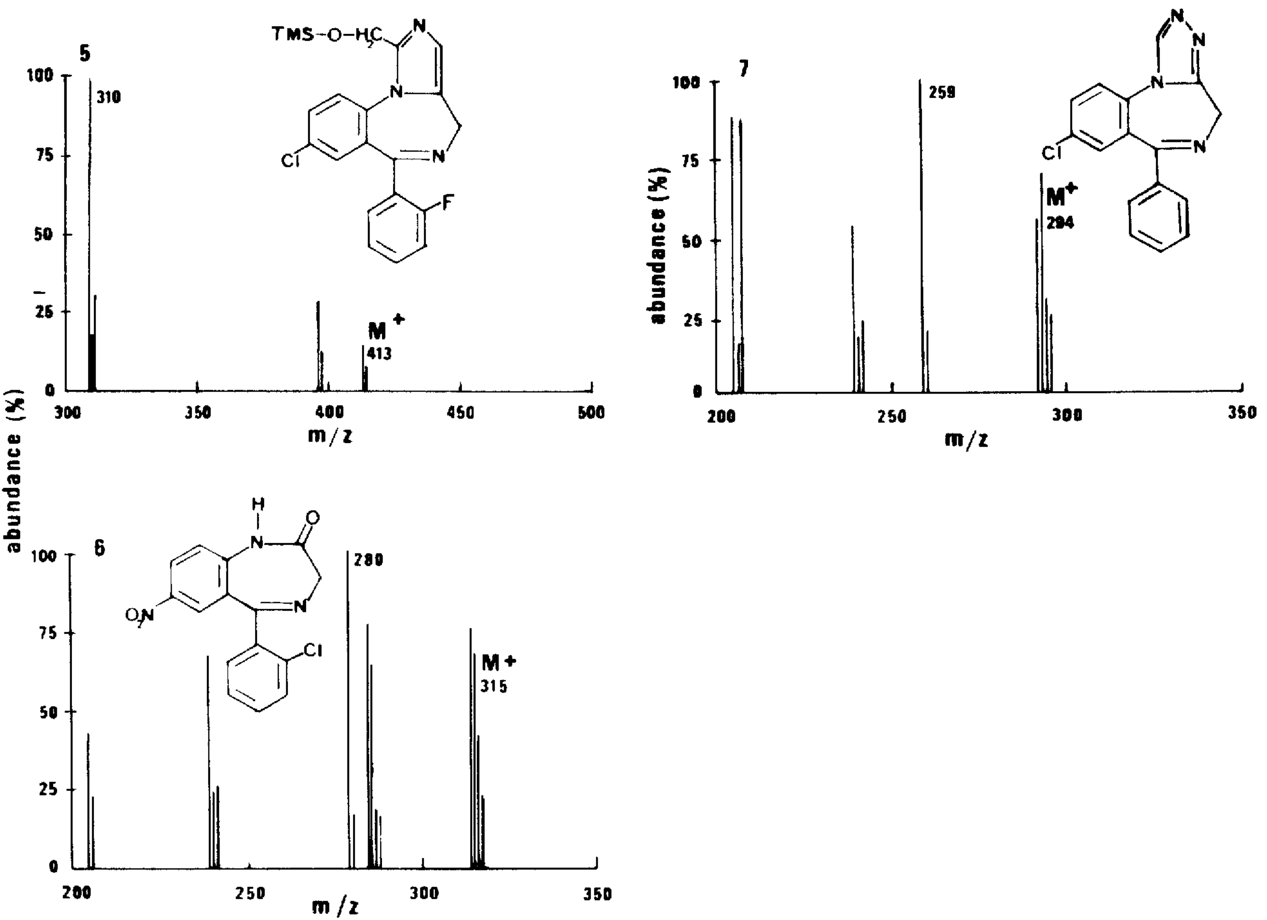

Fig. 4. Mass spectra of some other BZDs and hydroxy metabolites. See also Table IV for mass spectrum numbers.

ume of the silylating agent (BSTFA). The best reaction efficiency was obtained with the conditions described above, i.e., cyclohexane as solvent for BSTFA and heating at 60°C for 15 min.

Potential other applications of the GC–MS assay

As preliminary results, the mass spectra of some other non-hydroxylated BZDs (clonazepam, estazolam) and TMS derivatives of hydroxylated BZDs (4′-hydroxyclobazam, 4′-hydroxy-N-desmethylclobazam, α-hydroxymethyltriazolam, hydroxyethylflurazepam, α-hydroxymethylmidazolam), obtained from pure solutions in methanol, are presented in Table IV and Fig. 4.

The same extraction and derivatization procedure should be usable for their quantification in plasma, but the method has to be validated for these BZDs with the classical linearity and reproducibility tests.

CONCLUSIONS

The GC–MS method developed constitutes an improvement in chromatographic techniques as it allows the unequivocal identification of BZDs and their main metabolites (by direct comparison of the full mass spectra obtained from plasma extracts with the mass spectra of reference compounds) and their consecutive quantification in the same plasma extract. The extraction procedure used for the determination of intact BZDs in plasma makes the method simple, rapid and reliable, thus permitting its use in routine clinical situations such as therapeutic monitoring and overdose and dosage regimens. In addition, GC–MS is very useful when the specificities of the classical techniques, HPLC and GC, are insufficient. For example, in epileptic patients undergoing treatment with diazepam and clobazam, the quantification of N-desmethyldiazepam and clobazam cannot be performed by GC because these compounds show the same retention behavior. The other techniques, however, have sufficient specificity for classical pharmacokinetic studies in healthy volunteers not receiving interfering comedication.

An extensive report of the clinical applications of this work will be the subject of a further publication.

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