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HIGHLY SENSITIVE ASSAY OF A BENZODIAZEPINE ANTAGONIST IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION

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SUMMARY

A selective and highly sensitive capillary gas chromatographic method was developed for the determination of a benzodiazepine antagonist in human plasma. The analytical procedure involved extraction of the compound and its internal standard from basified plasma with *n*-butyl chloride—dichloromethane and chromatography of the extract on a DB-5 fused-silica column (30 m × 0.25 mm I.D.), applying automated splitless injection and nitrogen- phosphorus detection. The limit of quantification was about 50 pg/ml, using a 1-ml plasma specimen The mean inter-assay precision was 2.6% in the concentration range 0.5—10 ng/ml. The method was shown to be specific with respect to various benzodiazepines and their main metabolites. The practicability of the method was demonstrated by the analysis of more than 300 plasma samples from a dose proportionality study performed with human volunteers. Owing to its high sensitivity, the new method can be used to obtain pharmacokinetic parameters of the benzodiazepine antagonist in man after doses near the envisaged therapeutic intravenous dose of <1 mg.

INTRODUCTION

Certain recently discovered imidazobenzodiazepine derivatives selectively block the effects resulting from interactions of drugs with benzodiazepine receptors in the central nervous system, without themselves having major intrinsic activity [1]. A first representative of this novel class of drugs, ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-[1,5-*a*][1,4]benzodiazepine-3-carboxylate*, I (see Table I), has been selected for broad clinical evaluation as a selective benzodiazepine antagonist [2—7].

During the last few years, several methods have been developed for the

*Ro 15-1788, proposed trade name Anexate, F. Hoffmann-La Roche, Basle, Switzerland.

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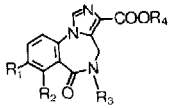
TABLE I

STRUCTURE OF THE BENZODIAZEPINE ANTAGONIST AND SOME RELATED COMPOUNDS



Compound	Description	R_1	R_2	R_3	R_4
I	Parent compound	F	H	CH_3	C_2H_5
II	Internal standard	H	F	CH_3	C_2H_5
III	Internal standard	H	Cl	CH_3	C_2H_5
IV	N-Desmethyl ester metabolite	F	H	H	C_2H_5
V	N-Desmethyl acid metabolite	F	H	H	H
VI	Acid metabolite	F	H	CH_3	H

determination of I in the plasma of dogs, rats and human volunteers. A selective high-performance liquid chromatographic (HPLC) assay with a limit of detection of 5—10 ng/ml was reported by our group in 1983 [8], and was successfully applied to more than 1000 plasma samples from various toxicokinetic and pharmacokinetic studies. The method was sensitive enough to evaluate pharmacokinetic parameters in man following a single intravenous dose of 20 mg of the antagonist [9]. In order to lower the limit of detection, this method was subjected to slight modifications by Klotz et al. [10] (2 ng/ml, modification of the extraction procedure and of the chromatographic system) and by Roncari and Zumbrunnen [11] (2 ng/ml, use of 3-μm instead of 5-μm reversed-phase material). More recently, a fully automated HPLC assay with a limit of detection of 5 ng/ml was developed by our group, applying column switching for direct injection of plasma samples [12]. A gas chromatographic (GC) assay using packed column technology and nitrogen—phosphorus detection was reported by Abernethy et al. [13] in 1983. The limit of detection (3 ng/ml, using 3-ml plasma specimens) was similar to that of the HPLC assay mentioned above.

None of these methods was sensitive enough to allow the determination of the pharmacokinetic parameters of I following studies in human volunteers near the envisaged therapeutic intravenous dose (<1 mg). Therefore, a new capillary GC assay, applying automated splitless injection and nitrogen-selective detection, was developed. Owing to the high sensitivity of the assay (about 50 pg/ml, using 1 -ml plasma specimens) it was possible to follow plasma levels of I for a period of seven elimination half-lives after a single 2-mg intravenous dose to human volunteers.

EXPERIMENTAL

Reagents and solvents

Dichloromethane (for pesticide residue analysis), ethanol (absolute, p.a.), *n*-butyl acetate (p.a.), sodium hydroxide (p.a.), sodium fluoride (p.a.) and potassium oxalate (p.a.) were obtained from E. Merck (Darmstadt, F.R.G.) and were used without further purification. *n*-Butyl chloride (HPLC grade)

was purchased from Fisons (Loughborough, U.K.) and re-distilled in all-glass apparatus before use. Water of HPLC-grade quality (Mallinckrodt, Paris, KY, U.S.A.) was used for the preparation of all aqueous solutions.

Preparation of plasma standards

Owing to the wide concentration range of I observed in biological samples, up to ten different plasma standards had to be prepared according to the following procedure. A stock solution was obtained by dissolving 10 mg of the pure drug in 10 ml of ethanol. Aliquots of the stock solution were diluted with ethanol to provide the working solutions listed in Table II. The plasma standards were prepared by spiking blank plasma (25 ml) with 100 μl of the corresponding working solution, providing concentrations between 0.0625 and 50 ng/ml.

TABLE II

CONCENTRATIONS OF THE BENZODIAZEPINE ANTAGONIST IN WORKING SOLUTIONS AND PLASMA STANDARDS

Working solutions (ng per 100 μl)	Plasma standards (ng/ml)
1.5625	0.0625
3.125	0.125
6.25	0.25
12.5	0.50
25.0	1.00
50.0	2.00
125	5.00
250	10.0
500	20.0
1250	50.0

The stock solution could be stored at 5°C for about four weeks. Working solutions were prepared prior to use. The plasma standards were divided into 2.5-ml aliquots and stored deep-frozen (—20°C) until required for analysis.

For the preparation of plasma standards, human blood was received from a blood bank (Blutspendezentrum, SRK, Basle, Switzerland) in a pre-cleaned glass vessel. A mixture of sodium fluoride and potassium oxalate was used as anticoagulant and esterase inhibitor at concentrations of 25 and 20 mg/ml blood, respectively. The blank plasma obtained by centrifugation (1000 *g*, 10 min) was tested for the absence of endogenous components interfering with the benzodiazepine antagonist I and/or the internal standards II and III.

Extraction procedure

A 1-ml aliquot of plasma sample was mixed with 50 μl of an ethanolic solution containing both internal standards at concentrations of 5 or 25 ng/ml, depending on the calibration range. The sample was vortex-mixed with 50 μl of aqueous sodium hydroxide solution (1 *M*) and then extracted with 5 ml of *n*-butyl chloride—dichloromethane (96:4) by shaking for 10 min at 15 rpm on a rotating shaker (Heidolph, F.R.G.). After centrifugation, the separated organic phase was evaporated to dryness at 50°C by means of a gentle stream of

pure (99.999%) nitrogen. The extraction residue was dissolved in 50 μl of *n*-butyl acetate by vortex-mixing for 15 s and then transferred into a 200-μl conical glass vial. A 1-μl aliquot was injected into the injector inlet using the automated splitless injection technique.

All operations were carried out with glassware that had been rinsed with ethanol and then air-dried prior to use.

Capillary gas chromatography

The GC analysis was performed with a Model 5880A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a split/splitless capillary inlet and a nitrogen—phosphorus detector.

Separations were performed on a 30 m × 0.25 mm I.D. fused-silica capillary column (J&W Scientific, Rancho Cordova, CA, U.S.A.). The stationary phase (DB-5, 95% dimethyl—5% diphenylpolysiloxane) was both cross-linked and chemically bonded to the fused-silica surface. The film thickness was 0.10 μm (β = 625). The separation number (Trennzahl [14]) was 44, measured isothermally at 90°C between methyl decanoate and methyl undecanoate, using hydrogen as the carrier gas at a linear velocity of 36 cm/s.

After injection, the initial temperature (120°C) was maintained for 1.75 min, then increased to 280°C at 30°C/min, held for 5 min at 280°C, and then decreased within 3 min to the initial temperature. The temperature of the nitrogen—phosphorus detector and injection port were adjusted to 300 and 280°C, respectively.

The linear velocity of the helium carrier gas was in the range 40—45 cm/s. The flow-rates of the auxiliary gases hydrogen and air were 3.0 and 50 ml/min, respectively. Helium was used as the make-up gas for the nitrogen—phosphorus detector at a flow-rate of 30 ml/min.

After approximately 100 injections of plasma extract, the inlet sleeve (8 cm × 2.2 mm I.D.) was cleaned to remove septum particles and the residue of non-volatile plasma constituents. Following a rinse with concentrated nitric acid and repeated flushing with dilute hydrochloric acid (1%), the liner was dehydrated at 280°C in an atmosphere of nitrogen, and finally re-deactivated. In the case of the glass sleeves, re-deactivation was carried out with Surfasil (Pierce, Rockford, IL, U.S.A.) at room temperature. The fused-silica liner was deactivated in the vapour phase by injecting 5 μl of pure 1,3-diphenyl-1,1,3,3-tetramethyldisilazane (Fluka, Buchs, Switzerland) ten times at 350°C in an atmosphere of nitrogen.

Immediately before injection of 1 μl of the reconstituted plasma extract by means of an automated sampler (HP 7672A), the split valve was closed for 60 s. Under these chromatographic conditions the benzodiazepine antagonist I and the internal standards II and III eluted after 8.43, 8.67 and 9.40 min, respectively. A complete analysis cycle lasted about 15 min.

Calibration and calculation

At least six different plasma standards covering the expected concentration range were processed as described above, together with the biological samples. The calibration graph was established by weighted quadratic least-squares regression (weighting factor = $1/y^2$) of the measured peak-height ratios I/II

or I/III (y) versus the concentrations of I (x) added to the plasma [15]. This regression equation was then used to calculate concentrations of I in unknown plasma samples from the measured peak-height ratios I/II or I/III.

Acquisition and on-line treatment of the data were performed by means of a computing integrator (Model SP4200, Spectra-Physics, Santa Clara, CA, U.S.A.) working with a BASIC program, recently developed in this laboratory*.

RESULTS AND DISCUSSION

Sample preparation, internal standard

The extraction conditions previously established for the HPLC procedure [8] were also suitable for the new GC method, with a few minor modifications. First, trisodium phosphate was replaced with sodium hydroxide, in order to avoid interferences caused by detector-sensitive impurities present in the phosphate. Second, a new internal standard was selected, which was more suitable for GC. Compounds II and III (see Table I) could both be used for this purpose, providing comparable results with respect to precision and accuracy. With respect to the chemical structure and the GC behaviour, the isomeric compound II had the properties of a nearly ideal internal standard. However, in more than half of the 32 clinical pre-dose samples that have been analysed so far an endogenous peak was observed, corresponding to a concentration of II in the range 50—500 pg/ml. For compound III no such endogenous interference was encountered. Fig. 1 shows a clinical plasma sample containing the two possible internal standards at concentrations of 5 ng/ml.

The extraction was carried out at pH 10.8 in order to suppress co-extraction of interfering plasma components, while the recovery of I (pK_a = 1.7 due to sp_2-hybridized nitrogen in the imidazo ring [17]) was not affected by selecting basic extraction conditions [8].

n-Butyl chloride—dichloromethane [8], diethyl ether [10] and ethyl acetate [13] have been used in previous methods for the extraction of I from plasma. The first solvent mixture was preferred in the method described here, as it yielded the best compromise between a high recovery of I (about 100%) and low co-extraction of interfering plasma components [8].

Solid—liquid extraction, making use of Extrelut columns (E. Merck), was found to be inferior to liquid—liquid extraction, for the following reasons. Prior to use, the columns had to be extensively washed with various solvents and then dried in a current of air [18]. Only after this time-consuming procedure did these columns generate comparable results to liquid—liquid extraction with respect to the blank. Additionally, co-extraction of lipophilic compounds from plasma was slightly increased, even when the same solvent was used and the same pH was applied as for liquid—liquid extraction.

The high sensitivity of the method could only be achieved by using thoroughly clean glassware. Absence of interfering compounds co-extractable

*The program makes use of a new procedure for chaining programs with the SP4200; this allows much larger programs to be executed than the resident memory is capable of storing. Additionally, a technique for handling multiple arrays in an efficient manner, with minimum use of memory, is demonstrated [16].

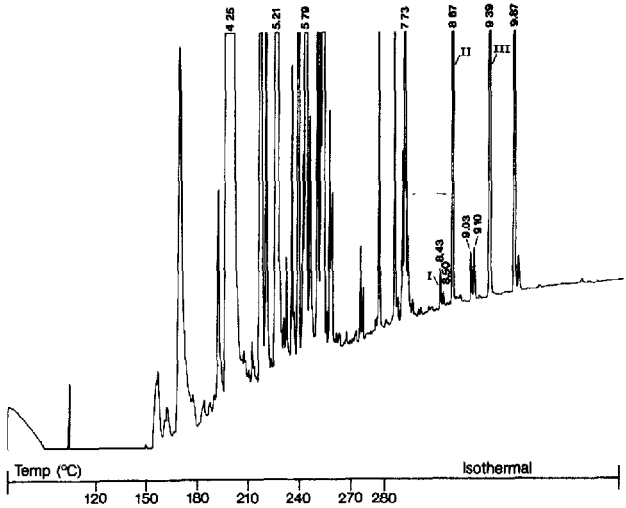


Fig. 1. Gas chromatogram of a human plasma sample taken 4 h after an intravenous dose of 1 mg of the benzodiazepine antagonist; calculated concentration of I, 0.35 ng/ml. For GC conditions, see text; chart speed, 2 cm/min; attenuation, 2^{-1}.

from the reagents was tested daily by running a reagent blank prior to the work-up of real plasma samples.

Choice of capillary column

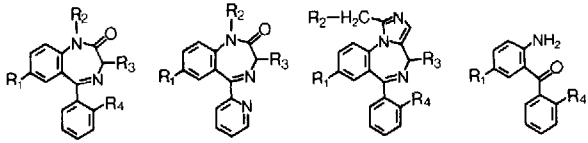
The intended use of I is as an antagonist against various benzodiazepine tranquilizers. Consequently, the chromatographic separation problems were more severe than usual, because of the presence not only of the antagonist, its metabolites, the internal standards and endogenous plasma components, but also of the benzodiazepine agonists and their metabolites.

In order to overcome this difficult separation problem, various stationary phases were tested: methylsilicone (OV-1), methylphenylsilicone with increasing phenyl content (SE-54, DB-5, OV-73, OV-3, OV-17) and a mixed phase (SE-54—5% Superox 0.1). As will be shown later, the antagonist could be separated from the benzodiazepines listed in Table III on all these phases, whereas great differences were observed in the ability to separate I from co-extracted endogenous components. The best results were obtained with the GC phase DB-5 and with a mixed phase consisting of SE-54– 5% Superox 0.1. The capillary column coated with DB-5 needed a high separation power (30 m) to avoid interference of I with endogenous components, while the capillary coated with the mixed phase was able to fulfil the same requirement with a shorter column, mainly owing to its adjustable polarity [19]. This resulted in a shorter analysis time of I compared with capillary columns coated with DB-5.

The preparation of this particular capillary column (15 m × 0.32 mm I.D.) was performed by deactivation [20] of an untreated fused-silica column with Carbowax 20M and static coating with the mixed phase [21]. The phase ratio (β) had to be adjusted to approximately 1500 to keep the elution temperature of I below 250°C. The retention time of I on this capillary column was 6 min,

TABLE III

STRUCTURE OF VARIOUS BENZODIAZEPINES AND THEIR MAIN METABOLITES TESTED FOR POSSIBLE INTERFERENCES WITH THE MEASURED SUBSTANCES I, II AND III



A B C D

Compound name	Compound No.	Structure type	R_1	R_2	R_3	R_4
Diazepam	1	A	Cl	CH_3	H	H
N-Desmethyldiazepam	2	A	Cl	H	H	H
Temazepam	4	A	Cl	CH_3	OH	H
Oxazepam	—	A	Cl	H	OH	H
Bromazepam	3	B	Br	H	H	—
Midazolam	6	C	Cl	H	H	F
1-Hydroxymethylmidazolam	7	C	Cl	OH	H	F
4-Hydroxymidazolam	5	C	Cl	H	OH	F
1,4-Dihydroxymidazolam (degradation products)	8, 9, 10	C	Cl	OH	OH	F
Flunitrazepam	12	A	NO_2	CH_3	H	F
7-Aminoflunitrazepam	13	A	NH_2	CH_3	H	F
7-Aminodesmethylflunitrazepam	14	A	NH_2	H	H	F
N-Desmethylflunitrazepam	15	A	NO_2	H	H	F
2-Amino-4-nitro-2'-fluorobenzophenone	11	D	NO_2	—	—	F

needing a final temperature of only 250°C in the temperature programme. In comparison with DB-5 alone, most of the co-extracted components were shifted to shorter retention times. However, as capillary columns with adjustable polarity cannot be prepared in every laboratory, a commercially available capillary (DB-5, J & W Scientific) was preferred for the analysis of clinical samples.

Interestingly, even within the group of methylsilicone—5%-phenylsilicones such as DB-5, CP-Sil 8 CB, SPB-5 and SE-54 (cross-linked), differences in the separation of I from endogenous compounds were observed, whereas the separation order of the investigated benzodiazepines and related metabolites was not affected.

Fig. 2 shows a typical chromatogram of a human blank plasma extract, demonstrating the absence of any endogenous peak co-eluting with I. Of the many human pre-dose plasma samples tested on DB-5, in no instance was an interfering plasma component found that corresponded to an equivalent concentration of more than 10 pg/ml I. However, even slight changes in the polarity of the column could cause interference problems between endogenous compounds and I. Interference problems were also observed when sodium citrate or ammonium oxalate was used as an anticoagulant. For this reason, sodium fluoride—potassium oxalate was preferred as the anticoagulant for all clinical studies.

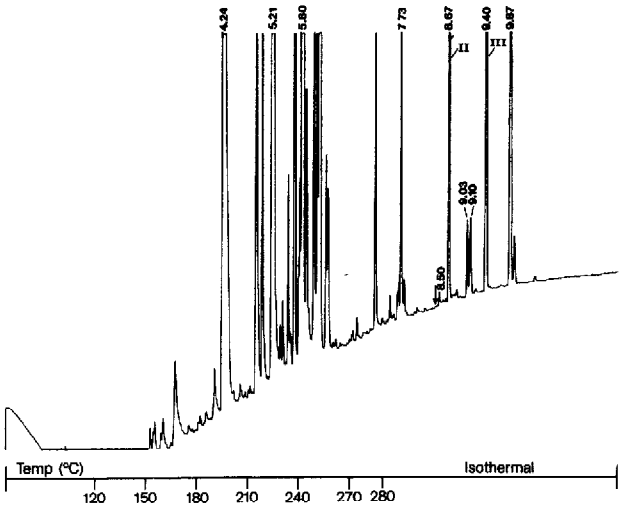


Fig. 2. Gas chromatogram of a typical human plasma sample taken before administration of I. The arrow indicates the retention time of I. For GC conditions, see text; chart speed, 2 cm/min; attenuation, 2^{-1}.

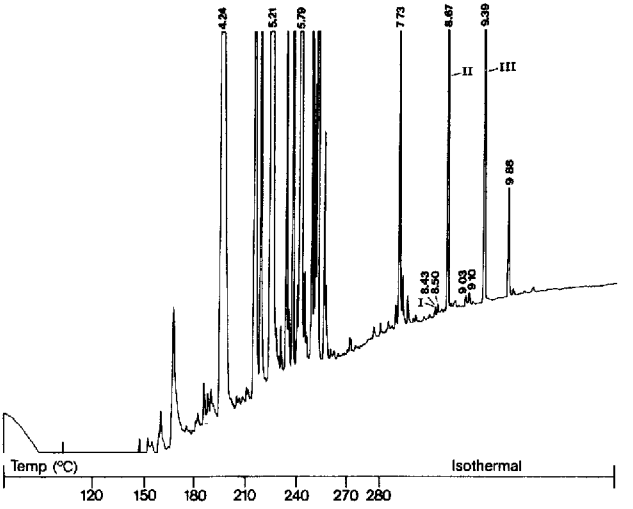


Fig. 3. Gas chromatogram of a human plasma standard spiked with 62.5 pg/ml I. For GC conditions, see text; chart speed, 2 cm/min; attenuation, 2^{-1}.

Fig. 3 shows the chromatogram of a volunteer blank plasma spiked with 62.5 pg/ml I. The antagonist was completely separated from an endogenous component with a retention time of 8.50 min.

Stability and performance of the capillary column

After about 400 injections, a distinct change in the adsorptive behaviour of the capillary column with respect to I could be observed, the peak of the antagonist showing slight tailing. Because of adsorptive losses on the capillary, the detection limit of I deteriorated by a factor of 5. In addition, a pronounced memory effect could be observed. In order to restore the initial inertness and

polarity, the column was shortened by about 50 cm at the injector end. This procedure was subsequently repeated every 300 injections*. The use of a retention gap [22] was not necessary because of the small volumes (1 μl) of reconstituted plasma extract injected on to the capillary column.

All chromatograms shown were obtained with a capillary column that had already been used for more than 1400 injections of plasma extracts. In spite of stress caused by the temperature programme and the plasma components, the absorptive behaviour of the capillary was virtually unchanged. That the performance was still excellent after so many injections could be attributed mainly to the fact that only one fiftieth of the reconstituted plasma extract was used per injection. The limit of quantification of about 50 pg/ml I in plasma could be achieved even after more than 1400 injections of plasma extract.

Injection system

Because of the time-consuming chromatographic procedure (15 min per cycle), an automated injection device was necessary in order to achieve an acceptable sample throughput per day. Using an automated splitless injector and making use of the solvent effect, it was possible to transfer I precisely and without loss into the capillary column. The precision of two consecutive automatic injections was about 1.5%. A further advantage of the splitless injector was the fact that, in contrast to the on-column injector, non-volatile plasma components and degradation products were retained in a removable injection liner instead of polluting the capillary column.

Choice of detector

As I had relatively poor electron-capture properties compared with other benzodiazepines [8], nitrogen—phosphorus detection was used. Because the NPD instrument is a mass flow dependent detector, make-up gas can be employed without loss of sensitivity. Therefore, the effective detector volume is very low, permitting the use of the full separation power of the capillary in contrast to an electron-capture detector.

The measured selectivity of the NPD instrument with respect to carbon compounds containing no nitrogen was 45 000 whereas that with respect to phosphorus-containing compounds was found to be better than 3. The selectivity ratios were measured between azobenzene and octadecane and between azobenzene and malathion, respectively. These excellent selectivities permitted the application of the simple sample work-up procedure described above, as it was not necessary to eliminate the whole bulk of interfering carbon compounds before analysis.

Selectivity of the method towards metabolites

The new method was shown to be specific with respect to the three known metabolites of I [23] (Table I). Fig. 4a demonstrates that the N-desmethyl

*The capillary may be shortened from 30 to about 25 m. The resulting loss of separation power is only 8.8% provided that the linear velocity of the carrier gas is maintained. The loss of separating power may be completely compensated for by reducing the linear velocity from 45 to approximately 30 cm/s.

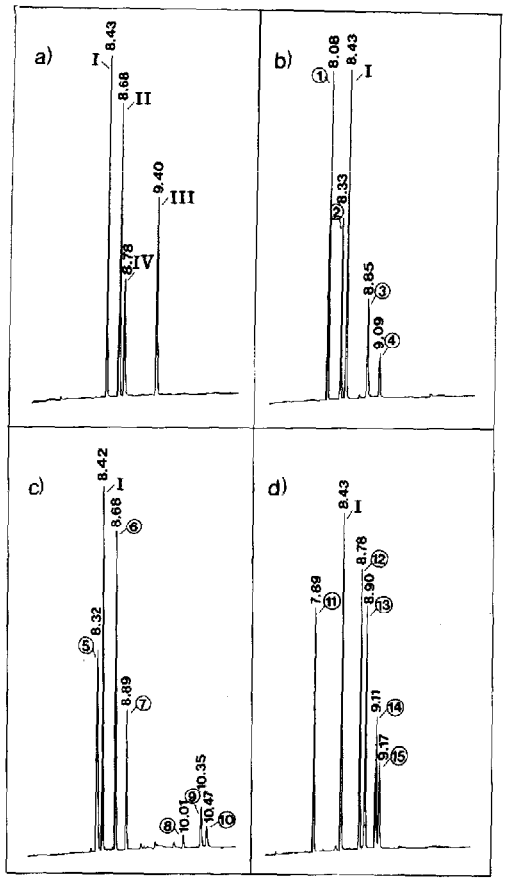


Fig. 4. Gas chromatograms of solutions containing various benzodiazepines and their main metabolites at concentrations of 250 pg/μl. For peak identification, see Tables I and III. For GC conditions, see text; chart speed, 2 cm/min; attenuation, 2^1.

ester metabolite IV was well separated from I and the two internal standards. Compared with on-column injection, no band broadening or decrease in response of the compounds could be observed. The acidic metabolites V and VI were not extracted from plasma at the adjusted high pH and, therefore, did not appear in the chromatogram.

Selectivity of the GC phase towards benzodiazepines

The stationary phase DB-5 was able to separate the antagonist from the various tested benzodiazepine agonists and related metabolites listed in Table III.

Diazepam and two of its metabolites, N-desmethyldiazepam and temazepam, were well separated from I (Fig. 4b). N-Desmethyldiazepam showed no peak tailing, whereas the response of temazepam was significantly reduced because of a loss of substance in the injector system. Oxazepam was not stable under the chosen chromatographic conditions. The rearrangement product formed [24] did not interfere with I, II or III. Fig. 4b also demonstrates that bromazepam did not interfere with the antagonist and its internal standards.

The elution pattern of midazolam together with three of its metabolites and I is shown in Fig. 4c. The peak shape for 1-hydroxymethylmidazolam was

good, but the 4-hydroxymetabolite showed band broadening, obviously caused by decomposition. The 1,4-dihydroxymetabolite degraded extensively in the injector system, generating three major decomposition products with unknown structure.

Fig. 4d shows that I was well separated from flunitrazepam and from four of its metabolites, whereas flunitrazepam co-chromatographed with the N-desmethyl ester metabolite of I.

In conclusion, compound I may be determined with high sensitivity in the presence of all tested benzodiazepine agonists. Further, the proposed capillary GC method shows potential for the determination of the antagonist and co-administered agonist in one analytical run.

TABLE IV

PRECISION OF THE CAPILLARY GC METHOD

Precision type	Concentration added (ng/ml)	Concentration found* (ng/ml)	Time interval	Number of replicates	Coefficient of variation (%)	Relative error** (%)
Intra-assay	0.050***	0.060	1 day	5	9.9	+19 6
	1.00	0.99		5	3.7	— 0.7
	10.0	10.5		5	2.3	+ 5.3
Inter-assay	0.50	0.50	4 weeks	6	2.7	— 0.4
	2.00	2.01		6	3.1	+ 0.3
	10.0	10 2		6	2.0	+ 2.1
Inter-assay	1.00	0.98	6 months	26	3.6	— 1.7
	10.0	10.4		23	3.3	+ 3.7
	100	102		9	1.9	+ 1.5

*Using compound III as internal standard.

**[(Concentration found — concentration added)/concentration added] · 100.

***Limit of quantification.

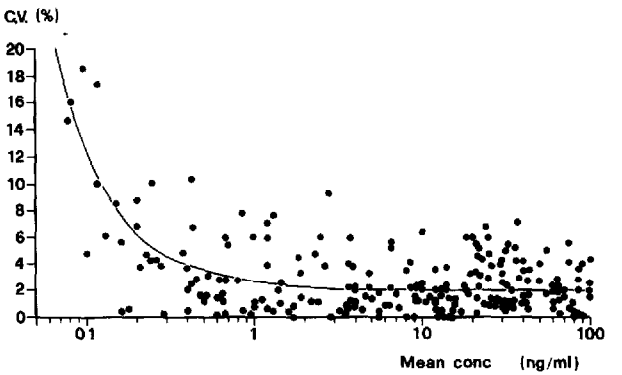


Fig. 5. Concentration dependence of the coefficient of variation (C.V.) obtained from 219 duplicate determinations of I in clinical samples.

Sensitivity, limit of detection, limit of quantification

Typical sensitivity values for the nitrogen—phosphorus detector are 0.4 pg/s of nitrogen, using azobenzene as the test compound.

The antagonist I was eluted with a peak width at half-height of 1 s. Thus a detection limit of 20 pg/ml I should theoretically be achieved, provided that the extract is dissolved in 50 μl of solvent and 1 μl is injected. By careful adjustment of the NPD bead and owing to the good response of I, 0.2 pg/s I could be detected experimentally with a signal-to-noise ratio of 3:1. Therefore, the limit of detection was 0.2 pg of I, which corresponded to a concentration of 10 pg/ml I in plasma. However, the practical limit of quantification, defined here as the minimum concentration that can be measured routinely with acceptable precision and accuracy, was 50 pg/ml (see Table IV and Fig. 5).

Linearity

In pharmacokinetic studies, plasma levels of I varied over a wide range (0.05—40 ng/ml) when a single intravenous dose of 2 mg of I was given to human volunteers. The calibration graphs were linear only up to 5 ng/ml, and a slight loss of linearity was observed in the extended range 0.0625—50 ng/ml.

Instead of dividing the calibration graph into several linear sub-ranges [25], a second-order polynomial fit was applied to the calibration data. A typical calibration graph could then be expressed by the equation $y = a + bx + cx^2$, whereby $a = 2.774 \cdot 10^{-4}$, $b = 5.024 \cdot 10^{-2}$ and $c = 2.195 \cdot 10^{-4}$.

According to Table IV, the precision of replicate measurements was nearly independent of concentration over a wide range, indicating that the variance was roughly proportional to the square of concentration. For this reason, the standard curve had to be calculated by means of a weighted least-squares regression procedure, using $1/y^2$ as weighting factor [15].

Precision

The precision of the method was evaluated by replicate analyses of spiked quality-control plasma samples, and by the analysis of clinical samples in duplicate over a wide concentration range [26].

The intra-assay precision was obtained by replicate analysis of control plasma samples on the same day. The inter-assay precision was determined by analysing the same control plasma sample on various days, using a separate calibration each day. The precision data (coefficient of variation of replicate analyses) and the accuracy data (relative error of the measurements) are given in Table IV.

Fig. 5 shows that the mean coefficient of variation of duplicate determinations performed on real samples was nearly constant at 2%, from 0.5 to 100 ng/ml. Below 0.5 ng/ml the coefficient of variation increased rapidly to 20% at the limit of quantification.

Application of the method to biological samples

The method has been applied successfully to the analysis of more than 300 plasma samples from a dose proportionality study performed in man. Fig. 1 shows a representative chromatogram from this study. The method was sen-

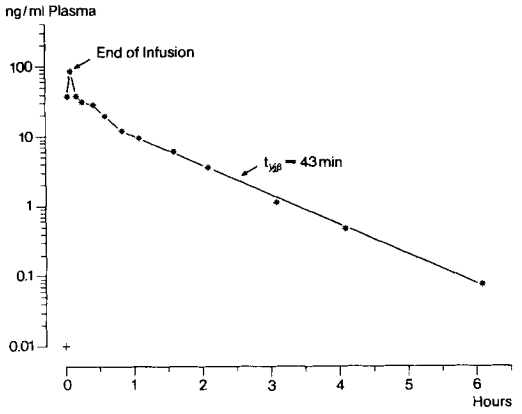


Fig. 6. Plasma concentration—time course of I following a single intravenous dose of 2 mg of the antagonist to a healthy male volunteer.

sitive enough to measure precisely the low concentrations of I in plasma for up to 6 h (corresponding to a period of approximately seven elimination half-lives) after a single intravenous dose of 2 mg to human volunteers (Fig. 6). As over 99% of the drug was eliminated within this period of time, the excellent sensitivity of the method allows the accurate determination of pharmacokinetic parameters from plasma concentration—time data even after doses close to the foreseen therapeutic intravenous dose (<1 mg).

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