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SIMULTANEOUS ASSAY OF TRIAZOLAM AND ITS MAIN HYDROXY METABOLITE IN PLASMA AND URINE BY CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A gas—liquid chromatographic method for the simultaneous determination of triazolam and its major hydroxy metabolite (1-hydroxymethyltriazolam) in human plasma and urine is described. After addition of two internal standards to the biological fluid, extraction at pH 9, acid washing, back-extraction, and derivatization, the analysis was performed on a wall-coated superior capacity open-tubular (WSCOT) CP-Sil 5 capillary column with electron-capture detection. The detection limit was 0.1—0.2 ng/ml; reproducibility was about 6—7% for plasma concentrations below 1 ng/ml. No interference from other possible minor hydroxy metabolites of triazolam was found. Gas chromatography coupled with mass spectrometry validated the chromatographic results. The method was successfully applied to plasma specimens collected from healthy human volunteers following a single intravenous administration of 1 mg of triazolam or 1-hydroxymethyltriazolam.

INTRODUCTION

Triazolam is a triazolobenzodiazepine derivative with sedative—hypnotic properties [1]. This compound is extensively metabolized in man, mainly by hydroxylation. The main metabolites (Table I) are 1-hydroxymethyltriazolam and 4-hydroxytriazolam [2, 3]. The 1-hydroxymethyl metabolite is reported to have 50—100% of the pharmacological activity of the parent compound [4].

Several methods have been described for the analysis of triazolam in biological fluids, including gas—liquid chromatography (GC) [5, 6], high-performance liquid chromatography (HPLC) [7, 8], radioimmunoassay (RIA) [9] and radioreceptorassay (RRA) [10]. RIA and RRA lack specificity as far as active metabolites are concerned, and GC and HPLC methods allow the

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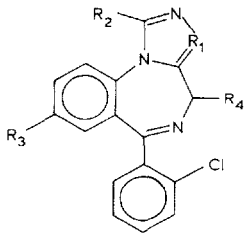
TABLE I

CHEMICAL STRUCTURES OF TRIAZOLAM, ITS HYDROXYLATED METABOLITES AND INTERNAL STANDARDS USED



Compound	Peak No.*	R_1	R_2	R_3	R_4
Triazolam	I	N	CH_3	Cl	H
1-Hydroxymethyltriazolam	II	N	CH_2OH	Cl	H
4-Hydroxytriazolam	III	N	CH_3	Cl	OH
1-Hydroxymethyl-4-hydroxytriazolam	IV	N	CH_2OH	Cl	OH
Estazolam	V	N	H	H	H
Ro 21-6962	VI	C-CH_2OH	CH_3	Cl	H

*Refers to labelled peaks in Figs. 1 and 3.

determination of triazolam only. It was considered important for the elucidation of the global pharmacokinetic behaviour of such a benzodiazepine to be able to determine both the parent drug and the 1-hydroxy active metabolite.

In this paper, a method is described for the simultaneous assay of triazolam and 1-hydroxymethyltriazolam in plasma and urine by GC. The method was validated by GC—mass spectrometry (MS), and was applied to pharmacokinetic studies in healthy volunteers.

EXPERIMENTAL

Reagents

Reagent-grade chemicals were used to prepare the following: phosphate buffer, 1.7 *M*, pH 10.5, prepared by dissolving 30 g of anhydrous K_2HPO_4 (Merck, Darmstadt, G.F.R.) in 100 ml of water and adjusting the mixture to pH 10.5 with KOH; buffer pH 9, obtained by a 15% dilution of concentrated buffer Titrisol (Merck); sulphuric acid 0.1 *N* from H_2SO_4 Ultrex. All these aqueous reagents were prepared with deionized and quartz-glass redistilled water, and then washed with diethyl ether agitation for approximately 2 h before use. Other reagents included diethyl ether, hexane, acetonitrile and toluene, all Pestipur brands supplied from SDS (Peypin, France). Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.).

Instrumentation

The GC apparatus employed was a Girdel (Suresnes, France) Model 330, equipped with a solid injection system (glass moving needle injector from

Girdel) and a 15 mCi electron-capture detector (ECD) operating with a pulse interval of 200 μsec. The column was a glass wall-coated superior capacity open-tubular (WSCOT) capillary column (25 m × 0.32 mm) with a polydimethylsiloxane (CP-Sil 5) as stationary phase (Chrompack, Orsay, France). The operating temperatures were injection port and detector 320°C, oven 280°C. Helium was used as carrier gas at a column head pressure of 0.8 bar. Argon—methane (95:5), at a flow rate of about 20 ml/min, was auxiliary gas. New capillary columns were conditioned before use by slow temperature programming (2°C/min) from 150 to 300°C and repeated injections of 2—3 μl of a 1% solution of cholesterol in toluene over 24—28 h. For GC—MS an HP 5985 connected to a data system HP 1000 (Hewlett-Packard, Les Ulis, France) was used with the same injection port and column model.

Standards

The compounds used were triazolam (T), 1-hydroxymethyltriazolam (1-HMT), 4-hydroxytriazolam (4-HT) and 1-hydroxymethyl-4-hydroxytriazolam (1-HM-4-HT) (Table I). All these compounds were supplied from Hoffmann-La Roche (Basle, Switzerland). Stock solutions were prepared by carefully weighing about 10 mg of each compound into a 10-ml volumetric flask and dissolving in methanol (Uvasol, Merck). Working solutions containing about 10 and 1 ng/ml were obtained by sequential dilutions of the respective stock solutions in methanol.

Internal standards

Estazolam (ET), supplied from Cassenne (Paris, France), was used for the triazolam assay, and Ro 21-6962, a hydroxylated imidazobenzodiazepine (Table I), supplied by Hoffmann-La Roche, was used for the 1-hydroxymethyltriazolam assay. For estazolam, stock and working solutions were prepared as described above for standards.

Ro 21-6962 after silylation of its hydroxy group by BSTFA gave two resolved peaks on a CP-Sil 5 capillary column. This compound was purified by HPLC as follows. Injections of stock solutions were performed on a Hewlett-Packard 1084 LC instrument equipped with an RP-18 column and UV detection (254 nm). The mobile phase was methanol—water (70:30) at a flow-rate of 1.5 ml/min. The two peaks observed under these conditions were collected separately and an aliquot was derivatized by BSTFA, analysed by GC, and identified by GC—MS in the chemical-ionization (CI) mode. The second HPLC peak corresponded to pure Ro 21-6962 and this fraction was used to prepare the stock solution. Working solutions were 0.5% solutions in methanol.

Preparation of samples

To 50 μl of methanol containing both internal standards estazolam and Ro 21-6962, 1 or 2 ml of buffer pH 9 and 0.5 or 1 ml of plasma, depending on expected concentrations, were added. After homogenization, the sample was extracted for 10 min with 10 ml of diethyl ether, then centrifuged for 5 min at 2500 *g*. The organic phase was evaporated to dryness under a stream of nitrogen and reconstituted in 1.5 ml of 0.1 *N* sulphuric acid. This was then washed for 2 min with 3 ml of hexane. The acid layer was adjusted to pH 9

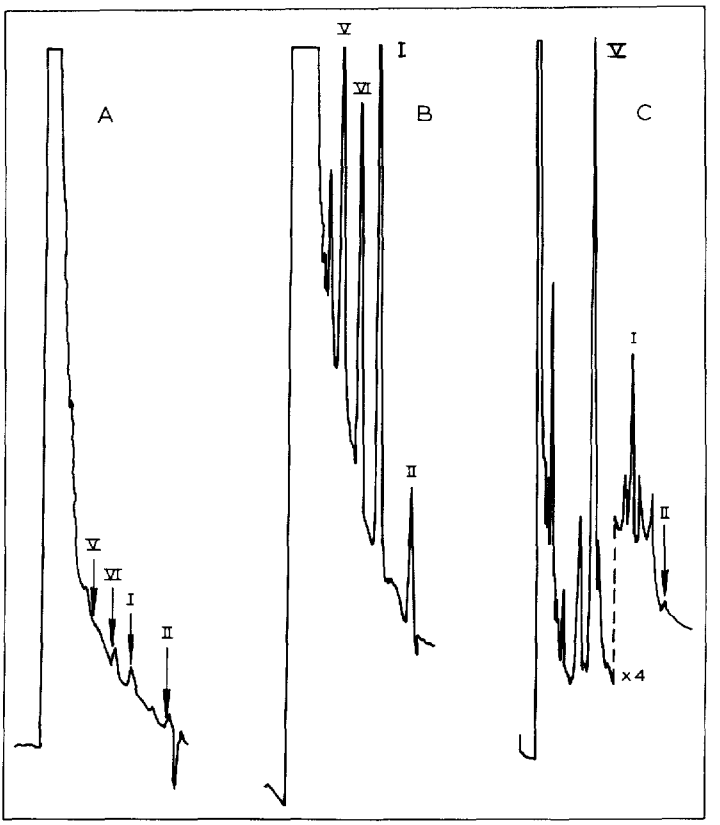


Fig. 1. Chromatograms obtained by GC—ECD after extraction and derivatization from: (A) control subject plasma; (B, C) subject plasma 1.5 h (9.3 ng triazolam; 1.2 ng 1-hydroxymethyltriazolam) and 12 h (0.18 ng triazolam; 1-hydroxymethyltriazolam not detectable) following a 1-mg intravenous bolus of triazolam. For peak labelling see Table I.

with phosphate buffer (approx. 1.5 ml), back-extracted for 10 min with 10 ml of diethyl ether and centrifuged for 15 min at 2500 *g*. The ether layer was concentrated to 500 μl, transferred to a 1.0-ml minivial (from Pierce) and evaporated to dryness under a gentle stream of pure nitrogen at 30—35°C, taking care that all water was removed. The residue was dissolved in 100 μl of acetonitrile; 10 μl of BSTFA were added as silylating agent and the minivial heated at 65°C for 15 min with continuous stirring (Reacti-Therm Heating Stirring module supplied from Pierce). After evaporation to dryness and reconstituting the residue in 50 μl of toluene, 2—3 μl were injected for GC analysis. Fig. 1 shows typical chromatograms, for control plasma extract (A), extracts of human plasma 1.5 h (B) and 12 h (C) following intravenous administration of 1 mg of triazolam.

Quantitation of unknown samples

Along with each set of unknowns, one blank plasma and two control plasmas to which had been added different concentrations of T and 1-HMT in the range to be determined were assayed. These calibration standards were used to establish daily a least-squares linear regression curve (from the peak area ratios

of T/ET and 1-HMT/Ro 21-6962 versus the respective plasma concentrations of T and 1-HMT. Peak areas were computed by means of a Hewlett-Packard 3388 A integrator. This internal standard curve was then used to interpolate unknown concentrations of triazolam and its hydroxylated metabolite in biological samples. Same calculations could be performed using peak height ratios instead of peak area ratios.

RESULTS AND DISCUSSION

Recovery, reproducibility, linearity, and sensitivity

The recovery of T and 1-HMT was calculated by comparing peak area ratios of T/ET and 1-HMT/Ro 21-6962 before extraction and after extraction of T and 1-HMT from human plasma. Then, the internal standards were added to the last extract. The extraction yield (Table II) was found to be satisfactory taking into account the extraction complexity, glass adsorption at low concentrations, and compound losses during the washing step due to the formation of an emulsion.

The reproducibility of the assay for T and 1-HMT is presented in Table III. The analysis precision given by the confidence interval of the mean value of each tested concentration was satisfactory for both compounds over the concentrations investigated. The analysis accuracy was obtained from the difference between expected and found concentrations, referring to a mean of three calibration curves established in the same period of time. These results, which appeared less acceptable than precision data, especially for low concentrations, reflect the variation of column conditioning and then of the column response from one day to another day. Thus, it seemed necessary, as indicated above, to undertake each day a limited two-point calibration curve with one blank in addition for the calculation of unknown plasma concentrations.

On the other hand, the linearity of the method was satisfactory for both compounds over the concentration ranges studied. In each case, one blank plasma and eight control plasmas, to which had been added four different concentrations (in duplicate) of T and 1-HMT, were simultaneously extracted

TABLE II

EXTRACTION YIELD OF TRIAZOLAM AND 1-HYDROXYMETHYLTRIAZOLAM FROM HUMAN PLASMA

$n = 6$.

Triazolam			1-Hydroxymethyltriazolam		
Concentration (ng/ml)	Recovery (%)	C.V.* (%)	Concentration (ng/ml)	Recovery (%)	C.V.* (%)
17.0	76	4	16.0	66	2
6.8	68	3	6.4	65	4
3.4	55	4	3.2	58	3
1.7	59	3	1.6	54	4

*C.V. = coefficient of variation.

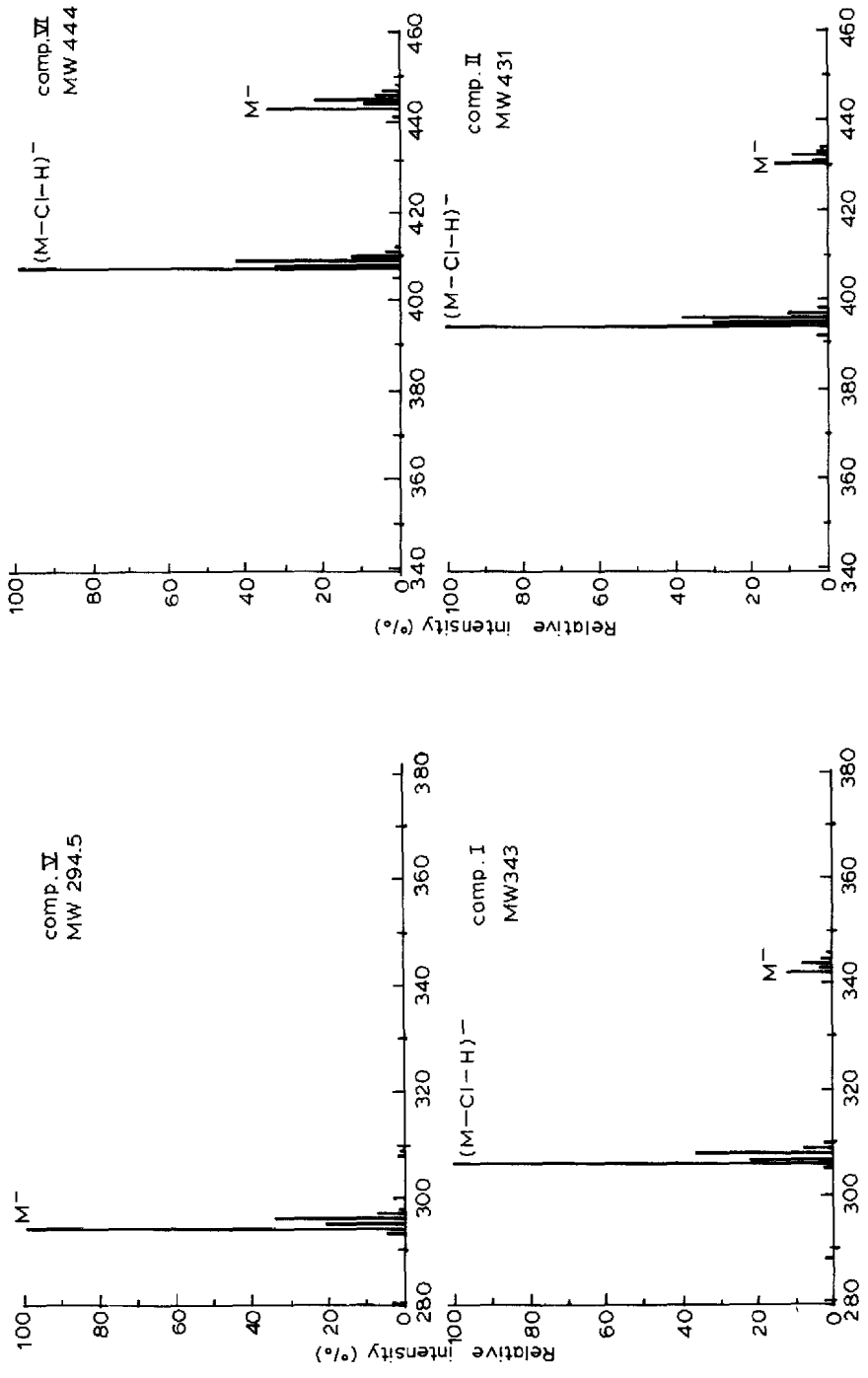


Fig. 2. Molecular mass spectra of analyzed compounds (T and 1-HMT) and respective internal standards (ET and Ro 21-6962).

TABLE III

INTRA-DAY REPRODUCIBILITY OF TRIAZOLAM AND 1-HYDROXYMETHYLTRIAZOLAM PLASMA ASSAY

n varies from 6 to 8.

Triazolam			1-Hydroxymethyltriazolam		
Concentration added (ng/ml)	Concentration found (ng/ml)	C.I.M.* (%)	Concentration added (ng/ml)	Concentration found (ng/ml)	C.I.M.* (%)
5.40	5.18	3	4.30	4.14	3.3
2.70	2.83	4.7	2.15	2.40	3.4
2.15	2.42	6.1	1.07	1.28	3.6
1.07	1.19	6.1	0.53	0.62	7.5
0.53	0.60	7.0			

*C.I.M. = confidence interval of mean (significance level = 0.05).

and derivatized according to the described procedure and chromatographed in triplicate. From the mean of peak area ratios T/ET and 1-HMT/Ro 21-6962 obtained for each investigated concentration, the equation of a typical calibration curve for triazolam concentrations of 2.15—21.5 ng/ml (estazolam: 11.6 ng) was $y = 0.8493 \cdot 10^{-2}x - 0.003$ (correlation coefficient $r = 0.9995$) and for 1-hydroxymethyltriazolam concentrations of 1.07—8.56 ng/ml, $y = 0.3530x + 0.005$ ($r = 0.9999$) (Ro 21-6962: 50 μl of a 0.5% methanol dilution of working solutions).

The sensitivity limit (Fig. 1) for plasma determinations, defined by a signal/noise ratio of about 3, was about 0.1—0.2 ng/ml with a 1.0-ml plasma specimen (this corresponds to an absolute amount of 10 pg of T or 1-HMT per injection). At this concentration, the relative error of the measurements, determined by analysing six control plasmas containing 0.2 ng/ml of standards, was about 15%.

Specificity

In relation to other hydroxylated metabolites of triazolam, the interference of 1-HM-4-HT can be neglected since this compound is, at most, present in trace amounts, while the formation of 4-HT seems to be a minor pathway of triazolam metabolism in man. This latter compound was incompletely derivatized using the conditions described. The retention time was slightly lower than that of triazolam and the detection limit higher. Moreover, during routine determinations on subject plasma, no interference near to the triazolam peak was observed. The best evidence for specificity came from the good agreement between the results obtained from the analysis of samples by both GC and GC—MS (Fig. 4), as mentioned below.

Validation of assay by GC—MS

The validity of the assay developed for plasma determinations of T and its main hydroxylated metabolite 1-HMT was confirmed by GC combined with an

identification of peak compounds by mass spectrometry. The GC—MS apparatus was operated in the negative CI mode. GC separations were carried out using a fused-silica wall-coated open tubular capillary column (12 m × 0.2 mm) with OV-101 as stationary phase. Oven temperature was programmed during analysis from 180°C to 280°C at a step rate of 15°C/min. The solid injector, ion source, and GC—MS interface were held at 300°C, 150°C and 270°C, respectively. Helium was used as carrier gas at a head column pressure of 0.5 bar. Methane was the reagent gas with an ion source pressure of 1 Torr. The mass spectrometer was operated with an emission current of 300 μA and an electron energy of 150 eV.

Under these conditons, mass spectra of T and 1-HMT with their respective internal standards, ET and Ro 21-6962, were obtained as presented in Fig. 2.

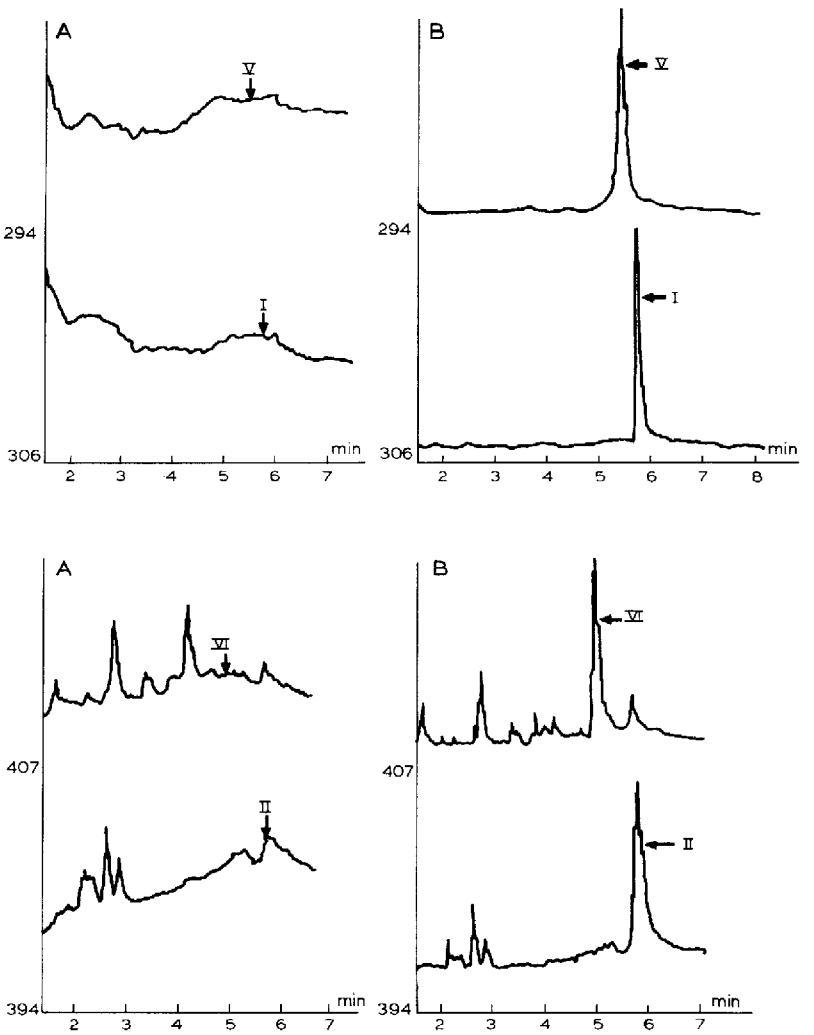


Fig. 3. Chromatograms obtained by GC—MS—CI after extraction and derivatization from (A) control subject plasma; (B) subject plasma 1.5 h following a 1-mg intravenous bolus of triazolam (same concentrations as in Fig. 1B). For peak labelling see Table I.

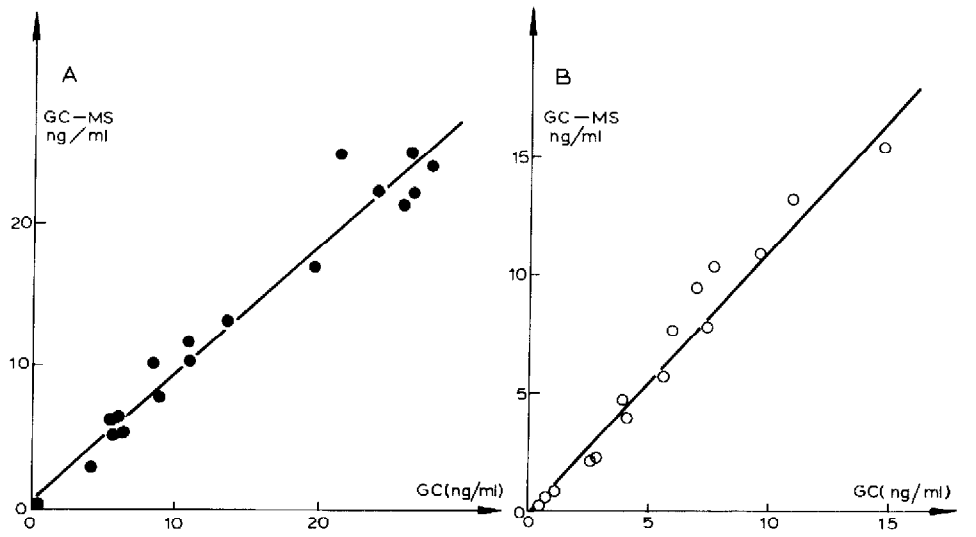


Fig. 4. Correlation between GC and GC—MS—CI results for triazolam (A) and 1-hydroxymethyltriazolam (B) plasma measurements.

These mass spectra were characterized by base isotopic peaks $(M - Cl - H)^-$ at 306, 394 and 407, respectively, for T and the TMS derivatives of 1-HMT and Ro 21-6962, and $(M)^-$ at 294 for ET. These ions, being the most intense, were used for quantitative determinations by mass fragmentography.

Typical chromatograms obtained following extraction and derivatization of subject plasmas, before administration of triazolam (blank), and 1.5 h after intravenous administration of 1 mg of triazolam are presented in Fig. 3. The same extracts were simultaneously analyzed by GC with ECD and MS detection. Correlations between GC—ECD and GC—MS—CI for 20 measurements are reported in Fig. 4A for triazolam determinations (regression curve $y = 0.8798x + 0.695$, $r = 0.978$) and in Fig. 4B for 1-HMT determinations (regression curve $y = 0.9319x - 0.166$, $r = 0.974$). The experimentally investigated concentrations ranged from 0.2 to about 20 ng/ml for each of these two compounds.

Discussion of application

The sensitivity of detection by ECD or MS operating in the negative CI mode, combined with the high efficiency of capillary columns, is sufficient to allow the pharmacokinetics in man of a benzodiazepine such as triazolam, under a very low dose administration (0.5—1.0 mg), to be followed. If only triazolam is to be determined, then the extraction procedure described may be used without derivatization and with different chromatographic conditions such as 290° C oven temperature and 0.9 bar column head pressure. This should allow a lower detection limit for plasma assay, the sensitivity limiting factor being glass adsorption during extraction and column adsorption. However, if simultaneous determination of T and 1-HMT is required, an extraction procedure with an acid-wash step and derivatization is imperative to prevent plasma extract contamination.

Application

Preliminary pharmacokinetic studies in six healthy volunteers following cross-over intravenous bolus of 1 mg of triazolam and 1 mg of 1-hydroxymethyltriazolam, with a one-week interval between the two administrations, were performed. Extensive treatment of these experimental data will be the subject of a further publication.

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