

Journal of Chromatography, 494 (1989) 119-134
Biomedical Applications
Elsevier Science Publishers B V, Amsterdam — Printed in The Netherlands

CHROMBIO 4849

DETERMINATION OF THE PARTIAL BENZODIAZEPINE RECEPTOR AGONIST Ro 16-6028 IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION AFTER CONVERSION INTO THE ETHYL ESTER DERIVATIVE

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(Received April 7th, 1989)

SUMMARY

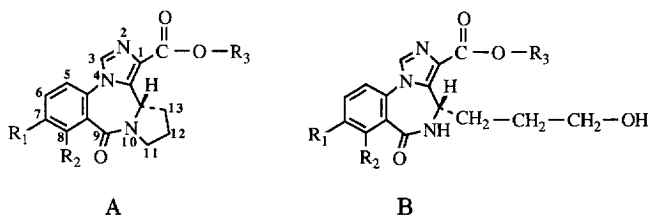
A highly sensitive capillary gas chromatographic method was developed to determine plasma levels of a novel partial benzodiazepine receptor agonist in man following the very low therapeutic doses required for anxiolysis. The compound was isolated from plasma by liquid-liquid extraction at basic pH, converted into the ethyl ester analogue by a two-step procedure, separated from plasma constituents by capillary gas chromatography and quantified by means of nitrogen-selective detection. Because of the thermolabile *tert*-butyl ester function, the agonist could not be gas chromatographed without degradation. Formation of the far more stable ethyl ester analogue was achieved by treatment with hydrogen chloride in ethanol, followed by an ethylation step with diazoethane. The high sensitivity of the new method (about 100 pg/ml, using 1-ml plasma specimens) allowed the monitoring of plasma levels of the agonist for up to 8 h (about three elimination half-lives) after a single 0.1-mg oral dose to human volunteers. The practicability of the procedure was demonstrated by the analysis of more than 600 plasma samples from clinical studies performed with human volunteers.

INTRODUCTION

tert-Butyl-(*S*)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*][1,4]benzodiazepine-1-carboxylate (Ro 16-6028, I, Table I, F Hoffmann-La Roche, Basle, Switzerland) is a tetracyclic imidazobenzodiazepine acting as a partial agonist at benzodiazepine receptors (BZR). Partial agonists yield a good separation between the pharmacological effects observed in animals with full agonists at BZR, such as diazepam. Thus, I has pronounced

TABLE I

STRUCTURES OF COMPOUNDS I-VIII



No	Compound	Type	R ₁	R ₂	R ₃
I	Parent compound	A	H	Br	C(CH ₃) ₃
II	Metabolite	B	H	Br	C(CH ₃) ₃
III	Metabolite	A	H	Br	CH ₂ C(CH ₃) ₂ OH
IV	Internal standard	A	Cl	Cl	C(CH ₃) ₃
V	Carboxylic acid of I and III	A	H	Br	H
VI	Ethyl ester of I and III	A	H	Br	CH ₂ CH ₃
VII	Ethyl ester of II	B	H	Br	CH ₂ CH ₃
VIII	Ethyl ester of IV	A	Cl	Cl	CH ₂ CH ₃

anticonflict and anticonvulsant activity, but is a much poorer sedative and muscle relaxant than diazepam [1]. In addition, I antagonizes various sedative effects of full agonists, but not their anticonvulsant activity [1]. Further, compound I exhibits an attenuated ethanol potentiation and a virtual lack of physical dependence liability [2], together with an absence of anticonvulsant tolerance, i.e., there is no loss of anticonvulsant effect with prolonged treatment, in contrast to full agonists at BZR [3]. Ongoing clinical studies have already clearly shown the beneficial effects of compound I in treating panic attacks and generalized anxiety disorders [1].

For pharmacokinetic studies, a sensitive method for the determination of compound I in plasma was required. First, a high-performance liquid chromatographic (HPLC) method with UV detection was developed by our group which could be used for toxicokinetic studies in dogs and rats [4]. However, the sensitivity of the method (2 ng/ml) was not sufficient to evaluate pharmacokinetic parameters of compound I in man near the envisaged therapeutic oral dose (1 mg). However, capillary gas chromatography (GC) with nitrogen-selective detection was shown to be very sensitive with respect to imidazobenzodiazepines, as described previously [5,6]. For this reason, a new GC method, involving splitless injection and nitrogen-selective detection, was developed. Because of the high sensitivity of the method, it was possible to monitor plasma levels of compound I for up to three elimination half-lives after a single 0.1-mg oral dose to human volunteers.

One major problem had to be overcome during development of the capillary

GC method Because of the thermolabile *tert*-butyl ester group, compound I could not be gas chromatographed in the picogram range without degradation, even when the on-column injection technique was applied for sample introduction To take advantage of the high sensitivity and separation power of capillary gas chromatography, compound I had to be converted into a stable derivative prior to GC analysis The ethyl ester VI, which could easily be formed from compound I by treatment with ethanolic hydrogen chloride and subsequent ethylation, proved to be suitable under splitless injection conditions.

EXPERIMENTAL

Reagents and solvents

Analytical-reagent grade chemicals were used, except where indicated otherwise

Dichloromethane (for pesticide residue analysis), ethanol, diethyl ether, *n*-hexane (for pesticide residue analysis), *n*-butyl acetate, sodium hydroxide and potassium hydroxide were obtained from E. Merck (Darmstadt, F.R.G). *n*-Butyl chloride (HPLC grade) was purchased from Fisons (Loughborough, U.K.) and acetyl chloride and *N*-nitroso- β -ethylaminoisobutyl methyl ketone from Fluka (Buchs, Switzerland).

n-Butyl chloride was carefully redistilled in an all-glass apparatus before use Doubly distilled water was used for the preparation of all aqueous solutions Sodium hydroxide solution (1 *M*) was purified by extraction with *n*-hexane. All other reagents were used without further purification

Hydrogen chloride in anhydrous ethanol (5 *M*) was freshly prepared daily by adding 713 μ l of acetyl chloride dropwise to 2 ml of cold ethanol (ice-bath) with stirring During preparation and storage the reagent was carefully protected from moisture

Diazoethane in diethyl ether was freshly prepared daily by the following procedure *N*-Nitroso- β -ethylaminoisobutyl methyl ketone (2 g) was dissolved in 18 ml of diethyl ether and treated at 0°C with 0.4 g of potassium hydroxide in 10 ml of ethanol-water (90:10, v/v) After standing for 5 min at room temperature, the ethereal diazoethane solution was separated by distillation at 45°C and stored at -20°C until used for ethylation

Preparation of plasma standards

Plasma standards used for calibration and quality control were prepared according to the following procedure A stock solution was obtained by dissolving 5 mg of compound I in 50 ml of ethanol Aliquots of the stock solution were diluted with ethanol to provide the working solutions Plasma standards were obtained by spiking blank plasma (20 ml) with 0.2 ml of the appropriate working solution, providing concentrations between 0.1 and 50 ng/ml

The stock solution could be stored in a refrigerator (4°C) for about 4 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 obtained from a blood bank (Blutspendezentrum, SRK, Basle, Switzerland), using heparin or sodium fluoride-potassium oxalate as anticoagulant. The blank plasma obtained by centrifugation (1000 g, 20 min) was tested for the absence of endogenous components interfering with the ethyl derivatives VI and/or VIII.

Extraction procedure

Frozen plasma samples were thawed at room temperature and homogenized by vortex-mixing. An aliquot of 1 ml of plasma was mixed with 50 μl of ethanolic internal standard solution (containing 0.5 μg IV per ml) and 50 μl of sodium hydroxide solution (1 M). The sample was then extracted with 5.5 ml of *n*-butyl chloride-dichloromethane (96:4, v/v) by shaking for 15 min at 15 rpm on a rotating shaker (Heidolph, Kelheim, F.R.G.). After centrifugation, 5 ml of the separated organic phase were carefully evaporated to dryness at 50°C by means of a gentle stream of pure (99.999%) nitrogen.

Derivatization procedure

The derivatization was carried out in disposable crimp-top autosampler vials (2 ml, 11 mm I.D., Hewlett-Packard, U.S.A.) crimp-sealed with a PTFE-lined cap. The plasma extract was dissolved in 100 μl of ethanol and quantitatively transferred to the vial. After adding 100 μl of hydrogen chloride in ethanol (5 M), the vial was carefully crimp-sealed and heated in a dry block heater, set at 90°C , for 30 min (Silli-Therm heating module; Pierce, Rockford, IL, U.S.A.). After opening the vial, the ethanolic hydrogen chloride was removed by evaporation for 90 min in a vacuo-centrifuge (Speed-Vac concentrator; Savant, Farmingdale, NY, U.S.A.) at 45°C and 250 mTorr.

The dried residue was dissolved in 100 μl of ethanol and then mixed with 100 μl diazoethane in diethyl ether. After standing for 15 min at room temperature the excess of reagent was evaporated for 15 min in the vacuo-centrifuge at room temperature and 250 mTorr.

Capillary gas chromatography

A Hewlett-Packard Model 5880A gas chromatograph equipped with a nitrogen-phosphorus detector, a split-splitless injector and a Model 7673A automatic sample injector was used.

The fused-silica capillary (30 m \times 0.32 mm I.D.) was coated with a dimethyldiphenylpolysiloxane liquid stationary phase of thickness 0.1 μm (DB-5; J&W Scientific, Rancho Cordova, CA, U.S.A.), both cross-linked and chemically bonded to the fused-silica surface. An uncoated, but deactivated, pre-column (1.2 m \times 0.32 mm I.D.) was attached to the analytical column by means

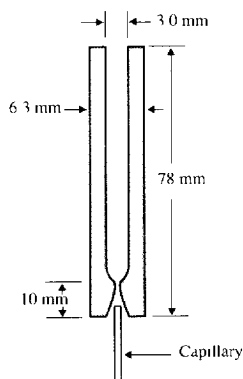


Fig 1 Modified injection liner

of a conical press-fit connector (Schmidlin, Zug, Switzerland), acting as a retention gap

A modified injection liner of fused silica or borosilicate glass was used according to Fig 1. The radial restriction of the liner was designed to be smaller than the outer diameter of the precolumn to facilitate the alignment of the capillary at 2–3 mm below the restriction point. Both the fused-silica liner (Hewlett-Packard) and the borosilicate glass liner were modified in-house, and a similar glass liner is commercially available (Amchro, Sulzbach, F R G).

The liner had to be carefully deactivated to prevent partial degradation of the analytes in the injector. In the case of the glass liner, a surface leaching procedure with hydrochloric acid was applied first to remove metal oxides and to form a silica gel layer on the surface by using the following procedure [7]. The liner was soaked in a solution of 20% hydrochloric acid in a polypropylene culture tube, placed in a glass flask fitted with a PTFE-faced cap and heated at 80°C overnight. Subsequently the liner was rinsed with dilute hydrochloric acid (1%) and dehydrated in an old GC injector at 280°C under an atmosphere of nitrogen. Both the fused-silica and the glass liner were deactivated in the vapour phase by injecting 5 µl of pure 1,3-diphenyl-1,1,3,3-tetramethyldisalanazane (Fluka) fifteen times within 1 h at 400°C, and then maintaining the liner at 400°C for an additional 3 h.

Helium was used as the carrier gas at inlet pressure of 140 kPa (1.4 bar), giving rise to a mean linear velocity of approximately 40 cm/s. The make-up gas was helium at a flow-rate of 30 ml/min. Hydrogen and air were used as detector gases at flow-rates of 3.0 and 50 ml/min, respectively.

The oven was operated isothermally at 120°C for 1 min after injection, then heated at 30°C/min to 280°C and held there for 7 min, and finally returned to 120°C. The injector and detector temperatures were maintained at 280 and 300°C, respectively.

Splitless injections were carried out with the automatic sample injector, us-

ing a split delay of 60 s. The inlet purge was adjusted to approximately 50 ml/min and a septum purge of 3 ml/min was employed.

Gas chromatographic analysis

The derivatized plasma extract was reconstituted in 25 μ l of *n*-butyl acetate, vortex-mixed for 10 s and finally transferred to a 200- μ l conical sample vial. An aliquot of 1.5 μ l was injected by means of the automatic sample injector adjusted to the fast injection mode.

The retention times for the derivatives VI and VIII were 8.6 and 9.15 min, respectively (Fig. 2). An analytical run lasted 15 min.

Calibration and calculation

Together with the unknown samples, five to seven plasma standards covering the expected concentration range were carried through the procedure. The calibration graph was obtained by weighted quadratic least-squares regression (weighting factor = $1/y^2$) of the measured peak-height ratios of the ethyl derivatives VI and VIII (y) versus the concentration of I (x) added to the plasma [8]. This regression equation was then used to calculate concentrations of compound I in unknown and quality control samples from the measured VI/VIII peak-height ratios.

Acquisition and on-line treatment of the data were carried out by means of a Model 4430 XWZ data system (Perkin-Elmer Nelson Systems, Cupertino, CA, U.S.A.) working with a software package recently developed in our laboratory. The chart speed of the recorder was adjusted to 2 cm/min for optimum reading of the peaks on the chromatograms.

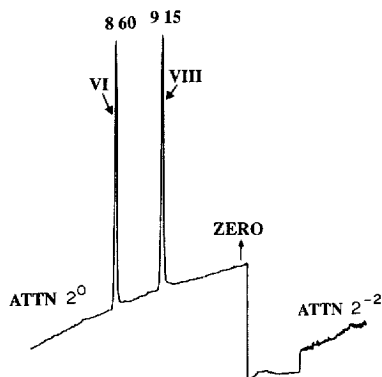


Fig. 2. Gas chromatogram of a pure solution containing compounds VI and VIII at concentrations of 40 pg/ μ l. For GC conditions, see *Capillary gas chromatography*, chart speed, 2 cm/min. The numbers above the peaks indicate the retention times of the compounds.

RESULTS AND DISCUSSION

Extraction and derivatization

The extraction conditions previously optimized for other imidazobenzodiazepines were also suitable for the isolation of I from plasma [9]. *n*-Butyl chloride–dichloromethane (96:4, v/v) as extractant yielded a high recovery of compound I and relatively clean plasma blanks. Co-extraction of interfering plasma constituents was further suppressed by choosing alkaline extraction conditions.

Not many procedures for converting a *tert*-butyl ester directly into the corresponding ethyl ester have been described. The only reaction suitable for micro-scale applications seems to be a transesterification by means of anhydrous ethanol in the presence of a strong acid as catalyst [10]. However, on applying this method to picogram amounts of compound I, not only the ethyl ester VI, but also considerable amounts of the corresponding carboxylic acid V were formed. Choosing more drastic reaction conditions (higher acid strength, higher temperature, longer reaction time) produced a shift of the V/VI ratio further towards VI, but also the overall yield of V and VI decreased because more side-products were formed. The problem was overcome by keeping the conditions of the transesterification as mild as possible, and converting any V formed into VI by means of an additional ethylation step.

The transesterification conditions were carefully optimized by altering the acid strength in the reaction mixture (1, 2, 2.5, 3 and 4 *M*), reaction time (15, 30, 45 and 60 min) and temperature (20, 60, 70, 80 and 90°C). It was found that treating compound I with 2.5 *M* hydrogen chloride in ethanol at 90°C for 30 min yielded the optimum compromise between a high overall yield of compounds V and VI and low formation of side-products. Complete removal of the hydrogen chloride after the first reaction proved to be an essential step. Any traces of hydrogen chloride that were left, not only deactivated the diazoethane during the ethylation but also, even worse, damaged the capillary column after only a few injections. Removal of the hydrogen chloride was performed most conveniently by means of a vacuo-centrifuge, whereas evaporating the acid by means of a stream of nitrogen was found to be less efficient and less reliable.

Diazoethane was preferred to other ethylation agents, because the reaction can be performed under mild conditions and the excess of reagent is readily removed by evaporation. The ethylation step was optimized by changing the volume of ethereal diazoethane (50, 100, 150, 200 and 300 μl), the reaction time (10, 20 and 40 min) and the volume of ethanol (0, 50 and 100 μl) used for increasing the rate of ethylation [11]. The best result was obtained by dissolving compound I in 100 μl of ethanol and agitating the solution with 100 μl of diazoethane in diethyl ether for 15 min at room temperature. To concentrate the sample, the reaction mixture was evaporated to dryness and the residue dissolved in a very small volume (25 μl) of *n*-butyl acetate.

Good precision and accuracy of the three-step procedure were achieved by

selecting a structurally related imidazobenzodiazepine as internal standard (IV), which also was converted to an ethyl ester (VIII) and showed, compared with compound I, almost identical behaviour during extraction, transesterification and ethylation

Capillary gas chromatography of compounds I and VI

Initial investigations indicated excessive degradation of I during the chromatographic process, even when on-column injection was employed. Because of the thermally controlled elimination of isobutene, the free acid V was formed, giving rise to a broad, distorted peak in the chromatograms (Figs. 3 and 4).

Preliminary trials to apply pyrolysis or reaction gas chromatography to the determination of underivatized I failed. No breakdown product could be detected with the nitrogen-phosphorus detector, which provided a reproducible, symmetrical peak in the lower nanogram range.

For these reasons, the *tert*-butyl ester I was converted into the far more stable ethyl ester VI. Because of the high sensitivity required for the determination of compound I in biological samples, any loss of substance during the introduction of VI into the capillary had to be avoided. With this and a high sample throughput in mind, on-column injection, programmed-temperature vaporization (PTV) and splitless injection were considered as possible injection techniques.

Unfortunately, at the beginning of this work, a PTV injector was not readily available. The on-column injection technique proved to be unsuitable because of impurities present in the extracts, after only 30 on-column injections the



Fig. 3 Gas chromatogram of a pure solution containing compound I at a concentration of 10 ng/ μ l demonstrating decomposition. Splitless injection mode, for GC conditions, see *Capillary gas chromatography*, chart speed, 2 cm/min. The numbers above the peaks indicate the retention times of the compounds.

Fig. 4 Gas chromatogram of an extract from a human plasma taken 3 h after an oral dose of 6 mg of compound I, containing I and III in their underivatized forms. On-column injection mode, for GC conditions, see *Selectivity*, chart speed, 2 cm/min, attenuation, 2^0 . The numbers above the peaks indicate the retention times of the compounds.

performance and the adsorption behaviour of the capillary column changed dramatically, resulting in a reduced sensitivity and a decrease in separation power, even though a retention gap was used.

For these reasons, the splitless injection mode was selected. However, when injecting pure solutions of picogram amounts of compound VI in the splitless mode into a split-splitless injector fitted with a conventional liner, an additional peak was detected in the chromatogram which did not occur under on-column injection conditions, indicating partial degradation of compound VI in the injector. To overcome this adverse injector effect with respect to reliable quantification of compound VI, a modified injection liner with a radial restriction was applied (Fig. 1) [12]. This modified liner had to be deactivated very carefully according to the procedure described above. Further, injections were performed by means of an automatic sampler in the fast injection mode, which reduced the actual dwell time of the needle in the injection port to only 100 ms, thus minimizing possible decomposition of sample constituents in the needle.

Fig. 2 depicts a chromatogram of a pure solution containing compounds VI and VIII at concentrations of 40 pg/ μ l of *n*-butyl acetate after splitless injection, demonstrating both the inertness of the liner and the high sensitivity of the GC system.

Performance and maintenance of the injector

The transfer of sample vapour from the vaporizing chamber (conical liner) into the column is the most critical factor in the splitless injection technique when applied to the quantification of analytes. Therefore, the applied injection volume selected was as small as possible to ensure rapid and almost complete sample transfer [13].

Under the given conditions, an injection volume of 1.5 μ l of *n*-butyl acetate produces a vapour volume of approximately 340 μ l. In addition, because the "solvent effect" was used, the vapour transfer is accelerated when the solvent recondenses in the column inlet [14]. Thus, the adjusted mean flow-rate of 48 μ l/s through the capillary allowed the transfer of more than six times the liner volume into the column during the splitless period of 60 s. This ensured a high yield of compound VI entering the column, provided that no decomposition of the analyte occurred.

There is a remote possibility that septum particles could partially plug the conical restriction, giving rise to irreproducible sample transfer. This can be avoided by positioning a light plug of silanized glass-wool just above the conical outlet.

To maintain optimum sample recovery and chromatographic efficiency, the liner was cleaned after about every 100 injections of samples by removing septum particles, residues of non-volatiles and decomposition products. Deactivation of the liner with 5% Surfasil[®] normally proved to be sufficient to restore the original inertness. In cases where the procedure failed, the liner had to be

discarded or immersed in concentrated sulphuric acid overnight and then treated by the deactivation procedure described above

Reproducibility of splitless injection

The reproducibility of the VI/VIII peak-height ratio for replicate injections was less than 1.5% for pure solutions and about 2% when plasma extracts were injected. For this reason, it was possible to perform only single sample injections, thereby allowing a high sample throughput per day.

Although raising the initial column temperature from 120 to 150°C resulted in a shorter analysis time, this was not used because of the resulting decrease in precision from 1.5 to 3.3%.

Stability and performance of the capillary column

The lengthy extraction and derivatization procedure restricted the number of samples that could be treated to 70 every 2 days, and these had to be analysed overnight. Therefore, the capillary had to be equipped with a retention gap [15–17], not only to re-concentrate the “bands broadened in space”, but also to spread involatile constituents of the sample along the uncoated pre-column, thereby enhancing the number of injections that could be made before the chromatographic system started to degrade [18]

During routine use, the pre-column was shortened by 50 cm on the injector side after approximately 100 injections. With this procedure, any accumulated non-volatiles were removed from the pre-column, provided that the flooded zone was less than 50 cm long, which was normally the case

The analytical column had to be exchanged only when its adsorptive behaviour towards compound VI became unacceptable with respect to reliable quantification. Usually more than 2000 analytical runs could be performed before the capillary started to deteriorate

Depending on the type of individual samples, a slight shift towards longer retention times of compounds VI and VIII in the range 1–2 s could sometimes be observed. This effect was probably caused by temporary coating of the pre-column with high-boiling compounds. However, under the conditions described here, this effect had no influence on the quality of the quantification.

A decrease in the analysis time for one analytical run from 15 to 13 min can be achieved by raising the final temperature from 280 to 310°C immediately after elution of the internal standard, to remove high-boiling co-extracted sample components from the capillary. However, the lifetime of the analytical column will also be adversely affected in that the inertness of the column will start to degrade much earlier as a result of this additional temperature stress.

Detector optimization

The selectivity of the nitrogen–phosphorus detector for nitrogen and phosphorus-containing compounds with respect to hydrocarbons was found to be

approximately 50 000 (determined between azobenzene and malathion versus octadecane) The excellent selectivities permitted the application of the relatively simple clean-up procedure described above, as it was not necessary to eliminate the whole bulk of interfering carbon compounds before analysis.

Recovery

Human blank plasma was spiked with compound I at concentrations of 0.5, 2.5 and 25 ng/ml. The plasma was divided into 1-ml aliquots, extracted and derivatized as described but without adding the internal standard IV. The residues obtained after ethylation were dissolved in *n*-butyl acetate containing the ethyl ester VIII as internal standard and chromatographed as described.

A second series of control samples, providing the 100% values, was prepared by extracting and derivatizing 1-ml aliquots of human blank plasma and then adding equimolar amounts of VI and the internal standard VIII in *n*-butyl acetate to the dry residues.

The analytical recovery was calculated by comparing the VI/VIII peak-height ratios of the extracted and derivatized samples to the ratios obtained from the control samples to which compound VI had been added after extraction and derivatization. The overall recovery was calculated by correcting the analytical recovery by the aliquot factors.

The data in Table II indicate a satisfactory overall recovery of compound I from human plasma of ca. 60–70%, which decreased slightly with increasing concentration. Choosing milder reaction conditions during the transesterification step resulted in a decrease in recovery from about 60% to 35% (Table III).

Selectivity

The method was very specific with respect to endogenous components co-extracted from plasma. In the more than 100 clinical blank plasma samples (pre-dose, placebo) analysed so far, in no case could interfering compounds be

TABLE II
RECOVERY OF COMPOUND I FROM HUMAN PLASMA

Concentration added (ng/ml)	Concentration found (ng/ml)	Number of replicates (<i>n</i>)	Relative standard deviation (%)	Recovery (%)
0.5	0.36	5	15.3	72.1
2.5	1.68	5	7.8	67.1
25.0	15.2	4	1.8	60.9

TABLE III

RECOVERY OF COMPOUNDS I AND III FROM HUMAN PLASMA UNDER DIFFERENT TRANSESTERIFICATION CONDITIONS ($n=2$)

Conditions during transesterification			Recovery of I (%)	Recovery of III (%)	Ratio ^a
Temperature (°C)	HCl (M)	Time (min)			
90 ^b	2.5 ^b	30 ^b	59.8	2.8	21.4
90	2.0	30	67.6	3.2	21.1
90	2.5	20	57.6	2.0	28.8
80	2.5	30	43.8	1.8	24.3
80	2.0	20	37.9	1.6	23.7
80	1.5	20	35.6	1.3	27.4

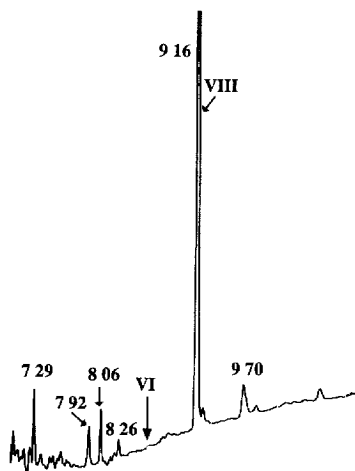
^aRatio = recovery of I/recovery of III^bStandard conditions

Fig 5 Gas chromatogram of a typical human blank plasma taken before administration of compound I. The arrow indicates the retention time of compound VI, for GC conditions, see *Capillary gas chromatography*, chart speed, 2 cm/min, attenuation, 2⁰. The numbers above the peaks indicate the retention times of the compounds.

detected that co-eluted with the ethyl ester VI. Fig 5 shows a typical chromatogram of a plasma sample before drug administration.

Metabolic investigations on compound I are continuing, so that the specificity of the method towards metabolites could not be fully evaluated. Six metabolites have been identified by Jauch [1], using the isolated perfused liver. As primary targets of oxidative metabolic attack, the carbon atom at position 11 and the *tert*-butyl side-chain of compound I were recognized. Further metab-

olism occurs by additional oxidative and reductive processes. Conjugation plays only a minor role in the overall biotransformation of the substance [19]. The metabolites II and III have been synthesized and could be used to test the specificity of the method.

Metabolite II also forms an ethyl ester (VII) during the derivatization procedure, which was well separated from compound VI during capillary gas chromatography.

The structure of metabolite III differs from the parent compound only in the ester side-chain and, therefore, the identical ethyl ester (VI) was formed during treatment with hydrogen chloride in ethanol. Fortunately, this lack of selectivity of the method towards compound III did not prove to be a critical factor in practice. It could be demonstrated that the contribution of compound III to the ethyl ester VI was small (<1%), in the following way. First, the hydroxy isobutyl ester side-chain of compound III was much more resistant to acids than the *tert.*-butyl ester group, so that, compared with compound I, the recovery of compound III was at least 20-fold less under all tested transesterification conditions (Table III). Second, metabolite III appeared in human plasma at concentrations that were at least ten times lower than those of the parent compound. This was determined using plasma samples from a high-dose tolerance study; concentrations of compound I were determined by HPLC (which separates I and III), and III was determined by capillary GC as follows.

Because the thermolability of compound III was less pronounced than that of compound I, the former could be gas chromatographed without derivatization by applying cold on-column injection and choosing a capillary with an excellent adsorptive behaviour towards polar solutes, as judged by the Grob test [20,21]. The selected capillary (30 m × 0.32 mm I.D.) was coated with a dimethylpolysiloxane liquid stationary phase of thickness 0.1 μm (DB-1; J&W Scientific), connected to a deactivated precolumn (1.2 m × 0.32 mm I.D.) by means of a deactivated glass press-fit connector. All other conditions were the same as described for compound VI.

A typical chromatogram of a human plasma extract is shown in Fig. 4. The metabolite III has a retention time of 8.95 min and was well separated from endogenous compounds. The broad distorted peak eluting beyond 7.03 min corresponds to the decomposition product of compound I, representing 39.1 ng I per ml. The quantification limit of compound III in human plasma was only 0.5 ng/ml because of the lower extraction yield of compound III compared with compound I. Nevertheless, the sensitivity for compound III was sufficient to calculate the possible error contribution to the quantification of compound VI in human plasma.

Limit of quantification

The detection limit, defined by a signal-to-noise ratio of 3:1, was 20 pg/ml, using a 1-ml specimen of plasma. The limit of quantification, defined here as the limit at which the procedure will be sufficiently precise and accurate to

TABLE IV

PRECISION

Type	Concentration added (ng/ml)	Concentration found (ng/ml)	Time interval	Number of replicates (n)	Relative standard deviation (%)	Difference between found and added concentration (%)
Intra-assay	0.1	0.102	1 day	6	11.3	+1.5
Inter-assay	0.5	0.499	5 weeks	6	9.4	-0.2
	2.5	2.56		6	4.6	+2.5
	5.0	4.94		6	5.0	-1.2
	25.0	26.0		6	5.0	+4.1

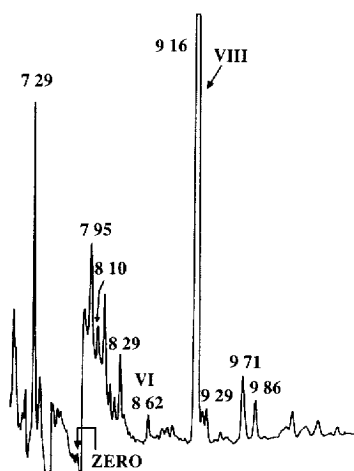


Fig 6 Gas chromatogram of a human plasma sample taken 8 h after an oral dose of 0.1 mg of compound I, calculated concentration of I (determined as VI), 125 pg/ml, for GC conditions, see *Capillary gas chromatography*, chart speed, 2 cm/min, attenuation, 2⁰. The numbers above the peaks indicate the retention times of the compounds.

yield a satisfactory quantitative estimate of the unknown concentration, was 100 pg/ml (Table IV). Fig 6 shows a chromatogram of a clinical plasma sample containing compound I at a concentration near the limit of quantification.

Owing to this high sensitivity, it was possible to measure precisely the low concentrations of compound I in plasma for up to 6–8 h (corresponding to a period of approximately three elimination half-lives) after a single oral dose of 0.1 mg to human volunteers (Fig 7).

Linearity

The correlation between the VI/VIII peak-height ratio versus the concentration of compound I in plasma was slightly non-linear in the range 0.1–25

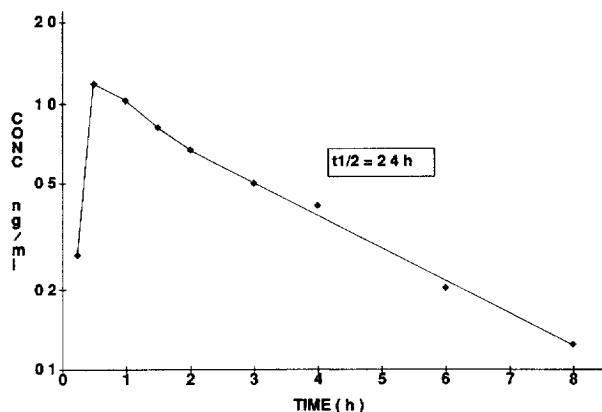


Fig 7 Plasma concentration-time course of compound I following a single oral dose of 0.1 mg of I to a healthy male volunteer

TABLE V

STABILITY OF COMPOUND I IN HUMAN PLASMA

Storage conditions	Concentration added (ng/ml)	Change of concentration after storage (%)	90% Confidence interval (%)	Number of replicates (n)
3 months at -20°C	1	-1.0	-6.3 to +4.7	4
	10	+0.2	-2.1 to +2.5	4
	100	-4.1	-6.6 to -1.6	6
	500	-1.9	-4.5 to +0.8	6
24 h at $+25^{\circ}\text{C}$	100	-1.0	-2.1 to +0.1	6
	500	+1.1	-0.4 to +2.6	6

ng/ml Applying a second-order polynomial fit to the data, a typical calibration graph could be expressed by the equation $y = a + bx + cx^2$, whereby $a = 0.0089257$, $b = 0.6758$ and $c = 0.0065536$. On the other hand, the calibration graph could also be divided into two parts (0.1-5 and 2.5-25 ng/ml), each of which was sufficiently linear

Precision

The inter-assay precision was measured at different concentration levels around therapeutic concentrations in human plasma. For each level a spiked plasma sample was prepared and analysed on different days (using a separate calibration graph on each day). The data in Table IV demonstrate that the precision of the three-step procedure was good over the whole concentration range

Stability in plasma

The partial benzodiazepine agonist I was added to human plasma at four different concentrations (1, 10, 100 and 500 ng/ml) and stored under two different conditions (3 months at -20°C , 24 h at 25°C) prior to analysis. The statistical interpretation of the data followed a procedure developed recently [22]. The data in Table V indicate that compound I was stable in human plasma under these conditions. All confidence intervals for the percentage changes in concentration after storage were narrow, demonstrating the good precision of the method.

ACKNOWLEDGEMENTS

The authors thank Dr R Jauch and Dr L Pieri for helpful advice and Dr D Dell for fruitful discussions and for correcting the manuscript. Thanks are due to Dr W Hunkeler for supplying the reference compounds and the internal standard.

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