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# DETERMINATION OF A NEW NON-BENZODIAZEPINE ANXIOLYTIC AND ITS O-DEMETHYL METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN-SWITCHING

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## SUMMARY

An highly sensitive and fully automated high-performance liquid chromatographic assay was developed for the determination of a novel non-benzodiazepine anxiolytic (I) {(*R*)-2-(methoxymethyl)-1-[(7-oxo-8-phenyl-7H-thieno[2,3-*a*]quinolizin-10-yl)carbonyl]pyrrolidine} and its O-demethyl metabolite (II) in plasma, using column-switching for direct injection of plasma samples. After dilution in internal standard solution, the sample was injected onto a pre-column (17 mm × 4.6 mm) dry-packed with pellicular $C_{18}$ reversed-phase material. Polar plasma components were removed by flushing the pre-column with water–acetonitrile (90:10, v/v). Retained substances, including I and II, were backflushed onto an analytical column, separated by gradient elution and detected by means of fluorescence detection (excitation, 304 nm; emission, 475 nm). After washing the analytical column and re-equilibrating the pre-column, the system was ready for the next injection. The limit of quantification for I and II was 0.25 and 0.5 ng/ml, respectively, using a 350-$\mu$l specimen of plasma. The practicability of the new method was demonstrated by analysis of more than 300 plasma samples from a tolerance study performed with human volunteers. Owing to its high sensitivity, the method can be used to calculate pharmacokinetic parameters of compounds I and II in man after a single oral dose of about 1 mg of I.

## INTRODUCTION

(*R*)-2-(Methoxymethyl)-1-[(7-oxo-8-phenyl-7H-thieno[2,3-*a*]quinolizin-10-yl)-carbonyl]pyrrolidine, I (Ro 19-5686; F. Hoffmann-La Roche, Basle, Switzerland) (see Fig. 1), is a novel substituted thienoquinolizinone acting as a partial agonist at the benzodiazepine receptor complex. This property suggests good anxiolytic action, together with a reduced side-effect profile such as reduced motor impairment, decreased ethanol potentiation and less physical dependence liability[1]. Metabolic investigations

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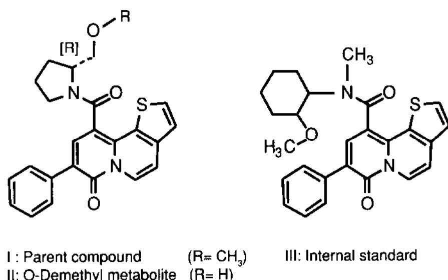


Fig. 1. Chemical structures for the compounds referred to in the text.

have shown that the O-demethyl derivative (II) is the major metabolite in plasma of rat, dog and man, with a similar pharmacological profile to that of the parent compound[2].

For pharmacokinetic studies, a precise method for the determination of plasma concentrations of compounds I and II was needed, taking account of the marked light sensitivity of the thienoquinolizinones. Fully automated high-performance liquid chromatographic (HPLC) column-switching techniques offer certain advantages for the analysis of photolabile compounds[3,4]. Compared with other techniques, minimum sample handling is involved where the samples have to be protected from light, while the on-line clean-up procedure can be carried out under normal laboratory light conditions.

In this report a highly sensitive HPLC column-switching method with fluorimetric detection is described for the determination of compounds I and II in plasma.

## EXPERIMENTAL

### *Laboratory precautions*

All handling of reference compounds and solutions was performed in a darkened laboratory. Stock solutions of compounds I–III were prepared in amber-glass volumetric flasks and kept in the dark at 4°C under an helium atmosphere. Working solutions were always freshly prepared prior to use. Plasma standards were prepared in amberized glassware. Pre-treatment of biological samples was carried out in a darkened laboratory. Plasma standards and biological samples were protected from light during storage in the freezer by wrapping the racks in aluminium foil.

### *Reagents and solvents*

Acetonitrile (HPLC grade S) was obtained from Rathburn (Walkerburn, U.K.), and tetrahydrofuran (unstabilized, HPLC grade) from Fisons (Loughborough, U.K.). Methanol (HPLC grade) was obtained from Fluka (Buchs, Switzerland) and water (HPLC grade) from Baker (Deventer, The Netherlands). Plasma standards were prepared using pre-tested fresh frozen plasma from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland).

### *Solutions and plasma standards*

A stock solution of the parent compound and its O-demethyl metabolite was prepared by dissolving 10 mg of compound I and 10 mg of compound II in 10 ml of

methanol. Aliquots of the stock solution were diluted in methanol, providing the working solutions. The plasma standards were obtained by spiking blank plasma (20 ml) with 100 $\mu$l of the corresponding working solution, providing concentrations between 0.25 and 2500 ng/ml of the two compounds. The standards were divided into aliquots of 1.5 ml and stored deep frozen (−20°C) until required for analysis.

*Instrumentation*

A schematic representation of the column-switching system is given in Fig. 2. The single-piston pump P1 (Model 414; Kontron, Zürich, Switzerland) delivered the purge solvent mixture M1 at a flow-rate of 2 ml/min. Aliquots (100–500 $\mu$l) of diluted plasma samples were injected by means of an automatic sample injector I1 [Model WISP 710B assembled with a cooling module (10°C); Waters, Milford, MA, U.S.A.] onto the pre-column PC. Injection of plasma samples larger than 200 $\mu$l was achieved by using a 1-ml syringe and the built-in auxiliary loop, and by decreasing the syringe motor rate to 1.85 $\mu$l/s. An UV detector D1 (Model Spectroflow 773; Kratos, Westwood, NJ, U.S.A.), operating at 254 nm, together with a recorder R (Model W + W

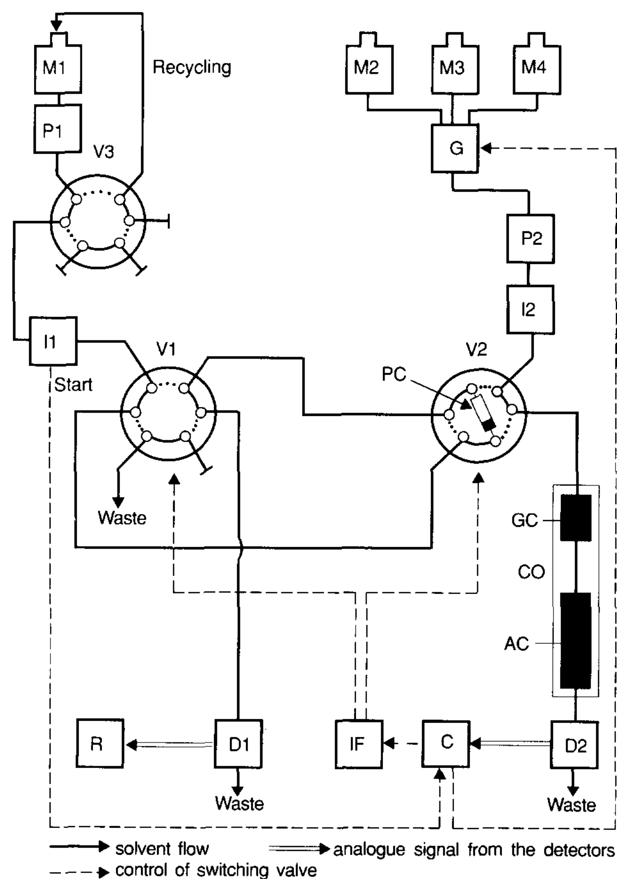


Fig. 2. Schematic representation of the column-switching system. The valves are shown in position 0.

600; Kontron) was used to monitor the removal of plasma components from PC during the purge step. A low-pressure gradient system G (Model Spectroflow 430, Kratos) together with a double-piston pump P2 (Model Spectroflow 400; Kratos) delivered the gradient mobile phases M2–M4 at a flow-rate of 1.5 ml/min for chromatography of the retained substances on the analytical column AC. A column oven CO (Model 7930; Jones, U.K.) allowed the analytical separation to be performed at increased temperature (35°C). A manual injector I2 (Model 7125) equipped with a 100-$\mu$l loop (Rheodyne, Cotati, CA, U.S.A.) was used for injection of control solutions directly onto the analytical column. Detection of the eluted compounds was carried out by means of a fluorescence detector D2 (Merck; excitation, 304 nm; emission, 475 nm; sensitivity 1–5). Data handling was carried out by means of a computing integrator C (Model SP 4200; Spectra Physics, San José, CA, U.S.A.) working with a special BASIC program, originally developed for the integrator SP 4100[5]. The gradient former G and the three air-actuated switching valves V1—V3 (Model 7000 assembled with a Model 7001 pneumatic actuator and a Model 7163 solenoid valve; Rheodyne) were controlled by the external time events of the computing integrator. To achieve compatibility, a laboratory-made interface IF was placed between the integrator output and the solenoid valve input[6].

*Columns and mobile phases*

The pre-column PC (17 mm × 4.6 mm; Bischoff-Analysentechnik, Leonberg, F.R.G.) was dry-packed with Bondapak $C_{18}$ Corasil, 37–50 $\mu$m (Waters). In order to prevent blockages caused by viscous or solid plasma constituents, PC was used only with metal sieves (3 $\mu$m, two pieces on each side), without fibre-glass filters[7]. The packing was first rinsed with methanol (removal of impurities, activation of the reversed-phase material) and then conditioned with M1 prior to use.

The analytical column AC (250 mm × 4 mm, E. Merck) was packed with $\mu$Bondapak $C_{18}$, 10 $\mu$m (Waters) by means of the usual slurry technique. A small guard column GC (17 mm × 4.6 mm; Bischoff), slurry-packed with the same material, was used to extend the lifetime of the analytical column.

The pre-column was replaced daily, or after 50 injections. The guard column was exchanged weekly, or after 250 samples. The analytical column had a lifetime of about 750 injections.

Four different mobile phases were used: M1, water–acetonitrile (90:10, v/v); M2, water–acetonitrile–tetrahydrofuran (62:36:2, v/v/v); M3, water–acetonitrile–tetrahydrofuran (50:40:10, v/v/v); M4, water–acetonitrile (15:85, v/v). All mobile phases were degassed with helium prior to use. The gradient system is described under *Analytical procedure*.

*Pre-treatment of samples*

The samples were thawed at room temperature and homogenized by ultrasonication (2 min). An aliquot of 350 $\mu$l plasma was diluted in an equal volume of a solution containing an appropriate amount of internal standard in water–acetonitrile (80:20, v/v). After vortex-mixing for 15 s, the resulting mixture was centrifuged at 1700 *g* for 5 min. The clear supernatant was transferred to the injection vial and stored at 10°C in the autosampler cabinet prior to injection.

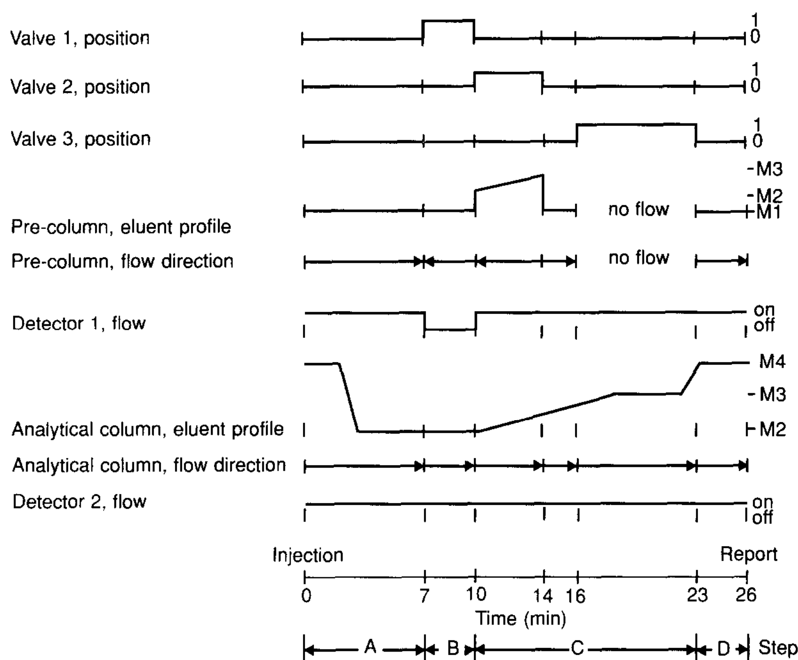


Fig. 3. Flow diagrams of the column-switching procedure (see text for further details).

*Analytical procedure*

A complete automated sample analysis lasted 26 min, and included the following four column-switching steps (see also flow diagrams in Fig. 3).

*Step A (0–7 min; V1 = 0, V2 = 0, V3 = 0).* An aliquot of a diluted and centrifuged plasma sample was injected onto PC. Proteins and other hydrophilic compounds were flushed with M1 to waste, while lipophilic compounds (including I–III) were retained on the top of PC. Progress of the clean-up process could be monitored by means of D1. Parallel to this process, GC and AC were washed for 2 min with M4 in order to remove strongly retained plasma components from the previous run. After changing within 1 min from 100% M4 to 100% M2, GC and AC were then reconditioned for 4 min with M2.

*Step B (7–10 min; V1 = 1, V2 = 0, V3 = 0).* PC was backflushed with M1 in order to remove trapped solid material (abrasion, precipiated proteins, etc.) without washing out the analyte. In order to prevent blockages, the purged fraction was led directly to waste without passing through the detector cell. The reconditioning process of GC and AC with M2 was continued in parallel.

*Step C (10–14 min; V1 = 0, V2 = 1, V3 = 0; 14–16 min, V1 = 0, V2 = 0, V3 = 0; 16–23 min, V1 = 0, V2 = 0, V3 = 1).* M1 was passed directly to waste. Enriched components were desorbed and transferred from PC to GC/AC in the backflush mode by means of the analytical mobile phase. After 4 min, PC was separated from GC/AC, purged for 2 min with M1 and then left without flow for 7 min. The transferred material was chromatographed on GC/AC using the following gradient system: from 100% M2 to 40% M2–60% M3 (10–16 min), to 100% M3 (16–19 min) and 100% M3 (19–23 min). The separated compounds were quantified by means of D2.

*Step D (23–26 min; V1 = 0, V2 = 0, V3 = 0).* PC was reconditioned with M1. At the same time, strongly retained components were removed from GC/AC by the following gradient system: from 100% M3 to 100% M4 (23–24 min), and 100% M4 (24–26 min). At the end of Step D the report was generated by the computing integrator and the system was ready for the next injection.

*Calibration and calculation*

Along with the unknown samples, five to seven plasma standards with appropriate drug concentrations were carried through the procedure. The calibration graph for the parent compound was obtained by weighted linear least-squares regression (weighting factor = $1/y^2$) of the measured peak height ratios of I/III *versus* the concentrations of compound I added to the plasma. In the same way, the calibration graph for the metabolite was established using the peak height ratios of II/III. These calibration graphs were then used to interpolate the concentrations of compounds I and II in biological samples from the measured peak height ratios I/III and II/III, respectively.

## RESULTS AND DISCUSSION

*Pre-treatment of samples*

The fully automated column-switching method required only a minimum of sample pre-treatment: the plasma was diluted in 20% acetonitrile in water (containing compound III as the internal standard), centrifuged and stored in the dark at decreased temperature prior to injection.

Diluting the plasma in water decreased the viscosity of the samples, which had a positive effect on the lifetime of PC. Acetonitrile was added to the samples to improve the recovery of compounds I–III from plasma. Use of the internal standard improved the reliability and accuracy of the method, especially when long sample sequences had to be processed. The centrifugation step was necessary to remove solid material from the sample, thereby preventing premature blockage of PC. Finally, use of a cooled autosampler improved the stability of the compounds in plasma during overnight storage in the automatic sample injector[3].

A detailed study on the photochemical behaviour of compound I had shown that, in the presence of oxygen (air), the compounds were sensitive to light. Not only daylight but also light from neon tubes was absorbed by the yellow compounds, which, consequently, lead to at least twelve photodegradation products[8]. (Under the chromatographic conditions described in this report, these degradation products were not detected.) For this reason, plasma samples had to be carefully protected from light during collection, storage and analysis.

As already pointed out[3,4], column-switching offers certain advantages during the analysis of photo-labile substances. Compared with other techniques, *i.e.*, liquid–liquid extraction, only a few pre-treatment steps are involved in which the samples have to be protected from light. In the case of the method described here, only thawing, dilution and centrifuging of the samples needed to be performed in the darkened laboratory, while the on-line clean-up procedure and chromatography could be carried out under normal laboratory conditions, since the samples are not exposed to light.

*Optimization of the on-line clean-up procedure*

Various reversed-phase materials with a particle size between 30 and 50 $\mu$m were tested as potential sorbents in the precolumn, such as Corasil $C_{18}$, Phenyl-Corasil (Waters), Nucleosil $C_{18}$ (Macherey-Nagel), Vydac RP (Separations Group), Sepralyte $C_{18}$, $C_2$, PH and 2 PH (Analytichem). All these materials were able to retain compounds I–III sufficiently from plasma. Sorbents of the porous layer bead (PLB) type could be cleared more easily from plasma constituents than completely porous materials. Packing of PC was easier with PLB than with porous sorbents. The Sepralyte materials were mechanically less stable than Corasil and Nucleosil, which have been especially designed for high-pressure applications. For these reasons, Corasil $C_{18}$ was selected as the most suitable material for routine applications.

In most applications, water is used (without any additives) as a purge solvent M1 in column-switching systems. However, it has been shown that better recoveries of strongly protein-bound pharmaceuticals can be obtained by adding a small percentage of acetonitrile to the water[4,9]. For this reason, and also to facilitate the removal of undesired plasma components from PC, a mixture of 10% acetonitrile in water was used as M1 during the enrichment step.

The quality of the water used in M1 was a critical factor during the assay. Organic impurities dissolved in the water were trapped on PC during the reconditioning and the enrichment phase, giving rise to interference problems after transfer to GC/AC. For this reason, only special water of HPLC-grade quality was used when low-concentration samples had to be measured. Also the volume of M1 passing through PC during the reconditioning step was limited to a minimum of 10 ml. This was achieved by introducing an additional valve (V1) into the system which enabled PC to be taken out of the stream of M1 for a certain time (7 min) (see diagrams in Fig. 3).

The optimum purge time for complete removal of proteins and other polar plasma constituents from PC was determined by monitoring the purge process by means of D1. By injecting 500-$\mu$l samples and adjusting the purge flow-rate to 2 ml/min, the clean-up process was completed in less than 7 min.

Under routine conditions, problematic material such as particulate matter from seal abrasion and precipitated proteins settled during the purge process at the inlet of PC. Since the subsequent transfer step was performed in the backflush mode this material was partly mobilized and then accumulated at the top of GC, thereby adversely affecting the lifetime of the packing. To circumvent this problem, an additional purge step, B, was inserted between the enrichment and transfer phases, in which the *loaded* PC was backflushed with M1 for 3 min. It was demonstrated that only deposited solid material was directed to waste, while the zone of retained components remained unaffected on PC.

*Internal standard*

HPLC column-switching methods which almost completely avoid conventional sample work-up often do not need an internal standard to improve precision of the measurements. However, as already pointed out elsewhere[6], the introduction of an internal standard can be very useful in compensating for slight changes in enrichment behaviour and chromatographic performance of PC, especially when long sample sequences are processed. For this reason, as well as to have a better control of the

sophisticated three-valve column-switching process, compound III was used as an internal standard in the method.

*Limit of detection, limit of quantification*

The two thienoquinolizinones I and II showed a strong native fluorescence ($\lambda_{em}$ = 475 nm) when excited with UV light at 304 nm. Operating with the Merck-Hitachi fluorescence detector at sensitivity range 5, injection of 2.5 ng I and II in 100 $\mu$l mobile phase M2 directly onto GC/AC gave peaks of approximately 95 and 85% full-scale deflection (input 8 mV), respectively. The detection limit, defined by a signal-to-noise ratio of *ca.* 3:1, was about 0.1 ng/ml (equivalent to an absolute injected amount of 25 pg) for both compounds. However, the practical limit of quantification defined here as the minimum concentration that could be measured routinely with acceptable precision and accuracy for compounds I and II was 0.25 and 0.5 ng/ml, respectively (see Table I).

*Recovery*

Recovery was measured on blank plasma samples spiked with compounds I and II at different concentrations. These spiked samples were analyzed in replicate as described and compared with spiked aqueous solutions, directly injected onto GC/AC via port I2, providing the 100% values. For both compounds, high recoveries (>88%) from human plasma were obtained. No species dependence was detected when recovery from human, dog and rat plasma was compared.

First experiments with spiked plasma samples, diluted 1:1 in water and using twice distilled water as the purge solvent M1, resulted in low recoveries, especially for the internal standard (<50%). It appeared that the interaction of the compounds with the plasma proteins was more pronounced than the affinity towards the PC

TABLE I

PRECISION OF THE METHOD IN THE LOW CALIBRATION RANGE ($n$ = 5)

| *Compound* | *Concentration (ng/ml)* | | *C.V. (%)* | *Difference between found and added concentration (%)* |
|---|---|---|---|---|
| | *Added* | *Found* | | |
| I | 0.25* | 0.245 | 7.4 | −2.0 |
| | 0.50 | 0.490 | 6.8 | −2.0 |
| | 1.00 | 0.995 | 1.7 | −0.5 |
| | 2.50 | 2.52 | 4.2 | +0.8 |
| | 5.00 | 4.92 | 1.4 | −1.6 |
| | 10.0 | 10.3 | 3.0 | +3.0 |
| | 25.0 | 24.4 | 1.2 | −2.4 |
| II | 0.25** | 0.275 | 9.3 | +10.0 |
| | 0.50* | 0.510 | 16.1 | +2.0 |
| | 1.00 | 1.02 | 5.0 | +2.0 |
| | 2.50 | 2.51 | 4.1 | +0.4 |
| | 5.00 | 4.95 | 1.9 | −1.0 |
| | 10.0 | 10.3 | 2.6 | +3.0 |
| | 25.0 | 24.5 | 1.5 | −2.0 |

* Limit of quantification.
** Below limit of quantification.

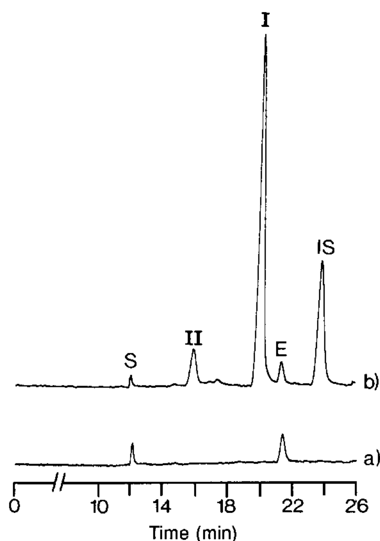


Fig. 4. Chromatograms of human plasma samples: (a) before dose; (b) collected 30 min after a single oral dose of 1 mg of compound I; measured concentrations 21.4 ng I/ml and 2.25 ng II/ml. Peaks: I = parent compound; II = metabolite; IS = internal standard; S = system peak; E = endogenous plasma component. Detector D2, sensitivity 5, attenuation 32.

packing material. This was confirmed by protein binding studies which showed that the parent compound was extensively bound to the plasma proteins with values of ≥99% for man, ≥96% for rat and >91% for dog[10]. In order to "loosen" the protein drug binding to some extent it was necessary to add small quantities of acetonitrile to the plasma samples as well as to the purge solvent M1[4,9]. The percentage of acetonitrile had to be limited to about 10% to prevent any precipitation of plasma proteins which would be deleterious in a column-switching system.

*Selectivity*

Simple column-switching methods with UV detection often suffer from decreased selectivity in comparison to extraction methods[11]. Using common packing materials such as $C_{18}$ or $C_8$, only proteins, salts and other highly polar plasma constituents are flushed to waste, while the majority of endogenous compounds are retained on PC and transferred to AC, together with the substances of interest. The application of fluorimetric detectors considerably improves the overall selectivity of column-switching methods, mainly because two analytical wavelengths are used and relatively few compounds have a native fluorescence in the UV region.

More than 50 blank plasma samples from different human volunteers have been analyzed up to the present time. No interfering plasma components were detected co-eluted with compound I, II or III. Fig. 4a shows a typical blank plasma extract.

*Precision*

The precision of the method was evaluated for the parent compound and the main metabolite over the concentration range 0.25–25 ng/ml by replicate analysis of each concentration over a period of several weeks. The data shown in Table I demonstrate the good precision of the fully automated method.

*Linearity*

The linear dynamic range of fluorescence detectors is generally less than that of UV detectors. In order to follow plasma concentrations of the parent compound from 2500 ng/ml down to the limit of quantification, the calibration range had to be divided into two linear sub-ranges. For both compounds in each sub-range, excellent linearity and a negligible $y$-intercept were found routinely.

According to Table I, the coefficient of variation (C.V.) of replicate measurements was nearly independent of concentration in each sub-range, indicating that the variance was roughly proportional to the square of concentration. For this reason, the calibration graphs were calculated by means of weighted least-squares regression, using $1/y^2$ as a weighting factor[12]. The standard software of the computing integrator provided only conventional linear regression and had, therefore, to be modified by means of additional programs[5].

*Stability*

The anxiolytic and its O-demethyl metabolite were added to human plasma at four different concentrations (1, 10, 100, 1000 ng/ml) and stored for different time intervals (2, 6, 24 h) at room temperature prior to analysis. During storage and analysis the samples were carefully protected from light.

The statistical interpretation of the data followed the procedure recently developed[13]. All measured concentrations indicated that compounds I and II were stable in human plasma at room temperature for at least 24 h. In Table II the stability of the two compounds at the concentration 1 ng/ml is shown.

*Practicability*

The use of column-switching for on-line clean-up of plasma samples led to a considerable saving of both chemicals and time. A skilled technician was able to analyze more than 40 samples per day and still had sufficient time for laboratory management work, such as documentation of the results, preparation of new precolumns etc.

TABLE II

STABILITY IN HUMAN PLASMA AT 25°C

| *Compound* | *Exposure time (h)* | *Concn. found (ng/ml)* | *Change of concn. after storage (%)* | *90% Confidence interval (%)* |
|---|---|---|---|---|
| I | 0 | 1 | | |
| | 2 | 1.00 | +0.1 | −2.6 to +2.9 |
| | 6 | 0.98 | −2.3 | −5.4 to +1.0 |
| | 24 | 0.97 | −2.6 | −5.6 to +0.6 |
| II | 0 | 1 | | |
| | 2 | 1.06 | +5.6 | +1.7 to +9.6 |
| | 6 | 1.02 | +1.9 | −2.6 to +6.7 |
| | 24 | 0.99 | −0.8 | −8.7 to +7.8 |

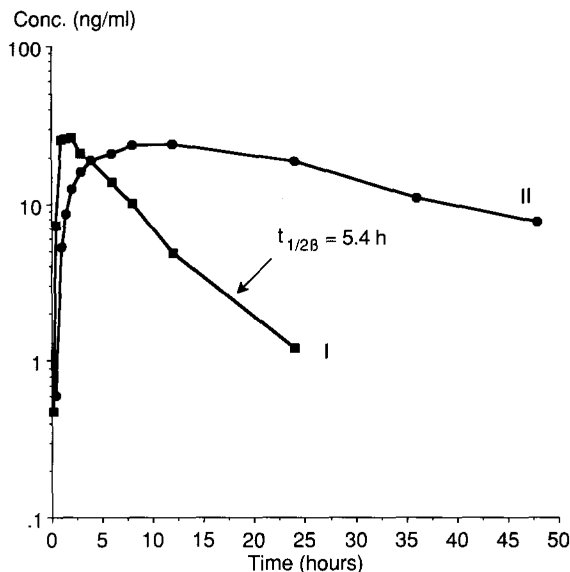


Fig. 5. Plasma concentration–time course of compounds I and II following a single oral dose of 1 mg of I to an healthy male volunteer. $t_{1/2\beta}$ = terminal elimination half-life.

*Application to clinical samples*

The new method has been applied successfully to the analysis of more than 300 plasma samples from a tolerance study performed on human volunteers. Fig. 4b shows a representative chromatogram. The method was sensitive enough to measure precisely the low concentrations of compound I in plasma for up to 36 h (corresponding to a period of approximately 5 elimination half-lives) after a single intravenous dose of 1 mg to human volunteers (Fig. 5).

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