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DETERMINATION OF TELENZEPINE IN HUMAN SERUM BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method for determining telenzepine in human serum is described. Analytes are obtained from alkalinized serum by extraction of the drug using reversed-phase octadecylsilane-bonded silica cartridges. Telenzepine and a desmethyl analogue added to serum as internal standard are retained on the C_{18} cartridge and recovered by elution with methanol. The gas chromatographic properties of telenzepine and the internal standard are improved by a two-step derivatization involving a benzodiazepinone–benzimidazole rearrangement and simultaneous formation of a methyl ester function. The processed extract is analysed by gas chromatography–mass spectrometry with selected-ion monitoring. Quantification is linear over the range 2–40 ng/ml. Inter-day precision is within 7%, except at the detection limit of 2 ng/ml (16%). Application of this assay to routine analysis is limited by the extensive sample pretreatment essential for derivatization of telenzepine.

INTRODUCTION

Telenzepine [1], 4,9-dihydro-3-methyl-4-[(4-methyl-1-piperazinyl)acetyl]-10*H*-thieno[3,4-*b*][1,5]benzodiazepin-10-one (**1a**, Fig. 1) is a new antisecretory agent whose efficacy in the treatment of gastric and duodenal ulcers [2, 3] and in the inhibition of acid secretion [4–6] has been demonstrated. A high-performance liquid chromatographic (HPLC) method involving extraction of the drug from serum into dichloromethane–2-propanol, reversed-phase chromatography and UV detection has been successfully employed in monitoring serum levels of **1a** in rat and dog. However, serum concentrations in humans following administration of therapeutic doses of **1a** are expected to be in the region of $\leqslant$30 ng/ml, which is at or below the detection limit of the HPLC assay. Therefore two approaches were used in parallel to measure human serum concentrations of **1a**: a radioreceptor assay [7] and a gas chromatographic–mass spectrometric (GC–MS) method. This report describes the analytical conditions for the extraction, derivatization and detection of **1a** utilizing GC–MS.

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EXPERIMENTAL

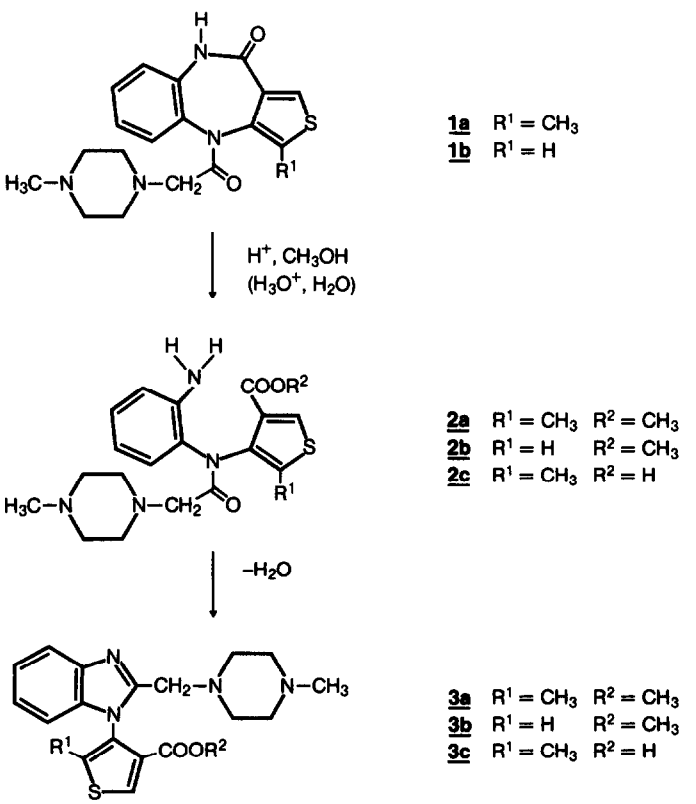
Materials

Telenzepine (**1a**), ^{3}H-labelled **1a** (specific activity $2.1 \cdot 10^{12}$ Bq/mmol), the desmethyl analogue (**1b**, Fig. 1), which was used as internal standard, and the derivatives **3a** and **3b** were obtained from Byk Gulden (Konstanz, F.R.G.). Bistrimethylsilyltrifluoroacetamide (BSTFA, silylation grade), methanol, acetonitrile and acetic acid, all of analytical grade, were obtained from Merck (Darmstadt, F.R.G.). Dichloromethane (solvent grade) was distilled before use. Methanol containing hydrochloric acid (30%, w/w) was prepared by passing gaseous hydrogen chloride through sulphuric acid and subsequently introducing it into methanol. Disposable Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Instrumentation

Recovery of ^{3}H-labelled **1a** was determined with a Kontron MR 300 automatic liquid scintillation system.

A Pye Unicam 204 gas chromatograph fitted with a flame ionization detector was used to monitor the derivatization process. Compounds **1a** and **1b** and their



1a $R^1 = CH_3$
1b $R^1 = H$

2a $R^1 = CH_3$ $R^2 = CH_3$
2b $R^1 = H$ $R^2 = CH_3$
2c $R^1 = CH_3$ $R^2 = H$

3a $R^1 = CH_3$ $R^2 = CH_3$
3b $R^1 = H$ $R^2 = CH_3$
3c $R^1 = CH_3$ $R^2 = H$

Fig. 1. Benzodiazepinone–benzimidazole rearrangement of **1**.

respective derivatives were separated on a glass column (1m×2 mm I.D.) packed with 3% OV-17 on Chromosorb W-AW-DMCS (80–100 mesh). The oven temperature was programmed from 150 to 300°C at 8°C/min. The injector and detector temperatures were 300°C. The helium flow-rate was set to 20 ml/min.

GC–MS was carried out using a VG MM 7070F instrument interfaced to a VG 2025 data system and equipped with a Pye Unicam 204 gas chromatograph. The chromatographic column (glass, 1.5 m ×1.5 mm I.D.) was packed with 3% OV-17 on Volaspher A4, 80–100 mesh (Merck).

The column outlet was connected to the jet separator interface using a deactivated fused-silica capillary. The temperatures of the injector, transfer line and ion source were 300, 280 and 250°C, respectively. Helium was used as carrier gas at a flow-rate of 15 ml/min. Sample injections (3–4 μl of processed serum extracts) were made at an oven temperature of 190°C. After injection the column oven was programmed to 315°C at 12°C/min. The final temperature was held for 3 min. The mass spectrometer was operated under electron-impact (EI) conditions at 70 eV electron energy and 4 kV acceleration voltage. Full scan mass spectra were recorded at a scan rate of 3 s/decade. For selected-ion monitoring (SIM) recordings a lock mass and two selected masses were sequentially and repetitively monitored, each for 0.2 s. The lock mass was 267.825 (molecular ion of CH_2I_2, which was permanently introduced through the reference inlet) and the selected masses were 288.081 for the derivative **3b** of the internal standard **1b** and 302.096 for the derivative **3a** of telenzepine **1a**. The resolution was adjusted to 2000 (10% valley) with the 302-a.m.u. ion.

Quantification was based on the heights of the chromatographic peaks recorded on a four-channel pen recorder (BBC Servogor 460). A linear regression of peak-height ratios (drug/internal standard) versus concentration ratios (drug/internal standard) was used to calculate concentrations.

Procedures

Extraction. To a 2-ml serum sample, 25 μl of methanol containing 50 ng of internal standard **1b** and 8 ml of distilled water were added. The sample was mixed by magnetic stirring, and the pH was adjusted to 9.5–10.0 by adding 1 *M* sodium hydroxide. C_{18} cartridges were conditioned by washing with 10 ml of methanol and 10 ml of distilled water. The sample was passed through the cartridge via a syringe. Subsequently the cartridge was rinsed with 10 ml of distilled water and dried by forcing air through it with a syringe until no further water was removed. Thereafter the sample was eluted with 3 ml of methanol. The eluate was collected in a 3-ml vial, and the solvent was removed in a vacuum centrifuge. Recovery was checked by extraction of serum spiked with ^{3}H-labelled **1a** (20 ng/ml) and liquid scintillation counting of the eluate and of the rinsing water.

Derivatization. Conditions for derivatization were optimized with respect to reaction time and temperature by treatment of pure samples of **1a** and **1b** (50–500 μg), which were obtained from methanolic solution after removing the solvent under a stream of nitrogen. Almost quantitative formation of the derivatives **3a** and **3b** (Fig. 1) was obtained as follows: **1a** and **1b** were treated with methanol–hydrochloric acid (0.5 ml) at 60°C in a 3-ml screw-capped vial sealed with

a PTFE-faced rubber septum (Aldrich, Milwaukee, WI, U.S.A.), and the solvent was removed after 16 h in a vacuum centrifgue (Bachofer, Reutlingen, F.R.G.) able to process twelve samples simultaneously. Acetic acid (0.5 ml) was added, and the sample was heated to 60 °C for 4 h. Subsequently the solvent was removed and the free bases **3a** and **3b** were liberated by adding aqueous potassium carbonate solution (10%, 0.5 ml) and extracted with dichloromethane (0.5 ml). The organic layer was removed by means of a pipette, placed in a glass-stoppered conic tube (100 mm × 12 mm) and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with acetonitrile (25–250 μl), and 1 μl of the solution was analysed by GC with flame ionization detection. Serum extracts were processed according to this procedure. The final residue was reconstituted with acetonitrile (7.5 μl) and BSTFA (7.5 μl) by heating at 60 °C for 10 min, and 3–4 μl were injected for SIM recordings.

Standard calibration curves. To establish a calibration curve, a solution of **1a** (0.2 ng/μl) was prepared in methanol. Appropriate aliquots (20–400 μl) were added to 2 ml of serum, and the samples were extracted and derivatized as described above. Standard curves were run on three days within a period of two weeks to obtain inter-day precision and accuracy data.

RESULTS AND DISCUSSION

The extraction efficiency for **1a** was determined using ^{3}H-labelled material: 84 ± 5% of the activity was recovered from serum containing 20 ng/ml [^{3}H]telenzepine by eluting the loaded cartridge with 3 ml of methanol. A small part of the activity (10%) was not retained on the cartridge during sample application, probably because of protein binding of the drug. A recovery of at least 80% at 25 ng/ml of **1a** and **1b** was estimated by comparing the peak heights of **3a** and **3b** in derivatized serum extracts with those of reference mixtures of **3a** and **3b**.

Underivatized **1a** is a thermostable compound, which can be analysed by GC in microgram amounts per injection. However, its response is non-linear and severe adsorption losses are observed at lower levels on packed columns as well as on fused-silica OV-1 wide-bore capillaries. Trimethylsilylation was tried in order to reduce the high polarity of **1a**, but this did not markedly improve its GC properties. Another disadvantage of this derivatization process was that minor and variable amounts of unreacted **1a** were always detected in addition to the trimethylsilyl derivative.

A promising approach for derivatization of **1a** was discovered when the product **3c** (Fig. 1), formed from **1a** by prolonged heating in aqueous hydrochloric acid, was identified. These reaction conditions were modified using methanol–hydrochloric acid in order to obtain directly the methyl ester **3a**. In this medium, formation of **3a** is slow and not completed in less than ca. 170 h at 60 °C. As outlined in Fig. 1, formation of **3** proceeds via the intermediate **2**. Now **2a** has been isolated and characterized as a by-product from preparative scale synthesis of **3a** in methanol–hydrochloric acid.

The low rate of the ring closure of **2a** to give the benzimidazole derivative **3a**

is probably due to protonation of the anilino nitrogen under these conditions, thereby inhibiting its nucleophilic attack on the amide carbonyl group. As expected, the rate of formation of **3a** was increased by removing the methanolic hydrochloric acid from the reaction mixture after formation of **2a** and replacing it with the weaker acetic acid. As illustrated in Fig. 2, generation of **3a** was found to be almost quantitative after treatment of **1a** with methanol–hydrochloric acid for 16 h and subsequently with acetic acid for 4 h, each step at 60 °C.

Analogous results were obtained for the transformation of the internal standard **1b** to **3b**. Compared with **1a** and **1b**, the retention times of **3a** and **3b** are markedly shorter, indicating a considerable decrease in polarity. Furthermore, adsorption losses were much less for **3a** and **3b**.

The electron-impact (EI) mass spectrum of underivatized **1a** (not shown) contained the following major ions (m/z) and relative abundances (in parentheses): 370, M^+ (5); 113 (100); 99 (5); 98 (5); 70 (37); 56 (5); 43 (7); 42 (11). It is dominated by the base peak. An advantage of the derivatives **3a** and **3b** is that they have sufficiently abundant fragment ions in their EI mass spectra (Fig. 3) at higher m/z values where interfering ions are less likely to occur.

Assignment of fragment identities (Fig. 4) was based on comparison with deu-

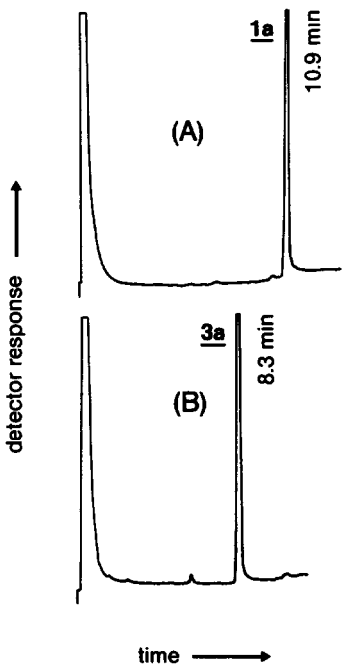


Fig. 2. Gas chromatograms of (A) underivatized **1a** (retention time 10.9 min) and (B) its derivative **3a** (8.3 min). For conditions see text. The corresponding retention times of **1b** and **3b** were 10.8 and 8.6 min, respectively.

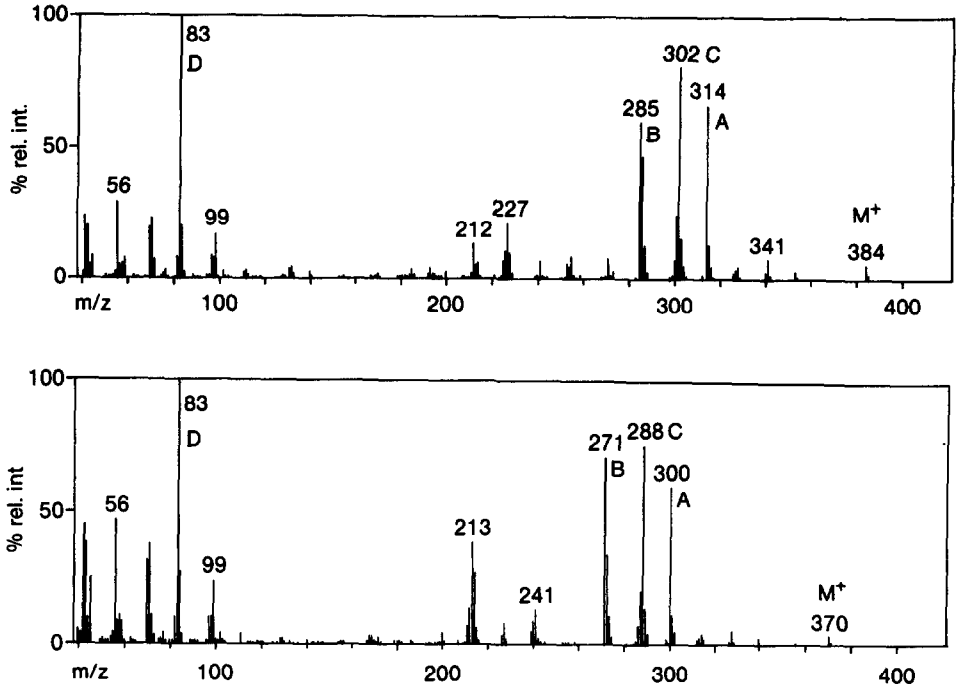


Fig. 3. Electron-impact (70 eV) mass spectra of **3a** (top) and **3b** (bottom).

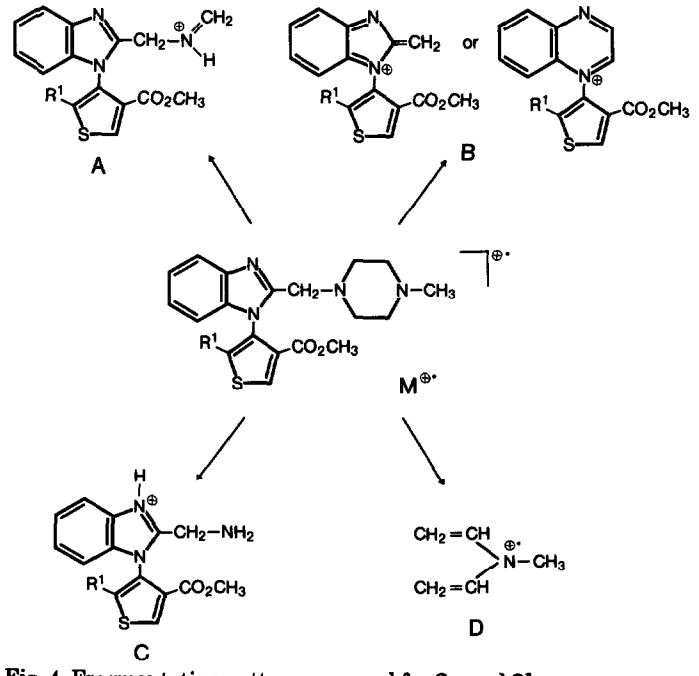


Fig. 4. Fragmentation pattern proposed for **3a** and **3b**.

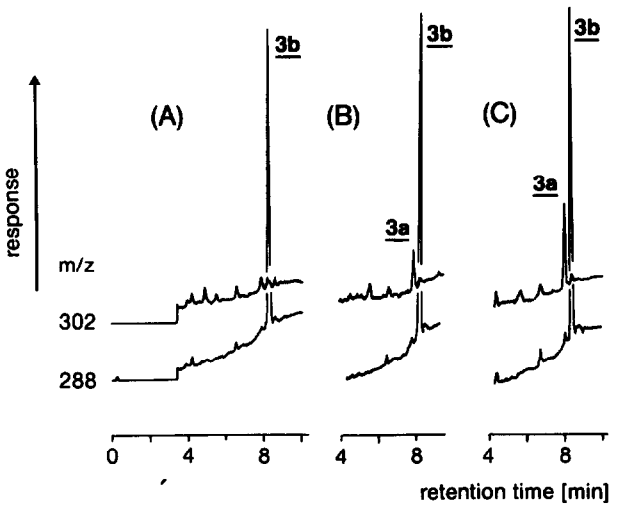


Fig. 5. Selected-ion chromatograms of serum extracts. (A) Processed blank spiked with 25 ng/ml internal standard only. (B) Processed standard spiked with 2 ng/ml telenzepine and 25 ng/ml internal standard. (C) Processed standard spiked with 5 ng/ml telenzepine and 25 ng/ml internal standard. **3a** is the derivative of telenzepine **1a**, and **3b** is the derivative of internal standard **1b**.

TABLE I

MEANS, STANDARD DEVIATIONS, COEFFICIENTS OF VARIATION AND ACCURACY OF BACK-CALCULATED VALUES OF STANDARD CURVES OF TELENZEPINE IN HUMAN SERUM

Drug added (ng/ml)	Drug level observed* (mean ± S.D.) (ng/ml)	*n*	Coefficient of variation (%)	Accuracy** (%)
2.0	1.79 ± 0.29	9	16.2	−10.5
5.0	5.20 ± 0.31	7	6.0	4.0
10.0	10.03 ± 0.65	8	6.5	0.3
20.0	20.84 ± 0.91	5	4.4	4.2
40.0	40.15 ± 1.63	3	4.1	0.4

*The mean of all individual values from determinations on three days.
**Defined as observed mean minus added drug level divided by added drug level × 100%.

terated analogues of **1a** (data not shown) [8]. As a process commonly observed in the mass spectra of N,N-disubstituted piperazines [9, 10], internal α-cleavage combined with hydrogen migration is responsible for the generation of ion A. Ion B is easily explained by neutral loss of the piperazinyl radical. The rather unexpected ions C and D seem to originate from related processes, both involving

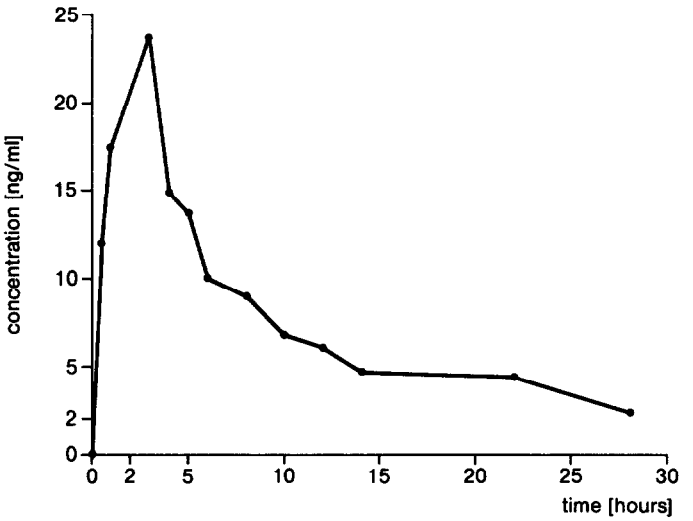


Fig. 6. Serum concentration–time profile from a young, healthy subject following administration of 3 mg of telenzepine dihydrochloride given as an infusion (0–2 h).

migration of either two or three hydrogens and, in addition, an unusual cleavage of two adjacent C–N bonds, whereby the charge can reside with either one of the two piperazine nitrogens.

The mass chromatograms of the ions A, B and C were checked for the level of interfering peaks. The best results with regard to selectivity and sensitivity were obtained by focusing on the most abundant ions C at m/z 302 and m/z 288, respectively, which were selected for quantification of **1a**. A mixture of BSTFA and acetonitrile was used for the final reconstitution of serum extracts in order to prevent the occurrence of a ghost peak at m/z 302, which was observed after repeated injection of extracts dissolved in pure acetonitrile. We may assume that silylation of an unknown serum component by BSTFA changes the chromatographic behaviour and/or fragmentation of this component, thereby eliminating its disturbing side-effect.

Typical chromatograms of spiked and blank sample extracts resulting from the method are presented in Fig. 5. A small interfering peak was produced by blank samples at the retention time of the derivative **3a**, which is responsible for the y-intercept of the calibration line. A further separated peak was observed at the trace m/z 302, which is due to an isotope peak of the abundant m/z 300 ion of the derivative **3b**. The linearity of the method was studied over the range 0–40 ng/ml. Calibration curves run on three days in a period of two weeks were linear. The linear regression line, which represented the best fit of all these data ($n=47$), had an equation of $y=0.987x+0.052$ ($y=$ peak-height ratio of drug to internal standard, $x=$concentration ratio of drug to internal standard). The correlation coefficient of the three-day composite curve was 0.997. Data for the precision and

accuracy are shown in Table I. The relative standard deviations of the back-calculated concentrations were within 7% except at the detection limit of 2 ng/ml (16%).

This procedure has been used successfully to analyse clinical and non-clinical human serum samples [6]. Fig. 6 shows a serum concentration–time profile from a healthy subject following administration of a 3-mg dose of telenzepine dihydrochloride given as an infusion. Application of this assay to routine analysis is limited by the extensive sample pretreatment essential for the derivatization of **1a**.

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