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# DETERMINATION OF THE *R*(—)- AND *S*(+)-ENANTIOMERS OF γ-VINYL-γ-AMINOBUTYRIC ACID IN HUMAN BODY FLUIDS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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## SUMMARY

An analytical procedure, which allows the determination and quantitation of the *R*(—)- and *S*(+)-enantiomers of γ-vinyl-γ-aminobutyric acid (γ-vinyl-GABA; MDL 71.754) in body fluids was developed. The method is based on a combined gas chromatographic—mass spectrometric technique. A glass capillary column coated with a chiral phase enabled the separation of the enantiomers of γ-vinyl-GABA as their N-trifluoroacetyl-O-methyl ester derivatives. This was followed by quantitation using a selected ion monitoring technique in the electron-impact mode of ionization. The internal standard, γ-acetylenic GABA, was included throughout the work-up of the samples. The assay was shown to be reproducible, specific and sensitive. No interferences were encountered from plasma, urine or cerebrospinal fluid constituents. The method was applied to the analysis of plasma samples obtained from a human volunteer who had received racemic γ-vinyl-GABA. Significant differences in the plasma concentrations and plasma half-lives of the two enantiomers were seen, clearly illustrating the need for a specific assay technique capable of distinguishing between the enantiomers of this drug.

## INTRODUCTION

Increasing the concentration of γ-aminobutyric acid (GABA) in the central nervous system has possible therapeutic potential for a number of neurologic disorders including epilepsy [1], tardive dyskinesia [2], Huntington's chorea [3], Friedreich's ataxia [4, 5] and Parkinsonism [6]. γ-Vinyl-GABA (Fig. 1) has been shown to be a potent and selective enzyme-activated inhibitor of the GABA-degrading enzyme, GABA-α-oxoglutarate aminotransferase (GABA-T) in vitro [7]. When administered orally to patients suffering from various neurologic disorders it was shown to cause dose-dependent increases of GABA in their cerebrospinal fluid [8, 9].

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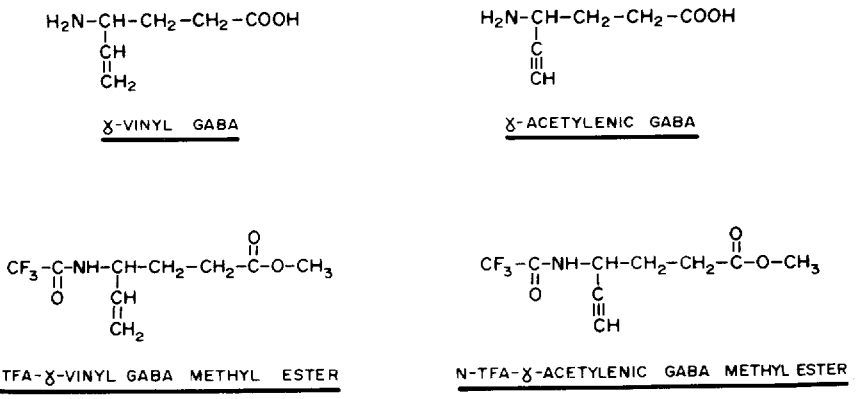


Fig. 1. Structures of γ-vinyl-GABA and γ-acetylenic GABA and of their N-trifluoroacetyl- (N-TFA-) and methyl ester derivatives.

Due to its synthetic route [10], the drug is presently supplied as the racemic *R,S* mixture. In animal experiments, however, it has been shown that only the *S*(+)-enantiomer of γ-vinyl-GABA is an inhibitor of mouse brain GABA-T, whereas the *R*(—)-enantiomer is devoid of any activity [11]. It is well known that the administration of enantiomeric mixtures of drugs such as propranolol [12—15], hexobarbital [16], methadone [17], *p*-chloroamphetamine [18], metoprolol [19], alprenolol [19], disopyramide [20] and oxaprotiline [21] can result in differing blood levels and pharmacokinetics of the enantiomers. These pharmacokinetic differences may result in differing concentration—effect relationships and, thus, be of clinical importance.

Our objective was to develop an analytical method capable of separating the *R*(—)- and *S*(+)-enantiomers of γ-vinyl-GABA. Successful separations of amino acid enantiomers have been described by Frank et al. [22, 23] by capillary gas chromatography using a chiral phase. The inclusion of an internal standard in the procedure makes it possible to quantitate the enantiomers in biological fluids and thus makes future pharmacokinetic studies feasible.

## EXPERIMENTAL

### *Reagents*

*R,S*-γ-Vinyl-GABA (MDL 71.754; Merrell-Dow Pharmaceuticals) and the internal standard *S*(+)-γ-ethynyl-GABA (*S*(+)-γ-acetylenic GAMA; MDL 71.667; Merrell-Dow Pharmaceuticals) (Fig. 1) were supplied as crystalline powders. For oral use, hard gelatin capsules containing 250 mg of drug per capsule were provided. Reagent-grade trifluoroacetic anhydride (E. Merck, Darmstadt, G.F.R.) and diazomethane prepared from N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich-Europe, Beerse, Belgium) were used in the derivatization.

### *Gas chromatography—mass spectrometry*

Mass spectrometric analysis was carried out on a combined gas chromatograph—mass spectrometer—computer system (Ribermag R-10-10, Nermag S.A., France).

Separation of the derivatized enantiomers of *R,S*-γ-vinyl-GABA and of the internal standard *S*(+)-γ-acetylenic GABA (Fig. 1) was achieved using a 25 m × 0.92 mm O.D. capillary column coated with L-valine—*tert.*-butylamide coupled to an alkylsiloxane polymer (Chirasil-Val; Applied Science Labs., Oud-Beijerland, The Netherlands).

The sample was injected onto the column using a Ros injector, allowing solvent-free injections (Girdel, Suresnes, France). The gas chromatograph (Serie 32, Girdel) was coupled directly to the mass spectrometer without the use of a molecular separator. The injector port temperature was maintained at 270°C, the interphase temperature was 270°C and the ion-source temperature was kept at 180°C. Separation of the compounds was achieved using helium as carrier gas at a column pressure of 0.8 bar. The initial column temperature of 90°C was maintained for 1 min after the injection and then increased at 4°C/min to 130°C. Mass spectra were acquired in the electron-impact mode of ionization using an electron energy of 70 eV.

*Work-up of samples*

To 100 μl of plasma were added 20 μl of an aqueous solution containing the internal standard *S*(+)-γ-acetylenic GABA. Protein was precipitated by addition of 200 μl of methanol. After centrifugation the supernatant was evaporated to dryness with a stream of nitrogen. The residue was redissolved in 200 μl of dichloromethane and 200 μl of trifluoroacetic anhydride and left to react for 1 h at room temperature. The solvent was then evaporated with a stream of nitrogen and the residue redissolved in 100 μl of methanol. To this solution was added, dropwise, a solution of diazomethane in diethyl ether until the sample solution had a distinctly yellow colour. Excess diazomethane was destroyed by addition of a droplet of acetic acid, resulting in a colorless solution which was evaporated to dryness. The dry residue was partitioned between 1 ml of water and 2 ml of benzene and the organic phase transferred to another vial for evaporation to dryness. Before analysis, the sample was redissolved in 10—100 μl of benzene of which 1—2 μl were deposited on the injection capillary of the Ros injector. After evaporation of the solvent the sample was brought into the heated injector port and thus injected into the column.

*Calibration curves*

Calibration curves were obtained for stock solutions of the products to determine linearity of response and for spiked human plasma, urine and cerebrospinal fluid.

## RESULTS AND DISCUSSION

*Reproducibility*

Spiking of human plasma with 200 nmol/ml of racemic *R,S*-γ-vinyl-GABA plus 200 nmol of the internal standard, *S*(+)-γ-acetylenic GABA, and subsequent analysis yielded good reproducibility (Table I).

On separate occasions, distributed over a 45-day period, six different sets of

TABLE I

ASSAY REPRODUCIBILITY AND DAY-TO-DAY VARIABILITY IN HUMAN PLASMA SPIKED WITH 200 nmol/ml OF *R,S*-γ-VINYL-GABA AND 200 nmol OF *S*(+)-γ-ACETYLENIC GABA

Each time 100 μl of plasma were used. Values are expressed in nmol/ml ± standard deviation.

| | γ-Vinyl-GABA | | |
|---|---|---|---|
| | *R*(—)-Enantiomer | *S*(+)-Enantiomer | |
| Reproducibility | 104.2 ± 4.0 | 103.7 ± 4.3 | ($n$ = 5) |
| Day-to-day variability (45-day period) | 100.6 ± 16.6 | 99.6 ± 16.3 | ($n$ = 6) |

plasma samples with two samples in each set were spiked with the same quantity of *R,S*-γ-vinyl-GABA and internal standard. This day-to-day variability is also presented in Table I.

*Calibration curves*

A calibration curve for human plasma was obtained using as internal standard 20 nmol of *S*(+)-γ-acetylenic GABA added per 100 μl of plasma, while the *R,S*-γ-vinyl-GABA concentration varied from 2.5 to 20 nmol. By linear regression analysis the line was calculated to be $C = -0.01926 + 0.006179 \cdot$ ng *R*(—)-γ-vinyl-GABA and $C = -0.01363 + 0.006165 \cdot$ ng *S*(+)-γ-vinyl-GABA with a correlation coefficient for both curves of —0.9992. Since the two curves were essentially identical a mean calibration curve of $C = 0.01652 + 0.006172 \cdot$ ng with a correlation coefficient of —0.9993 may be used to determine either the *R*(—)- or the *S*(+)-enantiomer. Expanding the calibration to higher plasma concentrations of γ-vinyl-GABA yielded a lesser degree of linearity; such concentrations thus require dilution before analysis or, alternatively, a smaller amount of plasma may be used. Calibration curves for urine and cerebrospinal fluid also showed excellent linearity for the same concentration range.

*Mass spectrometry*

Fig. 2 shows the electron-impact mass spectra of N-trifluoroacetyl(TFA)-γ-vinyl-GABA methyl ester and of the corresponding derivative of γ-acetylenic GABA. The molecular ions, *m/e* 239 for N-TFA-γ-vinyl-GABA-$OCH_3$ and *m/e* 237 for N-TFA-γ-acetylenic GABA-$OCH_3$ were both weak. In the following discussion, ions for the corresponding γ-acetylenic GABA derivative are shown in parentheses. Loss of $CH_3O^{\bullet}$ from the molecular ions leads to *m/e* 208 (206) and elimination of $CH_3OH$ to *m/e* 207 (205). Expulsion of $HCOOCH_3$ leads to *m/e* 179 (177). A McLafferty rearrangement yields the ions at *m/e* 165 (163) and *m/e* 74 (74). The base peak of the spectrum is produced through a loss of $^{\bullet}CH_2—CH_2—COOCH_3$ to give the ion at *m/e* 152 (150). The fragmentation scheme of the two derivatives is presented in Fig. 3.

For quantitative determination of γ-vinyl-GABA in human body fluids, a selected ion monitoring (SIM) technique was employed. For this purpose, the ions at *m/e* 152 (γ-vinyl-GABA) and *m/e* 150 (γ-acetylenic GABA) were

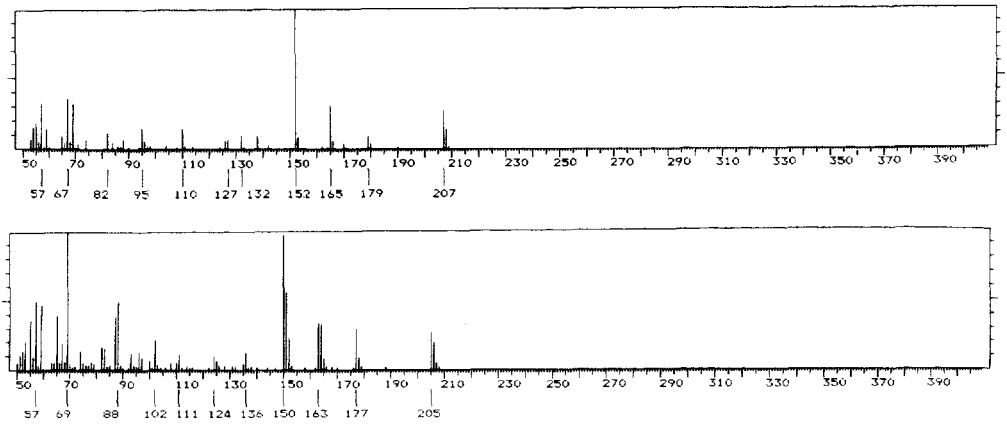


Fig. 2. 70 eV mass spectra of N-trifluoroacetyl-γ-vinyl-GABA methyl ester (top) and of the corresponding derivative of γ-acetylenic GABA (bottom). Column, 25 m Chirasil-Val; temperature, 130°C isothermal; pressure, 0.8 bar.

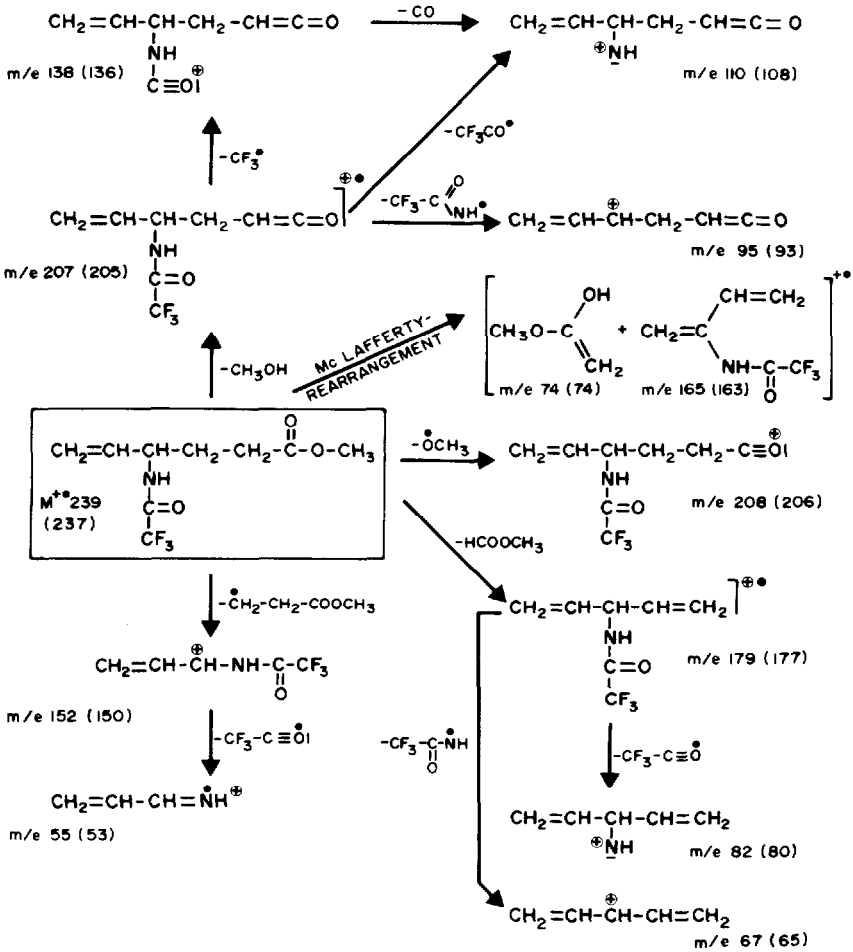


Fig. 3. Mass spectrometric fragmentation scheme of the derivatives of γ-vinyl-GABA and γ-actylenic GABA.

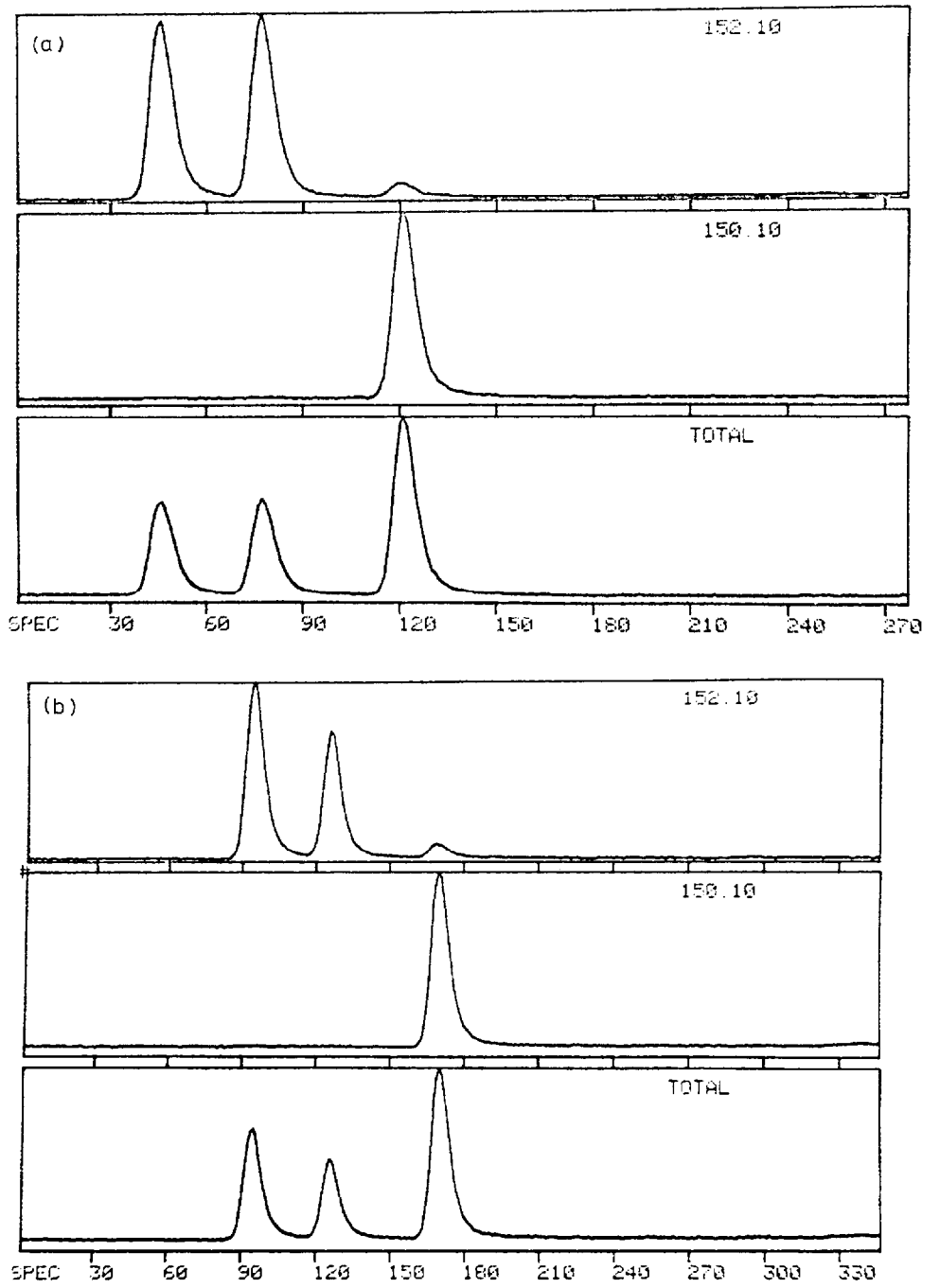


Fig. 4. SIM chromatograms of *m/e* 152 for γ-vinyl-GABA and *m/e* 150 for the internal standard: (a) standard mixture; (b) urinary sample obtained from a volunteer after a single oral dose of 500 mg of *R,S*-γ-vinyl-GABA.

chosen. A SIM chromatogram of a standard mixture is shown in Fig. 4a and that of a urinary sample of a volunteer who had received a single oral dose of 500 mg of *R,S*-γ-vinyl-GABA is presented in Fig. 4b. No interferences from body constituents were encountered.

*Time course of R(—)- and S(+)-γ-vinyl-GABA in a human volunteer*

A healthy, male volunteer received five doses of 500 mg of racemic *R,S*-γ-vinyl-GABA given at 12-h intervals. The drug sample was shown to contain equal amounts of the two enantiomers. Blood samples were obtained just prior to the last dose and at 30 min, 45 min, 1, 2, 3, 4, 6, 8 and 12 h after the last dose and the enantiomers of γ-vinyl-GABA were determined quantitatively. The result is shown in Fig. 5. It is evident that γ-vinyl-GABA was rapidly absorbed, peak plasma concentrations being observed at 1 h post dose. Plasma concentrations of the two enantiomers differ substantially from one another for the first 4 h post dose, and subsequently seem to approach equal levels. The

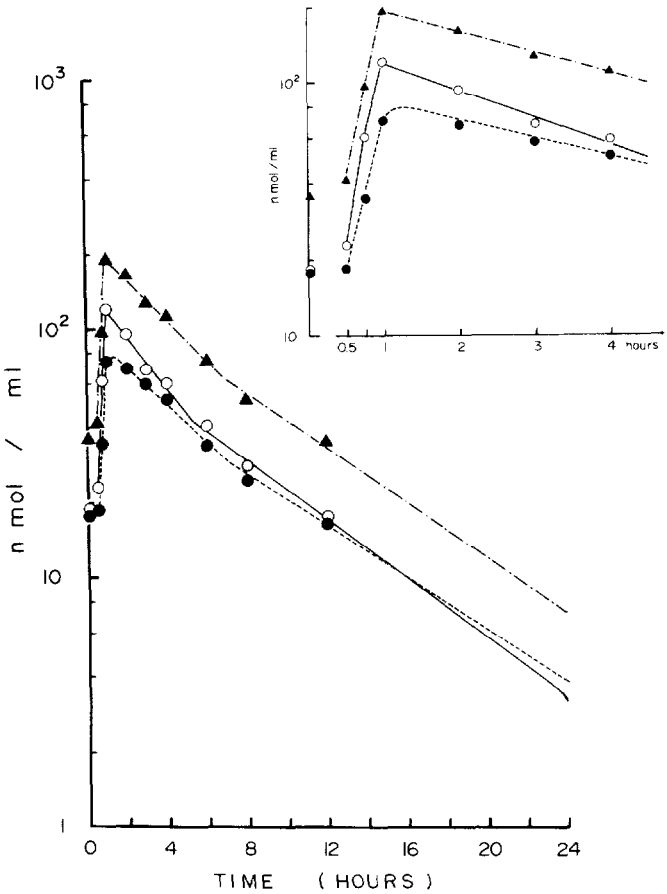


Fig. 5. Time course of *R*(—)- and *S*(+)-γ-vinyl-GABA in the plasma of a volunteer who had ingested a total of five doses each of 500 mg of racemic γ-vinyl-GABA at 12-h intervals when followed after the last dose. (▲), Sum of *R*- and *S*-enantiomers; (○), *R*(—)-enantiomer; (●), *S*(+)-enantiomer. Half-lives: *R*(—): $t_{½\alpha}$ = 2.8 h, $t_{½\beta}$ = 5.0 h; *S*(+): $t_{½\alpha}$ = 3.9 h, $t_{½\beta}$ = 5.7 h; *R* + *S*: $t_{½\alpha}$ = 3.6 h, $t_{½\beta}$ = 5.3 h.

peak plasma concentration of the biologically inactive *R*(−)-enantiomer was 85.1 nmol/ml, whereas the concentration of the biologically active *S*(+)-enantiomer was only 40.7 nmol/ml. Thus, of a total of 125.8 nmol/ml of γ-vinyl-GABA present in this sample less than one third corresponded to the biologically relevant form of this GABA-transaminase inhibitor.

These results clearly demonstrate the necessity of a specific assay technique capable of separating the enantiomeric forms of drugs. Such a technique becomes obligatory in the case where the enantiomers of a drug administered as the racemic mixture possess different pharmacological properties. The described method will be applied to future pharmacokinetic studies.

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