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Note

Gas chromatographic method for the determination of progabide (SL 76.002) in biological fluids

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Progabide (Sl 76.002) is a novel γ-aminobutyric acid receptor agonist which easily crosses the blood—brain barrier and has shown a broad anticonvulsant spectrum in various animal models [1—4]. In man, progabide appears to possess an interesting therapeutic action in epilepsy and in spastic syndromes, as indicated by open pilot [5] and controlled clinical trials [6, 7].

The knowledge of the pharmacokinetic profile of any given drug is these days considered a necessary step for a better understanding of its pharmacodynamic profile, as well as for the rational definition of its therapeutic regimen. For these reasons, a rapid, sensitive and specific method for the determination of progabide in biological fluids has been developed using gas—liquid chromatography with electron-capture detection (GLC-ECD).

EXPERIMENTAL

Standards and reagents

Progabide, 4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenyl)methylene]amino}butanamide, and the internal chromatographic standard SL 78.050, 4-{[(4-chlorophenyl-5-chloro-2-hydroxyphenyl)methylene]amino}butanamide, were synthesised by Dr. Kaplan of the Department of Chemistry at L.E.R.S. [3]. Their structural formulae are shown in Fig. 1.

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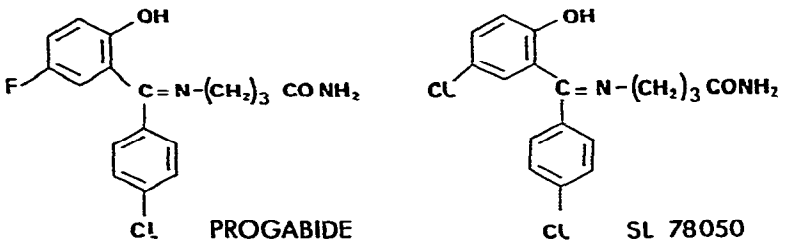

Fig. 1. Structural formulae of progabide and SL 78.050 (internal standard).

The solvents used were toluene, ethanol and *n*-hexane (analytical reagent grade, E. Merck, Darmstadt, G.F.R.); the derivatising reagent heptafluorobutyric anhydride (HFBA) was purchased from Fluka, Buchs, Switzerland.

Gas—liquid chromatographic conditions

Analyses were performed under isothermal conditions in a Perkin-Elmer Model 3920B gas chromatograph equipped with a ^{63}Ni linear electron-capture detector operating at —55 V (pulse current) with a 250-nsec width. The glass column (2 m × 2 mm I.D.) was packed with Gas-Chrom Q (80—100 mesh) coated with 3% OV-17 (Applied Science Labs., State College, PA, U.S.A.). The column was conditioned for 1 h at 270°C (nitrogen flow-rate 40 ml/min), for 4 h at 320°C (no nitrogen flow) and, finally, for 24 h at 280°C (nitrogen flow-rate 40 ml/min). The column temperature was 230°C, injection port and detector 275°C, and carrier gas (nitrogen) flow-rate 40 ml/min.

Mass spectrometric conditions

A Hewlett-Packard 5703 gas chromatograph (Avondale, PA, U.S.A.) coupled to a VG Micromass 70-70 mass spectrometer (Altringham, Great Britain) which was in turn connected to a VG 2050 series data system were used. The data system had a dynamic range of twelve bits (1:4095).

The GLC conditions were as described above. The mass spectrometer conditions were as follows: trap current 200 μA, source temperature 200°C, interface temperature 220°C, electron beam energy 70 eV.

Calibration graph and quantitation

Standard solutions of progabide (10 μg/μl) and of internal standard SL 78.050 (10 μg/μl) were prepared in ethanol. These solutions were stored at 4°C and under these conditions were stable for at least fifteen days. However, fresh solutions were prepared every week. Further dilutions were made to obtain lower concentrations for the standard graph. The standard graphs were prepared by adding 10, 30, 50, 100, 300, 500, 1000 ng of progabide and 500 ng of SL 78.050 to 1 ml of blank plasma. The samples were extracted according to the method described below and the extract derivatised by heating with HFBA at 60°C for 20 min. In order to remove excess reagent, the solution was evaporated to dryness under nitrogen at 60°C; 500 μl of hexane were added and 1 μl of this solution injected onto the column. The graphs were prepared by plotting the ratios of the peak height of progabide to the internal standard, against the known amounts of progabide. This curve was used to calculate the amount of progabide in unknown samples.

Extraction procedure

Spiked plasma samples for the calibration graph (see above) and plasma samples to be quantified were extracted at the same time and the procedure was as follows. SL 78.050 (500 ng) as internal standard, 100—500 μl of plasma (adjusted to 1 ml with the same acetate buffer), 1 or 2 ml of 0.2 *M* acetate buffer (pH 4.7) and 5 ml of toluene were added to a 10-ml stoppered tube. The tubes were gently agitated on a rotating mixer for 15 min. After centrifugation at 4°C for 10 min at 1000 *g*, 4.5 ml of the organic phase were transferred to a second series of test-tubes and then evaporated to dryness under nitrogen at 60°C. A solution of HFBA (10%, v/v) in ethyl acetate (200 μl) was then added to the dry residue and the derivatisation was carried out as described above.

An identical procedure was used for the analysis of urine.

RESULTS AND DISCUSSION

Representative GLC traces obtained from plasma samples of a dog which was given 300 mg/kg progabide orally, and a blank plasma spiked with the internal standard, are shown in Figs. 2A and B, respectively. The two peaks, one of derivatised progabide, retention time 2.4 min, and that of the derivatised internal standard SL 78.050, retention time 4.2 min, were not interfered with by peaks formed by any endogenous substances.

Calibration graphs were prepared as described above. A linear response was obtained up to 1000 ng with a regression coefficient of 0.998 (twelve points), a slope of 0.0043 ng^{-1}, and an intercept of 0.0026. The minimum concentration of progabide detectable in plasma was 1 ng/ml.

The reproducibility of the method was checked by repeating the analyses of plasma samples to which known amounts of progabide had been added (see Table I). A very small variation was observed (less than 2.5%).

GLC—mass spectrometric analysis of plasma samples confirmed the identity of the peaks with standards, and an investigation was made of the chemical identity of the HFB derivative of progabide. The mass spectrum of progabide derivatised with HFBA as above is given in Fig. 3. The molecular ion of *m/z* 512 is 18 a.m.u. less than expected for the monoacyl derivative.

There are several examples in the literature of the conversion of primary amides to nitrile upon treatment with either a perfluoroacyl anhydride or a silanising reagent [8, 9]. In this case, the formation of a nitrile would explain the observed molecular ion, and the unexpectedly good chromatographic properties. The derivatised authentic nitrile, analysed under exactly the same conditions as the derivatised progabide, gave the same spectrum with the same retention time (2.4 min). The principal high mass ions may be explained by a straightforward fragmentation: *m/z* 511, M—hydrogen; 493, M—fluorine; 477, M—chlorine; 458 (M—54), M—(CH_2CH_2CN).

Possible routes of the rearrangement ions *m/z* 125 (base peak) and *m/e* 138 are given in Fig. 4. These two assignments are supported by accurate mass determinations.

The described procedure has been applied to the analysis of several animal plasma and urine samples as well as of human plasma specimens from

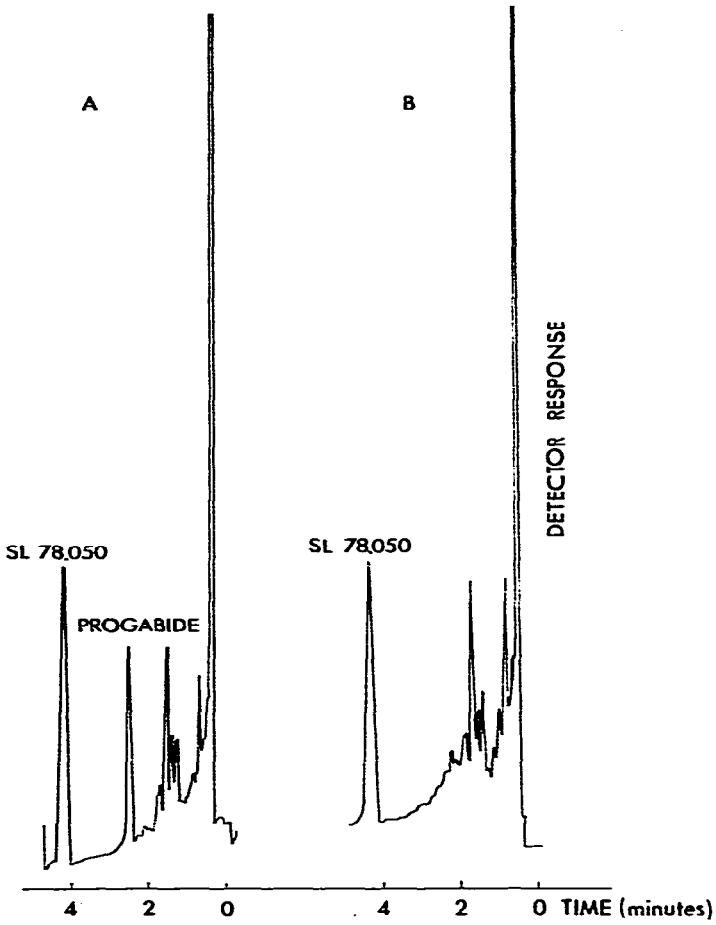

Fig. 2. Gas chromatograms obtained after extraction of plasma (200 μl) of a dog given 300 mg/kg progabide orally (A), and a blank dog plasma spiked with SL 78.050 (B). The tracings represent a concentration of 30 ng/ml progabide and 500 ng/ml internal standard.

TABLE I

CONCENTRATION OF PROGABIDE FOUND IN PLASMA FOR KNOWN AMOUNTS OF THE ADDED DRUG

Calculations were made on more than three determinations.

Amount added to plasma (ng/ml)	Amount recovered (ng/ml, mean ± S.D.)	Coefficient of variation* (%)
10	8.7 ± 0.34	3.9
30	27.6 ± 1.40	5.0
50	49.1 ± 3.40	6.9
100	104.0 ± 4.10	3.9
500	501.7 ± 15.50	3.1
1000	973.2 ± 41.30	4.2

*(S.D./mean) x 100.

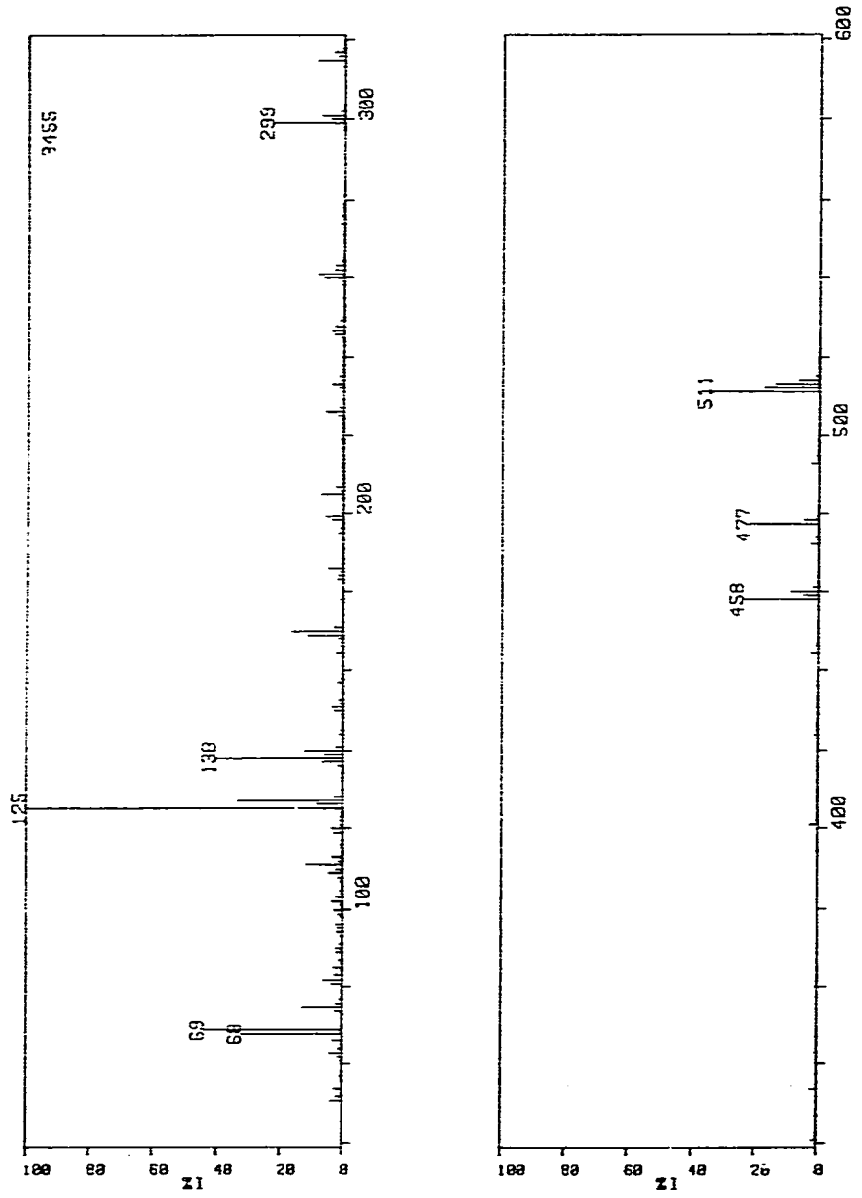

Fig. 3. Mass spectrum of progabide derivatised with HFBA.

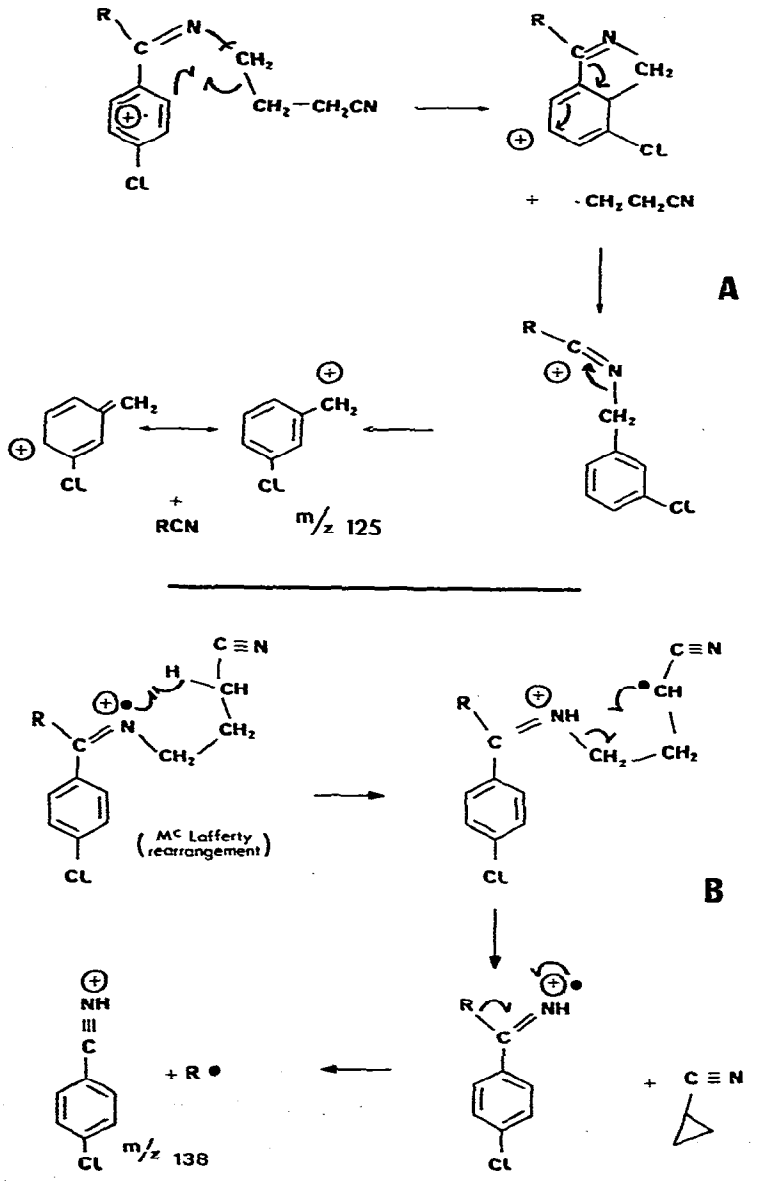

Fig. 4. Possible route of rearrangement of ions *m/z* 125 (A) and *m/z* 138 (B).

volunteers and patients. Representative plasma concentration curves over time following a single oral dose in rat, dog and man are reported in Fig. 5. Furthermore, endogenous substances and commonly used antiepileptic drugs were found not to interfere.

A skilled technician can run 35—40 samples a day.

Because of its simplicity, sensitivity and specificity, the described methodology has been found very suitable for both pharmacokinetic studies in experimental animals and man and for routine therapeutic drug monitoring during chronic treatment with progabide.

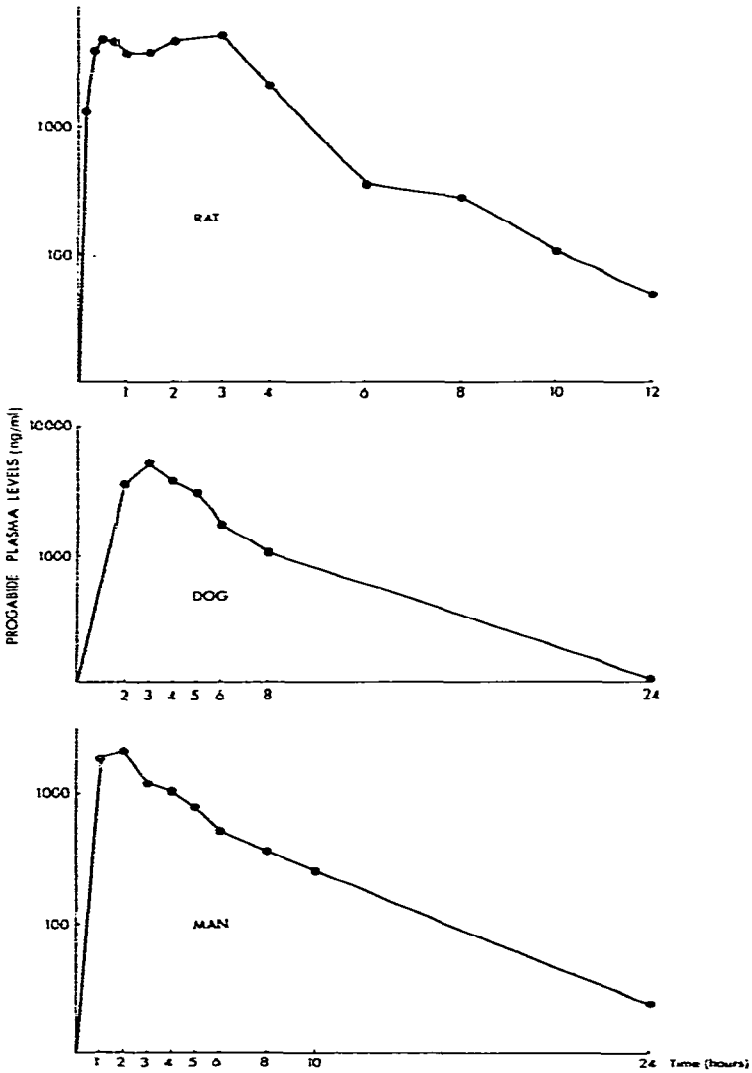

Fig. 5. Plasma concentration—time curves obtained after oral administration of progabide to rats (200 mg/kg), a dog (300 mg/kg) and a man (300 mg).

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