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DETERMINATION OF PROGABIDE AND ITS ACID METABOLITE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON SILICA COLUMN WITH ULTRAVIOLET DETECTION

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of progabide and its main acid metabolite in blood, serum and plasma. The assay involved a single and rapid extraction of drug and metabolite into toluene from the biological matrix buffered at pH 4.8, evaporation of the organic phase, and chromatography of the extracts on a silica column with UV detection. SL 81 0142 was used as internal standard. The method was specific for unchanged drug and metabolite and had a sensitivity of ca. 50 ng/ml of biological fluid for both the compounds. The method was successfully applied to the analysis of progabide and its metabolite in biological fluids of patients administered orally with progabide for clinical pharmacokinetic studies and drug monitoring.

INTRODUCTION

Progabide (SL 76 002) (Fig. 1, I) is a new γ-aminobutyric acid receptor agonist. It readily crosses the blood—brain barrier, and shows a large spectrum of antiepileptic activity in epileptic patients [1—3]. The drug is metabolized to an acid compound (PGA) (Fig. 1, II) that has the same pharmacological activity as the parent compound. As previously reported [4, 5], I is unstable in aqueous media, undergoing rapid hydrolysis to benzophenone. The instability of the imine bond, which is typical of Schiff bases, prevents the drug from being chromatographed successfully in a reversed-phase system.

The methods used for the determination of I in biological fluids are gas—liquid chromatography (GLC) with electron-capture detection of the heptafluorobutyryl derivative, and reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection of I and II reduced [5, 6].

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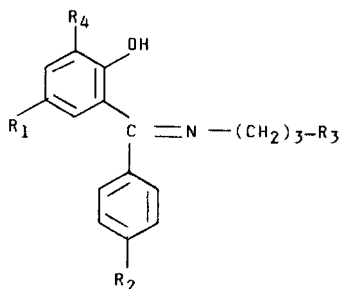

Compound		R_1	R_2	R_3	R_4
I	Progabide	F	Cl	$-CONH_2$	H
II	Acid metabolite (PGA)	F	Cl	$-COOH$	H
III	Internal standard (SL 810142)	Cl	H	$-CONH_2$	$-CH_3$

Fig. 1. Structures of progabide (I), its metabolite (II) and the internal standard (III).

The first method is unsuitable for the determination of the active metabolite II. The second allows the determination of I and II, after reduction of the imine double bond with sodium borohydride (after the reduction, the substance becomes stable in aqueous medium); however, this method is time-consuming because of the liquid—liquid purification necessary.

This paper describes a new HPLC method, which consists of a single and rapid extraction, chromatography on a silica column and UV detection.

EXPERIMENTAL

Reagents and solvents

Acetate buffer (2 *M*; pH 4.8) prepared from acetic acid, sodium acetate p.a. grade, methylene chloride and methanol, HPLC grade, were from E. Merck (Darmstadt, F.R.G.). Nanograde toluene was from J.T. Baker (Deventer, The Netherlands). The water used for the preparation of reagent solutions was HPLC grade, produced by the Milli Q-4 system (Millipore, Bedford, MA, U.S.A.). Progabide (I), PGA (II) and the internal standard (SL 81 0142) (Fig. 1, III), were of pharmaceutical grade and provided by the L.E.R.S. Chemistry Department (Paris, France).

Standard solutions

Stock solutions of compounds I—III were prepared by dissolving 5 mg of each compound in 10 ml of methanol. Standard solutions were prepared from stock solutions, by suitable dilution with methanol. Stock solutions are stable for at least two weeks if stored at 0—5°C.

HPLC equipment and operating conditions

The chromatographic system consisted of: a Model 414-T constant-flow pump (Kontron, Zürich, Switzerland); a Model PU 4020 UV spectrophotometric liquid chromatography detector (Pye Unicam, Cambridge, U.K.) operating at a wavelength of 340 nm and at a sensitivity of 0.02 a.u.f.s.; a Sedex 100

automatic sample injector (Sedere, Vitry-sur-Seine, France) with an automatic valve and a loop capacity of 100 μl; an analytical column, 30 × 0.39 cm I.D., filled with 10-μm μPorasil™ (Millipore-Waters, Milford, MA, U.S.A.). The mobile phase was methanol—acetic acid—water (2.6:0.3:0.15) diluted to 100 ml with methylene chloride (v/v). The flow-rate was 1.5 ml/min. The UV detector was coupled to an SP 4270 chromatographic computer (Spectra-Physics, San Jose, CA, U.S.A.) for the determination of peak height and subsequent calculations using the internal-standard method and a multi-point linear calibration.

Under these conditions, the retention times of I, II and III were ca. 8.5, 4 and 6.2 min, respectively.

Before a long period of non-use, the column was flushed in the following way: first with 50 ml of methylene chloride—methanol (97:3), then with 50 ml of methylene chloride and finally with 50 ml of *n*-hexane, which was the solvent for column storage.

Procedure for blood and plasma samples

A 50-μl volume of internal standard solution (Table I, No. 5) was transferred to a screw-topped test-tube (PTFE-lined caps, Sovirel 15) for each unknown sample, 1 ml of biological fluid was added and the samples were mixed well. A separate set of standards was prepared by transferring 50-μl aliquots of mixed standard solutions (Table I, Nos. 1—4) into separate screw-topped test-tubes; 1 ml of control (pre-dose) plasma or blood was then added and mixed well.

To all samples, 0.5 ml of acetate buffer (2 *M*; pH 4.8) was added, and the samples were homogenized on a vortex mixer for few seconds; finally 6 ml of toluene were added and all tubes were shaken on a tumble extractor (5 min at 40 rpm). The samples were then centrifuged at 1500 *g* for 3 min at 5°C on a refrigerated centrifuge, Model K110 (Jouan, Saint-Nazaire, France); after this operation, 5 ml of supernatant were carefully transferred to conical test-tubes (Sovirel 13) and evaporated to dryness under a gentle stream of pure nitrogen in a thermostatted water-bath at 60 ± 2°C.

The residues were dissolved in a suitable volume of HPLC mobile phase (150—700 μl, depending on the expected concentration of the compounds

TABLE I

STANDARD SOLUTIONS USED FOR ANALYSIS

Standard solution No.*	Concentration (μg per 50 μl)		
	Compound I	Compound II	Compound III
1	0.25	0.25	0.50
2	0.50	0.50	0.50
3	1.00	1.00	0.50
4	2.00	2.00	0.50
5	0	0	0.50

*Solutions 1—4 are mixed standard solutions to be added to control plasma or blood for calibration. Solution 5 is an internal standard solution to be added to unknown samples.

of interest), aliquots were transferred to conical vials (autosampler vials with conicals inserts, cat. no. 10375; Chrompack, Middelburg, The Netherlands). An aluminium disk was crimped into each vial and all samples were set in a sample tray for automatic injection.

Calculation

Peak height ratios I:III and II:III obtained from plasma or blood standards plotted versus concentration of I and II were used to generate the linear least-squares regression line. The concentration of I and II in the unknowns were determinated by interpolation from the calibration curve using the peak-height ratios I:III and II:III, obtained from unknown specimens.

RESULTS

Addition of water to the mobile phase

Adding water to the mobile phase is very important for the deactivation of the absorbent, which greatly improves the separation and peak symmetry of the tested compounds, especially I. The presence of water prevents the variation of retention times from run to run, reduces band tailing and increases the maximum sample loading.

Column mobile phase equilibrium is reached rapidly and is maintained for long time. According to our experience with the μPorasil packing, the optimum content of water in the eluent for absorbent deactivation is 0.15% (v/v); if necessary, it can be increased to 0.2% (after ca. 500 injections of biological extracts) in order to improve the symmetry of the peak of compound I. Very useful information and data concerning the addition of water or polar modifiers to the mobile phase during liquid—solid chromatography are available in the literature [7].

Stability in organic solvents

The stability of compounds I—III in toluene was checked because in particular situations it may be necessary to stop the sample preparation for some time (after liquid—liquid extraction and before extract evaporation). No problems of instability for the tested compounds arise when toluene solutions are maintained at room temperature or at +5°C for 3 h.

As the method entails automatic injection overnight, the stability of the compounds was investigated. All three were dissolved in eluent mixture in a wide range of concentrations and kept at room conditions for 15 h (in order to mimic and stress the conditions of sample extracts set in the sample tray for automatic injection); all showed good stability when compared with similar freshly prepared solutions.

Linearity

A linear correlation between the peak-height ratios I:III and II:III and the concentrations of I and II, respectively, was found in the range 0.05—5 μg/ml of plasma; the linear least-squares regression calculation performed on the peak-height ratios versus concentration lines the following equations: for I: $y = 0.8x - 0.006$ (r=1, n=6); for II: $y = 0.58x - 0.008$ (r=1, n=6).

Sensitivity

The detection limit was ca. 50 and 30 ng/ml in plasma or blood, with a signal-to-noise ratio of ca. 3:1, for I and II, respectively.

Selectivity

Several blank plasma and blood samples from different humans were tested

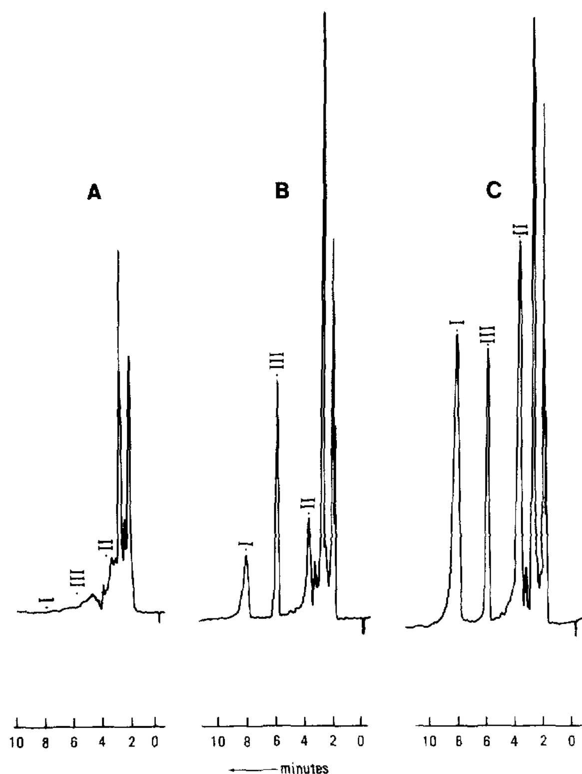

Fig. 2. (A) Chromatogram of drug-free plasma extract. (B) Chromatogram of authentic standards recovered from human drug-free blood; nominal concentrations of I and II were 0.25 μg/ml of biological sample. (C) Chromatogram of blood extract from a patient administered orally with a 600-mg dose of progabide (single administration, sample taken 3 h after drug intake).

TABLE II

INTRA-ASSAY PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH COMPOUND I (n=6)

Amount added (μg/ml)	Amount found (μg/ml)	Accuracy* (%)	Coefficient of variation (%)
0.15	0.15	102.0	2.2
0.50	0.48	97.0	1.9
1.00	0.98	97.8	1.0
2.00	2.00	100.8	1.0
Mean			1.5

*Accuracy defined as: (amount found/amount added) · 100.

for the absence of interfering endogenous components. Fig. 2A shows a typical chromatogram of a drug-free plasma extract: there are no interfering peaks at the retention times of the relevant compounds.

Statistical validation of the method

Before the statistical evaluation of the method, by using the internal standard, was performed, the absolute recoveries of compounds I, II and III were investigated from plasma and blood spiked with the compounds; the absolute recovery ranged between 80 and 90% for the three substances over a wide range of concentrations. Intra-assay and inter-assay precision studies were performed on control plasma spiked with different amounts of I and II (compound III was the internal standard), processed as described in Experimental. Intra-assay

TABLE III

INTRA-ASSAY PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH COMPOUND II (n=6)

Amount added (μg/ml)	Amount found (μg/ml)	Accuracy (%)	Coefficient of variation (%)
0.15	0.16	105	1.9
0.50	0.50	101	1.7
1.00	1.01	101	0.5
2.00	2.10	103	1.4
Mean			1.4

TABLE IV

INTER-ASSAY PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH COMPOUND I

Amount added (μg/ml)	Amount found (μg/ml)	Accuracy (%)	n	Coefficient of variation (%)
0.25	0.24	96	4	5.9
0.50	0.49	98	5	4.6
1.00	1.04	104	3	5.1
2.00	2.04	102	6	4.8
Mean				5.1

TABLE V

INTER-ASSAY PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH COMPOUND II

Amount added (μg/ml)	Amount found (μg/ml)	Accuracy (%)	n	Coefficient of variation (%)
0.25	0.23	92	3	4.3
0.50	0.50	100	6	5.1
1.00	1.00	100	5	4.4
2.00	2.04	102	6	5.0
Mean				4.7

precision was obtained by replicate analyses of plasma samples on the same day (Tables II and III). Inter-assay precision was determined by analysing the same plasma samples on various days over three weeks (Tables IV and V). The results demonstrate acceptable precision of the method over the concentration ranges investigated. Fig. 2B shows the relevant chromatogram.

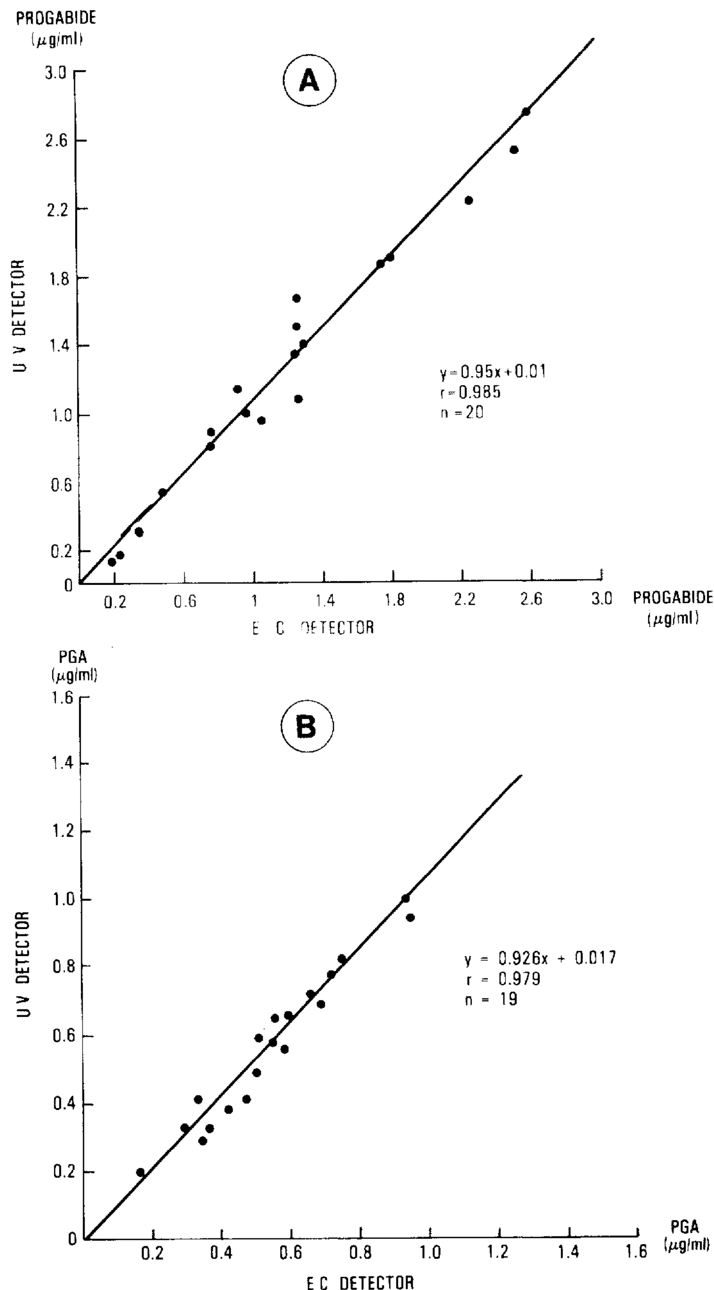

Fig. 3. Comparison of electrochemical and UV detection for (A) progabide and (B) PGA.

TABLE VI

BLOOD LEVELS OF COMPOUNDS I AND II IN A PATIENT (S.R.) ADMINISTERED ORALLY WITH PROGABIDE

A 1200-mg dose at t = 0 and 600 mg of drug every 6 h were given.

Time (h)	Blood levels (μg/ml)		Observations
	I	II	
0	N.D.*	N.D.	1200-mg Single administration
0.5	0.54	N.D.	
1.0	2.12	0.22	
1.5	2.58	0.35	
2.0	2.46	0.40	
3.0	1.96	0.49	
4.0	1.50	0.46	
5.0	1.25	0.47	
24.0	1.21	0.70	Samples taken before administration (minimum steady-state value)
48.0	1.89	0.91	
72.0	1.96	1.07	
96.0	2.02	1.49	
120	1.88	1.59	

*N.D. = Not detectable.

Comparison between this method and HPLC with electrochemical detection

For further validation of this method, it was compared with the HPLC with electrochemical detection [5], which is the method generally used for the determination of compound I in biological fluids. The results obtained from more than twenty samples (plasma and blood) obtained from clinical studies processed simultaneously by two analysts, were compared using Student's t-test for paired data. No statistical difference was found between the two methods at 95% confidence level for both compounds I and II (see Fig. 3). The correlation of the two methods by linear regression analysis gave $r = 0.985$ for I and $r = 0.979$ for II; the equations were $y = 0.95x + 0.01$ and $y = 0.926x + 0.017$, respectively.

Application of the method to biological specimens

The assay was applied to the quantitation of I and II in blood and plasma samples of patients dosed orally with I. Some results are reported in Table VI, and a typical chromatogram is shown in Fig. 2C.

DISCUSSION

Other types of silica column were tested, but μPorasil showed the best chromatographic properties with respect to the retention of compound I, which was too strong with other silica columns so that peak tailing occurred and the results were less reproducible.

The solvent and the buffer used for the extraction of compounds from the biological matrix were the same as those used by Padovani et al. [5]; however, the extraction conditions for this method are different because we used 6 ml of

toluene for a 5-min extraction (single), while Padovani et al. used 8 ml of toluene for a 20-min extraction (followed by clean-up). The choice of the wavelength of the detector (340 nm) allows the method to be specific, thus avoiding time-consuming purification of the biological samples; hence the sensitivity is lower than that of the other method [5], but, in most cases, the requirements of single-dose kinetic studies and drug monitoring are satisfied.

During work on blood samples, an extraneous peak was found in the chromatogram at retention times in the range 30—60 min; the peak is very broad and so does not affect the quantitation of the compounds of interest even if automatic injection is used with a fixed run time of 12 min. This extraneous peak was never found in plasma or serum samples.

The product of hydrolysis of I, benzophenone, if it is present in biological sample, is eluted at ca. 2 min; it cannot be quantitated but does not affect the determination of II.

The procedure described is rapid because there are no back-extractions to be performed; it does not need special apparatus, as it does use a very popular HPLC column that gives no problems under the described conditions. The column does not need any reactivation agent; the same column has been working for eight months, and has been used to process more than 1500 plasma and blood extracts without any trouble (no column damage or efficiency loss).

This method, based on liquid—solid chromatography, seems suitable for the quantitation of a compound that is unstable in aqueous medium. We have tried reversed-phase HPLC, but unsuccessfully. The reversed-phase HPLC method developed by Padovani et al. [5] was performed on the reduced compounds (the imine bond >C=N— being stabilized by hydrogenation), but this method is complicated because of the reduction and purification steps necessary before the chromatography with electrochemical detection (amperometric type, which we found very troublesome).

The method proposed has been used successfully for the determination of I and II in a large number of biological samples arising from clinical studies.

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