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Research Article

A Reversed-Phase High-Performance Liquid Chromatography Assay Procedure for Progabide and Its Related Metabolic Derivatives

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A reversed-phase high-performance liquid chromatography (HPLC) assay procedure for Progabide, its active acid metabolite (PGA), and its hydrolytic degradation product (SL79.182) has been developed. This highly specific technique has allowed the simultaneous determination of these drugs in aqueous samples, and when coupled with a single and easy extraction step, spiked plasma samples could also be analyzed. The method had a sensitivity of about 30, 45, and 100 ng/ml for Progabide, SL79.182, and PGA, respectively.

KEY WORDS: Progabide; reversed phase; high-performance liquid chromatography (HPLC); plasma; analysis.

INTRODUCTION

The rationale behind the synthetic development of Progabide, by Kaplan and co-workers (1), was the delivery of γ-aminobutyric acid (GABA) into the brain across the lipophilic blood–brain barrier.

Progabide is a Schiff's base derived from γ-aminobutyramide (GABAmide) and 2-hydroxy-5-fluoro-4′-chlorobenzophenone (SL79.182). Chemically, Progabide is 4-[((4-chlorophenyl) (5-fluoro-2-hydroxyphenyl)methylene)-amino]butanamide and its acid metabolite (PGA) is 4-[((4-chlorophenyl) (5-fluoro-2-hydroxyphenyl)methylene)-amino]butyric acid (Fig. 1).

The imine link (C = N) of both Progabide and PGA can be broken down *in vivo* to release GABAmide and GABA, respectively (Fig. 2). However, it was found (2) that Progabide was converted to PGA at a much faster rate than any of the other changes. Both Progabide and PGA display intrinsic GABA-mimetic activity, with PGA having about 50 times more potency than Progabide in displacing ^{3}H-GABA from binding sites in human cerebellum preparations. The order of potency determined for the above drugs was GABA > PGA > GABAmide >= Progabide (1,2). Thus, in order to elucidate the role played *in vivo* by the above compounds and also to be able to assess the bioavailability of Progabide in different animal models, a sensitive, reproducible, and preferably specific assay procedure was required.

Previously, Gillet *et al.* (3) reported an electron capture–gas-liquid chromatography (GLC) method for the analysis of Progabide which was used for the determination of the pharmacokinetic profile of Progabide in the rhesus monkey (4). The procedure involves extraction of Progabide followed by derivatization with heptafluorobutyric anhydride (HFBA). An analogue of Progabide, SL78.050 (Fig. 1), was used as the internal standard. Our preliminary investigations with the above method indicated the poor stability of the internal standard chosen under the derivatization conditions employed and the difficulty in the complete removal of HFBA from reaction mixtures by evaporation. The method is also unsuitable for the determination of PGA. More recently, two reversed-phase high-performance liquid chromatography (HPLC) assay procedures which employ electrochemical detection have been published (5,6). Both methods offer excellent sensitivity to Progabide and PGA but involve lengthy derivatization procedures following the initial extraction step, aimed at preventing any in-process degradative drug losses. In our own experience, any serious drug losses normally occur before the initial extraction procedure and not after, unless vigorous conditions are employed. Further, numerous liquid–liquid purification procedures were necessary to facilitate the detection of SL79.182 (6). In comparison, Ascalone *et al.* (7) used a less sensitive straight-phase HPLC method with uv detection which required only a single and easy extraction step. However, their method did not detect SL79.182.

This paper describes a new reversed-phase HPLC method with uv detection for the simultaneous analysis of Progabide, PGA, and SL79.182 in clean aqueous solutions or in plasma following a single and easy extraction step.

MATERIALS

Progabide, SL79.182, and PGA sodium salt were supplied by LERS, Paris, France, and used as received. A

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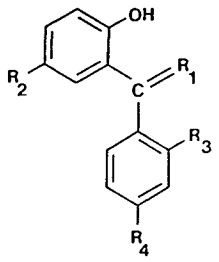

Compound	R_1	R_2	R_3	R_4
Progabide	$N(CH_2)_3.CONH_2$	F	H	Cl
SL75.102 (PGA)	$N(CH_2)_3.COOH$	F	H	Cl
SL78.050	$N(CH_2)_3.CONH_2$	Cl	H	Cl
SL79.182	O	F	H	Cl
Internal Standard	O	Br	Cl	H

Fig. 1. Structural formulas of Progabide and related compounds.

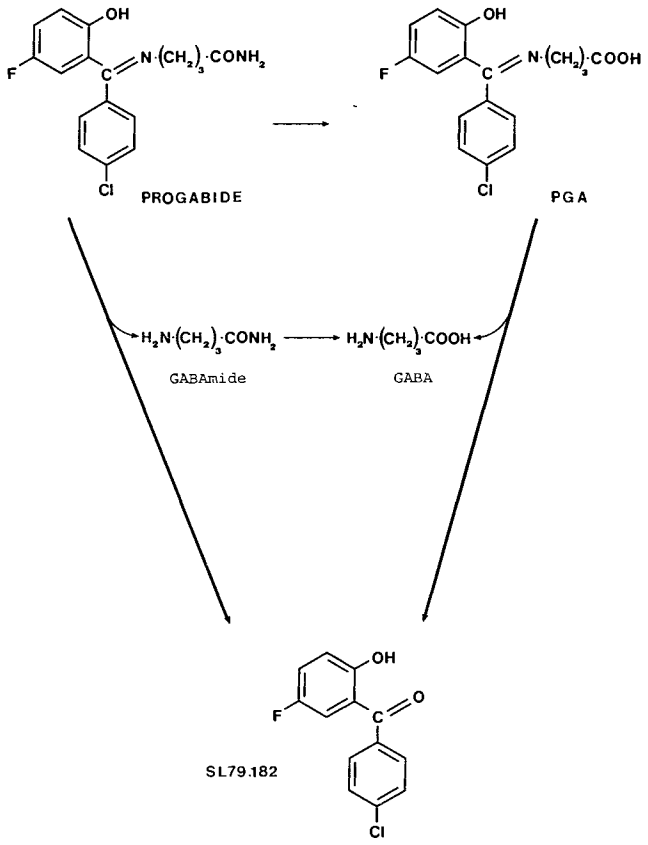

Fig. 2. Main biotransformation routes of Progabide.

structural analogue of SL79.182, namely, 2-hydroxy-5-bromo-2'-chlorobenzophenone was also provided through LERS for use as an internal standard (Fig. 1). Methanol and acetonitrile, both of HPLC grade, were purchased from Blackford Wells, U.K. Ethyl acetate (AnalaR) was obtained from B.D.H. Unless otherwise specified, all other reagents were of AnalaR quality.

Blank plasma was obtained by centrifuging at 3000 rpm pooled blood samples from lop-eared male rabbits and then stored at −20°C.

INSTRUMENTATION

A Cecil HPLC system was used. This consisted of a reciprocating pump (Cecil CE 2010) together with a Cecil CE 2112 variable-wavelength spectrophotometer fitted with an 8-μl flow-through cell of 10-mm path length. The signal was recorded on a J.J. CR 652 recorder and integrated by a Pye Unicam DP 88 computing integrator. The analytical wavelength used was determined by scanning for the uv spectra of the compounds on a Kontron Uvikon 810 spectrophotometer with a Uvikon 21 recorder. The chromatographic separation was performed on a 247 × 4.5-mm-i.d. stainless-steel column packed with Spherisorb ODS1 of 5 μm (Jones Chromatography). The mobile phase was maintained particle-free by an in-line 70 × 4.5-mm-i.d. stainless-steel column packed with Co:Pell ODS (Whatman Chemical Separations) chemically bonded to 30- to 38-μm glass beads. The Rheodyne injection port assembly comprised a 20- or a 100-μl fixed-volume stainless-steel loop for sample injection. For the sample preparation procedure, two MT16 minor vortex mixers (Chiltern Scientific Enterprises) and an MSE super minor centrifuge were used. A rotary evaporator (Rotavapor-R, Buchi) served to remove the organic solvents from the sample flasks.

METHODS

Analysis of Biological Samples

Determination of the Analytical Wavelength

Separate solutions of Progabide, SL79.182, and PGA were prepared in the mobile phase. These were scanned between 225 and 500 nm and a suitable common analytical wavelength was determined.

Chromatographic Conditions

Optimizing performance of an HPLC system involves a good combination of separation and compactness of peaks together with a high speed of elution. Various solvent combinations were attempted, and of these the system chosen for use had the following composition: acetonitrile, 35%/methanol, 30%/McIlvaine buffer (pH 5.25), 35% (8). Prior to use, the mobile phase was filtered free from particulate matter on a glass ceramic sinter and then degassed with helium. No further degassing was deemed necessary during the chromatography. The Spherisorb ODS1 5-μm column was used with a mobile-phase flow rate of 1.4 ml/min at a pressure of 150 bar. The uv detector was set at 255 nm with an AUFS of 0.05 except for the calibration curve of Progabide in the range 0–10 μg/ml, for which an AUFS of 0.2 was used.

Standard Solutions

Stock solutions of Progabide, SL79.182, PGA, and the internal standard were prepared in methanol. For Progabide

and PGA, the stock solutions were prepared freshly every 3 days; for SL79.182 and the internal standard, stock solutions were prepared every 2 weeks. All the solutions were stored at 4°C until required for use, at which time they were allowed to reach room temperature before aliquots were removed.

For the bioavailability studies (9), two calibration curves with different amounts of the internal standard were constructed for Progabide, whereas a single calibration curve was sufficient for PGA and SL79.182.

Sample Preparation Procedure

The procedure outlined below minimizes the in-process degradative losses that can occur due to hydrolysis (10). The plasma was allowed to thaw and 0.5-ml aliquots were transferred into 15-ml screw-neck glass extraction tubes placed in crushed ice. A 100-μl quantity of the internal standard solution (5 μg/ml) was added to each tube except for the Progabide calibration curve in the range 0.8–10.4 μg/ml, for which 80 μl of a 25 μg/ml solution of the internal standard was used. The plasma was then spiked with the solution of the drug under study in such a manner as to cover the concentration range desired. Next, 2 ml of ethyl acetate was added to each tube, which was then sealed with a PTFE-lined cap and vortexed for 2 min before returning it into the crushed ice bath. Following centrifugation at 3000 rpm for 5 min, the ethyl acetate layer was removed into a 5-ml quick-fit round-bottom flask and the organic solvent was removed on the rotary evaporator. The solid residue in each flask was reconstituted in 150 μl of the mobile phase and 100 μl was injected onto the column.

Appropriate blanks were carried through the same procedure and all samples were performed in triplicate.

Efficiency of the Sample Preparation Procedure

On the assumption of insignificant losses during the extraction procedure, amounts of Progabide equivalent to those that would have been obtained if 100% recovery was achieved were directly added to the 5-ml flasks from the stock solution and then followed by 100-μl quantities of the 5 μg/ml internal standard solution to cover the Progabide 0.1–4 μg/ml calibration curve. As given above, the solid residue obtained was reconstituted and 100 μl injected for analysis.

Analysis of Nonbiological Samples of Progabide

Aqueous samples of nonbiological origin do not require pretreatment before they are applied onto the HPLC column. Standard solutions of Progabide in a 0.1 *M* citric acid–sodium citrate buffer of pH 6.0 were prepared by spiking the buffer with a 5 mg/ml Progabide stock solution, and 20 μl of the resultant solutions was immediately analyzed by HPLC. An appropriate blank was also injected onto the column. All samples were performed in duplicate. The chromatographic conditions were as given earlier except that the flow rate of the mobile phase was increased to 1.7 ml/min.

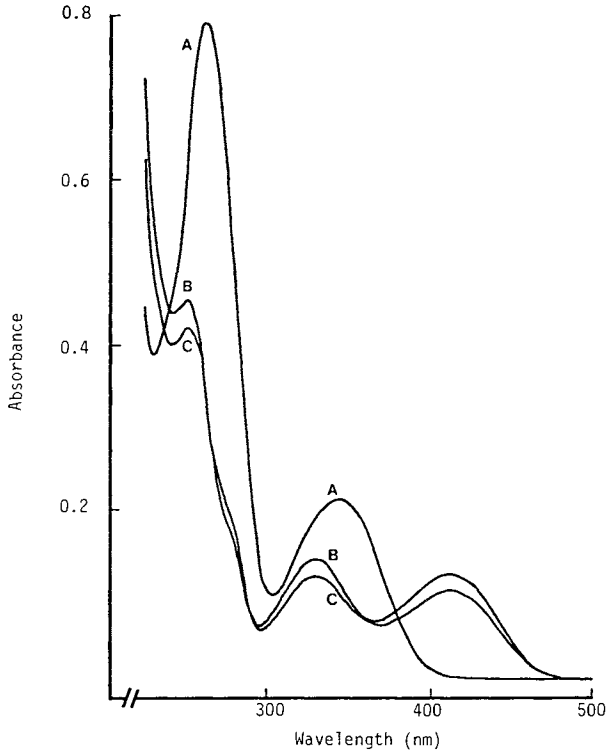

Fig. 3. Visible/uv absorption spectra of Progabide, SL79.182, and PGA in the mobile phase of the HPLC system: (A) SL79.182, (B) Progabide, and (C) PGA.

Calibration Curves—Construction and Use

Standard curves were obtained by plotting the peak area ratios (drug/internal standard) or peak area (where no internal standard was used) against the concentration of the drug examined. The concentration of drug in unknown samples was determined by interpolation from the standard curves.

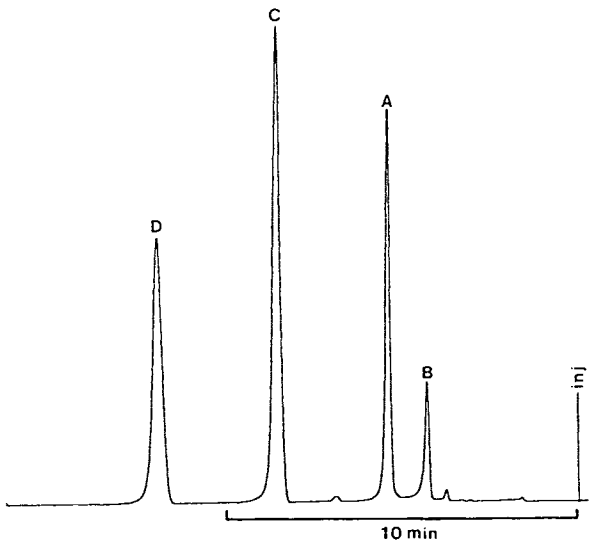

Fig. 4. Typical example of the RP-HPLC chromatogram of the detected compounds: (A) Progabide, (B) PGA, (C) SL79.182, and (D) internal standard.

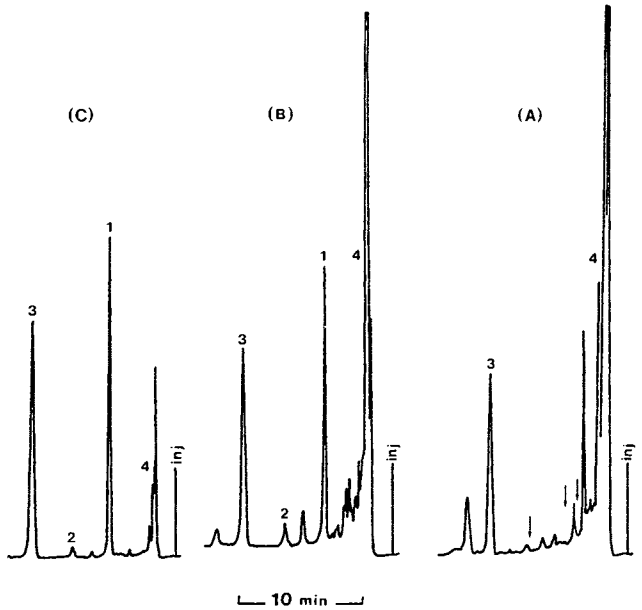

Fig. 5. Typical RP-HPLC chromatograms of blank plasma (A), spiked plasma with Progabide (B), and an equivalent unextracted sample of Progabide (C): (1) Progabide, (2) SL79.182, and (3) internal standard.

RESULTS AND DISCUSSION

In Fig. 3, the spectra of the three compounds are shown. The absorption maximum at 255 nm was chosen as the analytical wavelength in preference to that at 330 nm because of the increased sensitivity offered.

Figure 4 shows a typical chromatogram obtained when a 10-μl quantity of a mixture of all the compounds was injected onto the column. The retention times were 4.2 min for PGA, 5.4 min for Progabide, 8.5 min for SL79.182, and 11.7 min for the internal standard.

The sample preparation procedure adopted was based on a thorough knowledge of the stability–pH profiles of the drugs investigated and their partition–pH behavior (10). Although it is a standard practice (11) to precipitate the proteins prior to the extraction procedure and to attempt by a variety of methods, such as osmotic shock, hydrolysis, and filtration, to purify the sample, such treatment cannot be adopted in the case of Progabide and PGA because the rigorous conditions employed can result in degradative losses of these compounds. Emphasis was also placed on optimizing the experimental procedure used. For example, although both toluene and ethyl acetate can equally be used for the exraction step, ethyl acetate was used preferentially because it is much easier to remove on the rotary evaporator.

Table II. Intraassay Reproducibility and Accuracy for Spiked Plasma Samples

Compound	Sample concentration (μg/ml)	Average of 6 assays (μg/ml)	Coefficient of variation (%)
Progabide	0.20	0.20	2.1
	8.00	7.95	1.6
PGA	2.00	2.06	1.9
	8.00	8.10	1.4
SL79.182	0.20	0.20	1.3
	2.00	1.95	1.8

Unfortunately, however, avoiding a rigorous cleanup pretreatment leaves a number of contaminants in the samples. In Fig. 5A, the chromatogram of the blank plasma displays a number of unidentified peaks, particularly at early elution times. The arrows indicate the elution times of the three drugs: Progabide, SL79.182, and PGA. Only with the latter compound did the peak lie close to interfering peaks of significant size and this reflected in the calibration curve of PGA (Table I), where a positive appreciable intercept was obtained. The linear regression analysis results for the standard curves are summarized in Table I.

The developed procedure can detect very low concentrations of pure compounds but the practical limits of determination for plasma samples were found to be 30, 45, and 100 ng/ml for Progabide, SL79.182, and PGA, respectively, with a signal-to-noise ratio of ca. 5:1 and with only 0.5 ml of plasma carried through the analysis. This sensitivity is lower than the 10-ng/ml limit achieved by gas chromatography (3) and by HPLC with electrochemical detection (6) but is equivalent to that reported for HPLC with ultraviolet detection (7). However, it is adequate for monitoring the blood levels attained in therapeutically relevant clinical studies (12) and in single-dose pharmacokinetic studies (13). If required, the sensitivity can be further increased by processing larger volumes of plasma.

Although the extraction step was extremely efficient, the in-process losses resulted in an overall recovery of about 85% for the above drugs and 83% for the internal standard. In Figs. 5B and C, the recovery of Progabide at 0.75 μg/ml is illustrated. Comparison of the theoretical 100% recovery of

Table I. Least-Squares Linear Regression Analysis of the Calibration Curves

Calibration curve	Concentration range (μg/ml)	Regression equation	Correlation coefficient
Progabide theoretical 100% recovery	0.1–4.0	$Y = 0.9596(X) + 0.034$	0.9998
Progabide in plasma	0.1–4.0	$Y = 0.9775(X) - 0.008$	0.9998
Progabide in plasma	0.8–10.4	$Y = 0.2418(X) - 0.014$	0.9990
Progabide in buffer	4.0–30.0	$Y = 1997.4(X) + 661.8$	0.9998
SL79.182 in plasma	0.1–2.0	$Y = 1.6725(X) + 0.055$	0.9987
PGA in plasma	2.0–8.0	$Y = 0.3773(X) + 0.321$	0.9999

Table III. Interassay Reproducibility and Accuracy for Spiked Plasma Samples

Compound	Sample concentration (μg/ml)	Average of 6 assays (μg/ml)	Coefficient of variation (%)
Progabide	0.20	0.20	6.1
	8.00	7.89	4.6
PGA	2.00	2.09	3.1
	8.00	7.92	5.2
SL79.182	0.20	0.21	7.9
	2.00	2.08	3.9

Progabide with the plasma standard curve over the concentration range 0.1–4 μg/ml demonstrates that the two curves are well matched (Table I), indicating negligible in-process degradation of Progabide and consistent relative recovery of Progabide and the internal standard over the investigated concentration range. The within-day and between-day precision of the assay was determined at the low and high ends of the standard curves of the three compounds (Tables II and III). The results demonstrate the acceptable precision of the procedure. Unlike blank plasma, the injected blank buffer solution gave a chromatogram (Fig. 6A) with a small solvent front and a few early peaks of unretained solutes. This permitted an increase in the mobile phase flow rate to give retention times of 2.6, 3.7, and 5.1 min for PGA, Progabide, and SL79.182, respectively (Fig. 6B). A small peak, corresponding in position to the authentic PGA, was present in all the chromatograms. This peak increased as the amount of Progabide in the original aqueous solution was increased. Since Progabide does not degrade to PGA *in vitro* (10), it can be assumed that the small quantity of PGA present is an impurity in the original Progabide sample. The calibration curve obtained (Table I) clearly indicates the linearity of the detection system over the concentration range examined.

In conclusion, an optimized reversed-phase HPLC procedure has been developed for the simultaneous analysis of Progabide, PGA, and SL79.182 in aqueous solutions and also in plasma. This has been applied in kinetic and biopharmaceutical studies which are reported elsewhere (9,10).

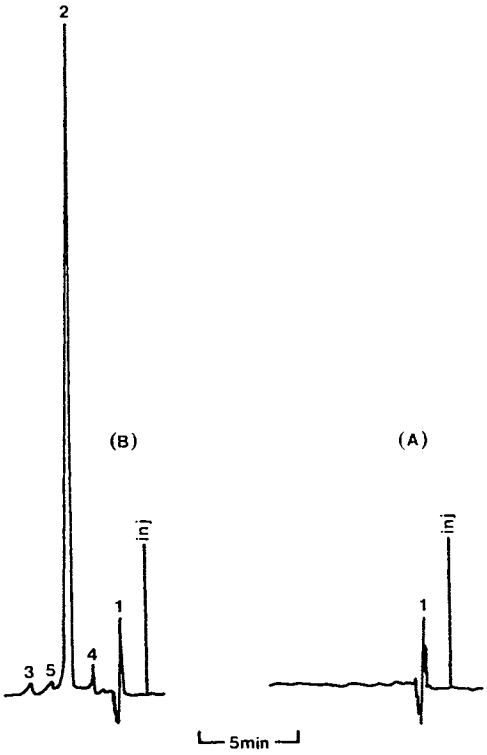

Fig. 6. Typical chromatograms obtained after injection of a blank buffer solution (A) and an aqueous buffered solution of Progabide (B): (1) solvent front containing unretained solutes, (2) Progabide, (3) SL79.182, (4) PGA, and (5) unidentified peak.

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