Journal of Chromatography, 276 (1983) 103—110
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1728

MEASUREMENT OF PROGABIDE AND ITS DEAMINATED METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

WAYNE YONEKAWA*, HARVEY J. KUPFERBERG and THOMAS LAMBERT

Epilepsy Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20205 (U.S.A.)

(First received November 9th, 1982; revised manuscript received March 23rd, 1983)

SUMMARY

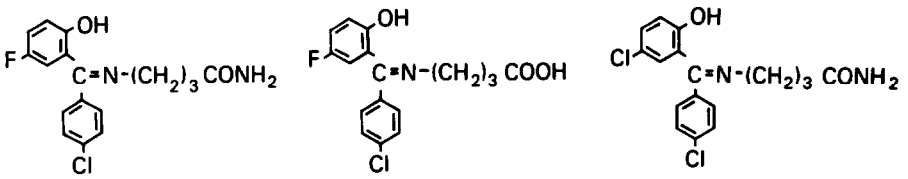
Progabide (4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenol)-methylene]amino}butanamide) and its deaminated metabolite were measured simultaneously in plasma by high-performance liquid chromatography. Both compounds were extracted from plasma and the molecules were stabilized at the methylene—amino double bond with sodium borohydride reduction. Oxidative electrochemical detection was used for final quantitation. The method was used to measure progabide and progabide acid in plasma from a healthy volunteer who received a single 1200-mg dose of progabide orally. Lower limits of detection for progabide and progabide acid were 30 and 15 ng/ml, respectively. Coefficient of variation was less than 5% for both compounds.

INTRODUCTION

Progabide (4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenol)-methylene]-amino}butanamide) is a recently introduced compound with possible γ-aminobutyric acid (GABA) mimetic and anticonvulsant properties [1—9]. The structures of progabide (SL-76002), the deaminated acid metabolite (SL-75102), and a dichloro analogue of progabide (SL-78050) used as the internal standard (I.S.) are shown in Fig. 1.

Methods currently available for the quantitation of progabide either lack sensitivity or do not allow concurrent measurement of the active acid metabolite [2, 10, 11]. Also, an inherent problem is the instability of both the parent drug and the acid metabolite at the methylene—amino double bond [12]. The use of standard plasma extraction procedures tends to hydrolyze this bond, yielding the benzophenone and GABA amide in the case of progabide or the benzophenone and GABA in the case of progabide acid.

0378-4347/83/$03.00



PROGABIDE (SL 76002) | PROGABIDE ACID (SL 75102) | I.S. (SL 78050)

Fig. 1. Chemical structures of progabide, progabide acid, and internal standard (I.S.).

This paper describes a quantitative high-performance liquid chromatographic (HPLC) procedure with electrochemical detection (LCEC) for the measurement of nanogram quantities of both progabide and progabide acid in plasma. By employing a sodium borohydride reduction of the double bond to stabilize the molecule, routine plasma extraction procedures can be utilized.

MATERIALS AND METHODS

Apparatus

The instruments used in this study were an Altex Model 110A pump (Altex, Berkeley, CA, U.S.A.) and a BAS LC-4A amperometric detector equipped with a TL-5 glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) for oxidative electrochemical detection. The injector was a Model 7125 Rheodyne equipped with a 10-μl loop (Rheodyne, Cotati, CA, U.S.A.).

A RAC® Partisil 5 ODS-3 column (5-μm particle size), 10 cm × 9.4 mm I.D., with >90,000 theoretical plates per m (Whatman, Clifton, NJ, U.S.A.) was found to give good separation and ideal retention times for all compounds chromatographed, and back pressure was minimal. A Spheri-5® (5-μm particle size) RP-18 cartridge, 3 cm × 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.) served as guard column.

All chromatograms were recorded on a Beckman 10-in. strip-chart recorder (Beckman, Fullerton, CA, U.S.A.).

Reagents and chemicals

Hexane (UV), methanol, propanol-2, and methylene chloride distilled-in-glass were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and were used without further purification. HPLC grade water (J.T. Baker, Phillipsburg, NJ, U.S.A.) was used to prepare all eluent buffers. Buffers for extraction required deionized water only.

Sodium citrate, monopotassium phosphate (HPLC grade), dipotassium phosphate, sodium hydroxide, and sodium acetate (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Sodium borohydride, >99% purity, was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. Some of the glassware required pretreatment with triethylamine, Sequanal grade® (Pierce, Rockford, IL, U.S.A.) to eliminate adsorptive losses [13].

Stock standard solutions with a concentration of 100 ng/μl were prepared by using acetonitrile as solvent for progabide and I.S. and methanol for progab-

ide acid. From this stock solution, working standard solutions of 10 ng/μl (progabide), 5 ng/μl (progabide acid), and 20 ng/μl (I.S.) were prepared. These were kept at —4°C when not in use and were replaced every three weeks.

Extraction

Working standard solutions were added to 16 × 125 mm glass culture tubes in amounts ranging from 30 to 1500 ng for progabide and 15 to 750 ng for progabide acid. After 400 ng of I.S. were added, all traces of solvent were removed under nitrogen and 0.5 ml of blank plasma was added. These standards were then extracted concurrently with the patient samples by the following procedure.

A 0.5-ml volume of plasma, 0.5 ml of 2 *M* sodium acetate (adjusted to pH 4.9 with hydrochloric acid), and 9 ml of hexane—propanol-2 (96:4) were added to tubes containing 400 ng of I.S. previously dried under nitrogen. The tubes were capped with PTFE-lined screw caps, shaken for 5 min, and centrifuged. The organic layer was transferred to clean 16 × 125 mm tubes pretreated with 20% triethylamine in methanol [13].

The reduction step consisted of adding 0.5 ml of 0.4% sodium borohydride in ethanol (w/v) to each tube. After a brief vortex mixing, the tubes were allowed to set at room temperature. After exactly 10 min, 2 ml of 0.25 *M* sodium citrate (adjusted to pH 2 with hydrochloric acid) were added to each tube. The tubes were shaken for 5 min, centrifuged, and the hexane—propanol-2 was removed completely by aspiration. Another 9 ml of hexane—propanol-2 were added, the tubes were shaken for 5 min, centrifuged, and the organic layer was discarded.

To the sodium citrate remaining in the tube, 200 μl of 5 *N* sodium hydroxide, 0.5 ml of 1 *M* sodium citrate (adjusted to pH 4.8 with hydrochloric acid), and 9 ml of methylene chloride were then added. The tubes were shaken for 5 min, centrifuged, and the organic layer was transferred to clean tubes. The methylene chloride was then dried under nitrogen. The dried residue was reconstituted in 400 μl of methanol, and 10 μl were used for HPLC analysis.

Chromatographic conditions

The mobile phase for HPLC was a methanol—buffer (70:30) solution degassed with an ultrasonic bath after mixing. The buffer was a 33.3 m*M* monopotassium phosphate solution adjusted to pH 5.06 with 33.3 m*M* dipotassium phosphate. The buffer was replaced weekly. The eluent flow-rate was adjusted to 2 ml/min.

The potential of 1 V was selected on the amperometric detector electrode because it was the lowest voltage that gave acceptable sensitivity. The glassy carbon electrode was polished daily to preclude the possibility of passivation due to the high voltage required for maximum sensitivity.

RESULTS AND DISCUSSION

The ultraviolet (UV) spectra of the unreduced progabide and its reduced form are shown in Fig. 2. With use of the 0.4% sodium borohydride solution under the conditions described, the reaction was completed in less than 10

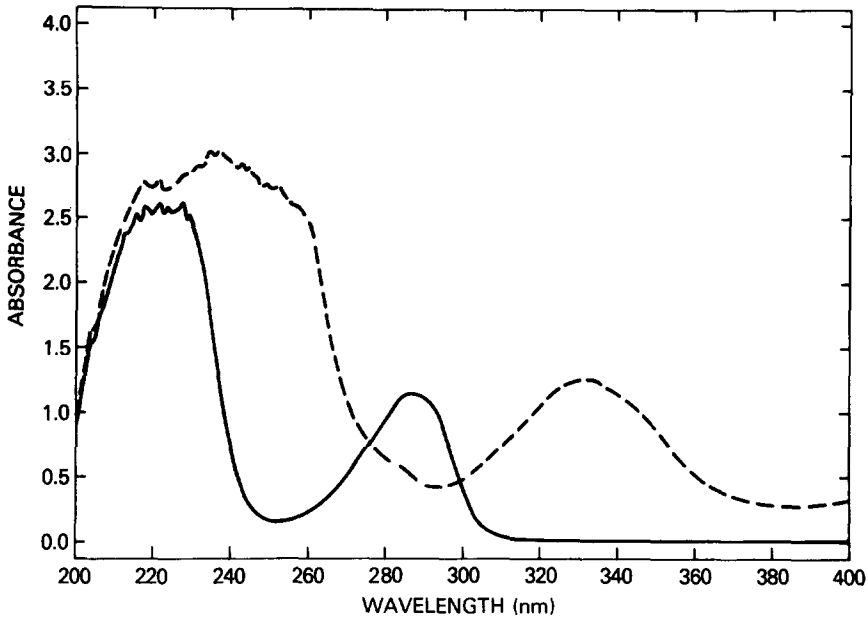


Fig. 2. UV spectra of 0.3 m*M* solution of progabide (— — —) and progabide after sodium borohydride reduction (——).

min, i.e., the maxima disappeared at ca. 332 nm, indicating the reduction of the methylene—amino double bond. The spectrum remained unchanged for at least 30 min, the maximum time the reaction was followed. The appearance of the maxima at 288 nm is thought to reflect additional ring resonance arising from the partial dissociation of the phenolic hydrogen. The electronegativity of fluorine may contribute to this dissociation. The UV spectra of progabide acid and the I.S. were similar but not identical to those of progabide. The relatively high UV absorption of these compounds indicated that UV quantitation might be a reliable method of analysis. The plasma extracts, however, manifested numerous interfering peaks throughout the UV range investigated, especially when the reduction and purification steps were not employed. The introduction of purification procedures yielded samples clean enough to allow detection at 280—290 nm, but the sensitivity was greatly attenuated. For these reasons, LCEC was chosen as the method of analysis.

The addition of 0, 100, 250, 500, or 1000 μl of ethanol in the hexane—propanol-2 (96:4) extraction solvent was compared with the percent drug reduced and showed that the reaction was facilitated by the addition of 250—1000 μl of ethanol. Therefore, 500 μl of sodium borohydride in ethanol was arbitrarily chosen as the amount exceeding the minimal effective alcohol concentration for the reaction. The effective range of the sodium borohydride concentration was also evaluated. All concentrations from 0.2% to 1% were equally effective for a complete reaction within 10 min. The 0.4% concentration was selected because it exceeded the minimal effective concentration yet was low enough to be easily neutralized in subsequent steps. It was also thought that the lower the sodium borohydride concentration, the less the possibility that the compounds might break down.

Progabide and progabide acid calibration curves obtained through linear regression analysis are described below. Each calibration curve is derived from the data of eight individual standard curves obtained over a 1-month period. These individual curves each consisted of at least six points covering the assay range.

Progabide: $Y = 0.0294 + 0.0028X$, $r = 0.9967$
95% confidence intervals: slope = 0.0028±0.0001, intercept = 0.0294±0.0469

Progabide acid: $Y = 0.0336 + 0.0035\,X$, $r = 0.0079$
95% confidence intervals: slope = 0.0035±0.0001, intercept = 0.0336±0.0432

The range of 30—1500 ng for progabide may have approached the maximal linear range of the electrochemical detector cell; occasionally a negative deviation from linearity was encountered at the high end. By diluting the final extract residue in a relatively large volume of 400 μl of methanol and injecting a constant 10 μl of this solution, several conditions that could possibly lead to this nonlinearity were minimized. Cell overloading, for example, was decreased by limiting the injection concentration to less than 40 ng. Under these dilution conditions, however, very low levels of progabide (<60 ng/ml) were difficult to quantitate unless the detector was kept meticulously clean. Also, so-called uncompensated resistance or IR drop effects were minimized by limiting the amounts of compound injected. Finally, any possible problems caused by injection volume differences were eliminated by keeping volumes constant.

The coefficient of variation was found to be <1% in a comparison of the peak height ratio between progabide or progabide acid and I.S. after repeated injections of the same sample. The coefficients of variation for a low and high determination of progabide and progabide acid extracted five times on three separate days and quantitated using three separate standard curves are shown in Table I.

A representative HPLC trace of a plasma extract from a healthy volunteer given a single oral dose of 1200 mg of progabide is shown in Fig. 3. The peaks correspond to plasma levels of 400 ng/ml for progabide and 829 ng/ml for progabide acid. The quantitation of plasma progabide levels versus time in nine healthy volunteers after single 1200-mg oral doses indicated that progabide

TABLE I

COEFFICIENTS OF VARIATION (%) OBTAINED FROM REPEATED EXTRACTIONS OF POOLED PLASMA SAMPLES (n = 15)

Coefficient of variation (%) = S.D./$\bar{X}$ × 100.

	Low	High
Progabide*	4.7	2.3
Progabide acid**	3.1	2.8

*Progabide concentration was <300 ng/ml for the low value and >2000 ng/ml for the high value.

**Progabide acid was <400 ng/ml for the low value and >1000 ng/ml for the high value.

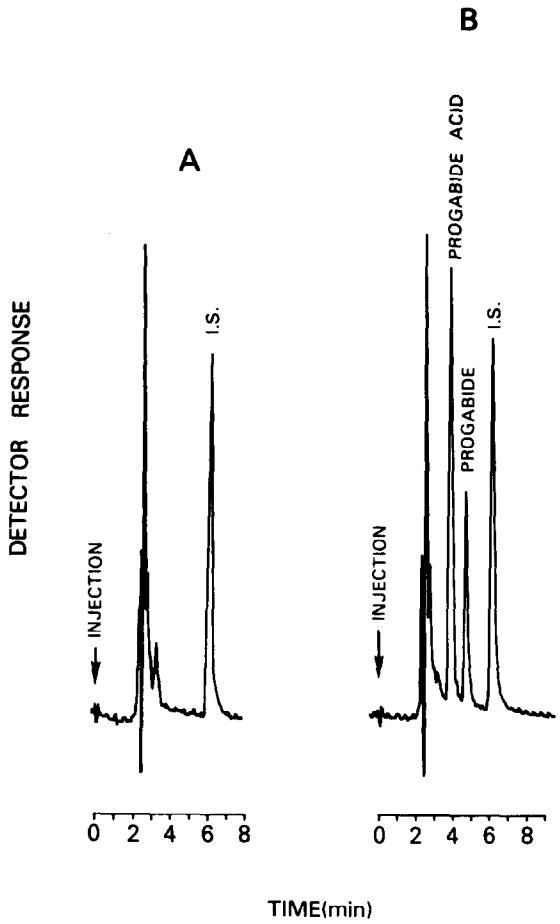


Fig. 3. Typical high-performance liquid chromatograms of plasma extracts from a volunteer before receiving progabide (A) and 12 h after orally receiving 1200 mg of progabide (B). Plasma progabide level at this time is approximately one-tenth of the peak plasma progabide level found at 3 h. The internal standard (I.S.) response is equal to 2.0 nA.

levels could reach as high as 6700 ng/ml within 2 h after administration. However, plasma progabide levels were not detectable after 48 h. The study also indicated that progabide acid levels could reach highs of 1400 ng/ml in 8 h, with detectable levels sometimes remaining after 48 h.

Fig. 4 shows a typical plasma decay curve in one of these volunteers. Progabide was rapidly absorbed, with peak plasma levels occurring 90 min after administration. Progabide also disappeared from plasma rapidly, with approximately a 2-h half-life. Progabide acid reached peak plasma levels at approximately 8 h, and plasma levels could be detected for a longer period of time.

It is obviously impossible to quantitate progabide in 0.5 ml of plasma when plasma levels of the drug are $\geqslant$3000 ng/ml; these levels exceed the highest standards, and linearity at this level is uncertain. In these cases, 200 μl of plasma were used for analysis, since our studies indicated that there is no difference in recovery when either 0.2 ml or 0.5 ml of plasma is used. However,

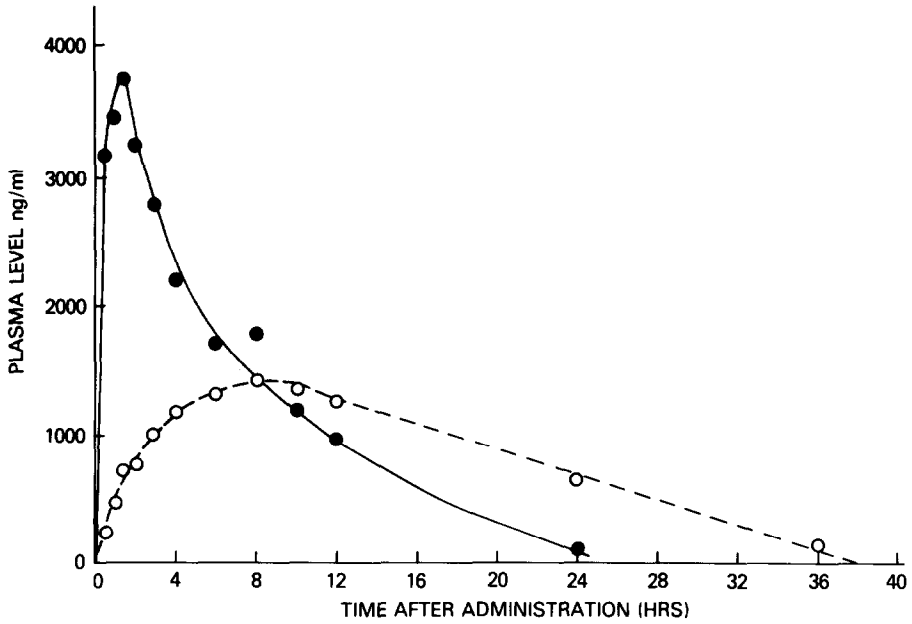


Fig. 4. Plasma progabide (•) and progabide acid (○) levels versus time after administration of a single 1200-mg oral dose of progabide to a healthy volunteer.

blank plasma was added to these samples to keep all volumes constant. When plasma progabide and progabide acid levels were low, 1 ml of plasma was used for analysis of both standards and unknowns. Recovery will decrease when more than 0.5 ml of plasma is used, but levels as low as 30 ng/ml of progabide and 15 ng/ml of progabide acid can be detected.

ACKNOWLEDGEMENTS

We wish to thank Dr. Kenneth Dudley and Daniel Bius for consultation during this study and Ms. B.J. Hessie for editorial advice and assistance.

REFERENCES

1 P. Loiseau, B. Cenraud, L. Bossi and P.L. Morselli, in M. Dam, L. Gram and J.K. Penry (Editors), Advances in Epileptology: The XIIth Epilepsy International Symposium, Raven Press, New York, 1981, pp. 135—139.
2 I. Johno, B.T. Ludwick and R.H. Levy, J. Pharm. Sci., 71 (1982) 633—636.
3 K.G. Lloyd and P.L. Morselli, in D.M. Woodbury, J.K. Penry and C.E. Pippenger (Editors), Antiepileptic Drugs, Raven Press, New York, 2nd ed., 1982, pp. 839—858.
4 P. Worms, H. Depoortere, A. Durand, P.L. Morselli, K.G. Lloyd and G. Bartholini, J. Pharmacol. Exp. Ther., 220 (1982) 660—671.
5 K.G. Lloyd, S. Arbilla, K. Beaumont, M. Briley, G. de Montis, B. Scatton, S.Z. Langer and G. Bartholini, J. Pharmacol. Exp. Ther., 220 (1982) 672—677.
6 B. Scatton, B. Zivkovic, J. Dedek, K.G. Lloyd, J. Constantinidis, R. Tissot and G. Bartholini, J. Pharmacol. Exp. Ther., 220 (1982) 678—688.
7 B. Scatton and G. Bartholini, J. Pharmacol. Exp. Ther., 220 (1982) 689—695.
8 N.G. Bowery, D.R. Hill and A.L. Hudson, Neuropharmacology, 21 (1982) 391—395.

9 B. Scatton, B. Zivkovic, J. Dedek, P. Worms, H. Depoortere, K. Lloyd and G. Bartholini, in R. Canger, F. Angeleri and J.K. Penry (Editors), Advances in Epileptology: The XIth Epilepsy International Symposium, Raven Press, New York, 1980, pp. 445—448.
10 G. Gillet, J. Fraisse-André, C.R. Lee, L.G. Dring and P.L. Morselli, J. Chromatogr., 230 (1982) 154—161.
11 J.T. Burke and B. Ferrandes, personal communication.
12 Preliminary Report on SL-76002, Synthélabo, Paris, 1980, 4 pp.
13 E. Antal, S. Mercik and P.A. Kramer, J. Chromatogr., 183 (1980) 149—157.