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DETERMINATION OF PROGABIDE AND ITS MAIN ACID METABOLITE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

APPLICATION TO THE MEASUREMENT OF BLOOD/PLASMA PARTITION RATIO

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SUMMARY

A method for the measurement in plasma, blood and urine of progabide, its main acid metabolite, and the corresponding benzophenone is described. This assay allows the determination of progabide and its acid metabolite for therapeutic drug monitoring, and with a minimum detectable concentration of 1—10 ng/ml for progabide and its acid metabolite, it is sensitive enough for pharmacokinetic studies. Progabide and its metabolites are extracted from biological samples with toluene at pH 4.5. Following reduction of the imine bond with sodium borohydride, the reduced drugs are back-extracted into an aqueous phase at acid pH and reextracted by diethyl ether at alkaline pH.

Progabide, its acid metabolite and the benzophenone are separated by high-performance liquid chromatography using a 3-μm ODS column with a quaternary solvent mixture of methanol—acetonitrile—phosphate buffer (0.033 *M*, pH 5.5)—sodium chloride (1.5 *M*) (30:30:40:9, v/v), and detected electrochemically at a potential of +850 mV vs. an Ag/AgCl electrode.

Antiepileptic drugs like carbamazepine, carbamazepine epoxide, phenytoin, valproic acid and ethosuximide do not interfere with the assay. Blood/plasma partition ratios of 0.69 and 0.55 for progabide and its acid metabolite, respectively, indicate that the former but not the latter is present in red blood cells.

INTRODUCTION

Progabide (Fig. 1, I), a new γ-aminobutyric acid (GABA) mimetic compound, which has been shown to possess a large spectrum of antiepileptic activity in

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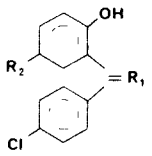

	R_1	R_2	
I	$N(CH_2)_3CONH_2$	F	PROGABIDE
II	$N(CH_2)_3$-COOH	F	PGA
III	$N(CH_2)_3CONH_2$	Cl	INTERNAL STANDARD 1
IV	O	F	BENZOPHENONE
V	O	Cl	INTERNAL STANDARD 2

Fig. 1. Structure of progabide, its metabolites and the internal standards

resistant epileptic patients [1—3], is metabolized to other GABA agonists: progabide acid metabolite (PGA), gabamide and GABA (Fig. 1). PGA exerts the same pharmacological activity as the parent compound.

Progabide undergoes rapid hydrolysis into benzophenone in aqueous solution at acidic pH and in methanolic solution. The instability of the imine bond presents the main difficulty in the quantification of the drug. Hence, great care must be taken in the collection of the blood or plasma samples, and it is necessary to prevent the hydrolysis of the imine bond occurring naturally during the extraction and work-up procedure. This is made possible by reducing the double bond with sodium borohydride, as described by Yonekawa et al. [4]. The stable reduced compounds can then be purified by back-extraction in acidic conditions. However, Yonekawa's procedure, developed for plasma samples, required modifications for the determination of progabide in blood.

The present paper describes a method for the quantification of progabide, its acid metabolite (PGA) and, if needed, the benzophenone, in plasma, blood and urine with an optimized chromatographic separation under reversed-phase conditions. It also describes the determination of the blood/plasma partition ratio of progabide and of its acid metabolite PGA.

EXPERIMENTAL

Chemicals

Sodium borohydride, sodium chloride, sodium acetate and sodium citrate, p.a. grade, were purchased from Merck (Darmstadt, F.R.G.). Methanol (UV grade) and diethyl ether (p.a. grade) were obtained from Carlo Erba (Milan, Italy). The latter solvent should be freshly distilled. Acetonitrile (HPLC grade) and toluene (p.a. grade) were purchased from Baker (Deventer, The Netherlands).

Progabide, 4-{[(4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methylene]-amino}butanamide, SL-75102, 4-{[(4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)-methylene]amino}butyric acid and their internal standard, SL-78050, 4-{[(4-chlorophenyl)(5-chloro-2-hydroxyphenyl)methylene]amino}butan-amide, were provided by the LERS Chemistry Department.

Standard solutions

Progabide and its internal standard were dissolved in ethyl acetate at a concentration of 10 ng/μl, whilst the solution of PGA at the same concentration was prepared in methanol. These solutions were stable for one month when kept at −20° C.

Apparatus and conditions

The liquid chromatograph consisted of a Model 750 Micromeritics pump (Norcross, GA, U.S.A.), a Waters Wisp automatic injector (Milford, MA, U.S.A.) and a Kipp Analytica Model 9205 (Emmen, The Netherlands) coulometric detector.

Separations were carried out on a Hypersil ODS 3-μm (150 × 4.6 mm) reversed-phase column packed according to the technique described by Broquaire [5]. Chromatograms were recorded using a linear stripchart recorder; peak areas were measured by a Perkin-Elmer Model Sigma 10 integrator.

The mobile phase was prepared by diluting 400 ml of 0.033 *M* phosphate buffer pH 5.05 with 300 ml of methanol and 300 ml of acetonitrile. The ionic strength was adjusted by adding 90 ml of 1.5 *M* sodium chloride. The mobile phase was adjusted to a flow-rate of 1.00 ml/min through the column, which was maintained at a constant temperature of 54° C using a water bath.

The column effluent was monitored electrochemically at an oxidation potential of +850 mV vs. an Ag/AgCl reference electrode.

Assay procedure

In the extraction procedure (Fig. 2), 1 ml of plasma, blood, or urine was added to a conical tapered tube containing 50 μl of a methanolic solution of internal standard (Fig. 1, III) (10 ng/μl) and 0.5 ml of 2 *M* acetate buffer pH 4.5. The mixture was extracted with toluene (8 ml) on a rotary shaker for 20 min. The two phases were then separated by centrifugation at 4° C (1000 *g* for 10 min).

A 0.5-ml volume of 0.5% sodium borohydride in ethanol was added to the organic phase. The mixture was vigorously shaken on a vortex mixer and the reduction proceeded during 20 min at room temperature. The reduced drugs were back-extracted for 20 min with 2 ml of 0.25 *M* citrate buffer pH 1.8. The organic layer contained the reduced benzophenone.

The pH of the aqueous phase was adjusted in the range 6.5—7.7 by adding 200 μl of 5 *M* sodium hydroxide and 500 μl of 1 *M* citrate buffer pH 7.7. This mixture was then extracted for 20 min with 7 ml of freshly distilled diethyl ether, and the organic phase was evaporated to dryness at 37° C under a gentle stream of nitrogen.

Following dissolution with 200 μl of methanol—0.015 *M* phosphate buffer pH 7.1 (4:6, v/v), the solution was transferred to an injection vial, and 100 μl were injected into the liquid chromatograph.

In order to quantify the benzophenone with its precursors (progabide, PGA) in one single chromatographic run, the organic extracts containing the reduced compounds were pooled prior to the HPLC separation (Fig. 2). The accuracy of the determination was improved by adding a second internal standard (Fig. 1, V) to blood samples prior to extraction.

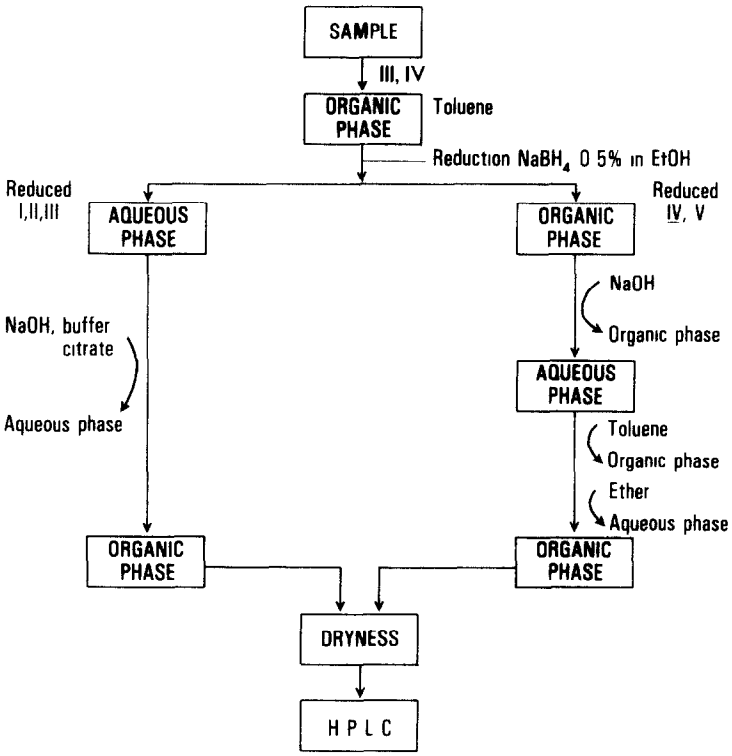

Fig. 2. Flow-chart of the extraction and reduction of progabide (I), its acid metabolite (II), and their corresponding benzophenone (IV). Compounds III and V are internal standards (see Fig. 1).

Peak area ratios progabide/internal standard, and PGA/internal standard were used to construct standard curves. The slope, intercept and correlation coefficient were determined by linear least-squares regression analysis.

Blood/plasma partition ratio

In order to determine the distribution ratio of progabide and PGA between blood and plasma, 600 mg of progabide were administered orally to three healthy volunteers (mean age 35 ± 8.7 years, mean body weight 60.3 ± 6.2 kg). Blood (15 ml) was collected at 0, 1, 2, 4, 6, and 8 h following drug administration. Half of the blood sample was immediately frozen, the second half was centrifuged and the plasma collected. Progabide and PGA were determined in blood and plasma, and the concentration ratio experimentally measured.

The relationship between blood and plasma concentrations is $C_b = C_p(1 - H) + C_eH$, where C_b, C_p and C_e are respectively blood, plasma and erythrocytes concentrations, and H is the haematocrit. For a haematocrit mean value of 0.45, C_b/C_p is superior or equal to 0.55. Only when $C_e = 0$ does C_b/C_p equal 0.55.

RESULTS

Stability

The stability of progabide and PGA was studied at 37° C. As shown in Fig. 3, the half-lives of progabide and PGA were 3.8 and 14.4 h, respectively. This indicates that it would be preferable to determine progabide in blood rather than in plasma samples in order to eliminate possible artefacts during the centrifugation step. From a clinical viewpoint it is also more practical to freeze blood immediately following sampling.

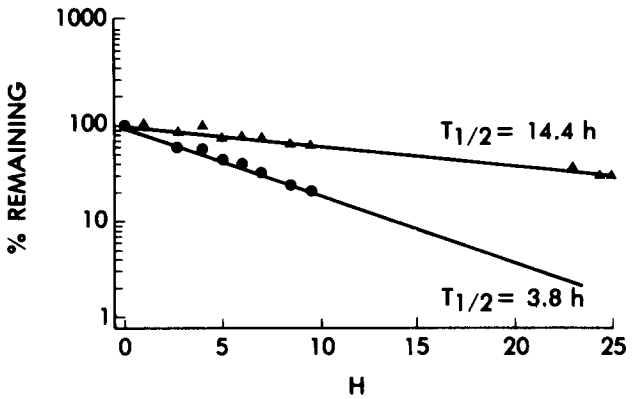

Fig. 3. Stability of progabide (•) and PGA (▲) in human blood at 37° C.

Extraction and reduction

Extraction curves for non-reduced compounds are shown in Fig. 4A and those of the reduced compounds in Fig. 4B. The pH value chosen for the initial extraction was in the pH range in which progabide is the most stable.

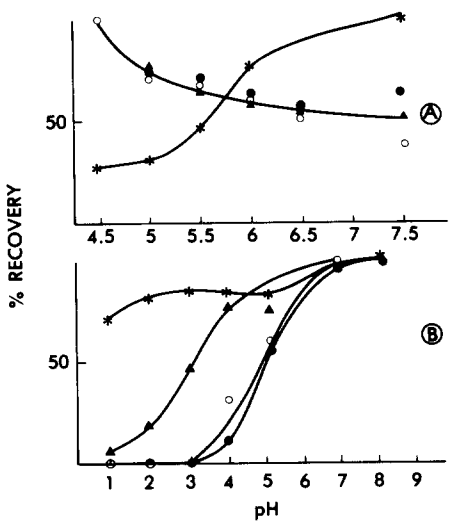

Fig. 4. Plots of percentage recovery vs. pH of (A) non-reduced compounds, and (B) reduced compounds. Recovery of progabide (•), PGA (▲), the internal standard 1 (○) and benzophenone, IV (*) as a function of pH (A) before and (B) after reduction with sodium borohydride.

Many solvents were tested for the extraction process, and diethyl ether proved to be the best for the non-reduced compounds; unfortunately it also extracted too many endogenous compounds to be useful, and the best compromise was provided by toluene. For the reduced compounds a more polar solvent was necessasry and diethyl ether gave the best recovery.

The influence of sodium borohydride reduction time was investigated and it was observed that yields, expressed as peak heights, were optimal for a 10—20 min reduction time, then decreased in a parabolic manner. However, peak height ratios between progabide, PGA and the benzophenone remained constant with time.

Chromatographic separation

The chromatogram obtained by high-performance liquid chromatography (HPLC) of unreduced progabide showed a large tailing peak. A marked improvement of the chromatographic properties was observed after reduction of the imine bond, but a simple binary mobile phase was not suitable to obtain a good separation of all the compounds. Thus each chromatographic parameter was investigated, and the solvent composition was optimized by the chromatographic resolution function introduced by Kaiser [6] using a two-factorial analysis.

TABLE I

OPTIMIZATION OF SOLVENT COMPOSITION — CHROMATOGRAPHIC RESOLUTION FUNCTION VALUES

KH_2PO_4 molarity			pH	NaCl molarity				CH_3CN (%)
0 01	0 033	0 1		0	0 041	0 082	0 1239	
−0 019	−100 0	−100 0	4 5	−0.233	−0 729	−0 1023	−0 0173	0
−0 116	−0 183	−0 105	5 2	−0 8493	−100	−0 9038	−0 0830	15
−100 0	−2 470	−100 0	5 9	−100 0	−0 771	−0 1431	−0 0841	30

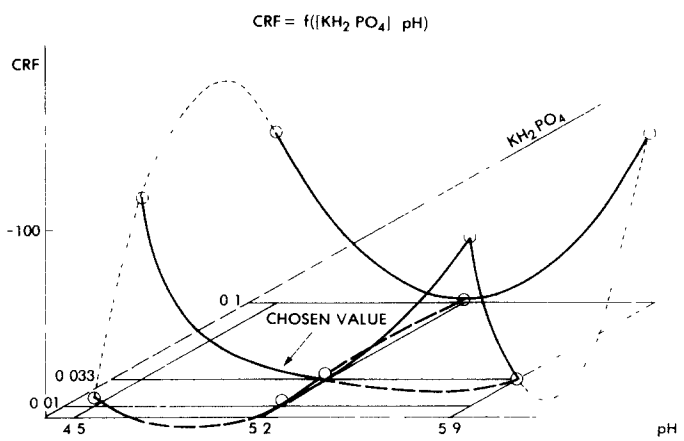

Fig. 5. Chromatographic resolution function (CRF) as a function of pH and molarity of phosphate buffer in the mobile phase.

The first step was optimization of the pH of the solution and the molarity of phosphate buffer; the second was to optimize the acetonitrile–methanol and the sodium chloride molarity. No mathematical development was used and the surface equation near the optimum was not calculated; thus, only qualitative results could be obtained. However, using the results from Table I, parabolic and cubic regressions were determined to draw Figs. 5 and 6. The chosen values were the experimental values near the surface minimum. It can be seen (Figs. 5 and 6) that the two minima were close to pH 5.1, 0.033 *M* KH_2PO_4, 30% acetonitrile, and 0.083 *M* sodium chloride.

Temperature had little effect on retention times of progabide and PGA, but benzophenone was greatly influenced by small variations of temperature. The results, illustrated in Fig. 7, indicate that the best resolution observed in the shortest analysis time was obtained at 54° C.

Fig. 8 shows a chromatogram of blood from a subject following administration of 900 mg of progabide. The retention times were, respectively, 3.1 min, 4.1 min and 5.9 min for PGA, progabide and their internal marker, and 5.0 and 7.0 min for benzophenone and its internal marker. The time interval between two injections was set at 8 min.

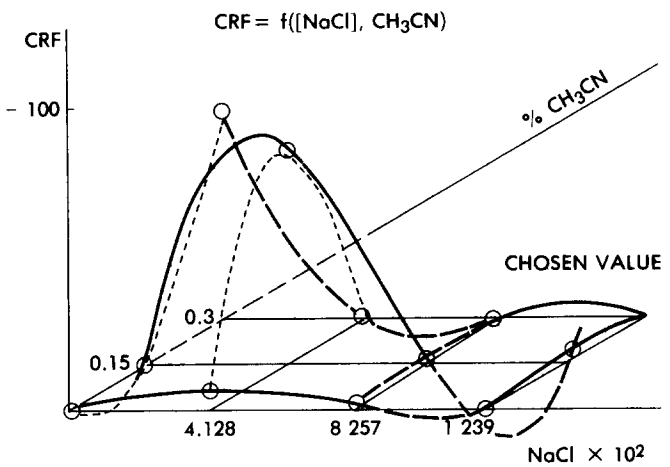

Fig. 6. Chromatographic resolution function as a function of sodium chloride molarity and percentage of acetonitrile in the mobile phase.

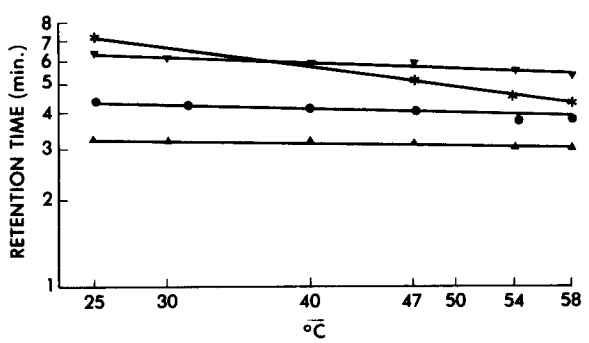

Fig. 7. Relationship between retention times of progabide (•), PGA (▲), their internal standard (▼) and benzophenone, IV (*), and the temperature of the mobile phase.

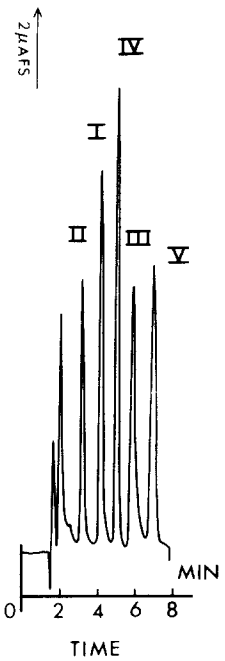

Fig. 8. Chromatogram obtained after extraction of blood following administration of 900 mg of progabide in one subject. Peaks: I = PG (559 ng); II = PGA (632 ng); III = internal standard 1 (500 ng); IV = benzophenone (500 ng); V = internal standard 2 (500 ng).

Assay linearity and precision

Calibration curves were constructed by analysing a series of blood samples of known progabide and PGA concentrations. Each data point was the mean of five measurements. The response was linear over the range 10—1500 ng/ml. Although the minimum detectable concentration was 1 ng/ml (three times the baseline noise), the practical limit was around 10 ng/ml.

TABLE II

REPRODUCIBILITY OF THE METHOD FOR THE MEASUREMENT OF PROGABIDE AND PGA AT DIFFERENT CONCENTRATIONS

	Spiked concentration (ng/ml)	No of observations	Concentration observed (ng/ml)	Standard deviation (ng/ml)	C V. (%)
Progabide	50	10	48	1 6	10
	250	5	259	2 5	2.2
	500	10	504	3 0	1 9
	750	5	745	4 0	1 2
	1000	10	999	2 9	0 9
PGA	50	10	51	1 3	8 0
	250	5	248	2.3	2.1
	500	10	499	2.6	1 7
	750	5	737	1.2	0.4
	1000	10	1008	2.2	0.7

The reproducibility and accuracy of the method are shown in Table II. The coefficient of variation (C.V.) ranged between 0.9% and 10% for concentrations of progabide of 1000 and 50 ng/ml, and between 0.7% and 8.0% for concentrations of PGA of 1000 and 50 ng/ml, respectively. Day-to-day variation of the slopes of the calibration curves was less than 5%.

Oxidation potentials

In order to obtain the best sensitivity, the resulting peak height was recorded for each compound as a function of the electrode potential. In the potential range explored (600—1000 mV) no limiting current was clearly visible except for the benzophenone. Moreover, two oxidative processes were visible for progabide, PGA and their internal marker (Fig. 9).

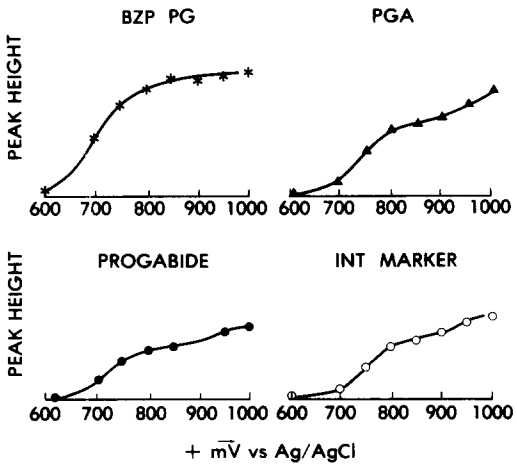

Fig. 9. Detector response, expressed as chromatographic peak height, for progabide (•), PGA (▲), benzophenone, IV (*) and the internal standard 1 (○) as a function of the electrode potential at pH 5.05.

Interferences

Progabide may be administered in combination with other antiepileptic drugs. For that reason 1-ml blood samples containing progabide and PGA (500 ng) were spiked separately with the most widely prescribed antiepileptic drugs (2 μg each of carbamazepine, carbamazepine epoxide, phenobarbital, phenytoin, ethosuximide and 5 μg of valproic acid), and were then processed according to the described method. No chromatographic interference could be observed.

HPLC—GLC correlation

This HPLC method was compared with a gas—liquid chromatographic (GLC) procedure previously used in our laboratory for the quantification of progabide alone [7]. The values obtained with 30 samples analysed with both methods were compared using Student's t test for paired data. This statistical analysis indicated a good correlation between the values calculated with both methods. The observed differences were not statistically significant. However, Fig. 10 shows that at concentrations higher than 1500 ng/ml experimental data tend to scatter.

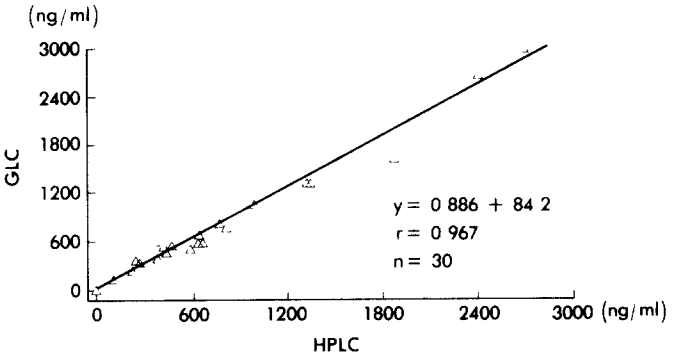

Fig. 10. Correlation of plasma concentrations of progabide obtained with a GLC method [7] and with the described HPLC method.

Application to blood/plasma partition ratio

The blood/plasma concentration ratio at 1, 2, 4, 6, and 8 h following administration of 600 mg of progabide was 0.68 for progabide and 0.55 for PGA (Table III). Two-way analysis of variance (subject, times) indicated that the experimental differences were not statistically significant. It is interesting to point out that the blood/plasma partition ratio of PGA of 0.55 is the theoretical value of a compound restricted to plasma. This was confirmed in a subsequent metabolic study in which progabide labelled with ^{14}C was administered to one volunteer. Progabide but not PGA was then detected in packed erythrocytes.

TABLE III

BLOOD/PLASMA CONCENTRATION RATIOS OF PROGABIDE AND PGA AFTER ORAL ADMINISTRATION OF 600 mg OF PROGABIDE

	Subjects	Time (h)				
		1	2	4	6	8
Progabide	1	0.964	0.736	0 803	0 689	0 737
	2	0.532	0.639	0.634	0.623	0.672
	3	0.707	0.662	0.733	0.552	0 611
	$\bar{M}$	0.734	0.679	0.723	0.621	0.673
	S.E.M.	0.125	0.029	0.049	0.040	0.036
	C.V. (%)	29.6	7.5	11 7	11 0	9 4
PGA	1	0.579	0.553	0.606	0.533	0 520
	2	0 400	0.538	0.474	0.521	0.500
	3	0.688	0 533	0.513	0 651	0 594
	$\bar{M}$	0.556	0.541	0.530	0.568	0.538
	S E M.	0.084	0 006	0.039	0.041	0.029
	C.V. (%)	26.2	1.92	12.8	12.6	9.6

DISCUSSION

Reduction of progabide, PGA and benzophenone yields stable products that can be back-extracted in acidic aqueous solutions and kept at room tempera-

ture for several hours during automatic injection of a large number of samples. It should be noted that PGA cyclizes during the reduction step to form a lactam; this is fortunate for it loses its amphoteric property. The decrease of peak height observed with reduction times longer than 20 min is puzzling; it may be due to oxidation of the phenol group at alkaline pH.

The chromatographic separation function developed by Kaiser [6] allows optimization of the separation without extensive calculation. In the present case more experimental data points would be needed to obtain a good resolution of the function. Nevertheless, this empirical determination of the function allowed an estimation of the optimal separation.

Blood/plasma partition coefficients indicate that progabide but not PGA diffuses into red cells. This does not seem to be due to insufficient time for equilibrium; 8 h should be more than necessary to reach equilibrium between plasma and red cells and there is no visible trend in the partition ratio as a function of time. However, it should be pointed out that outlying values were observed 1 h following oral administration.

The method described herein is time-consuming but its main advantage lies in the possibility of quantifying with precision and accuracy progabide, its acid metabolite and benzophenone in blood, plasma and urine. Progabide and PGA are normally not excreted in urine so the reduction step is necessary only for plasma and blood samples.

With automatic injection 30—40 samples and the corresponding calibration curves can be processed daily if the electrodes are rejuvenated every week by polishing them with alumina.

This method has been routinely used in our laboratory for over a year.

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