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Note

Simultaneous determination of progabide and its acid metabolite by reversed-phase high-performance liquid chromatography

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Progabide, 4-(4-chlorophenyl) - (5-fluoro-2-hydroxyphenyl) methylen-aminobutanamide (PG), is a γ-aminobutyric acid mimetic compound that is of interest in the treatment of refractory epilepsy [1]. The most important side-effects observed with progabide were hepatic (e.g. cytolytic hepatitis, transaminases increase). When administered with other antiepileptic drugs, such as phenytoin or carbamazepine, plasma levels are significantly decreased [2], whereas high levels may be observed in the case of renal failure [3]. Consequently, there is a widespread interest in the isolation and quantification of this compound and of its acid metabolite, which possess the same activity and the same toxicity.

A sensitive gas chromatographic method was proposed [4], but it was not suitable for the determination of the active metabolite. The methods described by Padovani et al. [5] and Ascalone et al. [6], both using high-performance liquid chromatography (HPLC), represent advances but are time-consuming and not easy to use in a routine clinical laboratory.

This report describes a simple, selective and reproducible method using UV detection and a reversed-phase column, which are more widely available in drug assay laboratories. The first results of a clinical trial are presented, and these confirm the suitability of the method.

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EXPERIMENTAL

Apparatus

The HPLC system used consisted of a Constametric III pump and a variable-wavelength SpectroMonitor D detector operating at a wavelength of 340 nm (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Model 7126 variable-loop (20 μl) injector (Rheodyne, Cotati, CA, U.S.A.) and a Model CR3A integrator–recorder (Shimadzu, Tokyo, Japan).

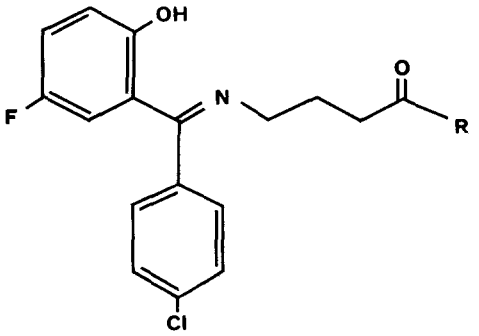
The separations were performed on a 300 mm × 4.6 mm I.D. Nucleosil C_{18} (5-μm) reversed-phase column (Macherey-Nagel, Düren, F.R.G.).

Chemicals

HPLC-grade methanol, acetonitrile and toluene were obtained from E. Merck (Darmstadt, F.R.G.). Phosphate buffer used for elution was prepared by dissolving 9.00 g of potassium dihydrogenphosphate and 0.14 g of disodium hydrogenphosphate heptahydrate in a final volume of 1 l of tridistilled water. The resulting pH was 5.05. Acetate buffer used for extraction was 0.1 *M* acetic acid–0.1 *M* sodium acetate (76:24), pH 4.5.

Standards

PG and acid progabide (APG) were kindly donated by the L.E.R.S. Chemistry Department (Paris, France). The structures of the compounds are given in Fig. 1. Stock solutions were prepared monthly by dissolving 100 mg of each compound in 100 ml of methanol and were stored at −20 °C. Working solutions containing 10 μg/ml were obtained by dilution of the stock solution in distilled



	R
Progabide	NH2
Acid progabide	OH

Fig. 1. Structures of progabide and acid progabide.

water. Thiopental, provided by Specia (Paris, France), was used as an internal standard and was prepared in the same way.

Chromatographic parameters

The mobile phase was phosphate buffer–methanol–acetonitrile–0.15 *M* sodium chloride (36:27:27:10), and was filtered (Whatman No. 2 filter) and degassed ultrasonically before use. The flow-rate was 2.5 ml/min at room temperature.

Extraction procedure

Plasma (2 ml) was spiked with 70 μl of internal standard working solution, then acidified with 1.2 ml of acetate buffer. After gentle mixing, one 5-min extraction with 10 ml of toluene was performed using an alternating shaker (Robin, Angers, France). When an emulsion occurred, it was broken ultrasonically. After centrifugation (1000 *g* for 10 min), the supernatant was transferred to a conical glass vial and evaporated to dryness under a stream of air, the glass tube being kept at 37°C in a thermostatted bath. The residue was dissolved in 100 μl of methanol, and 50 μl were injected into the chromatograph.

Calibration curves

Standard concentration curves were obtained by adding known amounts of standard solutions in the range 100–1500 ng/ml to blank human plasma. Extractions followed the experimental procedure described above. Calculations were based on the peak-height ratios of PG to thiopental and of APG to thiopental.

RESULTS

Chromatography

Fig. 2 illustrates typical chromatograms of plasma extracts from an untreated patient and from an epileptic patient treated with PG. The compounds are well separated with no interferences. The retention times of PG and APG are 7.9 and 6.9 min, respectively. The internal standard, thiopental, is eluted at 4 min. Analysis of plasma samples spiked with other antiepileptic drugs produced no interfering peaks.

Recovery and precision

Intra-assay coefficients of variation (C.V.) and accuracy (recovery) were checked by analysing plasma samples spiked with PG or APG. The results (Table I) indicate that both recovery and precision are good over the concentration range studied.

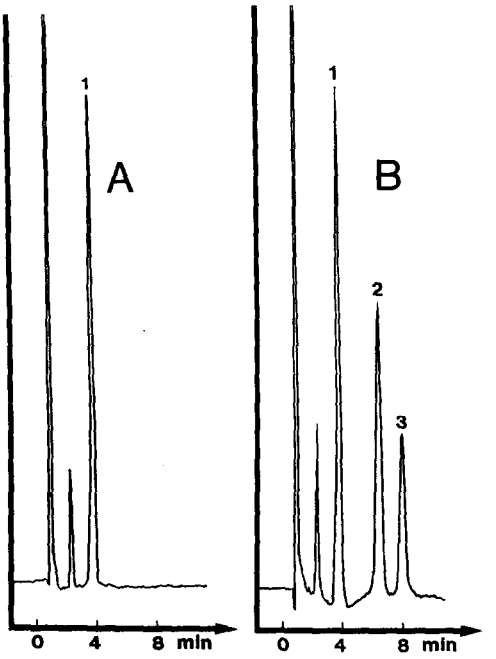


Fig. 2. Chromatograms of (A) a drug-free extract of plasma and (B) a sample containing 280 ng/ml PG and 720 ng/ml APG. Peaks: 1 = thiopental (internal standard); 2 = APG; 3 = PG.

TABLE I

INTRA-ASSAY PRECISION AND ANALYTICAL RECOVERY FOR ASSAY OF PG AND APG IN PLASMA ($n=8$)

Concentration (ng/ml)	PG		APG	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
100	97.0	5.2	101.5	6.5
300	96.2	3.4	103.6	4.8
600	100.4	2.6	107.2	3.2
900	98.3	7.2	104.8	6.3
1200	97.4	5.6	103.0	5.2
1500	98.7	8.0	105.8	7.6

Linearity and limit of detection

The linearity of this assay was evaluated by analysing human plasma spiked with PG and APG in the concentration range 100–1500 ng/ml. From the detector responses, calibration lines were calculated using the linear least-squares

TABLE II

LINEAR REGRESSION DATA FOR CALIBRATION CURVES ($n = 10$)

	PG			APG		
	Slope	y-Intercept	r	Slope	y-Intercept	r
Mean	0.095	0.038	0.991	0.070	0.080	0.995
S.D.	0.016	0.020	0.003	0.011	0.022	0.004

method for peak detector response ratio (drug/internal standard) versus concentration. Linear regression data for the consecutive calibration curves performed over a six-month period are shown in Table II, and these indicate good linearity and reproducibility of the calibration curves. Under these conditions limits of detection (at a signal-to-noise ratio of 2) of 20 and 8 ng/ml could be reached in plasma for progabide and acid progabide, respectively.

Clinical study assay results

The assay was used to determine the plasma concentrations of thirty epileptic patients receiving 30 mg/kg per day orally and presenting no more seizure. The average plasma levels observed in this group of patients were 584 ± 90 ng/ml for PG and 1140 ± 62 ng/ml for APG.

DISCUSSION

It must be said in their favour that HPLC methods proposed previously [5–7] established the essential toxicological and pharmacokinetic data of this drug. Nevertheless, these methods are not easy to use in a clinical laboratory since they involve either electrochemical detection or the use of a silica column. Electrochemical detection increases the sensitivity, but makes the method complicated and time-consuming because reduction and purification steps are necessary. The use of a silica column needs particular care, such as precise control of the content of water in the eluent [6] and periodical regeneration of the column [8].

The method described here, which is simple and rapid, is also reproducible and sensitive enough for therapeutic monitoring and for most pharmacokinetic studies. Thiopental was selected as the internal standard for several reasons: it was easily extracted by toluene in acidic conditions, its maximum UV absorption level (340 nm) was similar to those of the drugs under test, and its chromatographic peak was well separated from those of the other compounds. Other solvents were tested for the extraction procedure. The best selectivity under our experimental conditions was observed with toluene. However, use of this solvent frequently led to emulsion formation, which badly affected the

recovery. The most effective way we found for breaking this emulsion was to plunge the vials into an ultrasonic bath for 30 s.

Even though PG is more stable in plasma than in phosphate buffer and since it is quite stable at 4°C, blood and plasma samples should be kept in an ice-bath from the time of collection.

The extraction procedure can be performed in ca. 20 min and chromatography takes 10 min.

The described method has been successfully used in our laboratory for monitoring epileptic patients treated with progabide and may be useful for routine use in clinical laboratories.

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