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EFFECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GLAFENINE IN PLASMA: PHARMACOKINETIC APPLICATION

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SUMMARY

A high-performance liquid chromatograpic method for an effective determination of glafenine and its main metabolite, glafenic acid, is described. The assay involves separate extraction procedures for glafenine and for its metabolite, but the same internal standard (floctafenine) and the same chromatographic conditions (including a 5-μm C_8 column, a quaternary solvent mixture of water–acetonitrile–diethylamine–acetic acid and an ultraviolet detector set at 360 nm). For 1 ml of plasma, the detection limit is 0.05 mg/l for glafenine and 0.25 mg/l for glafenic acid. Compared with previously described techniques, this assay uses a very low glafenine linearity range, which allows the true pharmacokinetics of this drug to be described for the first time.

INTRODUCTION

Glafenine, 2-(7-chloro-4-quinolinyl)amino-benzoic acid 2,3-dihydroxypropyl ester, is a widely used analgesic. Its pharmacological activity has been demonstrated in clinical trials with placebo or with reference analgesic drugs [1–3]. If the tolerance of this drug is generally good (while keeping in mind the possibility of immunoallergic accident), the risk of acute renal insufficiency after massive absorption is real, and the number of cases reported in literature is probably an underestimate. It is commonly assumed that glafenic acid, its major metabolite, is involved in this toxicity but very little information is available on glafenine itself [4–6]. This reflects the poor knowledge of glafenine pharmacokinetics in humans because of the delicacy needed in this analysis. Generally, pharmacokinetic data on glafenine are obtained from glafenic acid measurements [7,8].

Only a few techniques have been published. The spectrophotometric method [9], the first available, is not sensitive and specific enough for clinical studies.

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Two high-performance liquid chromatographic (HPLC) methods have been proposed. The first [10] is not sensitive enough and does not allow effective quantitation of glafenine, and the second [11] gives only limited data owing to an apparent fragility of this molecule in plasma left at room temperature. Radiometric assays [12] have been described for following the biotransformations of glafenine in the rats and in humans but these techniques cannot be used in common medical practice.

To improve knowledge of glafenine pharmacokinetics we have developed a selective and sensitive HPLC method. This paper describes the method and the first results of a clinical trial that confirmed its suitability.

EXPERIMENTAL

Chemicals

Diethylamine, sodium hydroxide (8 *M*), hydrochloric acid (12 *M*), chloroform (all analytical-reagent grade) and acetonitrile (spectroscopic grade) were obtained from E. Merck (Darmstadt, F.R.G.). Glacial acetic acid, ammonia solution (28%) (analytical grade) and glycine (crystalline) were provided by Touzart et Matignon (Vitry, France).

The aqueous reagents were all prepared with distilled water. Glafenine, glafenic acid, 2-(7-chloro-4-quinolinyl)aminobenzoic acid and floctafenine, 2-(8-trifluoromethyl)-4-quinolinyl-aminobenzoic acid 2,3-dihydroxypropyl ester, used as internal standard, were kindly supplied by Roussel Labs. (Romainville, France).

Standard solutions

Stock solutions of each of the three compounds were prepared by dissolving 10 mg of crystalline powder in 100 ml of methanol, with 0.5 ml of ammonia solution added to give a final pH of ca. 11. Solution left at 4 °C remained stable throughout the experiments.

Chromatographic conditions

The high-performance liquid chromatograph used was a Shimadzu LC6-A pump, coupled to a Shimadzu SPD2A spectrophotometric detector. The recorder was a Kipp and Zonen BD40. Samples were injected through a Rheodyne 7125 injector with a 20-μl loop. This equipment was supplied by Touzart et Matignon. Separations were carried out on a 5-μm Spherisorb C_8 column (150 mm $\times$ 4.6 mm I.D.) (SFCC, Gagny, France). The mobile phase was prepared by diluting 550 ml of acetonitrile with 400 ml of distilled water and 3 ml of diethylamine. The pH was adjusted to 4.5 with ca. 5 ml of glacial acetic acid, then this solution was filtered (Whatman No. 2 filter) and degassed ultrasonically. The flow-rate was 1 ml/min through a column left at room temperature. The absorption was measured at 360 nm, based on the respective maximum wavelengths of glafenine (362 nm), glafenic acid (364 nm) and floctafenine (358 nm), and observed with stock solutions on a Spectronic 1201 spectrophotometer.

Sample preparation

From a chemical point of view, quantitative and simultaneous extraction of an ester and an acid seemed unlikely to be effective (see Results and Discussion), so separate extraction of each compound was undertaken.

Glafenine. Plasma (1 ml) was spiked with 50 μl of internal standard solution (10 mg/l, dilution with methanol of the stock solution), then made alkaline with 1 ml of glycine buffer (pH 11). After gentle mixing, two 10-min extractions with 5 ml of chloroform were performed using an alternating shaker (Toulemonde, Paris, France). The organic layers obtained after 10 min centrifugation at 1000 *g* were combined and evaporated to dryness under a stream of air, the glass tubes being kept at 37 °C in a thermostatted bath. The residue was dissolved in 100 μl of mobile phase and quickly homogenized on a vortex mixer, and 20 μl were injected into the chromatograph.

The glycine buffer used was a mixture of 51.2 ml of 0.1 *M* glycine solution (7.507 g of glycine plus 5.884 g of sodium chloride in 1 l of distilled water) with 48.8 ml of 0.1 *M* sodium chloride solution. This buffer is not stable and must be freshly prepared.

Glafenic acid. Plasma (1 ml) was spiked with 50 μl of internal standard stock solution, then acidified with 200 μl of 0.1 *M* hydrochloric acid solution. The chloroform extractions were carried out as described for glafenine.

Calibration

Standard concentration curves were obtained by adding known amounts of standard solutions in the range 0.05–2.5 mg/l for glafenine and 0.25–20 mg/l for glafenic acid to blank human plasma. Extractions followed the experimental procedures described above. Calculations were based on the peak-height ratios of glafenine to floctafenine and of glafenic acid to floctafenine.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 1 shows glafenine chromatograms obtained from one volunteer's plasma 30, 45 and 60 min after a single 400-mg oral dose. Fig. 2 shows glafenic acid chromatograms from the same volunteer (same timings).

The substances are well separated with no interferences. The retention times of glafenine and of glafenic acid are 8 and 3.5 min, respectively. The internal standard, floctafenine, is eluted at 11 min. Chromatographic conditions have been set up keeping in mind the low solubility of glafenine in water. Best results are obtained with the conditions listed above. Acetonitrile gives thinner peaks than methanol, and the C_8 column results in shorter retention times than C_{18} columns under the same chromatographic conditions.

It is worth noting that a mobile phase containing more than 80% water alters the elution sequence to glafenic acid, floctafenine and glafenine, with the third peak becoming very large and tailing.

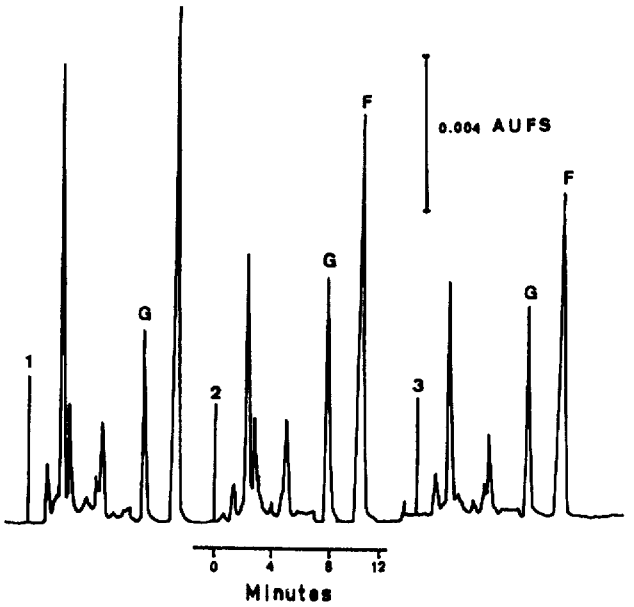

Fig. 1. Chromatograms of glafenine extracted from 1-ml plasma samples, collected from a healthy volunteer after a single oral dose of 400 mg of glafenine. 1 = 0.29 mg/l, 30 min; 2 = 0.42 mg/l, 45 min; 3 = 0.45 mg/l, 60 min. Peaks: G = glafenine, F = floctafenine.

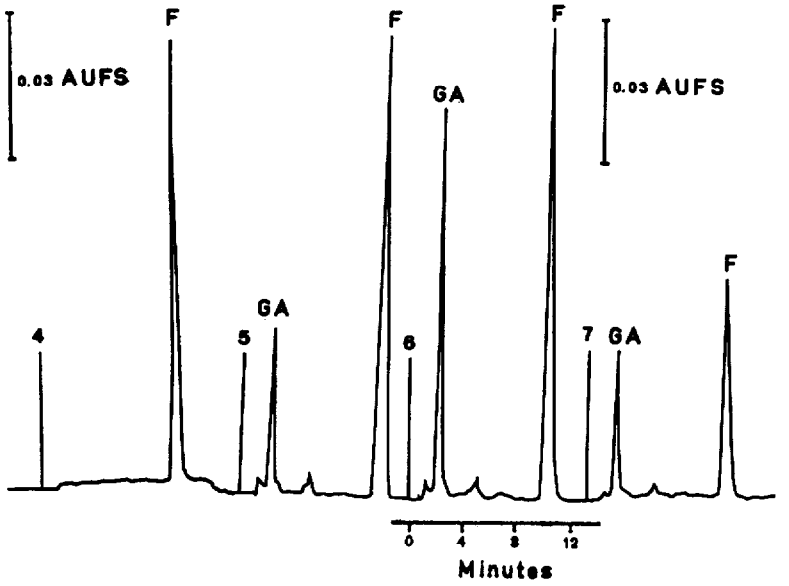

Fig. 2. Chromatograms of glafenic acid extracted from 1-ml plasma samples, collected from the same healthy volunteer. 4 = blank, zero time; 5 = 2.01 mg/l, 30 min; 6 = 5.38 mg/l, 45 min; 7 = 4.30 mg/l, 60 min. Peaks: GA = glafenic acid, F = floctafenine.

Analytical evaluation

The main analytical characteristics of this assay are reported in Table I. The detector response is linear over the given ranges, and the detection limits are easily achieved with a signal-to-noise ratio of 2.

TABLE I

MAIN ANALYTICAL CHARACTERISTICS OF GLAFENINE AND GLAFENIC ACID HPLC ASSAY

Parameter	Glafenine	Glafenic acid
Linearity range (mg/l)	0.05–2.5	0.25–20.0
Detection limit* (mg/l)	0.05	0.25
Mean equation**	$y=0.168\cdot10^{-2}x-0.01$	$y=0.332\cdot10^{-3}x-0.24$
C.V. of slope (%)	9.8	9.1
n	8	8

*Detection limit is twice the baseline noise.

**Mean equation: $y=\frac{\text{glafenine}}{\text{internal standard}}$ or $\frac{\text{glafenic acid}}{\text{internal standard}}$ and x=plasma (glafenine or glafenic acid) concentration.

TABLE II

REPRODUCIBILITY OBSERVED AFTER MEASUREMENT OF GLAFENINE AND GLAFENIC ACID AT THREE DIFFERENT CONCENTRATIONS ($n=10$)

Compound	Concentration (mg/l)	C.V.* (%)	
		Individual values	Mean
Glafenine	0.1	13.7	10.0
	0.5	8.1	
	1.0	8.1	
Glafenic acid	0.5	10.8	8.9
	5.0	8.3	
	10.0	7.7	

*C.V.$=(\frac{\text{S.D.}}{\text{mean}})100$.

The within-day reproducibility has been checked at three plasma concentrations for each compound, and results are presented in Table II.

It is worth comparing the mean coefficients of variation (C.V.) obtained with the respective slope C.V. (see Table I) illustrating the inter-day reproducibility, to see that they are very close.

Stability

Though glafenine was launched in 1965, very little information about its pharmacokinetics is available. We approached the development of one technique on three fronts: the extraction procedure, the chromatographic conditions and the dosage range of glafenine.

Moolenaar et al. [10] and Tournet et al. [11] undertook a simple removal of proteins from samples to determine glafenine. In both studies, after a single 400-mg oral dose, results were poor with, respectively, no measurement of glafenine [10] and only partial quantitation [11] due to supposed hydrolysis of the mole-

cule by a plasma esterase. This led us to study the solubility and stability of glafenine. As the water solubility of these drugs is very low, methanol–ammonia (99.5:0.5) appeared to be a suitable solvent. The stability of standard solutions (100 mg/l) was estimated by comparing the peak heights obtained (i) after direct injection of daily prepared solutions and (ii) after direct injection of solutions stored at 4 °C for more than one month. For glafenine, glafenic acid and floctafenine the variation found was always below 12%, suggesting a good stability of all three molecules.

To obtain a quantitative extraction we tested five organic solvents: acetone, chloroform, diethyl ether, ethyl acetate and methylene chloride. The best results were obtained with chloroform (recovery > 98% for the three compounds), but a simultaneous extraction of glafenine and glafenic acid was not effective. A highly alkaline environment is required for glafenine whereas glafenic acid needs an acid environment. As we are dealing with an ester and its hydrolysis salt, this is not surprising; what is surprising, however, is that floctafenine, which differs from glafenine in one substituent only ($-CF_3$ for Cl), is very well extracted in both cases, which suggests that the trifluoromethyl substituent enhances the lipophilicity.

Finally, we studied the role of the plasma esterase described by Tournet et al. [11]. For this purpose, we used duplicate blood samples taken at various times following a 400-mg oral dose to one healthy volunteer. One sample was prepared at 4 °C and injected into the chromatograph, and the duplicate was treated in the same way but at room temperature. No significant statistical difference was observed; furthermore, we have extracted many blood samples several hours after collection with no significant difference in the results. This does not prove the absence of this esterase, but it may indicate that glafenine is much more stable than previously thought.

Clinical application

The last point of discussion concerns the dosage range for glafenine. In the literature, we have found three papers discussing glafenine pharmacokinetics. Using a conversion factor of 1.25 (the molecular mass ratio of glafenine to glafenic acid), according to the model proposed by Rondelet et al. [9], Mallein et al. [7] obtained an average C_{max} of 14.42 ± 1.24 mg/l after a 400-mg oral dose. Later, Moolenaar et al. [10] assessed the concentration of unchanged glafenine as low, probably less than 0.5 mg/l, following the same oral dose; however, the detection limit of their technique prevented them from going any further. Finally, Tournet et al. [11] obtained a C_{max} value of ca. 8 mg/l in only one healthy volunteer (400 mg glafenine per os) but they reported analytical difficulties.

From all these considerations, we decided to determine glafenine in the range 0.05–2.5 mg/l and glafenic acid in the usual range 0.25–20 mg/l. A pharmocokinetic study of glafenine was performed after informed consent in ten healthy volunteers. The medication supplied was two Glifanan® 200-mg tablets. The sensitive and reliable assay described here successfully determined glafenine and glafenic acid. Fig. 3 shows the plasma concentration–time mean curves.

The time course for glafenic acid is quite similar to those previously described,

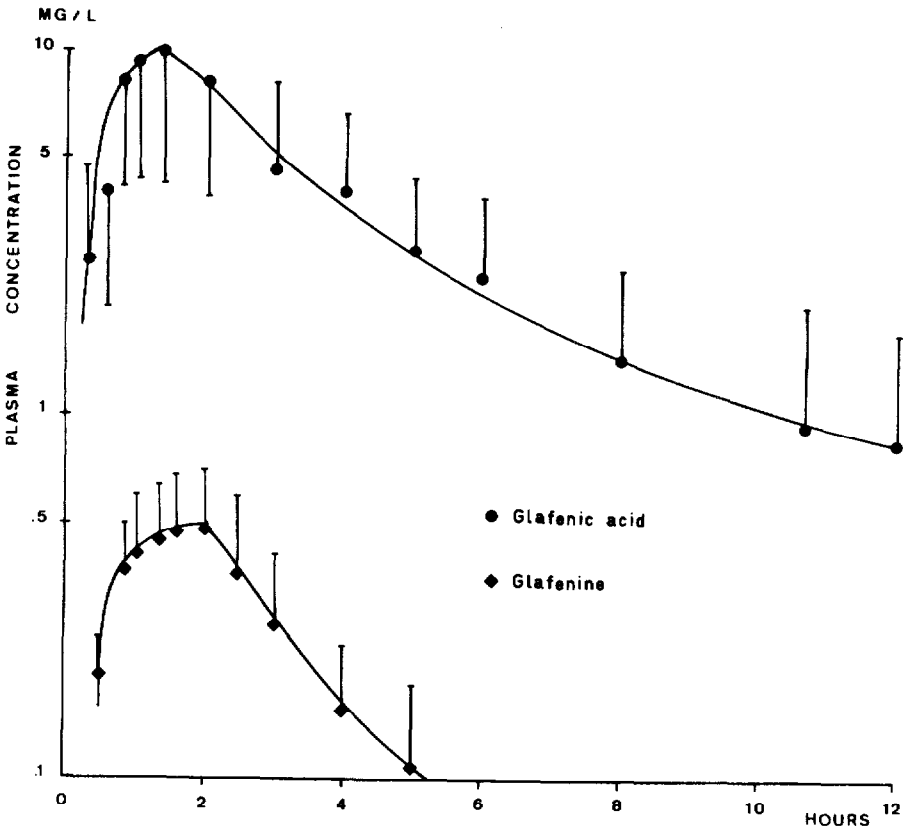

Fig. 3. Plasma concentration–time mean curves of glafenine (♦) and glafenic acid (●) following a 400-mg oral dose (mean + S.D.; $n = 10$).

but that for glafenine is clearly different. It shows that glafenine and glafenic acid have distinct pharmacokinetic behaviour in the absorption, distribution and elimination phases. Consequently, it appears an error to deduce the pharmacokinetics of glafenine based on data for glafenic acid. Using the improved HPLC assay described here, real pharmacokinetic parameters of glafenine will be available, leading to a better understanding of its pharmacology.

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