Journal of Chromatography, 223 (1981) 103—110
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 775

DETERMINATION OF ANTRAFENINE AND ITS MAIN ACID METABOLITE, 2-{[7-(TRIFLUOROMETHYL)-4-QUINOLINYL] AMINO}-BENZOIC ACID, IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LARGE VOLUME AUTOMATIC INJECTION AND GAS—LIQUID CHROMATOGRAPHY WITH DERIVATIVE FORMATION

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(First received March 31st, 1980; revised manuscript received November 18th, 1980)

SUMMARY

Specific and sensitive analytical methods have been developed for the measurement of antrafenine and its main acid metabolite, 2-{[7-(trifluoromethyl)-4-quinolinyl]amino} benzoic acid (FQB), at therapeutic concentrations in plasma and urine.

Following the addition of internal standards (the methyl ester of FQB and 2-{[8-(trifluoromethyl)-4-quinolinyl]amino}benzoic acid) the parent drug and the metabolite were extracted from biological material with diethyl ether at a weakly acid pH. Drug extracts were evaporated to dryness prior to chromatographic analysis.

Antrafenine was measured by high-performance liquid chromatography using a Spherisorb 5-μm ODS column with acetonitrile—0.1 *M* sodium acetate as the mobile phase. Samples were injected automatically using a 500-μl injection loop. The detector wavelength was 353 nm corresponding to the maximum UV absorption of both drug and internal standard. The coefficient of variation (C.V.) for the determination of antrafenine concentrations between 5 and 250 ng/ml ranged between 24 and 3%, respectively.

The acid metabolite of antrafenine was measured by gas—liquid chromatography with electron-capture detection using a 1-m column packed with 3% OV-225 on Gas-Chrom Q (100—120 mesh) at 240°C and on-column methylation with trimethylphenyl ammonium hydroxide. The C.V. of the method for the analysis of metabolite concentrations between 10 and 500 ng/ml ranged between 3 and 9%, respectively.

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0378-4347/81/0000—0000/$02.50

INTRODUCTION

Antrafenine (Stakane®) is an ester type non-narcotic analgesic and anti-inflammatory drug (Fig. 1) which is hydrolyzed in vivo to the corresponding acid and alcohol metabolites, which are also pharmacologically active [1–3].

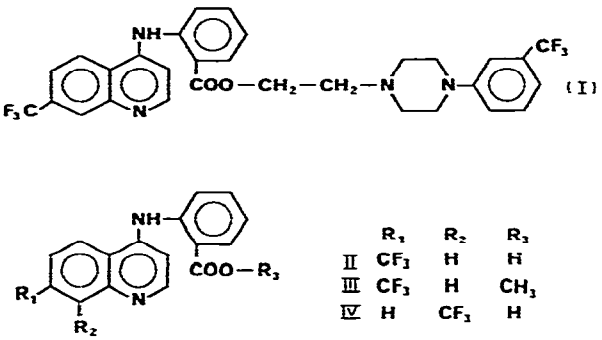

Fig. 1. Structure of antrafenine (I), the acid metabolite, FQB (II); the internal standard of antrafenine, EFQB (III); and the internal standard of FQB, AFQB (IV).

After the oral administration of antrafenine, the parent drug and its acid metabolite are detectable in plasma for at least 48 h, whereas the alcohol metabolite is only detectable in low concentrations in the form of a conjugate. Almost no unchanged antrafenine is excreted in urine. Following oral administration of [^{14}C]antrafenine labelled in the quinolyl and piperazine parts of the molecule, approximately 40% of the dose was recovered in the urine and 60% in the faeces. Most of the drug was found to be excreted as conjugated acid and alcohol metabolites.

In an earlier method (unpublished findings) antrafenine was hydrolyzed to the corresponding acid and alcohol products, both compounds being separated by thin-layer chromatography. These were then scraped off the thin-layer plate and following derivative formation, quantified by gas chromatography. This technique was found to be very laborious and unsuitable for pharmacokinetic studies.

In this paper, we describe an analytical procedure for the measurement of antrafenine and its main acid metabolite in plasma and urine using high-performance liquid chromatography (HPLC) with automatic injection (antrafenine) and gas–liquid chromatography (GLC) with on-column methylation (metabolite).

MATERIALS AND METHODS

Hydrochloric acid, distilled diethyl ether, methanol (analytical grade) and acetonitrile (LiChrosolv) used in this study were purchased from Merck (Darmstadt, G.F.R.). Trimethylphenyl ammonium hydroxide (TMPAH) 0.002 *M* (methanolic solution) was obtained from Pierce (Rockford, IL, U.S.A.).

Pure standards of antrafenine, its acid metabolite, 2-{[7-(trifluoromethyl)-4-quinolinyl]amino}benzoic acid (FQB), and their respective internal standards, the methyl ester of FQB (EFQB) and 2-{[8-(trifluoromethyl)-4-quinolinyl]amino}benzoic acid (AFQB) were synthesized in the Department of Chemistry of Lers-Synthélabo. Their structures are presented in Fig. 1.

The analysis of antrafenine was carried out on a Micromeritics 7000B liquid chromatograph equipped with a Micromeritics 785 UV visible spectrophotometer and a slightly modified Micromeritics 725 automatic injector with a 500-μl injection loop. After the last analysis the liquid chromatograph was switched off by the automatic injector, the detector and the recorder were switched off by a timer.

The mobile phase, acetonitrile—0.1 *M* sodium acetate (72.5:27.5, v/v) was adjusted to a flow-rate of between 2.00 and 1.25 ml/min (depending on the age of the column) through a 15 cm × 4.6 mm I.D. stainless-steel column, packed according to the technique described by Broquaire [4] with Spherisorb 5-μm ODS, batch 17/3 (Soparès, Gentilly, France). The detector wavelength was set at 353 nm, corresponding to the maximum UV absorption of both antrafenine and its internal standard (Fig. 2). Analysis of the acid metabolite (FQB) was performed on a Perkin-Elmer 3920 B gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD).

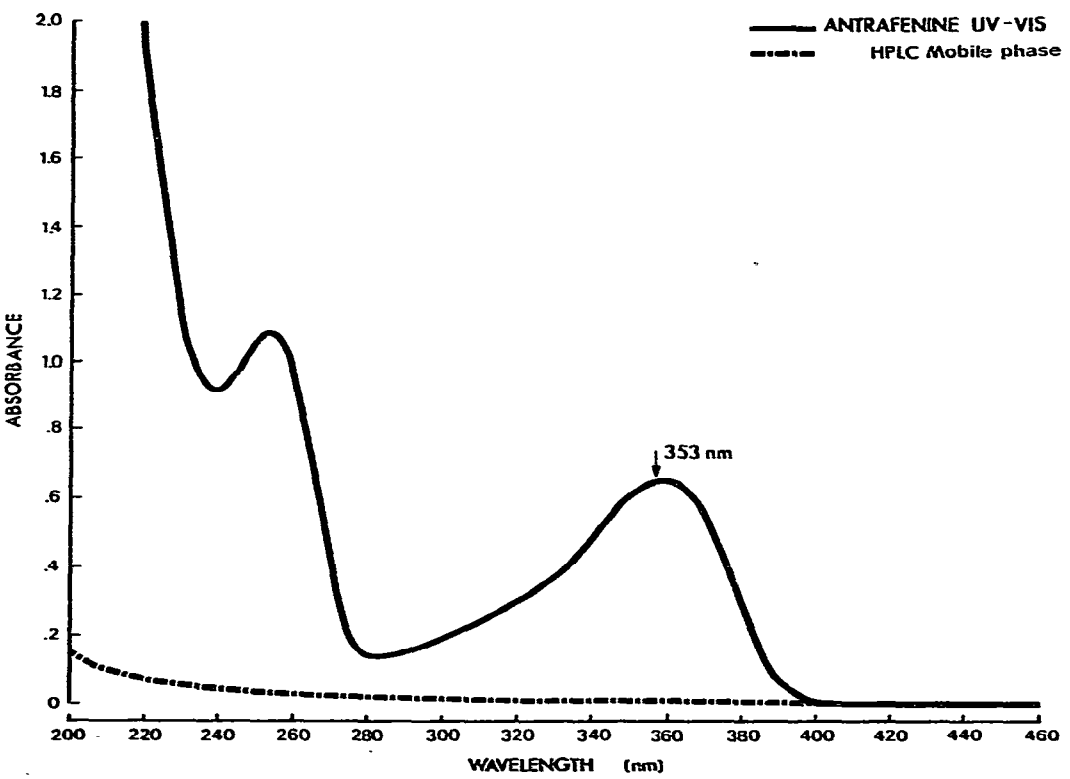

Fig. 2. Absorbance of antrafenine relative to that of HPLC mobile phase at different wavelengths.

A 1 m × 4 mm I.D. glass column was packed with 3% OV-225 coated on Gas-Chrom Q (100—120 mesh) and conditioned for 24 h at 245°C with an argon—methane (95:5) flow-rate of 65 ml/min. The operating conditions were: column temperature, 240°C; injector and detector temperatures, 300°C; and argon—methane carrier gas flow-rate, 65 ml/min.

Extraction procedures

Antrafenine. Plasma, blood or urine (2 ml) was added to a conical tapered tube containing 25 μl of an acetonitrile solution of internal standard (5 ng/μl). A 1-ml aliquot of a solution of 0.02 *M* potassium chloride acidified to pH 2.0 with hydrochloric acid was then added, the tube was vigorously shaken on a Vortex mixer and the pH adjusted to 2.8—3.2 with 10% hydrochloric acid, if necessary. The final solution was extracted with diethyl ether (8 ml) on a rock'n roll shaker for 20 min. The two phases were then separated by centrifugation at 4°C (1000 *g* for 5 min). The aqueous phase was discarded, and the ether phase transferred to another tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The dry extract was then dissolved in 870 μl of an acetonitrile—0.1 *M* sodium acetate solution (1:1) and each tube was shaken twice on a Vortex mixer for 10 sec. An aliquot of 860 μl of this final solution was transferred into an injection vial. The vial was placed on the automatic injector and 500 μl were injected into the liquid chromatograph.

FQB metabolite. A 25-μl aliquot of the AFQB internal standard solution (10 ng/μl in 0.1 *N* sodium hydroxide) was transferred to a conical tapered tube and mixed with 1.0 ml of 0.1 *N* hydrochloric acid and 1 ml of plasma. For urine 1 ml of the acidified potassium chloride solution was added. The final pH was approximately 4. The mixture was extracted twice with 2.5 ml of diethyl ether on a rock'n roll shaker for 20 min and then centrifuged (1000 *g* for 10 min). The two ether extracts were combined in a second tube and evaporated to dryness in a water bath (37°C) under a gentle stream of nitrogen. The TMPAH solution (250 μl) was added to the drug extract, Vortex mixed for 15 sec and 0.5—1.0 μl were injected into the chromatograph.

RESULTS

Antrafenine

Chromatograms from the analysis of plasma samples obtained after the oral administration of 450 mg of antrafenine are presented in Fig. 3a and b. The peaks corresponding to antrafenine and the internal standard were well resolved. Comparison with the blank plasma extracts showed that endogenous compounds present in plasma did not interfere with the analysis. A plasma pH of 2.8—3.2 was selected because under these conditions the extraction of endogenous interfering compounds was minimized (Fig. 3). The retention times were maintained at approximately 3.00 and 6.00 min for the internal standard and antrafenine, respectively, by decreasing the flow-rate from 2.00 to 1.25 ml/min depending on the age of the column. At 1.25 ml/min the reduced height equivalents to a theoretical plate (HETP) were 5.7 for the internal standard and 6.2 for antrafenine (mean diameter of the stationary phase was 5.0 μm, determined with a Coulter Counter). Time of analysis was 7

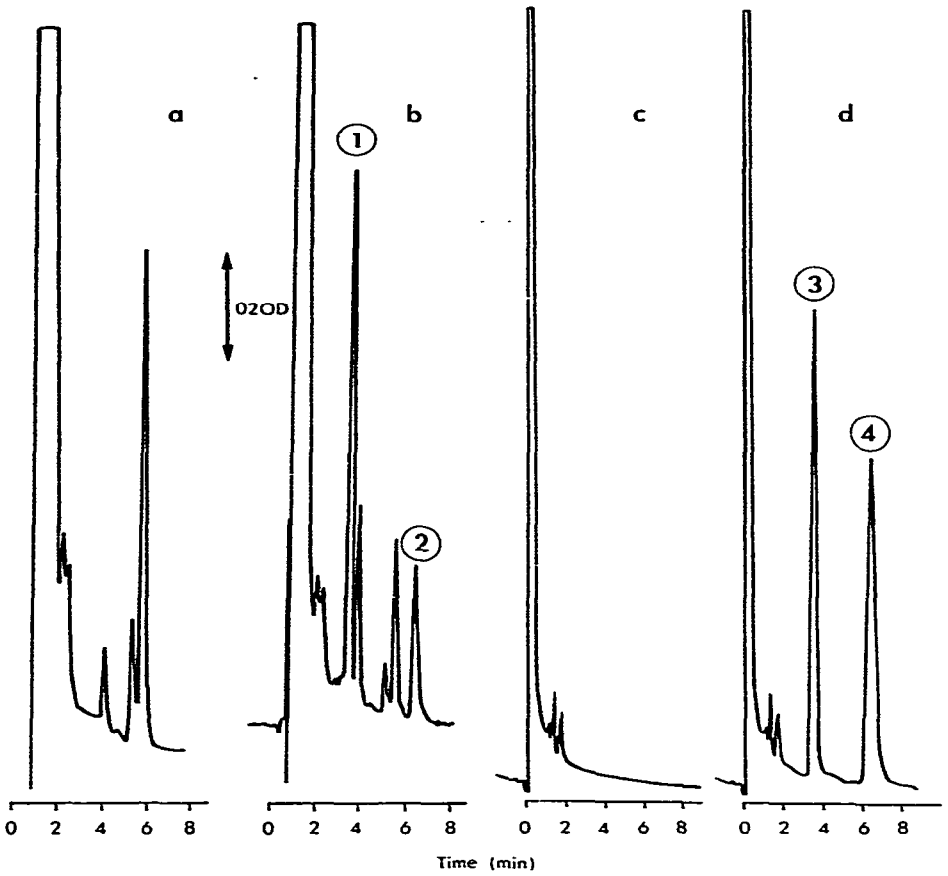

Fig. 3. Typical chromatograms after oral administration of antrafenine: "old column" HPLC of plasma extract, (a) blank); (b) containing antrafenine (2) and EFQB (1); GLC of plasma extract (c) blank, and (d) containing FQB (3) and AFQB (4). For conditions see text.

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD FOR THE MEASUREMENT OF ANTRAFENINE AT DIFFERENT CONCENTRATIONS

Spiked concentration (ng/ml)	No. of observations	Found concentration (ng/ml)	Standard deviation (ng/ml)	C.V. (%)
5	4	6.2	1.5	24
10	6	10.1	1.6	16
25	9	24.7	2.5	10
50	8	48.8	3.9	8
100	11	100.1	7.5	7.5
250	8	250.3	7.5	3

min between two injections. No strongly retained peaks appeared after this time.

The procedure was quantified using the peak ratio method. The calibration curve was linear between 5 and 1000 ng/ml (higher concentrations were not investigated). The limit of sensitivity was 5 ng/ml with a relative standard deviation of 24%. The reproducibility and the accuracy of the method were established by the injection of several samples at known concentrations. The number of analyses, the mean values, the standard deviation and the coefficient of variation (C.V.) are listed in Table I: the C.V. varied between 24 and 3% for concentrations of antrafenine between 5 and 250 ng/ml. Day-to-day variations of the calibration curve slope were less than ± 6%.

FQB metabolite

A typical GLC analysis of FQB and its internal standard (AFQB) in plasma is shown in Fig. 3c and d. Under the GLC conditions used, the retention times of FQB and AFQB were 3.45 and 6.25 min, respectively. The HETP was 2.25 mm for both compounds and the symmetry factors were 0.94 (FQB) and 0.75 (AFQB). The injector temperature was found to be a critical parameter because the dimethylation was quantitative only at temperatures above 300°C [3].

The response of the electron-capture detector was linear over the range 10—500 ng/ml for the biological specimens (blood, plasma, urine) analysed.

TABLE II

REPRODUCIBILITY OF THE GLC METHOD FOR THE MEASUREMENT OF FQB AT DIFFERENT CONCENTRATIONS

No. of observations = 6.

Spiked concentration (ng/ml)	Found concentration (ng/ml)	Standard deviation (ng/ml)	C.V. (%)
10	10.0	0.92	9.2
50	48.9	2.6	5.3
250	252.0	7.1	2.8
500	499.0	15.5	3.1

For higher concentrations of FQB the sample was diluted with TMPAH and reinjected. The C.V. was between 3.1 and 9.2% over the concentration range considered (Table II).

A typical plasma drug concentration—time curve of antrafenine and its acid metabolite in an adult subject following a single oral dose of 900 mg of antrafenine is shown in Fig. 4.

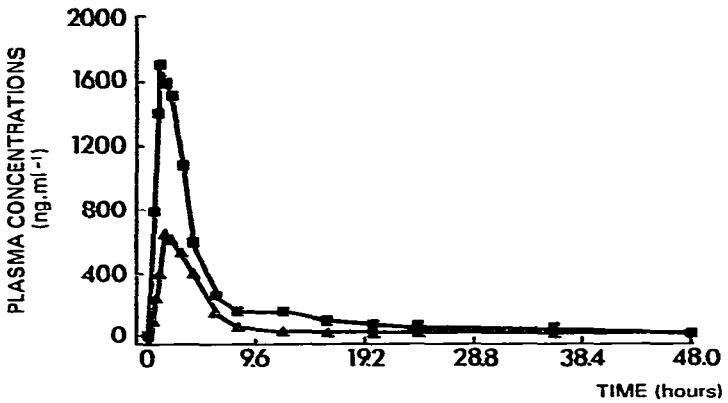

Fig. 4. Pharmacokinetic profile of antrafenine (▲) and its acid metabolite (■) in a healthy volunteer following single oral dosage of 900 mg.

DISCUSSION

Under the analytical conditions used for the HPLC analysis of antrafenine, the retention times were variable, depending on the age of the column. The flow-rate necessary to maintain constant retention times decreased from 2.0 ml/min for a new column to 1.25 ml/min for an old column. At this point an equilibrium appeared and the retention time did not decline further. A new column needed the injection of about 200 samples before reaching equilibration, and at that point, after more than 300 analyses, there was no loss in the efficiency of the column. The use of a large injection volume of non-eluting solvent (500 μl) did not alter the chromatographic separation and the reduced efficiencies were superior to those obtained with a classical injection valve [5]. The non-eluting solvent [acetonitrile—sodium acetate (1:1, v/v)] maintained antrafenine and its internal standard in a sharp band on the top of the column during all the injection time. Thus, there was no band-broadening due to the injected volume [6].

In a previous method [7] using manual injection, the sensitivity was 10 ng/ml of plasma, while with the automatic injector, the sensitivity was increased down to 5 ng/ml, using 2 ml of plasma, after injection of half of the total extract. The accuracy of the automatic injection method was twice that of the manual injection method. The improvement in accuracy of this method, considering that the same equipment was used in both cases, can be explained by a better dissolution in the large sample volume (870 μl) used for the automatic injection method than in a very small injection volume (25—50 μl) used in the manual technique.

Furthermore, in the present method, the injection of a large volume of solvent, different from that of the mobile phase, did not require a "saturation period" which was observed for other experiments with a large injection volume [5].

FQB and the isomer used as internal standard were chromatographed as their dimethyl derivatives.

The on-column methylation performed with TMPAH gave not only the methyl ester but also the methylamino derivative of FQB and AFQB, which was confirmed by GLC—mass spectrometry [3]. Derivatization was necessary to increase the volatility of FQB and AFQB, in order to make possible their analysis by GLC. The best results in terms of peak symmetry, resolution and sensitivity were obtained using a GLC column packed with 3% OV-225. The presence of a CF_3 group in the molecule of FQB and AFQB made the analysis possible using an electron-capture detector. The minimum sensitivity of this analysis was 10 ng/ml of FQB with a C.V. of ± 10% when 1 ml of plasma was analysed. The response of the electron-capture detector was linear for concentrations of FQB from 10—500 ng/ml of plasma. However, with higher concentrations of FQB (after oral doses of 300—600 mg) the analysis was performed by decreasing the volume of sample injected into the gas chromatograph (0.2—0.5 μl). In this case, the response of the electron-capture detector remained linear up to 2000 ng/ml.

In conclusion, the methods described, using both HPLC and GLC procedures, are suitable for the specific analysis of both antrafenine and its acid metabolite, FQB, at therapeutic concentrations in plasma and urine.

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