Journal of Chromatography, 616 (1993) 67–71
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6837

Simultaneous determination of amfenac sodium and its metabolite (7-benzoyl-2-oxindole) in human plasma by high-performance liquid chromatography

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(First received July 28th, 1992; revised manuscript received March 9th, 1993)

ABSTRACT

A simple, selective, sensitive and rapid high-performance liquid chromatographic method for the simultaneous determination of amfenac sodium (AMF) and its metabolite (7-benzoyl-2-oxindole, S_1) in plasma was established. To 100 μl of plasma, purified water (100 μl), ammonium sulphate (0.2 g) and ethanol (100 μl) containing fenbufen (100 μg/ml, internal standard) were added. After centrifugation the ethanol layer was directly injected into a reversed-phase ODS column. AMF and S_1 were eluted using a gradient buffer system of 20–80% acetonitrile–phosphate buffer (pH 7.0), and detected at 245 nm. The analytical recoveries of AMF at concentrations of 0.5, 2 and 10 μg/ml in plasma were *ca.* 92.1, 95.4 and 94.6%, respectively. The coefficients of variation of the AMF recoveries were below 4.6%. The S_1 recoveries at the concentrations of 40, 200 and 400 ng/ml were *ca.* 93.4, 98.7 and 95.3%, and the coefficients of variation were below 6.0%. The coefficients of variation for intra- and inter-day variation of AMF (5 μg/ml) and S_1 (100 ng/ml) were 4.5 and 3.6% and 4.2 and 4.2%, respectively. The detection limits of AMF and S_1 in plasma were as low as 0.1 μg/ml and 20 ng/ml, and the coefficients of variation were 4.0 and 4.4%, respectively. The method was applied to determine the plasma concentrations of AMF and S_1 after oral administration of AMF capsules (100 mg of AMF) to human volunteers.

INTRODUCTION

Amfenac sodium, sodium 2-amino-3-benzoylphenyl acetate (AMF), is a non-steroidal anti-inflammatory drug [1] used to treat chronic articular rheumatism [2], arthritis deformans [3] and lumbago [4]. AMF is metabolized to several compounds, including 2-amino-3-benzoylbenzoic acid and 1,4-dihydro-2-oxo-4-phenyl-2*H*-3,1-benzoxazine-8-carboxylic acid, in humans [5,6] and animals [5,7,8]. It has been reported that AMF is converted easily into 7-benzoyl-2-oxindole (S_1) by dehydrated ring-closure in acidic solutions (pH < 5) *in vitro* [8]. For structures see Fig. 1.

In our preliminary results from the dissolution test of AMF from the capsules, AMF showed a pH-dependent dissolution in various pH media. The percentage of 30-min dissolution of AMF from the capsules was 24.9% at pH 4.0 and only 5.3% at pH 1.2; at both pH 7.2 and 9.2 it was almost 100%. These results suggest that AMF is converted easily in the stomach into S_1, which may be absorbed intact from the intestinal tract

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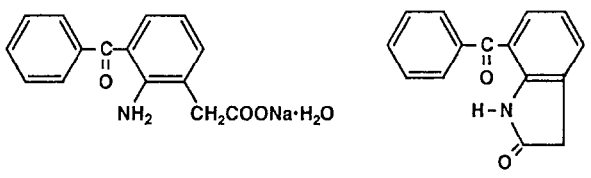
0378-4347/93/$06.00



AMF S_1

Fig. 1. Structures of amfenac sodium (AMF) and 7-benzoyl-2-oxindole (S_1).

and then enter into the circulatory system, and that S_1 is not reconverted into AMF at the systemic pH (pH 7.4). Murata *et al.* [5] and Kageyama and Sugawara [9], however, have reported that no S_1 could be detected in plasma following an oral dose of two 50-mg capsules of AMF.

Gas chromatography (GC) [9], gas chromatography–mass spectrometry (GC–MS) [5,6] and high-performance liquid chromatography (HPLC) [5] have been used for the detection of AMF and its metabolites in human and animal plasma and urine. These methods, however, have the following disadvantages: the GC and GC–MS methods involve repeated extraction by organic solvent, fractionation and derivatization, and the HPLC method has low sensitivity and poor baseline resolution.

This paper describes an HPLC method for the simultaneous determination of AMF and S_1 in human plasma after oral administration of AMF capsules (100 mg of AMF).

EXPERIMENTAL

Reagents and chemicals

AMF capsules were obtained from Meiji Seika (Tokyo, Japan; Fenazox, lot 41, 50 mg of AMF per capsule). Standard AMF powder was supplied by the same manufacturer (lot RFZ 1600). S_1 was recrystallized from methanol after treatment of an aqueous AMF solution with concentrated hydrochloric acid, which showed one spot on a thin-layer chromatogram (TLC) and one peak in HPLC. S_1 was estimated to be over 99.5% pure by HPLC. The molecular structure of S_1 was confirmed by high-resolution mass spectrometry (HRMS) and elemental analysis. TLC was performed on Kieselgel 60 F_{254} precoated glass plates (Merck, Darmstadt, Germany). Fenbufen (internal standard, I.S.) was purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and ethanol were purchased from Wako (Osaka, Japan). All other chemicals were of reagent grade.

S_1 crystallized as light orange needles (m.p. 158°C; R_F 0.34 [$CHCl_3$–MeOH (98:2, v/v)]); HRMS of $C_{15}H_{11}O_2N$: calculated, 237.0790; found, 237.0793; analysis of $C_{15}H_{11}O_2N$: calculated, C, 75.93, H, 4.67, N, 5.90; found, C, 75.85, H, 4.43, N, 5.91.

Instrumentation and chromatographic conditions

A Shimadzu (Kyoto, Japan) HPLC system, consisting of a Model LC-6A pump with a gradient elution system, a Model SPD-6AV selectable-wavelength UV detector, a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA) and a Model C-R6A Chromatopac integrator was used. The detector wavelength was set at 245 nm with a sensitivity of 0.01 a.u.f.s. The HPLC column was stainless steel (250 mm × 4.6 mm I.D.) packed with 5-μm Bensil-ODS (Bentec, Chiba, Japan). Solvent A was 0.05 *M* potassium phosphate buffer (pH 7.0)–acetonitrile (8:2, v/v). Solvent B was acetonitrile. Both solvents were degassed by the reduced pressure method using an ERC-3322 degasser (Erma, Saitama, Japan). Solvent A was eluted first for 10 min, followed by a linear gradient elution of 20 to 80% of solvent B for 20 min.

Standard solutions

A stock solution containing AMF (100 μg/ml in purified water) or S_1 (5 μg/ml in methanol) was used after further dilution with purified water or methanol to the desired concentrations. A stock solution (100 μg/ml) of the I.S. was prepared in ethanol. All solutions were stored at −20°C and showed insignificant degradation over a period of two months.

Assay procedure

To 100 μl of plasma sample, 100 μl of purified water, 0.2 g of ammonium sulphate and 100 μl of

ethanol containing fenbufen (100 μg/ml, I.S.) were added. The solution was vortex-mixed for 30 s, then centrifuged at 6000 *g* for 5 min for deproteinization and extraction of AMF, S_1 and the I.S. The supernatant was filtered through a 0.22-μm membrane filter, and a portion (20 μl) was directly injected into the HPLC column. All samples were prepared in triplicate.

Quantification

Peak areas (μV · s) were used for quantitation. To prepare a calibration curve, the standard samples of AMF or S_1 were spiked into drug-free plasma by adding known amounts of AMF or S_1 to give final concentrations ranging from 0.1 to 10 μg/ml for AMF and 20 to 400 ng/ml for S_1. A calibration curve for AMF or S_1 was obtained by plotting the AMF/I.S. or S_1/I.S. peak-area ratio *versus* various concentrations of AMF or S_1. The concentrations of AMF and S_1 in human plasma were calculated from the calibration curve using linear least-squares regression analyses.

Recovery and reproducibility

The analytical recoveries of AMF and S_1 from human plasma were obtained as follows. Plasma samples containing standard AMF or S_1 at three different concentrations (0.5, 2 and 10 μg/ml for AMF and 40, 200 and 400 ng/ml for S_1) were analysed by HPLC. The reference samples were prepared in purified water instead of plasma. The peak areas of the plasma samples were compared with those of the references after complete ethanol extraction procedures, and the absolute recovery was calculated.

Reproducibility studies were performed by analysing aliquots of a pooled plasma containing AMF (5 μg/ml) or S_1 (100 ng/ml) every six days during two months, five times the same day (every 2 h).

Clinical study

Four healthy male volunteers, ranging in age from 29 to 35 years (median 32 years) and in weight from 62 to 75 kg (median 67 kg), participated in this study. No abnormalities from clinical examinations or hematological and biochemical profiles were found. The subjects fasted from 12 h before the study until 6 h after receiving AMF. They were permitted to drink water only. The subjects took two 50-mg capsules of AMF with 200 ml of water. Blood samples were obtained from an antecubital vein through a heparin lock before and at 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 2.85 h after administration of the drug. Blood samples were immediately centrifuged at 2000 *g* for 10 min, and the separated plasma samples were stored at −20°C until analysis.

Pharmacokinetic analysis

Estimation of pharmacokinetic parameters was performed using a linear one-compartment model with first-order absorption process. The MULTI computer program proposed by Yamaoka *et al.* [10] was used throughout. The least-squares algorithm used was the Simplex method, weighting = 1. The slope (K_{el}) of the terminal log-linear portion of the plasma AMF concentrations *versus* time curve was determined by a log-linear least-squares fit. The elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/K_{el}$. The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal rule, with the area extrapolated to infinity. The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were obtained directly from the observed data.

RESULTS AND DISCUSSION

Chromatography

AMF, S_1 and the I.S. exhibited symmetrical peaks (Fig. 2) with baseline resolution, and no interfering peaks from endogenous components in human plasma were observed (Fig. 2A). Fig. 2B shows a typical chromatogram form a volunteer's plasma sample 1 h after dosing. The retention times of AMF, the I.S. and S_1 were 5.2, 8.5 and 22.1 min, respectively, and their capacity factors (k') were 1.86, 3.14 and 11.14, respectively. The separation factors (α) between AMF and the I.S., the I.S. and S_1, and AMF and S_1 were 1.69, 3.55 and 6.0, respectively.

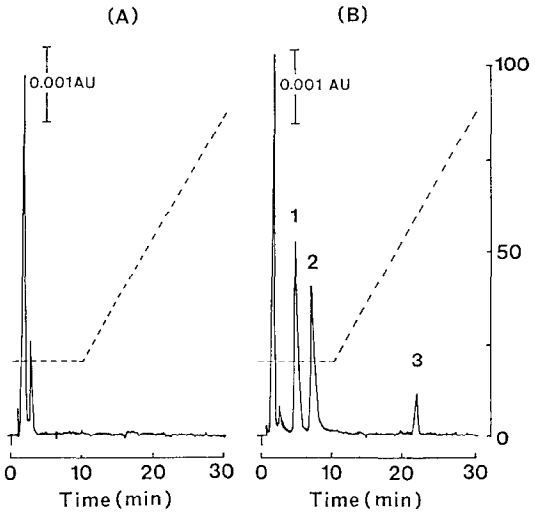


Fig. 2. HPLC of amfenac sodium (AMF), 7-benzoyl-2-oxindole (S_1) and fenbufen (I.S.). (A) Extract of blank plasma; (B) extract of a healthy volunteer's plasma 1 h after dosing with 100 mg of AMF, in which the concentrations or AMF (peak 1), I.S. (peak 2) and S_1 (peak 3) were estimated at 6.3 μg/ml, 1.1. μg/ml and 60 ng/ml, respectively.

Linearity

The calibration curves for AMF (0.1–10 μg/ml) and S_1 (20–400 ng/ml), prepared as described above, were apparently linear and passed through the origin: $y = 0.018x - 0.013$, $r = 0.998$ for AMF and $y = 3.123x - 0.521$, $r = 0.981$ for S_1.

Assay procedure

The accuracy of the method was evaluated using plasma samples added at three different concentrations of the reference AMF (0.5, 2 and 10 μg/ml) or S_1 (40, 200 and 400 ng/ml). The results were 0.5 ± 0.1, 2.0 ± 0.3 and 10.0 ± 1.0 μg/ml for AMF, and 40 ± 5, 200 ± 11 and 400 ± 29 ng/ml for S_1, respectively.

Recovery, reproducibility and sensitivity

The recovery ratio of AMF at concentrations of 0.5, 2 and 10 μg/ml and S_1 at concentrations of 40, 200 and 400 ng/ml were 92.1 ± 4.2, 95.4 ± 2.1 and 94.6 ± 2.2% and 93.4 ± 5.6, 98.7 ± 1.3 and 95.3 ± 2.1%, respectively. The coefficients of variation (C.V.) for intra- and inter-day variabilities of AMF (5 μg/ml) and S_1 (100 ng/ml) were 4.5 and 3.6% and 4.2 and 4.2%, respectively. The detection limits of AMF and S_1 were in concentrations as low as 0.1 μg/ml and 20 ng/ml, respectively, at a signal-to-noise ratio greater than 5. However, AMF and S_1 could be detected in concentrations below 0.1 μg/ml and 20 ng/ml when plasma sample and injection volumes were increased. The C.V. of AMF and S_1 for the detection limit were 4.0 and 4.4%, respectively.

Clinical applicability

AMF is administered to patients in a hard gelatin capsule, not an enteric-coated capsule. Therefore, when an AMF capsule is administered orally, it is expected that most AMF may be converted easily into S_1 by acidic fluids in the stomach, and whether S_1 has a pharmacological or toxicological activity has not yet been established. If AMF can be converted into S_1 in the stomach, S_1 must be detected in plasma, because the dissolution test of AMF capsules showed that S_1 is not reconverted into AMF at physiological pH (7.4).

Fig. 3 shows the mean plasma concentrations of AMF and S_1 after oral administration of two capsules (100 mg of AMF) to the human volunteers. Some pharmacokinetic parameters of AMF in human plasma are summarized in Table I. We did not calculate the pharmacokinetic parameters of S_1, because data points were too few and some points were below the detection limit.

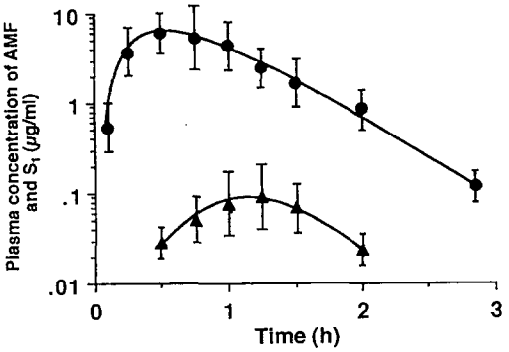


Fig. 3. Plasma concentrations of amfenac sodium (●) and 7-benzoyl-2-oxindole (▲) after oral administration of AMF (100 mg) to four human subjects. Each point represents a mean ± S.D.

TABLE 1

KINETIC PARAMETERS OF AMF AND S_1

Data were obtained after oral administration of 100 mg of AMF to four healthy subjects.

Parameter[a]	Unit	Value	
		AMF	S_1
k_{el}	1/h	0.521 ± 0.104	
$t_{1/2}$	h	1.3 ± 0.4	
T_{max}	h	0.5 ± 0.2	1.3 ± 0.4
C_{max}	μg/ml	5.62 ± 4.14	0.12 ± 0.11
AUC	μg/ml · h	6.11 ± 5.12	0.12 ± 0.10

[a] Abbreviations: k_{el} = first-order elimination constant; $t_{1/2}$ = elimination half-life; C_{max} = maximum plasma concentration; T_{max} = time to reach C_{max}; AUC = area under the plasma concentration–time curve from zero to infinity.

We confirmed the presence of S_1 in plasma, but its levels were much lower than those of AMF. The C_{max} and T_{max} of S_1 were very different from those of AMF (see Fig. 3): the mean C_{max} and T_{max} of S_1 were 0.12 μg/ml and 1.3 h, and those of AMF were 5.62 μg/ml and 0.5 h. Interestingly, the appearance of S_1 with lower T_{max} than AMF may suggest that AMF absorbed from a digestive tract might be converted into S_1 enzymically in the body. A more detailed investigation will be necessary to solve the riddle of why S_1 was detected in plasma, especially in view of the toxicological effect of S_1.

CONCLUSION

The method described here permits the simultaneous determination of AMF and S_1 in plasma with a high specificity and sensitivity. It is useful for clinical pharmacokinetic studies, drug monitoring and dosage control of AMF in patients' plasma treated with AMF.

ACKNOWLEDGEMENTS

The technical assistance given by Miss Sachiko Ohtsuka is gratefully appreciated. The authors thank Prof. Yoichi Yoshimoto, Department of Pharmaceutics, Meiji College of Pharmacy (Tokyo, Japan) for his valuable suggestions and discussion.

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