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SIMULTANEOUS DETERMINATION OF OFLOXACIN, FENBUFEN AND FELBINAC IN RAT PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Ofloxacin, fenbufen and its active metabolite, felbinac, were simultaneously extracted from 50 μl of rat plasma and analysed by high-performance liquid chromatography on a reversed-phase column. Quantitative and reproducible determinations were possible for ofloxacin, fenbufen and felbinac over the concentration ranges 0.15–40, 0.3–80 and 0.45–45 μg/ml, respectively. The detection limits for all the drugs were lower than those reported previously. The recovery of ofloxacin, fenbufen and felbinac added to plasma was nearly 100% with a coefficient of variation of less than 3.0%. This method was found to be applicable to pharmacokinetic studies of each drug after the concomitant administration of ofloxacin and fenbufen.

INTRODUCTION

Quinolones are new antibacterial agents with a broad spectrum of activity [1]. Recently, it was reported that the concomitant administration of a new quinolone antibacterial agent (enoxacin) and non-steroidal anti-inflammatory agent (fenbufen) induced convulsions in several cases [2]. Similar cases may be presumed to be likely for other new quinolones with fenbufen. However, neither pharmacokinetic nor pharmacodynamic interactions between both drugs have been clarified yet.

Fenbufen is known to be a "pro-drug", which is metabolized to an active compound, 4-biphenylacetic acid (felbinac) [3–5]. Therefore, it is important to clarify the pharmacokinetics of any new quinolone, fenbufen and felbinac after the concomitant administration of the new quinolone and fenbufen. In order to study these pharmacokinetics, it is necessary to develop a simple, sensitive and selec-

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tive assay method for these drugs in biological fluids, as there is no adequate method for determining them either simultaneously or readily.

In this work we employed ofloxacin, which has a good bioavailability after oral administration [6], as a new quinolone antibacterial agent and developed a specific method for the simultaneous determination of ofloxacin, fenbufen and felbinac in rat plasma by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals

Ofloxacin was kindly supplied by Daiichi Seiyaku (Tokyo, Japan) and fenbufen and felbinac by Lederle Japan (Tokyo, Japan). Nalidixic acid and N-phenylanthranilic acid (internal standards) of analytical-reagent grade were purchased from Nakarai Chemicals (Kyoto, Japan). Methanol, distilled water, sodium laurylsulphate and dichloromethane were of liquid chromatographic grade. Other reagents were of analytical-reagent grade.

Chromatographic conditions

A Shimadzu Model LC-4A pump (Shimadzu, Kyoto, Japan), equipped with a Shimadzu Model SPD-2AS variable-wavelength spectrophotometric detector, was used. Samples were introduced with a syringe into a Rheodyne Model 7125 injector with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.). The stationary phase was Chemcosorb 5-ODS-H (5 μm particle size) (Chemco, Osaka, Japan), slurry-packed in a stainless-steel column (150 mm$\times$4.6 mm I.D.). The mobile phase was methanol–0.005 *M* sodium laurylsulphate (2:1, v/v) adjusted to pH 2.5 with 85% phosphoric acid. The flow-rate was 0.6 ml/min and separation was performed at 40°C. The eluate was monitored at 300 nm (0–8 min) and 275 nm (8–20 min) with a sensitivity of 0.01 a.u.f.s. The chromatographic data were calculated with a Shimadzu Model C-R2AX data module.

Standard solutions

Stock solutions of ofloxacin (100 μg/ml), fenbufen (200 μg/ml), felbinac (200 μg/ml) and the internal standard (nalidixic acid, 300 μg/ml; N-phenylanthranilic acid, 200 μg/ml) were prepared by dissolving each drug first in a small volume (less than 1.0 ml for 10 mg of any drug) of 0.1 *M* sodium hydroxide solution and diluting with distilled water. Working standard solutions of the drugs and internal standards were prepared by diluting the stock solutions with distilled water.

Extraction procedure

To 50 μl of rat plasma were added 1.0 ml of 0.1 *M* dipotassium phosphate solution adjusted to pH 7.0 with 85% phosphoric acid and 100 μl of the internal standard solution. This mixture was extracted with 3.0 ml of dichloromethane–isoamyl alcohol (9:1) in a 10-ml glass tube, which was shaken vigorously for 10 min. After centrifugation at 3500 rpm (2270 *g*) for 10 min, 2.0 ml of the organic phase were transferred into another tube and evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The residue was reconstituted in 100 μl of meth-

anol–0.05 *M* sodium hydroxide solution (2:1) by vortexing. A 10-μl aliquot was injected into the chromatograph.

Calibration graphs

To blank plasma were added known amounts of ofloxacin, fenbufen and felbinac in the ranges 0.15–40, 0.3–80 and 0.45–45 μg/ml (in plasma), respectively. These plasma samples were treated according to the above determination procedure. Peak-height ratios of ofloxacin to nalidixic acid were measured and plotted against the concentration of ofloxacin in plasma. For fenbufen and felbinac, peak-height ratios with respect to N-phenylanthranilic acid were plotted in the same way as for ofloxacin.

Extraction efficiency

In preliminary experiments with the internal standards, the absolute recoveries determined by comparing the peak-height obtained from the extracted sample with that obtained after direct injection of a standard solution were 103.7 ± 0.8 and $100.2 \pm 1.1\%$ for nalidixic acid and N-phenylanthranilic acid, respectively ($n=6$). The extraction efficiencies of ofloxacin, fenbufen and felbinac were determined by comparing the peak-height ratio (drug versus internal standard) obtained after extraction of these drugs from the spiked plasma sample with that obtained after the direct injection of an equivalent amount of each drug.

Drug administration to rat

Male Wistar rats (8–10 weeks old, 160–240 g) were used for studies of the reproducibility and accuracy of the assay and another rat (10 weeks old, 275 g) was used for the preliminary kinetic study. Each rat was first cannulated in the right jugular vein. A bolus dose of 5 mg/kg ofloxacin with 10 mg/kg fenbufen was administered intravenously via the jugular vein cannula. Blood (about 0.13 ml for the preliminary kinetic study and 0.5–1 ml for other purposes) was withdrawn from the cannula periodically into a heparinized (1 unit per 0.1 ml of blood) tube. The plasma was immediately separated and kept frozen until the analysis.

Reproducibility

Blood samples were obtained from the rats at appropriate times after the concomitant administration of ofloxacin and fenbufen. Aliquots (50 μl each) of the plasma samples were analysed repeatedly according to the above procedure.

Accuracy

The accuracy of the assay was evaluated by measuring the recovery of known amounts of each drug added to rat plasma. To the plasma samples obtained from the rat after the concomitant administration of ofloxacin and fenbufen were added known amounts of ofloxacin, fenbufen and felbinac and these three compounds were then determined. The recovery of each drug was calculated by comparing the experimental value with the corresponding theoretical value.

RESULTS

Fig. 1 shows representative chromatograms for a plasma blank, a plasma blank spiked with ofloxacin, fenbufen and felbinac and a plasma sample obtained from the rat which was given ofloxacin and fenbufen. There was no peak of endogenous material in the rat plasma. The peaks for ofloxacin, fenbufen and felbinac were well separated from each other. The retention times (t_R) for ofloxacin, fenbufen and felbinac were 5.9, 8.9 and 9.8 min, respectively, and those of the internal standards, nalidixic acid and N-phenylanthranilic acid, were 5.0 and 15.0 min, respectively. Although a few small peaks that seemed to be derived from some minor metabolites of fenbufen were detected in the plasma of the rat, no interferences occurred among the peaks for ofloxacin, fenbufen, felbinac and the internal standards.

Calibration graphs for ofloxacin, fenbufen and felbinac were linear over the ranges 0–40, 0–80 and 0–45 μg/ml, respectively. The regression equations were as follows: ofloxacin, $y=0.002+0.241x$ ($r=1.000$); fenbufen, $y=0.086+0.475x$ ($r=1.000$); and felbinac, $y=0.009+0.215x$ ($r=1.000$), where y is the peak-height ratio of the drug to the internal standard, x is the concentration (μg/ml) of the drug in plasma and r is the correlation coefficient. The limits of determination were 0.15 μg/ml for ofloxacin, 0.3 μg/ml for fenbufen and 0.45 μg/ml for felbinac. These calibration and sensitivity data for each drug were not affected by the presence of other drugs in the plasma.

The extraction efficiency was examined by adding known amounts of ofloxacin, fenbufen and felbinac to blank plasma at high, medium and low concentrations. The results are summarized in Table I. For these three drugs, the extents

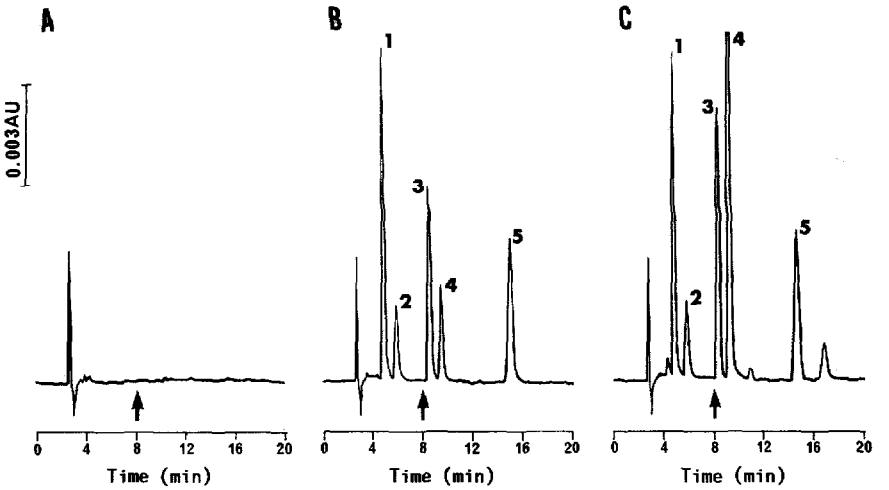

Fig. 1. Chromatograms of (A) a plasma blank, (B) a plasma blank spiked with ofloxacin, fenbufen and felbinac and (C) a plasma sample 2 h after bolus intravenous administration of 5 mg/kg ofloxacin and 10 mg/kg fenbufen to a rat. Peaks: 1 = nalidixic acid (internal standard); 2 = ofloxacin; 3 = fenbufen; 4 = felbinac; 5 = N-phenylanthranilic acid (internal standard). The arrows indicate the time of automatic changing of the detection wavelength from 300 to 275 nm.

TABLE I

EFFICIENCY OF EXTRACTION OF OFLOXACIN, FENBUFEN AND FELBINAC FROM RAT PLASMA

Drug	Added (μg/ml)	Extraction (mean ± S.D., $n=6$) (%)	C.V. (%)
Ofloxacin	0.15	100.9 ± 7.4	7.3
	1.00	95.3 ± 1.0	1.1
	20.0	94.9 ± 0.5	0.5
Fenbufen	0.30	98.0 ± 4.8	4.9
	3.00	101.5 ± 1.0	1.0
	60.0	101.9 ± 1.1	1.1
Felbinac	0.45	91.9 ± 3.3	3.5
	3.00	94.1 ± 0.7	0.8
	30.0	94.4 ± 1.0	1.0

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF OFLOXACIN, FENBUFEN AND FELBINAC IN RAT PLASMA

Drug	Plasma level (mean ± S.D., $n=10$) (μg/ml)	C.V. (%)
Ofloxacin	0.20 ± 0.01	5.6
	0.93 ± 0.02	1.9
	6.15 ± 0.11	1.8
Fenbufen	0.73 ± 0.03	3.5
	4.03 ± 0.06	1.5
	50.9 ± 1.1	2.2
Felbinac	0.69 ± 0.03	4.8
	4.85 ± 0.07	1.4
	20.7 ± 0.3	1.2

of extraction from the plasma ranged from 91.9 to 101.9% with coefficients of variation (C.V.) of less than 8%.

The precision of the determination of ofloxacin, fenbufen and felbinac in plasma was examined by performing ten replicate analyses at each of the three different concentrations of the drugs in plasma (Table II). The C.V. values ranged from 1.2 to 5.6%.

Table III shows the recovery data for ofloxacin, fenbufen and felbinac when each drug was added to the rat plasma at two different concentrations. The average recovery was nearly 100% with little variation for each drug.

Plasma concentrations of ofloxacin, fenbufen and felbinac after bolus intra-

TABLE III

RECOVERY OF OFLOXACIN, FENBUFEN AND FELBINAC ADDED TO RAT PLASMA

Drug	Added (μg/ml)	Found (mean, $n=6$) (μg/ml)	Recovery (mean $\pm$ S.D., $n=6$) (%)	C.V. (%)
Ofloxacin	None	0.95	—	—
	0.30	1.31	105.1 $\pm$ 2.2	2.1
	3.00	4.02	101.8 $\pm$ 1.1	1.1
Fenbufen	None	7.28	—	—
	1.00	8.41	101.6 $\pm$ 1.9	1.8
	10.00	17.06	98.7 $\pm$ 0.9	0.9
Felbinac	None	9.15	—	—
	1.00	10.33	101.8 $\pm$ 1.0	1.0
	10.00	18.88	98.6 $\pm$ 1.3	1.4

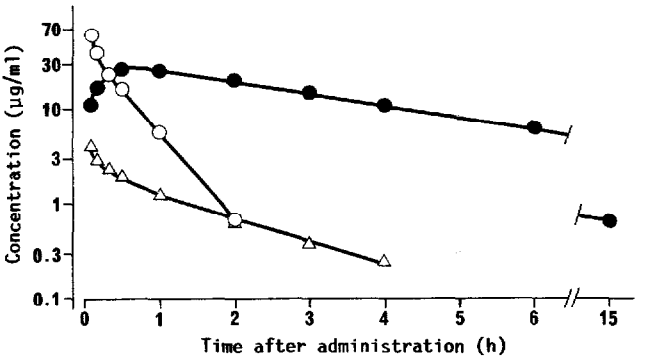

Fig. 2. Plasma concentrations of (△) ofloxacin, (○) fenbufen and (●) felbinac after bolus intravenous administration of 5 mg/kg ofloxacin and 10 mg/kg fenbufen to a rat.

venous administration of 5 mg/kg ofloxacin and 10 mg/kg fenbufen to a rat were determined, and the time courses of the plasma levels are shown in Fig. 2.

DISCUSSION

Various methods, including thin-layer chromatography [7], gas chromatography [8] and HPLC [9,10], have been developed to determine fenbufen and felbinac separately. For the determination of ofloxacin in biological fluids, one study employing microbiological assay and HPLC [11] and three employing HPLC [12–14] have been reported. A survey of these papers suggested that ofloxacin, fenbufen and felbinac may be determined simultaneously and separately by means of the reversed-phase HPLC. The HPLC conditions described in this paper are based on the method used for the determination of another new quinolone, norfloxacin [15]. The original conditions reported by Pauliukonis et al. [15] were found to give a suitable retention time for ofloxacin, but too long

for fenbufen and felbinac. We were able to establish a simple and reliable method for determining ofloxacin, fenbufen and felbinac simultaneously under improved conditions in which we increased the percentage of methanol in the mobile phase and employed sodium laurylsulphate as the ion-pairing reagent.

In spite of the small volume of the plasma sample (50 μl), the limits of determination of ofloxacin, fenbufen and felbinac by the present method were 0.15, 0.3 and 0.45 μg/ml, respectively, all of which are below the therapeutic range. In contrast, the published HPLC methods [9,10] for fenbufen and felbinac require 1–2 ml of plasma to detect 0.5 μg/ml as the lower limits. On the other hand, the previous HPLC methods for ofloxacin yielded a limit of detection for this drug as low as 0.03 μg/ml [12] or 0.5 μg/ml [13] by using 1 ml of serum sample or 0.15 μg/ml by using 0.5 ml of plasma sample [14]. These aspects demonstrate that the present method has a significant advantage with respect to both sensitivity and selectivity over the previous methods, which were developed independently for the determination of ofloxacin, fenbufen and felbinac.

As is obvious from the present results, a high extraction efficiency was obtained for each drug in spite of the single extraction procedure. Further, both the reproducibility and recovery in the determination of each drug were satisfactory over a wide concentration range. The method is simple, sensitive, precise and accurate enough to be utilized for detailed pharmacokinetic studies of the drugs, as exemplified in Fig. 2.

Further, we have found that the method is also applicable to the separation of other two new quinolones, enoxacin (t_R=6.2 min) and norfloxacin (t_R=6.5 min), from fenbufen and felbinac in plasma samples.

CONCLUSIONS

We have developed a simple, sensitive, precise and accurate HPLC method for the simultaneous determination of ofloxacin, fenbufen and felbinac in rat plasma. This method is useful for pharmacokinetic studies of these compounds and will facilitate detailed investigations into the interactions between new quinolones and fenbufen.

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