Jpn. J. Hosp. Pharm.
一 般 論 文
15(4) 292—298 (1989)

Simplified Procedures for the Simultaneous Determination of Enoxacin, Fenbufen and Felbinac in Rat Plasma by High-performance Liquid Chromatography

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(Received April 17, 1989)

A high-performance liquid chromatography has been employed for a simplified simultaneous determination of enoxacin, fenbufen and its active metabolite, felbinac, in rat plasma. Enoxacin, fenbufen and felbinac were simultaneously extracted from 50 μl of rat plasma and analysed by using liquid chromatography equipped with a reversed-phase column. By the present method, reproducible quantitative determinations were possible for enoxacin, fenbufen and felbinac over the concentration range of 0.2-20, 0.2-120 and 0.4-40 μg/ml, respectively. The recovery of enoxacin, fenbufen and felbinac added to plasma was 93% to nearly 100% with the coefficient of variation less than 3.0%. This method is applicable to the detailed pharmacokinetic studies of each drug after a concomitant administration of enoxacin and fenbufen.

Keywords—HPLC; enoxacin; fenbufen; felbinac; rat; plasma; simultaneous determination; coadministration

Introduction

Enoxacin is a new broad-spectrum antibacterial agent of the quinolone class[1]. It has been recently reported that a severe convulsion was induced in several cases when enoxacin and a non-steroidal anti-inflammatory agent, fenbufen, were administered concomitantly[2]. Fenbufen is known to be a "Pro-drug", which is readily metabolized to an active compound, 4-biphenylacetic acid (felbinac)[3-5]. Therefore, the severe convulsion is likely to be induced and/or enhanced by an increase of the cerebrospinal level of enoxacin due to possible pharmacokinetic interaction with fenbufen or felbinac. Basic investigation for the pharmacokinetics of enoxacin when coadministered with fenbufen is required to clarify or predict the possible interaction between both drugs. However, pharmacokinetic interaction between both drugs has not been clarified yet in either human or experimental animals. In order to study the pharmacokinetics of

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enoxacin, fenbufen and felbinac, it is necessary to develop a simple, sensitive and selective assay method for these drugs in the biological fluids, since there was no adequate method to determine them simultaneously as well as readily.

In the present paper, we described a new specific method for the simultaneous determination of enoxacin, fenbufen and felbinac in rat plasma by high-performance liquid chromatography (HPLC).

Experimental

1. Chemicals

Enoxacin was kindly supplied by Dainippon Pharmaceutical Co. Ltd., (Osaka, Japan) and fenbufen and felbinac were by Lederle Japan (Tokyo, Japan). Nalidixic acid and N-phenylanthranilic acid, as the internal standards, of analytical grade were purchased from Nakarai Tesque Inc. (Kyoto, Japan). Methanol, distilled water, sodium laurylsulfate and dichloromethane were of liquid chromatographic grade. Other reagents were of analytical grade.

2. 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 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 x 4.6 mm I.D.). The mobile phase was methanol-0.005M sodium laurylsulfate (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 275 nm with a sensitivity of 0.01 a.u.f.s. The chromatographic data were calculated with a Shimadzu Model C-R2AX data module.

3. Standard solutions

The stock solution of enoxacin (100 μg/ml), fenbufen (200 μg/ml), felbinac (200 μg/ml) or the internal standard (nalidixic acid, 300 μg/ml; N-phenylanthranilic acid, 200 μg/ml) was prepared by dissolving each drug first in a small volume (less than 1.0 ml for 10 mg of any drug) of 0.1M sodium hydroxide and diluting with the distilled water. Drug standard solution and internal standard solution were prepared by diluting the stock solution with distilled water to appropriate concentrations.

4. Extraction procedure

To 50 μl of rat plasma were added 1.0 ml of 0.1M dipotassium phosphate 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-isoamylalcohol (9:1) in a 10 ml glass tube, which was shaken vigorously for 10 min. After centrifugation at 3500 rpm (2270x*g*) for 10 min, 2.0 ml of the organic phase was transferred to another tube and

evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The residue was reconstituted in 100 µl of methanol-0.05M sodium hydroxide (2:1) by vortexing. A 10 µl aliquot was injected into the chromatograph.

5. Calibration curves

To blank plasma were added known amounts of enoxacin, fenbufen and felbinac in the range of 0.2-20, 0.2-120 and 0.4-40 µg/ml (in plasma), respectively. These plasma samples were treated according to the above determination procedure. Peak-height ratios of enoxacin to nalidixic acid were measured and plotted against the concentrations of enoxacin in plasma. For fenbufen and felbinac, peak-height ratios to N-phenylanthranilic acid were plotted in the same way as for enoxacin.

6. Drug administration to rat

Male Wistar rats (10 weeks old, 250-280 g), which were precedingly cannulated in the right jugular vein, were used. A bolus dose of 5 mg/kg of enoxacin with 10 mg/kg of fenbufen was administered intravenously *via* the jugular vein cannula to rat. The blood (about 0.13 ml for the preliminary kinetic study or 0.5-1 ml for other purposes) was withdrawn from the cannula periodically into the heparinized (1 unit per 0.1 ml of the blood) tube. The plasma was immediately separated and kept frozen until the analysis was conducted.

7. Reproducibility and accuracy

The blood sample was obtained from the rat at an appropriate time after the concomitant administration of enoxacin and fenbufen. Aliquots (50 µl each) of the plasma samples were analysed repeatedly according to the above determination procedure.

Accuracy of the assay was evaluated by the recovery of a known amount of each drug added to rat plasma. To the plasma samples obtained from the rat after the concomitant administration of enoxacin and fenbufen were added known amounts of enoxacin, fenbufen and felbinac. The determination of enoxacin, fenbufen and felbinac in these plasma samples was then carried out. 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 enoxacin, fenbufen and felbinac and a plasma sample obtained from the rat to which was given enoxacin and fenbufen. There was no peak of endogenous material in rat plasma. The peaks for enoxacin, fenbufen and felbinac were well separated from each other. The retention times (t_R) for enoxacin, fenbufen and felbinac were 6.2, 9.0 and 10.0 min, respectively. The internal standards, nalidixic acid and N-phenylanthranilic acid, had t_R of 5.0 and 15.8 min, respectively. Though a few small peaks which seemed to be derived from some minor metabolites of fenbufen were detected in the plasma of the rat, no interference was involved among the peaks for enoxacin, fenbufen, felbinac and the internal standards.

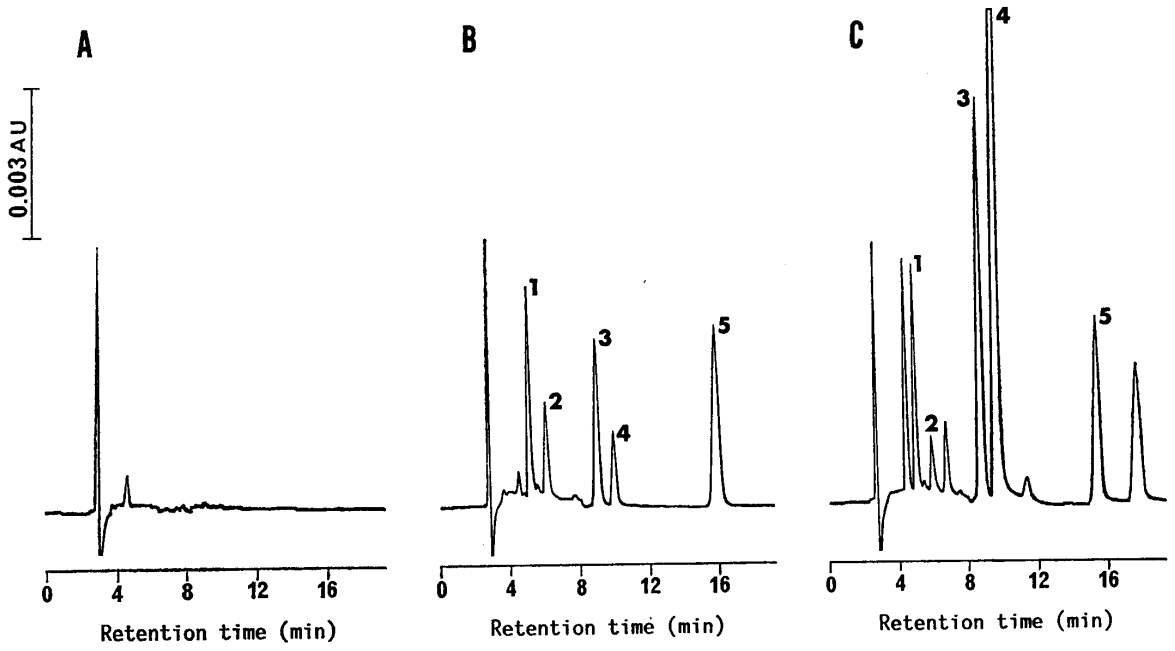

Fig.1. Chromatograms of (A) a Plasma Blank, (B) a Plasma Blank Spiked with Enoxacin, Fenbufen and Felbinac, (C) a Plasma Sample 1 hour after Bolus Intravenous Administration of 5 mg/kg of Enoxacin and 10 mg/kg of Fenbufen to a Rat
Peaks: 1, Nalidixic Acid (Internal Standard); 2, Enoxacin; 3, Fenbufen; 4, Felbinac; 5, N-Phenylanthranilic Acid (Internal Standard)

Calibration curves for enoxacin, fenbufen and felbinac were linear over the range of 0.2-20, 0.2-120 and 0.4-40 μg/ml, respectively. The regression equations were as follows: enoxacin, $y=0.495x+0.110$ ($r=1.000$); fenbufen, $y=0.431x+0.061$ ($r=1.000$); felbinac, $y=0.205x-0.009$ ($r=1.000$), 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 coefficient of correlation. The coefficients of variation at 0.2 and 20 μg/ml of enoxacin were 4.1 and 0.8% (n=5), respectively. The lower limits of the determination were 0.2 μg/ml for enoxacin, 0.2 μg/ml for fenbufen and 0.4 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 precision for the determination of enoxacin, fenbufen and felbinac in plasma was examined by performing ten replicate analyses at each of three different concentrations of drugs in plasma (Table 1). The coefficients of variation were 2.1, 2.4 and 2.7% at 0.48, 1.45 and 4.78 μg/ml of enoxacin in plasma, respectively. The coefficients of variation for fenbufen and felbinac ranged from 0.8 to 2.3%. Table 2 shows the recovery data for enoxacin, fenbufen and felbinac when the rat plasma was added with each drug at two different concentrations. The recovery of enoxacin added to plasma in the concentration of 0.3 and 3.0 μg/ml was 93.2 and 93.7%, respectively, with the coefficient of variation less than 3%. The average recovery for fenbufen and felbinac was approximately 100% with little variation.

Plasma concentrations of enoxacin, fenbufen and felbinac after bolus intravenous administration of 5 mg/kg of enoxacin and 10 mg/kg of fenbufen in a rat were determined, and the time-courses of these plasma levels are shown in Fig.2.

Table 1. Reproducibility of the Determination of Enoxacin, Fenbufen and Felbinac in Rat Plasma

Drug	Plasma level[a], μg/ml		C.V. (%)
	Mean ± S.D.	Range	
Enoxacin	0.48 ± 0.01	0.45 - 0.50	2.1
	1.45 ± 0.03	1.40 - 1.51	2.4
	4.78 ± 0.13	4.62 - 5.03	2.7
Fenbufen	0.50 ± 0.01	0.49 - 0.51	2.0
	6.10 ± 0.06	6.00 - 6.20	1.0
	81.0 ± 0.7	80.2 - 82.0	0.9
Felbinac	0.84 ± 0.02	0.82 - 0.88	2.3
	6.71 ± 0.06	6.63 - 6.78	0.8
	25.5 ± 0.2	25.1 - 25.8	0.9

a) n=10

Table 2. Recovery of Enoxacin, Fenbufen and Felbinac Added to Rat Plasma

Drug	Added (μg/ml)	Found[a] (μg/ml)	Recovery[b] (%)	C.V. (%)
Enoxacin	None	2.24	-	-
	0.30	2.37	93.2 ± 1.9	2.0
	3.00	4.91	93.7 ± 2.4	2.6
Fenbufen	None	7.76	-	-
	1.00	8.78	100.2 ± 1.0	1.0
	10.00	17.64	99.3 ± 0.5	0.5
Felbinac	None	7.03	-	-
	1.00	8.06	100.3 ± 1.1	1.1
	10.00	17.12	100.5 ± 0.7	0.7

a) Each value represents the mean of 6 determinations.
b) Each value represents the mean ± S.D. of 6 determinations.

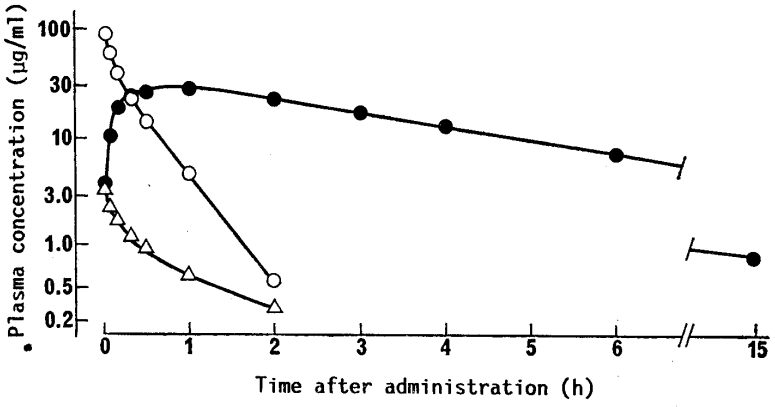

Fig.2. Plasma Concentrations of Enoxacin, Fenbufen and Felbinac after Bolus Intravenous Administration of 5 mg/kg of Enoxacin and 10 mg/kg of Fenbufen to a Rat
Symbols: △, Enoxacin; ○, Fenbufun; ●, Felbinac

Discussion

HPLC procedures were reported for the separative determination of fenbufen and felbinac in biological fluids[6,7]. Enoxacin in plasma was also determined by HPLC[8,9]. However, these reported methods proved unsuitable for the simultaneous determination of enoxacin, fenbufen and felbinac. Pauliukonis et al. described a specific method for the determination of another new quinolone, norfloxacin, in plasma and urine by reversed-phase ion-pair HPLC[10]. A survey of this report has suggested that enoxacin, fenbufen and felbinac may be determined simultaneously and separately by means of the reversed-phase HPLC method. The original condition reported by Pauliukonis et al.[10] was found to give a suitable retention time for enoxacin, but too long for fenbufen and felbinac. We could establish a simple and reliable method to determine enoxacin, fenbufen and felbinac simultaneously under the improved condition in which we increased the percentage of methanol in the mobile phase and employed sodium laurylsulfate as the ion-pairing reagent.

The proposed method could be adapted for smaller sizes of plasma samples such as less than 50 or 100 μl. In spite of a small volume of the plasma sample (i.e. 50 μl), the lower limits to determine enoxacin, fenbufen and felbinac by the present method were 0.2, 0.2 and 0.4 μg/ml, respectively. All of these levels were far below the therapeutic range. These aspects demonstrate that the present method has a significant advantage in both sensitivity and selectivity over the previous methods which were developed independently for the determination of enoxacin, fenbufen and felbinac.

The recovery data showed that the assay values for all three drugs were reliable in both low and high drug concentrations. The replicate analyses of rat plasma, which was obtained after the concomitant administration of enoxacin and fenbufen, indicated very low coefficients of variation at each concentration determined for three drugs. As is obvious from the present results, both reproducibility and recovery in the determination of each drug were in satisfactory level over the wide concentration range. The present method which we developed for the simultaneous determination of enoxacin, fenbufen and felbinac is simple, sensitive, precise and accurate enough to utilize for the detailed pharmacokinetic studies of these drugs, as exemplified in Fig.2.

In conclusion, we developed a simple, sensitive, precise and accurate method for the simultaneous determination of enoxacin, fenbufen and felbinac in rat plasma. This method was found to be useful for the pharmacokinetic studies of enoxacin, fenbufen and felbinac and will facilitate the detailed investigation into the interaction between the new quinolones and fenbufen.

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