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High-performance liquid chromatographic determination of pioglitazone and its metabolites in human serum and urine

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Abstract

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of pioglitazone and its metabolites (M-I to M-V) in human serum and urine was developed. The method for serum involved the solid-phase and liquid–liquid extraction. Urine with and without enzymatic hydrolysis using β-glucuronidase was treated with liquid–liquid extraction. The compounds in the extract were analyzed using HPLC with UV detection at 269 nm. The detection limits of pioglitazone, M-I, M-II, M-III, M-IV and M-V in serum were 0.01–0.05 μg/ml, those in urine were 0.1–0.5 μg/ml, and those in urine after enzymatic hydrolysis were 0.3–0.5 μg/ml, respectively. The method was applied to the clinical trials of pioglitazone.

Keywords: Pioglitazone

1. Introduction

Pioglitazone, (±)-5-[*p*-[2-(5-ethyl-2-pyridyl)-ethoxy]benzyl]-2,4-thiazolidinedione (AD-4833) (Fig. 1) has been shown to have hypoglycemic effects in animal models of non-insulin-dependent diabetes mellitus [1–12] and is now undergoing clinical trials. Zhong and Lakings reported an assay method for pioglitazone alone in dog plasma [13]. However, for the pharmacokinetic studies of pioglitazone in clinical trials, the determination of metabolites as well as pioglitazone was required. In this paper, we describe a high-performance liquid chromatographic (HPLC) method for the simultaneous determination of pioglitazone and its metabolites (M-I to M-V) in human serum and urine.

2. Experimental

2.1. Reagents and materials

Pioglitazone, its metabolites (M-I to M-V) and compound AD-4875 used as an internal standard (I.S.) were all synthesized in the Pharmaceutical Research Division, Takeda Chemical Industries (Osaka, Japan). Methanol, acetonitrile, diethyl ether

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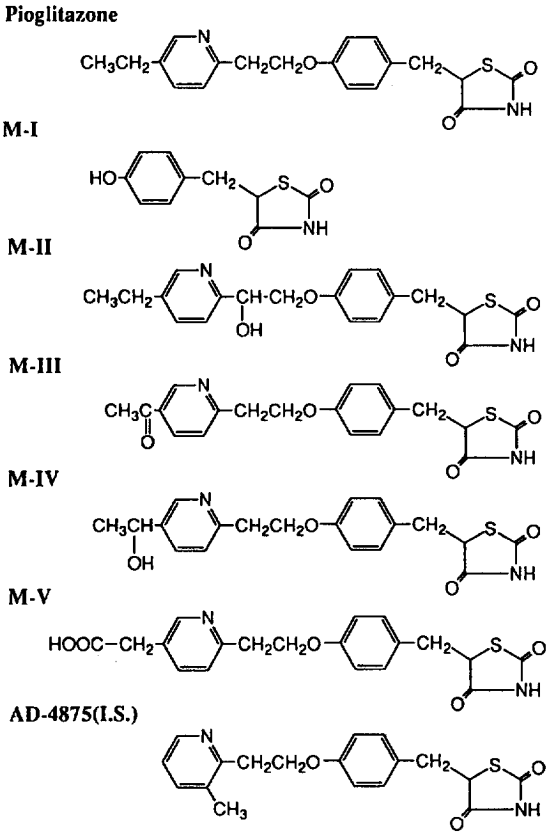

Fig. 1. Structures of pioglitazone, metabolites (M-I to M-V) and AD-4875 (I.S.)

and dichloromethane were of HPLC grade (Wako, Osaka, Japan). Solid-phase extraction (SPE) was performed using Bond Elut C_{18} cartridge (200 mg/3 ml; Varian, Harbor City, CA, USA). β-Glucuronidase (Type X-A) was obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical-reagent grade (Wako) and were used without further purification.

I.S. was dissolved in a mixture of methanol and 0.05 *M* potassium dihydrogenphosphate solution (2:3, v/v) to obtain an I.S. solution (4 μg/ml for serum and 10 μg/ml for urine sample). Enzyme solution (10 000 U/ml) was prepared by dissolving β-glucuronidase in 0.1 *M* phosphate buffer (pH 7.0). Phosphate buffer was prepared by mixing potassium dihydrogenphosphate and disodium hydrogenphosphate solution to obtain the required pH.

2.2. Instruments and conditions

The HPLC system consisted of an SCL-6A controller, two LC-6A pumps, a CTO-6A column oven, an SIL-6A autoinjector, an SPD-6A UV detector and a C-R4AX integrator (all from Shimadzu, Kyoto, Japan). The column was Inertsil ODS-2 (5-μm particle size, 150 × 4.6 mm I.D.; GL Sciences, Tokyo, Japan). The mobile phase (A) (MP(A)) was 0.05 *M* phosphate buffer (pH 6.0)–methanol (9:1, v/v) and MP(B) was 0.05 *M* phosphate buffer (pH 6.0)–methanol–acetonitrile (4:2:4, v/v). The gradient elution was performed, whose time program is described later. The temperature and the flow-rate were 40°C and 1.0 ml/min, respectively. Detection was carried out at UV 269 nm.

2.3. Extraction procedure and time programme for HPLC

2.3.1. Serum sample

The SPE column (Bond Elut C_{18}) was pre-activated with methanol (3 ml, twice) and water (3 ml, twice). To 200 μl of serum was added 800 μl of 0.05 *M* phosphate buffer (pH 3.0) and the mixture was applied to the activated SPE column. The column was washed twice with 3 ml of water. The analytes were eluted with 2 ml of a mixture of methanol and 0.02 *M* sodium acetate (9:1, v/v). The eluate was added with 100 μl of acetic acid and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 1 ml of 0.1 *M* potassium dihydrogenphosphate. The solution was extracted twice with 4 ml of a mixture of diethyl ether and dichloromethane (4:1, v/v). The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of I.S. solution and an aliquot of 100 μl was injected into the HPLC system.

The time program for the gradient elution was as follows: The concentration of MP(B) was linearly increased from 20% to 45% and finally to 100% over a period of 18 and 24 min, respectively. The MP(B) concentration was held at 100% for 7 min and cycled back to the initial condition (20%) in 0.1 min. The system was equilibrated at the initial mobile phase composition for 17 min before injecting the next sample. The analysis time was 66 min.

2.3.2. Urine sample

To 200 μl of urine was added 1 ml of 0.1 *M* potassium dihydrogenphosphate. The solution was extracted twice with 4 ml of a mixture of diethyl ether and dichloromethane (4:1, v/v). The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 500 μl of I.S. solution and an aliquot of 50 μl was injected into the HPLC system.

The time program for the gradient elution was as follows: The concentration of MP(B) was linearly increased from 5% to 100% over a period of 42 min, held for 7 min and cycled back to the initial condition (5%) in 0.1 min. The system was equilibrated at the initial mobile phase composition for 17 min before injecting the next sample. The analysis time was 66 min.

2.3.3. Urine sample after enzymatic hydrolysis

To 100 μl of urine was added 400 μl of the enzyme solution (β-glucuronidase, 10 000 U/ml). The mixture was incubated for 3 h at 37°C and added with 1 ml of 1 *M* phosphate buffer (pH 3.5). The resultant solution was extracted twice with 4 ml of a mixture of diethyl ether and dichloromethane (4:1, v/v). The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 500 μl of I.S. solution and an aliquot of 50 μl was injected into the HPLC system.

The time program for the gradient elution was as follows: The concentration of MP(B) was linearly increased from 20% to 45%, to 47% and finally to 100% over a period of 15, 10 and 15 min, respectively. The MP(B) concentration was held at 100% for 5 min and cycled back to the initial condition (20%) in 0.1 min. The system was equilibrated at the initial mobile phase composition for 21 min before injecting the next sample. The analysis time was 66 min.

2.4. Validation

Drug-free serum or urine spiked with known amounts of pioglitazone and its metabolites were analyzed according to the respective analytical methods described above. Peak-height ratios of each compound to I.S. were plotted against the respective concentrations to give the calibration graphs. The percentage recoveries of extraction for each compound from serum or urine were calculated from the peak-height ratios of spiked samples relative to directly injected standard solutions. Precision and accuracy were assessed by back-calculating the concentrations of the analyte from the peak-height ratio in the calibration graph of the spiked samples.

3. Results and discussion

A preliminary study showed that M-II gave a badly tailing peak on the chromatogram. In order to improve the peak shape of M-II, various columns were investigated: YMC A-302 ODS (150 × 4.6 mm I.D.; YMC, Kyoto, Japan), YMC AM-302 ODS (150 × 4.6 mm I.D.; YMC), Inertsil ODS-2 (150 × 4.6 mm I.D.; GL Sciences), Capcell Pak C_{18} SG120 (150 × 4.6 mm I.D.; Shiseido, Tokyo, Japan), LiChrospher RP-Select B (250 × 4.0 mm I.D.; E.Merck, Darmstadt, Germany) and Wakopak Nucleosil $5C_{18}$ (150 × 4.0 mm I.D.; Wako). Of these columns, only Inertsil ODS-2 and Capcell Pak C_{18} SG120 gave a sharp peak with a minimum tailing. Considering the column efficiency, the former column was adopted. Furthermore, the addition of methanol to the mobile phase improved the peak shape for M-II compared with acetonitrile alone.

As the clean-up method for serum sample, the combination of solid-phase and liquid–liquid extraction at ca. pH 4 was required to eliminate the interferences at the retention time of each compound on the chromatogram. Each extraction method alone could not eliminate the interferences. For urine samples, only liquid–liquid extraction was sufficient for the present purpose. For the simultaneous extraction of all analytes of interest, the pH of the aqueous layer needed to be maintained between 3 and 5.

A preliminary investigation using a urine (0 to 24 h sample from a volunteer who ingested 60 mg pioglitazone in the clinical trials) showed the existence of the M-IV conjugate in urine. Therefore the condition of the enzymatic hydrolysis was optimized using the content of M-IV formation in urine by the various conditions concerning the concentration of the enzyme (β-glucuronidase) and incubation time. The β-glucuronidase concentration in the added

enzyme solution between 5000 to 20 000 U/ml with the incubation time of 2 to 24 h at 37°C gave an almost similar M-IV concentration. From these findings, the enzyme concentration of 10 000 U/ml with the incubation time of 3 h at 37°C was adopted as the enzymatic hydrolysis condition.

The gradient elution mode for HPLC was employed in order to obtain a better separation of each compound from endogenous interferences. The gradient time program was optimized for serum and urine samples, separately. Figs. 2–4 show the chromatograms with minimum interferences at the retention time of each compound.

The calibration graphs were obtained by analyzing spiked serum and urine samples. The least-squares regression fit showed good linearity, passing through the origin (correlation coefficient >0.997) for each compound in serum and urine, up to 5 μg/ml and 50 μg/ml, respectively. The extraction recoveries for the compounds from serum or urine were all above 60%. The intra- and inter-assay data for accuracy and precision are presented in Table 1. The detection limits for each compound were as follows, at a signal-to-noise ratio of 3 and also considering the existence of small interferences at the retention time of each compound; pioglitazone, M-I, M-II, M-III, M-IV and M-V in serum were 0.01, 0.05, 0.02, 0.01, 0.01 and 0.02 μg/ml, those in urine were 0.1, 0.5, 0.2, 0.1, 0.1 and 0.1 μg/ml and those in urine after enzymatic hydrolysis were 0.5, 0.5, 0.5, 0.5, 0.3 and 0.3 μg/ml, respectively. Pioglitazone and its metabolites were all stable in serum and urine for at least 2 months at −20°C (Table 2).

The method has been applied to the clinical trials of pioglitazone. Fig. 5 shows the serum levels of pioglitazone and its metabolites in a volunteer who ingested 30 mg of pioglitazone. Pioglitazone, M-III, M-IV and M-V were observed in serum. Almost no increase in the concentration of each compound in

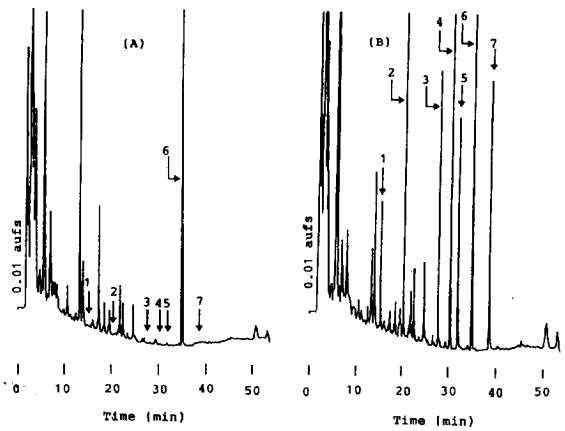

Fig. 3. Chromatograms of (A) drug-free urine and (B) urine spiked with pioglitazone and its metabolites (each 5 μg/ml). The arrows show the retention times of the compounds; 1 = M-I, 2 = M-V, 3 = M-IV, 4 = M-III, 5 = M-II, 6 = I.S., 7 = pioglitazone

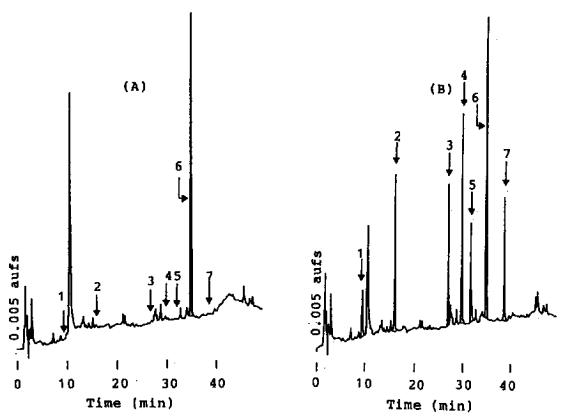

Fig. 2. Chromatograms of (A) drug-free serum and (B) serum spiked with pioglitazone and its metabolites (each 0.5 μg/ml). The arrows show the retention times of the compounds; 1 = M-I, 2 = M-V, 3 = M-IV, 4 = M-III, 5 = M-II, 6 = I.S., 7 = pioglitazone

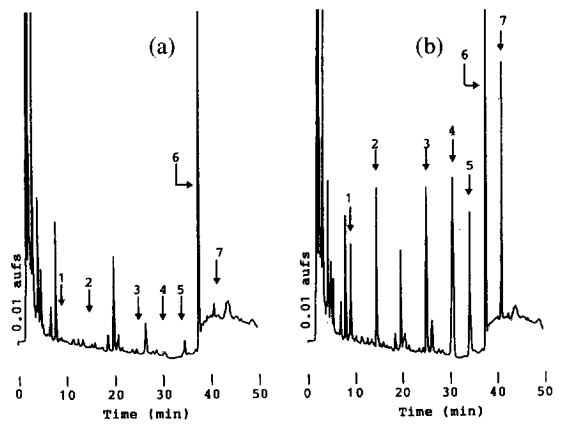

Fig. 4. Chromatograms of (A) drug-free urine and (B) urine spiked with pioglitazone and its metabolites (each 10 μg/ml) and treated with β-glucuronidase. The arrows show the retention times of the compounds; 1 = M-I, 2 = M-V, 3 = M-IV, 4 = M-III, 5 = M-II, 6 = I.S., 7 = pioglitazone

Table 1
Accuracy and precision data for pioglitazone and its metabolites added to human serum and urine

Compound	Added concentration (μg/ml)	Mean found concentration (μg/ml)					
		Intra-assay (n=5)			Inter-assay (n=3)		
		Mean (μg/ml)	C.V. (%)	Bias (%)	Mean (μg/ml)	C.V. (%)	Bias (%)
Serum							
Pioglitazone	0.5	0.481	2.1	−3.8	0.487	1.2	−2.6
M-I	0.5	0.483	1.2	−3.4	0.508	7.2	1.6
M-II	0.5	0.472	1.5	−5.6	0.478	1.1	−4.6
M-III	0.5	0.490	2.0	−2.0	0.496	1.2	−0.8
M-IV	0.5	0.482	0.6	−3.6	0.488	1.5	−2.4
M-V	0.5	0.481	2.2	−3.8	0.488	2.2	−2.4
Urine							
Pioglitazone	5	4.92	1.1	−1.6	5.29	9.2	5.8
M-I	5	4.98	0.7	−0.4	5.05	1.9	1.0
M-II	5	4.85	1.0	−3.0	5.00	4.8	0.0
M-III	5	4.93	0.7	−1.4	5.09	6.2	1.8
M-IV	5	4.96	0.5	−0.8	5.17	5.9	3.4
M-V	5	4.96	2.2	−0.8	5.27	6.2	5.4
Urine[a]							
Pioglitazone	10	10.7	4.5	7.0	10.3	3.8	3.0
M-I	10	10.2	1.4	2.0	10.1	1.0	1.0
M-II	10	9.84	2.8	−1.6	9.87	1.2	−1.3
M-III	10	10.4	3.9	4.0	10.1	2.4	1.0
M-IV	10	10.4	3.2	4.0	10.2	2.3	4.0
M-V	10	10.4	3.2	4.0	10.1	2.9	1.0

[a] Urine after enzymatic hydrolysis.

serum was observed after enzymatic hydrolysis under conditions similar to those described for urine. In this volunteer, 28% of the administered dose was excreted in urine in 48 h. M-IV and M-V were the main compounds excreted in urine. M-IV existed mostly in the conjugated form in urine. The details of the results from the clinical trials will be reported elsewhere in the future.

Table 2
Stability of pioglitazone and its metabolites in human serum and urine stored at −20°C for 2 months

Compound	Residual content (n=3) (%)	
	Serum (1 μg/ml)	Urine (10 μg/ml)
Pioglitazone	101.6	80.6
M-I	96.7	101.9
M-II	96.8	96.8
M-III	98.1	86.4
M-IV	98.4	92.6
M-V	98.7	95.7

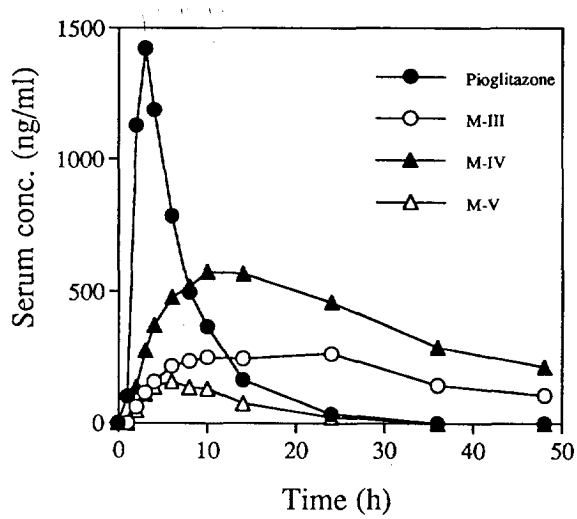

Fig. 5. Serum levels of pioglitazone and its metabolites in a volunteer after oral administration of 30 mg of pioglitazone.

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