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

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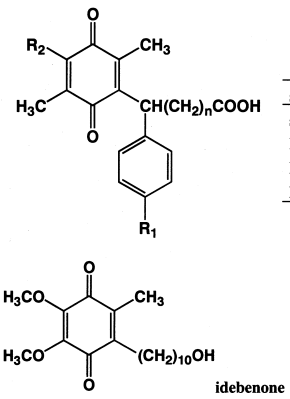
**Abstract**

A high-performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of seratrodast, a new antiasthmatic drug, and its metabolites (M-I to M-III) in human serum and urine. The method for serum and urine with and without enzymatic hydrolysis using β-glucuronidase involved liquid–liquid extraction and chemical oxidation with iron(III) chloride. The compounds in the extract were analyzed using HPLC with UV detection at 266 nm. The detection limits of seratrodast, M-I, M-II and M-III in serum and urine were 5–10 and 5–20 ng/ml, respectively, and those of deconjugated compounds in urine were 10–50 ng/ml. The method was applicable for human serum and urine from clinical trials. © 1997 Elsevier Science B.V.

*Keywords:* Seratrodast

## 1. Introduction

Seratrodast ((±)-7-(3,5,6-trimethyl-1,4-benzoquinon-2-yl)-7-phenylheptanoic acid) (Fig. 1), a novel, long-acting, potent thromboxane $A_2$/prostaglandin endoperoxide receptor antagonist, is now commercially available as an antiasthmatic drug [1–12]. To characterize the clinical pharmacokinetics, an analytical method for seratrodast and its metabolites in biological fluids was required. In this paper, we describe a high-performance liquid chromatographic (HPLC) method for the simultaneous determination




| substance | n | $R_1$ | $R_2$ |
|---|---|---|---|
| seratrodast | 5 | H | $CH_3$ |
| M-I | 3 | H | $CH_3$ |
| M-II | 5 | OH | $CH_3$ |
| M-III | 5 | H | $CH_2OH$ |

Fig. 1. Structures of seratrodast, metabolites (M-I to M-III) and idebenone (I.S.).

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of seratrodast and its metabolites (M-I to M-III) in human serum and urine.

## 2. Experimental

### *2.1. Reagents and materials*

Seratrodast, its metabolites (M-I to M-III) and idebenone (Fig. 1) used as an internal standard (I.S.) were all synthesized in the Pharmaceutical Research Division, Takeda Chemical Industries (Osaka, Japan). Acetonitrile and ethyl acetate were of HPLC grade (Wako, Osaka, Japan). β-Glucuronidase (type B-3, H-1 and IX) and sulfatase (type VIII) were 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 ethyl acetate to obtain an I.S. solution (0.167 μg/ml). Enzyme solutions (667 U/ml for β-glucuronidase and 33.3 U/ml for sulfatase) were prepared by dissolving each enzyme in 0.2 *M* phosphate buffer (pH 5.0). Phosphate buffer was prepared by mixing potassium dihydrogenphosphate and disodium hydrogenphosphate solution to obtain the required pH. Iron(III) chloride solution was prepared by dissolving $FeCl_3 \cdot 6H_2O$ in water (1 mg/ml). Propylene glycol solution was prepared by dissolving propylene glycol in ethyl acetate (5%, w/v).

### *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, a C-R3A integrator (all from Shimadzu, Kyoto, Japan) and a U-228 recorder (Nippon Denshi Kagaku, Kyoto, Japan). The column was YMC Pack ODS (A-302, 5 μm particle size, 150×4.6 mm I.D.; YMC, Kyoto, Japan). The mobile phase A (MP(A)) was 0.05 *M* potassium dihydrogenphosphate for serum and 0.05 *M* phosphate buffer (pH 3.0) for urine. The mobile phase B (MP(B)) was acetonitrile–0.05 *M* potassium dihydrogenphosphate (6:4, v/v) for serum and acetonitrile–0.05 *M* phosphate buffer (pH 3.0) for urine (6:4, v/v). A gradient elution was performed, the time program for which was as follows: the concentration of MP(B) was linearly increased from 50 to 100% over a period of 50 min. The MP(B) concentration was held at 100% for 5 min and cycled back to the initial condition (50%) in 5 min. The system was equilibrated at the initial mobile phase composition for 15 min before injecting the next sample. The analysis time was 75 min. The temperature of the column was 25°C for serum and 40°C for urine. The flow-rate was 1.0 ml/min. Detection was carried out at UV 266 nm.

### *2.3. Extraction procedure and time program for HPLC*

The samples were handled using brown containers to avoid light-induced decomposition [13].

#### *2.3.1. Serum*

To 500 μl of serum was added 150 μl of 0.1 *M* hydrochloric acid and the mixture was extracted with 6 ml of I.S. solution. After the centrifugation, propylene glycol in ethyl acetate (200 μl) was added to the organic layer and evaporated to dryness under a stream of nitrogen at 40°C. Iron(III) chloride solution (2 ml) was added to the residue and vortexed for 10 s. The solution was extracted with 4 ml of ethyl acetate, and propylene glycol solution (200 μl) was added to the separated organic layer and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of a mixture of acetonitrile and 0.05 *M* potassium dihydrogenphosphate (11:9, v/v) and an aliquot of 100 μl was injected into the HPLC system.

#### *2.3.2. Urine*

Urine (500 μl) was extracted with 6 ml of I.S. solution. After the centrifugation, propylene glycol solution (200 μl) was added to the organic layer and evaporated to dryness under a stream of nitrogen at 40°C. Iron(III) chloride solution (2 ml) was added to the residue and vortexed for 10 s. The solution was extracted with 4 ml of ethyl acetate, and propylene glycol solution (200 μl) was added to the separated organic layer and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of a mixture of acetonitrile and 0.05 *M* phosphate buffer, pH 3.0 (11:9, v/v), and an

Table 1
Comparison of concentration of seratrodast and M-III in urine by enzymatic hydrolysis (ng/ml)

| Analyte | Before hydrolysis | Sulfatase (type VIII) | β-D-Glucuronidase (type B-3) | β-D-Glucuronidase (type H-1) | β-D-Glucuronidase (type IX) |
|---|---|---|---|---|---|
| Seratrodast | 87 | 290 | 550 | 520 | 470 |
| M-III | nd | 68 | 190 | 180 | 190 |

Incubated for 18 h at 37°C (sulfatase 25 U/0.5 ml urine, β-D-glucuronidase 500 U/0.25 ml urine). nd, not detected.

aliquot of 100 μl was injected into the HPLC system.

*2.3.3. Urine after enzymatic hydrolysis*

To 250 μl of urine was added 750 μl of the enzyme solution (β-glucuronidase, type B-3). The mixture was incubated for 1 h at 37°C, and extracted with 6 ml of I.S. solution. After the centrifugation, propylene glycol solution (200 μl) was added to the organic layer and evaporated to dryness under a stream of nitrogen at 40°C. Iron(III) chloride solution (2 ml) was added to the residue and vortexed for 10 s. The solution was extracted with 4 ml of ethyl acetate, and propylene glycol solution (200 μl) was added to the separated organic layer and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of a mixture of acetonitrile and 0.05 *M* phosphate buffer, pH 3.0 (11:9, v/v), and an aliquot of 100 μl was injected into the HPLC system.

*2.4. Validation*

Drug-free serum or urine spiked with known amounts of seratrodast, its metabolites and their reduced products which were prepared with sodium

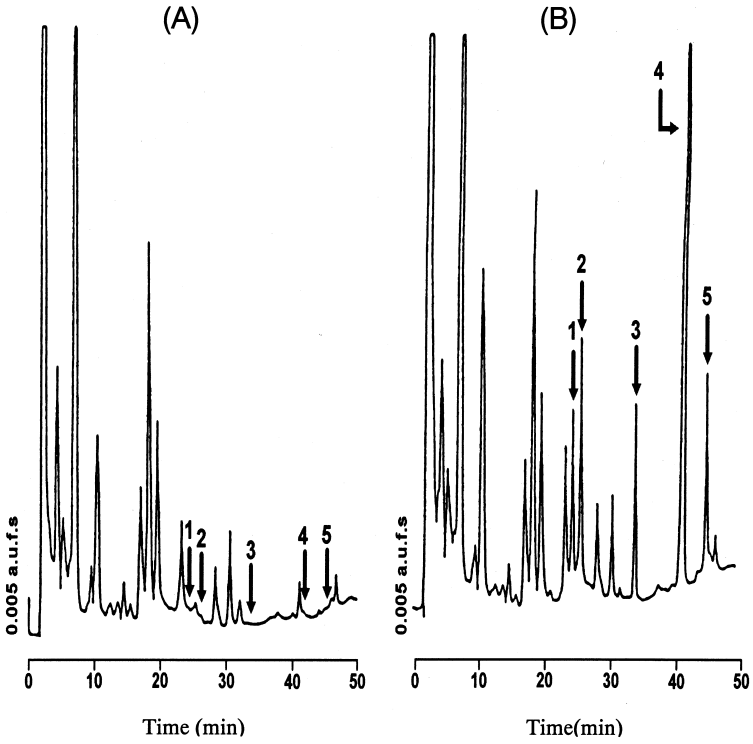


Fig. 2. Chromatograms of (A) drug-free serum and (B) serum spiked with seratrodast and its metabolites (200 ng/ml each). The arrows show the retention times of the compounds: 1, M-III; 2, M-II; 3, M-I; 4, I.S.; 5, seratrodast.

borotetrahydrate in ethanol solution (0.04%, w/v) 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

The pharmacokinetic studies of seratrodast [14,15] demonstrate the possibility for hydroquinone and quinone forms of seratrodast and its metabolites to exist in serum and urine. They are supposed to be in equilibrium. Because determination of seratrodast and its metabolites as hydroquinone forms is difficult, the analytes were oxidized to measure as corresponding quinone forms.

To analyze all of these compounds simultaneously, liquid–liquid extraction, oxidation with iron(III) chloride, and HPLC with gradient elution were appropriate. As a clean-up procedure, liquid–liquid extraction with ethyl acetate at lower than pH 7 was required for the simultaneous extraction of all the analytes. Iron(III) chloride was selected to avoid interference to chromatograms and prevent M-II (containing phenolic group) oxidation [16].

A preliminary study using urine (0–5 h sample from a volunteer who ingested 20 mg seratrodast in clinical trials) showed the existence of conjugated seratrodast and M-III in urine. Therefore, the conditions of enzymatic or acid hydrolysis were optimized using the urine sample by the various conditions such as hydrolyzing reagents, concentrations

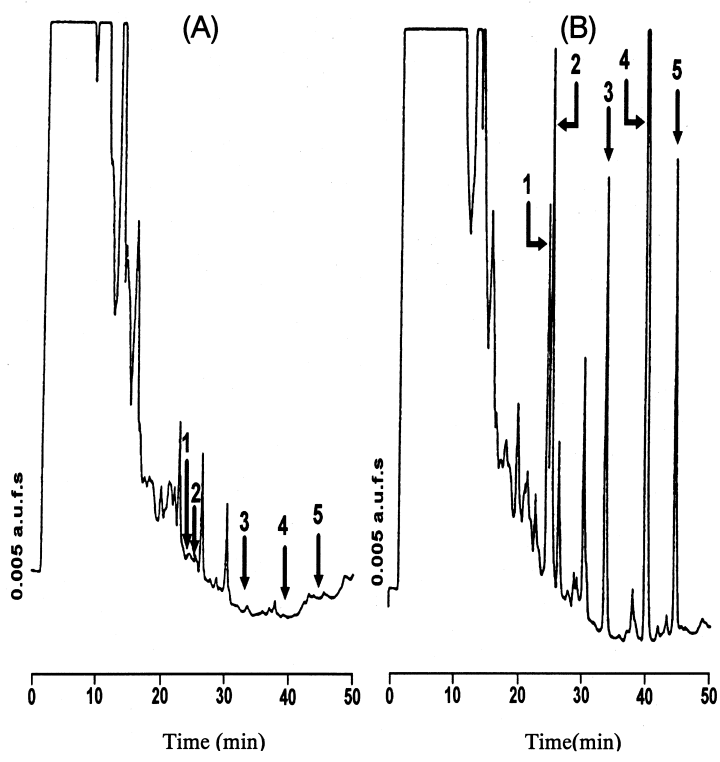


Fig. 3. Chromatograms of (A) drug-free urine and (B) urine spiked with seratrodast and its metabolites (200 ng/ml each). The arrows show the retention times of the compounds: 1, M-III; 2, M-II; 3, M-I; 4, I.S.; 5, seratrodast.

of the agent and incubation time. Of these reagents, hydrochloric acid was excluded because of interferences on chromatograms. Considering the amount of seratrodast and M-III deconjugated (Table 1), type B-3 β-D-glucuronidase was adopted. The incubation with this enzyme (500 U/0.25 ml urine) for 1–24 h at 37°C gave almost equal concentrations of unconjugated seratrodast and M-III. Therefore, the enzyme concentration of 500 U/0.25 ml urine with an incubation time of 1 h at 37°C was adopted as the conditions for enzymatic hydrolysis.

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.

Table 2 shows the comparison of preliminary intra-day variation for oxidized and reduced forms of seratrodast and its metabolites in serum and urine. There were no differences with results between the sample spiked with oxidized or reduced forms at two concentrations. Therefore, reduced forms of seratrodast and its metabolites were utilized for intra-day and inter-day validation.

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 $>0.9999$) for each compound in serum, urine and hydrolyzed urine, up to 4, 1, and 4 μg/ml, respectively. The extraction recoveries for the compounds from serum or urine were all above 89%. The intra- and inter-assay data for accuracy and precision are presented in Table 3. On the basis of a signal-to-noise ratio of 3 and the existence of a small amount of interferences at the retention time of each compound, the detection limits for seratrodast, M-I, M-II, and M-III were concluded to be 5, 5, 5 and 10 ng/ml in serum, 5, 10, 10 and 20 ng/ml in urine and 10, 30, 50 and 50 ng/ml in urine after enzymatic hydrolysis. Seratrodast and its metabolites were all stable in serum and urine for at least 2 months at −20°C (Table 4).

The described method was applied to the de-

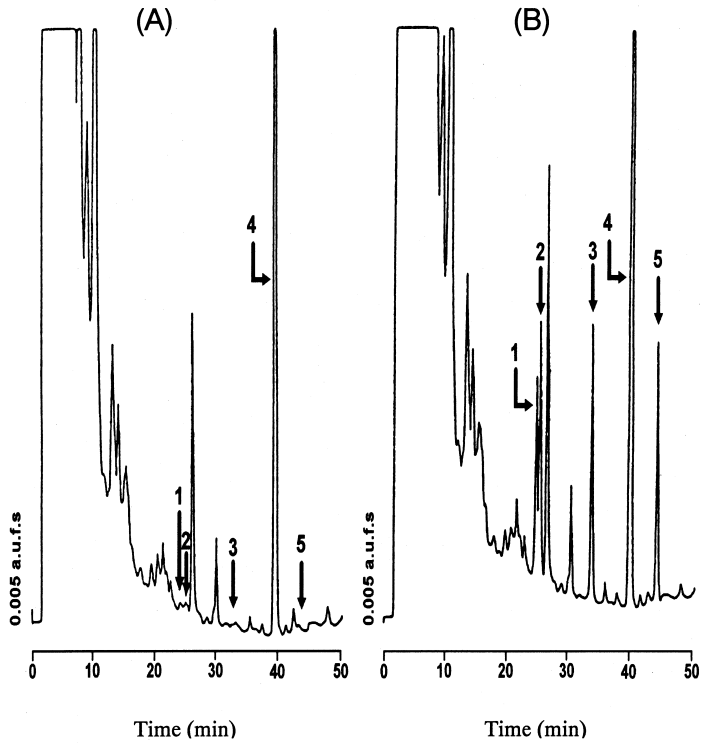


Fig. 4. Chromatograms of (A) drug-free urine and (B) urine treated with β-glucuronidase after being spiked with seratrodast and its metabolites (200 ng/ml each). The arrows show the retention times of the compounds; 1, M-III; 2, M-II; 3, M-I; 4, I.S.; 5, seratrodast.

Table 2
Comparison of intra-day variation of seratrodast and its metabolites to their reduced compounds in serum and urine ($n=3$)

| Compound | Added (ng/ml) | Oxidized form | | | Reduced form | | |
|---|---|---|---|---|---|---|---|
| | | Mean found | C.V. (%) | Bias (%) | Mean found | C.V. (%) | Bias (%) |
| *Serum* | | | | | | | |
| Seratrodast | 50 | 47.0 | 0.6 | −6.0 | 47.8 | 0.6 | −4.4 |
| | 500 | 459.0 | 1.4 | −8.2 | 516.5 | 4.5 | 3.3 |
| M-I | 50 | 49.4 | 1.6 | −1.2 | 49.8 | 0.7 | −0.4 |
| | 500 | 515.5 | 1.2 | 3.1 | 549.0 | 2.8 | 9.8 |
| M-II | 50 | 47.6 | 0.5 | −4.8 | 46.2 | 1.2 | −7.6 |
| | 500 | 518.5 | 1.3 | 3.7 | 544.5 | 2.6 | 8.9 |
| M-III | 50 | 50.0 | 0.9 | 0.0 | 48.8 | 0.4 | −2.4 |
| | 500 | 528.0 | 0.7 | 5.6 | 551.0 | 2.5 | 10.2 |
| *Urine* | | | | | | | |
| Seratrodast | 50 | 48.0 | 4.2 | −4.0 | 47.9 | 2.4 | −4.2 |
| | 500 | 495.5 | 2.1 | −0.9 | 494.5 | 0.8 | −1.1 |
| M-I | 50 | 50.0 | 2.8 | 0.0 | 50.1 | 0.8 | 0.2 |
| | 500 | 508.5 | 0.7 | 1.7 | 508.0 | 0.8 | 1.6 |
| M-II | 50 | 49.5 | 2.9 | −1.0 | 50.2 | 0.3 | 0.4 |
| | 500 | 505.0 | 1.2 | 1.0 | 505.5 | 1.1 | 1.0 |
| M-III | 50 | 49.6 | 2.7 | −0.8 | 49.9 | 0.8 | −0.2 |
| | 500 | 503.0 | 1.1 | 0.6 | 505.5 | 1.0 | 1.1 |

termination of seratrodast and its metabolites in human serum and urine. Fig. 5 shows the mean concentrations of seratrodast and its metabolites in serum samples from three volunteers who ingested 20 mg of seratrodast. An increase in the concentration of each compound in serum was hardly observed after enzymatic hydrolysis under the conditions described for urine. In these volunteers, 5–15% of the dose was excreted in urine within 48 h, mostly as conjugates. The pharmacokinetic profiles in clinical trials using this method were reported elsewhere [17].

Table 3
Accuracy and precision data for seratrodast and its metabolites, added to human serum and urine

| Compound | Concentration (ng/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Intra-assay ($n=5$) | | | | Inter-assay ($n=3$) | | | |
| | Added | Mean found | C.V. (%) | Bias (%) | Added | Mean found | C.V. (%) | Bias (%) |
| *Serum* | | | | | | | | |
| Seratrodast | 200 | 190.4 | 2.9 | −4.9 | 160 | 154.2 | 5.5 | −3.6 |
| M-I | 200 | 203.6 | 4.8 | 1.8 | 160 | 159.7 | 6.5 | −0.2 |
| M-II | 200 | 202.3 | 4.0 | 1.2 | 160 | 157.9 | 8.5 | −1.3 |
| M-III | 200 | 203.6 | 2.2 | 1.8 | 160 | 156.5 | 6.6 | −2.2 |
| *Urine* | | | | | | | | |
| Seratrodast | 200 | 194.0 | 0.8 | −3.0 | 160 | 155.0 | 2.8 | −3.1[a] |
| M-I | 200 | 197.4 | 0.9 | −1.3 | 160 | 161.8 | 0.3 | 1.1[a] |
| M-II | 200 | 197.9 | 2.8 | −1.1 | 160 | 160.0 | 1.6 | 0.0[a] |
| M-III | 200 | 199.3 | 5.6 | −0.3 | 160 | 163.8 | 2.6 | 2.4[a] |
| *Urine after enzymatic hydrolysis* | | | | | | | | |
| Seratrodast | 1000 | 950 | 2.1 | −5.0 | 320 | 318.4 | 4.5 | −0.5 |
| M-I | 1000 | 990 | 1.1 | −1.0 | 320 | 324.5 | 4.8 | 1.4 |
| M-II | 1000 | 1019 | 1.8 | 1.9 | 320 | 335.0 | 2.2 | 4.7 |
| M-III | 1000 | 1025 | 1.3 | 2.5 | 320 | 322.2 | 2.8 | 0.7 |

[a] $n=2$.

Table 4
Stability of seratrodast and its metabolites in human serum and urine stored at −20°C for 1 and 2 months

| Compound | Added concentration (ng/ml) | Residual content (%) | | | |
|---|---|---|---|---|---|
| | | Serum | | Urine | |
| | | 1 month | 2 months | 1 month | 2 months |
| Seratrodast | 500 | 100.4 | 100.5 | 96.5 | 94.6 |
| M-I | 120 | 107.0 | 101.4 | 97.2 | 95.0 |
| M-II | 120 | 106.9 | 97.4 | 96.4 | 91.4 |
| M-III | 120 | 99.0 | 85.7 | 95.8 | 95.4 |

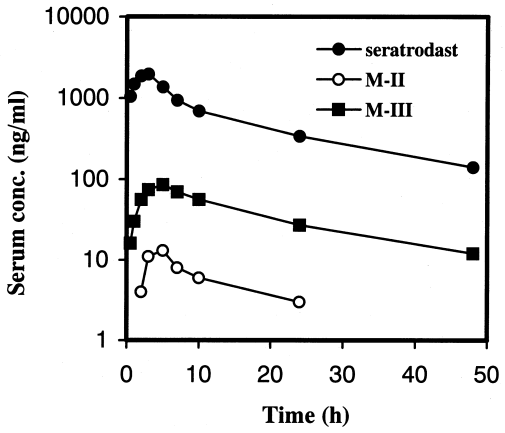


Fig. 5. Mean serum levels of seratrodast and its metabolites in three volunteers after oral administration of 20 mg of seratrodast.

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