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# Achiral and chiral determination of ciprofibrate and its glucuronide in human urine by capillary electrophoresis

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**Abstract**

A method for achiral separation of the racemic hypolipidaemic agent ciprofibrate and its main metabolite ciprofibrate glucuronide by capillary electrophoresis (CE) was developed. The glucuronide was isolated from urine by chromatographic procedures and characterized by alkaline as well as enzymatic hydrolysis and mass spectrometric and nuclear magnetic resonance experiments. Chiral discrimination of the ciprofibrate enantiomers and their diastereomeric glucuronides by CE was achieved by the use of γ-cyclodextrin as buffer additive. The fractionated crystallization of ciprofibrate yielded the *R*-(+)-enantiomer as less soluble diastereomeric salt with (+)-1-phenylethylamine. This allowed the identification of the enantiomers of ciprofibrate as well as the diastereomeric glucuronides of ciprofibrate by CE. In a study with three volunteers inter- and intra-individual differences of ratios of both ciprofibrate glucuronides in urine were observed. After oral administration of a single dose of the racemate the *S*-ciprofibrate glucuronide was mainly excreted in the first time intervals, in the last time intervals the *R*-glucuronide dominated. 

*Keywords:* Enantiomer separation; Ciprofibrate

## 1. Introduction

The hypolipidaemic agent ciprofibrate is used to reduce the risk of coronary heart disease. Cholesterol is lowered by 25% and triglycerides by 30 to 40%. After oral application ciprofibrate is mainly conjugated with glucuronic acid. The glucuronide is excreted in urine and represents more than 90% of the administered dose of the drug [1] (Fig. 1).

Due to the cyclopropyl substituent, ciprofibrate ($pK_a$=3.65) is chiral. The drug is marketed as racemate. Ciprofibrate enantiomers are known to have different therapeutic effects. After a single oral dose to male Fisher F344 rats, *S*-(−)-ciprofibrate produced slightly but statistically significantly greater reductions in plasma concentrations of cholesterol than *R*-(+)-ciprofibrate. After multiple dosing plasma concentrations of *R*-(+)-ciprofibrate were higher than those of its enantiomer [2]. In humans substantially higher concentrations of *R*-(+)-ciprofibrate were found in plasma after multiple dosing. Small amounts of ciprofibrate were detectable in urine due to enzymatic hydrolysis by endogenous β-glucuronidase present in urine [3]. In Ref. [4] a high-performance liquid chromatographic (HPLC) method was used for the determination of diastereo-

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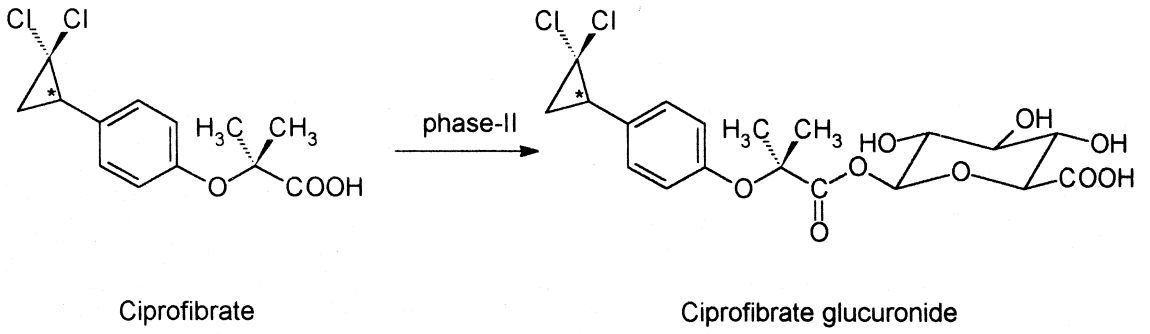


Fig. 1. Ciprofibrate and its main metabolite.

meric ciprofibrate glucuronides in human urine. However, time consuming derivatization steps of the polar glucuronides were necessary.

Recently, CE has been well established as a powerful tool for the analysis of drugs and their metabolites in body fluids [5–8]. CE is especially useful for the determination of polar compounds such as glucuronides, e.g. paracetamol glucuronides in [9]. The addition of cyclodextrins (CDs) to the running buffer enables chiral separations [10].

The aim of our investigations was to develop methods for achiral and chiral determination of ciprofibrate and its glucuronide in urine by CE. γ-CD was used as chiral selector. Using this method diastereomeric glucuronides in urine after the application of ciprofibrate were analyzed. In order to identify both ciprofibrate glucuronides in electropherograms they were isolated from urine by HPLC and characterized by MS, $^1$H-NMR and after hydrolysis. By fractional crystallization of the diastereomeric (+)-1-phenylethylamine salts of ciprofibrate, samples enriched in *R*-(+)- and *S*-(−)-ciprofibrate were obtained. Thus, the migration order of ciprofibrate enantiomers and ciprofibrate glucuronide diastereomers in electropherograms could be determined.

## 2. Experimental

### 2.1. Chemicals and reagents

Ciprofibrate was purchased from Sigma-Aldrich (Deisenhofen, Germany). The glucuronide of ciprofibrate was isolated from a urine sample as described in Section 2.3. Celite 545, Amberlite XAD-2, (+)-1-phenylethylamine and petrolether were supplied by Fluka (Buchs, Switzerland). Cyclodextrins were supplied by Wacker (Munich, Germany). Methanol and orthophosphoric acid were obtained from Baker (Deventer, The Netherlands). β-Glucuronidase was purchased from Sigma (Deisenhofen, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

### 2.2. Electrophoretic equipment and operating conditions

Achiral and chiral separations were performed on a P/ACE 2100 instrument (Beckman Instruments, Munich, Germany) with GOLDSOFTWARE 7.11. The electric field strength was 500 V/cm and the temperature of the capillary cooling system was 20°C. The untreated fused-silica capillary (Grom, Herrenberg, Germany) had the dimensions of 47 cm (40 cm to detector)×50 μm I.D. Detection was carried out at a wavelength of 230 nm near the cathodic end of the capillary.

Achiral separations were carried out with 50 m*M* sodium tetraborate buffer (borax), pH 9.4 according to Ref. [9]. Between each run the capillary was rinsed with 0.1 *M* sodium hydroxide for 2 min followed with the run buffer for 2 min. Urine samples were introduced at the anodic side into the capillary by hydrodynamic injection with 0.5 p.s.i. (1 p.s.i.=6894.76 Pa) for 3 s followed by the injection of water for 1 s.

Chiral separation was performed in a 100 m*M* $NaH_2PO_4$ buffer containing 7.5 m*M* γ-CD. The pH had been adjusted to 6.0 with 100 m*M* phosphoric acid. After each run the capillary was rinsed with 100 m*M* phosphoric acid and run buffer for 2 min

each. The samples were introduced at the anodic side by hydrodynamic injection at 0.5 p.s.i. for 4 s.

### 2.3. Isolation of ciprofibrate glucuronide from urine

Urine of a volunteer was collected for 24 h after oral administration of 200 mg of ciprofibrate. A 850-ml volume of the urine sample was adjusted to a pH of 2.5 with orthophosphoric acid. The urine sample was cleaned-up by adding 40 g of Celite 545 and subsequent filtration. A slight turbidity was removed by the use of a cellulose acetate membrane filter (Schleicher & Schuell, Dassel, Germany) with a pore diameter of 0.45 μm. The filtered urine sample was directly used for solid-phase extraction (SPE) on a preparative column (45×3.5 cm, packed with 250 g of Amberlite XAD-2) according to Ref. [11]. Elution was performed with methanol–1% aqueous acetic acid mixtures followed by ethyl acetate as final step.

The highest concentration of glucuronide was found by CE in the ethyl acetate fraction. This fraction was evaporated under reduced pressure below 40°C. The residue was dissolved in methanol and fractionated by HPLC.

The HPLC system consisted of a Beckman Model 110 A pump equipped with a Rheodyne 7010 injector, a 200-μl loop, a Merck-Hitachi 665 A variable-wavelength UV monitor set at 232 nm and a Merck-Hitachi D 2500 Chromato Integrator. Separation was performed on a Merck LiChrospher 60 RP-8 select B column 250×4 mm, 5 μm with a guard column 25×4 mm packed with the same adsorbent. The mobile phase consisted of acetonitrile–double distilled water–glacial acetic acid (35:64.5:0.5, v/v/v). The flow-rate was 1.0 ml/min. In the collected fractions the acetonitrile was evaporated at 40°C. Finally the residual solution was lyophilized.

### 2.4. Identification of the isolated ciprofibrate glucuronide

#### 2.4.1. Mass spectrometry

An ion trap mass spectrometer (Finnigan, Branford, CT, USA) equipped with an electrospray interface (Finnigan) was used in the negative ion mode with a spray voltage of −2 kV. Prior to analysis the glucuronide was dissolved in methanol and injected into the system.

Fig. 2 shows the mass spectrum of ciprofibrate glucuronide ($C_{19}H_{22}Cl_2O_9$, $M_r$ 465.3). The analyte was deprotonated and consequently detected as M-1 ion. Three different signals with a mass difference of two units are due to the isotope ratios of the two chlorine atoms in the molecule. The $m/z$ values were 463.5 (100%), 465.3 (80%) and 467.3 (13%). Signals at a higher $m/z$ ratio like 2M-1 represent dimers of the glucuronides formed during electrospray ionization.

#### 2.4.2. Nuclear magnetic resonance

$^1$H-NMR spectra were recorded on a Varian unity plus 600 MHz spectrometer (Varian, Darmstadt, Germany) on solution in $d_4$-methanol. The internal standard was tetramethylsilane. The $^1$H-NMR spectra (δ, ppm) of ciprofibrate glucuronide are assigned as shown in Fig. 3; 1.58 (ss, 6H, $CH_3$), 1.91 (m, 2H, $CH_2$), 2.89 (dd, 1H, CH), 3.84 (m, 1H, H5′), 5.56 (d, 1H, H1′), 6.93 (m, 2H, $H_a$ aromatic) and 7.15 (m, 2H, $H_b$ aromatic).

#### 2.4.3. Alkaline hydrolysis

To an aqueous solution of the glucuronide 0.1 *M* NaOH was added. After a reaction time of 1 h at 37°C, 0.1 *M* HCl was added to neutralize the solution which was used directly for CE analysis.

#### 2.4.4. Enzymatic hydrolysis

The glucuronide was dissolved in sodium acetate buffer, pH 4.6, and β-glucuronidase type H-1 from *Helix pomatia* was added. After incubation for 1 h at 37°C the reaction was stopped by adding acetonitrile. The precipitation was completed by storage for 3 h at −20°C. The proteins were precipitated by centrifugation (10 min at 1500 *g*) and the solution was evaporated by a gentle stream of nitrogen at room temperature. The residue was reconstituted in water and analyzed by CE.

### 2.5. Sample preparation for determination of R- and S-ciprofibrate glucuronides in urine

A single dose of 200 mg ciprofibrate was given to each of the three volunteers. Urine was collected for eight 12-h intervals and adjusted to pH 2.5 with

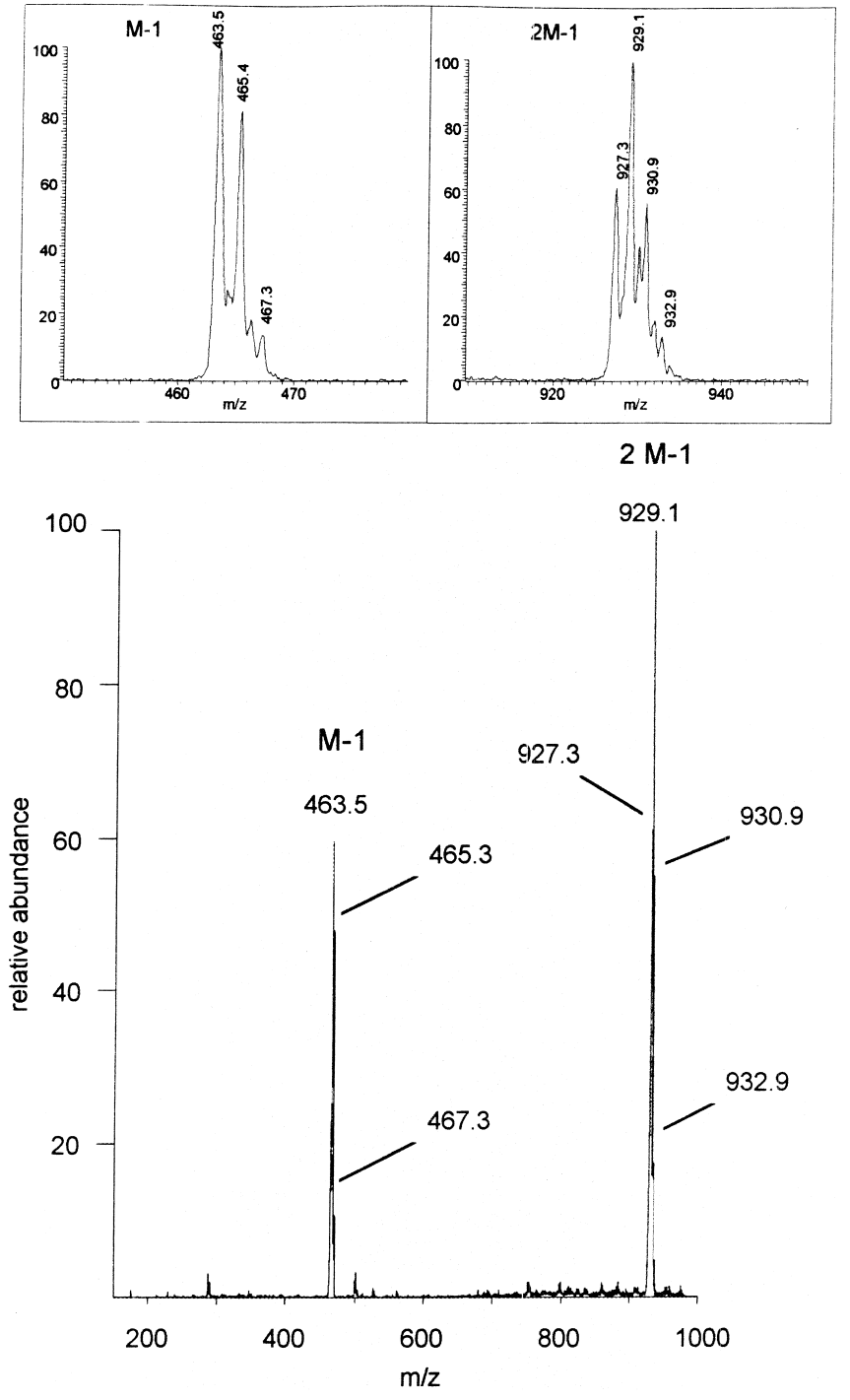


Fig. 2. Mass spectrum of the isolated ciprofibrate glucuronide M-1 and its dimer 2M-1. The different signals with a mass difference of two units are due to the isotopic chlorine atoms in the molecule.

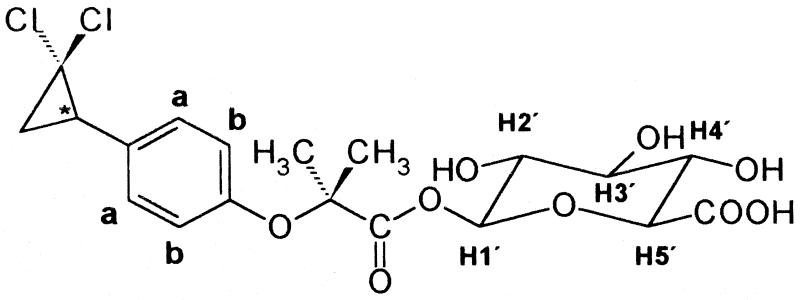


Fig. 3. Ciprofibrate glucuronide: assignment used in NMR analysis.

orthophosphoric acid. The samples were stored at −20°C. Prior to extraction the samples were diluted with the same amount of water and the pH was adjusted to 2.0 with orthophosphoric acid. SPE was carried out on Varian-CN columns with 500 mg adsorbent (Varian CN-N 1210-2034, Darmstadt, Germany) using the SPE apparatus of Baker. The columns were conditioned with 2 ml methanol and 2 ml water. Urine (6 ml) prepared by the procedure given above was extracted. The columns were washed with 2 ml of water and finally eluted with 2 ml of methanol. The elute solvent was evaporated under a stream of nitrogen, the residue was dissolved in 200 μl of water and analyzed by CE.

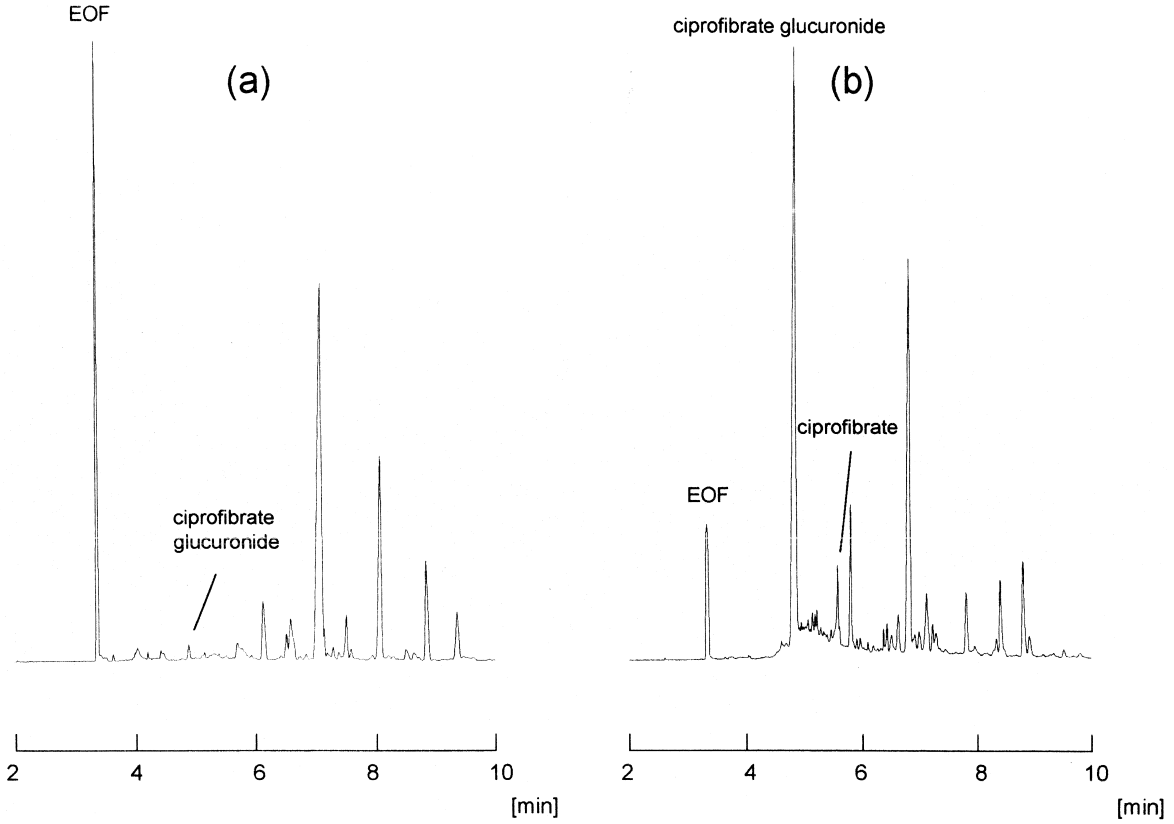


Fig. 4. Electropherogram of urine after oral administration of ciprofibrate before (a) and after (b) SPE.

### 2.6. Fractional crystallization

Racemic ciprofibrate (60 mg) and (+)-1-phenylethylamine (24 mg) were dissolved in 1.5 ml ethyl acetate. After the addition of a small amount of petrolether the solution was kept for 24 h at −20°C. The precipitate suspended in water was acidified with $H_2SO_4$ to liberate the free acid from the diastereomeric salt, which was extracted with diethylether. The organic phase was dried with $Na_2SO_4$, filtered and evaporated to dryness. The optical rotation of the residue was $[\alpha]_D^{20} = +19.3°$ ($c = 0.3$ g/100 ml ethanol).

## 3. Results and discussion

### 3.1. Achiral determination of ciprofibrate and its glucuronide in urine by CE

The electrophoretic separation of ciprofibrate and its glucuronide was performed in a capillary filled with sodium tetraborate buffer, pH 9.4. Peak tailing could be eliminated by injection of a water zone directly after the sample [9,12].

After a single dose of ciprofibrate (200 mg), the urine of a volunteer was collected for 24 h. The urine sample was analyzed by direct injection into the capillary. In Fig. 4a the separation of ciprofibrate glucuronide is presented. Ciprofibrate was not detected under these conditions. In order to minimize interferences with endogenous compounds and to preconcentrate ciprofibrate as well as its glucuronide the SPE procedure as described in Section 2.5. was used. Fig. 4b shows the CE electropherogram after SPE. The concentration of ciprofibrate glucuronide in the sample is much higher using this enrichment procedure. Ciprofibrate also could be detected (Fig. 4a).

### 3.2. Chiral determination of ciprofibrate enantiomers and their diastereomeric glucuronides by CE

Separation of the ciprofibrate enantiomers and diastereomeric glucuronides was achieved by using γ-CD as chiral selector. Diastereomers as ciprofibrate glucuronides could be separated in principle without a chiral selector. However, CE is often not suitable to separate diastereomers because the chiral centers of the ciprofibrate and the glucuronic acid moiety of the molecule are too far apart to interact. Therefore, chiral selectors were used. A 100 m*M* $Na_2HPO_4$ buffer, pH 6.0, containing γ-CD at a concentration of 7.5 m*M* was used to analyze the enantiomeric composition of ciprofibrate and the diastereomeric ciprofibrate glucuronides simultaneously (Fig. 5). The migration order of ciprofibrate enantiomers was determined with the enriched samples described before. The dextrorotary compound corresponds to *R*-ciprofibrate [2,3]. In Fig. 6, CE separations of ciprofibrate racemate (a) and the dextrorotary sample after fractional crystallization (b) are shown. *S*-(−)-Ciprofibrate is detected before *R*-(+)-ciprofibrate. In Fig. 7 the peaks of the isolated ciprofibrate glucuronides in urine now can be assigned to *R*- and *S*-ciprofibrate glucuronide by their hydrolysis products. The electropherogram shows more *S*-(−)-ciprofibrate after hydrolysis of the diastereomeric mixture of the glucuronides in the urine sample originating from an

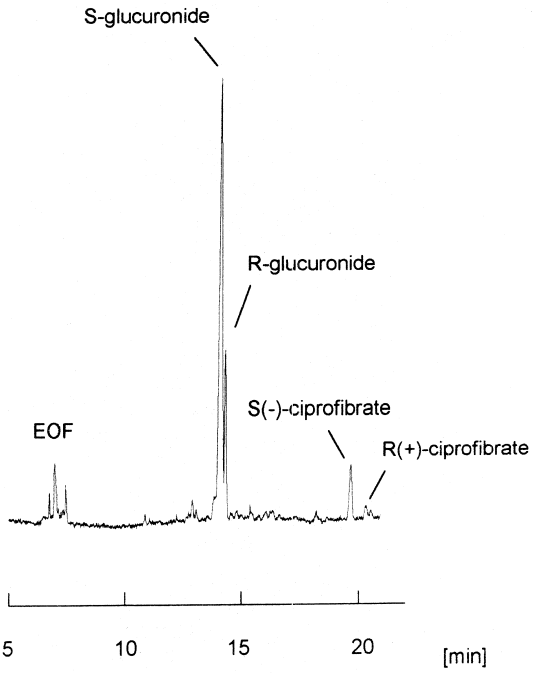


Fig. 5. Electropherogram of a urine sample 12 h after receiving 200 mg of ciprofibrate; γ-CD was used as chiral selector.

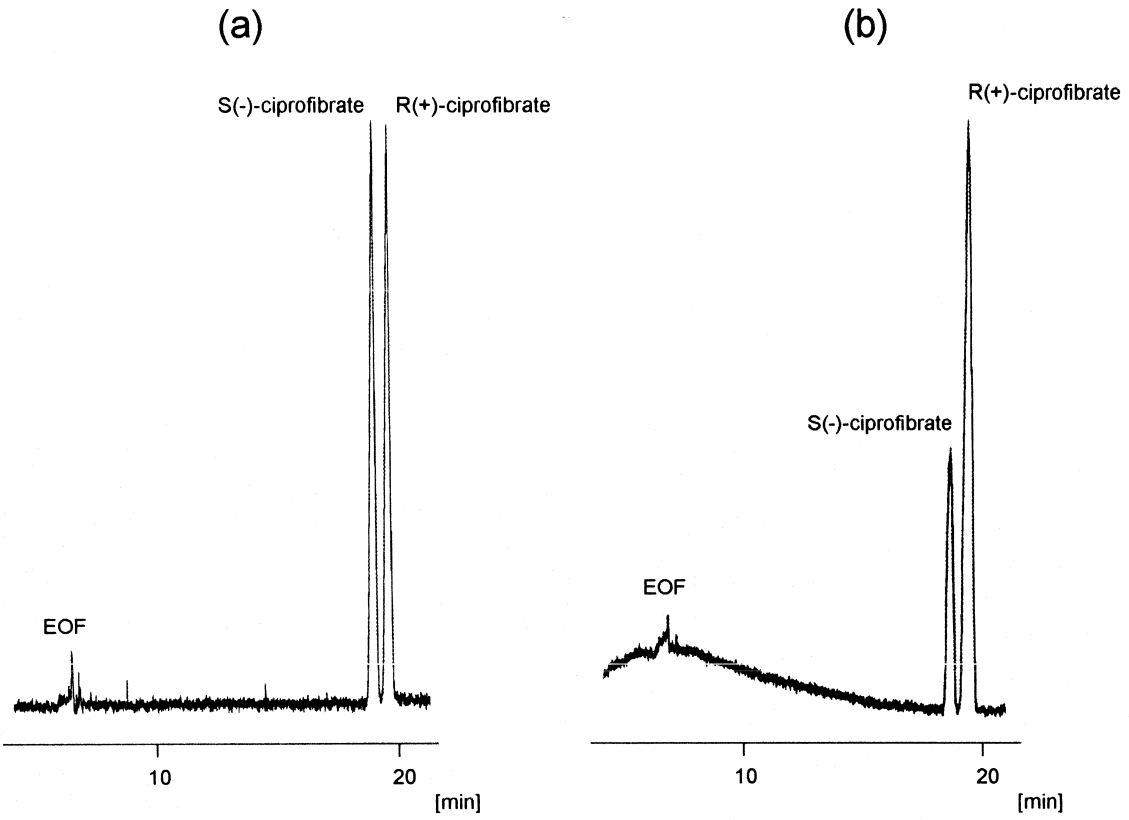


Fig. 6. Electropherograms of ciprofibrate enantiomers: before (a) and after (b) fractionated crystallization.

excess of the *S*-glucuronide. The different ratios of the diastereomeric ciprofibrate glucuronides are due to stereoselective metabolism and/or excretion processes.

### *3.3. Determination of the time dependent excretion of R- and S-ciprofibrate glucuronides in urine*

The CE method for chiral determination was used to determine the time dependent excretion of the diastereomeric ciprofibrate glucuronides in urine. After application of a single dose of 200 mg ciprofibrate to three volunteers, urine was collected at 12-h intervals for 96 h. The urine samples were extracted by SPE and analyzed as described above. A quantitative determination was not performed. Fig. 5 shows an electropherogram of a extracted urine 12 h after oral application of ciprofibrate. *R*- and *S*-ciprofibrate glucuronides and small amounts of ciprofibrate were detected. The ratios of time normalized peak areas of *R*- and *S*-ciprofibrate glucuronides were similar to the corresponding ratios of the small amounts of *R*- and *S*-ciprofibrate present in the urine sample which apparently originated from partial hydrolysis of the glucuronides.

In Table 1 the ratio of diastereomeric ciprofibrate glucuronides is shown for different time intervals. Varying ratios of the *R*- and *S*-glucuronides can be observed. Fig. 8 reveals that in the first 36 h each volunteer excreted more *S*-ciprofibrate glucuronide, whereas in intervals after 72 h mainly *R*-ciprofibrate glucuronide was excreted. Both inter- and intra-individual time dependent differences in the ratios of ciprofibrate glucuronides in urine of the three volunteers were observed.

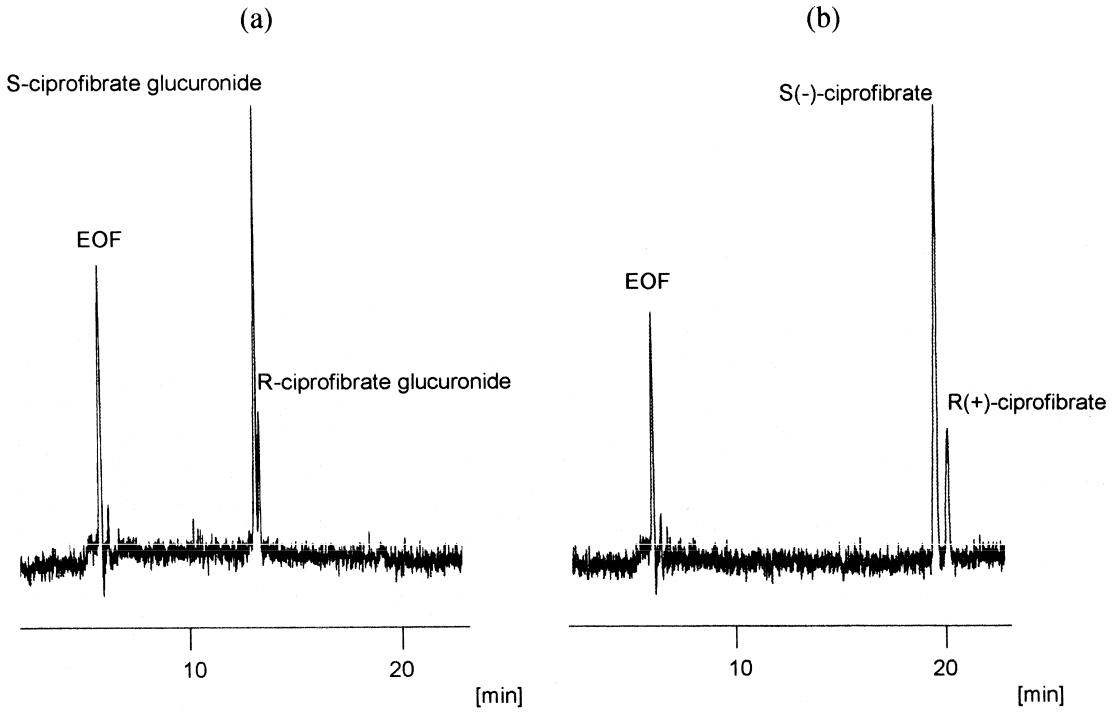


Fig. 7. Ciprofibrate glucuronide before (a) and after (b) alkaline hydrolysis. γ-CD as chiral buffer additive allows separation of diastereomeric ciprofibrate glucuronides as well as ciprofibrate enantiomers.

## 4. Conclusion

Diastereomeric ciprofibrate glucuronides were isolated from urine by chromatography and characterized by MS and $^{1}$H-NMR. Ciprofibrate and its glucuronides were determined in urine after SPE by an achiral as well as a chiral assay. The chiral method was used for the determination of diastereomeric ratios of ciprofibrate glucuronides in urine which varied with time.

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Table 1
Time dependent ratios of *R*- and *S*-ciprofibrate glucuronide in urine of three volunteers after oral application of 200 mg ciprofibrate

| Volunteer | Ratio | Urine sample (h) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0–12 | 12–24 | 24–36 | 36–48 | 48–60 | 60–72 | 72–84 | 84–96 |
| 1 | *R*:*S* glucuronides (%) | 17.29:82.71 | 20.24:79.76 | 24.80:75.20 | 29.55:70.45 | 38.29:61.71 | 44.46:55.54 | 56.52:43.48 | 62.65:37.35 |
| | SD | 1.41 | 2.16 | 0.86 | 0.99 | 0.82 | 2.25 | 1.36 | 1.56 |
| 2 | *R*:*S* glucuronides (%) | 13.33:86.87 | 18.60:81.40 | 24.22:75.78 | 29.77:70.23 | 37.88:62.12 | 48.42:51.58 | 51.55:48.45 | 63.92:36.08 |
| | SD | 0.90 | 0.81 | 0.56 | 2.20 | 0.85 | 0.47 | 1.27 | 3.14 |
| 3 | *R*:*S* glucuronides (%) | 18.76:81.24 | 28.65:71.35 | 44.32:55.68 | 57.14:42.86 | 71.53:28.47 | 83.95:16.05 | 86.95:13.05 | 93.32:6.68 |
| | SD | 1.00 | 0.72 | 1.28 | 0.72 | 1.73 | 1.15 | 0.67 | 0.29 |

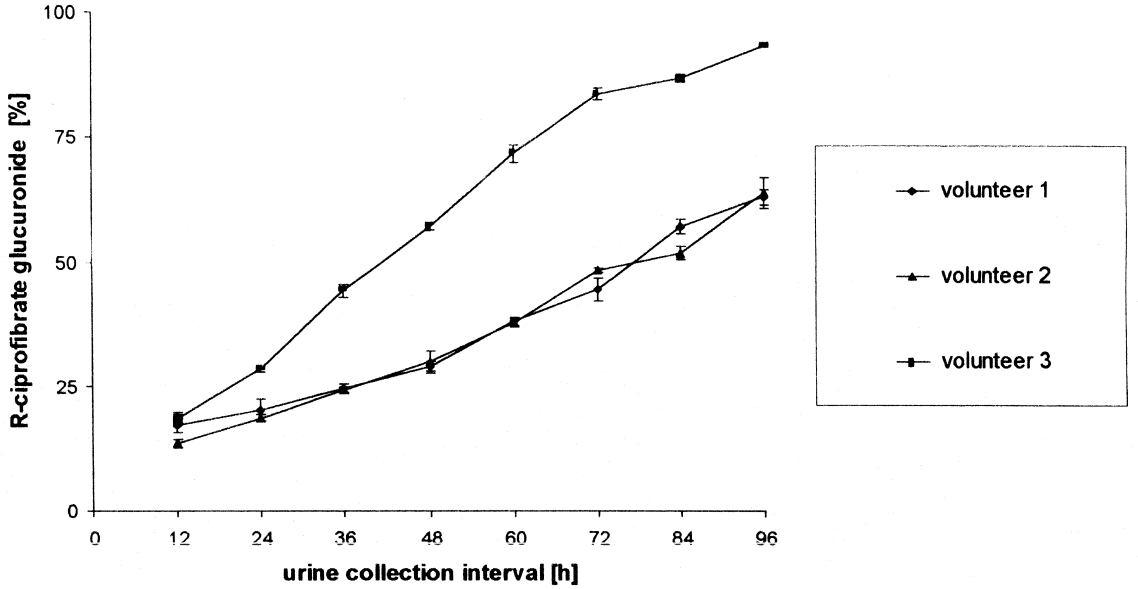


Fig. 8. Time dependent change of ratios of diastereomeric ciprofibrate glucuronides in urine of three volunteers (fraction of *R*-ciprofibrate glucuronide in the diastereomeric mixture).

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