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Determination of troglitazone stereoisomers in rat plasma using semi-micro HPLC with electrochemical detection

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Abstract

A highly sensitive determination method for troglitazone stereoisomers was developed by high-performance liquid chromatography with electrochemical detection (HPLC–ECD). The oxidation behavior of troglitazone was investigated for the application of ECD by measuring the cyclic voltammogram. The separation was performed on a semi-micro chiral column (Chiralcel OJ-RH) using a mobile phase consisting of methanol–acetic acid (1000:1, v/v) containing 50 mM $LiClO_4$ at a flow rate of 20 μl/min. The peak areas of the stereoisomers separated from 0.1 to 50 ng/ml of troglitazone had good linearity with correlation coefficients of >0.999, and had similar response. The limit of detection was 1.3 fmol (signal-to-noise ratio of 3). This method was applied to the determination of troglitazone stereoisomers in rat plasma. The levels of troglitazone stereoisomers in rat plasma could be monitored until 24 h after the oral administration.

Keywords: Chiral separation; Electrochemical detection; Rat plasma; Semi-micro-HPLC; Troglitazone

1. Introduction

Troglitazone [1,2] has two asymmetric carbons, one at the 2-position of the chroman ring and the other at the 5-position of the thiazolidine ring in its molecule. It is produced as a mixture of equal amounts of four stereoisomers, whose structures are shown in Fig. 1. Some HPLC methods have been reported and validated for the analysis of troglitazone in pharmaceutical and biological samples [3–6], however, it would be impossible to detect each stereoisomer of troglitazone using these methods. In general, separation of stereoisomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite different pharmacokinetic properties and different pharmacological or toxicological effects [7–9]. Furthermore, stereoselectivity was predicted for the pharmacokinetics and metabolism of troglitazone in humans [10–12]. That is one of the most important reasons why the regulatory authorities demand more stringent investigations for evaluating the safety and effectiveness of drugs containing asymmetric centers.

Determination for troglitazone stereoisomers had been developed by a reversed-phase chiral HPLC-UV method [13]. However, a lot of blood is needed for the therapeutic drug monitoring considering the detection sensitivity. And complex sample pretreatment would be required to prevent interference from other substances for the determination by UV detection, especially in biological fluids. Troglitazone has a chroman ring in its molecule like vitamin E whose redox behaviors had been studied [14]. So it was considered that sensitive and selective determination could be developed by HPLC with electrochemical detection.

This paper describes the development and validation of a highly sensitive and specific method for the determination of troglitazone stereoisomers by semi-micro HPLC with electrochemical detection. Furthermore, we applied this method to the evaluation of the stereoisomers of troglitazone in

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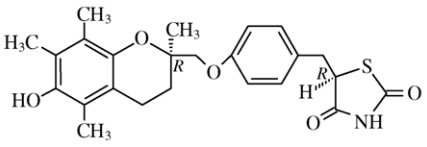
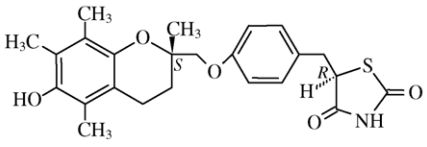
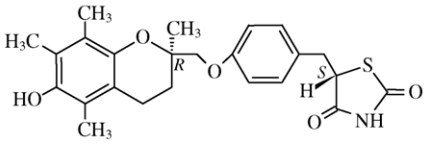
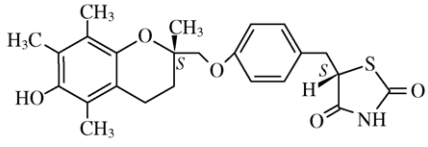
Stereoisomer	Chemical structure
2*R*-5*R*	
2*S*-5*R*	
2*R*-5*S*	
2*S*-5*S*	

Fig. 1. Compound names and chemical structures of troglitazone stereoisomers.

biological fluids, and to the monitoring of the levels of troglitazone stereoisomers in rat plasma after oral administration.

2. Materials and methods

2.1. Sample and reagents

Troglitazone bulk drug substance (Lot No. NR312) used in this study was synthesized by the Process Development Laboratories, Sankyo Co. Ltd. All other reagents and solvents were commercially available and of analytical reagent grade. Water for preparing buffers and mobile phases was used after purification with a NANOpure II system (Barnstead, USA).

2.2. Apparatus

Cyclic voltammetry was performed in a 50-ml glass beaker-type cell with a conventional potentiostat (HAB-151, Hokuto Denko, Tokyo, Japan) and an X–Y recorder (Model 3086, Yokogawa Electrics, Tokyo, Japan). The working, counter and reference electrodes were plastic formed carbon (3.0 mm i.d.), platinum wire and Ag/AgCl electrodes, respectively. The plastic formed carbon electrode (Tsukuba Materials Information Laboratory, Ibaraki, Japan) was polished using 0.3 μm lapping film (Maruto Instrument, Tokyo, Japan) and sonicated in water. Pure argon gas was passed through the test solution for 15 min to remove oxygen and kept over the solutions during measurements.

The HPLC–ECD with a conventional column consisted of a Jasco PU-880 pump (Jasco, Tokyo, Japan), a 7125 injector fitted with a 5-μl injection loop (Rheodyne, CA, USA), a Chiralcel OJ-RH column (4.6 mm i.d. × 150 mm, particle size: 5 μm, Daicel Chemical Industries, Tokyo, Japan), a column oven (CTO-10ASVP, Shimadzu, Kyoto, Japan) and an EDP-1 electrochemical detector (Kotaki, Tokyo, Japan).

The HPLC–ECD with a semi-micro-column consisted of an LC-26A vacuum degasser (BAS, Tokyo, Japan), a BAS LC-100 pump (BAS, Tokyo, Japan), a 7125 injector fitted with a 5-μl injection loop (Rheodyne, CA, USA), a Chiralcel OJ-RH column (1.0 mm i.d. × 150 mm, particle size: 5 μm, Daicel Chemical Industries, Tokyo, Japan), an FT-1 column oven (BAS, Tokyo, Japan) and an LC-4C electrochemical detector (BAS, Tokyo, Japan).

2.3. HPLC conditions

Separation of troglitazone stereoisomers was carried out using the following HPLC–ECD conditions. The conventional HPLC was performed using a mobile phase composed of methanol–acetic acid (1000:1, v/v) containing 50 mM $LiClO_4$ at a flow rate of 0.4 ml/min. The column temperature was controlled at 25 °C with an injection volume of 5 μl. And the applied potential was settled at +0.7 V versus Ag/AgCl.

The corresponding semi-micro HPLC was performed at a flow rate of 20 μl/min because the inside diameter was 20-fold smaller than the conventional column. The other conditions were the same as the conventional HPLC conditions described above.

For the measurement of the rat plasma sample, the HPLC conditions were refined because of interference from the matrix. The mobile phase was composed of methanol–water–acetic acid (930:70:1, v/v/v) containing 50 mM $LiClO_4$. The applied potential was changed at +0.38 V versus Ag/AgCl.

2.4. Rat plasma sample preparation

Rat plasma (100 μl) was vortex-mixed with 200 μl of ethyl acetate for 2 min and then centrifuged at 5000 × *g* for 5 min and the supernatant was collected. This procedure was repeated three times and all of the supernatant was mixed and dried with nitrogen gas. The residue was reconstituted with 100 μl of mobile phase and filtered through a 0.45 μm of membrane filter. A volume of 5 μl of the clear filtrate was then injected into the HPLC–ECD system.

2.5. Animal experiments

Male Wistar-Imamichi rats (255–265 g, 8 weeks of age) were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were allowed free access to food and drink for more

than 1 week. And before the experiments, each animal was fasted overnight.

Five mg/kg of troglitazone in 0.5% carboxymethyl cellulose suspension was orally administered. About 300 μl of blood samples were taken at 0, 1, 2, 3, 4, 5, 6, 8, and 24 h post-dosing, and plasma was immediately separated from heparinized blood by centrifugation (1300 × *g*, 10 min).

3. Results and discussion

3.1. Oxidation property of troglitazone

The oxidation behavior of troglitazone was examined at various pH values between 2 and 9 by cyclic voltammetry. In this study, the measurements were carried out in a methanol–Britton Robinson buffer or Tris–buffer (4:1, v/v) containing 50 mM $LiClO_4$ as a supporting electrolyte. The mixed solvent was chosen because it was also used as a mobile phase in the HPLC experiments afterwards. Fig. 2 shows the cyclic voltammograms of 1 mM troglitazone at various pH conditions, in which potential scanning was started from 0.0 V to the positive direction and reversed repeatedly at +1.0 and −1.0 V. During the potential scan, an oxidation peak appeared at around +0.4 V, but no significant reduction peak was observed. Fig. 3 shows the pH dependencies of the oxidation peak potential of troglitazone and α-tocopherol. The relation between pH and the potential of troglitazone at low pH gave a slope of −0.028 V/pH and the relation of α-tocopherol gave −0.035 V/pH, which were close to half of −0.059 V/pH. The number of protons involved in the electrode reaction of both compounds could be deduced to be one by using two electrons in the oxidation process. Troglitazone has the same group in its molecule as the chroman ring of α-tocopherol,

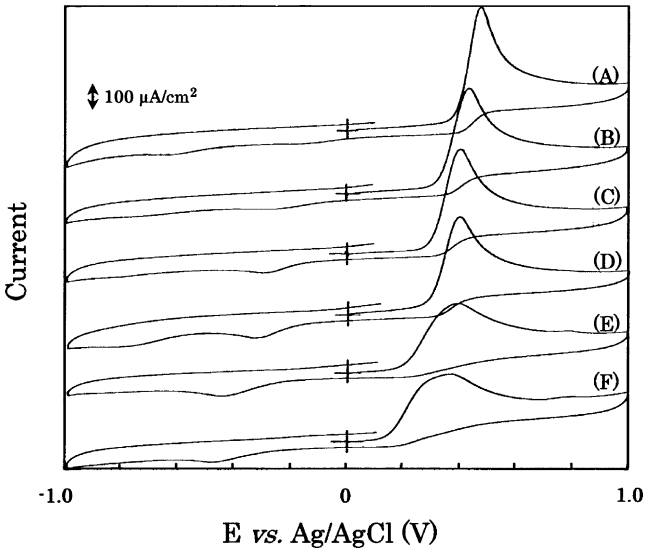


Fig. 2. Cyclic voltammograms of troglitazone in various pH buffer/methanol containing 50 mM $LiClO_4$ (1:4): (A) pH 2.1; (B) pH 3.7; (C) pH 4.3; (D) pH 5.3; (E) pH 8.0; (F) pH 9.0. Scan rate: 0.05 V/s.

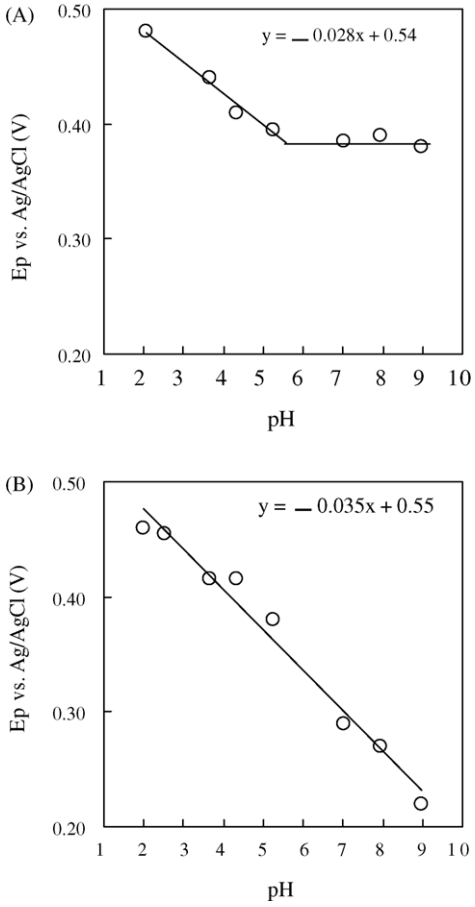


Fig. 3. pH dependencies on oxidation peak potential of (A) troglitazone and (B) α-tocopherol.

which plays a role of oxidation [14]. However, the value of the slope in Fig. 3(A) was changed to 0 V/pH at pH above 6, suggesting that the protons participating in the oxidation process were moved to a certain functional group, for example, the dissociation group in its molecule. Troglitazone is an acidic compound whose pK_a of the imino group in the thiazolidine ring is 6.1 by the titration method [15]. According to the oxidation reaction of α-tocopherol, the presumed reaction of troglitazone under high pH conditions is presented in Fig. 4.

3.2. Optimization of semi-micro HPLC–ECD conditions

Consideration of the redox property shows that the oxidation mode can be selected on the determination of troglitazone by electrochemical detection. The hydrodynamic voltammogram obtained with 110 ng of troglitazone from +0.2 to 0.9 V is shown in Fig. 5. As can be seen in the figure, each isomer underwent oxidation above +0.5 V and maximum current was attained at +0.8 V. However, the detection potential for analysis of troglitazone stereoisomers was usually maintained at +0.7 V, which produced a stable peak signal with low noise level.

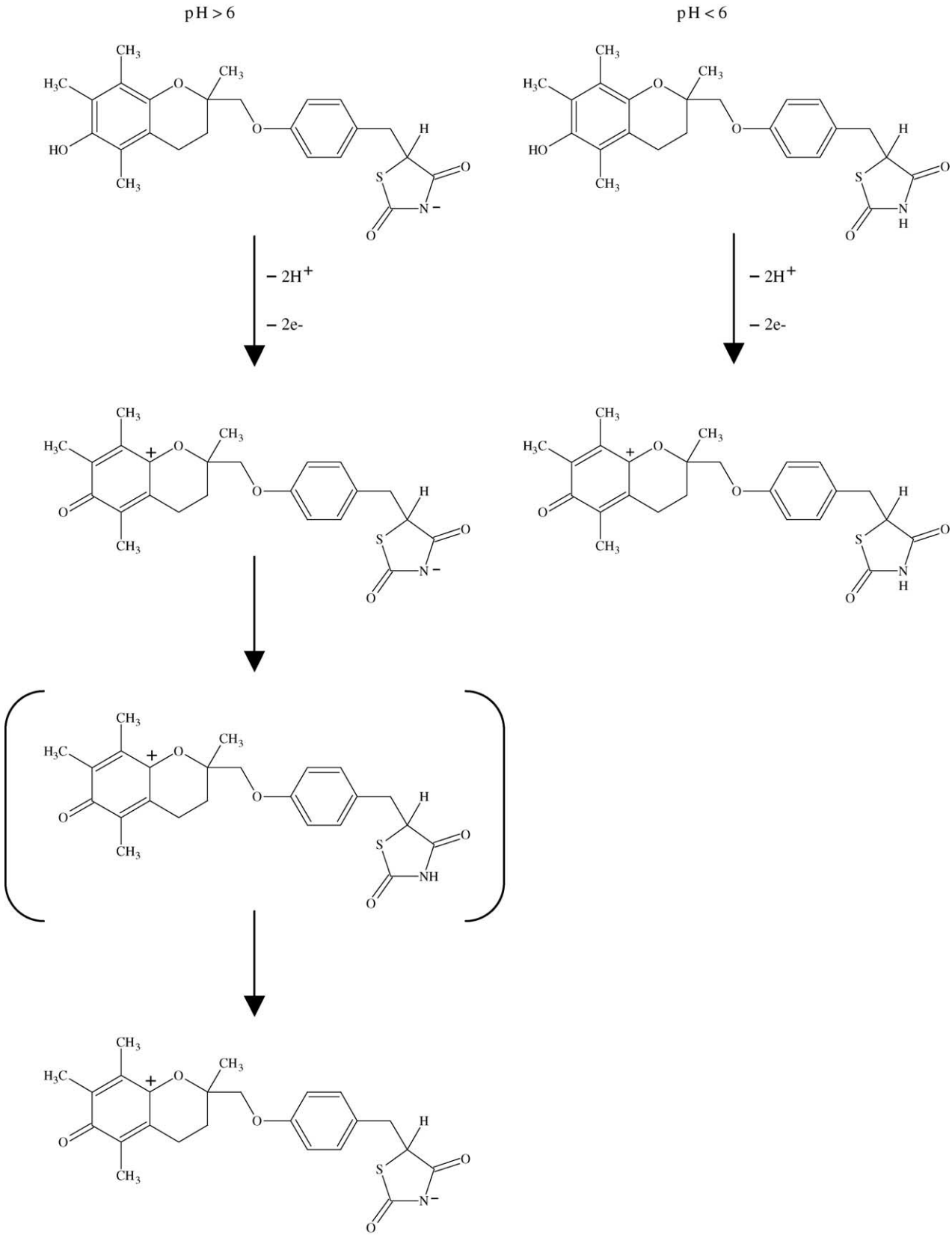


Fig. 4. Presumed oxidation reaction scheme of troglitazone under high pH conditions.

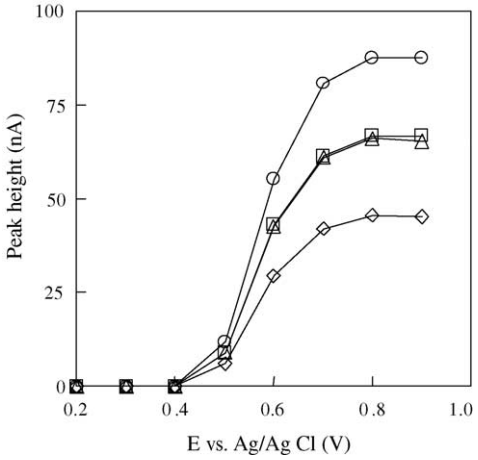


Fig. 5. Hydrodynamic voltammogram of oxidation peak potential on each stereoisomer of troglitazone, (◯) 2*R*–5*R*, (□) 2*S*–5*R*, (△) 2*R*–5*S*, (◊) 2*S*–5*S*.

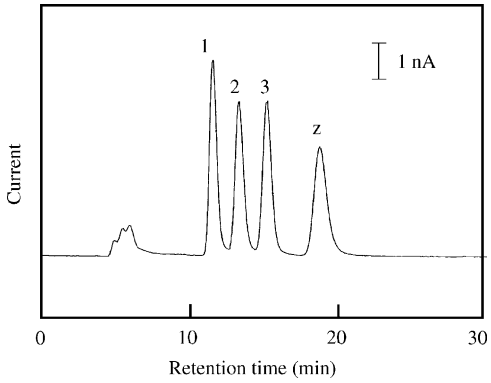


Fig. 6. Typical chromatograms of troglitazone stereoisomers by semi-micro-column HPLC–ECD: (1) 2*R*–5*R*; (2) 2*S*–5*R*; (3) 2*R*–5*S*; (4) 2*S*–5*S*.

Theoretically, separations that are carried out on a conventional size HPLC are superior to that with miniaturized HPLC systems. However, the use of semi-micro HPLC columns with a typical diameter of 1–2 mm is advantageous due to the reduction of sample size that enables the analyst to use fewer available biological samples, such as blood of small animals drawn frequently to get a time profile. Moreover, the use of lower flow rates of mobile phase in comparison with conventional HPLC results in a significant improvement in the detection sensitivity due to the lower diluting effect by the eluent. Recently, several semi-micro chiral columns have been commercially available and application to biological samples has been reported with the merit of absolute sensitivity [16].

Fig. 6 shows a typical chromatogram of troglitazone stereoisomers with semi-micro HPLC–ECD. The 2*R*–5*R*, 2*S*–5*R*, 2*R*–5*S* and 2*S*–5*S* stereoisomers of troglitazone were eluted in this order with resolutions (*R*s) of more than 2.0. So good separation of stereoisomers, which is similar to that with HPLC-UV [13] are obtained and there is no interference from $LiClO_4$ as a supporting electrolyte. The peak area of each stereoisomer on the chromatogram was plotted against amounts between 1.25 and 625 fmol in the sample solution. Good linearity ($r > 0.999$) for troglitazone stereoisomers were obtained with the equations of $y = 2.699x - 0.175$ (2*R*–5*R*), $y = 2.540x - 0.119$ (2*S*–5*R*), $y = 2.663x - 0.574$ (2*R*–5*S*), $y = 2.756x - 0.317$ (2*S*–5*S*). Each stereoisomer had linearity with almost the same value of slope, corresponding to each respective electrochemical response. A repeatability study was also carried out using 125 fmol of each stereoisomer (2*R*–5*R*, 2*S*–5*R*, 2*R*–5*S*, 2*S*–5*S*) in the sample solution. Relative standard deviation of the percentage of each stereoisomer was below 0.71% ($n = 6$). The detection limits of the troglitazone stereoisomers in this method were calculated at a signal-to-noise ratio of 3:1, and compared with the conventional HPLC-UV [13] and the conventional HPLC–ECD methods. As shown in Table 1, the sensitivity of the semi-micro HPLC–ECD is 3000 times higher than that of conventional HPLC-UV, and about 500 times higher than that of conventional HPLC–ECD. It is considered that the low flow rate enables much higher efficiency of electrochemical reaction on the surface of the electrode than a simple calculation (20-fold) of downsizing. In this way, the semi-micro HPLC–ECD conditions for the determination of troglitazone stereoisomers was optimized with good selectivity, sensitivity, linearity and precision.

3.3. Application to rat plasma and therapeutic dose monitoring

In order to demonstrate the applicability of the proposed method, the method was applied to the analysis of rat plasma. Rat plasma was provided for the confirmation of the separation by the standard addition method. For the liquid–liquid extraction on the sample preparation and mixing water into the mobile phase, good separation and no other interference peaks were observed from the presence of the matrix derived from rat plasma. Furthermore, the applied potential was changed at +0.38 V versus Ag/AgCl, so that the interference peaks derived from the matrix were disappeared. Although troglitazone was not detected at the potential by the conventional HPLC (Fig. 5), the semi-micro-HPLC successfully detected all troglitazone stereoisomers.

Table 1
Comparison of detection limits of troglitazone stereoisomer (2*S*–5*S*) among improved HPLC systems

System	Column size	Detection	Detection limit (on-column) (fmol)
1	4.6 mm i.d. × 150 mm	UV (285 nm)	3900
2	4.6 mm i.d. × 150 mm	EC (+0.7 V vs. Ag/AgCl)	600
3	1.0 mm i.d. × 150 mm	EC (+0.7 V vs. Ag/AgCl)	1.3

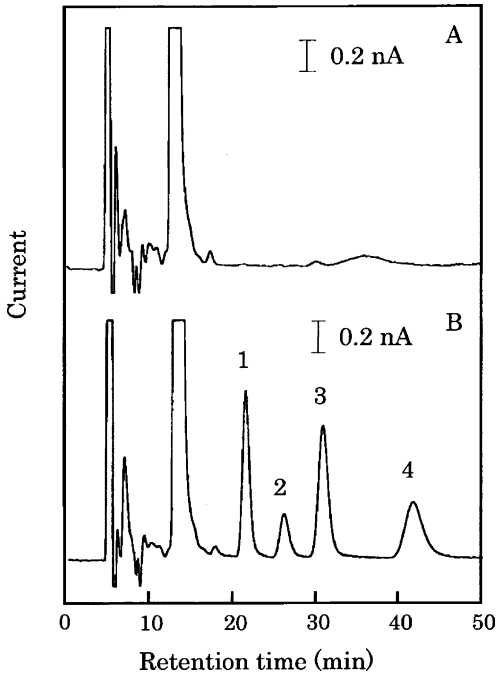


Fig. 7. Chromatograms of troglitazone stereoisomers in rat plasma (A) before and (B) 4 h after administration by semi-micro HPLC–ECD: (1) 2*R*–5*R*; (2) 2*S*–5*R*; (3) 2*R*–5*S*; (4) 2*S*–5*S*.

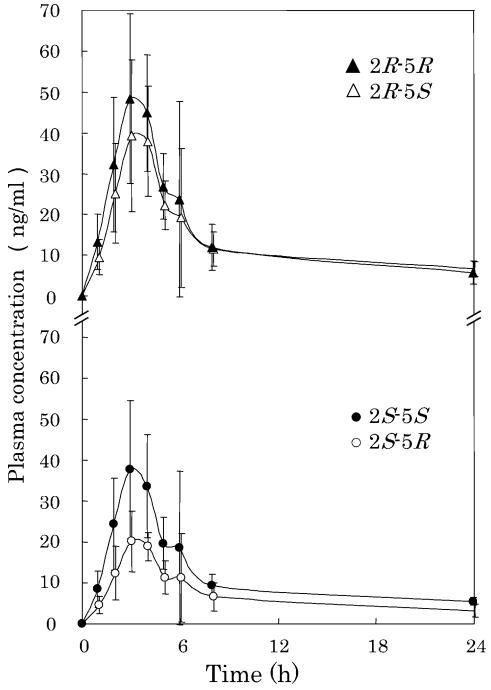


Fig. 8. Plasma concentration-time profiles for troglitazone stereoisomers after oral administration ($n = 3$).

Each stereoisomer had good linearity ($r > 0.999$) within the range of 15–625 fmol, which corresponds to 1.4–55 ng/ml in rat plasma. The equations were $y = 1.078x - 0.426$ (2*R*–5*R*), $y = 0.905x - 0.365$ (2*S*–5*R*), $y = 1.040x + 0.731$ (2*R*–5*S*), $y = 1.055x + 0.230$ (2*S*–5*S*). Three different amounts of troglitazone stereoisomers (about 0.55, 2.8, and 5.5 ng), which are equivalent to the concentrations of 5.5, 28, and 55 ng/ml in plasma, respectively, were spiked to 100 μl of rat plasma. Good recovery data (84.8–106.0%) of each stereoisomer was obtained. Thus, we confirmed that this method is reliable for measuring troglitazone stereoisomers in rat plasma.

This HPLC–ECD method was applied to pharmacokinetic studies. Chromatograms of troglitazone stereoisomers in rat plasma before and after oral administration is shown in Fig. 7, the plasma concentration-time profiles for the troglitazone stereoisomers are shown in Fig. 8 and the pharmacokinetic parameters of each stereoisomer are shown in Table 2. Each isomer reached the highest concentration at about 4 h after administration. The AUC and C_{max} of 2*R*–5*S*, 2*R*–5*R*, 2*S*–5*S* and 2*S*–5*R* troglitazone were found to be greater in this order, which may have been due to species specific stereoselective metabolism [12] and differences of protein binding [11]. The high sensitivity of this method made possible the reduction of the sampling volume of rat blood, previously requiring frequent drawing of blood, and accurate calculation of the kinetic parameters. In this way, this chiral HPLC–ECD method would be a useful method available for the pharmacokinetic study of troglitazone stereoisomers.

Table 2
Pharmacokinetic parameters of troglitazone stereoisomers after oral administration of 5 mg/kg troglitazone (racemate) to rats ($n = 3$)

Stereoisomer	C_{max} (ng/ml)	T_{max} (h)	AUC (ng × h/ml)	MRT (h)
2*R*–5*R*	45.6	4.0	319.8	8.5
2*S*–5*R*	24.1	4.0	169.1	8.3
2*R*–5*S*	57.0	4.0	353.8	7.6
2*S*–5*S*	44.5	4.0	280.1	8.2

4. Conclusions

The newly developed HPLC–ECD method for troglitazone stereoisomers has superior sensitivity and selectivity to the other HPLC conditions compared in this study. And this method is applicable and useful for evaluation of troglitazone stereoisomers in biological samples with good linearity and precision. As high sensitivity as 1.3 fmol on a column by HPLC–ECD could be achieved for monitoring the enantiomeric levels of troglitazone in rat plasma up to 24 h using only a small sample.

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