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High-performance frontal analysis for the study of protein binding of troglitazone (CS-045) in albumin solution and in human plasma

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Abstract

An on-line frontal analysis HPLC system was developed for the determination of the unbound concentration of troglitazone (CS-045), a new oral antidiabetic agent, in human serum albumin (HSA) solution and in human plasma. This system consists of a high-performance frontal analysis (HPFA) column, an extraction column, and an analytical column, which are connected via two switching valves. After the direct injection of the sample solution into the HPFA column, the drug was eluted as a zonal peak with a plateau region. The unbound drug concentration was determined as the drug concentration in the plateau. As low as 0.3 n*M* unbound CS-045 was determined with good reproducibility. It was found that CS-045 strongly binds with HSA, and the bound fraction in the 550 μM HSA solution was 99.93%, which was very close to that in human plasma (99.89%). The bound fractions were constant within the total drug concentration range of 1–10 μM in the HSA solution and 250 n*M*–10 μM in human plasma.

1. Introduction

Troglitazone (CS-045, Fig. 1) is a novel oral antidiabetic agent which enhances insulin action at the receptor and postreceptor levels in both peripheral and hepatic tissues, while it does not enhance insulin secretion [1]. CS-045 is now under development for the treatment of non-insulin-dependent diabetes mellitus. CS-045 is highly bound to plasma protein, and the unbound CS-045 concentration could not be determined precisely using any conventional method.

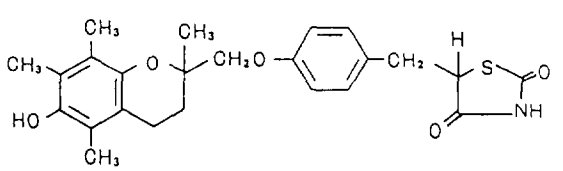

Fig. 1. Molecular structure of troglitazone (CS-045).

The quantitative investigation of drug–protein binding is essential in effective drug development and its safe use in clinical applications, because protein binding plays an important role in the pharmacokinetics and pharmacodynamics of the drug [2–4]. Equilibrium dialysis and ultrafiltration followed by HPLC analysis have been widely used for this purpose. However, these conventional analytical methods are not applicable for the analyses of strongly bound drugs,

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because of the errors caused by drug adsorption onto the membrane and by the leakage of the bound drug from the membrane as well as the difficulty in determining low concentrations of unbound drug. To overcome this problem, we have developed high-performance frontal analysis (HPFA), a chromatographic method which allows simple and easy determination of unbound drug concentrations after direct sample injection [5–13]. HPFA is advantageous for the analysis of strongly bound drugs because (i) HPFA is free from the problems which arise using a membrane (adsorption of drug and leakage of bound drug), and (ii) the bound drug is transformed into the unbound form in the HPFA column, which improves the measurement of low levels of unbound drug [13]. Details of the principle and features of the HPFA method were mentioned previously [6]. The reliability of this method has been confirmed by comparing the analytical results with those obtained by the conventional methods, such as ultrafiltration, using many kinds of drugs (indometacin, salicylate, acetazolamide, diclofenac, carbamazepine, warfarin, ketoprofen and fenoprofen) as model samples.

This paper deals with the development of an on-line frontal analysis HPLC system, in which the HPFA method was incorporated, to determine the unbound CS-045 concentrations in human serum albumin (HSA) solution and in human plasma.

2. Experimental

2.1. Reagent and materials

CS-045 was a product of Sankyo (Tokyo, Japan). Human serum albumin (HSA, fatty acid free) was purchased from Sigma (St. Louis, MO, USA). Human plasma was prepared from fresh human blood.

2.2. Preparation of sample solutions

The stock solution of CS-045 was made up in ethanol. An appropriate volume of the stock solution was transferred to a 10-ml screw-capped glass vial, and the solvent was evaporated under a nitrogen gas stream. An appropriate volume of the HSA solution (in 67 m*M* phosphate buffer, ionic strength 0.17) or human plasma was added to the vial, and was shaken gently for 2 h at 37°C.

2.3. Determination of unbound CS-045 concentration

Fig. 2 shows the schematic diagram of the on-line frontal analysis HPLC system, where the HPFA column (F) (Develosil 100Diol5, 30 cm × 8 mm I.D., Nomura, Seto, Japan), an extraction column (G) (Develosil ODS-10, 1 cm × 4 mm I.D., Nomura), and an analytical column (H) (Cosmosil $5C_{18}$-AR, 15 cm × 4.6 mm I.D., Nacalai Tesque, Kyoto, Japan) were connected via a four-port switching valve (I) and a six-port switching valve (J). The operating conditions are given in Table 1.

The sample solution was directly injected onto the diol-silica column through the injector loop. CS-045 was eluted as a zonal peak with a plateau region, as the drug–protein binding equilibrium in the sample solution was regenerated at the top of the diol-silica column. As a result, the drug concentration in the plateau region represents the unbound drug concentration in the sample

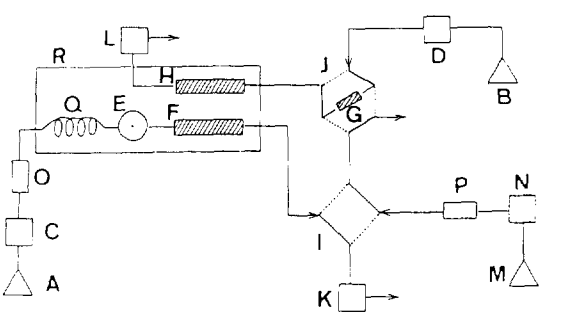

Fig. 2. Schematic diagram of the on-line HPLC system. (A) Mobile phase for HPFA; (B) mobile phase for chiral separation; (C,D) pump; (E) sample injector; (F) column for HPFA; (G) column for extraction; (H) analytical column; (I) four-port switching valve; (J) six-port switching valve; (K,L) UV detector; (M) distilled water to wash extraction column; (N) pump; (O,P) ODS column for mobile phase purification; (Q) 5 ml loop for mobile phase preheating; (R) column oven.

Table 1
Conditions of HPFA–HPLC system

Subsystem		Condition
HPFA	Column	Develosil 100Diol5 (30 cm × 8 mm I.D.)
	Mobile phase	67 m*M* sodium phosphate buffer (pH 7.4, ionic strength 0.17)
	Flow-rate	1.0 ml/min
	Detection	UV 230 nm
	Temperature	37°C
Extraction	Column	Develosil ODS-10 (1 cm × 4 mm I.D.)
Analytical HPLC	Column	Cosmosil $5C_{18}$-AR (15 cm × 4.6 mm I.D.)
	Mobile phase	Water–acetonitrile–acetic acid (500:500:1, v/v)
	Flow-rate	1.2 ml/min
	Detection	UV 230 nm
	Temperature	37°C

solution. While the plateau region was being eluted from the HPFA column, the valve I was switched from the solid line to the broken line for a given period of time. By this heart-cut procedure, a known volume of eluent in the plateau region was transferred into the extraction column G where the drug was adsorbed and concentrated. By switching the valve J, the extracted drug was desorbed and transferred into the analytical column H, and the peak area was measured at UV 230 nm. The extraction column was washed with water for 3 min before the heart-cut procedure and for 1 min after the heart-cut procedure. The 5 ml loop Q was used for preheating the HPFA mobile phase. For the purification of the mobile phase, small ODS columns O and P (Develosil ODS-10, 1 cm × 4.0 mm I.D., Nomura Chemical) were put in the line. A typical time schedule for valve switching is shown in Fig. 3.

The instruments used are as follows: pumps C and D (LC-6A, Shimadzu), pump P (Twincle, Jasco, Tokyo, Japan), UV detectors K and L (SPD-2A and SPD-6A, Shimadzu), injector E (Rheodyne Type 8125, equipped with a 20 μl or 2.5 ml loop) and an integrated data analyzer (Chromatopac C-R6A, Shimadzu).

The calibration line was prepared as follows. The standard solutions of CS-045 were made up in ethanol at the concentrations of 0.415, 1.02, 2.44, 5.06, 10.1, 14.9 and 20.0 μ*M* for the analysis of the CS-045–HSA mixed solutions, and at 0.232, 0.58, 1.16, 2.31, 4.62, 8.06, 11.5 and 16.0 μ*M* for the analysis of the CS-045–plasma samples. The diol-silica column was off-line, and the volume of the injector loop was changed from 2.5 ml to 20 μl. A 5-μl portion of standard solution was injected directly into the extraction column which had been previously washed with water for 3 min. After perfusing the extraction column with water for 1 min, the adsorbed CS-045 was back-flushed into the analytical column by the column switching procedure. The calibration line was prepared by plotting the peak area (average of two runs) vs. the amount of CS-045 (picomole) injected. The

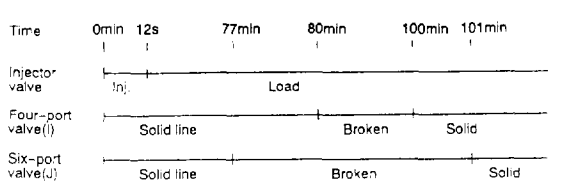

Fig. 3. Typical time program for valve switching. The heart-cut time is 80–100 min.

calibration lines showed good linearity (correlation coefficient, >0.999). The amount of CS-045 calculated from the calibration line was divided by the heart-cut volume to give the unbound drug concentration.

The peak area of CS-045 obtained by injecting a 5-μl portion of 14.9 μM CS-045 solution in ethanol into extraction column G were 96% compared to the direct injection of the same volume of the same CS-045 solution into the analytical column H. This indicates the complete extraction of CS-045 onto column G and the complete transfer of the extracted CS-045 into the analytical column.

3. Results and discussion

3.1. Determination of unbound CS-045 in HSA solution

It is essential that the binding equilibrium in the drug–protein sample solution is regenerated in the HPFA column. In order to maintain the equilibrium state, it is preferable to use an aqueous buffer solution without organic modifier in the mobile phase. However, it is difficult to elute a hydrophobic drug from a restricted-access type stationary phase containing hydrophobic ligands using an aqueous buffer solution. Therefore, a hydrophilic diol-silica column was used in the present study. This column allows the elution of CS-045 by phosphate buffer containing no organic modifier.

Fig. 4 shows the chromatograms of 10 μM CS-045 and 550 μM HSA mixed solution. When 5 μl of the mixed solution were injected into the diol-silica column, CS-045 gave a single peak well separated from the protein peak (Fig. 4A). However, when a 200-μl portion of the mixed solution was injected, CS-045 was eluted as the zonal peak with a plateau region (Fig. 4B), which can be found more clearly from the subtraction chromatogram (Fig. 4D). The plateau height was not changed even when the injection volume of the same solution was decreased to 100 μl. When CS-045 standard solution containing no protein was injected, CS-045

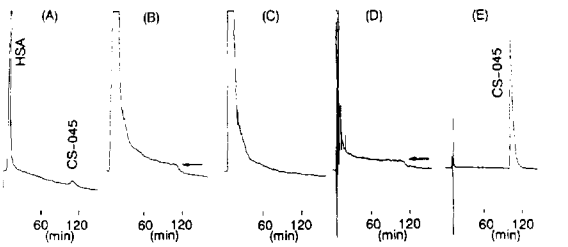

Fig. 4. Elution profile of CS-045 from the diol-silica column. Sample: (A,B) 10 μM CS-045–550 μM HSA solution; (C) 550 μM HSA; (E) 1.39 mM CS-045 in ethanol. Injection volume: (A) 5 μl, (B) 200 μl, (C) 200 μl, (E) 5 μl. Chromatogram (D) is the subtraction of (C) from (B). HPLC conditions, see Table 1. The a.u.f.s. of chromatogram (E) is eight times larger than those of (A)–(D).

was eluted as a single sharp peak (Fig. 4E). This clearly indicates that the protein binding is responsible for the zonal elution of this drug. From this result, the injection volume was determined to be 200 μl.

Preventing diffusion of the sample solution in the injector loop is essential for HPFA, because otherwise the binding equilibrium is disturbed and the plateau region may disappear. The injector-reswitching technique [12] is useful to overcome this problem. In this study, the injector loop was loaded with a 300-μl portion of the CS-045–HSA solution, and connected to the mobile phase flow (1.0 ml/min) for 12 s. This resulted in 200 μl sample injection. By this technique, the sample input could be regarded as an ideal rectangular shape.

Fig. 5 shows the relation between the elution time and the CS-045 concentration in the eluent. The eluent was heart-cut every 5 min, and was subjected to the on-line preconcentration and HPLC analysis. Each point was plotted at the end of the heart-cut time. This result clearly indicates that a wide range of the plateau region appeared between 35–115 min. The unbound concentration, estimated as the average CS-045 concentration in this region (35–115 min, 16 data points), was 7.28 ± 0.187 nM, that is, the bound fraction (bound:total concentration ratio) was 99.93%.

It is interesting that a plateau volume as large as 80 ml was obtained by injecting only a 200 μl portion of the sample volume. This is called

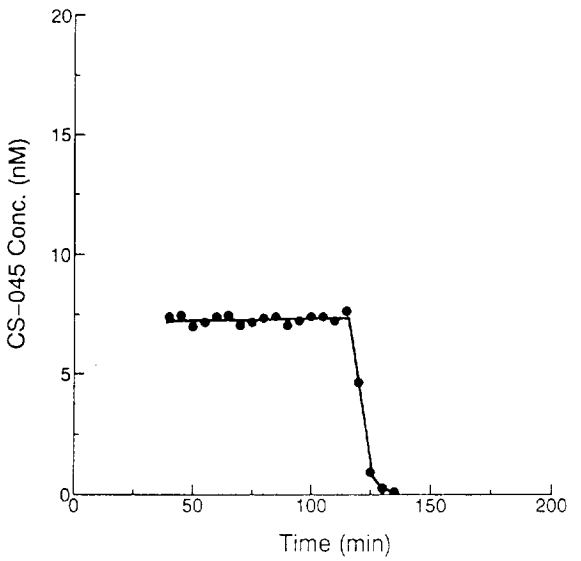

Fig. 5. Relation between the elution time and the CS-045 concentration for human plasma. Sample: 10 μM CS-045–550 μM HSA solution. Injection volume: 200 μl. HPLC conditions, see Table 1. Heart-cut volume, 5 ml. Each point was plotted at the end of the heart-cut time.

'regulation effect' [13]. In HPFA, the bound drug is not separated from the unbound drug, but is converted into the unbound form. Then, the whole (bound and unbound) drug fraction is eluted as a single zonal peak, and the drug concentration in the plateau region is regulated by the protein binding to be the same as the unbound drug concentration. Because of this 'regulation effect', the plateau volume becomes larger than the injection volume. This effect increases when the drug binds more strongly to the protein.

Table 2 lists the unbound CS-045 concentrations in 550 μM HSA solutions determined by the present HPLC system. Fig. 6 shows the

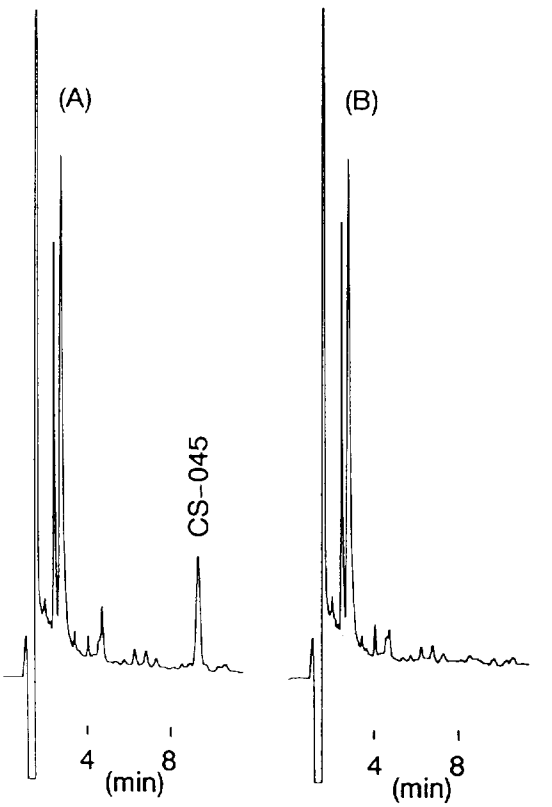

Fig. 6. Analysis of unbound CS-045 in HSA solution on the analytical column. Sample: (A) 1 μM CS-045–550 μM HSA solution; (B) 550 μM HSA solution. HPLC conditions, see Table 1.

chromatogram obtained from the sample solution containing 1 μM CS-045. As low as 0.655 nM of the unbound concentration was determined by UV detection (230 nm), because CS-045 in a large volume (20 ml, heart-cut time 80–100 min) of the plateau region was concentrated in the extraction column. The reproducibility was good (C.V. = 5.10%, $n = 5$). The

Table 2
Unbound CS-045 concentrations in human serum albumin solutions

Sample solution	Unbound concentration (nM)	Bound fraction
1 μM CS-045–550 μM HSA	0.655 ± 0.033	99.93 ± 0.003%
10 μM CS-045–550 μM HSA[a]	7.28 ± 0.187	99.93 ± 0.002%

Mean ± S.D. ($n = 5$).
[a] Determined from the results of Fig. 5 ($n = 16$).

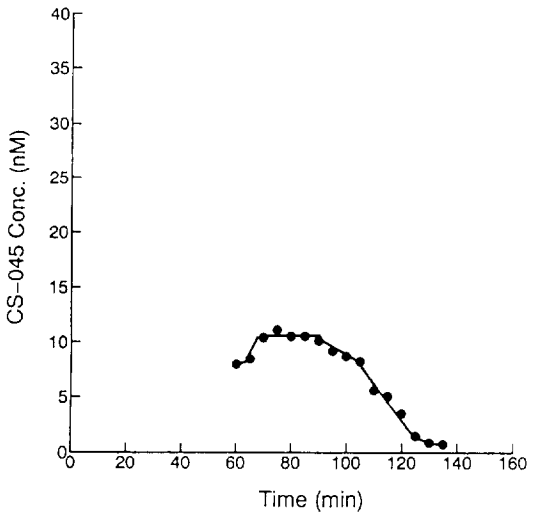

Fig. 7. Relation between elution time and CS-045 concentration in the eluent. Sample: 10 μM CS-045 in human plasma. Injection volume: 1.33 ml. HPLC conditions, see Table 1. Heart-cut volume, 5 ml. Each point was plotted at the end of the heart-cut time.

bound fraction in this sample solution was 99.93%, which was the same as in the 10 μM CS-045–550 μM HSA solution.

3.2. Determination of unbound CS-045 in human plasma

The present system was applied to the analysis of plasma samples. As reported previously, the injection volume for HPFA depends on the unbound drug fraction; a larger injection volume is required if the unbound drug fraction is higher [8]. In the analyses of plasma samples, the sample injection volume was selected to be 1.33 ml, because the unbound fraction in human plasma was higher than that in the 550 μM HSA solution as mentioned later. The injection procedure was as follows. A 2-ml portion of the sample solution was loaded into the injector loop, and the loop was then connected to the mobile phase flow for 80 s. Therefore, the actual injection volume was 1.33 ml. Fig. 7 shows the relation between the elution time and the concentration of CS-045 in the eluent after direct injection of 10 μM CS-045 in human plasma. The CS-045 concentration in each of the eluent fractions between 65 and 90 min was almost the same. That is, the plateau region continued for 25 min. The unbound CS-045 concentration, averaged over 65–90 min (5 data points), was 10.5 ± 0.36 nM, that is, the bound fraction was 99.89%.

Table 3 lists the unbound CS-045 concentrations in human plasma determined by the present system. The chromatograms obtained are shown in Fig. 8. No significant interference by the endogenous plasma components was observed. Very low unbound drug concentrations in the plasma samples containing therapeutic levels of CS-045 (total drug concentration, 250 nM and 1 μM) were determined (heart-cut volume, 10 ml) with good reproducibility (C.V. $< 9.31\%$, $n = 5$). The unbound fractions in the human plasma samples containing 250 nM, 1 μM and 10 μM CS-045 were 0.110%, 0.106% and 0.105%, respectively. These results indicate that the unbound CS-045 concentration in human plasma was proportional to the total concentration ranging from 250 nM to 10 μM. From the results in Table 2, it is obvious that HSA plays a major role in the protein binding of CS-045 in human plasma.

Table 3
Unbound CS-045 concentrations in human plasma

Total concentration	Unbound concentration	Bound fraction
250 nM CS-045	0.274 ± 0.026 nM	$99.89 \pm 0.010\%$
1 μM CS-045	1.06 ± 0.075 nM	$99.89 \pm 0.007\%$
10 μM CS-045[a]	10.5 ± 0.363 nM	$99.90 \pm 0.004\%$

Mean ± S.D. ($n = 5$).
[a] Determined from the result of Fig. 7 ($n = 5$).

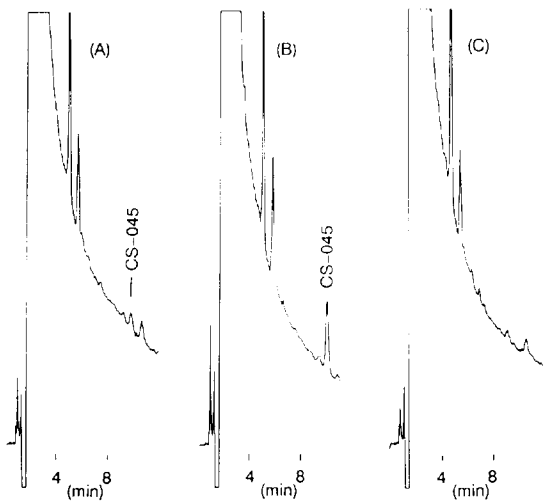

Fig. 8. Analysis of unbound CS-045 in human plasma on the analytical column. Sample: (A) 250 nM CS-045 in human plasma; (B) 1 μM CS-045 in human plasma; (C) human plasma. HPLC conditions, see Table 1.

4. Conclusion

The unbound CS-045 concentrations in the 550 μM HSA solution and in human plasma were determined using an on-line frontal analysis HPLC system. CS-045 was strongly bound to plasma protein; the bound CS-045 fraction was 99.89%. The bound CS-045 fraction was constant within a wide range of total drug concentration (250 nM–10 μM). HSA plays a major role in the protein binding of CS-045.

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