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Enantioselective protein binding of semotiadil and levosemotiadil determined by high-performance frontal analysis[1]

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Abstract

An on-line frontal analysis HPLC system was developed for the determination of the unbound concentrations of semotiadil, a new calcium antagonist with non-dihydropyridine structure, and its antipode (levosemotiadil), and was applied to the enantioselective investigation of their plasma protein binding properties. 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 1.04 nM of the unbound drug was determined with good reproducibility. Semotiadil (*R*-isomer) and levosemotiadil (*S*-isomer) are bound strongly and enantioselectively to human serum albumin (HSA) and human α_1-acid glycoprotein (AGP), and the enantioselectivity was reversed between these plasma proteins. While HSA binds *S*-isomer more strongly than the antipode, human AGP binds *R*-isomer more strongly. In human plasma, the unbound drug fraction was less than 1%, and the enantioselectivity was similar to that observed in AGP solution. © 1997 Elsevier Science B.V.

Keywords: Semotiadil; Levosemotiadil; Protein binding; Albumin; α_1-acid glycoprotein; HPLC; High-performance frontal analysis

1. Introduction

When a drug is administered it enters the blood stream, where it binds to plasma proteins such as albumin and α_1-acid glycoprotein [1–3]. Plasma protein binding of a drug is a reversible and kinetically rapid interaction. Unbound drug in plasma transfers freely to the target organ, whereas bound drug can only pass with difficulty through the blood capillary wall to reach the action site. Unbound drug concentration shows better correlation to pharmacological activity than the total (bound + unbound) drug concentration. Some important pharmacokinetic properties such as hepatic metabolism rate, renal excretion rate, biomembrane permeation rate and

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steady-state distribution volume are the function of unbound drug fraction (unbound/total concentration ratio). This is why the quantitative and enantioselective investigation of plasma protein binding is essential to pharmacokinetic studies and therapeutic dosing regimens. However, a binding study of a highly bound drug has often been hindered by the difficulty in the determination of low levels of unbound drug concentrations. Development of a sensitive analytical method is, therefore, an integral part of the study on strong drug-protein binding. In addition, a chiral drug often exhibits different binding properties between the enantiomers [4,5], and these are important in the investigation of their pharmacokinetic and pharmacodynamic properties.

Semotiadil and levosemotiadil (chemical structure, see Fig. 1) are an enantiomeric pair of drugs under development. Semotiadil (*R*-isomer) is a new calcium antagonist with non-dihydropyridine structure [6–10] and shows antihypertensive [11,12] and antianginal activities [13,14]. Levosemotiadil (*S*-isomer) is an antiarrhythmic drug with sodium and calcium channel blocking action [15–20], as well as potassium blocking activity [21]. Both enantiomers are highly bound to plasma proteins, and the unbound concentration could not be determined precisely using any conventional method.

High-performance frontal analysis (HPFA) is a novel chromatographic method developed for the determination of unbound drug concentration [22–33]. HPFA has several unique features:

1. It allows direct injection analysis.
2. It does not suffer from any errors due to leakage of protein through membrane and adsorption of drug to the membrane.
3. It allows simultaneous determination of total and unbound drug concentrations, so far as the drug peak is well separated from the protein peak.
4. It is easy to incorporate into an on-line HPLC system. By coupling with a chiral HPLC column, the unbound concentration of a chiral drug can be determined stereoselectively.
5. It is useful especially for the analysis of a strongly bound drug, because the 'regulation effect' serves to determine unbound drug concentration as low as a few nM or lower [29].

The reliability of HPFA has been confirmed by comparing the analytical results with those obtained by a conventional ultrafiltration method, using several drugs of different natures such as indomethacin [22], carbamazepine [23], warfarin [24], salicylate [25], acetazolamide [25], ketoprofen [26], fenoprofen [28] and diclofenac [29].

In this study, a novel on-line HPFA-HPLC system was developed for the determination of unbound concentrations of semotiadil and levosemotiadil, and was applied to the characterization of their plasma protein binding properties.

2. Experimental

2.1. Reagents and materials

Semotiadil fumarate and levosemotiadil fumarate were provided by Santen Pharmaceutical (Osaka, Japan). Human serum albumin (HSA, Ca. No. A-3782, fatty acid free), bovine serum albumin (BSA, Ca. No. a-7511, fatty acid free) and human α_1-acid glycoprotein (AGP, Ca.No. G-9885) were purchased from Sigma. The drug-protein mixed solutions were prepared in sodium phosphate buffer (pH 7.4, I = 0.17), and were kept at 37°C before analysis.

The diol-silica column (Develosil 100 Diol 5) and the analytical column (Develosil 300 ODS UG 5) were purchased from Nomura (Seto, Japan). C4 packing material (Wakosil 5C4) was purchased from Wako (Osaka, Japan).

2.2. Principle of HPFA

In HPFA, drug-protein mixed solution is injected directly into a restricted-access type HPLC

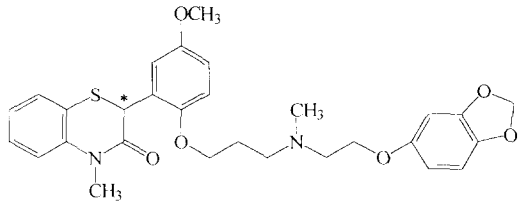

Fig. 1. Chemical structure of semotiadil (*R*-isomer) and levosemotiadil (*S*-isomer).

column, which excludes large molecules such as proteins but retains drugs of small molecular size on a stationary ligand in the micropores of the packing materials [34,35]. If the injection volume is large enough, the release of bound drug from protein is apparently suppressed, and an equilibrium zone is generated in the interstices of packing material near the top of the column. In this equilibrium zone, the drug in the interstices reaches the same protein binding equilibrium as in the sample solution. The drug concentration in the micropores becomes equal to the unbound drug concentration in the interstices, and the drug in the micropores reaches chromatographic partition equilibrium. The protein peak is eluted first from the column and the unbound drug is eluted as a trapezoidal peak having a plateau region. This plateau drug region is formed due to the elution of the unbound drug in the equilibrium zone. Therefore, the unbound drug concentration can be determined from the plateau height or by the heart-cut of this plateau region followed by the on-line HPLC analysis. This is the principle of the HPFA method [23,29,33], and hence the appearance of the plateau region is essential to this method.

2.3. Determination of unbound drug concentrations by on-line HPLC/HPFA system

Table 1 lists the HPLC conditions. Fig. 2 shows the schematic diagram of on-line HPLC system. HPFA column F, extraction column G and analytical column H were connected via a four-port switching valve I and a six-port switching valve J. The instruments used are as follows: pumps C and D (LC 6A, Shimadzu), pump N (Twincle, Jasco, Tokyo, Japan), UV detectors K and L (SPD-2A and SPD-6A, Shimadzu), injector E (Rheodyne Type 8125, equipped with a 5-ml loop) and two integrated data analyzer connected with K and L (Chromatopac C-R3A and C-R6A, Shimadzu, Kyoto, Japan).

The sample solution was directly injected onto the diol-silica through the injector E. The drug was eluted as a zonal peak with a plateau region. The concentration in the plateau region can be regarded equal to the unbound drug concentration in the sample solution. A given volume of the eluents in the plateau region was then heart-cut by switching the valve I and transferred into the extraction column G, where the drug was concentrated. By switching the valve J, the extracted drug was desorbed and transferred into the analytical column H. The peak areas were measured at 294 nm. The extraction column was washed with distilled water for 1 min before and 30 s after

Table 1
HPLC conditions

Subsystem	Condition	
HPFA	Column	Develosil 100 Diol 5 (5 cm × 4.6 mm i.d.)
	Mobile phase	Phosphate buffer (pH 7.4, I = 0.17)
	Flow rate	1.0 ml min^{-1}
	Detection	UV 294 nm
	Temperature	37°C
Extraction	Column	Wakosil 5C4 (1 cm × 4 mm i.d.)
Analytical	Column	Develosil 300 ODS UG 5 (15 cm × 4.6 mm i.d.)
HPLC	Mobile phase	40 mM NaH_2PO_4:MeOH = 4:6 (v:v)
	Flow rate	1.0 ml min^{-1}
	Detection	UV 294 nm
	Temperature	37°C

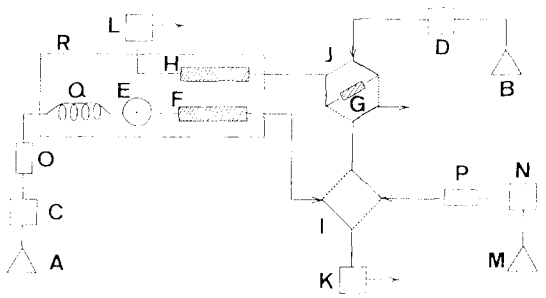

Fig. 2. Schematic diagram of the on-line HPFA-HPLC system: (A) mobile phase for HPFA; (B) mobile phase for analytical column; (C, D) pump; (E) sample injector; (F) column for HPFA; (G) column for extraction; (H) analytical column; (I) four-port switching valve; (J) six-port switchng valve; (K, L) UV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

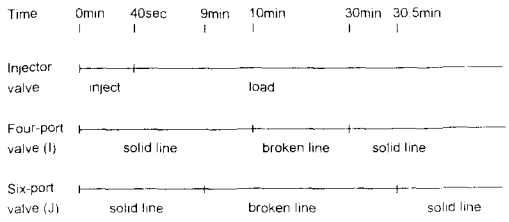

Fig. 3. Typical time program of valve switching.

the heart-cut procedure. The 5-ml loop O was used for preheating the mobile phase for HPFA. A typical time program of valve switching is shown in Fig. 3, where the heart-cut time is 10–30 min.

The injection volume of HPFA depends on the unbound drug fraction; a larger injection volume is required if the unbound drug fraction is higher [25,33]. The actual injection volume of HSA and AGP solutions and of human plasma samples was selected to be 667 μL, while that of drug solutions in 600 μM BSA and in 1% BSA was selected to be 2 and 4.5 ml, respectively. These injection volumes were selected so as to obtain a clear plateau region.

The calibration lines were prepared as follows. A series of eight standard solutions containing 2–500 μM of semotiadil or levosemotiadil were made up in methanol. The diol-silica column was removed from the line and the injector loop volume was changed from 5 ml to 20 μl. Each 5-μl portion of the mixed standard solution was injected directly into the extraction column which had been previously washed with distilled water for 1 min. After perfusing the extraction column with the same solution for 30 s, the adsorbed drug was transferred (back-flashed) into the analytical column by the column switching procedure. The column temperature was 37°C. The calibration lines were prepared by plotting the peak areas (average of three runs) vs the amount of drug injected. Good linearity ($R > 0.999$) was obtained. The unbound drug concentration was then calculated from the amount of the drug divided by the heart-cut volume.

The slope of the calibration line was the same as that of the calibration line prepared by injecting the drug standard solutions directly into the analytical column without using the extraction column, which indicates the complete drug adsorption on and the complete desorption from the extraction column.

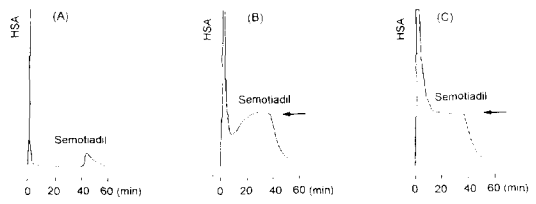

Fig. 4. Elution profiles of 50 μM semotiadil and 600 μM HSA mixed solution. Injection volume: (A) 20; (B) 333 and (C) 667 μl.

3. Result and discussion

The sample injection volume in HPFA should be large enough to obtain a plateau drug region. Fig. 4 shows the effect of sample injection volume upon the elution profile of 50-μM Semotiadil and 600 μM HSA mixed solution. A physiological pH of sodium phosphate buffer was used as the mobile phase for HPFA without adding any organic modifier so as not to disturb the drug-protein binding equilibrium. A diol-silica column was used as the HPFA column because it allows the elution of hydrophobic drugs under such a mild mobile phase condition. In the case of a small injection volume (20 μl), semotiadil was eluted as a sharp peak (Fig. 4A). However, as the injection volume increased, the peak height reached the maximum level, and a plateau region appeared (Fig. 4B and C). The plateau height did not increase with the further increase in the injection volume.

It is necessary to prevent the diffusion of sample solution in the injector loop, because otherwise the binding equilibrium would be disturbed and the plateau region would disappear. The 'injector-reswitching technique' is useful to overcome this problem [26,29]. The sample solution was injected as follows. The injector loop was loaded with a 1-ml portion of the sample solution, and connected to the mobile phase flow (1 ml min^{-1}) for 40 s. Therefore, the actual injection volume

Table 2
Unbound drug concentrations in 600 μM HSA solutions

Total drug concentration (μM)	Unbound drug concentration[a]		R/S
	R-isomer (nM)	S-isomer (nM)	
1	6.93 ± 0.19	2.50 ± 0.10	2.77
0.5	3.39 ± 0.03	1.22 ± 0.02	2.78

Heart-cut volume, 10–25 ml.
[a]Mean ± S.D. ($n = 5$).

was 667 μl. Owing to this injection procedure, the sample input was regarded as an ideal rectangular shape. The injection volume shown in Fig. 4 (except for 20 μl) was also the actual sample volume introduced by the 'injector-reswitching technique' with appropriate loading volume and reswitching time.

A given volume (1–25 ml) of the plateau region was transferred (heart-cut) to the extraction column by switching of the 4-port valve. The trapped drug was then eluted out from the extraction column into the analytical column by switching of the 6-port valve. Semotiadil or levosemotiadil is eluted at 7.9 min. The drug amount in the heart-cut plateau region was determined from the peak area, and the amount was then divided by the heart-cut volume to calculate the unbound drug concentration.

Table 2 shows the unbound drug concentrations in a physiological concentration (600 μM) of HSA solutions determined using the present HPLC/HPFA system. Both enantiomers were bound to HSA very strongly (bound drug fraction, 99% or more) and enantioselectively. The unbound concentration of *R*-isomer was about three times larger than the antipode. As low as 1.22 nM of unbound concentration was determined with good reproducibility by use of a common UV detector, because the unbound drug in a large volume of plateau region (25 ml) was subjected to UV detection after on-line concentration.

HSA and BSA are widely used in protein binding study as model plasma proteins. HSA and BSA are very similar in primary structure [36], but sometimes exhibit different binding properties. Therefore, it is necessary to investigate the difference in binding properties between these albumins. Table 3 compares the unbound drug concentrations in HSA and BSA solutions determined by the present HPFA method. The unbound concentrations in 600 μM BSA solution (total drug concentration, 50 μM) were much higher than those in 600 μM HSA solution, which means that both enantiomers are bound to BSA less strongly than to HSA. In addition, the enantioselectivity in the BSA solution (*R*/*S* ratio of unbound concentration, 1.17) is less prominent than that shown in the HSA solution (*R*/*S* ratio, 3.33). The reversed order of enantioselectivity between HSA and BSA has been also found in case of warfarin [37] and disopyramid [38].

Table 3
Unbound drug concentrations in serum albumin solutions

Sample solution	Unbound drug concentration[a]		R/S
	R-isomer (nM)	S-isomer (nM)	
50 μM drug in 600 μM HSA	386 ± 4.39	116 ± 1.15	3.33
50 μM drug in 600 μM BSA	2060 ± 12	1760 ± 24	1.17

Heart-cut volume, 1 or 5 ml.
[a]Mean ± S.D. ($n = 5$).

Table 4 shows the unbound drug concentrations in physiological concentration (25 μM) of human AGP solution. The actual injection volume was the same as in the HSA binding study (667 μl). As low as 1.04 nM of unbound concentration was determined with good reproducibility. The unbound concentration of *R*-isomer is lower

Table 4
Unbound drug concentrations in 25 μM human AGP solutions

Total drug concentration (μM)	Unbound drug concentration[a]		R/S
	R-isomer (nM)	S-isomer (nM)	
1	1.78 ± 0.08	1.98 ± 0.04	0.90
0.5	1.04 ± 0.08	1.18 ± 0.06	0.88

Heart-cut volume, 20 or 22 ml.
[a]Mean ± S.D. ($n = 5$)

Table 5
Unbound drug concentrations in human plasma

Total drug conc. (μM)	Unbound drug concentration[a]		R/S	Bound fraction[a]	
	R-isomer (nM)	S-isomer (nM)		R-isomer	S-isomer
1	4.35 ± 0.09	6.11 ± 0.04	0.71	99.57 ± 0.009%	99.39 ± 0.004%
0.5	3.02 ± 0.07	4.32 ± 0.06	0.70	99.40 ± 0.016%	99.14 ± 0.013%

Heart-cut volume, 20 ml.
[a]Mean ± S.D. ($n = 5$).

than that of S-isomer, and the R/S ratio of the unbound concentration is about 0.9. This enantioselectivity is opposite to that shown in albumin solution. Comparing the samples containing the same total concentration of drug, the unbound drug concentration in AGP solution was lower than that in HSA solution (see Table 2), although the AGP concentration was much lower than the HSA concentration (600 μM). This means that AGP binds to these enantiomers much more strongly than HSA.

The present method was applied to the analysis of human plasma samples. Table 5 shows the unbound drug concentrations in human plasma samples. The total drug concentrations are in their therapeutic levels. The bound fractions of both enantiomers are more than 99%. Likewise in the AGP solutions, the unbound concentration of R-isomer in human plasma was lower than that of S-isomer. This means that the AGP plays a dominant role in the enantioselective plasma protein binding. The unbound drug concentrations in the human plasma were higher than those in the physiological concentration of AGP solution. This suggests that the binding can be inhibited by certain endogenous compounds. It is reported, for example, that nicardipine, a hydrophobic basic drug, is considerably bound to plasma lipoprotein [39]. Since semotiadil and levosemotiadil are hydrophobic basic drugs, it is likely that these drugs are also bound to lipoprotein in human plasma. Further study on this point is in progress.

4. Conclusion

The present on-line HPLC/HPFA system enables sensitive determination of unbound semotiadil (R-isomer) and levosemotiadil (S-isomer) concentrations. As low as 1.04 nM of unbound drug was determined with good reproducibility. Both drugs are bound strongly to HSA and human AGP. While HSA binds S-isomer more strongly than the antipode, human AGP binds R-isomer more strongly. In human plasma, the bound drug fractions are more than 99%, and the enantioselectivity in human plasma is similar to that observed in AGP solution.

References

[1] M.C. Meyer and D.E. Guttman, J. Pharm. Sci., 57 (1968) 895–918.
[2] J.J. Vallner, J. Pharm. Sci., 66 (1977) 477–465.
[3] G.T. Tucker and M.S. Lennard, Pharmacol. Ther., 45 (1989) 309–329.
[4] T. Noctor, in I.W. Wainer (Ed.), Drug Stereochemistry, Ch. 12., Dekker, NY, 1993.
[5] T.C. Kwong, Clin. Chem. Acta, 151 (1985) 193–216.
[6] G. Stark, K. Kasper, U. Stark, N. Miyawaki, M. Decrinis and H.A. Tritthart, Eur. J. Pharmacol., 286 (1995) 71–78.
[7] M. Kageyama, T. Yanagisawa and N. Taira, Br. J. Pharmacol., 114 (1995) 1289–1295.
[8] N. Miyawaki, T. Furuta, T. Shigei, H. Yamauchi and T. Iso, J. Cardiovasc. Pharmacol., 16 (1990) 769–775.
[9] N. Teramoto, Jpn. J. Pharmacol., 61 (1993) 183–195.
[10] K. Nishimura, N. Miyawaki, H. Yamauchi and T. Iso, Arzneimittelforschung., 40 (1990) 244–248.
[11] M. Kageyama, K. Nishimura, T. Takada, N. Miyawaki and H. Yamauchi, J. Cardiovasc. Pharmacol., 17 (1991) 102–107.
[12] T. Takada, N. Miyawaki, M. Kageyama, K. Matsuno, N. Ishida, H. Yamauchi and T. Iso, J. Cardiovasc. Pharmacol., 18 (1991) 855–862.
[13] T. Mori, K. Irie and S. Ashida, Jpn. J. Pharmacol., 55 (Suppl. I) (1991) 328.
[14] T. Mori, K. Irie, F. Ishii and S. Ashida, Jpn. J. Pharmacol., 52 (Suppl. I) (1990) 203.
[15] I. Kodama, R. Suzuki, K. Maruyama and J. Toyama, Br. J. Pharmacol., 114 (1995) 503–509.
[16] K. Nakayama, K. Morimoto, Y. Nazawa and Y. tanaka, J. Cardiovasc. Pharmacol., 20 (1992) 380–391.
[17] N. Miyawaki, F. Yamazaki, T. Furuta, T. Shigei, H. Yamauchi, Drug Dev. Res., 22 (1991) 293–298.
[18] S. Nagashima, T. Uematsu, T. Araki, M. Matsuzaki, H. Fukuchi and M. Nakashima, Naunyn-Schmiedeberg's Arch. Pharmacol., 345 (1992) 688–695.
[19] A. Hirasawa, A. Haruno, T. Matsuzaki and K. Hashimoto, Jpn. Heart J., 33 (1992) 851–861.
[20] M. Fukuchi, T. Uematsu, S. Nagashima and M. Nakashima, Naunyn-Schmiedeberg's Arch. Pharmacol., 341 (1990) 557–564.
[21] Y. Hara and H. Nakaya, Br. J. Pharmacol., 116 (1995) 2750–2756.
[22] A. Shibukawa, T. Nakagawa, N. Nishimura, M. Miyake and H. Tanaka, Chem. Pharm. Bull., 37 (1990) 443–447.
[23] A. Shibukawa, N. Nishimura, K. Nomura, Y. Kuroda and T. Nakagawa, Chem. Pharm. Bull., 38 (1990) 443–447.
[24] A. Shibukawa, M. Nagao, Y. Kuroda and T. Nakagawa, Anal. Chem., 62 (1990) 712–716.
[25] N. Nishimura, A. Shibukawa and T. Nakagawa, Anal. Sci., 6 (1990) 355–359.
[26] A. Shibukawa, A. Terakita, J. He and T. Nakagawa, J. Pharm. Sci., 81 (1992) 710–715.
[27] A. Terakita, A. Shibukawa and T. Nakagawa, Anal. Sci., 9 (1993) 229–232.
[28] A. Shibukawa, M. Nagao, A. Terakita, J. He and T. Nakagawa, J. Liq. Chromatogr., 16 (1993) 903–914.
[29] A. Shibukawa, C. Nakao, T. Sawada, A. Terakita, N. Morokoshi and T. Nakagawa, J. Pharm. Sci., 83 (1994) 868–873.
[30] A. Terakita, A. Shibukawa and T. Nakagawa, Anal. Sci., 10 (1994) 11–15.
[31] A. Shibukawa, M. Kadohara, J. He, M. Nishimura, S. Naito and T. Nakagawa, J. Chromatogr. A, 694 (1995) 81–89.
[32] A. Shibukawa, T. Sawada, C. Nakao, T. Izumi and T. Nakagawa, J. Chromatogr. A, 697 (1995) 337–343.
[33] A. Shibukawa and T. Nakagawa, Anal. Chem., 68 (1995) 447–545.
[34] T.C. Pinkerton, J. Chromatogr., 544 (1991) 13–23.
[35] D.J. Anderson, Anal. Chem., 65 (1993) 434 R–443 R.
[36] U. Kragh-Hansen, Pharmacol. Rev., 33 (1981) 17–53.
[37] C. Lagerorantz, T. Larsson and I. Denfors, Comp. Biochem. Physiol., 69 C (1981) 375–378.
[38] J.J. Lima, Drug Metab. Dispos., 16 (1988) 563–567.
[39] S. Urien, Interaction of Drugs with Human Plasma Lipoproteins, In: J.P. Tillement and E. Lindenlaub (Eds.), Proc. Symp. Protein Binding and Drug Tansport, F.K. Schattauer Verlag, Stuttgart, NY, 1986, pp. 63–75.