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STEREOSELECTIVE PROTEIN BINDING OF VERAPAMIL ENANTIOMERS

ANNETTE S. GROSS,* BARBARA HEUER† and MICHEL EICHELBAUM*‡
*Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie Stuttgart, and †Medizinische Klinik, Universität Bonn, Bonn, Federal Republic of Germany

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Abstract—The binding of the (+)- and (−)-enantiomers of verapamil (V) to purified albumin (40 g/L), α_1-acid glycoprotein (0.55 g/L) and fresh serum has been studied over a wide range of verapamil concentrations (0.055 to 22 μM). The free fraction of the pharmacologically more potent (−)-V was always greater than that of (+)-V. Similar free fractions were observed in solutions of α_1-acid glycoprotein ((+)-V 0.079 ± 0.016; (−)-V 0.142 ± 0.020) and fresh serum ((+)-V 0.096 ± 0.009; (−)-V 0.136 ± 0.006), however the free fraction was higher in a solution of albumin ((+)-V 0.400 ± 0.030; (−)-V 0.572 ± 0.029). Saturation of verapamil binding sites was observed for α_1-acid glycoprotein only. Enantioselective verapamil serum binding was also noted in samples collected from five healthy volunteers following oral and intravenous verapamil administration. The free fraction of the individual isomers *in vitro* when added to predose serum as the pseudoracemic drug ((+)-V 0.06 ± 0.01, (−)-V 0.12 ± 0.02) was similar to that observed for the enantiomers when studied separately *in vitro*, indicating that the binding of each enantiomer is independent of the other optical isomer. The free fraction *ex vivo* after intravenous therapy ((+)-V 0.06 ± 0.01, (−)-V 0.12 ± 0.02) was similar to that observed *in vitro* in that subjects pre-dose serum. The free fraction of both enantiomers, however, was higher after oral drug therapy ((+)-V 0.13 ± 0.02, (−)-V 0.23 ± 0.03). The lower binding noted may be a result of competition for serum binding sites by verapamil metabolites, which attain higher concentrations following oral dosing.

Twenty-five percent of all chemically synthesised drugs have a chiral centre and up to 82% of certain synthetic chiral pharmaceuticals, for example β-adrenoceptor antagonists, are administered as racemates [1]. The enantiomers contained in a racemic drug mixture often exhibit substantial differences in activity and toxicity [1]. Various aspects of drug disposition may also be stereoselective. Stereoselective metabolism has been studied in some detail; however, protein binding has received less attention [2]. Only free drug is available to exert pharmacological effects, and different concentration–effect relationships may result for each enantiomer if stereoselective binding occurs. In addition differences in protein binding may influence the renal excretion of the individual enantiomers or the hepatic metabolism of low clearance drugs such as warfarin [1].

The calcium antagonist verapamil is approximately 90% bound to plasma proteins [3–7]. Interindividual differences in verapamil protein binding can be attributed, like other basic drugs [8, 9], to variations in the concentrations of albumin [6, 7], α_1-acid glycoprotein [6, 7] and possibly other plasma proteins [6]. Verapamil is administered as a racemic mixture; however, the bioavailability [10], pharmacokinetics [11] and pharmacological activity [12] of the individual (+)- and (−)-enantiomers differ. Following intravenous administration of (+)- and (−)-verapamil stereoselective plasma protein binding has also been observed [11]. It was therefore decided to study in greater detail the binding of (+)- and (−)-verapamil to albumin, α_1-acid glycoprotein and drug-free serum. In addition the protein binding of the enantiomers of verapamil has been investigated *ex vivo* utilising a stable labelled isotope technique. Healthy volunteers were administered (+)-verapamil labelled with two deuterium atoms and unlabelled (−)-verapamil. The two enantiomers could subsequently be analysed separately in biological samples using a gas-chromatography mass-spectroscopy analytical technique. Verapamil protein binding has been studied in serum samples from these subjects prior to verapamil administration (*in vitro* study) and after both intravenous and oral dosing (*ex vivo* study).

MATERIALS AND METHODS

(+)- and (−)-verapamil and dideuterated (d_2) (+)-verapamil were synthesised as previously described [11]. Protein binding was determined by equilibrium dialysis at 37° using perspex dialysis cells separated by a Visking cellophane membrane (Serva, Heidelberg, F.R.G.). Crystalline α_1-acid glycoprotein (0.55 g/L, Lot No. 26F-9330, Sigma Chemical Company, St. Louis, U.S.A.) and crystalline human serum albumin (40 g/L, Lot No. 55F-9326, Sigma Chemical Company, St. Louis, U.S.A.) were reconstituted in 0.067 M pH 7.4 Sorensen's phosphate buffer.

The binding of both the verapamil enantiomers to albumin, α_1-acid glycoprotein and fresh serum *in vivo* was initially studied over a wide range of concentrations. Pre-dialysis verapamil concentrations ranged from 0.055 to 22 μM (25 to 10,000 μg/

[‡] Author to whom correspondence should be addressed at Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Auerbachstr. 112, 7000 Stuttgart 50, F.R.G.

L) and total equilibrium verapamil concentrations ranged between 0.04 and 17 μM (18 to 7730 μg/L). For these experiments each unlabelled enantiomer was added separately to the protein solution.

Verapamil protein binding was also studied in serum samples collected from five healthy male volunteers (aged 22 to 25 years) who were administered equimolar amounts of unlabelled (−)-verapamil and d_2-(+)-verapamil. Protein binding was examined firstly by *in vitro* addition of 0.44 nmol (200 ng) of both enantiomers to samples collected prior to verapamil dosing. Verapamil free fraction was determined *ex vivo* in serum samples collected after simultaneous oral administration of 175 μmol (80 mg) of d_2(+)-verapamil and unlabelled (−)-verapamil and separate intravenous administration of 11 μmol (5 mg) of each enantiomer. In these *ex vivo* samples verapamil concentrations up to 2.2 μM (1000 ng/ml) were present prior to equilibrium dialysis.

Verapamil free fraction was determined by dialysing 1 ml of protein solution or serum containing verapamil against 1 ml of Sorensen's phosphate buffer. After 16 hr of gentle shaking, the verapamil concentrations in suitable aliquots of both the protein and buffer solutions was measured. Both d_2- and unlabelled verapamil concentrations, which in the *ex vivo* study corresponded to the concentrations of (+)- and (−)-verapamil respectively, were determined using a gas chromatographic-mass spectroscopic technique [11, 13]. Higher concentrations of unlabelled (+)- and (−)-verapamil used individually in the *in vitro* investigations were determined by high-performance liquid chromatography with fluorescence detection [14]. The two assay techniques have been extensively characterised and verified against each other [unpublished observation]. Protein concentrations in serum before and after dialysis were determined by the method of Lowry [15] to establish the magnitude of any shifts in volume during dialysis [16]. The concentration of α_1-acid glycoprotein in the purified protein solution prepared was determined by radial immunodiffusion using M-Partigen plates (Behringwerke AG). The α_1-acid glycoprotein concentration measured (0.55 g/L) was only 75% of that anticipated (0.75 g/L).

The free fraction of verapamil (fu) was calculated as the ratio of the concentration in the buffer compartment (free) to that in the protein compartment (total). All experiments were performed in duplicate and the mean free fraction is reported. The methodology used was reproducible ([albumin] = 40 g/L, [(+)-verapamil] = 0.15 μM, fu = 0.378 ± 0.030, CV = 8.0%, $n = 6$).

If saturation of protein binding was observed over a wide range of verapamil concentrations, the data were fitted using nonlinear least squares regression analysis [17] to the following expression [18] for i classes of binding sites, where D_f is the molar free drug concentration, r is the ratio of the bound drug concentration to the molar concentration of protein, K_i is the Scatchard association constant for the ith class of binding sites and n_i is the number of binding sites of that class per molecule of protein:

$$r = \sum_{i=1}^{i} \frac{n_i K_i D_f}{1 + K_i D_f} \qquad (1)$$

The overall free fraction of (+)- and (−)-verapamil in protein solutions or serum is reported as the mean ± SD. Differences were assessed using the Wilcoxon matched-pairs signed-ranks test or the Friedman two-way analysis of variance [19]. A probability of less than 0.05 was considered significant.

RESULTS

The free fraction of (+)- and (−)-verapamil in solutions of albumin, α_1-acid glycoprotein and human serum *in vitro* is given in Table 1.

Albumin

The binding of both enantiomers to albumin was independent of verapamil concentration. At all concentrations the free fraction of (+)-verapamil (0.400 ± 0.030) was substantially lower ($P < 0.005$) than the free fraction of (−)-verapamil (0.572 ± 0.029), indicating that verapamil binding to albumin is stereoselective. The highest equilibrium concentration of both (+)- and (−)-verapamil did not appear to saturate albumin binding sites. These sites could therefore not be characterised in detail.

α_1-acid glycoprotein

Both enantiomers were highly bound to α_1-acid glycoprotein (Table 1), confirming that verapamil binds to this protein in the absence of albumin [4]. The free fraction of both (+)- and (−)-verapamil in solutions of α_1-acid glycoprotein (0.55 g/L) was substantially lower than that observed in the albumin solution, indicating higher binding to the acute phase protein. Over the range of concentrations studied, the free fraction of (+)-verapamil was lower ($P < 0.005$) than (−)-verapamil and consequently the binding of verapamil with α_1-acid glycoprotein is stereoselective. At the higher equilibrium concentrations of both enantiomers (>4 μM), verapamil free fraction increased with increasing concentration. The free fraction at equilibrium concentrations less than 4 μM was independent of verapamil concentration and for (+)- and (−)-verapamil was 0.079 ± 0.016 ($N = 8$) and 0.142 ± 0.020 ($N = 8$) respectively.

The binding of verapamil enantiomers with α_1-acid glycoprotein could be characterised further using Scatchard analysis [18] as saturable binding was observed. A model incorporating one, rather than two classes of α_1-acid glycoprotein binding sites was more appropriate for fitting the data to Eqn (1). The relationships observed for both (+)- and (−)-verapamil are shown in Fig. 1. The number of binding sites determined was similar for (+)- and (−)-verapamil: 0.76 (Coefficient of variance 6.9%) and 0.77 (6.1%), respectively. The affinity constant for (+)-verapamil, 1.21 μM^{-1} (coefficient of variance 19.5%), was nearly double that of (−)-verapamil, 0.74 μM^{-1} (15.4%).

Fresh serum in vivo

The binding of (+)- and (−)-verapamil in fresh serum *in vitro* also differed (Table 1). Over the entire range of concentrations studied, (+)-verapamil was more highly bound ($P < 0.001$) than the (−)enantiomer. The mean free fraction of (+)- and

Table 1. Free fraction (fu) of (+)- and (−)-verapamil (v) in solutions of purified human serum albumin (40 g/L), α_1-acid glycoprotein (0.55 g/L) and fresh human serum. Total pre-dialysis verapamil concentrations ranged from 0.055 to 22 μM and total equilibrium verapamil concentrations ranged from 0.04 to 17 μM

Pre-dialysis concentration μmol/L	Albumin		α_1-acid glycoprotein		Serum	
	(+)v fu	(−)v fu	(+)v fu	(−)v fu	(+)v fu	(−)v fu
0.055	n.d.*	n.d.	0.077	0.144	0.088	0.138
0.110	0.391	0.619	0.052	0.118	0.089	0.115
0.220	0.427	0.539	0.104	0.125	0.102	0.123
0.440	0.421	0.570	0.073	0.132	0.089	0.139
1.100	0.411	0.549	0.068	0.148	0.085	0.138
1.650	0.378	0.542	0.078	0.159	n.d.	n.d.
2.200	0.332	0.609	0.092	0.180	0.092	0.140
5.500	0.383	0.568	0.086	0.131	0.107	0.134
11.00	0.407	0.548	0.156	0.198	0.105	0.141
16.50	0.430	0.598	0.233	0.270	n.d.	n.d.
22.00	0.415	0.577	0.298	0.367	0.139	0.202

* Not determined.

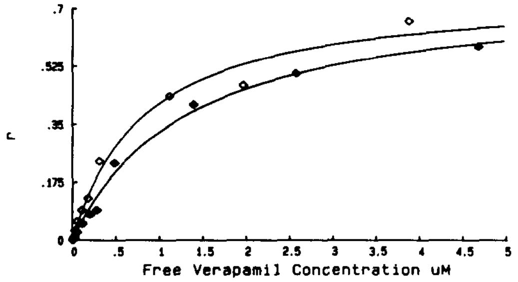

Fig. 1. The binding of (+)-(◇) and (−)-(◆) verapamil to purified α_1-acid glycoprotein (0.55 g/L). Pre-dialysis verapamil concentrations ranged from 0.1 to 22 μM and free verapamil concentrations at the end of dialysis ranged from 0.003 to 4.7 μM. The curves shown are the binding isotherms obtained using Eqn (1) and assuming one class of α_1-acid glycoprotein binding sites. From these relationships the number of binding sites per mole of α_1-acid glycoprotein and the association constants for (+)- and (−)-verapamil were calculated. The number of moles of verapamil bound per mole of α_1-acid glycoprotein is designated *r*.

(−)-verapamil at concentrations less than 10 μM was 0.096 ± 0.009 and 0.136 ± 0.006 respectively. At therapeutic verapamil concentrations the free fractions of both enantiomers were similar in serum and solutions of α_1-acid glycoprotein (Table 1). However, unlike the binding of verapamil with α_1-acid glycoprotein, the free fraction in serum increased only at the highest equilibrium verapamil concentration (16 μM), suggesting that serum binding sites were not as readily saturated.

Ex vivo *study*

The free fractions of (+)-verapamil and (−)-verapamil in the serum of five volunteers prior to and following oral and intravenous administration of equal amounts of labelled (+)-verapamil and unlabelled (−)-verapamil are given in Table 2. In all serum samples the free fraction of (−)-verapamil was higher ($P < 0.05$) than that of (+)-verapamil. Following *in vitro* addition of 0.44 nmol of pseudoracemic verapamil to predose serum, the free fraction of (+)-verapamil (0.066 ± 0.011) was lower ($P < 0.05$) than that of (−)-verapamil (0.120 ± 0.020). The *in vitro* serum binding of the individual enantiomers when added together in serum as pseudoracemic verapamil is the same as that determined previously *in vitro* with the isolated enantiomers. The binding *ex vivo* in each subject following separate intravenous administration of the enantiomers [(+)-verapamil 0.064 ± 0.011, (−)-verapamil 0.120 ± 0.022] is also identical with that observed *in vitro* in the same subject using pseudoracemic verapamil. However following oral administration of the drug the free fraction of both (+)-verapamil (0.130 ± 0.022) and (−)-verapamil (0.226 ± 0.032) was substantially greater ($P < 0.05$).

DISCUSSION

Stereoselective verapamil binding has been observed in serum *in vitro* and *ex vivo* and in solutions of purified α_1-acid glycoprotein and albumin. The free fraction of the pharmacologically more potent (−)-verapamil is always greater than that of (+)-verapamil and for both enantiomers is independent of verapamil concentration over the therapeutic range. The binding of the individual enantiomers within this concentration range is the same for both (+)- and (−)-verapamil when studied either separately or as the pseudoracemate. Consequently the binding of each individual enantiomer is not affected by the presence of the other enantiomer. The free fraction of both enantiomers observed in serum from five subjects *ex vivo* after intravenous administration (Table 2) or in the *in vitro* studies (Table 1) is similar to that noted previously *ex vivo* [11] in healthy subjects following the intravenous

Table 2. Serum protein binding of d_2(+)-verapamil[(+)V] and (−)-verapamil[(−)V] in healthy subjects following *in vitro* addition of pseudoracemic verapamil to predose serum and *ex vivo* in samples collected after both intravenous and oral administration of both enantiomers

	Predose *in vitro*		Intravenous *ex vivo*		Oral *ex vivo*	
Subject	(+)V	(−)V	(+)V	(−)V	(+)V	(−)V
TK	0.07	0.11	0.05	0.09	0.14	0.23
ML	0.07	0.15	0.06	0.13	0.13	0.21
PE	0.08	0.13	0.08	0.15	0.10	0.21
JS	0.05	0.11	0.06	0.12	0.16	0.28
CL	0.06	0.10	0.07	0.11	0.12	0.20
Mean	0.07	0.12	0.06	0.12	0.13	0.23
±SD	0.01	0.02	0.01	0.02	0.02	0.03

administration of (+)-verapamil (fu = 0.063 ± 0.022) and (−)-verapamil (fu = 0.115 ± 0.016). The lower serum binding of (−)-verapamil presumably contributes to the larger volume of distribution of this enantiomer [11].

The binding of verapamil with albumin is not saturable over the range of concentrations studied, suggesting that the binding sites are of high capacity and relatively low affinity. Enantioselective albumin binding of both acidic and basic drugs has been reported previously [2]. Both verapamil enantiomers are bound to a greater extent to α_1-acid glycoprotein than albumin and the binding sites on the acute phase protein could be characterised in further detail. The number of binding sites for both (+)- and (−)-verapamil was similar (0.76). A noninteger number of binding sites on the α_1-acid glycoprotein molecule has been previously observed for other basic compounds including imipramine [20] and lignocaine [21]. Present data suggest that basic drugs bind to a single common binding site on the α_1-acid glycoprotein molecule [22] and the verapamil data substantiate results with propranolol and disopyramide which indicate that drug binding to this site can be stereoselective [23–25]. The binding affinity for (+)-verapamil was greater than that of the (−) enantiomer.

Using the mean data of the observations from the *in vitro* investigations, the ratio of the free fraction to the bound fraction, of the more highly relative to the more weekly bound isomer was calculated. The enantioselective ratio was similar for verapamil binding with albumin (2.01) and α_1-acid glycoprotein (1.97); however, it was lower in fresh serum (1.47). The lower ratio observed in fresh serum suggests that factors in addition to albumin and α_1-acid glycoprotein concentrations influence verapamil serum binding. For example verapamil may associate with further proteins, such as lipoproteins [6], in a nonstereoselective manner.

For both enantiomers the free fraction in serum is similar in the same subject when determined after *in vitro* addition or *ex vivo* after intravenous administration; however, the free fraction is higher after oral administration (Table 2). As extensive verapamil metabolism occurs during absorption and the first-pass through the liver, verapamil metabolites are formed in greater amounts following oral administration [3]. The protein binding of only a small number of verapamil metabolites has been examined [4, 5]. Norverapamil appears not to influence the binding of verapamil; however, other metabolites have not been studied in detail. It is apparent, however, that the free fraction of verapamil determined either *in vitro* or *ex vivo* following intravenous administration does not represent the free fraction following oral drug administration. Verapamil free fraction should therefore be determined *ex vivo* in samples obtained from subjects during administration of the dosage form being studied. Verapamil free fraction cannot be extrapolated from data obtained in the same subject using other routes of administration. The addition factors which influence verapamil binding after oral therapy have as yet to be identified; however, higher serum concentrations of verapamil metabolites following oral drug administration may be responsible.

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REFERENCES

1. Williams K and Lee E, Importance of drug enantiomers in clinical pharmacology. *Drugs* **30**: 333–354, 1985.
2. Drayer DE, Pharmacodynamic and pharmacokinetic differences between drug enantiomers in humans: an overview. *Clin Pharmacol Ther* **34**: 718–723, 1983.
3. Schomerus M, Spiegelhalder B, Stieren B and Eichelbaum M, Physiological disposition of verapamil in man. *Cardiovasc Res* **10**: 605–612, 1976.
4. Yong CL, Kunka RL and Bates TR, Factors affecting the plasma protein binding of verapamil and norverapamil in man. *Res Commun Chem Pathol Pharmacol* **30**: 329–339, 1980.
5. Keefe DL, Lee YG and Kates RE, Verapamil protein binding in patients and in normal subjects. *Clin Pharmacol Ther* **29**: 21–26, 1981.
6. McGowan FX, Reiter MJ, Pritchett ELC and Shand DG, Verapamil plasma binding: relationship to α_1-acid glycoprotein and drug efficacy. *Clin Pharmacol Ther* **33**: 485–490, 1983.
7. Giacomini KM, Massound N, Wong FM and Giacomini JC, Decreased binding of verapamil to plasma proteins

in patients with liver disease. *J Cardiovasc Pharmacol* **6**: 924–928, 1984.

8. Piafsky KM, Disease-induced changes in the plasma binding of basic drugs. *Clin Pharmacokinet* **5**: 246–262, 1980.
9. Routledge PA, The plasma protein binding of basic drugs. *Br J Clin Pharmacol* **22**: 499–506, 1986.
10. Vogelgesang B, Echizen H, Schmidt E and Eichelbaum M, Stereoselective first-pass metabolism of highly cleared drugs: studies of the bioavailability of L- and D-verapamil examined with a stable isotope technique. *Br J Clin Pharmacol* **18**: 733–740, 1984.
11. Eichelbaum M, Mikus G and Vogelgesang B, Pharmacokinetics of (+)-, (−)- and (±)-verapamil after intravenous administration. *Br J Clin Pharmacol* **17**: 453–458, 1984.
12. Echizen H, Vogelgesang B and Eichelbaum M, Effects of d,l-verapamil on atrioventricular conduction in relation to its stereo-selective first-pass metabolism. *Clin Pharmacol Ther* **38**: 71–76, 1985.
13. Spiegelhalder B and Eichelbaum M, Determination of verapamil in human plasma by mass-fragmentography using stable-labelled verapamil as internal standard. *Arzneimittel-Forsch* **27**: 94–97, 1977.
14. Kuwada M, Tateyama T and Tsutsumi J, Simultaneous determination of verapamil and its seven metabolites by high-performance liquid chromatography. *J Chromatogr* **222**: 507–511, 1981.
15. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, Protein measurement with the folin-phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Lima JJ, MacKichan JJ, Libertin N and Sabino J, Influence of volume shifts on drug binding during equilibrium dialysis: correction and attenuation. *J Pharmacokinet Biopharm* **11**: 483–498, 1983.
17. Peck CC and Barrett BB, Nonlinear least-squares regression programs for microcomputers. *J Pharmacokinet Biopharm* **5**: 537–541, 1979.
18. Fletcher JE, Ashbrook JD and Spector AA, Computer analysis of drug-protein binding data. *Ann NY Acad Sci* **226**: 69–81, 1973.
19. Siegel S, *Nonparametric Statistics for the Behavioural Sciences*. McGraw-Hill Kogakusha Ltd., Tokyo, 1956.
20. Kornguth ML, Hutchins LG and Eichelman BS, Binding of psychotropic drugs to isolated α_1-acid glycoprotein. *Biochem Pharmacol* **30**: 2435–2441, 1981.
21. Krauss E, Polnaszek CF, Scheeler DA, Halsall HB, Eckfeldt JH and Holtzman JL, Interaction between human serum albumin and α_1-acid glycoprotein in the binding of lidocaine to purified protein fractions and sera. *J Pharmacol Exp Ther* **239**; 754–759, 1986.
22. Muller WE and Stilbauer AE, Characterisation of a common binding site for basic drugs on human α_1-acid glycoprotein. *Naunyn-Schmiedeburgs Arch Pharmacol* **322**: 170–173, 1983.
23. Walle UK, Walle T, Bai SA and Olanoff LS, Stereoselective binding of propranolol to human plasma, α_1-acid glycoprotein and albumin. *Clin Pharmacol Ther* **34**: 718–723, 1983.
24. Albani F, Riva R, Contin M and Baruzzi A, Stereoselective binding of propranolol enantiomers to human α_1-acid glycoprotein and human plasma. *Br J Clin Pharmacol* **18**: 244–246, 1984.
25. Lima JJ, Jungbluth GL, Devine T and Robertson LW, Stereoselective binding of disopyramide to human plasma protein. *Life Sci* **35**: 835–839, 1984.