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Stereoselective binding of chiral anti-diabetic drug nateglinide to plasma proteins

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Abstract

The binding of nateglinide (NA) enantiomers with human plasma (HP), human serum albumin (HSA) and bovine serum albumin (BSA) was investigated. The protein binding was studied over a drug concentration range of 5–100 μM at a protein concentration of 600 μM. Unbound drug concentrations were determined by direct chiral liquid chromatography using chiralcel OJ-RH column. At therapeutic drug concentrations, the protein binding of each enantiomer was >98%. The results showed that the binding of NA enantiomers was stereoselective, mutually competitive and non-linear. The binding characteristics were, however, opposite for the two most important plasma binding proteins. Opposite stereoselectivity was observed between BSA and HSA while stereoselectivity was identical between HSA and HP. Scatchard analysis was used to illustrate the different binding affinities of NA enantiomers to BSA, HSA and HP. The interaction between enantiomers observed in HP and serum albumins was confirmed as a competitive type interaction at the high affinity site. Scatchard analysis was used to illustrate the different binding affinities of NA enantiomers to BSA, HSA and HP.

Keywords: enantiomer; nateglinide; plasma protein binding.

Introduction

Plasma protein binding of drugs is a reversible and kinetically rapid process, which plays an important role in pharmacokinetics and pharmacodynamics of drugs (1–3). The protein binding of enantiomers of a chiral drug is potentially different, because of the inherent differences in protein affinity for them (4, 5). Enantioselective drug action and disposition may be due to the difference in the serum protein binding of the enantiomers (6). Nateglinide (NA) is a chiral novel oral glucose regulator, which was recently approved for the treatment of type 2 diabetes mellitus (7, 8). The drug increases insulin release from the pancreatic β-cells though inhibition of ATP-dependent potassium channels. The R-NA (Figure 1) has been in the market because it exhibits a 20% blood glucose decrease at an oral dose of 1.6 mg kg^{-1}. However, the S-enantiomer requires a dose of more than 100 mg kg^{-1} to show equal potency (9). Peak plasma concentrations of single therapeutic dose (120 mg) are reached after 0.5–1.0 h after oral administration and the elimination half-life of NA is approximately 1.4 h (10).

NA is approximately 99% bound to plasma proteins (11, 12) and it can be stereoselective as a consequence of selective transport characteristics of R-NA and S-NA in the duodenal, jejunal and ileal segments of rat intestine (13). The difference in the protein binding may influence the bioavailability, pharmacokinetics and pharmacological activity of the individual R- and S-enantiomers. Recently protein binding of NA in human plasma (HP) albumin has been studied by high performance capillary electrophoresis frontal analysis (14) but no report so far has been published on the protein binding of NA individual enantiomers. Therefore, in this experiment we have studied the influence of stereoselectivity on the protein binding of racemic NA and its individual enantiomers. Experiments were carried out to discover the binding of NA enantiomers when they bind individually and from the racemate.

Materials and methods

Reagents

R-NA was kindly provided by Aurobindo Pharma. Ltd. (Hyderabad, India). S-NA was synthesized in the Department of Pharmaceutical Chemistry, School of Pharmacy, Jena, Germany. Human serum albumin (HSA) (fatty acid free) and bovine serum albumin (BSA) (fatty acid and globulin free) were purchased from Sigma-Aldrich Logistic GmbH, Schnelldorf, Germany. HP was supplied by the Institute for Transfusion Medicine, Friedrich Schiller University, Jena, Germany. Buffers and solutions were prepared with double deionized water (MilliQ, Millipore, Billerica, MA, USA) and were filtered through a membrane filter (0.45 μm) before use.

Stock and standard solutions

Stock solutions of NA racemate and enantiomers were prepared by dissolving the required amount of racemate and individual enantiomers in phosphate buffer solution (67 mM, pH 7.4) to yield concentration of 1 mM. Standard solutions for calibration curves were

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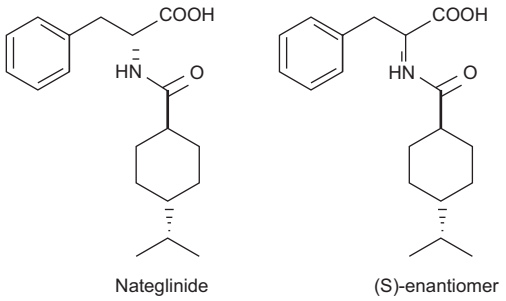

Figure 1 Structure of nateglinide and its enantiomer.

prepared by diluting the appropriate amounts of each stock solution respectively. All the solutions were stored in the standard flasks and wrapped with aluminum foil and kept in a refrigerator at 4–8°C. The calibration curve was used for the estimation of protein binding in the test samples.

HPLC analysis

The Shimadzu HPLC system (Kyoto, Japan) consisting of an LC-10AS HPLC pump and SPD-10A UV/VIS detector, and an SK-10A Auto injector with a 20 µL loop was used for the resolution of NA enantiomers. The class LC 10 Software version 1.6 (Shimadzu) was used for data analysis. Chiralcel OJ-RH column (150×4.6 mM, 5 µ) was delivered with mobile phase consisting of a solution of 100 mM potassium dihydrogen phosphate, pH 2.5, and acetonitrile (68:32 v/v), at a flow rate of 1 mL min^{-1}. The temperature of 33°C was maintained constant throughout the experiment for good separation. Analytes were monitored at 210 nM. The injected volume was 20 µL.

Protein binding studies

A rotative dianorm equilibrium dialyser (Bachofer Laboratories, Reutlingen, Germany) consisting of two Teflon microcells, each separated by dialysis membrane (12,000 molecular weight cut-off), was used to study the protein binding of racemic NA and individual enantiomers. The dialysis membrane was initially boiled for 5 min in double distilled water. After two sequential rinses in double distilled water, the sacks were placed in isotonic buffer (pH 7.4) for 10 min before being cut and fitted between 1 mL Teflon dialysis cells. The whole equilibrium dialyzer was placed in a temperature controlled water bath at 37°C and the cells were rotated about their axis at a speed of 20 rpm. Buffer solutions of racemic NA, R-NA and S-NA were mixed appropriately with HP, BSA and HSA solutions to yield a final concentration of 600 µM of proteins and 5, 10, 20, 50, and 100 µM of racemic NA, R-NA and S-NA, respectively.

In vitro samples (0.5 mL of protein+0.5 mL of test compound) in the donor compartment were dialyzed against drug free phosphate buffer (1 mL, 0.67 mM, pH 7.4) in the receiver compartment with constant stirring until the equilibrium was achieved. Following an equilibration time of 10 h, the drug in the receiving compartment was collected and frozen at –20°C until the analysis. Concentrations of racemate and individual enantiomers of NA in the dialysates were determined by enantioselective HPLC assay as described in the earlier section. The bound concentration of racemate and individual enantiomers was calculated according to

$$\% \text{ binding}=(Ct-Cu/Ct)\times 100 \quad [1]$$

$$C_u=Ct-C_b \quad [2]$$

where T denotes total drug concentration introduced into the system and F denotes free drug concentration. These values were averaged then used to calculate the bound concentrations of racemate and individual enantiomers. The binding parameters were determined according to the following scatchard equation (15):

$$r/C_u=-Kr+nK \quad [3]$$

where r is the number of mols of bound drug per mol protein, C_u is the free drug concentration, C_b is the bound drug concentration, K is the binding constant and n the number of binding sites. The equivalent binding affinity k_A derived from the following formula was used to determine the binding affinity to serum albumin under the assumption that binding occurs exclusively to albumin.

$$\text{Log } k_A=[\log \{(f_b)/1-(f_b)\}]-[\log (\text{HSA})] \quad [4]$$

The experimental data were fitted by non-linear least-square regression analysis.

Statistical analysis

Data are expressed as mean±SD. Statistical comparisons between the R-NA and S-NA group or rac-NA group were performed by paired t-test. A probability of p=0.05 was considered statistically significant.

Results

Enantioselective analysis and method validation

The separation of the enantiomers of NA by HPLC has been achieved using Chiralcel OJ-RH (13) column. Following initial screening using various pHs and temperatures, pH 2.5 was selected and a temperature of 33°C gave baseline separation of racemic NA with a resolution of R_s=2.4. (R)-NA (Rt: 15.9 min) migrated before the (S)-NA (Rt: 16.9) (Figure 2A). The method was subsequently validated using racemic NA with respect to linearity and precision. Under the above experimental conditions the calibration lines were obtained by measuring the peak area of R- and S-NA standard solutions of 1, 5, 10, 25, 50 and 100 µM. Calibration curves were linear for both enantiomers with regression coefficient of at least r^2=0.997. The linear regression equations for R-NA and S-NA were y=19054x–11034 and y=20215x–10178, respectively, where y represents the peak area and x represents sample concentration. All coefficients of variation (CV) were below 10%, indicating the precision of the present method. Concentrations falling above the range were diluted and analyzed. No degradation of NA during analysis and in the experiment indicated the stability of the compound under experimental conditions.

Drug protein binding to serum albumins and human plasma

The results for the binding of NA enantiomers by BSA, HSA and HP as determined by equilibrium dialysis of the proteins in the presence of racemic NA or individual enantiomers are summarized in Table 1 and Figures 3–5. Figure 2B shows a chromatogram of unbound NA enantiomers in an incubation of 50 µM racemic NA in the presence of 600 µM BSA.

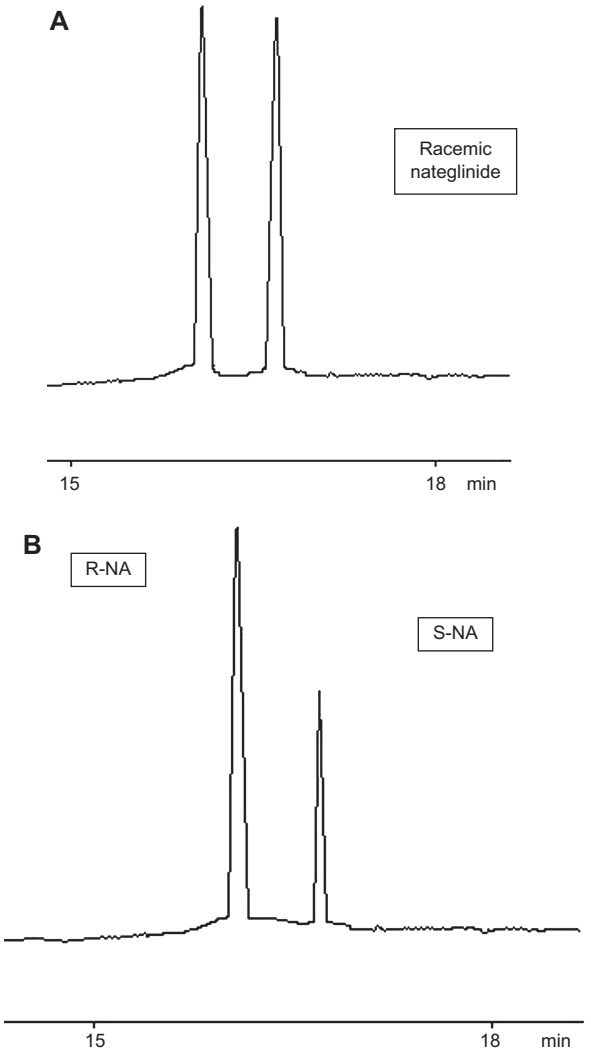

Figure 2 Chromatograms of (A) racemic nateglinide and (B) incubations of 50 µM of racemic nateglinide in the presence of 600 µM BSA.
For experimental conditions, see experimental part.

The protein binding of enantiomers was stereoselective and saturated at the higher concentration tested, as can be seen from Figures 3 and 4. For all proteins, binding decreased with increased concentration of NA (Table 1). BSA displayed stereoselectivity for the enantiomers, with S-NA being bound stronger compared with R-NA either incubated with the single enantiomer or with the racemate. In contrast, HSA and HP showed opposite stereoselectivity. It was clear that the percentage of bound drug did not appear to increase linearly with the unbound drug concentration over the concentrations studied. The binding of both enantiomers to serum albumins and HP is dependant on NA concentration.

The protein binding of enantiomers measured with the racemate was lower than the protein binding of single enantiomers incubated individually, indicating that enantiomers from racemate may have competitive interaction at the binding site. Specific binding constants for the HSA, BSA and HP could not be derived exactly from the scatchard plots (Figures 3

Table 1 Protein binding and the approximate effective binding affinity of Rac-NA, R-NA and S-NA to BSA, HSA and HP.

Concentration, µM	Protein	R-NA	S-NA	R/S	Log k_A		R-NA (rac)	S-NA (rac)	R/S	Log k_A	
					R-NA	S-NA				R-NA	S-NA
5	BSA	99.02±0.21	99.51±0.04	0.995	2.20	2.51	99.06±0.35	99.76±0.10	0.992	2.26	2.70
	HSA	99.60±0.10	98.86±0.06	1.007	2.50	2.17	99.17±0.84	97.89±0.12	1.013	2.21	1.73
	HP	99.70±0.31	98.51±0.43	1.012	–	–	99.05±1.07	98.14±2.27	1.009	–	–
10	BSA	98.80±0.03	99.50±0.08	0.992	2.13	2.49	98.79±1.09	99.34±0.06	0.994	2.10	2.21
	HSA	99.30±0.19	98.98±0.63	1.004	2.27	2.18	98.91±1.43	96.00±1.20	1.030	2.05	1.60
	HP	99.60±0.53	99.01±0.98	1.005	–	–	99.06±0.32	96.41±1.63	1.027	–	–
20	BSA	98.35±2.04	98.99±1005	0.993	1.98	2.17	98.09±0.76	98.95±1.04	0.991	1.77	1.93
	HSA	98.78±0.93	98.04±0.48	1.007	2.10	1.91	98.89±0.47	95.98±2.02	1.030	1.63	1.49
	HP	98.88±1.36	98.16±1.54	1.007	–	–	98.66±0.64	96.07±1.41	1.026	–	–
50	BSA	97.00±2.09	98.66±1.86	0.983	1.72	2.06	96.02±1.54	97.80±1.08	0.981	1.60	1.72
	HSA	98.10±1.35	97.64±2.12	1.004	1.93	1.82	97.10±2.30	94.36±3.02	1.029	1.42	1.29
	HP	98.79±0.76	97.01±0.32	1.018	–	–	97.59±2.12	93.00±1.09	1.049	–	–
100	BSA	85.75±3.91	88.4±0.98	0.970	0.99	1.09	81.02±5.12	85.05±3.12	0.952	0.84	0.97
	HSA	88.50±1.65	85.34±1.08	1.037	1.10	1.58	83.70±3.20	77.66±6.09	1.075	0.81	0.75
	HP	88.95±4.06	85.78±3.12	1.020	–	–	83.64±7.70	79.64±8.12	1.055	–	–

The data are expressed as percent bound drug (mean±SD, n=6).

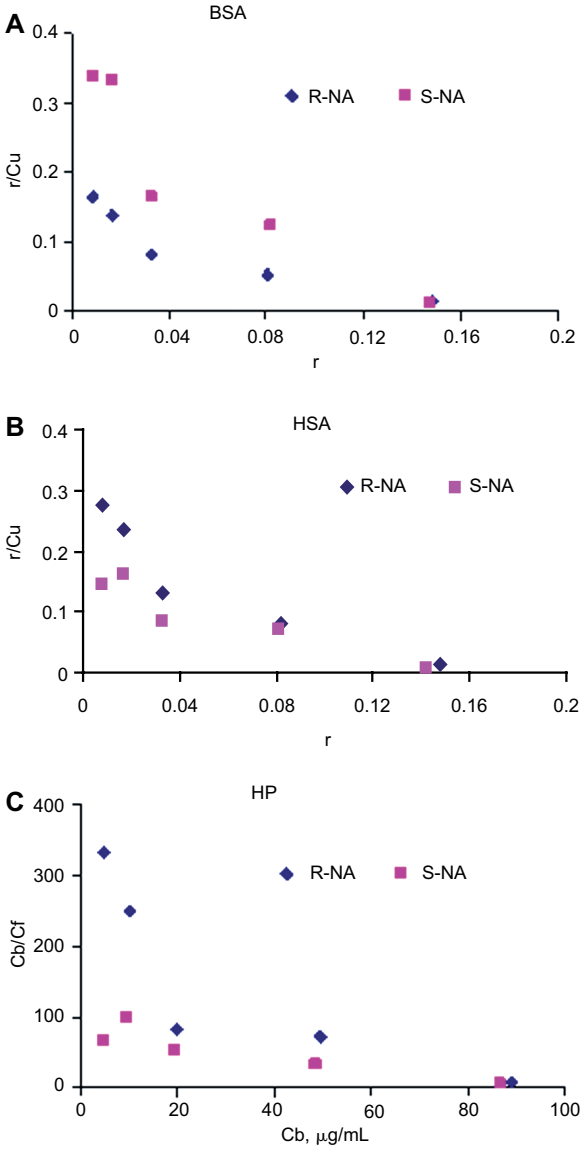

Figure 3 Scatchard plots of the binding of (R)-nateglinide and (S)-nateglinide individually.
The data points are expressed as mean of six separate experiments; the standard deviations are smaller than the symbols.

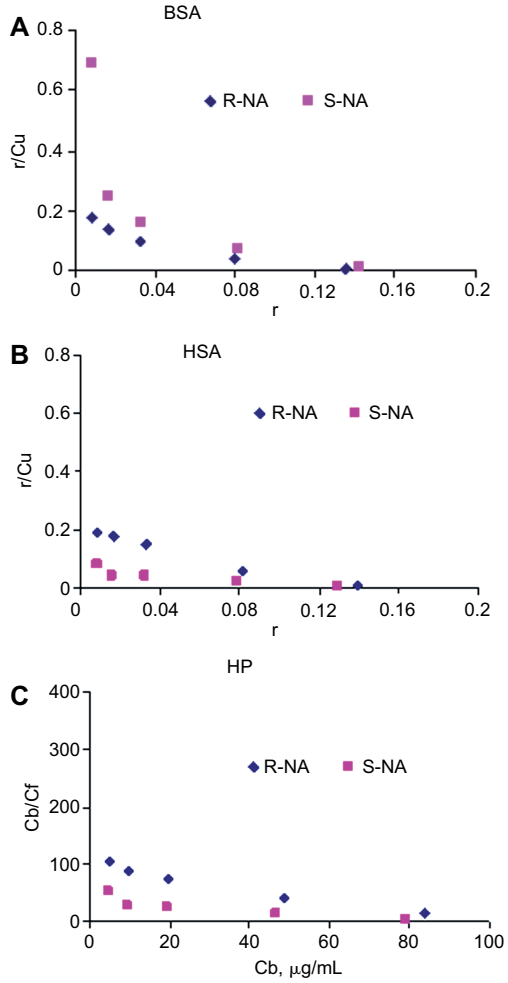

Figure 4 Scatchard plots of the binding of (R)-nateglinide and (S)-nateglinide from the racemate.
The data points are expressed as mean of six separate experiments; the standard deviations are smaller than the symbols.

and 4) because of the biphasic course of the curves. From the scatchard curve, each NA enantiomer interacted with two classes of binding sites: one with high affinity and small binding capacity, the other with low affinity and high binding capacity. NA mainly interacted with the former (which is the main cause for the stereoselective binding) at lower concentrations. As the drug concentration increased, the saturation of binding at the high affinity binding site led to bind with low affinity binding site, which usually has little stereoselectivity. Figure 5 shows a comparison of independently determined primary association constants of R-NA and S-NA to HSA and BSA. The data are reasonably well correlated with scatchard data and would allow us to differentiate the critical binding of highly bound NA enantiomers of a very narrow range.

Discussion

The protein binding of NA enantiomers upon incubation of racemate and of pure R-, S-NA has been investigated by equilibrium dialysis. A stereoselective HPLC assay was used to allow the determination of both enantiomers simultaneously. A previous study has analyzed the binding of R-NA to HP albumin solution by non-stereoselective high performance capillary electrophoresis frontal analysis by Yanyan et al. (14). Neither of the assays allowed us to studythe binding of NA enantiomers indivually or from the racemate. The reported binding rate was increased with the increase of protein concentration when the NA concentration is fixed. On the contrary, when the protein concentration is fixed, the binding rate decreased with the increased NA concentration. The binding values are not in good agreement with our values. The difference in the binding affinity between results reported by other authors and presented here could be due to different techniques used. It is well known that the racemate and enantiomers of meglitinide

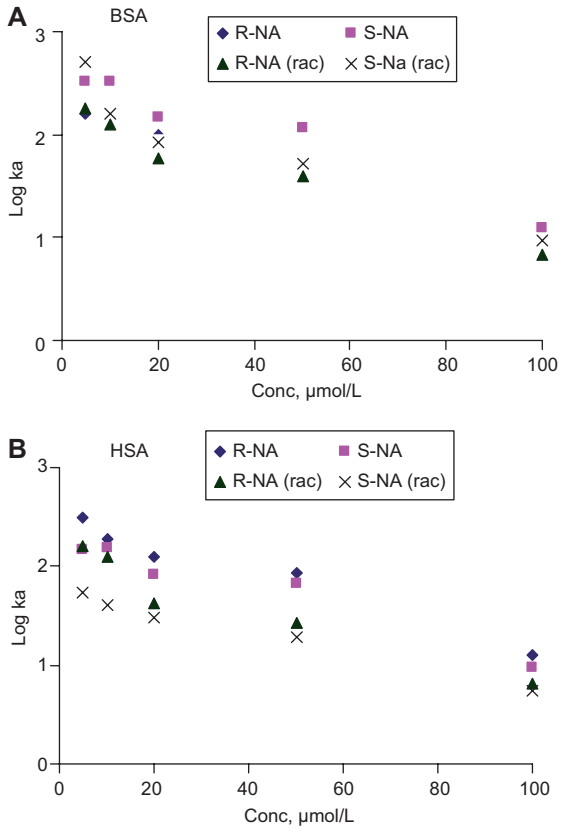

Figure 5 Comparison of approximate effective binding affinity of NA enantiomers in BSA, HSA and human plasma incubated with the single enantiomer and racemate (n=3).
The approximate binding affinity was calculated from percentage protein binding.

type antidiabetics are strongly bound to HSA stereoselectively (16).

After incubation of individual enantiomers and racemic NA in solutions of BSA, HSA and in the HP, it could be demonstrated that the binding of one enantiomer was affected by the presence of the other enantiomer, indicating that binding competition between enantiomers may occur at the high affinity binding site, while being independent from each other at the low affinity binding site. Figures 3 and 4 show the protein binding of enantiomers measured with individual enantiomers and racemate. Both R- and S-enantiomers in racemate decreased more or less, when compared with the data from incubation with enantiomers separately, indicating that S- and R-NA may have competitive interaction at the binding site. It should also be noted that the slopes of the scatchard plots of both enantiomers measured in racemate were quite different from those with R-NA or S-NA alone.

The stereoselective binding of NA enantiomers with lower drug concentration is significant, whereas at higher concentration it is insignificant when studied either invidually or as the racemate. This effect was more obvious when comparing the amounts of free fractions which increased proportionally by increasing drug concentrations from 5 µM to 100 µM using a fixed protein concentration of 600 µM. The smaller amounts of free R- and S-NA in the case of HP may have been caused by the presence of other proteins like globulins. Nevertheless, as shown in Figures 3 and 4, the descending parts of the BSA and HSA curves indicated the presence of approximately one specific saturable binding site.

Conclusions

The protein binding of NA is concentration dependant and stereoselective. Plasma levels of the pharmacologically active enantiomer R-NA would be affected significantly by plasma protein binding. So it seems evident that stereoselectivity in protein binding of NA depends significantly on the kind of protein. The recognized differences in the binding behavior of R- and S- enantiomers and the competition between them are important findings, since there is always a need to extrapolate the experimental data in order to predict the situation in vivo.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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