Differences in the serum binding determinants of isradipine and darodipine—consequences for serum protein binding in various diseases

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1 Serum protein binding of isradipine and darodipine, and serum concentrations of α_1 -acid glycoprotein (AAG), albumin (HSA) and non-esterified fatty acids (NEFA) were measured in three groups of patients, I: healthy subjects ($n = 20$); II: patients with inflammatory disorders ($n = 15$) and III: patients with hepatic insufficiency ($n = 17$).

2 AAG was increased significantly in group II patients ($P < 0.001$) and decreased in group III patients ($P < 0.001$); HSA was decreased significantly in group II and group III patients ($P < 0.001$).

3 The free percentage of isradipine was decreased significantly in group II patients ($P < 0.05$) and increased in group III patients ($P < 0.05$) and multivariate analysis showed that these variations were inversely related to changes in AAG concentration.

4 The free percentage of darodipine was increased significantly in group II and III patients ($P < 0.05$) due to a decrease in HSA concentration, as shown by multivariate analysis.

5 The changes in free serum percentages of isradipine and darodipine were inversely related to concomitant changes in the concentration of the serum protein for which they showed the highest affinity, AAG for isradipine and HSA for darodipine, respectively.

6 The unexplained variability in the binding data was greater when AAG was the major determinant of binding (isradipine).

Keywords isradipine darodipine protein binding α_1 -acid glycoprotein hepatic insufficiency inflammatory disorders

Introduction

Albumin and α_1 -acid glycoprotein (AAG) are the major binding proteins for drugs in plasma (Fehske *et al.*, 1980; Routledge, 1986a). The variation in plasma binding of drugs is generally mediated by variations in albumin concentration for acidic drugs and by variations in AAG concentration for basic drugs (Routledge, 1986b). Isradipine (PN 200-110) and darodipine (PY 108-068) are two calcium channel blockers related to dihydropyridines (Hof *et al.*, 1982,

1984). Their chemical structures are similar and they are neither acidic nor basic drugs. In a previous study, we have shown that these drugs were bound to AAG with very different association constants and to albumin with comparable affinities (Pinquier *et al.*, 1988). In keeping with these observations, it is possible that the determinants of variability in plasma protein binding of these drugs may differ.

The purpose of this study was to investigate

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the influence of inflammatory disorders and liver insufficiency on the serum protein binding of isradipine and darodipine. We also measured serum concentrations of AAG, albumin and non-esterified fatty acids (NEFA) in order to define quantitative relationships between these variables and serum binding of the drugs.

Methods

Subjects and sample collection

Sera from 52 healthy individuals and patients were studied.

Group I ($n = 20$). Twenty healthy subjects (10 men and 10 women) 19 to 60 years of age with no clinical or biochemical signs of illness and free of drug therapy.

Group II ($n = 15$). Eleven men and four women with inflammatory disorders defined by an erythrocyte sedimentation rate up to 50 mm h⁻¹ for more than 8 days. These patients had histologically proven neoplasia, temporal arteritis, polymyalgia rheumatica or rheumatoid arthritis. Their ages varied between 18 and 84 years. The serum sample was taken before the diagnosis, when the patient was receiving only simple analgesics, such as paracetamol, amido-pyrine or codeine.

Group III ($n = 17$). Eight men and nine women with histologically verified cirrhosis of the liver and hepatocellular insufficiency defined by a reduction in coagulation factor V to 50% of normal. The ages of the patient were between 39 and 71 years.

In both groups II and III, no patient was taking drugs known to be highly bound to AAG or HSA. Patients receiving acidic anti-inflammatory, anti-psychotic or steroid drugs were excluded.

Blood was drawn from all fasting participants into vacuum glass tubes between 07.00 h and 08.00 h. Samples were allowed to clot for 1 h and then centrifuged to yield serum. The separated serum was frozen at -30°C until used.

AAG and HSA concentrations were measured using Beckman kits (Ref. 662996 and 663010) with a Beckman Immunochemistry System ICS (Beckman Instruments, 93220, Gagny, France) based on laser nephelometry. The proteins are detected on the basis of changes in light-scattering properties due to formation of soluble immune complexes following the addition of specific antibody. Non-esterified fatty acids (NEFA) were measured by an enzymatic method (NEFA C kit, ref. 46551, Biolyon, 69572, Dardilly, France). Bilirubin was measured by a standard enzymatic method.

Drugs

Radiolabelled isradipine ($[^{14}\text{C}]$ -PN 200-110, 15.1 Ci mol⁻¹) and darodipine ($[^{14}\text{C}]$ -PY 108-068-n, 10.7 Ci mol⁻¹) were provided by Sandoz (Basel, Switzerland) and were stored as a solution in methanol at -30°C until used. The radiochemical purities of $[^{14}\text{C}]$ -isradipine and $[^{14}\text{C}]$ -darodipine were assessed by thin layer chromatography on silica plates with the following solvent system: methanol-water-conc. ammonia (70:30:1, vol/vol). The radiochemical purity of the stock solution was more than 98% and no change was observed in either the dialysate or dialysed serum after equilibrium dialysis.

Binding experiments

Three aliquots (0.2 ml) of each individual serum sample were added to Teflon cells (0.25 ml/side) and dialysed against Sørensen's buffer (67 mM pH 7.1, containing 500 nmol l⁻¹ of radiolabelled isradipine or darodipine ('therapeutic concentration', approximately 50 nmol l⁻¹, Internal Report, Sandoz, Basel, Switzerland). The dialysis was performed at 37 $^{\circ}\text{C}$ for 3 h with constant stirring (20 rev min⁻¹) and without apparent accumulation of fluid on the serum side of the dialysis chamber. The two chambers were separated by a semi-permeable membrane (Visking, pore diameter 15–20 Å, cutoff 12,000 Daltons).

Previous studies showed that equilibrium with respect to the free drug fraction was achieved within 2 h. As observed previously the pH of frozen serum samples was approximately 8 (Brørs & Jacobsen, 1985). Preliminary studies showed that dialysing the serum against buffer at pH 7.1 maintained a physiological pH in the system ($7.35 < \text{pH} < 7.40$). The drugs did not bind to the dialysis apparatus.

At equilibrium the radioactivity in each compartment was measured by liquid scintillation counting (Packard, Tricarb 460 CD).

Statistical analysis of results

Comparisons between means (control subjects and patients, men and women) were performed by standard two-way analysis of variance (ANOVA).

The free drug fraction (f_u) was calculated by division of the drug concentration in buffer by the concentration in serum at the end of dialysis. An analysis was also carried out to determine whether drug serum binding could be described by a linear model. The serum binding of isradi-

pine and darodipine was calculated in terms of the binding ratio of the molar concentration of bound (B) to free (F) drug, which should be linearly related to the binding protein concentration provided the constant of dissociation of the drug-protein complex is much higher than the free drug concentration. The analysis was done with a stepwise multiple linear regression technique using the following model:

$$\frac{B}{F} = \Sigma[(nK)_i \cdot P_i] + \Sigma[b_j \cdot V_j] + k \quad (1)$$

The terms $(nK) \cdot P$ represent a formulation in which the total binding constant (nK , product of the number of binding sites and the association constant) derived from the population is assumed to describe fully the relationship between P , the protein concentration and B/F . Since other variables (V), such as NEFA concentration or age have been shown to affect serum binding, the regression terms $b \cdot V$ were added. The ordinate intercept was k . A multiple linear regression with covariance analysis was also carried out to determine whether the multiple regression equations were significantly different between the groups (comparison of slopes, nK , and of ordinate intercepts, k).

Results

Laboratory tests and clinical details

No significant difference was apparent between sexes in the mean concentrations of AAG, HSA, NEFA and bilirubin (ANOVA). Therefore, the data for both sexes were combined. Patient *vs* control comparisons were made by the non-parametric test of Mann Whitney and the means and s.d. are summarized in Table 1.

The mean age in group III patients was greater statistically than that of the control group ($P < 0.05$). As expected, AAG was increased in inflammatory disorders and decreased in hepatic insufficiency; HSA was decreased in group II and III patients and to a greater extent in group III patients. Bilirubin concentrations were higher in group III patients.

Serum binding of isradipine and darodipine

The free percentage of isradipine was significantly lower in group II and higher in group III as shown in Table 1, whereas the free percentage of darodipine was significantly higher in groups II and III. The binding ratios of isradipine and darodipine were correlated positively with AAG and HSA concentrations, but not with age, bilirubin or NEFA (Table 2).

Multiple linear regression analysis of the data for all individuals indicated that the binding ratios of isradipine and darodipine were related significantly to AAG and HSA concentrations, but NEFA and bilirubin concentrations, and age were not significant determinants of binding (Table 3).

The partial regression coefficients for AAG and HSA shown in Table 3 are the apparent total binding constants for isradipine and darodipine. These estimates of nK for the interaction of the drugs with the proteins were clearly different between isradipine and darodipine.

Multiple linear regression with covariance analysis showed that the partial regression coefficients (slopes nK) were not significantly different between the groups and the surfaces described by them could be considered as parallel. Moreover the B/F intercepts (k) were not significantly different between the groups.

Table 1 Free serum percentages of isradipine and darodipine (500 nmol l^{-1}) and biological and clinical data of the groups studied (means $\pm$ s.d.).

	Control subjects (n = 20)	Patients with inflammatory disorders (n = 15)	Patients with hepatic insufficiency (n = 17)
% free isradipine	2.51 ± 0.60	$1.93 \pm 0.36^*$	$3.85 \pm 1.28^*$
% free darodipine	3.05 ± 0.40	$3.63 \pm 0.6^*$	$5.14 \pm 1.20^*$
AAG ($\mu\text{mol l}^{-1}$) ^a	23.5 ± 6.3	$47.4 \pm 12.8^{**}$	$7.7 \pm 4.4^{**}$
HSA ($\mu\text{mol l}^{-1}$) ^a	647 ± 59	$449 \pm 97^{**}$	$364 \pm 84^{**}$
NEFA ($\mu\text{mol l}^{-1}$)	491 ± 165	$570 \pm 370^{\text{NS}}$	$512 \pm 183^{\text{NS}}$
Bilirubin ($\mu\text{mol l}^{-1}$)	5.88 ± 3.66	$5.20 \pm 4.67^{\text{NS}}$	$74.4 \pm 65.7^{**}$
Age (years)	40.3 ± 12.1	$48.7 \pm 21.3^{\text{NS}}$	$52.3 \pm 9.2^*$

^aMolecular weights were assumed to be 66,000 Daltons for HSA and 40,000 Daltons for AAG.

^{NS}Not significantly different, $^*P < 0.05$, $^{**}P < 0.001$ (Mann Whitney test, patients *vs* controls).

Table 2 Linear correlation coefficients for biological variables, age and isradipine and darodipine binding ratio in all patients studied ($n = 52$)

Variable	AAG	HSA	NEFA	Age
AAG	1			
HSA	-0.179	1		
NEFA	+0.186	+0.087	1	
Age	+0.068	-0.226	-0.011	1
Isradipine binding ratio	+0.717**	+0.326*	+0.020	+0.042
Darodipine binding ratio	+0.435**	+0.732**	+0.126	-0.221

* $P < 0.01$; ** $P < 0.001$.**Table 3** Regression statistics for the relationships between isradipine and darodipine binding ratios (bound/free) and AAG and HSA concentrations in all of the sera studied ($n = 52$)

Drug	Independent variable	Partial regression coefficient ($l \mu\text{mol}^{-1}$) (estimate $\pm$ s.d.)	F ^{1,49} value	P value
Isradipine*	AAG	0.520 ± 0.075	47.5	< 0.001
	HSA	0.0193 ± 0.009	4.26	< 0.05
Darodipine#	AAG	0.120 ± 0.042	12.2	< 0.001
	HSA	0.032 ± 0.004	56.5	< 0.001

*Multiple $r = 0.7418$; ordinate intercept = 17.18 ± 4.8 (F value = 12.62, $P < 0.001$).#Multiple $r = 0.7951$; ordinate intercept = 7.57 ± 2.2 (F value = 11.76, $P < 0.001$).

Discussion

The differences in AAG and HSA plasma levels between the patients groups are in agreement with numerous other reports (Tillement *et al.*, 1978; Piafsky, 1980; Routledge, 1986b). AAG was increased in inflammatory disorders and decreased in hepatic insufficiency; HSA was decreased in both conditions. Although group III patients differed in age from the control group, the modifications observed in protein concentrations were related to the pathological condition rather than to the difference in age. AAG concentration should be increased in the oldest group, group III (Routledge, 1986b), but we observed the reverse.

It may, therefore, be assumed that the protein binding differences between the groups was related to their pathological state.

Multivariate analysis indicated that AAG is the main determinant of isradipine serum binding, whereas HSA is the main determinant of darodipine serum binding (Table 3). The results showed that AAG was responsible for 51.4% of the variability in the serum binding of isradipine

and that HSA accounted for only 10.6% of the variability. In contrast, HSA was responsible for 53.5% of the variability in the serum binding of darodipine, whereas AAG explained only 19% of the variability. The partial regression coefficients shown in Table 3 were very close to the binding parameters that were determined with isolated serum proteins in a previous study (Pinquier *et al.*, 1988). In this report, the nK values for AAG and HSA were $0.498 \pm 0.105 \text{ l } \mu\text{mol}^{-1}$ and $0.034 \pm 0.006 \text{ l } \mu\text{mol}^{-1}$, respectively, for isradipine, and $0.155 \pm 0.077 \text{ l } \mu\text{mol}^{-1}$ and $0.053 \pm 0.001 \text{ l } \mu\text{mol}^{-1}$, respectively, for darodipine.

The non-zero values of the ordinate intercepts indicate that other proteins must influence the serum binding of the drugs. According to the results of our previous study (Pinquier *et al.*, 1988), these are probably lipoproteins. Some of the variability in serum binding was not explained by AAG and HSA (38% for isradipine and 27.5% for darodipine). This may be explained, in part, by the variability necessarily added to

the data by binding to lipoproteins, and certain unmeasurable factors, such as differences in the structures of the binding proteins related to the pathological conditions (Rudman *et al.*, 1972). The covariance analysis did not indicate significant differences in nK between the groups, which would have confirmed differences in the properties of the proteins. However, qualitative differences are likely to exist between the groups, especially for AAG which has been shown to be a highly variable protein (Tinguely *et al.*, 1985; Pedersen *et al.*, 1987). The greatest unexplained variability in serum binding of isradipine (38%) compared with that of darodipine (27.5%) would then be the consequence of the greater contribution of AAG to the serum binding of isradipine.

As seen in Table 1, the serum binding of isradipine in the group of patients with inflammatory disorders was significantly higher than that in the two other groups. The increase in serum binding in this group may be reasonably ascribed to their high AAG concentrations relative to other groups, despite a net decrease in HSA concentration. This observation of increased

drug binding in inflammatory disorders has been observed for many basic drugs (Routledge, 1986b; Urien *et al.*, 1988). The serum binding of darodipine in group II and III patients was significantly lower than that in the control group (Table 1). This difference is probably related to the low HSA concentrations in groups II and III relative to group I, despite an appreciable increase in AAG concentration in group II.

Given the similar chemical structures of the drugs (they are optical isomers), similar serum binding might have been anticipated. On the contrary, our results show that AAG is the major determinant of isradipine binding to serum, as shown for most basic drugs (Piafsky *et al.*, 1978; Routledge, 1986b) and that HSA is the major determinant of darodipine binding to serum, as shown for most acidic drugs (Wanwimolruk *et al.*, 1982; Urien *et al.*, 1986).

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