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The effect of ageing on plasma albumin and plasma protein binding of diazepam, salicylic acid and digitoxin in healthy subjects and patients with renal impairment

A. VIANI[1], G. RIZZO[2], M. CARRAI[3] & G. M. PACIFICI[1]
Departments of [1]Biomedicine, Medical School, University of Pisa and [2]Nephrology and [3]Gastroenterology, Hospital of Pisa, Pisa, Italy

1 Plasma albumin concentration was measured in 118 healthy subjects (aged between 18 and 87 years), in 95 renal patients with creatinine clearances between 15 and 50 ml min^{-1} (aged between 14 and 79 years) and in 101 uraemic patients maintained on chronic haemodialysis (aged between 27 and 83 years).

2 There was a significant ($P < 0.001$) negative correlation between albumin concentration and age in healthy subjects, but no correlation in patients with low creatinine clearance or in uraemic patients.

3 The *ex vivo* plasma binding of diazepam (1 μM), salicylic acid (2 mM) and digitoxin (37 nM) was studied in groups of age-selected young and aged healthy subjects in patients with low creatinine clearance and in patients with uraemia. The unbound fractions of diazepam and salicylic acid were about double in old compared with young healthy subjects whereas they were similar in young and old patients with lowered creatinine clearance. In uraemic patients, ageing did not affect the binding of salicylic acid whereas the unbound fraction of diazepam was slightly but significantly greater in elderly subjects. The unbound fraction of digitoxin was independent of age in both healthy subjects and in those with renal disease.

4 Decreased plasma binding of diazepam and salicylic acid was partially corrected by extensive dialysis of plasma. The lower plasma binding of diazepam and salicylic acid associated with ageing may be ascribed to the effects of endogenous displacers and to hypoalbuminaemia. The influence of these two factors appears to be drug-dependent

Keywords binding diazepam salicylic acid digitoxin ageing renal failure

Introduction

Several studies (Andreasen, 1973; Borga *et al.*, 1976; Pacifici *et al.*, 1986, 1987; Veering *et al.*, 1990; Wallace & Whiting, 1976) have shown that hypoalbuminaemia is associated with both old age and renal disease. However, it is not known whether elderly renal patients have lower plasma albumin concentrations than healthy elderly subjects. We therefore measured the concentration of albumin in plasma from renal patients and healthy subjects as a function of age.

The plasma binding of drugs is also lower in the elderly (Davis *et al.*, 1985; Wallace & Verbeeck, 1987) and in patients with renal disease (Andreasen, 1974; Borga *et al.*, 1976; Henderson & Lindup, 1990; Kober *et al.*, 1979; Levy, 1979; Reidenberg & Drayer, 1984; Sjoholm *et al.*, 1976; Tiula *et al.*, 1987). Therefore, a second aim of this study was to define how ageing influences the plasma binding of drugs in patients with renal disease.

A classification of drug binding sites on human albumin (Sjoholm *et al.*, 1979; Tillement *et al.*, 1984) is based upon the binding of diazepam, warfarin and digitoxin (Sjoholm *et al.*, 1979). Salicylic acid binds to the warfarin site (Sjoholm *et al.*, 1979). Therefore, our study considered the binding of diazepam, salicylic acid and digitoxin.

Correspondence: Professor Gian Maria Pacifici, Department of Biomedicine, Medical School, via Roma 55, 1-56100 Pisa, Italy

Methods

Chemicals

[^{3}H]-Diazepam (specific activity 83.1 Ci mmol^{-1}), [^{3}H]-digitoxin (specific activity 15.8 Ci mmol^{-1}) and [^{14}C]-salicylic acid (specific activity 58.2 mCi mmol^{-1}) were obtained from Du Pont De Nemours, NEN Products Division (Florence, Italy). As received, the radiochemical purities of diazepam and salicylic acid were greater than 99% whereas that of digitoxin was 98.5%. After a few months of storage at −25° C, [^{3}H]-diazepam required purification which was performed by t.l.c. The chromatography was performed on 19-channel silical-gel plates (LK5DF, Whatman, Milan, Italy) using chloroform/acetone (9:1 v/v) as the eluent. The final radiochemical purity of [^{3}H]-diazepam was greater than 99%. Unlabelled salicylic acid and human serum albumin (lot 115 5-9330, product No 1883) were purchased from Sigma (St Louis, MO, USA). Unlabelled diazepam and digitoxin were generously supplied by Roche (Milan, Italy) and Nativelle (Florence, Italy), respectively.

Plasma samples

One hundred and eighteen healthy subjects aged between 18 and 87 years and 196 renal patients were included in the study. Of the latter, 95, aged between 14 and 79 years, had a creatinine clearance ranging between 15 and 50 ml min^{-1}. In the text, they are referred to as 'patients with renal failure'. The clearance of creatinine was measured using the following equation:

$$\text{Creatinine clearance} = (C_{ur} \cdot Q_{ur})/C$$

where C_{ur} and C are the concentrations of creatinine in urine and plasma, respectively and Q_{ur} is the urine flow rate.

The remaining 101 patients, aged between 27 and 83 years, had uraemia and were maintained on chronic haemodialysis. Blood samples (10 ml) were taken from an antecubital vein at 08.00 h after an overnight fast. In the patients maintained on chronic haemodialysis samples were taken just before haemodialysis. Blood was transferred into tubes containing 2 drops of heparin (5,000 u ml^{-1}) and centrifuged at 1000 *g* for 20 min. The plasma was collected and stored at −80° C for up to 6 months. Albumin concentration was measured by immunodiffusion according to Mancini *et al.* (1965) using nor-partigen SLB 03 plates obtained from Istituto Behring (Scoppito, Italy).

Dialysis of plasma

Two pools of plasma were made by combining 2 ml of plasma from individual samples from 10 young and 10 elderly blood donors who were not taking any medication. Aliquots (10 ml) of the pools were transferred to Visking® dialysis tubing (1-8/32″; molecular weight cutoff 5 KDalton; Medicell International Ltd, London, UK) and dialysed against 50 volumes of 0.067 M sodium phosphate buffer (pH 7.4) containing 1 g l^{-1} albumin for 18 h. Dialysis was carried out in an ice-bath with continuous stirring as described by Viani & Pacifici (1990). After dialysis, the plasma, referred to as 'dialysed plasma', was used for measuring the binding of diazepam and salicylic acid. Control plasma was kept on ice for 18 h. Binding was studied in quadruplicate both in control and in dialysed plasma.

Ultrafiltration

Pooled plasma (18 ml) was transferred to 'Centrifugal Ultrafree' cones (Millipore, Bedford, MA, USA) and centrifuged at 2500 *g* for 30 min. The plasma remaining in the filter was transferred to another cone and ultrafiltered again. This procedure was repeated four times until the volume of plasma water recovered was 13–15 ml, corresponding to 72–83% of the initial volume of plasma. Commercial human albumin was dissolved in the ultrafiltrate to give a final concentration of 40 g l^{-1} and the binding of diazepam (1 μM) and salicylic acid (2 mM) was measured.

Measurement of binding

Aliquots (0.7 ml) of plasma, or albumin solution were dialysed against the same volume of 0.067 M sodium orthophosphate buffer (pH 7.4) containing one of the following drugs: diazepam (1 μM), salicylic acid (2 mM) and digitoxin (37 nM). Dialysis was performed at 37° C for 4 h in a Diachema apparatus (Dianorm, Munich, Germany). Dialysis membranes (molecular weight cutoff 5 KDalton) were obtained from Dianorm (Munich, Germany). Diazepam was added in 20 μl ethanol to 20 ml of buffer and digitoxin was added in 20 μl dimethyl sulphoxide to 20 ml of buffer. These volumes of solvent had no effect on drug binding.

Statistical analysis

Differences between mean values were assessed by Student's *t*-test.

Results

There was a significant negative correlation between albumin concentration and age in healthy individuals but no correlation was observed in patients with renal failure or uraemia (Figure 1).

Table 1 summarises data on the plasma binding of diazepam in young and elderly healthy subjects and patients. The percentage of unbound diazepam was significantly greater in old compared with young healthy subjects. In contrast, old and young patients with renal failure had similar percentages of unbound diazepam. A slight but significantly higher percentage of unbound diazepam was found in old compared with young uraemics maintained on chronic haemodialysis.

Table 2 shows the percentage of unbound salicylic acid in healthy subjects and patients. Values were significantly greater in elderly compared with young healthy subjects whereas no difference was obtained as a function of age in patients with renal failure or in uraemics. The binding of digitoxin was not influenced

Table 1 Unbound percentage of diazepam in the plasma of healthy subjects and patients

	Healthy subjects		*Patients with renal failure*		*Patients maintained on chronic haemodialysis*	
	Young A	*Elderly* B	*Young* C	*Elderly* D	*Young* E	*Elderly* F
n	20	18	13	13	14	14
Age (years) (mean ± s.d.)	37 ± 10	78 ± 7	35 ± 10	65 ± 6	42 ± 6	69 ± 4
Albumin concentration ($g\ l^{-1}$) (mean ± s.d.)	49.5 ± 8.1	36.3 ± 8.4	37.8 ± 7.0	37.9 ± 7.6	46.4 ± 5.2	43.3 ± 9.8
	$P < 0.001$		NS		NS	
% Unbound diazepam (mean ± s.d.)	2.3 ± 0.6	4.7 ± 0.7	4.2 ± 0.5	4.4 ± 0.7	4.6 ± 0.8	5.4 ± 1.1
	$P < 0.001$		NS		$P < 0.05$	

% Unbound diazepam: A is different from C ($P < 0.001$); A is different from D ($P < 0.001$); A is different from E ($P < 0.001$); B is not different from C, D, E and F.

Table 2 Unbound percentage of salicylic acid in the plasma of healthy subjects and patients

	Healthy subjects		*Patients with renal failure*		*Patients maintained on chronic haemodialysis*	
	Young A	*Elderly* B	*Young* C	*Elderly* D	*Young* E	*Elderly* F
n	20	14	17	13	14	14
Age (years) (mean ± s.d.)	37 ± 10	75 ± 7	34 ± 11	67 ± 5	42 ± 6	69 ± 4
Albumin concentration ($g\ l^{-1}$) (mean ± s.d.)	49.5 ± 8.1	37.7 ± 7.5	37.8 ± 7.0	37.9 ± 7.6	46.4 ± 5.2	43.3 ± 9.8
	$P < 0.001$		NS		NS	
% Unbound salicylic acid (mean ± s.d.)	19.6 ± 3.1	30.9 ± 6.0	26.0 ± 5.4	29.3 ± 5.1	34.2 ± 4.6	36.3 ± 5.1
	$P < 0.001$		NS		NS	

% Unbound salicylic acid: A is different from C D, E and F ($P < 0.001$); B is not different from C, D, E and F.

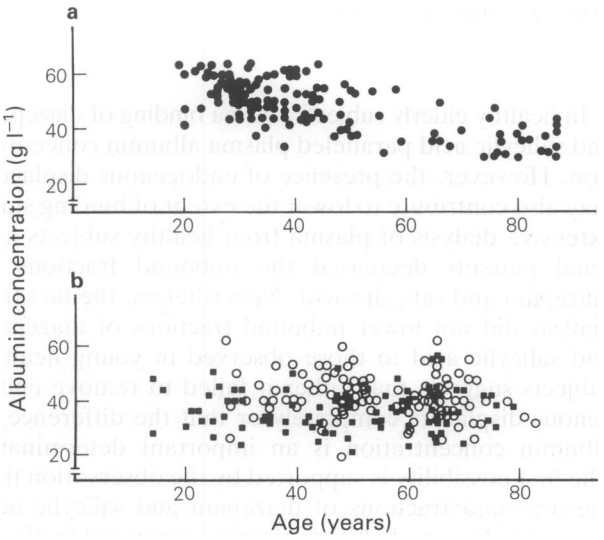

Figure 1 Plasma albumin concentration *vs* age in a) healthy subjects and b) renal patients. The open circles and squares refer to uraemic patients maintained on chronic haemodialysis and patients with low creatinine clearance, respectively. Albumin concentration correlates with age in a) ($P < 0.001$) but not in b).

by age either in healthy subjects or in renal patients (Table 3).

The percentages of unbound diazepam, salicylic acid and digitoxin in young healthy subjects were significantly lower than those in young and aged patients with renal failure and uraemia. The binding of diazepam and salicylic acid in plasma was no different in young and aged patients with renal failure or in young and aged patients with uraemia.

Extensive dialysis of plasma increased the binding of diazepam and salicylic acid. However, the percentages of unbound drug remained lower in young healthy subjects compared with those in the other groups (Table 4). These findings suggest that extensive dialysis failed to remove endogenous displacers completely and/or that albumin concentration is an important determinant of the binding of diazepam and salicylic acid. The latter possibility was verified by studying binding to purified human albumin. At a concentration of 37 $g\ l^{-1}$ albumin the percentages of unbound diazepam and salicylic acid were 0.71 ± 0.05 and 18.76 ± 1.32, respectively, whereas, at 50 $g\ l^{-1}$ albumin they were 0.58 ± 0.01 and 10.47 ± 0.20, respectively. Thus, by increasing the albumin concentration, the percentages of unbound

Table 3 Unbound percentage of digitoxin in the plasma of healthy subjects and patients

	Healthy subjects		*Patients with renal failure*		*Patients maintained on chronic haemodialysis*	
	Young A	*Elderly* B	*Young* C	*Elderly* D	*Young* E	*Elderly* F
n	20	14	17	13	14	14
Age (years) (mean ± s.d.)	36 ± 10	73 ± 8	36 ± 10	68 ± 5	42 ± 6	69 ± 4
Albumin concentration ($g\ l^{-1}$) (mean ± s.d.)	51.5 ± 8.3	37.9 ± 6.9	37.8 ± 7.0	37.5 ± 7.4	46.4 ± 5.2	43.3 ± 9.8
	$P < 0.001$		NS		NS	
% Unbound digitoxin (mean ± s.d.)	4.1 ± 0.5	4.5 ± 0.9	4.6 ± 0.7	4.7 ± 0.8	5.2 ± 1.0	5.9 ± 0.7
	NS		NS		$P < 0.05$	

% Unbound digitoxin: A is different from C ($P < 0.01$); A is different from D ($P < 0.02$); A is different from E and F ($P < 0.001$); B is different from E ($P < 0.05$); B is different from F ($P < 0.001$); B is not different from C and D.

Table 4 The effect of extensive dialysis on the binding of diazepam (1 μM) and salicylic acid (2 mM) in plasma from healthy subjects and renal patients

	Young subjects			*Elderly subjects*		
	Before dialysis	*After dialysis*	*'Before–After'*	*Before dialysis*	*After dialysis*	*'Before–After'*
Diazepam (% unbound)						
Healthy subjects	1.56 ± 0.36	0.95 ± 0.17[a]	0.61	2.59 ± 0.56	1.19 ± 0.09[b]	1.40
Patients with renal failure	2.10 ± 0.11	1.29 ± 0.10[c]	0.81	2.53 ± 0.19	1.27 ± 0.07[c]	1.26
Chronic uraemics on haemodialysis	2.67 ± 0.28	1.46 ± 0.17[b]	1.21	4.02 ± 0.15	2.40 ± 0.07[c]	1.62
Salicylic acid (% unbound)						
Healthy subjects	18.89 ± 1.41	15.94 ± 0.17[b]	2.95	39.43 ± 1.42	35.32 ± 0.95[b]	4.11
Patients with renal failure	30.16 ± 0.89	25.29 ± 1.55[b]	4.20	29.49 ± 1.41	23.81 ± 2.01[b]	5.68
Chronic uraemics on haemodialysis	32.25 ± 2.79	27.18 ± 4.42	5.07	34.21 ± 4.99	29.89 ± 1.21	4.32

Level of significance between 'before' and 'after' dialysis. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

diazepam and salicylic acid decreased by 22% and 79%, respectively.

The percentages of unbound diazepam and salicylic acid in solutions of albumin (40 $g\ l^{-1}$) in plasma ultrafiltrate obtained from young and old healthy subjects were 0.81 ± 0.07 (young) and 0.89 ± 0.02 (old) (NS) and 18.8 ± 0.28 (young) and 17.9 ± 0.32 (old) (NS), respectively.

Discussion

Plasma albumin concentration decreases with age and in renal disease (Gugler & Azarnoff, 1976; Lindup, 1987; Wallace & Verbeeck, 1987). It might therefore have been expected that elderly renal patients would have lower plasma albumin concentrations and show less plasma drug binding compared with healthy elderly subjects. Our findings indicate that this is not so.

In healthy elderly subjects plasma binding of diazepam and salicylic acid paralleled plasma albumin concentration. However, the presence of endogenous displacers may also contribute to lower the extent of binding since extensive dialysis of plasma from healthy subjects and renal patients decreased the unbound fractions of diazepam and salicylic acid. Nevertheless, the fact that dialysis did not lower unbound fractions of diazepam and salicylic acid to those observed in young healthy subjects suggests that either it failed to remove endogenous displacers completely or that the difference in albumin concentration is an important determinant. The first possibility is supported by the observation that the unbound fractions of diazepam and salicylic acid were greater in dialysed plasma compared with an equimolar solution of albumin in buffer. It was clear that the binding of diazepam was more influenced than that of salicylic acid by extensive dialysis. The two drugs bind to different albumin sites and their binding may be inhibited by different displacers.

Albumin concentration is a determinant of the extent of the plasma binding of the drugs studied although it is more important for salicylic acid than for diazepam. This finding may reflect the different affinities of the two ligands for albumin and/or the difference in the molar ratio of albumin and the two ligands. The association constant of diazepam (Kober *et al.*, 1978; Lucas *et al.* 1986; Muller & Wollert, 1973; Viani *et al.*, 1991) is two orders of magnitude greater than that of salicylic acid (Viani *et al.*, 1991; Zaroslinski *et al.*, 1974). The concentration of albumin was a quarter of that of salicylic acid, whereas albumin was in great excess compared with diazepam. It is likely that hypoalbuminaemia associated with ageing or renal disease accounts, at least in part, for the decreasing plasma binding of salicylic acid whereas it has a modest effect on the binding of diazepam.

The mechanism of changes in plasma drug binding associated with ageing and renal disease is multifactorial. Hypoalbuminaemia and endogenous displacers are two factors and they contribute to different extents to the decreased binding of diazepam and salicylic acid. The unbound fractions of diazepam and salicylic acid were similar in old healthy subjects and in young and old renal patients. The unbound fractions of diazepam, salicylic acid and digitoxin were significantly lower in young healthy subjects than in young and aged renal patients.

The concentration of digitoxin used in the present study was about four orders of magnitude lower than the physiological concentration of albumin. Thus, binding sites for this drug are in great excess and differences in albumin concentration as well as the presence of endogenous displacers would be expected to have little effect on binding. This may explain the lack of a difference in young and elderly healthy subjects and the modest effect of renal failure.

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