Br. J. clin. Pharmac. (1983), **16**, 233–239

DRUG BINDING IN SERA DEFICIENT IN LIPOPROTEINS, ALBUMIN OR OROSOMUCOID

EVA PIKE[1], PETER KIERULF[1], BJØRN SKUTERUD[1], JAN ERIK BREDESEN[1] & PER KNUT M. LUNDE[2]

[1]The Central Laboratory, Ulleval Hospital, University of Oslo, Oslo 1, Norway and [2]Department of Pharmacotherapeutics, University of Oslo, Oslo, Norway

1 The relative role of lipoproteins, albumin and orosomucoid in the serum binding variations of various drugs was examined by separate removal of these proteins.

2 Lipoproteins were removed from serum by ultracentrifugation, albumin by affinity chromatography and orosomucoid by immunoprecipitation.

3 Removal of the lipoproteins did not affect the serum binding of the acidic (phenytoin) and neutral (digitoxin) drugs tested, nor the basic drugs disopyramide, quinidine or propranolol. A reduction in binding of amitryptyline, nortriptyline, doxepin and desmethyldoxepin was observed.

4 Removal of albumin did, with some exception for nortriptyline, not affect the serum binding of the basic drugs tested. A pronounced reduction in the binding of phenytoin and digitoxin was observed.

5 Removal of orosomucoid did not affect the binding of the acidic and neutral drugs tested. A reduction in the binding of all the basic drugs tested was observed, especially for disopyramide whose binding almost disappeared.

6 Quinidine, propranolol, phenytoin and digitoxin all bound to isolated lipoproteins, but the removal of lipoproteins had no effect on the total serum binding for these drugs.

7 Hence, the use of deficient sera provides valuable information as to the quantitative role of the various proteins in drug binding, whereas studies using purified proteins are often necessary to examine the mechanisms of the drug protein interactions.

Keywords drug binding lipoproteins albumin orosomucoid

Introduction

Studies on the binding of drugs to isolated serum proteins may provide information as to the qualitative binding characteristics (Brinkschulte & Breyer-Pfaff, 1980; Nilsen & Jacobsen, 1975). However, due to the functional interplay between serum proteins (Chen & Danon, 1979; Costello *et al.*, 1982) extrapolation from such studies as to the relative quantitative role of the various proteins in the total serum binding capacity must be done with great caution. To avoid these problems we examined the relative role of albumin, lipoproteins and orosomucoid in the serum binding of various basic, neutral and acidic drugs, using a serum pool from which the proteins in question were separately removed. Preliminary data have been presented elsewhere (Pike *et al.*, 1982a).

Methods

Serum pool

A serum pool was obtained from two fasting healthy volunteers by venepuncture. The blood was collected into glass containers, and all contact with plastics and plasticizers was avoided. Serum was stored in glass tubes at −20°C until analysed (within 1 month and re-tested 9 months later). Previous studies (Pike & Skuterud, 1983) revealed no difference in serum binding characteristics before and after freezing.

Drugs

A range of basic drugs was selected in order to compare their relative binding characteristics. In addition,

one neutral and one acidic drug were included. The drugs (and concentrations used) were: amitriptyline (357 nmol/l), nortriptyline (1400 nmol/l), doxepin (357 nmol/l), desmethyldoxepin (370 nmol/l), quinidine (10 μmol/l), propranolol (0.2 μmol/l), disopyramide (10 μmol/l), digitoxin (9.2 nmol/l) and phenytoin (40 μmol/l). Radioactive drugs ([^{14}C]-phenytoin and nortriptyline, the others [^{3}H]-labelled) were used for all drugs except for disopyramide. All radioactive compounds were examined and purified by thin layer chromatography using at least two different solvent systems.

Deficient sera

Lipoprotein deficient serum was prepared by adding solid potassium bromide (KBr) and centrifuging (123 000 *g*) at 4° C for 56 h (Pike *et al.*, 1982b). To correct for the removal of lipoproteins, deficient serum was volume adjusted with Krebs Ringer bicarbonate buffer. A KBr control serum was similarly prepared except that centrifugation was omitted. Prior to binding studies both deficient serum and KBr control serum were dialysed against Krebs Ringer bicarbonate buffer.

Albumin deficient serum was prepared by a modified affinity chromatography technique using Cibacron blue—Sepharose 6B (Travis *et al.*, 1976). Preliminary experiments showed that some of the lipoproteins were always removed together with albumin. This was avoided when lipoprotein deficient serum (44 ml) was applied to the column (2.5 cm × 40 cm) and the column washed with 0.05 M Tris/HCl/0.05 M NaCl, pH 8.0 (3 ml/10 min). Albumin deficient eluates thus prepared, were monitored with OD_{280}, pooled, concentrated (Millipore ® filtration cell with PSED 02510 filters, Bedford, Mass., USA) and dialysed (against 100 volumes of Krebs Ringer bicarbonate buffer pH 7.4). Subsequently this albumin deficient serum was readjusted with the removed lipoproteins (stored at 4° C for 48 h) and diluted with buffer to reach KBr control serum concentrations of lipoproteins (cholesterol and triglycerides) and proteins other than albumin.

Orosomucoid deficient serum was prepared as previously reported (Pike *et al.*, 1981) by adding concentrated anti-human-orosomucoid antibodies from rabbits (DAKO, Copenhagen, Denmark). Pilot experiments using trace amounts of [^{125}I]-orosomucoid showed such sera to contain less than 2% of the original orosomucoid concentration. Orosomucoid deficient control serum was prepared by adding Krebs Ringer bicarbonate buffer to the same volume as concentrated antibodies (= 'diluted control serum', see Table 1a).

Analytical methods

Drug serum binding was measured in duplicate by equilibrium dialysis at 37° C, against Krebs Ringer bicarbonate buffer. The equilibrium pH in serum was 7.4–7.5 (Pike *et al.*, 1981; Pike & Skuterud, 1983). The percentage unbound drug was found by using radioactive labelled drugs and scintillation counting for all drugs, except disopyramide, which was determined by gas chromatography (Bredesen *et al.*, 1982).

Protein concentration measurements and lipid electrophoresis were performed as previously described (Pike & Skuterud, 1982; Pike *et al.*, 1982b). In order to quantitate the low albumin concentration in albumin deficient serum a modified nephelometric method was used. Thus diluted anti-albumin antibodies were boosted with polyethyleneglycol 4% (w/v), enabling concentration measurements of albumin down to 1 mg/l. Serum electrophoresis on agarose and cellulose acetate was performed according to standard methods (Johanssen, 1972; Kohn, 1958).

Control experiments

Stability of isolated lipoproteins Since preparation of albumin deficient serum depends on removal and subsequent restoring of the serum lipoproteins, the binding stability of these isolated lipoproteins was examined for drugs whose binding in total serum is also determined by the lipoprotein content (Pike *et al.*, 1982b).

Regeneration of normal serum Since preparation of deficient sera generally involved various analytical procedures including several separation techniques, concentration, dialysis, storage at 4°C for 48 h, and resubstitution with separated lipoproteins (albumin deficient serum) a regenerated normal control serum was prepared.

Regenerated normal serum was prepared by restoring removed lipoproteins (stored 8 days at 4°C) and albumin (stored 6 days at 4°C) to values equal to the KBr control. The regenerated normal serum was compared to KBr control serum (stored at 4°C for 0 as well as 8 days) with respect to drug binding, total protein, albumin, orosomucoid and lipoprotein concentrations, as well as to electrophoretic behaviour in agarose.

KBr control serum compared to original serum The KBr control serum was compared to the original pooled serum, as well as to original serum, diluted to equal total protein concentration in the KBr control, with respect to drug binding, protein concentrations and electrophoretic behaviour.

The reproducibility of methods Serving as a control for the lipoprotein deficient serum, the albumin de-

ficient serum and the regenerated normal serum, the KBr control serum was examined by drug binding properties after 0, 2 and 8 days storage at 4° C. In addition the KBr serum was re-examined after storing for 9 months at −20°C. The following mean values (± 1 s.d.) of per cent unbound drug were found ($n = 8$): amitriptyline = 9.1% (± 0.2), nortriptyline = 12.2% (± 0.1), doxepin = 22.4% (± 0.5), desmethyldoxepin = 23.9% (± 0.6), quinidine = 21.6% (± 0.5), propranolol = 17.8% (± 1.0), disopyramide = 41.3% (± 2.1), digitoxin = 5.9% (± 0.1) and phenytoin = 17.6 (± 0.3).

There was no difference in protein concentrations or electrophoretic behaviour of the proteins during this period of storage.

Results

The deficient sera presently prepared to evaluate the relative importance of lipoproteins, albumin and orosomucoid in drug binding, all showed a composition compatible with selective removal of the protein in question. Thus, concentration estimates on total protein, albumin, orosomucoid and lipoproteins for these sera compared favourably with their respective controls (Table 1a). Furthermore, qualitative examinations of deficient sera and regenerated normal serum, by agarose gel electrophoresis, demonstrated similar electrophoretic behaviour of the various serum fractions in those sera, as compared to the KBr control serum (Figure 1). The α_1-fraction in the regenerated serum, however, has a slightly more catodic movement than the KBr control fraction.

Lipoprotein deficient serum demonstrated a decrease in serum binding of the tricyclic antidepressant drugs, but no effect for the other drugs (Table 1b). Albumin deficient serum demonstrated a pronounced decrease in serum binding of the neutral drug digitoxin and the acidic drug phenytoin, and a minor decrease in the binding of nortriptyline (Table 1b). The regenerated normal serum generally demonstrated a slightly reduced binding capacity as compared to the KBr control serum (the difference is on the borderline of the analytical error). Orosomucoid deficient serum demonstrated a decrease in serum binding of all basic drugs examined (Table 1b). Furthermore, removal of orosomucoid from serum resulted in disappearance of binding for the basic drug disopyramide (Table 1b), whereas no effect was observed on the acidic and neutral drug tested.

Isolated lipoproteins were suspended in Krebs Ringer bicarbonate buffer to 'reference' serum levels of cholesterol and triglycerides (Table 2a). As shown by protein estimates, agarose gel and lipid electrophoresis, there were no proteins other than lipoproteins present in detectable amounts, and the electrophoretic behaviour of the isolated proteins was similar to that of the lipoproteins in the KBr control serum (Table 2a and Figure 2). Isolated lipoproteins thus suspended, clearly bound or solubilized all drugs tested, except disopyramide (Table 2b). Storage of lipoproteins at 4°C for 48 h did not change the binding capacity towards amitriptyline and nortriptyline (79 and 85% bound before, and 79 and 86% after storage). Thus, the lipoproteins boosted to albumin deficient serum were intact with respect to binding.

When comparing dialysed KBr control serum to original serum, the protein concentrations in the KBr control serum were about 20% lower than in the original serum. Diluting original serum in buffer to the same protein concentration as obtained for the KBr control serum after removal of KBr by dialysis, gave similar reduction in binding for those drugs tested (amitriptyline, nortriptyline and quinidine).

Table 1a Protein (g l) and lipid (mmol/l) concentrations in serum before (control serum) and after (deficient serum) separate removal of lipoproteins, albumin and orosomucoid

	Lipoprotein deficient serum	*Albumin deficient serum*	*KBr* control serum*	*Regenerated** control serum*	*Orosomucoid deficient serum*	*Diluted*** control serum*
Total protein	48	23	49	53	64****	50
Albumin	34	0.2	31	32	35	33
Orosomucoid	0.6	0.7	0.7	0.8	~0	0.8
Cholesterol	0.4	5.6	5.7	5.5	6.5	6.3
Triglycerides	~0	1.2	1.1	1.3	1.2	1.1

* KBr control serum = original serum added KBr and dialysed against Krebs Ringer bicarbonate buffer

** Regenerated control serum = albumin deficient serum spiked with albumin and lipoprotein to KBr control serum concentrations

*** Diluted control serum = original serum added same volume of Krebs Ringer bicarbonate buffer as antihuman orosomucoid used to prepare orosomucoid deficient serum (see **Methods**)

**** Enrichment due to addition of concentrated antibodies (see **Methods** and Figure 1)

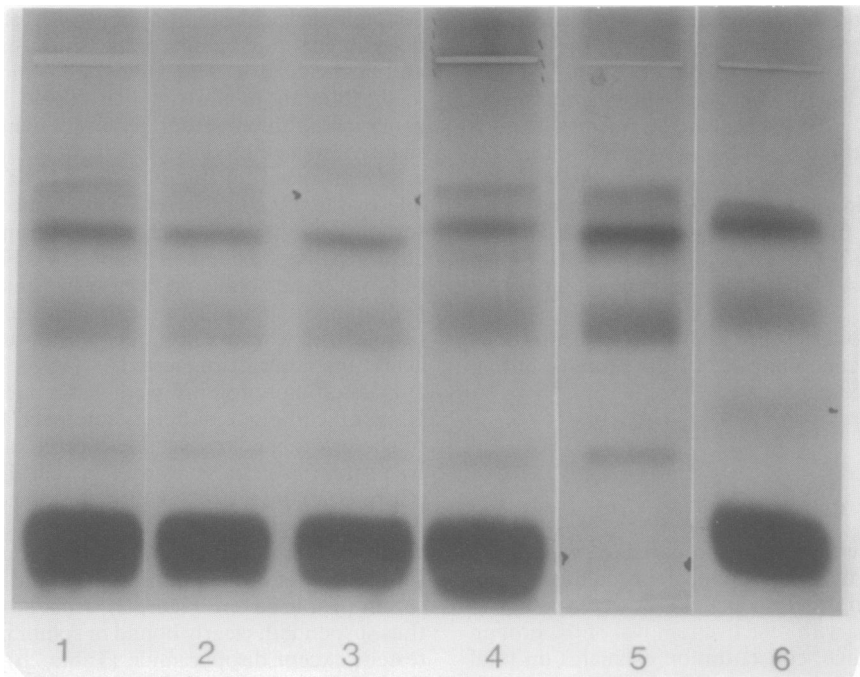

Figure 1 Agarose gel electrophoresis of original serum (1), KBr control serum (2), lipoprotein deficient serum (3), orosomucoid deficient serum (4), albumin deficient serum (5) and regenerated control serum (6).

Discussion

Our findings, based on separate removal of lipoproteins, albumin and orosomucoid from a serum pool, demonstrate that binding characteristics vary between as well as within classes of drugs.

The removal of lipoproteins from serum caused a varying decrease in the binding of the tricyclic antidepressant drugs tested. The effect was greater for amitriptyline and nortriptyline than for doxepin and its demethylated metabolite. This is in accordance with Gescher & Li Wan Po (1978), who found amitriptyline to be more lipophilic than doxepin. In our study nortriptyline was somewhat more dependent upon lipoproteins for serum binding than amitriptyline. This was shown also by Brinkschulte & Breyer-Pfaff (1980). In a previous study (Pike *et al.*, 1982b) we demonstrated a high degree of correlation

Table 1b Effect on serum binding of separate removal of lipoproteins, albumin and orosomucoid. (Single samples analysed in duplicate, for analytical reproducibility, see **Methods**)

	% unbound drug					
	Lipoprotein deficient serum	*Albumin deficient serum*	*KBr control serum*	*Regenerated control serum*	*Orosomucoid deficient serum*	*Buffer diluted control serum*
Amitriptyline	15.6	10.2	9.1	9.8	14.5*	8.9*
Nortriptyline	23	14.8	12.2	13.4	13.3*	11.3*
Doxepin	28	25	22	—	34	24
Desmethyldoxepin	30	26	24	—	34	26
Quinidine	24	24	22	22	51	21
Propranolol	18.7	18.9	17.8	—	44	18.7
Disopyramide	44	43	41	—	98*	44*
Digitoxin	5.8	44	5.9	6.3	5.9	5.5
Phenytoin	18.4	63	17.6	19.6	18.8	18.1

* $n = 4$

Table 2a Protein (g/l) and lipid (mmol/l) concentrations of isolated lipoproteins in Krebs Ringer bicarbonate buffer

Total protein	4
Albumin	0.3
Orosomucoid	< 0.1
Cholesterol	4.4
Triglycerides	0.6

between the serum binding of amitriptyline, nortriptyline and the lipoprotein concentration. Taken together, our present and previous findings substantiate the concept that lipoproteins contribute substantially to the serum binding of amitriptyline and nortriptyline.

The removal of lipoproteins had no effect on serum binding of any of the other drugs tested. It should be noted, however, that all drugs, except disopyramide, demonstrated a pronounced binding to or solubilization in isolated lipoproteins resuspended in Krebs Ringer bicarbonate buffer to 'reference' serum concentration of cholesterol and triglycerides. This is in line with several other authors who have demonstrated binding to lipoproteins of basic drugs in particular (for review see Piafsky, 1980). Thus, Nilsen & Jacobsen (1975) showed that quinidine bound to isolated lipoproteins and Sager *et al.* (1979) found that propranolol was distributed to lipoproteins. The latter concluded, however, that orosomucoid was the serum protein of importance for the binding of propranolol, although a more recent report (Sager *et al.*, 1981) may indicate some role of the lipoproteins as well. As to the importance of lipoproteins for the variation in total serum binding of quinidine the results have been more controversial (Kates *et al.*, 1978; Nilsen *et al.*, 1978; Pike *et al.*, 1982). The present and a previous study (Pike *et al.*, 1982) on quinidine, however, demonstrated that serum lipoproteins have little influence on the total serum binding neither of quinidine nor propranolol, although binding or solubilization of these substances by lipoproteins do occur (Table 1b *vs* Table 2b).

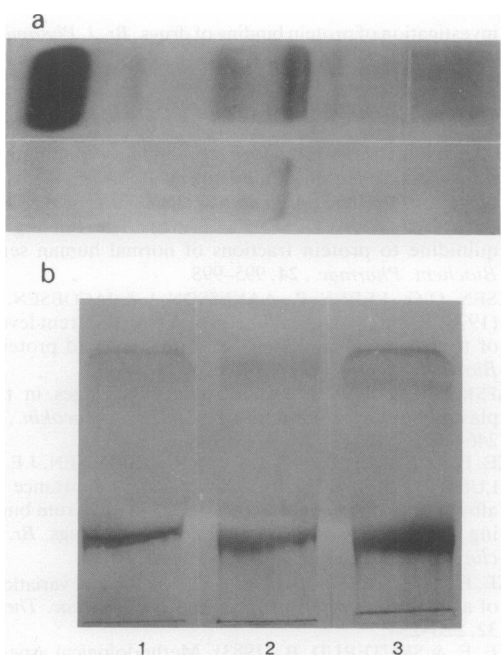

Figure 2 **(a)** Agarose gel electrophoresis of KBr control serum and isolated lipoproteins and **(b)** Lipid electrophoresis of original serum (1), isolated lipoproteins (2) and KBr control serum (3).

Table 2b Drug binding to isolated lipoproteins in Krebs Ringer bicarbonate buffer (single samples analysed in duplicate)

	% unbound drug
Amitriptyline	21
Nortriptyline	14
Doxepin	45
Desmethyldoxepin	32
Quinidine	78
Propranolol	53
Disopyramide	97*
Digitoxin	43*
Phenytoin	55*

* Confirmed in lipoproteins from another serum

At present there is limited information regarding the binding of acidic and neutral drugs to proteins other than albumin. Kramer & Richens (1972) demonstrated that phenytoin binds to β-lipoproteins. Our results show that phenytoin and digitoxin both are substantially bound to or solubilized in isolated lipoproteins, although when lipoproteins were removed from serum, no effect on total serum binding was observed.

As expected, removal of albumin from serum caused a pronounced decrease in serum binding for the acidic and neutral drugs tested. The albumin deficient serum (Table 1b) also gave a small decrease in serum binding for nortriptyline, but hardly any effect for amitriptyline. This may be in agreement with Sharples (1976) who concluded that the major factor influencing the binding of tricyclic drugs to human serum albumin was their ability to form a strong complex with one tryptophan residue in albumin. They suggested that the enhanced affinity for nortriptyline as compared to amitriptyline was due to the greater capacity for the secondary nitrogen of nortriptyline for side chain binding.

The other basic drugs showed no variation in serum binding before and after removal of albumin, although quinidine (Conn & Luchi, 1961), propranolol (Scott *et al.*, 1979), amitriptyline and nortriptyline (Brink-

schulte & Breyer-Pfaff, 1980; Pike & Skuterud, 1982) and to a minor extent, disopyramide (Lima & Salzer, 1981) have been shown to bind to albumin.

Binding to orosomucoid has been shown for many basic drugs (for review, see Piafsky, 1980). In the present study the removal of orosomucoid from serum caused a decrease in serum binding for all basic drugs tested, but not for digitoxin and phenytoin. Disopyramide was unique in that serum binding almost disappeared with the removal of orosomucoid. Disopyramide is known to have a concentration dependent serum binding within concentrations seen after clinical doses (Bredesen *et al.*, 1982). The present results, using orosomucoid deficient serum, indicate that the concentration dependent variations in serum binding for disopyramide might well be due to saturation of the binding capacity of orosomucoid.

Our results also demonstrate that amitriptyline and nortriptyline depend both on lipoproteins and orosomucoid for their variations in serum binding. Upon removal of orosomucoid serum binding of amitriptyline decreased more than for nortriptyline, indicating that amitriptyline is more prone to bind to orosomucoid than nortriptyline. This is in agreement with Brinkschulte & Breyer-Pfaff (1980). The binding of a basic drug to an acidic protein might suggest a binding of ionic character. The removal of the sialic acid residues from orosomucoid, however, gave only a small increase in unbound fraction of amitriptyline (Pike *et al.*, unpublished results). The secondary amine nortriptyline is also slightly more basic than the tertiary amine amitriptyline. Hence, more studies on the nature of the binding of basic drugs to orosomucoid are needed.

Our results, particularly evident when using lipoprotein deficient serum, clearly demonstrate that quantitative studies on drug binding to various serum proteins should preferably be performed on protein deficient sera, rather than on isolated proteins. However, important qualitative information may be obtained from the latter type of studies, including mechanisms of binding. Since the various compounds within classes of acidic, neutral and basic drugs may vary considerably in their binding characteristics, further comparative studies are necessary.

This study was supported by grants from the Norwegian Research Council for Science and the Humanities and the Norwegian Medicinal Depot.

We thank Pfizer Oslo for providing isotopes of doxepin and desmethyldoxepin, K. M. Olsen and Aa. B. Andersen for technical assistance, and B.I. Sandved for preparing the manuscript.

References

BREDESEN, J.E., PIKE, E. & LUNDE, P.K.M. (1982). Plasma binding of disopyramide and mono-*N*-dealkyldisopyramide. *Br. J. clin. Pharmac.*, **14**, 673–676.

BRINKSCHULTE, M. & BREYER-PFAFF, U. (1980). The contribution of α_1-acid glycoprotein, lipoproteins and albumin to the plasma binding of perazine, amitriptyline and nortriptyline in healthy man. *Naunyn. Schmiedeberg's Arch. Pharmac.*, **314**, 61–66.

CHEN, Z. & DANON, A. (1979). Binding of reserpine to plasma albumin and lipoproteins. *Biochem. Pharmac.*, **28**, 267–271.

CONN, H.L. & LUCHI, R.J. (1961). Some quantitative aspects of the binding of quinidine and related quinoline compounds by human serum albumin. *J. clin. Invest.*, **40**, 509–516.

COSTELLO, P.B., GREEN, F.A. & JUNG, C.Y. (1982). Free versus bound salicylate concentration in adults and children with chronic inflammatory joint disease. *Arthr. Rheum.*, **25**, 32–37.

GESCHER, A. & LI WAN PO, A. (1978). Correlation of physicochemical properties with absorption and metabolism of some tricyclic drugs. *J. Pharm. Pharmac.*, **30**, 353–358.

JOHANSSON, B.G. (1972). Agarose gel electrophoresis. *Scand. J. clin Lab. Invest.*, **29**, suppl. 124, 7–19.

KATES, R.E., SOKOLSKI, T.D. & COMSTOCK, T.J. (1978). Binding of quinidine to plasma proteins in normal subjects and in patients with hyperlipoproteinemias. *Clin. Pharmac. Ther.*, **23**, 30–35.

KRAMER, R.L. & RICHENS, A. (1972). Two dimensional immunoelectrophoresis of human serum proteins for the investigation of protein binding of drugs. *Br. J. Pharmac.*, **45**, 184P–185P.

KOHN, J. (1958). Small scale membrane filter electrophoresis and immunoelectrophoresis. *Clin. Chim. Acta*, **3**, 450–454.

LIMA, J.J. & SALZER, L.B. (1981). Preliminary communication. Contamination of albumin by alpha-1-acid glycoprotein. *Biochem. Pharmac.*, **30**, 2633–2636.

NILSEN, O.G. & JACOBSEN, S. (1975). The binding of quinidine to protein fractions of normal human sera. *Biochem. Pharmac.*, **24**, 995–998.

NILSEN, O.G., LEREN, P., AAKESSON, I. & JACOBSEN, S. (1978). Binding of quinidine in sera with different levels of triglycerides, cholesterol and orosomucoid protein. *Biochem. Pharmac.*, **27**, 871–876.

PIAFSKY, K.M. (1980). Disease-induced changes in the plasma binding of basic drugs. *Clin. Pharmacokin.*, **5**, 246–262.

PIKE, E., KIERULF, P., SKUTERUD, B., BREDESEN, J.E. & LUNDE, P.K.M. (1982a). The relative importance of albumin, lipoproteins and orosomucoid for serum binding variations of basic, neutral and acidic drugs. *Br. J. clin. Pharmac.*, **14**, 574P.

PIKE, E. & SKUTERUD, B. (1982). Plasma binding variations of amitriptyline and nortriptyline. *Clin. Pharmac. Ther.*, **32**, 228–234.

PIKE, E. & SKUTERUD, B. (1983). Methodological aspects of the determination of plasma binding of amitriptyline and nortriptyline. *Medd. Norsk Farm. Selsk.*, **45**, 33–72.

PIKE, E., SKUTERUD, B., KIERULF, P., FREMSTAD, D., ABDEL SAYED, S.M. & LUNDE, P.K.M. (1981). Binding

and displacement of basic, acidic and neutral drugs in normal and orosomucoid-deficient plasma. *Clin. Pharmacokin.*, **6**, 367–374.

PIKE, E., SKUTERUD, B., KIERULF, P. & LUNDE, P.K.M. (1982b). The significance of lipoproteins of the serum binding variations of amitriptyline, nortriptyline and quinidine. *Clin. Pharmac. Ther.*, **32**, 599–606.

SAGER, G., HANSTEEN, V., AAKESSON, I. & JACOBSEN, S. (1981). Effect of heparin on serum binding of propranolol in the acute phase of myocardial infarction. *Br. J. clin. Pharmac.*, **12**, 613–620.

SAGER, G., NILSEN, O.G. & JACOBSEN, S. (1979). Variable binding of propranolol in human serum. *Biochem. Pharmac.*, **28**, 905–911.

SCOTT, B.J., BRADWELL, A.R., SCHNEIDER, R.E. & BISHOP, H. (1979). Propranolol binding to serum orosomucoid. *Lancet*, **i**, 930.

SHARPLES, D. (1976). Factors affecting the binding of tricyclic tranquillizers and antidepressants to human serum albumin. *J. Pharm. Pharmac.*, **28**, 100–105.

TRAVIS, J., BOWEN, J., TEWKSBURY, D., JOHNSON, D. & PANNELL, R. (1976). Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochem. J.*, **157**, 301–306.

(Received March 8, 1983, accepted March 29, 1983)