Biochemical Pharmacology, Vol. 28, pp. 267–271.

0006-2952/79/0115-0267 $02.00/0

# BINDING OF RESERPINE TO PLASMA ALBUMIN AND LIPOPROTEINS

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(*Received* 23 *March* 1978; *accepted* 25 *May* 1978)

**Abstract**—The binding of reserpine to plasma albumin and lipoproteins was studied by equilibrium gel filtration. Reserpine was shown to be over 96 per cent bound to plasma proteins. Two discrete peaks of plasma–protein binding were identified, the one in the lipoprotein fraction and the other corresponding to plasma albumin. Binding to the lipoproteins showed large variations between normal subjects (mean extent of binding of 5.4 ± 0.8) and hyperlipoproteinemic patients (mean of 11.2 ± 0.9), and correlated very significantly with the triglyceride and total lipid concentration and, to a lesser extent, with the concentration of cholesterol in the plasma. It is concluded that plasma lipoproteins, as well as albumin, bind reserpine, and that the degree of lipoproteinemia may contribute to variations in plasma–drug binding.

The binding of drugs to plasma albumin has been studied extensively (for review, see Refs. 1–3). However, the possible interactions of drugs with other plasma proteins, more specifically with the lipoproteins (LP), are poorly understood. Rudman *et al.* [4] reported several years ago that a few lipid-soluble drugs, as well as several steroid hormones and fatty acids, may be associated with the lipoprotein fraction of human, rabbit and chicken plasmas. Subsequently, the binding of some other drugs to plasma LP was also reported [5–10], although several of these studies produced conflicting results.

While screening for the possible binding of lipid-soluble drugs to plasma LP, reserpine was found to exhibit outstanding association with both plasma albumin and LP. Although the pharmacological significance of such binding could be questioned vis-à-vis the "hit and run" hypothesis of the action of reserpine (for review, see Ref. 11), this binding was of interest as a model. Indeed, few drugs, if any, are bound to plasma LP as avidly as reserpine. Also, binding to LP could explain some aspects of the pharmacology of reserpine, as, for instance, its lower uptake into platelets when incubations are carried out in plasma, compared with plain buffer or even albumin solution [12]. Part of this work has been reported in abstract form [13].

## MATERIALS AND METHODS

Blood was collected in plastic test tubes containing EDTA (Overpelt-Plascobel, N.V., Belgium) and centrifuged at 1200 *g* for 10 min. The plasma was kept at 4° until utilized. Subsequent steps were carried out in glass containers, since in preliminary experiments reserpine was observed to adhere very avidly to plastic (results not shown). All experiments were carried out at room temperature, i.e. between 20 and 23°.

*Equilibrium gel filtration.* This method provides quantitative estimates of binding to LP and to albumin separately, while each is kept in equilibrium with a constant concentration of free drug in the buffer, with which the column is equilibrated. The column, 9 mm internal diameter, was packed with Bio-Gel A-0.5 m, 100–200 mesh (Bio Rad Laboratories, Richmond, CA) to a bed height of 75 cm. The gel had been equilibrated with phosphate–saline buffer, pH 7.4, containing [$^3$H]reserpine (general label, 1.65 Ci/mmole, Nuclear Research Center, Negev, Israel), at the estimated concentration of the free drug in the plasma (about 0.6 ng/ml). [$^3$H]reserpine was added to 1 ml plasma at a concentration of 20 ng/ml at least 30 min before loading the column. The same buffer with [$^3$H]reserpine that had been used for equilibration was then used to develop the column. Flow rate was 0.25 ml/min and the effluent was collected in fractions of 1 ml each. No significant adsorption of reserpine to the gel could be detected. The protein content of the effluent was analyzed by absorbance at 280 nm.

*Equilibrium dialysis.* This was carried out in cellulose dialysis tubing, 0.22 in. in diameter (Fisher Scientific Co., Pittsburgh, PA). One ml plasma was placed in each bag and dialyzed against 17 ml phosphate–saline buffer, pH 7.4, in a test tube at room temperature. The buffer was stirred continuously with a magnetic stirrer. Binding to the membrane was not significant, as determined by control runs, in which buffer was placed in the bag, instead of plasma. Equilibrium was shown to occur by 24 hr, but some experiments were extended to 44 hr. [$^3$H]reserpine was added to the plasma inside the bag, and radioactivity was determined at equilibrium both inside and outside the bag.

*Determination of radioactivity.* Radioactivity was analyzed in 0.5-ml samples of the column effluent after the addition of a 10-ml toluene–Triton scintillation mixture. Counting efficiency, as determined by automatic external standard, was approximately 34 per cent and did not change when either buffer or protein solutions from the effluent were analyzed.

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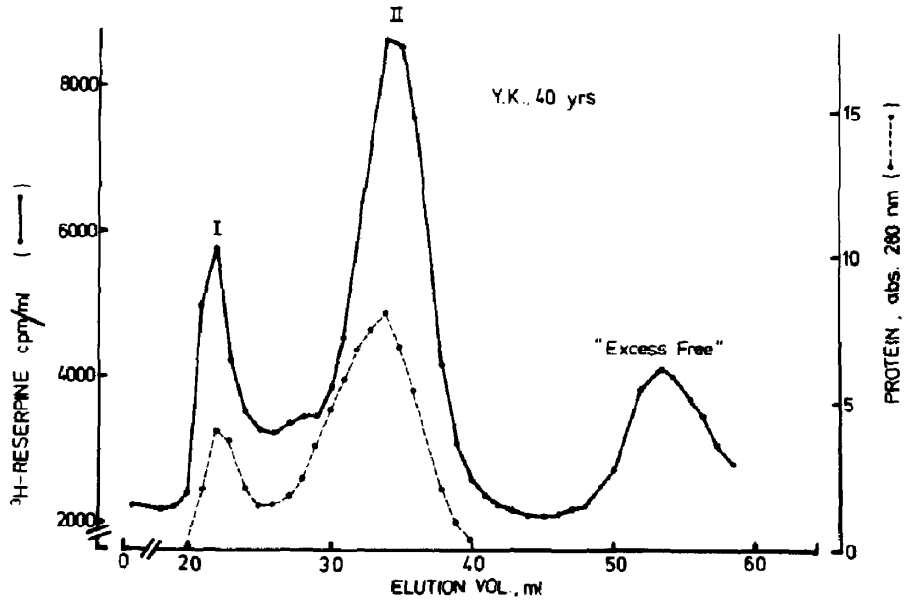


Fig. 1. Equilibrium gel filtration elution diagram of normal human plasma containing [$^3$H]reserpine. The broken line represents protein concentration, absorbance at 280 nm; the solid line, [$^3$H]reserpine, cpm/ml.

Radiopurity of the [$^3$H]reserpine was confirmed by thin-layer chromatography [14]. [$^3$H]reserpine that had been eluted through the column (with plasma) chromatographed as authentic reserpine (97 per cent).

Human serum albumin (HSA) and bovine serum albumin (BSA) were fraction V, 96–99 per cent pure (Sigma Chemical Co., St. Louis, MO).

*Calculations.* The bound drug in each protein peak of the gel filtration was calculated from the area of the peak derived from 1 ml plasma above the buffer baseline, in cpm. Per cent binding was calculated as the total of the peak areas divided by the sum of peak areas and free buffer concentration. The extent of binding in each protein peak was espressed as $B/F$, where $B$ is the amount of drug bound to proteins in 1 ml plasma and $F$ is the free drug concentration in the buffer, in cpm. In the dialysis experiments, the per cent binding was determined as the difference between plasma and buffer concentrations divided by the plasma concentration at equilibrium.

*Biochemical analyses.* Autoanalyser SMA 12/60 (Technicon Instruments Corp., Terrytown, NY) was used for the determination of plasma total proteins (worksheet no. N-14b I/II), cholesterol (worksheet no. N-77 I/II) and albumin [15]. Plasma total-lipids were determined according to Frings and Dunn [16] and triglycerides according to Martin [17].

## RESULTS

As the protein fractions emerged from the column, the free drug concentration was essentially the one used for equilibration of the column. Thus, any reserpine bound to protein emerged as a peak superimposed on the equilibrium concentration. The excess (or deficit) free drug, beyond the column "free" concentration, formed a late peak (or trough), which was not included in the calculation of the bound reserpine. The concentration of [$^3$H]reserpine added to the buffer was such as to avoid a late trough, which could cause some dissociation on the column.

The elution pattern of plasma proteins from a representative normal subject and the binding of [$^3$H]reserpine are shown in Fig. 1. The overall mean (+ S. E. M.) per cent binding in five normal individuals was 96.2 ± 0.1 per cent. The initial protein peak, containing most of the LP as well as macroglobulins, carried some 22 per cent of the radioactivity, while the greater part of the label was associated with the second peak, comprised mainly of albumin and some globulins [18]. Identification of the protein responsible for binding at the second peak was accomplished by comparison with the binding of reserpine to purified HSA. Binding to HSA (4.5 g/100 ml) was similar to that observed in the second plasma peak, suggesting

Table 1. Binding of [$^3$H]reserpine to plasma proteins from various species

| Species | $B/F$ peak I plasma | $B/F$ peak II plasma | $B/F$ purified albumin* | Per cent binding plasma | Per cent binding albumin* |
|---|---|---|---|---|---|
| Man | 5.4 | 19.2 | 16.0 | 96.1 | 94.1 |
| Bovine† | 10.0 | 7.0 | 4.4 | 94.4 | 81.5 |
| Ovine† | 2.0 | 7.0 | | 89.2 | |
| Rat† | 7.1 | 9.8 | | 94.4 | |

* Albumin concentration was 4.5 g/100 ml, dissolved in phosphate- saline buffer.
† Determinations were carried out in one sample each.

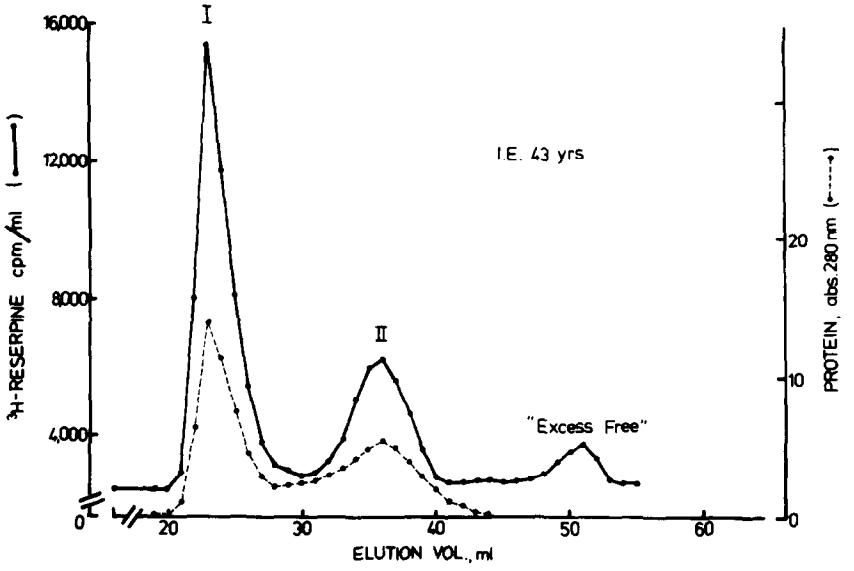


Fig. 2. Representative elution profile of plasma, containing [$^3$H]reserpine, from a hyperlipoproteinemic patient (type IV). The broken line represents protein concentration; the solid line, [$^3$H]reserpine, cpm/ml.

Table 2. Binding of [$^3$H]reserpine to plasma albumin and lipoproteins from normal and hyperlipoproteinemic subjects*

| | Plasma lipids | | | Plasma | Reserpine binding | | |
|---|---|---|---|---|---|---|---|
| | Total lipids (mg/100 ml) | Cholesterol (mg/100 ml) | Triglycerides (mg/100 ml) | albumin (g/100 ml) | *B/F* peak I | *B/F* peak II | Total per cent bound |
| Normals (n = 5) | 482 ± 49 | 192 ± 17 | 79 ± 9.3 | 4.5 ± 0.2 | 5.4 ± 0.8 | 20 ± 1.4 | 96.2 ± 0.11 |
| Hyperlipoproteinemic (n = 8) | 1185 ± 137 | 302 ± 15 | 341 ± 55 | 4.1 ± 0.2 | 11.2 ± 0.9 | 14.8 ± 1.2 | 96.3 ± 0.14 |
| P | < 0.005 | < 0.005 | < 0.005 | NS† | < 0.005 | < 0.02 | NS† |

* Values are expressed as mean ± S. E. M.
† NS = not significant.

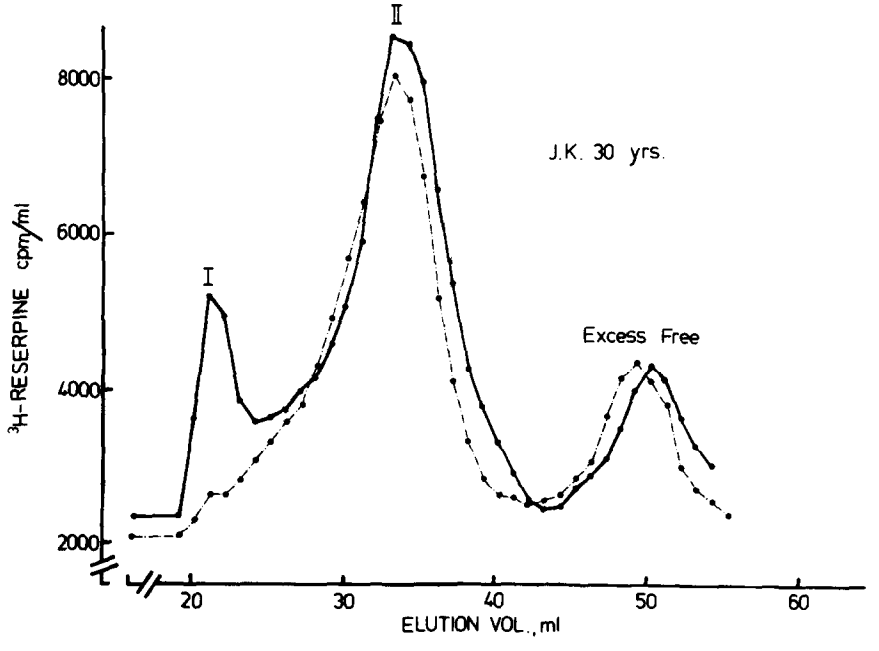


Fig. 3. Elution diagram of [$^3$H]reserpine added to normal plasma (solid line), and same plasma after removal of LP (broken line).

that albumin could account for the binding in the second plasma peak. For comparison, the binding of [$^3$H]reserpine to serum proteins from other species was also studied (Table 1). Results show that purified BSA (4.5 g/100 ml) bound a considerably smaller quantity of [$^3$H]reserpine than did HSA. Lower binding was also observed when the $B/F$ ratios in the second peak obtained from samples of bovine, ovine or rat plasmas were compared with the same in human plasma.

The identity of the reserpine-binding proteins in the early peak as LP was inferred from two lines of experimental evidence. First, we studied the binding of [$^3$H]reserpine to plasmas of hyperlipoproteinemic patients. As illustrated in Fig. 2, the first (LP) peak in hyperlipoproteinemic subjects was much larger than in normals. The data of the binding of [$^3$H]reserpine to plasmas of hyperlipoproteinemic and normal subjects are summarized in Table 2. The mean $B/F$ ratio in the peak representing binding to the LP shows more than a 2-fold greater binding to the lipoprotein fraction in the hyperlipoproteinemic subjects than in the normals. Moreover, regression analysis revealed a highly significant correlation between the extent of binding ($B/F$) in peak I and plasma triglyceride concentration ($r = 0.87$, $P < 0.0001$). Significant correlations were also observed between $B/F$ and plasma cholesterol ($r = 0.68$, $P < 0.01$) and $B/F$ and plasma total lipids ($r = 0.83$, $P < 0.0005$). Table 2 also shows that the binding of [$^3$H]reserpine to the albumin fraction was lower in the hyperlipoproteinemic subjects in spite of normal plasma albumin concentration, thus yielding overall binding not dissimilar to the control group.

Further support for the conclusion that lipoproteins are responsible for the binding in the early protein peak was derived from experiments in which the plasma very low density LP (VLDL) and low density LP (LDL) had been removed by precipitation with heparin and manganous chloride, as described by Burstein and Scholnick [19]. Figure 3 shows that the early radioactive peak was practically eliminated when these lipoproteins had been removed before adding the [$^3$H]reserpine. The same elution pattern was observed if precipitation of the LP was done after addition of the labeled drug. In addition, the lipoprotein precipitate acquired the label.

Equilibrium dialysis of whole plasma from three subjects yielded per cent binding similar to those calculated from equilibrium gel filtration (mean of 94.7 per cent bound by dialysis, compared with 96.2 per cent by gel filtration).

## DISCUSSION

The present results indicate that reserpine avidly binds to both plasma albumin and lipoproteins. Moreover, the latter binding varies considerably in the plasmas of individuals with normal or increased lipoprotein concentrations and correlates with the degree of lipoproteinemia. Unlike albumin, the concentration of which shows relatively little variation among individuals, lipoprotein concentrations may fluctuate many-fold, depending on constitution, age, sex, diet or disease. That lipoprotein binding could contribute to variations in plasma–drug binding has been postulated [9, 10], but never shown to actually occur. The results of the present report show a definitely higher binding of reserpine to the LP of hyperlipoproteinemic subjects, but the total per cent binding did not seem to vary between the two groups. It appears that the higher binding to the LP somehow effected lower binding to the albumin in the same plasmas. The mechanism of this interference is unclear. However, since the binding of drugs to LP may involve mechanisms dissimilar to those affecting binding to albumin, the availability of a drug bound to LP may not be the same as that bound to albumin.

Gel filtration under non-equilibrium conditions resulted in considerably decreased binding to the LP as well as to albumin (results not shown). It may be concluded, therefore, that the binding to both plasma protein fractions is reversible. Whether reserpine in the plasma LP is associated with the apoprotein or the lipid component cannot be concluded from the present data. Balzar *et al.* [20] studied the binding of reserpine to membrane lipids and concluded that the binding of reserpine to membranes of the sarcoplasmic reticulum took place in the unsaturated fatty acids of the membrane phospholipids. As to the nature of the specific plasma lipoprotein that binds reserpine, the data suggest that either VLDL or LDL or both are involved, since binding depended on the presence of these LP, and was abolished after precipitation of these fractions. Also, the extent of binding correlated best with the concentration of plasma triglycerides, the major constituent of VLDL. Although binding constants have not been determined, some speculation may be in order. Molar concentrations of VLDL and LDL are some 2–3 orders of magnitude lower than that of plasma albumin [21]. With binding to LP of the magnitude observed, one must assume either the affinity of reserpine for the LP or the number of binding sites or both to be much greater than those for the association of reserpine with albumin.

The large species variation observed in the binding of reserpine to serum proteins is of special interest and should be borne in mind when extrapolations are made from one species to another. Similar differences among species were reported for a few other drugs, including salicylate [22], chlorpromazine [23] and warfarin [24]. Although the present results on non-human species were obtained from one sample each, and subsequently may not be accurate estimates of the binding in those species, the differences are felt to be real.

Some of the earlier work on lipoprotein binding of drugs yielded quantitatively or qualitatively varied results. Thus, a 2-fold difference in the extent of binding of $\Delta^9$-tetrahydrocannabinol (THC) was reported by ultracentrifugal [6] and electrophoretic [5] methods. Also, while estradiol and dicumarol, among other molecules, were reported to be associated with plasma LP in one study [4], such binding was denied in another investigation which utilized a different methodology [6]. Gel filtration has been used extensively for protein separation or, alternatively, for protein binding studies, usually when one binding protein is involved. In the latter case, dissociation on the column has been noted [25, 26], unless equilibrium was carefully maintained [27]. Thus, equilibrium gel filtration has been shown to be a reliable as well

as a quantitative method for the study of protein–drug interactions [28–30]. The validity of the present method in the study of drug binding to LP was confirmed by our ability to demonstrate binding of oleic acid and $\Delta^9$-THC to LP as well as to albumin (results not shown). The reasonable agreement between gel filtration and equilibrium dialysis data lends further support to the validity of the former method. Additional merits include the small quantities of plasma required (1 ml) and relative speed of the procedure (about 4 hr).

*Acknowledgements*—This work was supported by a grant from the Chief Scientist's Office, Ministry of Health. We thank Drs. Y. Liel and Z. Canaani for providing plasma samples, and Mrs. Judith Knopf for excellent secretarial assistance.

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