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EQUILIBRIUM SATURATION CHROMATOGRAPHIC METHOD FOR STUDYING THE BINDING OF LIGANDS TO HUMAN SERUM ALBUMIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INFLUENCE OF FATTY ACIDS AND SODIUM DODECYL SULPHATE ON WARFARIN–HUMAN SERUM ALBUMIN BINDING

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SUMMARY

A size exclusion chromatographic method for studying ligand–macromolecule binding parameters is described. This equilibrium saturation method allows the determination of the concentrations of constituents in equilibrium and is specially useful for characterizing ligand–protein binding under conditions that can be compared with physiological conditions. The method has been used for measuring warfarin–human serum albumin (HSA) binding and for studying the influence of free fatty acids (FFA) and sodium dodecyl sulphate on warfarin–HSA binding. Some comparisons with the Hummel and Dreyer method are given. The influence of the FFA is strongly dependent on their chain length, with an inversion of the effect for a 10-carbon chain.

INTRODUCTION

Gel filtration has been widely used for the measurement of ligand–macromolecule binding, according to several methods reviewed by Wood and Cooper[1]. These methods include zonal separation, the Hummel and Dreyer method[14] and frontal analysis. Brumbaugh and Ackers[2] described an equilibrium saturation technique, based on the direct optical scanning of a Sephadex G-100 chromatographic column that has been saturated with a solution of a desired mixture of ligand and macromolecule; the ratio of the absorbance in the column bed to the absorbance of the solution above the bed provides the means for determining the free ligand concentration.

On the other hand, we have recently reported[3] the application of frontal analysis and the Hummel and Dreyer method in high-performance liquid chromatography (HPLC).

In this paper, we describe a size exclusion chromatographic method for studying ligand–macromolecule binding, which depends on the elution of a size exclusion chromatographic column with a phosphate buffered solution of ligand and macromolecule, and on monitoring of the eluting solution at the outlet of the column by a classical HPLC detector. The chromatographic support is chosen so as to exclude the protein and its complexes while permitting free entry of small ligand molecules, and so allows the separation of free ligand from protein and protein ligand complexes. The injection of a few microlitres of phosphate buffer on to the column leads to the appearance of two negative peaks on the chromatogram. The first such peak emerges at the void volume of the column and expresses the deficiency of protein and bound ligand caused by the buffer injection, and the second peak the deficiency of the free ligand. The area of the latter peak provides a means for determining the free ligand concentration. This method uses simpler instrumentation than the Brumbaugh and Ackers method, as only commonly used liquid chromatographic apparatus is needed.

Firstly, we tested the method by measuring warfarin–human serum albumin (HSA) binding and compared the results obtained with those previously reported by the use of the Hummel and Dreyer method[3]. Then we applied the method to the determination of the influence of free fatty acids (FFA) of various chain lengths and sodium dodecyl sulphate (SDS) on warfarin–HSA binding. The Hummel and Dreyer method was also used in a few instances.

The results obtained are compared with the modification of warfarin–HSA binding previously reported in the presence of some FFA by the use of some classical techniques of protein binding measurement, such as ultrafiltration[4], equilibrium dialysis[5,6] and fluorescence spectroscopy[7]. We present some new results on the influence of some FFA and SDS not yet studied on warfarin–HSA binding.

EXPERIMENTAL

Materials

HSA, an albumin essentially free of fatty acids, was obtained from Sigma (St. Louis, Mo., U.S.A.). The absence of FFA was verified by a gas chromatographic method and the total molar ratio of FFA to HSA was found to be lower than 0.03.

Warfarin, as the sodium salt, was donated by Merrel-Toraude Laboratories (Paris, France). HSA and warfarin were dissolved in phosphate buffer (0.067 *M*, pH 7.4).

The following fatty acids and salts (99% pure) were purchased from Sigma: hexanoic, octanoic, nonanoic, decanoic, lauric, myristic and oleic acid and sodium palmitate. All of the acids were dissolved in absolute ethanol as $4 \cdot 10^{-2}$ *M* solutions, and aliquots of the solutions were added to buffered warfarin or HSA solutions (the ethanol concentration did not exceed 0.1% in the final solutions). The sodium palmitate was dissolved in absolute ethanol as a 10^{-3} *M* solution, and appropriate volumes were poured into flasks. The ethanol was allowed to evaporate from these solutions at room temperature, then HSA solution was added to the flasks. All of the FFA–HSA solutions were mixed by sonication.

SDS was obtained from BDH (Pool, Great Britain) and was dissolved directly in the phosphate buffer.

Equipment

A 6000A pump, U6K injector and UV Type 440 detector with a 313-nm filter, all from Waters Assoc. (Milford, Mass., U.S.A.), were used. The wavelength used allowed the detection of HSA and warfarin in the eluent, but not FFA and SDS.

Column

A 15 cm × 4.7 mm I.D. column filled by a slurry packing technique with LiChrosorb Diol (10 μm) support (Merck, Darmstadt, G.F.R.) was used. In all of the experiments this support allowed HSA to elute very quickly from the column at the void volume (1.5 ml), but retained free warfarin longer. This support provides a better separation than the previously used Glycophase G support between HSA and warfarin[3].

In all of the experiments, the column temperature was maintained at 37°.

RESULTS AND DISCUSSION

Warfarin–HSA binding by the HPLC equilibrium saturation method

Fig. 1a shows a typical elution pattern, monitored at 313 nm, obtained when a few microlitres of phosphate buffer were injected on to a LiChrosorb Diol column and eluted with a buffered warfarin–HSA solution. The first negative peak, which emerges at the void volume of the column, represents the deficiency of HSA and warfarin–HSA complexes caused by the injection of buffer and the second peak corresponds to the deficiency of free warfarin. Thus, the area of the latter peak gives, after previous calibration, the free warfarin concentration, A, in the mobile phase.

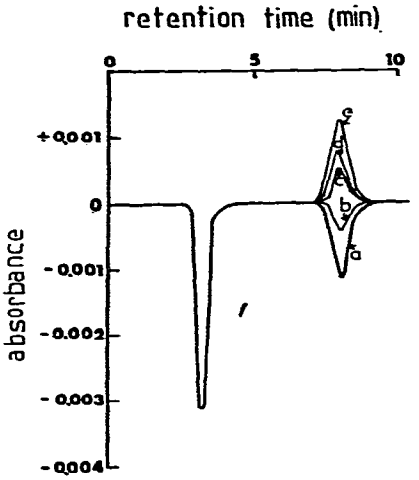


Fig. 1. Elution patterns obtained by the HPLC equilibrium saturation method for warfarin–HSA binding. Eluent: warfarin (2.5 μM)–HSA (5.8 μM) buffered solution. Flow-rate: 0.4 ml·min^{-1}. Samples: (a) 25 μl of phosphate buffer; (b), (c), (d) and (e) 25 μl of 0.75, 1.75, 2.0 and 2.5 μM warfarin solutions, respectively.

To determine A precisely, we use the following internal calibration process: we inject on to the column successive samples of the same volume, which contain increasing concentrations of warfarin in phosphate buffer. The chromatograms so obtained are represented by curves b, c, d and e in Fig. 1. The first peak remained unchanged, whereas the second one varies. By plotting the area of the latter peak as a function of the concentration of warfarin in the sample, and extrapolating it to zero, the corresponding concentration of warfarin needed to cancel the peak of the free ligand equals the free warfarin concentration, A (Fig. 2). Once this value has been determined, the mean number of moles of ligand bound per mole of macromolecule, $\bar{r}$, can easily be calculated as the total warfarin and HSA concentrations are known.

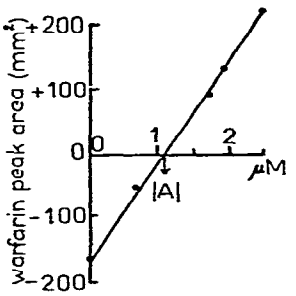


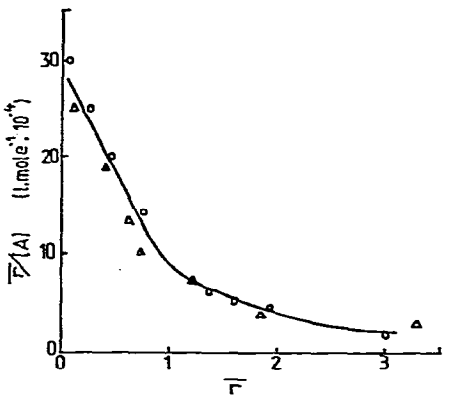
Fig. 2. Internal calibration for determining free warfarin concentration, A, in warfarin (2.5 μM)–HSA (5.8 μM) solution.

After having verified by UV spectrophotometry that at 313 nm the molar extinction coefficient of bound and free warfarin are identical, we have shown the correspondance of the measured first peak area with the area calculated from bound warfarin plus total HSA concentrations.

By varying the warfarin concentration in the eluent from 1.0 to 80 μM, the HSA concentration remaining equal to 5.8 μM and, calculating as described above the corresponding binding ratios, $\bar{r}$, it was possible to draw the Scatchard plot represented in Fig. 3 and to compare it with the Scatchard plot obtained by the Hummel and Dreyer method[3]. Both diagrams are in good agreement, and show the validity of the new method. We observed in these experiments that the second peak is eluted earlier as the total warfarin concentration in the mobile phase decreases, *i.e.*, when its extent of association with HSA increases, while the HSA peak always elutes at the void volume of the column. This phenomenon of the dependence of the retention volume of warfarin on its association with eluting HSA is the subject of a more detailed study[8].

Influence of FFA and SDS on warfarin–HSA binding

The HPLC equilibrium saturation method. Figs. 4 and 5 represent the chromatograms obtained when palmitic acid and octanoic acid, respectively are present in a warfarin (2.5 μM)–HSA (5.8 μM) eluting solution. When the molar ratio of palmitic acid to albumin, R, increases from 0 to 4, the first peak area is enhanced whereas the second decreases. The opposite phenomenon is produced in the presence of octanoic



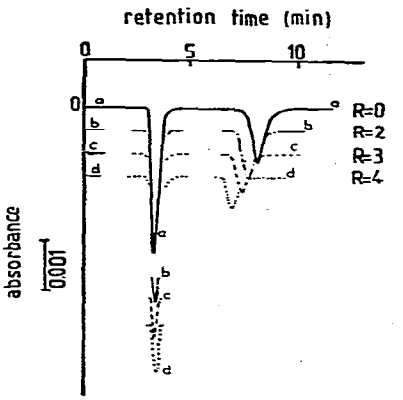
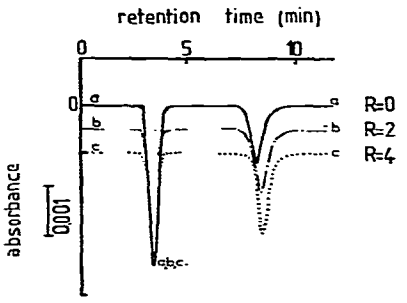


Fig. 3. Scatchard plots for warfarin–HSA binding. △, HPLC equilibrium saturation method; ○, Hummel and Dreyer method.

Fig. 4. HPLC equilibrium saturation chromatograms for warfarin–HSA binding in the presence of various amounts of palmitic acid. Sample: 25 μl of phosphate buffer. Eluents: warfarin (2.5 μM)–HSA (5.8 μM) solutions. R = Molar ratio palmitic acid/HSA.



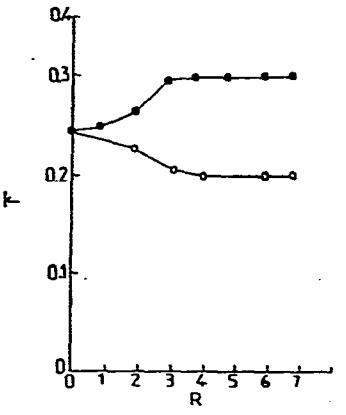


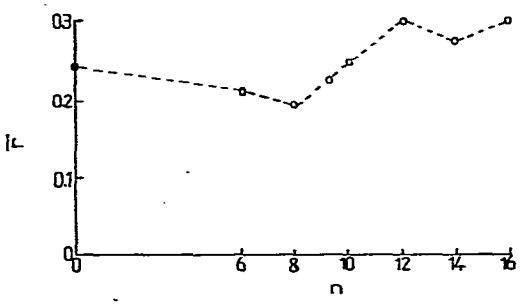
Fig. 5. HPLC equilibrium saturation chromatograms for warfarin–HSA binding in the presence of various amounts of octanoic acid. Sample: 25 μl of phosphate buffer. Eluents: warfarin (2.5 μM)–HSA (5.8 μM) solutions. R = Molar ratio octanoic acid/HSA.

Fig. 6. Binding ratios of warfarin to HSA in warfarin (2.5 μM)–HSA (5.8 μM) solutions containing various amounts of FFA. ●, Palmitic acid; ○, octanoic acid. R = Molar ratio FFA/HSA.

acid. Thus, the binding of warfarin to HSA is enhanced by palmitic acid and decreased by octanoic acid (Fig. 6). In both instances an increase in R from 4 to 8 causes no further change.

Similar experiments were carried out with a warfarin (2.5 μM)–HSA (5.8 μM) eluting solution, which contained other FFA with various chain lengths, at a molar ratio to albumin of 4. Fig. 7 shows the variation of the measured warfarin–HSA binding ratio, $\bar{r}$, *versus* carbon chain length, n, of the particular FFA. From $n = 6$ to 10 $\bar{r}$ decreases and for $n \geqslant 10$ $\bar{r}$ increases.

Fig. 8 represents the variation of the warfarin–HSA binding ratio when SDS is present at various molar ratios to albumin in the warfarin (2.5 μM)–HSA



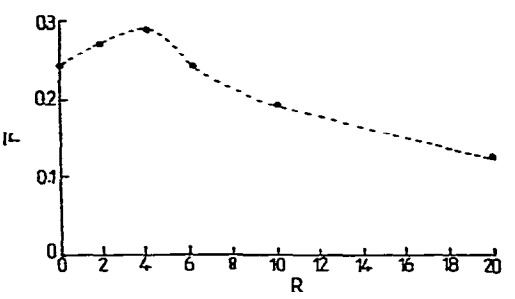
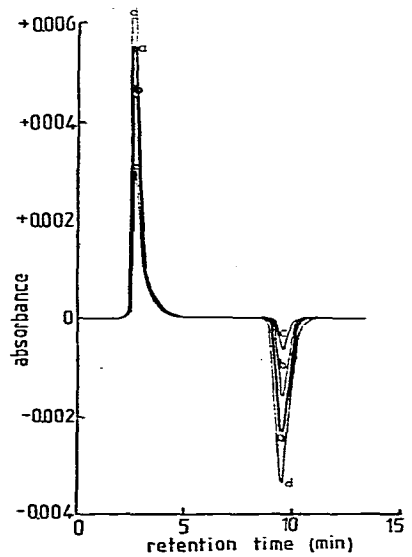


Fig. 7. Binding ratios of warfarin to HSA in warfarin (2.5 μM)–HSA (5.8 μM) solutions containing FFA of various chain lengths with $R = 4$.

Fig. 8. Binding ratios of warfarin to HSA in warfarin (2.5 μM)–HSA (5.8 μM) solutions containing various amounts of SDS. R = Molar ratio SDS/HSA.

(5.8 μM) eluting solution. For small R values (up to 5–6) the warfarin–HSA binding is enhanced and for R values higher than 6 it decreases.

We observed a general trend of the retention volume of the free ligand peak (as shown in Figs. 4 and 5) for all of the experiments reported here in the presence of FFA or SDS: the smaller the area of the warfarin peak, the earlier it is eluted. This phenomenon agrees with the earlier remark about the dependence of the retention volume of warfarin on its extent of association with HSA.



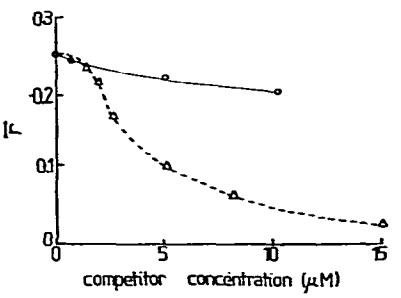


Fig. 9. Elution profiles obtained by the Hummel and Dreyer method for warfarin–HSA binding. Column: LiChrosorb Diol (15 cm × 4.7 mm I.D.). Flow-rate: 0.5 ml·min^{-1}. Eluents: (a) and (d), 1 μM warfarin solution; (b) and (c), 1 μM warfarin solution plus 26 μM octanoic acid and 15 μM SDS, respectively. Samples: (a), (b) and (c), 10 μl of HSA (2 g·l^{-1}) buffered solution; (d), 10 μl of HSA (2 g·l^{-1}) + $R = 4$ palmitic acid buffered solution.

Fig. 10. Binding ratios of warfarin to HSA (Hummel and Dreyer method) *versus* SDS (△) or octanoic acid (○) concentration.

Hummel and Dreyer method. We have reported previously[3] the Hummel and Dreyer method as a useful technique for measuring ligand–macromolecule binding by HPLC. We have now used this method to study the influence of some FFA and SDS on warfarin binding.

Fig. 9a shows the elution pattern obtained when a few microlitres of an HSA (2 g/l) buffered solution were injected on to the column and eluted with a warfarin (1 μM) buffered solution. The presence of octanoic acid (26 μM) or SDS (15 μM) together with warfarin in the eluent led to chromatograms b and c, respectively. Both peak areas are decreased in the presence of octanoic acid or SDS, and thus warfarin–HSA binding is decreased. Fig. 10 shows the variation in the calculated HSA–warfarin binding ratio *versus* the concentration of the competitor in the eluent. For high concentrations of the competitors, SDS displaces much more warfarin than does octanoic acid; on the other hand, the curve relative to SDS is sigmoidal. These phenomena agree with those observed when the saturation chromatographic method was used, but we should not compare both methods exactly because in the Hummel and Dreyer method the molar ratio of FFA or SDS to albumin, R, cannot be known accurately. Under these conditons a comparison with the physiological situation cannot be made easily.

For these reasons we tested another means of using the Hummel and Dreyer method, involving incubation of HSA with FFA or SDS, followed by injection of this mixture on to the column, which was eluted with a buffered solution containing only warfarin. Injections of various mixtures of palmitic or oleic acid with HSA gave chromatograms with larger peak areas for bound warfarin (Fig. 9d), indicating enhancement of warfarin–HSA binding, in complete agreement with the above results. On the other hand, injections of mixtures of octanoic acid or SDS with HSA did not show any change in the chromatograms obtained with injection of HSA alone (Fig. 9a). The octanoic acid– and SDS–HSA complexes are probably not strongly bound enough to emerge undissociated from the column and the albumin dissociates during the gel filtration process while the palmitic acid– or oleic acid–HSA complexes are strongly bound enough and emerge intact from the column. The presence of the latter FFA bound to HSA can influence the successive binding of warfarin to HSA. Our findings agree with Nimmo and Bauermeister's theoretical analysis[9], which showed that the zonal chromatographic behaviour of a reversible complex is related only to its kinetic characteristics, namely its association constant, K: the association constants are in the region of 10^6 l·mole^{-1} for SDS–HSA complexes and 10^3–10^4 l·mole^{-1} octanoic acid–HSA complexes[10], and for palmitic acid– or oleic acid–HSA complexes 10^7–10^8 l·mole^{-1} (ref. 11). This difference in the magnitudes of the association constants can explain the different chromatographic behaviours of the complexes.

Therefore, the latter method cannot be used for all binding studies because of the reversible nature of many binding equilibria. The equilibrium saturation method seems to be more convenient for such binding studies.

Discussion of results

So far, only the influence of lauric, oleic and palmitic acids on warfarin–HSA binding have been reported. The enhancement of warfarin binding that we measured in the presence of oleic and palmitic acids, for low R values agree with

the results of equilibrium dialysis[5,6] or fluorescence spectroscopic[7] experiments. For values of R higher than 4, we did not observe a further change in the affinity of warfarin for HSA, whereas the above techniques showed a decrease. Another point of discrepancy is given by the results of ultrafiltration[4], with a decrease in warfarin–HSA binding in the presence of lauric acid ($R = 1.8$), whereas we measured an increase in binding.

Our conclusions are similar to those of Soltys and Hsia[12,13], who measured by an electron spin resonance technique the influence of FFA on spin label ligand–HSA binding. They indicate that the FFA effect is dependent on carbon chain length: FFA with a carbon chain length of less than 10 displace the spin labels from HSA, whereas longer FFA chains enhance the spin label binding allosterically.

CONCLUSIONS

In the particular case application of the proposed method studied, *i.e.*, the binding of drugs to albumin, it was possible to determine precisely and very quickly the free drug concentration at equilibrium for given total albumin and drug concentrations in the eluent. Our results agree with those previously reported[3]. In the same way it is possible to study the effects of various substances (FFA and SDS) on the reversible binding of the drugs to albumin at an exactly known molar ratio of FFA to albumin.

We have shown the importance of the FFA carbon chain length, a decrease in drug binding occurring in the presence of short-chain FFA (less than 10 carbon atoms) and an increase in the presence of FFA with chain lengths above 10. The conformation of the albumin molecule is probably different in the presence of these types of FFA, and affects differently the subsequent binding of warfarin.

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