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DETERMINATION OF DRUG PROTEIN-BINDING BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A CHEMICALLY BONDED BOVINE ALBUMIN STATIONARY PHASE[a]

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SUMMARY

A liquid chromatographic method for the determination of the degree of protein-binding of drugs has been established, using a stationary phase to which bovine serum albumin has been bonded chemically In a structurally heterogeneous group of compounds, results of the method correlate well with protein-binding data obtained by equilibrium dialysis ($r=0\ 89, n=23, p<0\ 001$) Within a series of analogous piperazines a good correlation is found ($r=0\ 981, n=11, p<0\ 001$) The chromatographic method allows automation of the measurement of protein-binding of large series of compounds with protein-binding ranging between 10 and 99% The method is not expensive and is less time consuming than equilibrium dialysis Only 1 mg of technical-grade material is required to determine the protein-binding, and radioactive labelling of the material is not necessary

INTRODUCTION

The degree of protein-binding is a parameter of importance in the evaluation of the pharmacological and pharmacokinetic properties of potential drugs. Many methods for the determination of drug protein-binding have been reported. Usually the measurement is based on equilibrium dialysis [1–3]. Only a few authors [4,5] have used high-performance liquid chromatography (HPLC), employing size-exclusion techniques. Yoshida et al. [6] gave an im-

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0378-4347/89/$03 50

pulse for the determination of drug protein-binding using a physically protein-coated ODS column.

In this paper we describe the retention behaviour of drugs on a chemically bonded albumin stationary phase, which is commercially available. The aim was to obtain a direct chromatographic parameter for the representative drug protein-binding.

Allenmark et al. [7] investigated the effects of mobile phase parameters on retention behaviour. A buffer concentration of ca. 0.1 *M* is necessary to achieve hydrophobic interaction [8], which is of importance in drug protein-binding [9]. Addition of 1-propanol gives a decrease in retention for all solutes.

We studied the retention characteristics of a heterogeneous group of 23 drugs and compared the chromatographic results with reported plasma protein-binding data, obtained from equilibrium dialysis. Furthermore we compared the two methods for eleven analogous piperazines.

EXPERIMENTAL

Materials

The solvents used in HPLC were of analytical-reagent grade. All drugs, given in Tables II and III, were dissolved in 0.1 *M* phosphate buffer (pH 7.4) with 2% 1-propanol at a concentration of 0 2–0.5 nmol/μl.

The equilibrium dialysis was performed with human plasma. A sample of 21 ml of blood was collected in 4 ml of aqueous citrate solution (2.7% disodium citrate and 2.3% glucose). After centrifugation the plasma was diluted two-fold with 0.2 *M* phosphate buffer (pH 7 4). The total dilution was 2.67 The drugs were dissolved in 0.1 *M* phosphate buffer (pH 7.4) in a concentration range of 6000–75 ng/ml.

Column liquid chromatography

We used an LC pump with column oven and autosampler HP 1090 (Hewlett-Packard, Waldbronn, F.R G). The flow-rate was 0.5 ml/min and the column temperature was adjusted to 37 °C.

From the samples, 1 μl was injected into a 15 cm × 4 mm I D column packed with Resolvosil-BSA-7 (Macherey-Nagel, Duren, F R.G.), a chemically bonded bovine serum albumin stationary phase. Mixtures of 1-propanol and 0.1 *M* phosphate buffer (pH 7.4) were used as mobile phase Detection of the compounds was carried out with an SF 757 UV detector (Kratos, Ramsey, NJ, U.S A.) at 254 nm.

Equilibrium dialysis

The equilibrium dialysis was carried out with the Dianorm apparatus from Innovation Medizin (Zurich, Switzerland). The apparatus has twenty dialysis cells. Each dialysis cell consists of two compartments, separated by a dialysis

membrane (Diachema, MW cut-off 5000). One 1.3-ml compartment contained 1 ml of the above-mentioned drug solution and the other contained 1 ml of diluted human plasma. The dialysis cells were rotated in a water-bath at 37°C for 4 h Air present in the compartment improves mixing, which accelerates dialysis. All trials were done in duplicate and five concentrations of each drug were tested. After dialysis, the concentration of the drugs in the compartments was assayed by HPLC. The samples were preconcentrated on Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S A) and subsequently chromatographed on a reversed-phase system. This system consisted of a Zorbax column (Dupont, Wilmington, DE, U.S.A.) with acetonitrile–water as mobile phase The composition of the mobile phase was more or less adjusted to the retention of the analytes. The compounds were detected by a Kratos SF 757 UV detector at 254 nm

The concentration in the buffer compartment represents the amount of free drug and that in the plasma compartment the sum of free and bound drug. The fraction bound at infinite dilution was determined from the slope of 1/bound (1/b) versus 1/free (1/f) as described by Klotz and Hunston [10]. Bound is defined as the molar fraction of bound substance and free is defined as the molar concentration of free substance.

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram of some sulfa compounds. The retention behaviour is a function of the degree of protein-binding determined by equilibrium dialysis. As can be expected, drugs with a high degree of protein-binding give a high retention on the bovine serum albumin column. The injected amount was chosen to be very low, because above a level of 0.5 nmol overloading effects of the chromatographic peak occur, resulting in a smaller retention volume.

The best relation with the degree of protein-binding is found using the chromatographic parameter $k'/(k'+1)$, which expresses the fraction of the injected amount bound to the stationary phase, where k' is the capacity factor.

From the literature a group of eighteen drugs was chosen, for which the protein-binding was determined by equilibrium dialysis [11,12]. Additionally the dialysis results for five other drugs were determined as described in Experimental.

The chromatographic data of the heterogeneous group of drugs were measured with increasing percentages of 1-propanol being added to the mobile phase. The $k'/(k'+1)$ values were measured in two independent runs on two different columns. The results were correlated with the protein-binding results. The correlation coefficients and the standard deviations (S.D.) for the $k'/(k'+1)$ data are presented in Table I.

The addition of 1-propanol to the mobile phase in the range studied does not

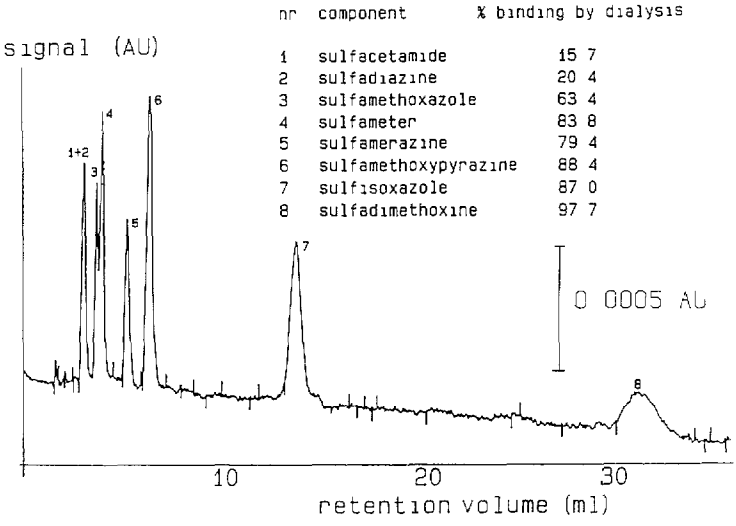


Fig 1 Chromatogram of sulfa drugs Column, 15 cm × 4 mm I D Resolvosil-BSA-7, mobile phase, 0 1 *M* phosphate (pH 7 4), flow-rate, 0 5 ml/min, injection volume, 1 µl, temperature, 37 °C, detector, UV (254 nm)

TABLE I

INFLUENCE OF MOBILE PHASE COMPOSITION ON THE CORRELATION BETWEEN $k'/(k'+1)$ AND THE DEGREE OF PROTEIN-BINDING AND THE CHROMATOGRAPHIC SPREADING FOR A NUMBER (n) OF HETEROGENEOUS DRUGS

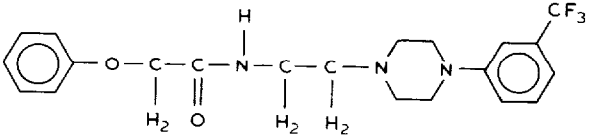
1-Propanol in mobile phase (%)	Correlation coefficient	n	S D in $k'/(k'+1)$
0	0 84	19	0 025
1 5	0 88	22	0 013
3	0 89	23	0 009
4 5	0 88	23	0 014

influence the correlation between chromatographic and equilibrium dialysis data However, at 0 and 1.5% 1-propanol, some compounds with high protein-binding are not eluted from the column. This is also reflected by the lower number (n) of measured drugs The mobile phase with 3% 1-propanol yields a lower S D for $k'/(k'+1)$ and is suitable for screening a large range of protein-binding

The protein-binding determined by dialysis and the $k'/(k'+1)$ values obtained with chromatography on the bovine serum albumin stationary phase with 3% 1-propanol in the mobile phase are shown in Table II The S D of the $k'/(k'+1)$ values of the individual drugs is also given, which reflects the re-

TABLE II

RESULTS OF $k'/(k'+1)$ AND PROTEIN-BINDING FROM LITERATURE FOR A HETEROGENEOUS GROUP OF 23 DRUGS

No	Component	Degree of protein-binding	$k'/(k'+1)$	S D in $k'/(k'+1)$
1	Befiperide[a]	99 5	0 988	0 001
2	 [a]	99 3	0 930	0 004
3	Phenylbutazone	99 1	0 981	0 001
4	Sulfadimethoxine	97 7	0 819	0 005
5	Chlorpromazine	95 1	0 944	0 006
6	Nitrosalicylic acid	94 5	0 945	0 003
7	Dipyridamole	91 7	0 941	0 003
8	Sulfinpyrazone	90 8	0 974	0 001
9	Buthalital	87 3	0 761	0 004
10	Sulfisoxazole	87 0	0 736	0 004
11	Sulfosalicylic acid	83 8	0 827	0 006
12	Sulfameter	83 0	0 541	0 006
13	Sulfamerazine	79 4	0 656	0 004
14	Sulfamethoxazole	63 4	0 516	0 011
15	Fluvoxamine[a]	54 0	0 614	0 009
16	Fluprazine[a]	53 5	0 628	0 001
17	Clovoxamine[a]	53 0	0 571	0 010
18	Hexobarbital	51 6	0 425	0 002
19	Salicylamide	50 8	0 563	0 009
20	Aniline	37 8	0 340	0 002
21	*p*-Aminobenzoic acid	31 7	0 377	0 008
22	Sulfadiazine	20 4	0 494	0 007
23	Sulfacetamide	15 7	0 416	0 019

[a]According to the equilibrium dialysis method described in Experimental

producibility of the chromatographic results Fig. 2 gives a graphical presentation of the results.

The occasional discrepancies between chromatographic data and equilibrium dialysis results are most probably due to the fact that with this chromatographic set-up only the binding with albumin is considered, whereas equilibrium dialysis is carried out with all plasma proteins Stationary phases with other chemically bonded plasma proteins would be desirable to improve the model.

The degree of protein binding within a group of analogous piperazines was determined with equilibrium dialysis according to the method described in Experimental as well as with the chromatographic method expressed as $k'/(k'+1)$. Table III and Fig. 3 report the results and indicate excellent correla-

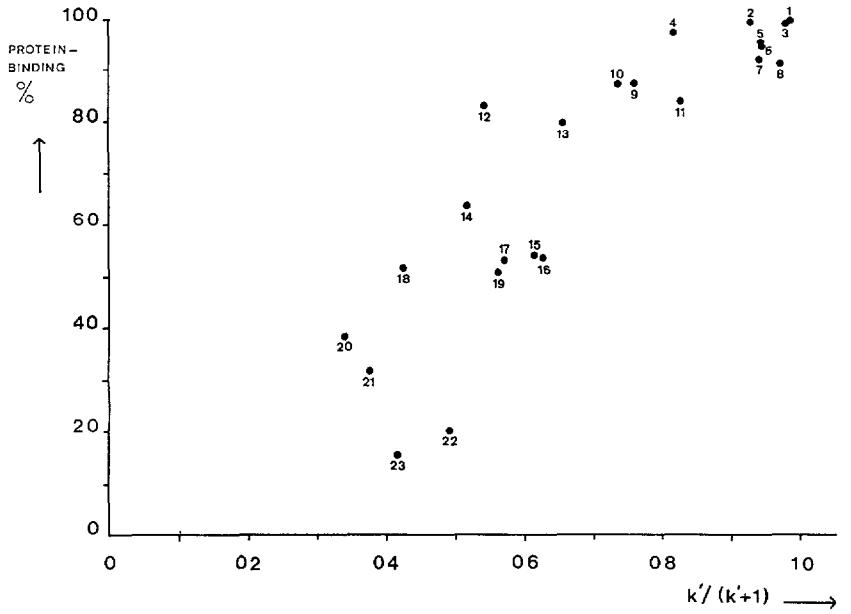


Fig 2 Relation between protein-binding (%) and $k'/(k'+1)$ for a heterogeneous group of 23 drugs For peak identification see Table II

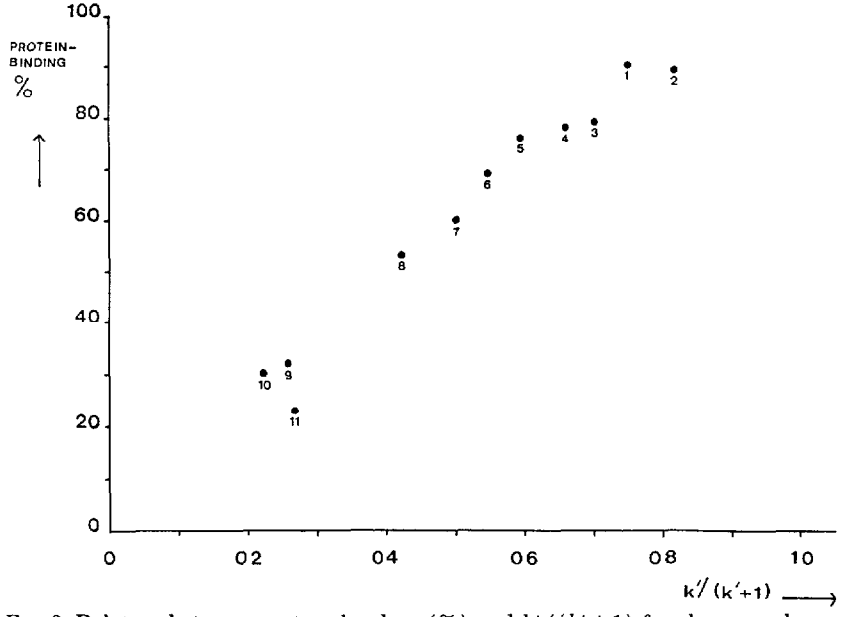
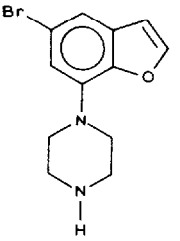
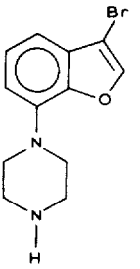
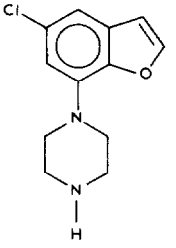
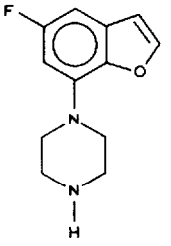


Fig 3 Relation between protein-binding (%) and $k'/(k'+1)$ for eleven analogous piperazines For peak identification see Table III

TABLE III

RESULTS OF $k'/(k'+1)$ AND PROTEIN-BINDING MEASURED BY EQUILIBRIUM DIALYSIS FOR ELEVEN ANALOGOUS PIPERAZINES

No	Component	Degree of protein-binding	$k'/(k'+1)$
1		90	0 749
2		89	0 816
3		79	0 701
4		78	0 660

(Continued on p 298)

TABLE III (continued)

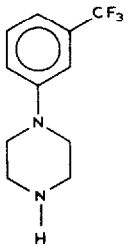
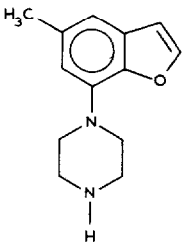
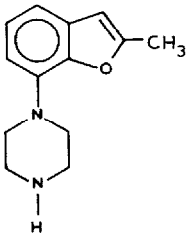
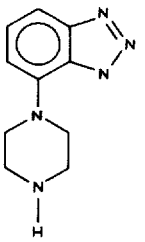
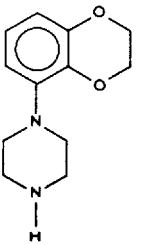
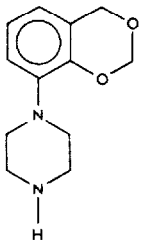
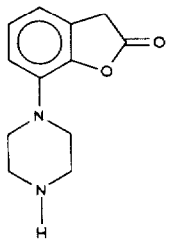
No	Component	Degree of protein-binding	$k'/(k'+1)$
5		76	0 595
6		69	0 547
7		60	0 501
8		53	0 423
9		32	0 258

TABLE III (continued)

No	Component	Degree of protein-binding	$k'/(k'+1)$
10		30	0 222
11		23	0 269

tion between chromatographic and equilibrium dialysis data. Within this group a coefficient of correlation of $r=0.981$ was obtained, indicating that the proposed method is very suitable for selecting compounds with either high or low protein-binding property from a group of analogous compounds. This may be of particular interest in the selection phase of new drug development

CONCLUSIONS

The chance of a drug being bound to a bovine serum albumin stationary phase, expressed as $k'/(k'+1)$, correlates well with protein-binding measured with conventional techniques based on equilibrium dialysis Within a group of analogous drugs a very good correlation is found, emphasizing the benefit of the method in the search for potential drugs with certain protein-binding properties. The method can be carried out as a routine HPLC analysis with a suitable detector Radioactive labelling is not necessary and only a small amount of technical grade material is required. Because the separation is based on the binding of the drug to the protein, determination of protein-binding of impure drugs is even possible. A limitation of the method is that binding a drug to albumin is not always the best model for plasma protein-binding. Chemical bonding of standard human plasma proteins on a matrix suitable for HPLC

will probably improve the model and accordingly the results Future studies on different types of protein-bonded phase will be performed

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