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CORRELATION BETWEEN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PLASMA PROTEIN BINDING

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SUMMARY

As hydrophobic interactions are involved in both reversed-phase liquid chromatography and plasma protein binding, the relationship between retention time and binding was investigated experimentally in two series of compounds. For betaxolol and its O-alkyl analogues, the nature of the O-alkyl group strongly influences the retention time on a Spherisorb CN 5-μm column. With 0.03 *M* acetate buffer (pH 5.6)-acetonitrile (60:40) as the mobile phase, k' values increase from 1.8 to 8.3 with a concomitant increase in plasma protein binding from 0.5% (R=H) to 88.2% (R=cyclopentylmethyl). The relationship between the free fraction and log k' is sigmoidal. In the second example, structural changes in the propyl side-chain of alpidem (a new anxiolytic) lead to minor variations in the protein binding: 98.9 to 84.9%. This slight decrease with the more polar metabolite is correlated with a sharp decrease in the k' values from 14.7 to 0.94 on a Supelcosil LC 18 DB column. Based on retention times, it should be feasible to predict qualitatively, if not quantitatively in some instances, plasma protein binding in a series of structurally similar compounds.

INTRODUCTION

Under reversed-phase conditions, hydrophobic forces are mainly responsible for retention in the stationary phase, and retention times increase with the lipophilicity of the molecule. Although the exact mechanism of retention are not fully elucidated as yet, alkyl-bonded phases have been widely used to assess the hydrophobicity of various classes of compounds [1–10].

In plasma many drugs are bound to proteins by electrostatic and/or hydrophobic interactions [11–13]. Hence, there should be a correlation between retention behaviour and protein binding for drugs that are mainly bound through hydrophobic interactions. Furthermore, in a series of analogues or for a drug and its metabolites, capacity factor (k') values should be of some predictive value for

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assessing the free fraction. This practical application was investigated with two examples: betaxolol (a β-blocker) and its O-alkyl analogues, and alpidem and its metabolites.

EXPERIMENTAL

Drugs and chemicals

Betaxolol, alpidem, their analogues and/or metabolites, [^{14}C]betaxolol and [^{14}C]alpidem were synthesized by the Chemistry Department of LERS. The structure of these compounds are given in Figs. 1 and 2.

Human serum albumin (HSA, 99% purity) and human α_1-acid glycoprotein (α-AGP, 99% purity) were obtained from Behring Institute (Hoechst Behring, France).

High-performance liquid chromatography

The liquid chromatographic system consisted of a pump module, Chromatem 380 (Touzart et Matignon, Paris, France), a WISP 710B automatic injector (Waters, France), and a fluorescence detector SFM 23 (Kontron, Switzerland). Retention times and peak areas were measured by the Laboratory Automated System from Hewlett-Packard, implemented on an HP 1000 computer.

Columns and chromatographic conditions

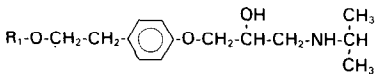
Betaxolol and its analogues. These were separated on a 15 cm$\times$0.46 mm I.D. stainless-steel column packed with 5-μm Spherisorb CN (Sopares-France). The mobile phase, a mixture of acetonitrile and 0.03 *M* acetate buffer (pH 5.6) (40:60, v/v) was pumped at a flow-rate of 1.0 ml min^{-1}. The compounds were detected by fluorimetry (excitation wavelength 275 nm and emission wavelength 305 nm).

Alpidem, its metabolites and analogues. Alpidem and its metabolites were separated under isocratic conditions. The 25 cm$\times$0.46 mm I.D. column and the 2 cm$\times$0.46 mm I.D. precolumn were packed with 5-μm Supelcosil LC 18 DB (Supelco-France). The flow-rate of the mobile phase, acetonitrile (pH 4.8)–0.025 *M* phosphate buffer–methanol (45:45:10, v/v/v) was 1.5 ml min^{-1}. The compounds were detected by fluorimetry (excitation wavelength 250 nm and emission wavelength 370 nm).

Determination of the protein binding

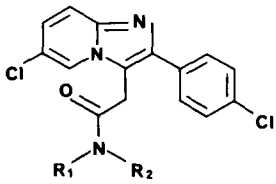
In vitro plasma binding of the drug was measured in triplicate by equilibrium dialysis, at 37°C, for 3 h in PTFE half-cells separated by a cellophane membrane (Diachema, Zürich, Switzerland). One compartment contained 0.7 ml of 0.02 *M* phosphate buffer (pH 7.4) and the other one (0.7 ml) the particular drug under study dissolved in plasma, human serum albumin (40 g l^{-1}) or α-acid glycoprotein solutions (1 g l^{-1}). The concentrations of betaxolol and alpidem were 50 and 100 ng ml^{-1}, respectively.

At equilibrium, drugs were determined from 0.5 ml in both compartments by reversed-phase liquid chromatography (RPLC) as described by Caqueret and Bianchetti [14] for betaxolol and its analogues and by Ascalone et al. [15] for



Compound	R_1
1	H
2 (Metoprolol)	CH_3
3	C_2H_5
4	CH_3–CH(CH_3)
5	C_3H_7
6 (Betaxolol)	cyclopropyl-CH_2
7	cyclobutyl-CH_2
8	cyclopentyl-CH_2

Fig. 1. Chemical structures of betaxolol and its analogues.



Compound	R_1	R_2
9	$-CH_2-CH_2-CH_2-OH$	$-CH_2-CH_2-CH_2-OH$
10	-H	$-CH_2-CH_2-CH_2-OH$
11	-H	$-CH_2-CHOH-CH_3$
12	-H	-H
13	-H	$-CH_2-C(=O)-CH_3$
14	$-CH_2-CH_2-CH_3$	$-CH_2-CH_2-CH_2-OH$
15	-H	$-CH_2-CH_2-CH_3$
16	$-CH_2-CH_2-CH_3$	$-CH_2-C(=O)-CH_3$
17	$-CH_2-CH_2-CH_3$	$-CH_2-CHOH-CH_3$
18 (Alpidem)	$-CH_2-CH_2-CH_3$	$-CH_2-CH_2-CH_3$

Fig. 2. Chemical structures of alpidem and its metabolites.

alpidem and its metabolites. The bound fraction was calculated as $(C_1-C_2)/C_1$, where C_1 is the concentration in the protein compartment and C_2 the concentration in the buffer at equilibrium.

RESULTS

Validation of betaxolol and alpidem protein binding

Betaxolol and alpidem bound fractions were also measured by use of ^{14}C-labelled molecules in order to validate the results obtained by RPLC. In this case, 0.2 ml

TABLE I

UNBOUND FRACTION AND k' VALUES OF BETAXOLOL AND ITS ANALOGUES

Compound*	k'	Free drug (%)		
		α_1-Glycoprotein	Albumin	Plasma
1	1.8	99.5	99.5	99.5
2 (metoprolol)	2.7	92.0	84.5	85.0
3	3.2	81.8	82.8	71.8
4	3.8	92.9	66.4	60.4
5	4.2	82.0	61.7	48.5
6 (betaxolol)	4.2	80.0	58.0	49.0
7	6.0	45.8	14.5	4.8
8	8.3	45.1	19.3	11.8

*Numbers refer to Fig. 1.

was sampled in each compartment after dialysis, and concentrations were calculated by radioactivity counting.

Plasma binding values of betaxolol and alpidem were 53.6 and 98.9%, respectively, with the ^{14}C-labelled molecules, and 51.0 and 98.9% with the RPLC method.

Betaxolol and O-alkyl analogues

The k' values for betaxolol and its analogues are reported in Table I; these data show that k' values vary from 1.8 to 8.3 and the binding to plasma proteins from 0.5% (R_1=H) to 88.2% (R_1=cyclopentylmethyl). For the binding to human serum albumin and α_1-acid glycoproteins the ranges are 0.5–80.7% and 0.5–54.9%, respectively.

An example of the chromatographic separation of these compounds is presented in Fig. 3.

By plotting the free fraction versus log k' values, sigmoidal curves are obtained in plasma and in pure protein solutions as shown in Fig. 4.

Alpidem and metabolites

As expected, alpidem which is less polar than its metabolites, is retained longer under reversed-phase conditions; it is also more extensively bound in plasma. Binding varies from 84.9% for the more polar compound to 98.9% for alpidem, with a concomitant increase in k' values from 0.94 to 14.7 (Table II).

DISCUSSION

Betaxolol and its analogues

The bound fraction values observed in this study for metoprolol and betaxolol are similar to those described in the literature [16,17]. The nature of the O-alkyl group in the chain opposite to the isopropyl amino radical strongly influences the binding to serum albumin, α_1-acid glycoprotein and plasma proteins. Substitu-

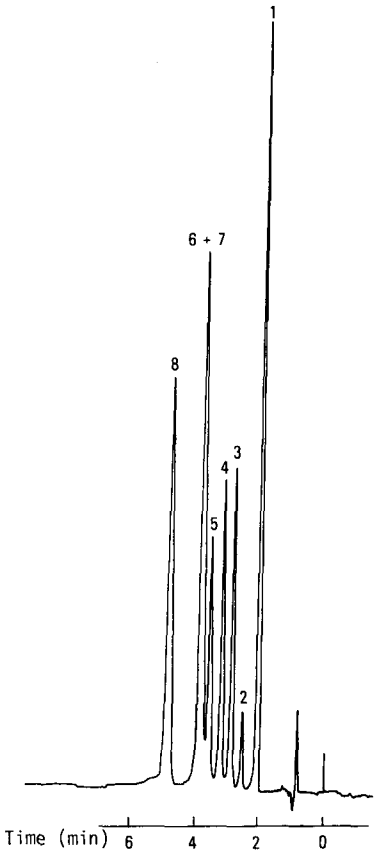


Fig. 3. RPLC of betaxolol and its analogues. The isocratic conditions are given in the text, and the structures of the compounds in Fig. 1.

tion of a hydrogen by a cyclopentylmethyl group transforms a totally free drug to one ca. 90% bound. This increase in binding is correlated with an increase in k' values; the relationship is sigmoidal in plasma as well as in HSA and α-AGP solutions. However, more scattering is observed with α-AGP. Retention times increase with the degree of substitution but binding tends to plateau as its value approaches 100%.

Alpidem and its metabolites

As expected, more polar metabolites are less retained than alpidem in RPLC. The span in k' values is quite large, from 0.94 to 14.7, so it is necessary to use a chromatographic system with a gradient in order to separate all the tested compounds in a reasonable time (36 min) for analytical purposes [18]. Structural changes in the propyl side-chain do not lead to major variations in the protein binding. This indicates that the aromatic rings are responsible for most of the protein binding, and this implies that hydroxylation of the aromatic moiety would result in a sharply decreased binding.

It should be noted that monosubstituted metabolites are less bound than disub-

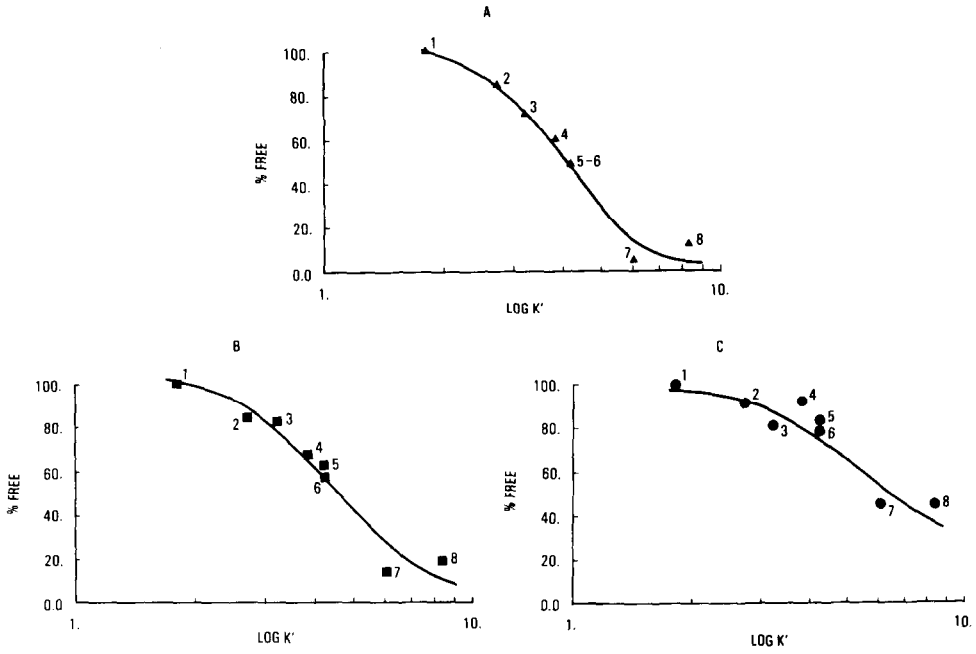


Fig. 4. Correlation between the free fraction and log k' for betaxolol and its O-alkyl analogues. (A) Plasma; (B) human serum albumin solution; (C) α_1-AGP solution. Numbers refer to Fig. 1.

stituted derivatives (Fig. 5). Hence there should not be any interaction between mono- and disubstituted metabolites at the binding sites.

These two examples show that in reversed-phase chromatography there is a good correlation, not necessarily linear, between retention behaviour and plasma protein binding. This correlation can be used to predict the binding of metabolites or analogues when hydrophobic forces are responsible for their binding to plasma

TABLE II

UNBOUND FRACTION AND k' VALUES OF ALPIDEM AND ITS ANALOGUES

Compound*	k'	Free drug (%)
9	0.94	15.1
10	1.17	8.3
11	1.27	12.6
12	1.30	9.4
13	1.89	5.5
14	3.17	3.6
15	3.69	3.3
16	4.87	2.5
17	5.30	2.4
18 (alpidem)	14.73	1.1

*Numbers refer to Fig. 2.

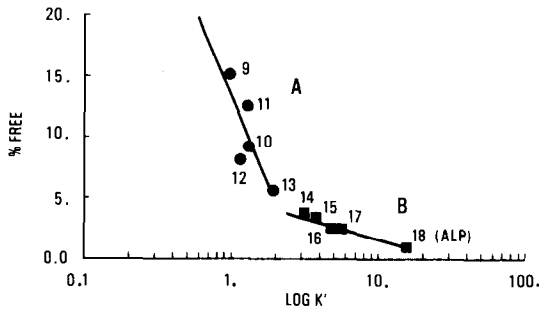


Fig. 5. Correlation between the free fraction and log k' for alpidem and its metabolites. ALP = alpidem; A = monosubstituted metabolites; B = disubstituted metabolites. Numbers refer to Fig. 2.

proteins. Knowing the binding values of metoprolol (compound 2) and betaxolol (compound 6), one would expect a negligible binding for compound 1, the O-dealkylated metabolite of both metoprolol [19] and betaxolol [20]. Indeed, the binding of compound 1 is less than 0.5%: the latter value is determined by the sensitivity limit of the betaxolol assay (1 ng ml^{-1}).

Obviously the correlation holds true for metabolites that are hydroxylated, but may no longer be valid for those containing a carboxylic acid, for which electrostatic forces would predominate, or for compounds that exhibit concentration-dependent binding.

The authors emphasize the empirical nature of the approach. It was not intended to correlate retention behaviour with binding thermodynamic parameters. For a summary of our knowledge on the determination of hydrophobic parameters by RPLC, the reader is referred to a recent review by Braumann [5]. In particular, the problem of the determination of the mobile phase hold-up time should be addressed, if one is interested in measuring k' values with great accuracy. For this type of work, *n*-alkyl-bonded columns are recommended (as shown with alpidem and its metabolites). The CN column was chosen in our study with betaxolol and its analogues solely on the basis of the separation power: an octadecyl column would give the same results but with less symmetrical chromatographic peaks.

In conclusion, even if retention behaviour may not always quantitatively predict the binding in plasma, it should give the ranking order of the binding of metabolites or analogues. This is particularly useful in the approximation of the free fraction of metabolites and the prediction of potential binding interactions between a drug and its metabolites. The metabolites are usually not available in sufficient amount to perform equilibrium dialysis experiments.

The present study corroborates the results of a previous one in which we have shown that phenytoin labelled with ten deuterium atoms is less retained and significantly less bound to plasma proteins than unlabelled phenytoin [20].

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