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Simple equilibrium dialysis–high-performance liquid chromatographic method for the *in vitro* assessment of 5-methoxypsoralen bound to human albumin

S. MAKKI*

*Laboratoires de *Chimie Analytique et de Dermatologie et Biologie Cutanée, Faculté de Médecine et Pharmacie, Place St. Jacques, 25030 Besançon Cedex (France)*

P. MURET

Laboratoires de Dermatologie et Biologie Cutanée et de Pharmacologie, Faculté de Médecine et Pharmacie, Place St. Jacques, 25030 Besançon Cedex (France)

A. RENAUD

Laboratoire de Pharmacologie, Faculté de Médecine et Pharmacie, Place St. Jacques, 25030 Besançon Cedex (France)

P. AGACHE

Laboratoire de Dermatologie et Biologie Cutanée, Faculté de Médecine et Pharmacie, Place St. Jacques, 25030 Besançon Cedex (France)

and

P. MAGNIN

Laboratoire de Pharmacologie, Faculté de Médecine et Pharmacie, Place St. Jacques, 25030 Besançon Cedex (France)

ABSTRACT

Increasingly used in therapeutics, 5-methoxypsoralen (5-MOP), a linear furocoumarin, associated with UVA irradiation (PUVA), is now an established treatment for skin diseases such as vitiligo, mycosis funcoides and particularly psoriasis. Succesful PUVA therapy depends on a sufficiently high peak 5-MOP plasma concentration coinciding with the UVA irradiation. However, as with most drugs, only the free plasma fraction is able to enter the target cells and has a pharmacological effect.

In this work, the binding of 5-MOP to human albumin was studied *in vitro*, using a dialysis chamber. Bound and free 5-MOP fractions were quantified by a modification of Stolk's high-performance liquid chromatographic method. Dialysis was performed at 37°C and pH 7.4 for 2 h, against a 4% albumin solution in phosphate buffer. The 5-MOP concentrations used were from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-2}$ g/l in $1 \cdot 10^{-1}$ g/l steps. The 5-MOP bound strongly to human albumin in an unsaturable way. The mean 5-MOP binding to albumin was 95.3%. These results are in accordance with those published by Artuc *et al.* and not with those of Veronese *et al.*, who found a lower saturable fixation (91%). These two research groups used tritiated 5-MOP. The technique used in this work is simple and inexpensive. It can be employed easily *in vivo*, *e.g.*, for the assessment of 5-MOP free fractions in different therapeutic conditions.

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INTRODUCTION

The psoralens, linear furocoumarins, are increasingly used in therapeutics. The psoralens employed in dermatology are 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) and trimethylpsoralen (TMP). The PUVA therapy, association of psoralens (P) with ultraviolet radiation (UVA) is one of the main treatments for psoriasis and vitiligo. 5-MOP (Fig. 1), the last to be introduced in this therapy, is the least studied, especially regarding its binding to serum albumin, the principal carrier of the molecule to the skin.

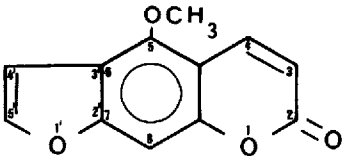

Fig. 1. 5-Methoxypsoralen (5-MOP).

Artuc *et al.* [1] and Veronese *et al.* [2] studied the relationship between 5-MOP and albumin. They used radiolabelled 5-MOP to determine its fixation to human albumin. However, their results concerning the saturation of the binding of 5-MOP to the albumin were contradictory.

In this paper, we present a simple method which employs equilibrium dialysis for the study of 5-MOP fixed to human albumin and the detection of the remaining non-linked, free drug. The determination of these fractions was carried out by high-performance liquid chromatography (HPLC). No radiolabelled 5-MOP was employed. Our results are in agreement with those reported by Artuc *et al.* [1].

EXPERIMENTAL

Equilibrium dialysis

The binding of 5-MOP was accomplished with a Dianorm apparatus [3]. The drug was placed in one half of each cell and the human albumin in the other half. The two halves were separated by a semi-permeable cellulose membrane (Diachema). The experiments were carried out under the following conditions: 37°C, pH 7.4, phosphate buffer (0.2 *M*) and incubation for 2 h with constant rotation at 20 rpm.

Extraction procedure

Sample volumes of 0.5 ml were withdrawn at different time intervals (0, 0.25, 0.50, 1, 2 and 18 h) from the two sides (halves) of the cells: the free 5-MOP from the first half of the cell and the free and bound 5-MOP from the second half. Each sample was extracted with 5 ml of heptane–methylene chloride (4:1, v/v). The mixture was stirred for 5 min and centrifuged at 1700 *g* for about 7 min. The organic layer was transferred to a test-tube and evaporated to dryness on a water-bath at 50°C under a stream of nitrogen [4].

HPLC

HPLC separations were carried out with a Merck–Hitachi Model 655A-11 instrument. The method was based on Stolk's technique [4], with some modifications. To increase the sensitivity, a spectrofluorimeter (excitation at 312 nm, emission at 490 nm [5]) was used instead of a UV detector. The mobile phase was methanol–water (60:40, v/v). The column was LiChrospher 100 RP-8 (5 μm) in LiChrocart 125-4 (Merck, Darmstadt, Germany). After having established the calibration graph with solutions of known concentrations, psoralens, extracted from phosphate buffer, were injected into the HPLC system.

Calibration graph

Basic solutions were prepared with 200 μg/ml of 5-MOP and 8-MOP in ethanol. The calibration graph was obtained by adding 25, 50, 75, 100, 300 and 600 ng/ml of 5-MOP and 200 ng/ml of 8-MOP to six human serum samples of 1 ml each, free of drug. 8-MOP was used as an internal standard. The calibration graph was a plot of peak-height ratios (5-MOP/8-MOP) *versus* 5-MOP concentrations in different samples.

RESULTS AND DISCUSSION

Determination of free (F) and bound (B) 5-MOP at different times

Table I shows the concentrations of F- and B-5-MOP as a function of time. It is noticeable that by the second hour (Fig. 2), an equilibrium between the two halves of the cell was obtained with equal concentrations of F-5-MOP and stabilization of 5-MOP bound to human albumin. The standard deviations are small. This demonstrates little variation and good reproducibility. The concentration of 5-MOP used for this study was 196 ng/ml. The free active fraction of 5-MOP was 8 ng/ml (about 5%) and there was no change in this value between 2 h and 18 h.

Assessment F- and B-5-MOP in relation to increasing concentrations

Table II shows that at a concentration of 50 ng/ml, the proportion of the 5-MOP bound to albumin was small. From 200 to $5 \cdot 10^4$ ng/ml the binding of 5-MOP to

TABLE I

DETERMINATION OF FREE (F) AND BOUND (B) 5-MOP AS A FUNCTION OF TIME

Each value represents the average of three dialyses. All samples were analysed twice by HPLC.

Time (h)	5-MOP (ng/ml)		
	Free (F)	Bound + free (B + F)	Bound (B), calculated
0	196	–	–
0.25	63.8 ± 0.8	85.8 ± 0.6	22.4 ± 1.6
0.50	27.7 ± 0.8	138.8 ± 2.0	111.2 ± 2.0
1	14.0 ± 1.4	152.7 ± 0.1	138.0 ± 0.5
2	8.0 ± 1.3	173.0 ± 0.1	165.0 ± 0.7
18	7.8 ± 1.2	170.8 ± 1.0	162.8 ± 1.0

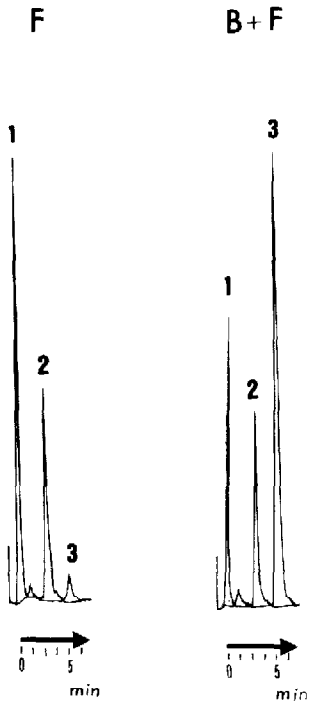

Fig. 2. Chromatograms of psoralens, detected for the determination of free 5-MOP (F-, peak 3) and bound and free 5-MOP (B + F-, peak 3) at equilibrium (2 h). Peaks: 1 = solvent (ethanol); 2 = 8-MOP (internal standard); 3 = 5-MOP.

albumin was stabilized at about 95% and B-5-MOP/F-5-MOP ratio remained constant (20.00 ± 0.36). The lower binding ability at low concentrations of 5-MOP could be due to fixation on a first type of receptor on the albumin molecule, quickly saturated, and hidden by the very large binding ability of a second receptor. Such a phenomenon has been described for some drugs bound to albumin in an unsaturable way such as weak acids [6].

TABLE II

ASSESSMENT OF F- AND B-5-MOP IN RELATION TO INCREASING CONCENTRATION

Concentration (ng/ml)	F (ng/ml)	B + F	B (ng/ml)	B/F	% of B
50	4.1	41	36.9	9	90
200	8.1	173.2	165.1	20.38	95.3
$1 \cdot 10^3$	40.3	851.4	811.1	20.12	95.2
$1 \cdot 10^4$	435.2	9438.6	9003.4	20.68	95.4
$5 \cdot 10^4$	2230.6	47 320.7	45 090.1	20.21	95.2

DISCUSSION

The aim of this work was to devise a simple technique for the *in vitro* determination of 5-MOP bound to human albumin. This would be of help in assessing the free 5-MOP concentration, which is the fraction responsible for the therapeutic effect.

The binding to human serum albumin of 8-MOP, the most commonly employed psoralen in PUVA therapy [7], has been evaluated by several investigations using liquid scintillation counting, and showed saturable binding [8–11]. For 5-MOP only a few studies are available, in which radio-labelled psoralen was used. Whereas Artuc *et al.* [1] found an unsaturable binding of 5-MOP to human albumin in the 5 ng/ml–230 μg/ml, Veronese *et al.* [2] reported saturable binding using a more limited range of concentration (30 ng/ml–30 μg/ml) (Table III). However, in the latter work, there was only a trend towards a saturation process visible at higher concentrations.

TABLE III

SUMMARY OF METHODS AND RESULTS

Step	Parameter	Artuc *et al.* [1]	Veronese *et al.* [2]	This work
Dialysis equilibrium	Time (h)	36–50	14	2
	Temperature (°C)	4	37	37
	Apparatus	Rotating	Agitated	Rotating
	Buffer	0.1 *M* phosphate (pH 7.2)	0.05 *M* phosphate (pH 7.4)	0.2 *M* phosphate (pH 7.4)
5-MOP determination	–	Liquid scintillation counting	Liquid scintillation counting	HPLC
Results	Binding (%)	98–99	91	95.3
	Saturation	Non-saturable	Saturable	Non-saturable
	Concentration range	5 ng/ml–230 μg/ml	30 ng/ml–30 μg/ml	50 ng/ml–50 μg/ml

The method proposed here is simple and rapid and does not require labelled drug. In 1984, Prognon [5] used this technique to study 3-carbethoxypsoralen fixation to albumin. Using this method, the *in vivo* fixation of psoralens on albumin can be assessed in clinical practice. This is of major importance as the strong correlation observed between 5-MOP plasma concentration and PUVA therapy efficiency [7] may be related only to the free 5-MOP concentration. Investigations of the influence of low- and high-fat meals on the fixation of 5-MOP to human albumin are in progress.

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REFERENCES

1 M. Artuc, G. Stuettgen, W. Schalla, H. Schaffer and J. Gazith, *Br. J. Dermatol.*, 101 (1979) 669.
2 F. M. Veronese, R. Bevilaequa, O. Schiavon and G. Rodighiero, *Farmaco, Ed. Sci.*, 34 (1979) 716.
3 M. Lecomte, R. Zini, P. d'Athis and J. P. Tillement, *Eur. J. Drug Metab. Pharmacokinet.*, 1 (1979) 23.
4 L. M. Stolk, *Pharm. Weekbl.*, 2 (1980) 281.
5 P. Prognon, *Thèse Série E*, No. 194. Université Paris Sud, Paris, 1984.
6 R. Zini, P. d'Athis, J. Barre and J. P. Tillement, *Biochem. Pharmacol.*, 28 (1979) 2661.
7 E. Andrew, A. Nilsen, P. Thune and I. Wilk, *Clin. Exp. Dermatol.*, 6 (1981) 591.
8 F. M. Veronese, R. Bevilacqua, O. Schiavon and G. Rodighiero, *Farmaco, Ed. Sci.*, 33 (1978) 667.
9 R. Bevilacqua, C. A. Benassi, O. Schiavon and F. M. Veronese, *Farmaco, Ed. Sci.*, 36 (1981) 598.
10 O. Schiavon, C. A. Benassi and F. M. Veronese, *Farmaco, Ed. Sci.*, 39 (1984) 109.
11 M. Pibonin, R. Zini, P. Nguyen, A. Renouard and J. P. Tillement, *Br. J. Dermatol.*, 117 (1987) 207.