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Protein binding of methohexital. Study of parameters and modulating factors using the equilibrium dialysis technique

I. Girard[a], S. Ferry[a,b]

[a] *Unité de Pharmacologie Clinique, Unité de Phase I: Hôpital Cardiologique, 28 avenue du Doyen Lépine, 69003 Lyon, France*
[b] *Laboratoire de Pharmacie Clinique et d'Evaluation du Médicament, Faculté de Pharmacie, 8 avenue Rockefeller, 69008 Lyon, France*

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Abstract

This paper describes the parameters that characterize methohexital–albumin binding and the influence of physiological or analytical factors on this binding. Two useful and reproducible methods for measuring the free concentration—equilibrium dialysis (ED) and ultrafiltration (UF)—are described and their performances are compared. Methohexital binds exclusively to albumin according to a two-class binding model. The first is a saturable class site of high affinity constant ($K_A = 11\,200\ M^{-1}$) and a number of sites per albumin molecule of 1. The second is a non-saturable site of poorer affinity ($nK_A = 810\ M^{-1}$). The bound fraction of methohexital in the therapeutic range and at physiological albumin concentration is $86.7 \pm 0.9\%$ in isolated albumin solution. In serum, it ranges from 80 to 84.5%, according to subjects ($n = 6$). Binding is inhibited by the presence of endogeneous compounds of serum (for a given albumin concentration the bound fraction decreases from 90.3% in isolated albumin solution to 82.6% in serum), probably by free fatty acids. An increase in the bound fraction is observed when the pH is increased from 7 to 9. This phenomenon may be explained by a higher affinity of the drug towards the basic (B-form) conformation of the albumin molecule, in analogy with the close barbiturate thiopental. A decrease in the bound fraction against temperature is shown, as though binding forces diminished with increase in temperature. Indeed, the binding modification is less pronounced in the presence of serum endogenous compounds. As expected, there is no evidence of any effect of heparin anticoagulant on the bound fraction. Methohexital binding is strongly modified by the albumin concentration; the bound fractions change from 67 to 91% in the albumin range 150–900 μM.

Keywords: Equilibrium dialysis; Methohexital; Protein binding; Serum; Ultrafiltration

1. Introduction

The binding of a drug to serum proteins influences its distribution, elimination and pharmacological activity [1,2]. Many workers have dealt with the importance and interest of protein binding for the interpretation of drug concentrations and pharmacokinetics [3–5]. The implications of plasma drug binding for anaesthesiologists have been reviewed by Wood [6]. Marked changes in

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plasma composition occur after trauma or surgery with increasing concentrations of albumin and free fatty acids and decreasing concentrations of globulin and α-glycoprotein acid, leading to altered binding of drugs such as lidocaine, phenytoin and quinidine.

The effect of cardiopulmonary bypass during cardiac surgery on drug protein binding are complex [6–10]. The associated haemodilution affects drug binding, not only because of dilution of endogeneous proteins, but also because of the non-physiological protein concentrations in the pump prime. Buylaert et al. [8] suggested that free instead of total drug concentrations should be considered for discussion of the pharmacodynamic consequences of pharmacokinetic changes occurring during the bypass procedure.

In addition, variation of plasma pH, with possible protein binding consequences, may occur during anaesthesia. Co-administration during anaesthesia of other drugs may also have consequences in protein binding. Volatile anaesthetics at high concentration can cause structural changes in proteins such as albumin and modifications of thiopental binding [11,12] were observed. Russo et al. [13] have studied the displacement of thiopental from its binding sites in albumin, by association with 11 drugs in 50 combinations of drugs and molar ratios. Bally et al. [14] observed a potentialization of the methohexital effect when associated with fentanyl, but they did not relate it with a possible binding displacement. For the same methohexital administration, when associated with fentanyl the recovery is longer and the concentration in plasma when awake is almost half.

Methohexital, an ultra-short acting barbiturate, is used as an intravenous anaesthetic barbiturate, together with thiopental. Methohexital may be likened to thiopental with regard to their physicochemical properties, and the methodology for methohexital binding measurements should be close to that of thiopental. Both drugs are weak acids ($pK_a = 7.9$ and 7.6 for methohexital and thiopental, respectively), little ionized at physiological pH (24% and 39% for methohexital and thiopental, respectively), highly lipophilic (oil/water partition coefficient 65% for methohexital and 89% for thiopental). Strongly bound to albumin, methohexital can be classified among type III drugs [2].

Recovery from anaesthesia is significantly shorter than after administration of thiopental [2,15–17]. This is largely a result of the rapid redistribution process to less perfused tissues, as is the case for thiopental and hexobarbital. Short recovery is also due to rapid inactivation of the drug by hepatic metabolism [2,18].

The hepatic extraction of methohexital (extraction coefficient 0.5) is less affected than that of thiopental (extraction coefficient 0.2) by protein binding variations.

The elimination rate of methohexital from plasma is faster than that of thiopental [2,15,16,19], with a higher clearance of 11 ml kg^{-1} min^{-1}, and a three times shorter half-life of 2–4 h.

The pharmacokinetic and clinical consequences of alterations of protein binding of thiopental [1–6] have been widely investigated by numerous workers. Less attention has been focused on methohexital protein binding.

During pharmacokinetic studies, Brand et al. [16] used ED for methohexital protein binding measurements with a much long dialysis time of 18 h. More recently, Bjorksten and co-workers [9,20] used UF at room temperature and LC assay for binding measurements of methohexital and thiopental during cardiopulmonary bypass. Redke et al. [21] used the Bjorksten UF assay [9,20] followed by gas chromatographic determination.

The aim of the present work was to determine the characteristics of methohexital–albumin binding and the influence on the bound fraction of determinant factors such as plasma pH, albumin concentration, temperature and heparin anticoagulant. The methodology for the determination of the free concentration and bound fraction of methohexital in plasma by ED and UF is described and the performances of both techniques are compared.

2. Experimental

2.1. Reagents

Sodium methohexital (MW = 284) was obtained from Lilly (France) and sodium secobarbi-

tal (MW = 260) from Roussel Uclaf (France). Potassium dihydrogenphosphate and disodium hydrogenphosphate, Suprapur grade, were supplied by Merck (Darmstadt, Germany). Acetonitrile was of Uvasol spectrophotometric grade (Merck). Water for the preparation of buffer and stock solutions was double distilled (Rathburn, Walkerburn, UK). Hexane, diethy ether, 2-propanol and other chemicals were of analytical grade from Merck. Albumin (human serum albumin, A 1887, essentially fatty acid free, prepared from fraction V) (MW 66 248) was supplied by Sigma Chemical (France).

2.2. Human serum

Human serum was obtained from normal healthy volunteers from 20 to 40 years old. Total protein and HSA concentrations were assayed in each serum or pool of sera after sampling and after thawing before use. Total protein and HSA were determined by the colorimetric biuret assay and the colorimetric BCP assay (Sigma diagnostic, France) respectively.

Serum was kept at −20°C. Before use, the pH was measured and adjusted to 7.4 if necessary by addition of lactic acid. Protein-free serum was obtained by ultrafiltration of human serum on an Ultrafree-CL system (Millipore, St. Quentin en Yvelines, France).

2.3. Standards and buffer preparation

Stock solutions of methohexital and secobarbital at concentrations of 3.5 mM (1 g l^{-1}) and 3.8 mM (1 g l^{-1}), respectively, were prepared in doubly distilled water and stored at 4°C for 3 weeks.

Dilute stock solutions of methohexital at 176 μM (50 mg l^{-1}) and of secobarbital at 7.7 μM (2 mg l^{-1}) and 0.77 μM (0.2 mg l^{-1}) were prepared in doubly distilled water and stored at 4°C for 3 weeks.

Before each experiment, calibration solutions of methohexital in phosphate buffer at 17.6 μM (5 mg l^{-1}) and 1.76 μM (0.5 mg l^{-1}) were prepared.

Sorensen buffer (pH 7.4, 0.067 M) was prepared with doubly distilled water by mixing 200 ml of 0.067 M KH_2PO_4 with 800 ml of 0.067 M Na_2HPO_4. The pH was adjusted by adding orthophosphoric acid.

Solutions of human serum albumin (HSA) were obtained by dissolving appropriate amounts of HSA in Sorensen buffer. If necessary, the pH was adjusted to 7.4 by adding lactic acid. HSA solutions were prepared before each experiment.

2.4. Equilibrium dialysis

2.4.1. Methodology

Equilibrium dialysis was performed using a Dianorm (Science Tech) apparatus with 20 cells of the macro 1S type (1 ml/1 ml) with a high membrane surface area. Experiments were carried out in a water-bath at 37°C and under constant stirring at 12 rpm. Each half-cell was filled with a volume of 950 μl instead of 1 ml, to avoid leakage problems encountered when cells were completely filled. In all experiments, the drug was introduced in the protein side of the cell.

Cell compartments were separated by Diachema membranes (Science Tech, Type 10.14), manufactured from natural cellulose, with a molecular weight cut-off of 5000. The membranes were rinsed with doubly distilled water for 15 min and incubated overnight with the buffer before use.

The time needed to reach dialysis equilibrium was 1 h, and the equilibrium remained unchanged for at least 5 h. All subsequent experiments were performed for 1.5 h. The dialysis buffer was 0.067 M Sorensen phosphate buffer (pH 7.4).

2.4.2. Influence of pH on drug–protein binding

Serum at various adjusted pH values (7.0, 7.4, 7.9, 8.95), spiked with 17.6 μM methohexital, were dialysed ($n = 2$). A plot of bound fractions versus pH was determined.

2.4.3. Comparison of methohexital binding in serum and in isolated protein

Solutions of methohexital (1.76, 17.6 and 35.2 μM) in serum (HSA 785 μM, free fatty acids 0.34 mM) and in isolated albumin (HSA 785 μM) were dialysed under the same conditions. The bound fractions found with the two matrices were compared.

2.4.4. Effect of temperature

The effect of dialysis temperature on the resulting bound fraction of methohexital in serum and in isolated albumin solution was studied in the range 20–45°C. The spiked methohexital concentration was 17.6 μM.

2.4.5. Effect of heparin anticoagulant:

For comparison of drug-protein binding in the presence and absence of heparin, serum and heparinized plasma from the same person were spiked with drug (17.6 μM) and dialysed under the described conditions.

2.4.6. Influence of albumin concentration

Solutions of HSA ranging from 151 μM (10 g l^{-1}) to 906 μM (60 g l^{-1}) were spiked with methohexital 17.6 μM ($n = 6$ for each HSA concentration) and dialysed under the same conditions as described.

2.4.7. Calculations

The unbound fraction (f_U) is calculated as $f_U = C_{Ud}/C_{Td}$, where C_{Td} is the total concentration of retentate in the protein side. To obtain the starting "in vivo" unbound concentration (C_U) of the drug, the total starting concentration (C_T) must also be measured: $C_U = C_{Ud}C_T/C_{Td}$. This equation assumes that cell volumes remain constant during dialysis and that binding is linear over the considered range of drug concentrations.

2.4.8. Calculation of binding parameters

The drug binding was studied over a wide range of methohexital concentrations (9–8900 μM) in isolated albumin solution (HSA 600 μM). The studied range of drug concentrations originate from the range 0.1 K_D–100 K_D. The dissociation constant K_D ($K_D = 1/K_A$) was estimated from the plot of $C_B/C_U = f$(albumin concentration), whose slope is representative of nK_A. The curve $C_B = f(C_U)$ was plotted. Binding was found to be a combined saturable–non saturable process. The association constants K_A for each class of site and the number of binding sites (n) per protein molecule were calculated according to the equation of saturable–non-saturable binding:

$$C_B = \frac{n_1 P K_1 C_U}{1 + K_1 C_U} + n_2 P K_2 C_U$$

where P represents the albumin concentration.

The binding parameters were calculated using a non-linear least-squares method based on a Gauss–Newton algorithm, with Micro Pharm software [22].

2.5. Ultrafiltration

Ultrafiltration was performed with the Ultrafree-CL filtration system, type UFC4 LGC 25, with a cellulose membrane with a molecular weight cut-off (MWC) of 10 000. Interfering chromatographic peaks were eliminated by rinsing the filters twice with 2 ml of water.

The filters were filled with 2 ml serum or protein containing solution and filtration was carried out by centrifugation in a 35° angle rotor at 3000 r.p.m. (1460 g) during 30 minutes. Centrifugation were performed at regulated temperature (22°C). 700 μl of ultrafiltrate were collected in these conditions. The unbound concentration of methohexital was determined by measuring the drug concentration in the ultrafiltrate.

2.6. Measurement of methohexital concentrations

The concentration of methohexital in plasma, albumin solutions, dialysates and ultrafiltrates was determined by high performance liquid chromatography (HPLC). The HPLC technique used [23] was slightly modified to enhance sensitivity.

Briefly, 0.5 ml of plasma spiked with 0.5 ml of internal standard solution (secobarbital, 0.77 or 7.7 μM or higher concentrations, depending on the measured methohexital concentrations) were extracted twice with 3 ml of hexane–diethyl ether–2-propanol (50:50:2, v/v/v). The organic layer was evaporated to dryness and the residue dissolved in 0.2 ml of mobile phase. An aliquot of 50 μl was analysed by HPLC.

HPLC assay was performed on a reversed-phase Novapak C_{18} column (150 × 4.6 mm i.d., 5 μm particle size; Waters, France), with a mobile phase consisting of acetonitrile–water (37:63,

v/v). The flow rate was 1.2 ml min^{-1} and detection was performed at 195 nm.

The method was linear over a wide range (0.17–1.700 μM (0.05–500 mg l^{-1}) of methohexital concentrations. The quantification limit was 0.09 μM (0.025 mg l^{-1}) (accuracy 97%, RSD = 6.6% ($n = 6$). The inter-assay RSD ranged from 5.8% ($n = 6$) at 0.176 μM methohexital to 7.4% ($n = 7$) at 1.76 μM and 5.8% ($n = 10$) at 17.6 μM.

Quality control samples of plasma spiked with known methohexital concentrations were included with each sample assay.

2.7. Data analysis

Results are expressed as mean ± standard deviation (SD). Differences in binding results were assessed by a paired t-test ($p < 0.05$).

3. Results and discussion

The time to attain equilibrium during ED studies if often considered to limit the usefulness of the method. The methohexital equilibrium time reported by Brand et al. [16] is 18 h, and reported equilibrium times for thiopental are variable: 3.5 h [13,24,25], 8 h [11,12], 12–16 h [26] and 24 h [27], depending on the experimental conditions. The use of high-diffusivity membranes and dialysis cells with high Q-factors efficiently reduces the equilibrium time to 1 h. For safety a time of 1.5 h was used in subsequent assays.

No important alteration of the cell volumes caused by osmotic effects of the protein molecules was observed, as expected for dialysis times shorter than 2.5 h. The dilution of total protein concentration found was only 4.5% (mean, $n = 6$). The absence of protein leakage through the membrane was verified.

The non-specific binding of the drug to the membrane and apparatus was studied in the absence of protein, as suggested by Fois and Ashley [28]. The results showed no adsorption of methohexital to 5000 MWC membranes and cells. Assays with Diachema membranes of 10 000 MWC, more difficult to manipulate, showed no adsorption, a shorter equilibrium time (30 min instead of 1 h), but poorer reproducibility, probably due to protein leakage.

The variability of the obtained bound fractions with different methodological factors, in particular different dialysis buffers [29–31], makes the inter-study comparison of results difficult, as has already been emphasized [31–33]. Moreover, methodological factors are often briefly or incompletely discussed. The suitability of Sorensen buffer for dialysis was validated by comparison of the bound fraction obtained by dialysis of serum versus Sorensen buffer and versus the same serum where proteins have been eliminated by ultrafiltration ("ideal" dialysis solution, owing to the same electrolyte composition as serum).

Comparison of the bound fraction of methohexital measured by UF and ED at 22°C showed unchanged results (Table 1). Jung et al. [26] also found similar results for thiopental ED and UF assay at 24°C, but differences were found at 37°C, probably owing to the greater protein leakage at this temperature. A comparison of both techniques at physiological temperature could not be made because of the unavailability of suitable materials.

The length of the centrifugation time also favours the protein leakage. Some cases of protein leakage after 30 min of centrifugation were observed in spite of the small MWC of membranes (10 000 Da).

When comparing the two separation techniques, it can be seen that the determination of free concentration is much simpler by UF. Determination of free concentration by ED is the result

Table 1
Comparison of unbound fractions of methohexital obtained after ED and UF at 22°C of 17.6 μM methohexital in serum and albumin solution

Matrix	Method	n	Unbound fraction ± SD (%)
Albumin solution (HSA 600 μM)	ED	4	6.1 ± 0.15
	UF	2	6.3 [a]
Serum (HSA 490 μM)	ED	5	17.7 ± 1.7
	UF	6	18.4 ± 0.77 [a]

[a] Difference ED-UF not significant, $p > 0.05$.

Table 2
Intra-assay and inter-assay reproducibility of methohexital free concentration (C_U) measurements by ED and UF

Technique	Assay	Matrix	Total methohexital concentration (μM)	n	RSD (%)
ED	Intra-assay reproducibility	Albumin solution (HSA 600 μM)	17.6	6	6.9
			1.76	8	8.7
		Serum (HSA 620 μM)	17.6	5	9.3
			1.76	5	6.3
	Inter-assay reproducibility	Albumin solution (HSA 600 μM)	17.6	10	6.7
			1.76	9	11.7
		Serum (HSA 600 μM)	17.6	6	8.0
UF	Intra-assay reproducibility	Albumin solution (HSA 600 μM)	17.6	8	4.0
			1.76	9	9.5
	Inter-assay reproducibility		17.6	8	7.7
			1.76	5	9.5

of three concentration measurements (C_{Ud}, C_{Td}, C_T) instead of one for UF. The reproducibility and limit of quantification of methods were similar (Table 2). The frequently observed protein leakage is a drawback of the UF system under the described conditions. ED, considered as the reference method, is to be preferred to UF for binding measurements under physiological conditions (equilibrium conditions, physiological temperature). UF will be better applied to measurements of free concentrations in pharmacokinetic or pharmacological studies, concerning mainly concentration variations.

The binding of methohexital in serum and in isolated albumin is shown in Table 3. The increase in the bound fraction in isolated albumin solution is representative of the phenomenon of inhibition of drug–albumin binding in the presence of serum endogenous compounds. Free fatty acids, although not present in excess, are probably responsible for drug displacement, as is often the case for drugs of group III [2]. This is consistent with the observations of Cherrah [34] on phenobarbital, whereas no significant difference was found between thiopental binding in serum and in albumin solutions [24], and no influence of free fatty acids on the binding of thiopental was observed by Morgan et al. [35].

An increase in the bound fraction of methohexital was obtained when the serum pH was increased from 7 to 9 (Fig. 1). This contrasted with the observation that the non-ionized form of acidic drugs has a higher affinity for HSA. Also, consider the N → B transition of albumin conformation between pH 6 and 9 [36], an increase in pH could be expected to result in a decrease in the albumin capacity to attract anions. However, for a number of anion drugs the binding has been found to increase with increasing pH: phenobarbital, phenytoin [30], thiopental [25], and bumetanide [37].

This behaviour could be explained in part by the assumption of ionic bonds and a higher affinity of the ionized form of the drug towards albumin. Another explanation, suggested by Christensen et al. [25] for thiopental, is that the binding takes place to the N- or B-form of HSA [36], independently of the ionization of the drug. Moreover, the binding between thiopental and albumin could be characterized best by the solubility of the drug in hydrophobic areas in the albumin rather than by a conventional binding model [24]. Thus, the increase in the bound fraction of methohexital with increase in pH could also be explained by a higher affinity of the drug towards the basic (B-form) conformation of albumin.

Table 3
Binding of methohexital in serum an in isolated protein solution (same HSA concentration: 785 μM)

	Serum			HSA solution		
	Methohexital conc. (μM)			Methohexital conc. (μM)		
	1.76	17.6	35.2	1.76	17.6	35.2
Bound	80.1	83.8	82.1	88.8	90.1	90.9
fraction (%)	82.7	83.0	84.1	90.4	91.1	90.4
Mean $\pm$ SD	82.6 $\pm$ 1.3			90.3 $\pm$ 0.74 [a]		

[a] Significantly different ($p < 0.01$).

As shown in Fig. 2, the observed free fraction increased in the range 20–45°C, maybe because the binding forces decrease with increase in temperature. The variation was more pronounced when methohexital was bound to isolated albumin. In the presence of serum endogeneous compounds, the variation of the unbound fraction was reduced. Similar results were reported for a study of imipramine in four healthy volunteers [31], where the free fraction increase between 25 and 45°C varied depending on the subject. For thiopental, contradictory results of an increased [26] and unchanged [9,25] free fraction with increase in temperature have been reported.

Heparin is widely used as an anticoagulant for blood sampling in pharmacokinetic studies. It has been reported that heparin may displace certain drugs from their protein-binding sites, as observed for imipramine [31] and quinidine. In this study, no significant difference ($p > 0.05$) was found for the unbound fraction of methohexital in serum (20.4 $\pm$ 0.83%, $n = 5$) and in heparinized plasma (21.7 $\pm$ 0.56%, $n = 5$). No influence of heparin on binding has also been reported for thiopental [26], bumetanide [37] and phenytoin [38].

Methohexital binding is strongly dependent on HSA. Fig. 3 shows the bound fraction of methohexital according to HSA concentration. Christensen et al. [25] even suggested that for very highly lipophilic drugs such as thiopental, the HSA concentration affected the magnitude of K_A and n values, and that conditions for specific binding could hardly be comparable in solutions with very different albumin concentrations.

Methohexital binding to isolated albumin was characterized by the usual parameters: bound fraction (f_B), affinity constant (K_A) and number of sites per albumin molecule (n). The bound

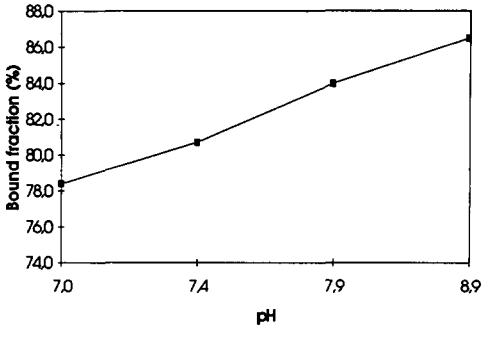

Fig. 1. Effect of plasma pH on the bound fraction of 17.6 μM methohexital solution.

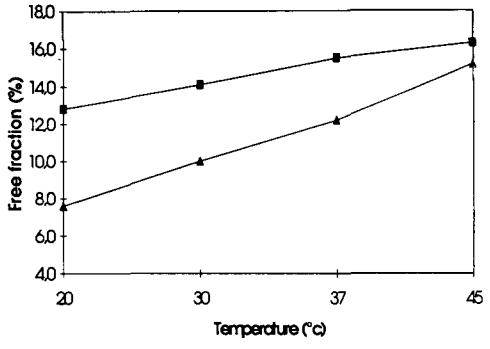

Fig. 2. Effect of temperature on the free fraction of methohexital in albumin solution (▲) and in serum (■). Methohexital concentration, 17.6 μM.

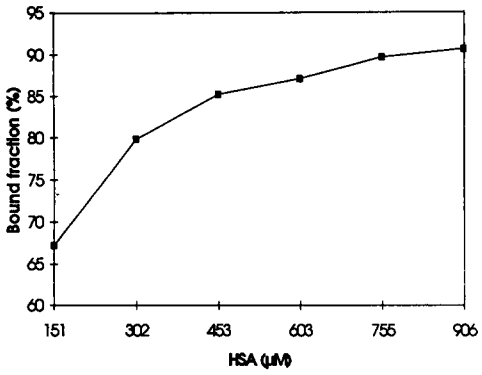

Fig. 3. Effect of human serum albumin (HSA) concentration on the bound fraction of methohexital. Methohexital concentration in isolated albumin solution, 17.6 μM.

fraction remained fairly constant at $86.7 \pm 0.9\%$ over the drug concentration range 9–200 μMm, that includes the expected therapeutic range [3,14, 39–41] and then decreased (45% for 4.150 μM methohexital concentration), as shown in Fig. 4.

Fig. 5 shows the free concentration versus the bound concentration of drug. Binding of methohexital to albumin appeared to be a two-class binding model. The first class of sites is saturable, characterized by a high affinity constant, $K_A = 11\,200 \pm 1030\ M^{-1}$, and a number of sites per protein molecule of 1 ($n = 1.01 \pm 0.08$). The second is a non-saturable class site of low affinity ($n'K'_A = 810 \pm 60\ M^{-1}$).

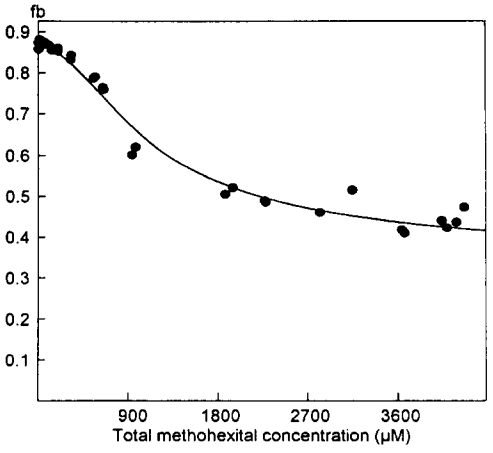

Fig. 4. Variation of the methohexital bound fraction (f_B) according to the total drug concentration.

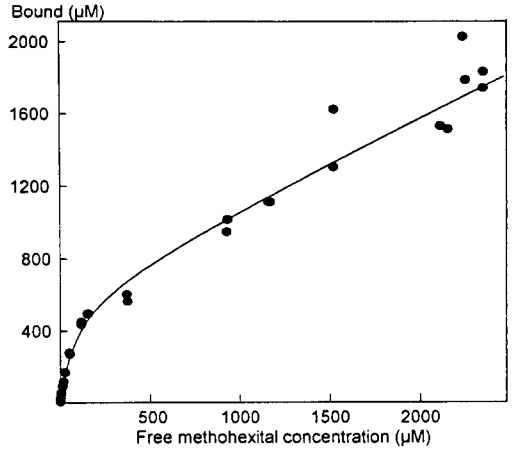

Fig. 5. Bound versus unbound methohexital concentration over a wide range of drug concentrations.

As expected, the found parameters are different from those of thiopental [24] ($K_A = 3 \times 10^6\ M^{-1}$, $n = 10^{-3}$), which is highly lipophilic, and from those of phenobarbital [34], which is poorly bound to albumin (58%) with a much lower affinity constant of about 500 M^{-1}.

The obtained bound fractions of methohexital in serum varied between subjects from 80 to 84.5% ($n = 6$). As in the serum pH was adjusted before the experiments, the variations in binding could be essentially attributed to inter-subject differences in albumin concentrations and endogenous inhibitory compounds.

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