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PROTEIN BINDING OF INDOMETHACIN IN HUMAN CEREBROSPINAL FLUID

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Abstract—The binding of the non-steroidal anti-inflammatory drug indomethacin to proteins in human cerebrospinal fluid (CSF), drawn during lumbar puncture from 10 patients affected by lumbosciatica, was measured by equilibrium dialysis and spectrofluorimetry. Similar binding studies on human serum albumin solutions (0.5 and 1 g/L) were performed using the same techniques. The mean binding percentage of indomethacin determined by equilibrium dialysis was 40%. The results obtained by both techniques allowed us to conclude that the binding of indomethacin in CSF was essentially due to albumin.

Since the protein content of normal cerebrospinal fluid (CSF†) is about 0.5% of that of serum, it is generally assumed that the CSF concentrations of lipophilic drugs accurately reflect their free (unbound) plasma concentrations at steady-state [1, 2], despite the known presence in CSF of proteins able to bind to drugs [3]. Several non-steroidal anti-inflammatory drugs (NSAIDs) were shown to diffuse into the CSF [4, 5]. However for indomethacin [6], pirprofen [7] and to a lesser extent ketoprofen [8] the level of the drug in the CSF, beyond the equilibrium point, was higher than the corresponding free concentration determined in plasma. These observations might be attributed to a protein binding of these drugs in CSF.

In order to assess this hypothesis, we studied *in vitro* the protein binding of indomethacin in CSF. Indeed, its diffusion into CSF was rapid due to its lipophilicity and despite its high protein binding in plasma (99.7 ± 0.1%), essentially to albumin [9]. The low protein content of CSF could have rendered the conventional equilibrium dialysis (ED) inaccurate. Therefore, we compared the CSF results to those obtained with low concentrations of human serum albumin (HSA) solution (0.5 and 1 g/L). We also applied the spectroscopic method (SF) fluorimetric quenching of tryptophan, as an additional technique with which to check the validity of the results obtained with ED [10, 11].

MATERIALS AND METHODS

Cerebrospinal fluid. An aliquot of normal CSF was obtained from each of 10 informed adults hospitalized for lumbosciatica and requiring a diagnostic lumbar puncture. Paracetamol was the only treatment authorized. The CSF samples were pooled and treated as if they had come from one subject. The total concentration of protein in the pool of CSF was 0.425 g/L and the level of albumin was 0.29 g/L (4.205 μM).

Products. Indomethacin was donated from Merck Sharp Dohme and Chibret. Paracetamol was obtained from the CHRU (Nancy, France). Albumin was purchased from Sigma (L'isle d'abeau, France; Ref. A 1887), essentially free of fatty acid (below 0.005%) and prepared from fraction V of HSA. Various concentrations of HSA (0.5 and 1 g/L) were prepared in a Sörensen phosphate buffer 67 mM, pH 7.4. All the products used in the study were of analytical grade and the water bidistilled. The acetonitrile used as eluent was of chromatographic grade.

Equilibrium dialysis. The free concentration of the drug in the CSF pool and in the 0.5 and 1 g/L HSA solutions was determined by equilibrium dialysis using Spectrapor 2 membranes for 3 hr against a 67 mM Sörensen phosphate buffer, pH 7.4, in 1 mL cells at 37°, using a Dianorm apparatus. The dialysis time of 3 hr was determined in a previous study as being sufficient for establishing equilibrium. The concentrations of indomethacin added to the CSF pool and to the HSA solutions varied from 0.5 to 5 μg/mL and from 1 to 200 μg/mL, respectively. The concentrations of paracetamol added to the 1 g/L HSA solution varied from 0.5 to 600 μg/mL. The free concentration of the drug was determined as its concentration in the buffer after dialysis. The binding values were corrected for volume shift during equilibrium dialysis [12, 13]. Assay for the free concentration of indomethacin was performed by HPLC as follows: The liquid–liquid extraction by diethyl ether in an acidified medium (pH 5) has been described previously [6]; chromatography was performed on a Waters apparatus (590 pump, 482

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† Abbreviations: CSF, cerebrospinal fluid; ED, equilibrium dialysis; SF, spectrofluorimetry; HSA, human serum albumin; DNSA, dansylamid; DS, dansylsarcosin; NSAID, non-steroidal anti-inflammatory drug; CNS, central nervous system.

spectrophotometer, 740 integrator, thermostatic oven at 35°) at a flow rate of 1.5 mL/min. The mobile phase consisted of acetonitrile/acetic acid (0.6%) (55:45, v/v). The column used was a C_{18} (Whatman Partisil 5 ODS-3, 250 × 6.35 mm). The detection was set at 240 nm. Phenylbutazone was used as the internal standard. The linearity was good from 1 ng/mL in buffer to 50 μg/mL. The reproducibility varied from 4.6 to 8.3% for high to low concentrations. The presence of paracetamol did not interfere in the assay of indomethacin.

Assay for the free concentration of paracetamol was performed by HPLC as follows: The liquid–liquid extraction of paracetamol is performed with 3 mL of ethyl acetate. Etophylline is used as an internal standard; chromatography was performed on the same Waters apparatus and C_{18} column as for indomethacin. The elution was obtained through a mixture of methanol/bidistilled water (25:75, v/v) as an eluent at a flow rate of 1.5 mL/min and the detection monitored at 245 nm. The linearity from 2 ng/mL to 2 μg/mL was good and the reproducibility varied from 1.9 to 4.8%. The retention time for paracetamol was 4.5 min and for etophylline 6.25 min.

Spectrofluorimetry. The spectroscopic measurements were performed with a fluorescence spectrophotometer Perkin–Elmer 240.

The protein ultraviolet fluorescence is due to its aromatic amino acids, tryptophan and tyrosine. HSA contains only one tryptophan residue (No. 214), whose exact localization in the primary sequence is known (near site I) [14]. Its fluorescence is at a maximum for an excitation wavelength, λ_{ex}, of 290 nm and an emission wavelength, λ_{em}, of 340 nm and can be quenched by ionic ligands binding near tryptophan 214 [15].

In addition, the binding of non-fluorescent drugs to protein can be studied by means of fluorescent probes such as dansylamid (DNSA), specific for site I and dansylsarcosin (DS), specific for site II. The emitted fluorescence of the complex DNSA/albumin (λ_{ex} = 340 nm; λ_{em} = 480 nm) and DS/albumin (λ_{ex} = 350 nm; λ_{em} = 480 nm) can be modified when drugs are added. It was verified that the drugs studied emitted no fluorescence at the wavelengths used for either the tryptophan quenching or the displacement experiments.

Circular dichroism. The pool of CSF was analysed by circular dichroism with a dichrograph Jobin Yvon IV. A solution of pentolactone was used to calibrate the apparatus. A cell of 0.5 cm with a sensitivity of 2×10^{-5} was used to study the conformation of the proteins present in CSF from 200 to 500 nm. The assay was performed at room temperature (25°).

Mathematical analysis. Two different mathematical models were used to fit the experimental data:

(1) The Scatchard plot for two classes of independent sites described by the equation [16]

$$\frac{B}{P} = \Sigma_{i=1}^{2} \frac{n_i k_i F}{1 + k_i F}$$

where F and B are the concentrations of free and bound drug, respectively; P is the albumin concentration; k_i is the association constant for the ith class and n_i the number of sites of the ith class.

(2) The parabolic model [17]; in many cases, the binding data were fitted using a parabolic relation, essentially when the binding saturation was not attained. This model is described by the relation

$$F = aT + bT^2$$

where T is the total equilibrium concentration of drug and a and b are two parameters without physiochochemical significance.

The binding parameters (i, n_i, k_i or a, b) were determined using an optimization method by quadratic polynomial interpolation with the least squares criterion on an IBM AT computer.

The parameters were compared with a Friedman test using the Statview SE + graphic software installed on a Macintosh Plus.

RESULTS AND DISCUSSION

The binding of indomethacin occurs mainly at albumin sites, that are in common with many other acidic drugs (e.g. NSAIDs, benzodiazepines). For this reason, only patients treated with paracetamol alone were included in this study and the absence of drugs was verified, essentially NSAIDs [18], since these are given usually to patients suffering from lumbosciatica.

Paracetamol was maintained as an analgesic for ethical considerations, despite its known diffusion into the CSF [9]; its level in the CSF pool was 2 μg/mL. The choice of this drug was made on the basis of its low protein binding in plasma, as illustrated in a previous study in which no binding of the drug to plasma occurred for paracetamol added up to 60 μg/mL [19]. For a much higher concentration (280 μg/mL), the binding of paracetamol was only 15–21%. However, a preliminary study was conducted on a 1 g/L HSA solution in order to verify paracetamol binding in low protein content media like CSF and thus a possible interference with the binding of indomethacin. For concentrations from 0.5 to 20 μg/mL, no binding was measured, whereas for higher concentrations (up to 600 μg/mL) the mean percentage of binding was near to 35%. Moreover, two series of equilibrium dialysis experiments were performed on a 1 g/L HSA solution with the concentrations of indomethacin added varying from 2 to 200 μg/mL; a constant concentration (50 μg/mL) of paracetamol (exceeding largely that found in CSF) was added in one series of experiments but not the other. The free dialysed concentrations of indomethacin were identical with or without paracetamol. This let us suppose that the low binding of paracetamol does not interfere with the binding of indomethacin.

The conformation of proteins in CSF was studied using circular dichroism (not shown). The spectrum of CSF was superimposable with that obtained for a solution of HSA (0.3 g/L), suggesting that the main part of the protein in CSF is albumin.

Equilibrium dialysis

Equilibrium dialysis studies were performed with indomethacin on CSF and HSA solutions (0.5 and 1 g/L). The experimental conditions were close to the limit for application of this technique, due to the

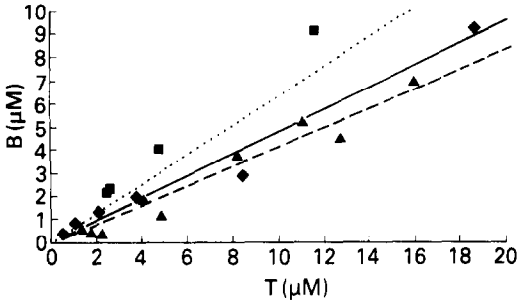

Fig. 1. Bound concentration (B) of indomethacin to HSA vs total drug concentration (T) dialysis. Experimental data for HSA solutions 0.5 (◆) and 1 g/L (■) and CSF (▲) and optimization fitting by parabolic model (—, ..., ---, respectively).

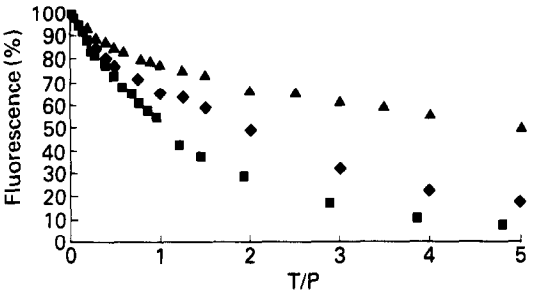

Fig. 2. Tryptophan quenching of HSA with indomethacin in albumin solutions 0.5 (◆) and 1 g/L (■) and in CSF (▲), for different molar concentration ratios of total drug concentration (T) to HSA (P).

low concentration of albumin present in the media. Low HSA solutions (0.5 and 1 g/L) were used to confirm that binding actually occurs at very low protein concentrations and that this technique is sufficiently sensitive that binding obtained in CSF cannot be attributed to an artefact caused by methodological problems.

Binding of the drug occurred both in HSA solutions at low concentrations and in CSF (Fig. 1). At first the experimental data were plotted using a Scatchard model with two classes of sites. This model, however, often led to unjustified deductions; there is a practical difficulty in calculating the four parameters (n_1, n_2, k_1, k_2) because of the infinite number of combinations that exist. Therefore, we chose to use this model simply as a mathematical tool and no real significance is to be given to the parameters. Moreover, this model is not adaptive when, as in our case, saturation of sites is not attained [20, 21]. Another mathematical model (Behm and Wagner [17]), with fewer parameters, was preferred for representing the variation of the free concentration of ligand as a function of the total quantity present at equilibrium. Nevertheless, the parameters of both models are given in Table 1. For the concentration range of indomethacin studied (up to 5 μg/mL), the mean percentage binding in CSF, calculated using the polynomial model (41%), was similar to that obtained from experimental data with equilibrium dialysis (39%).

This bound fraction is in good agreement with that found with HSA solutions: 47% for 0.5 g/L HSA and 74% for 1 g/L HSA. The binding capacity must increase with albumin level since in plasma it reached 99.7%. This fact was exposed previously for indomethacin [22] and many other drugs, e.g. salicylate [23].

Since indomethacin may be partly protein bound in CSF, it can be expected that its total CSF concentration at steady-state is higher than its free plasma concentration. Our data may explain, at least in part, why a single intramuscular injection of 50 mg indomethacin led to CSF concentrations that were elevated relative to concomitant free plasma levels, beyond the equilibration time [6].

Spectrofluorimetry

Spectrofluorimetry studies were performed to confirm the results obtained by equilibrium dialysis.

Tryptophan fluorescence quenching. The emitted fluorescence of tryptophan (in CSF and in the HSA solutions 0.5 and 1 g/L) was quenched when indomethacin was added up to a ratio of $T/P = 5$ (Fig. 2).

The possibility of interference of paracetamol present in CSF was checked in the spectrometric experiments, as for ED. When paracetamol, used as a potential quencher, was added to a 1 g/L solution of HSA, no modification of the fluorescence of tryptophan was observed over a wide range of

Table 1. Parameters a and b (parabolic model) and n_1, n_2, k_1 and k_2 (Scatchard model) for the binding of indomethacin in CSF and HSA solutions, as determined by equilibrium dialysis

	Behm and Wagner parameters		Scatchard parameters				
Medium	a	b (1/μM)	n_1	n_2	k_1 (μM)	k_2 (μM)	N
Alb (0.5 g/L)	0.6701 ± 0.0140	0.00023 ± 0.00001	2.605 ± 0.011	60.30 ± 0.02	0.105 ± 0.001	0.001 ± 0.001	20
Alb (1 g/L)	0.3578 ± 0.0327	0.00046 ± 0.00010	1.670 ± 0.169	25.72 ± 5.05	0.332 ± 0.036	0.003 ± 0.001	18
CSF	0.5810 ± 0.0205	0.00010 ± 0.00004	2.700 ± 0.009	32.30 ± 0.01	0.035 ± 0.001	0.004 ± 0.001	19

Values are means ± SE.
Alb, HSA solution.
N, number of experiments.

Table 2. Parameters n_1, n_2, k_1 and k_2 of the Scatchard model for the binding of indomethacin to CSF and HSA solutions, as determined by tryptophan quenching

Medium	n_1	k_1 (μM)	n_2	k_2 (μM)	N
Alb (0.5 g/L)	0.4099 ± 0.0389	8.8510 ± 2.5698	2.2690 ± 0.1859	0.0435 ± 0.0069	12
Alb (1 g/L)	0.3800 ± 0.0118	21.5329 ± 4.7351	1.2540 ± 0.0101	0.1414 ± 0.0046	18
CSF	0.5050 ± 0.0130	7.5930 ± 2.5800	2.2730 ± 0.5573	0.0390 ± 0.0162	14

Values are means ± SE.
Alb, HSA solution.
N, number of experiments.

paracetamol concentrations (1–25 μg/mL). Additionally, the quenching of a solution of HSA (1 g/L) was performed with indomethacin at concentrations varying from 1 to 50 μg/mL. This experiment was also performed in the presence of paracetamol at two different concentrations, the first near to (2 μg/mL) and the other greatly exceeding (20 μg/mL) that found in CSF. There was no difference in quenching with or without paracetamol. Therefore, we can conclude that paracetamol present in CSF does not interfere in the quenching of CSF-albumin by indomethacin.

Fluorescence quenching could be performed in a biological medium such as CSF since the maximum fluorescence (without any quencher) was fixed arbitrarily at 100%. This value could be attributed not only to the fluorescence emitted by the tryptophan of albumin but to other compounds present in the medium. We know, however, that in plasma indomethacin is bound almost exclusively to albumin. Therefore, the decrease in fluorescence can be attributed essentially to the tryptophan 214 present in albumin. This decrease in fluorescence due to quenching suggests three hypotheses:

(1) The emission of fluorescence is masked by the binding of the ligand to the area containing tryptophan.
(2) The fluorescence decreases due to an energy transfer from a fluorescent donor (tryptophan) to a non-fluorescent acceptor (ligand).
(3) A conformational change in the protein after ligand binding inhibits the fluorescence of the tryptophan.

However, the decrease in fluorescence induced by indomethacin can be analysed in terms of binding; using the method of Steiner *et al.* [24], the bound and free concentrations were calculated from the initial slope of the relative fluorescence decrease (quenching) versus the total amount of indomethacin added. The binding data were then represented according to classical binding models.

Saturation of the binding sites was attained in both CSF and HSA solutions. The data were plotted using a Scatchard representation and the best fit was obtained with two classes of independent sites. The corresponding parameters (Table 2) were not statistically different (P = 0.1738) leading to the conclusion that indomethacin binding in CSF occurs in a similar manner to that in HSA solutions and is relatively independent of the albumin content.

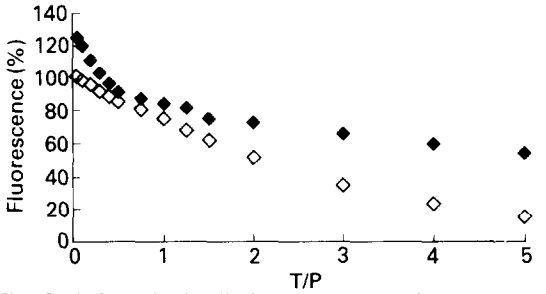

Fig. 3. Indomethacin displacement of specific markers, dansylamid (◆) and dansylsarcosin (◇) for different molar ratios of total drug concentration (T) to HSA (P).

Moreover, the quenching permitted us to appreciate not only the binding of the drug to the first class of site (site I containing tryptophan) but also to a second class of site located near the tryptophan.

Displacement experiments. It could be interesting to define the binding sites of indomethacin because of the discrepancies found in the literature, in the case of site I [25] or both sites I and II [14]. Therefore, displacement experiments with specific markers were performed, using DNSA and DS as sites I and II fluorescent probes, respectively, with HSA solution (1 g/L) and with probe to albumin ratios varying from 0.05 to 1. For indomethacin, the fluorescence emitted by the DNSA/albumin and DS/albumin complexes was decreased to about 50% and 85%, respectively, when the drug was added up to a concentration of about 26 μg/mL [$T/P = 5$, where T and P are the total drug and the protein concentration (μM), respectively] (Fig. 3). The decrease in fluorescence can be attributed to a specific competitive displacement of the markers from their binding sites by the drug. These data allow us to conclude that indomethacin binds to both sites I and II. These could be related to the two classes of sites shown by quenching data, since site II is located near tryptophan 214 [14], and explain the need of a Scatchard model with two classes of independent sites to model the corresponding results.

Importance of protein binding in CSF

The concentration of bound indomethacin per mole of albumin (or number of occupied sites) (B/P) was represented as a function of the total concentration of drug available per mole of albumin

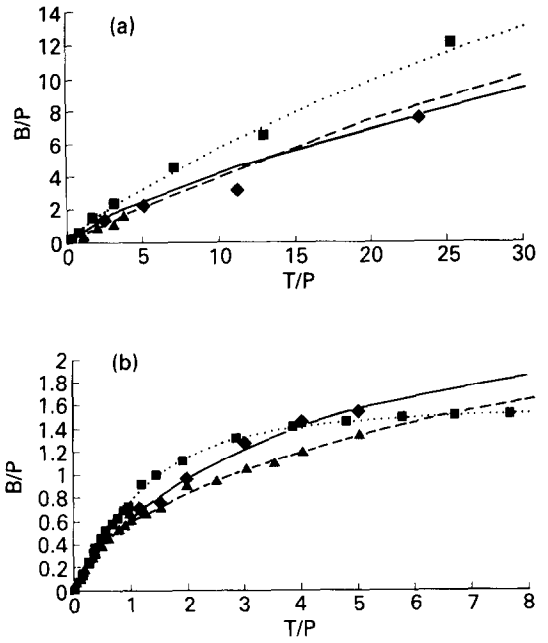

Fig. 4. Number of sites (B/P) of HSA as a function of the total concentration of indomethacin available per mole of albumin (T/P). Data obtained by equilibrium dialysis (a) and spectrofluorimetry (b) for HSA solutions 0.5 (◆) and 1 g/L (■) and CSF (▲) and optimization fitting by Scatchard model with two classes of sites (—, ..., – – –, respectively).

(T/P), as previously proposed, in order to take into account the change of drug environment when the protein concentration varies [26]. In the experiments using spectrofluorimetry, the graphs obtained with different concentrations of HSA and CSF were very similar (Fig. 4). We can conclude from this that the binding of indomethacin to protein present in CSF occurs essentially to albumin. The number of occupied sites (B/P) obtained with ED is higher than with SF. This result showed how complementary the two techniques were: the one (ED) took into account the totality of the binding sites, the other (SF) only specific binding sites, near tryptophan 214, like sites I and II. This hypothesis can be confirmed with the Scatchard binding parameters obtained for SF and for ED. The parameter n_1 for ED is close to the sum of the parameters n_1 and n_2 obtained for SF (Tables 1 and 2). We must, however, be careful with the interpretation of this result, due to the multiplicity of solutions that can be calculated using the Scatchard model with more than one class of sites [20].

Relatively few studies have been conducted to show the possibility of drug-binding to CSF due to ethical considerations and methodological difficulties. Alfredsson and Sedvall [27] using ED showed that chlorpromazine is bound to human CSF proteins (mean value 46%); their study concerned the CSF of patients treated with chlorpromazine. The variation in the bound fraction (19 to 72%) was not related to the total amount of protein in CSF and no binding parameters were deduced from their results. Our study proved that whatever the technique chosen, i.e. ED or SF, indomethacin binds to a high degree to proteins present in CSF, principally to albumin.

This conclusion is of great interest because the distribution of several NSAIDs in the CSF may be related to their antipyretic properties and/or to their side-effects on the central nervous system (CNS) [5]. For drugs like NSAIDs which are highly protein bound, the possibility of binding to protein in the CSF cannot be neglected. However, by taking this into account, one can estimate the concentrations of the drugs available for diffusion into the CNS. It is often supposed that drug present in CSF is equal to the free concentration of drug in plasma beyond the equilibrium point. Thus, if we consider that the binding of indomethacin in CSF is 41%, we have to accept, according to equilibrium laws, that there is almost twice as much drug in the CSF than binding in the plasma lets us suppose. This amount of drug in CSF is made up of both the free fraction (in equilibrium with the free fraction in plasma) and the bound fraction (in equilibrium with the free fraction in CSF).

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