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Plasma protein binding of sulphadiazine, sulphamethoxazole and trimethoprim determined by ultrafiltration

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Abstract: The plasma protein binding of trimethoprim, sulphadiazine and sulphamethoxazole was studied at 37°C by ultrafiltration. Plasma samples contained steady state levels of the drugs from ten volunteers from a cross-over comparative pharmacokinetic study on co-trimazine and co-trimoxazole. The three compounds were determined in plasma and ultrafiltrate by HPLC, the recoveries being close to 100% in each case. Freezing of spiked samples had no influence on the binding. Trimethoprim was 48.5–52.2% bound (mean 50.0%); sulphadiazine was 50.9–60.6% bound (mean 56.2%); and sulphamethoxazole was 74.3–80.8% bound (mean 76.9%). The significantly lower protein binding of sulphadiazine compared to sulphamethoxazole means that equivalent non-protein bound plasma levels of the two sulphonamides are achieved from smaller doses of co-trimazine than co-trimoxazole. Use of co-trimazine may thus minimize the risk of adverse reactions.

Keywords: *Protein binding; ultrafiltration; sulphadiazine; sulphamethoxazole; trimethoprim; high-performance liquid chromatography.*

Introduction

In antibacterial chemotherapy the free non-protein bound fraction of a drug accounts for the clinical effect. Thus it is essential to know accurately the extent of protein binding when evaluating the efficacy of a treatment. This is especially important in combination therapy in which the ratio between the free concentrations of two compounds must be within a certain range in order to give an optimally effective treatment. This principle is applicable to sulphonamide–trimethoprim combinations such as co-trimazine and co-trimoxazole.

A number of studies on the protein binding of the three compounds sulphadiazine, sulphamethoxazole and trimethoprim have been reported involving several types of sample. Techniques used have included: equilibrium dialysis [1–3], ultrafiltration [4–8] and a polyacrylamide batch technique [9].

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The choice of experimental technique is important, for it may be that variations in protein binding obtained in studies of the same compound are related to the techniques used. In this work it was found initially that experiments with equilibrium dialysis were not successful: the equilibration times were quite long (6 h), causing a significant decrease in the volume ($\geq$ 50%) on the buffer side of the dialysis cell by diffusion of water into the plasma compartment. Furthermore, albumin exists in two conformational states [10–12], and the transition between the two is strongly dependent on pH and on the concentration of other compounds, especially chloride and calcium. Recent studies [13] have indicated that the binding properties of the two forms of albumin may differ considerably. Consequently, in equilibrium dialysis experiments it is important to control the composition of the buffer. The present work utilized an ultrafiltration method in which no additions of external components (buffer or other) were made.

Materials and Methods

Samples

Preliminary studies to check the effects of freezing plasma samples on the degree of protein binding, and to check drug recoveries by quantitative determinations, were performed on fresh blood from drug-free blood donors. Sulphadiazine and sulphamethoxazole were added to a number of fresh plasma samples at room temperature, which were then incubated for 30 min at 37°C. The samples were analysed before and after freezing at −25°C overnight. In addition, plasma that had been frozen beforehand was thawed, the compounds added, and then analysed. The same plasma pool was used for all three studies, and the concentration of the sulphonamides was about 40 $\mu g\ ml^{-1}$.

Individual protein binding studies were performed on 10 male volunteers participating in a crossover study between co-trimoxazole (960 mg twice daily) and co-trimazine (1000 mg daily) under steady state conditions [14]. Samples were taken after the last dose on the last day. Half an hour before the sampling started, all volunteers had a standardized breakfast; two cups of coffee or tea, one sandwich and one egg. The volunteers then fasted until lunch, which was served at the earliest three hours after drug administration. After this period, free intake of food and fluid was allowed. In a limited study on the sulphonamides, and in order to reveal any variation in protein binding during the day, the 1 and 3 h samples, and the 8 and 12 h samples, respectively, were pooled. For the trimethoprim binding studies, no such determinations were made because adequate volumes of plasma were not available. Instead samples from several sampling times (3, 5, 8, 12, 13 and 15 h) were pooled in order to achieve the necessary volumes.

Equilibrium dialysis

Preliminary experiments with this technique were performed with a Dianorm apparatus (MSE, USA) equipped with rotating teflon cells and Spectrapor membranes (Spectrum Med., Ind., USA).

Ultrafiltration

Cellulose dialysis tubing (Union Carbide Corp., Chicago, USA) with flat diameter 10 mm, inner diameter 6 mm, wall thickness 51 μm and pore diameter of 24 Å was used. The tubing was soaked beforehand in water for 1 h to remove glycerine, carefully wiped dry with Kleenex tissues (Kimberly-Clark Ltd, Larkfield, Maidstone, Kent, UK), and

centrifuged for 5 min at about 300 *g* to remove the final excess of water. The remaining water content was 0.2 g per 17 cm tubing (the length of tubing used inside the centrifuge tube during the experiments), as determined by a Karl Fischer titration.

During storage of plasma samples, pH increases because of the loss of carbon dioxide. In order to restore the plasma pH to 7.4, a gas mixture containing 5% CO_2 in synthetic air was bubbled through the samples for about 10 min before starting the ultrafiltration procedure [15]. The centrifuge tubes used in the experiments were also filled with this gas mixture prior to the start of the study.

Plasma (2.0 ml) was pipetted into the tubing, which was attached inside the centrifuge tubes by a stopper that pressed the tubing tightly to the side. The samples were incubated for 30 min at 37°C before the first centrifugation (10 min at 300 *g*), yielding *ca.* 50 µl centrifugate which was discarded. The dialysis tubing was then transferred to a clean tube and a second centrifugation was performed (20 min at 300 *g*), giving about 150 µl centrifugate, which was analysed, along with the remaining contents of the tubing. The procedure was performed in a thermostatted (37°C) room.

It was assumed that the water initially present in the tubing was firmly bound by the cellulose and did not take part in the equilibrium process in the plasma. Equilibrium plasma concentrations were corrected for the volume of the plasma proteins [16] (correction factor = 1.05) and for a 10% loss of volume during the ultrafiltration. The percentage of drug binding is calculated according to the equation:

$$\% \text{ bound} = \frac{100\ (C_{pc} - C_u)}{C_{pc}}$$

where C_{pc} is the corrected plasma equilibrium concentration = 1.05 (0.9 C_p + 0.1 C_u); C_p is the plasma equilibrium concentration; C_u is the ultrafiltrate concentration.

The technique described is similar to that used by Schanker and Morrison [17] and later modified by Borgå *et al.* [18] and Lunde *et al.* [15].

Analytical methods

The concentrations of all three compounds were determined by modern liquid chromatographic methods both in the ultrafiltrate and in the plasma compartment. The analytical method for sulphadiazine has been described in detail by Westerlund and Wijkström [19]; sulphamethoxazole was determined by a modified version of a method described by Vree *et al.* [20]: sulphaethidole was used as an internal standard, and the mobile phase was phosphate buffer pH 4.0–ethanol (4:1 v/v). The column was 100 × 4.0 mm (1 × i.d.). For trimethoprim new analytical methods were developed [21]. The chromatography was performed on a bonded phase column (Nucleosil RP 18, 5 µm (Macherey-Nagel + Co, Düren, FRG), 100 × 4.0 mm, (1 × i.d.)). Mobile phases were: for plasma determinations — phosphate buffer pH 2.0 (ionic strength = 0.1 – acetonitrile (9:1 v/v) containing *N,N*-dimethyl-*N*-octylamine DMOA (1 mM), and for the ultrafiltrate — sulphate buffer pH 2.0 (ionic strength = 0.1) – acetonitrile (9:1 v/v). A 20 µl sample of the ultracentrifugate was injected directly on to the column, while an alkaline extraction–evaporation procedure was used for the plasma determinations.

Results

Preliminary studies

The possible influence of freezing on the degree of protein binding of the

sulphonamides was tested in plasma obtained from two healthy volunteers. Three different types of spiked plasma samples were investigated: (1) fresh — spiked and quantitated the same day; (2) frozen — stored for 24 h then thawed, spiked and quantitated; (3) fresh — spiked and frozen the same day, stored for 24 h, then thawed and quantitated. The results showed that freezing of plasma had no influence on the binding, since no significant differences were obtained in these experiments. A possible complication of ultrafiltration methods is the risk of adsorption of drug molecules to the membrane [22], a reported example being desmethylchlorimipramine [23]. No such effects, however, were observed for either the sulphonamides or trimethoprim (Table 1). Recoveries were close to 100% with high precision.

Table 1
Recoveries from spiked plasma samples after ultrafiltration

Compound	Added* (μg/ml)	Recoveries (%) Mean	S.E.M.
Sulphadiazine	86–94	98.2	0.64
Sulphamethoxazole	79–86	101.4	0.73
Trimethoprim	1.0–4.2	99.1	0.77

* Blood was collected from five different donors. Different amounts in plasma samples from the various donors.

Trimethoprim

Literature data on the binding of trimethoprim (TMP) to human plasma proteins varies widely, with values ranging from 31 to 76% bound [1, 2, 4, 5]. TMP has a dissociation constant, pK_a, of 7.2, which is close to the pH (7.4) of plasma. The degree of protein binding may thus be strongly influenced by the actual pH during the experiments. The importance of proper pH control is demonstrated in the literature data: the lowest degree of binding, 31%, was obtained after equilibrium dialysis with a pH 7.0 buffer, while 45% binding was obtained at pH 7.4. Higher degrees of binding have been reported with no mention of pH control. Since plasma pH will increase during storage due to loss of CO_2, the samples used in these studies probably had a raised pH: for many compounds the protonated forms bind to a lower extent than the corresponding neutral forms.

The present results (Table 2) on nine young, healthy volunteers ranged from 48.5 to 52.2% binding, with a mean of 50.9% with a relative standard deviation of 1.1%. These results are similar to those reported by Schwartz and Rieder [2]. For spiked plasma samples obtained from two blood donors and at two concentration levels, 1 and 4.1 μg/ml, respectively, a tendency towards a higher degree of binding was observed; mean value = 55.3% ($P < 0.05$, Mann–Whitney U-test). This may indicate an influence of trimethoprim metabolites and/or sulphonamides on the binding.

Sulphadiazine

Literature data on the binding of sulphadiazine [6–9] also show a variation (42–58%) although not as wide as that of TMP. The pK_a of sulphadiazine (6.4) is one unit lower than plasma pH; thus proper control of this parameter may not be as critical for

Table 2
Plasma protein binding (%) of trimethoprim

(A) Spiked samples from two blood donors. Concentration range: 1.0–4.1 μg ml^{-1}

Range	Median	Mean	S.E.M.	*n*
53.6–57.6	55.2	55.3	0.48	10

(B) Healthy male volunteers, age 30.4 years (s = 3.2). Concentration range: 0.67–1.33 μg ml^{-1}

Range	Median	Mean	S.E.M.	*n*
48.5–52.2	50.9	50.9	0.36	9

sulphadiazine. The binding decreases, however, with increasing drug concentration [9]. The binding is lowered in patients with chronic hepatic disease [6] and in undernourished people [7].

The present results (Table 3) show that the range of binding was from 50.9 to 62.5%, with a mean value of 56.2% and a relative standard deviation of 3.1%, and that there was no variation of the binding during the day.

Table 3
Plasma protein binding (%) of sulphadiazine

(A) Spiked samples from three blood donors. Concentration range: 43.2–47.0 μg ml^{-1}

Range	Median	Mean	S.E.M.	*n*
46.1–57.2	52.3	51.5	1.56	8

(B) Healthy male volunteers, age 30.4 years (s = 3.2). Concentration range: 14.1–39.7 μg ml^{-1}

Pool interval (hr)	Range	Median	Mean	S.E.M.	*n*
1 + 3	50.9–58.7	56.5	56.0	0.88	10
8 + 12	51.0–62.5	56.5	56.4	1.19	9
All samples	50.9–62.5	56.6	56.2	0.98	19

Two to four determinations were performed on each sample.

These results are in good agreement with literature data; there was a tendency towards a lower degree of binding in spiked samples compared with those from sulphadiazine-administered volunteers, although the difference was not statistically significant (Mann–Whitney *U*-test).

Sulphamethoxazole

Literature data on plasma and serum protein binding of this drug range from 62 to

73% [2, 4, 8, 24, 25], determined by both ultrafiltration and equilibrium dialysis methods. Sulphamethoxazole has a pK_a value of 5.6 and like sulphadiazine should be rather insensitive to small variations in plasma pH.

The present results indicate that the extent of binding of sulphamethoxazole is higher than that reported to date, with a range of 73.5–82.5%, and a mean value of 77%. The binding obtained from spiked samples was lower, mean = 74.1% ($P < 0.05$, Mann–Whitney U-test).

Table 4
Plasma protein binding (%) of sulphamethoxazole

(A) Spiked samples from three blood donors. Concentration range: 39.6–43.0 μg ml^{-1}

Range	Median	Mean	S.E.M.	*n*
72.2–76.9	73.1	74.1	0.54	11

(B) Healthy male volunteers, age 30.4 years (s = 3.2). Concentration range: 40.3–71.1 μg ml^{-1}

Pool interval (hr)	Range	Median	Mean	S.E.M.	*n*
1 + 3	73.5–82.5	76.6	77.5	0.91	9
8 + 12	74.6–78.2	76.1	76.4	0.44	9
All samples	73.5–82.5	76.5	77.0	0.51	18

Two to four determinations were performed on each sample.

Discussion

The protein binding values obtained for the ten male volunteers studied here showed inter-individual variations, which was largest for sulphadiazine (50.9–60.6%) and smallest for trimethoprim (48.5–52.2%). There is a large variation in mean values of protein binding reported in the literature for these three compounds. Intra-individual variations will certainly have an influence on data obtained in different studies. It is also important to control the experimental conditions carefully. Use of the equilibrium dialysis technique demands the utilization of a controlled buffer composition, with respect to pH, ionic strength, and the content of various electrolytes, especially chloride and calcium ions. Adsorption phenomena may complicate the interpretation of results from both ultrafiltration and equilibrium techniques. Protein binding may be further influenced by factors such as the presence of inhibitors (increased concentration levels of endogenous compounds, e.g. bilirubin; the simultaneous administration of other drugs, e.g. salicylate), inter- and intra-individual variations in protein concentrations, malnutrition, and liver and kidney diseases.

Results obtained using spiked plasma samples differed slightly from those obtained in plasma from volunteers. However, the number of experiments was limited, especially for the spiked samples, so even where statistically significant differences were obtained (trimethoprim and sulphamethoxazole), the results indicate only a tendency that must be confirmed using a larger number of samples.

There is a positive linear correlation between the degree of binding for sulphadiazine and sulphamethoxazole ($r = 0.73$) in the cross-over study, while no such correlation was found either between sulphadiazine and TMP ($r = -0.07$) or between sulphamethoxazole and TMP ($r = -0.06$). It is well known that albumin possesses several binding sites for sulphonamides, while TMP may be bound to a different protein, e.g. a glycoprotein, which is known to bind many basic compounds [26].

The clinical effect of a chemotherapeutic agent is assumed to be directly related to the non-protein bound plasma levels of the drug. A consequence of the higher degree of protein binding of sulphamethoxazole compared with that of sulphadiazine is that, since sulphamethoxazole is then less available as a free, active drug, considerably higher doses of sulphamethoxazole are required to give equivalent clinically effective plasma concentrations. This might increase the frequency of adverse effects obtained from sulphamethoxazole treatment.

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