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BINDING OF THEOPHYLLINE IN HUMAN SERUM DETERMINED BY ULTRAFILTRATION AND EQUILIBRIUM DIALYSIS

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1 Binding of theophylline (80 μmol/l) was determined in serum from healthy subjects by equilibrium dialysis and by ultrafiltration, using [^{3}H]-theophylline, at 22°C and at different pH-values. pH was regulated by gassing with CO_2 or by dialysing the serum against a phosphate buffer before use.

2 Binding of theophylline in serum was 34–38% determined by equilibrium dialysis and 41–45% determined by ultrafiltration at pH 7.4–7.5. The protein concentration in serum decreased by 12–16% during equilibrium dialysis and increased by 20% during ultrafiltration. The intersubject variation in binding was small.

3 Binding of theophylline in serum was pH-dependent with 25–30% bound at pH 7.0 and 58–60% bound at pH 8.1–8.3. Binding was significantly correlated to the fraction of ionized theophylline.

4 The binding of theophylline in normal human serum is about 35–40% at pH 7.4 and 22°C. The difference in binding observed between equilibrium dialysis and ultrafiltration may be explained by the opposite changes in protein concentration during the experiment.

5 Control of pH is necessary to obtain physiologically relevant data on drug binding in serum.

Introduction

The use of theophylline as an antiasthmatic drug has increased recently and a plasma concentration in the range 55–110 μmol/l has been recommended for optimal therapeutic effect (Hendeles *et al.*, 1978). Serious side effects have, however, been observed with plasma concentrations in the range 110–160 μmol/l (Zwillich *et al.*, 1975; Jacobs *et al.*, 1976; Hendeles *et al.*, 1977). To avoid toxic reactions, theophylline dosage has been monitored by plasma concentration measurements, especially when optimal therapeutic effect is required.

Only the unbound fraction of a drug in plasma/biophase is pharmacologically active. The data on protein binding of theophylline in human serum is conflicting. In healthy subjects, binding varies from 30–80% in different studies (Mangione *et al.*, 1978; Lesko *et al.*, 1981; Piafsky *et al.*, 1977; Simons *et al.*, 1979; Aslaksen *et al.*, 1981). This imposes considerable uncertainty upon the judgement of dosage regimen based on plasma concentration measurement in individual patients.

Considering that theophylline is a weak acid with a pK_a = 8.7, the observation that the binding of theophylline in serum is pH-dependent (Vallner *et al.*, 1978), is not surprising. The inconsistency in published data concerning the extent of binding of theophylline in serum might be explained by lack of pH control or by different binding techniques.

The aim of the present study was to determine the binding of theophylline in normal human serum with two different methods under controlled conditions, especially as to pH.

Methods

Chemicals

[8-^{3}H]-Theophylline (sp.act. 13.5 Ci/mmol) was obtained from Amersham Int. Ltd, England. Radiochemical purity was 96% determined by thin-layer chromatography (n-butanol water 4:1). Unlabelled theophylline was obtained from the Norwegian Drug Monopoly, Oslo, Norway. Other chemicals were of analytical grade.

Buffers

Krebs Ringer phosphate buffer:
NaCl 122 mM, KCl 4.9 mM,
$MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM,
Na_2HPO_4 15.9 mM. pH was adjusted by adding HCl or NaOH.

Krebs Ringer bicarbonate buffer:
NaCl 121 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM,
$MgSO_4$ 1.2 mM, $NaHCO_3$ 25.3 mM $CaCl_2$ 1.3 mM.
pH was adjusted by gassing with 5% CO_2 (v/v) in air.

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Serum

Whole blood was obtained from healthy individuals, 18–25 years of age. After 1 h at room temperature, the blood specimen was centrifuged at 2000 *g* for 0.5 h. Serum was aspirated and frozen immediately, and stored at −20°C until used.

Before use, serum pH was adjusted by 3 days dialysis against Krebs Ringer phosphate buffer at 4°C or by gassing with 5% CO_2 in air.

Equilibrium dialysis

Serum (500 μl) was dialyzed against buffer (500 μl) for 16–18 h at 22°C, using dialysis membrane 20/32 (Union Carbide Corp., Chicago, Illinois, USA) between two Perspex® cells. The declared pore size of the membrane was 24Å. Theophylline was added to the cell containing serum to obtain a concentration of about 80 μmol/l in serum at the end of dialysis. After dialysis, cell content was aspirated and [^{3}H]-theophylline determined in duplicate in serum and buffer.

Serum or buffer (50 μl) was added to counting vials containing 2 ml scintillation liquid (Hydrosolve®, Lumac Systems AG, Basel, Switzerland). Radioactivity was determined in a Packard Tri-Carb Scintillation Spectrometer Model 3 330. Counting efficiency was 25% in both serum and buffer as determined by [^{3}H]-toluene.

Concentration of theophylline was calculated from distribution of radioactivity, specific activity and added amount of drug. Protein binding was expressed by % bound = B/(B + F) × 100, B and F representing concentration of bound and unbound theophylline, respectively. Protein concentration was measured by the biuret reaction (Gornall *et al.*, 1949) before and after dialysis to determine dilution.

Ultrafiltration

Ultrafiltration of serum was performed at 22°C using ultrafilter cones (Centriflo, CF-50 A, Amicon, Lexington, Mass., USA). The cones were declared to be freely permeable for substances with molecular weight below 5000 and to retain more than 95% of substances with molecular weight higher than 50000. The cones were soaked in distilled water for 1 h and centrifuged to remove residual water. Serum 2.2 ml containing 80 μmol/l theophylline was loaded into the cones and gassed with 5% CO_2 in air or 100% CO_2 and covered with parafilm. After 0.5 h pH was determined and additional gassing performed if required. Centrifugation was performed for 10–15 min at 230 *g* to produce at least 200 μl ultrafiltrate, and pH determined in serum at the end of filtration. [^{3}H]-theophylline was determined as described in duplicate in serum before and after filtration and in the ultrafiltrate. Binding was calculated as % bound = B/(B + F) × 100, B being expressed as the mean concentration in serum before and after filtration, and F as the concentration in the ultrafiltrate.

The protein concentration in serum was determined by the biuret reaction before and after ultrafiltration, and in the ultrafiltrate by the method of Lowry *et al.* (1951).

Results

Binding at physiological pH

[^{3}H]-Theophylline was stable during the experiment as shown by thin-layer chromatography of extracts of dialysed serum and buffer. The serum binding of theophylline as determined by equilibrium dialysis was in the range 34–38% at a serum concentration of 80 μmol/l (Table 1). pH in serum was 7.40–7.43 at the end of dialysis. Serum dilution during dialysis was 12–16%.

When binding was determined by ultrafiltration, theophylline binding was 41–45% at a serum concentration of 80 μmol/l (Table 1). Thus, binding in serum was higher in all five subjects when determined by ultrafiltration, and the difference was statistically significant in four of them. pH in serum was 7.35–7.40 at the beginning and 7.45–7.56 at the end of filtration. Serum was concentrated during ultrafiltration by about 20%. The protein concentration in the ultrafiltrate, as determined by Lowry-reactive material, was usually 0.5–5% of that in serum. The concentration of theophylline in serum increased about 10% during filtration, showing the necessity of determining the serum concentration both before and after filtration.

No detectable binding of theophylline by the dialysis cell, by the dialysis membrane, or by the ultrafilter cones was observed.

The effect of pH on theophylline binding

When pH in serum was varied, a significant difference in binding of theophylline was observed, with decreased binding at lower pH and increased binding at higher pH.

For equilibrium dialysis serum was preequilibrated with Krebs Ringer phosphate buffer with various pH-values. The binding of theophylline was about 25% at pH 7.0 and about 45% at pH 7.8.

Figure 1 shows that the theophylline binding was significantly correlated with pH ($y = 21.4 \cdot x - 124.1$, $r = 0.976$, $n = 25$, $P < 0.001$) and with the ionized fraction of theophylline ($y = 250 \cdot x + 20.8$, $r = 0.983$, $n = 25$, $P < 0.001$), assuming that pK_a for theophylline is 8.7 (Florey, 1972; Windholz, 1976).

The theophylline binding was also determined by

Table 1 Protein binding of theophylline in serum from five healthy subjects at 22°C. Physiological pH was obtained by gassing serum with 5% CO_2 (v/v) in air. For equilibrium dialysis, Krebs Ringer bicarbonate buffer was also gassed. Values are given as mean ± s.d. with the number of separate determinations in parenthesis.

Subject	*Equilibrium dialysis (% bound)*	*Ultrafiltration (% bound)*	*Significance level* for difference*
IS	35.8 ± 0.6 (4)	45.0 ± 0.3 (2)	$P < 0.001$
GF	37.7 ± 4.4 (4)	41.6 ± 3.1 (4)	$P > 0.2$
RS	34.3 ± 0.7 (4)	44.7 ± 2.0 (4)	$P < 0.001$
TW	34.5 ± 0.7 (4)	41.7 ± 0.8 (2)	$P < 0.002$
HN	36.6 ± 1.1 (4)	40.9 ± 2.1 (4)	$P < 0.02$

*Student's *t*-test (two tailed)

equilibrium dialysis for gassed and ungassed serum/Krebs Ringer bicarbonate in three subjects. In the gassed samples, pH was in the range 7.35–7.40 and % bound was 35.0 ± 1.5 (mean ± s.d.) and in the ungassed samples pH was in the range 8.20–8.30 and % bound was 57.8 ± 3.5 (mean ± s.d.).

In the ultrafiltration studies, pH in serum was varied by gassing with CO_2 for different lengths of time. The binding was about 30% at pH 7.0 and about 60% at pH 8.1. Figure 1 shows that the binding of theophylline was significantly correlated with pH ($y = 30.1 \cdot x - 184.9$, $r = 0.930$, $n = 16$, $P < 0.001$) and

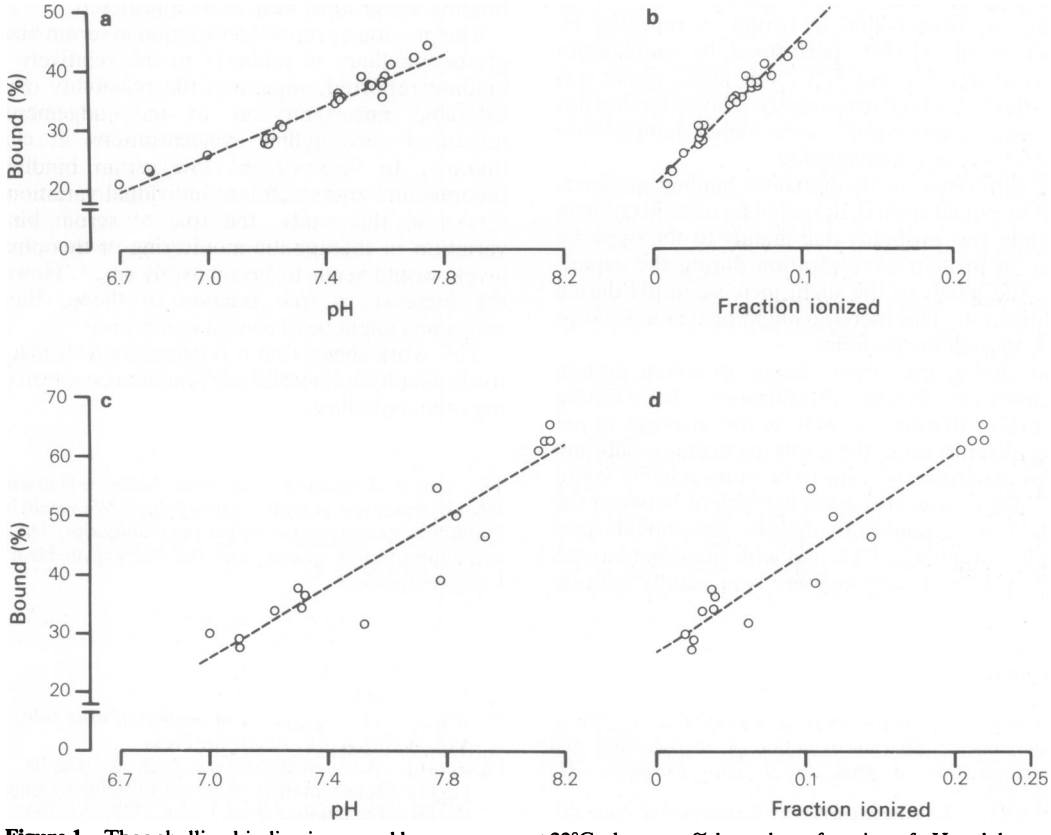

Figure 1 Theophylline binding in normal human serum at 22°C, shown as % bound as a function of pH and degree of ionization of theophylline, using equilibrium dialysis (a,b) and ultrafiltration (c,d). The least squares regression lines are shown.

with the ionized fraction of theophylline ($y = 167 \cdot x + 27.1$ $r = 0.953$, $n = 16$, $P < 0.001$), assuming that pK_a for theophylline is 8.7. These relationships between binding and pH and between binding and fraction ionized were not significantly different from those after equilibrium dialysis, due to the relatively large variation in values obtained after ultrafiltration.

Discussion

The binding of theophylline in normal human serum in the present study was 34–38% as determined by equilibrium dialysis and 41–45% as determined by ultrafiltration at physiological pH and a serum theophylline concentration of 80 μmol/l. The degree of binding observed in this study is similar to that reported by Fleetham *et al.* (1979), for healthy subjects, but much lower than that reported by Mangione *et al.* (1978), Simons *et al.* (1979), Koysooko *et al.* (1974), Lesko *et al.* (1981) and Aslaksen *et al.* (1981).

Vallner *et al.* (1978) observed that the binding of theophylline in serum is pH-dependent. The results of the present study confirm this observation. The binding of theophylline in serum as reported by Vallner *et al.* (1978), performed by equilibrium dialysis at pH 7.4 and 37°C, was 49%, which was higher than observed in our study. This difference has no obvious explanation, since higher temperature would tend to decrease binding.

The difference in theophylline binding as determined by equilibrium dialysis and by ultrafiltration in our study was probably due mainly to the opposite change in protein concentration during the experiment, and partly to the slight increase in pH during ultrafiltration. This increase might be due to leakage of CO_2 though the parafilm.

Considering the larger change in serum protein concentration during ultrafiltration than during equilibrium dialysis, as well as the increase in pH during ultrafiltration, the results from the equilibrium dialysis experiments seem to be more reliable in our study. There was also good agreement between the results from equilibrium dialysis experiments performed with HCO_3-/CO_2 and with phosphate as the buffer system. Taken together, our results indicate that the binding of theophylline in normal human serum at concentrations in the mid-therapeutic range is on the average slightly below 40% at 22°C and pH 7.4

The much higher binding values reported by some investigators may be explained by lack of pH control. Serum rapidly loses CO_2 resulting in an increase in pH. In our experiments with ungassed, previously frozen serum, pH was in the range 8.1–8.3 and the binding of theophylline, as determined by ultrafiltration or by dialysis against ungassed Krebs Ringer bicarbonate buffer, was about 58–60%, results which are similar to those reported in other studies in which pH control was not described (Mangione *et al.*, 1978; Simons *et al.*, 1979; Koysooko *et al.*, 1974; Lesko *et al.*, 1981; Aslaksen *et al.*, 1981).

The correlation of the degree of theophylline binding with pH and with the ionization degree of theophylline might suggest that binding is influenced by the ionization degree of theophylline. However, other explanations, such as pH-dependent changes in conformation of the albumin molecule, resulting in altered affinity for drugs (Wilting *et al.*, 1980), and changes in the degree of ionization of ionizable binding site groups, have to be considered.

The previously reported variation in serum binding of theophylline, in addition to the relatively high binding reported, suggested the possibility of considerable uncertainty as to the judgement of measured theophylline concentrations in clinical therapy. In view of the low serum binding of theophylline and small interindividual variation observed in this study, the role of serum binding variation in therapeutic monitoring of theophylline levels would seem to be relatively small. However, the increase in free fraction of theophylline in acidaemia might be of clinical significance.

This work shows that it is necessary to control pH to obtain physiologically relevant data on serum binding of theophylline.

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