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DETERMINANTS OF THE PLASMA PROTEIN BINDING OF THEOPHYLLINE IN HEALTH

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1 The plasma protein binding of theophylline was determined after addition of [^{14}C]-theophylline (15 μg/ml) to plasma from 24 healthy drug-free volunteers and equilibrium dialysis for 2 h at 37°C.

2 The percentage of drug unbound was 60.0% ± 2.2% (s.d.) with very little variation between individuals. The binding ratio of theophylline was not significantly related to the plasma albumin or α_1-acid glycoprotein (AAG) concentrations but was significantly, although weakly, negatively related to the logarithm of the non-esterified fatty acid concentration (NEFA) ($r = 0.443$, $P < 0.05$).

3 Intravenous administration of heparin (1000 units) caused a significant rise in plasma NEFA concentration and in the percentage of drug unbound in plasma after equilibrium dialysis.

4 In human serum albumin solutions, the binding ratio of theophylline was significantly related to the albumin concentration and at the albumin concentration seen in the 24 normal subjects, the percentage of drug unbound was almost identical. Addition of AAG in physiological concentrations did not enhance theophylline binding but oleic acid, and to a lesser extent palmitic acid, reduced binding significantly.

5 The percentage of theophylline unbound in plasma varied markedly with pH so that at pH7 the percentage unbound was 52% greater than at pH 8. There was no evidence of concentration dependence of binding up to 140 μg/ml theophylline.

6 Theophylline appears to bind almost exclusively to albumin and its plasma protein binding varies little in healthy subjects, showing no concentration-dependence over the therapeutic range of concentrations. The binding is affected by pH and by NEFA concentration, however, and these factors may be of greater importance in disease states. Caution should be employed in the use of heparin in studies of plasma protein binding of theophylline.

Introduction

The plasma protein binding of theophylline has been studied by several groups. Their results have varied considerably, both in the absolute extent of binding and in the degree of variation in binding between individuals. Simons and co-workers suggested, for instance, that the percentage of theophylline in the unbound form could vary more than two-fold in health (Simons *et al.*, 1979) and it has been reported that there might be a greater than four-fold variability in subjects with obstructive airways disease (Lesko *et al.*, 1981). This variability could be of great importance in monitoring theophylline therapy since it is likely that it is unbound rather than total plasma drug concentration that reflects the clinical effect. This study was therefore performed to determine the degree of theophylline plasma protein binding in health and the determinants of that binding.

Methods

Normal subjects

Twenty-four healthy drug-free non-fasting subjects (12 males) aged between 16 and 67 years were studied. Blood was collected by direct venepuncture into polypropylene syringes into polyethylene tubes containing lithium heparin 17.5 U/ml blood (Sarstedt). The tubes were placed in iced water and centrifuged within 30 min of collection at 4°C and 500 *g* for 15 min. The plasma was separated and immediately frozen at −20°C. Samples were then thawed within 4 weeks for estimation of protein binding, non-esterified fatty acid concentration (NEFA) and plasma albumin and α_1-acid glycoprotein (AAG) concentration.

The plasma protein binding of theophylline was then performed after addition of theophylline (15

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μg/ml) containing [^{14}C]-theophylline 180 ng/ml (Amersham). The purity of [^{14}C]-theophylline was assessed before and after the equilibrium dialysis procedure and was > 98% in both cases. Two 1 ml aliquots of plasma were added to teflon equilibrium dialysis cells (MSE Dianorm) and dialysed for 2 h at 37°C against Sorensen's phosphate buffer (Diem, 1962) containing 0.59% sodium chloride and to which the theophylline had been added. The compartments were separated by a Spectrapor 2 membrane (molecular weight cut-off, 12000 spectrum) and no leakage of albumin was detected during the procedure. After dialysis two 300 μl aliquots from each side of the cell were added to 5 ml Picofluor scintillation fluid (Packard) and the radioactivity measured using a Phillips PW 4540 scintillation counter. Quench correction was made by the external standards ratio method (counting efficiency approximately 83%) and the percentage of unbound drug in plasma calculated as the ratio of the absolute disintegration rates in buffer and plasma multiplied by 100. Binding of theophylline to the membrane was 2.96 ± 0.62% and equilibrium was achieved by 90 min. (The coefficient of variation of the duplicate samples was 2.04%). The mean ± s.d. dilution of human serum albumin solutions (range 12.5–50 g/l) was 3% ± 2.1% during the dialysis procedure.

Total non-esterified fatty acid concentrations in plasma were measured by a modification of the method of Duncombe (1964). Plasma albumin concentrations were measured by rocket electrophoresis (Laurell, 1972) and plasma AAG by single radial immunodiffusion (Mancini *et al.*, 1964), and cross-checked with the results obtained by an independent reference laboratory.

The effect of heparin

The effect of heparin was measured in eight healthy drug-free non-fasting normal volunteers. Blood was collected and the binding measured as previously described, both immediately before and 15 min after intravenous administration of 1000 units heparin. The possible *in vitro* effect of the concentration of heparin used in the heparinised tubes was measured in a separate experiment. Blood (50 ml) was collected by direct venepuncture from a non-fasting healthy normal volunteer and half added to heparinised tubes (7.5 U/ml blood) before separation of the plasma. The other half was allowed to clot at room temperature and the serum separated and treated in identical manner to the plasma before measurement of theophylline binding.

The effect of proteins and NEFA

In vitro studies were performed using essentially non-esterified fatty acid-free human serum albumin (less than 0.005% NEFA, Sigma) made up in solutions of the phosphate buffer described previously. To these solutions were added pure AAG which had been prepared by the method of Hao & Wickerhauser (1973), palmitic acid (Sigma) and oleic acid (Sigma). The non-esterified fatty acids were dissolved in ethanol, dried down before addition of HSA solution and gently mixed overnight until completely dissolved. Control solutions were treated in an identical manner.

The effect of pH

The pH of all solutions was measured using a pH meter model 7020 (Electronic Instruments Ltd). The effects of pH on plasma protein binding were measured in the plasma from one healthy volunteer. The plasma was adjusted with HCl or NaOH to the desired pH (between 7 and 8) and dialysed against Sorensen's phosphate buffers at the same pH range (Diem, 1962). Other aliquots were left untreated and dialysed against a range of the same phosphate buffers between pH 7 and 8.

The effect of theophylline concentration

Theophylline (Napp Laboratories) was added in ethanol solution to test tubes and dried down before addition of heparinised plasma from two healthy drug-free volunteers to achieve pre-dialysis concentrations between 0.1 and 200 μg/ml. Equilibrium dialysis was performed as previously described and the concentrations in the samples checked by high performance liquid chromatography (Soldin & Hill, 1977). The equilibrium concentration of theophylline was calculated using the mass balance equation and related to the unbound fraction of theophylline.

Statistical comparisons were performed using Student's *t*-test or Wilcoxon's rank sum tests when appropriate and one way analysis of variance and student Newman Keuls test for multiple comparisons.

Correlations were performed using simple least squares linear regression analysis or Spearman's rank correlation procedure and the minimum level of significance was taken as $P < 0.05$ (two-tailed) in all cases.

The relationship between binding and albumin, AAG and NEFA concentrations was assessed using the binding ratio (ratio of bound to free drug) which should be linearly related to protein concentration, provided the number of binding sites on the protein remains constant and that the dissociation constant of the drug-protein complex is much greater than the free drug concentration (Nilsen *et al.*, 1978). The NEFA levels, which were skewed to the right, were log-transformed in order to achieve a distribution consistent with normality.

Results

Normal subjects

The percentage of theophylline unbound in the plasma of the 24 subjects varied from only 55.2 to 63.9% (mean ± s.d. 60.0% ± 2.2%) their plasma albumin concentrations ranged from 45 to 63 g/l (mean ± s.d. 51.6 ± 5.8). The plasma AAG varied between 0.37 and 1.3 g/l (mean ± s.d. 0.71 ± 0.23) and the NEFA concentration was 190 to 1157 (mean 498, median 472) μmol/l. The relationship between the binding ratio of theophylline and albumin concentration in the subjects just failed to reach statistical significance ($r = 0.378$ $P > 0.05$ $P < 0.1$). The binding ratio of theophylline was not significantly related to plasma AAG concentration ($r = -0.167$, $P > 0.05$) but was significantly inversely related to the natural logarithm of the plasma NEFA concentration ($r = -0.443$, $P < 0.05$) although the relationship was rather weak (Figure 1).

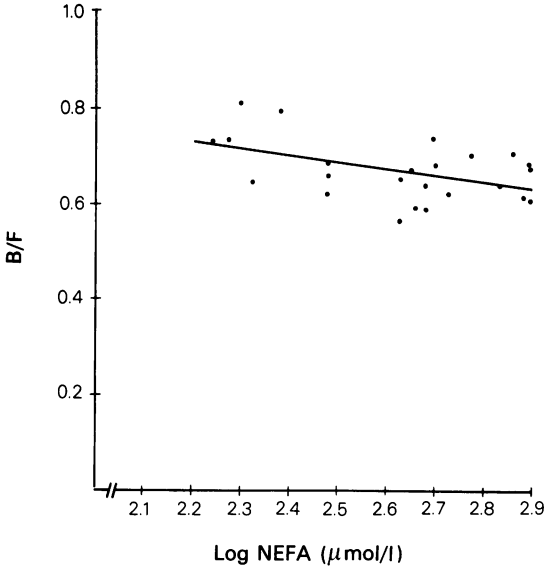

Figure 1 Relationship between the binding ratio of theophylline and log NEFA concentration ($r = -0.443$, $n = 24$ $P < 0.05$, m = −0.127, c = 1.006).

The effect of heparin

Fifteen minutes after intravenous administration of heparin, plasma NEFA rose from 303 (range 210–450) μmol/l to 995 (range 370–2030) μmol/l ($P < 0.05$, Wilcoxon's test) and the percentage of theophylline unbound from 59.2% to 68.5% ($P < 0.05$, Wilcoxon's test (Figure 2). There was no relationship, however, between the change in NEFA concentration and the change in the percentage unbound after heparin (Spearmans rho = 0.286 $P > 0.05$).

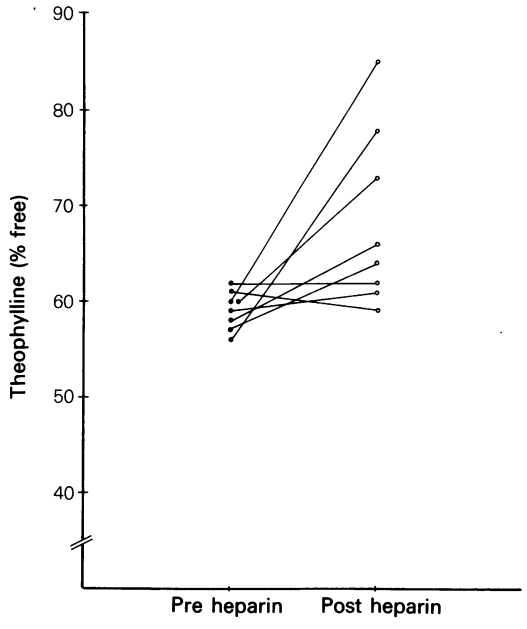

Figure 2 The percentage of theophylline free (unbound) in plasma before (●) and 15 min after heparin 1000 units i.v. (○) in eight normal volunteers ($P < 0.05$).

The effect of proteins

The binding ratio of theophylline in human serum albumin solutions correlated closely with albumin concentration up to 70 g/l ($r = 0.998$, $n = 6$, $P < 0.001$) (Figure 3) and the calculated percentage free theophylline at 51.6 g/l (the mean albumin concentration in the 24 subjects) was 59.5%, very similar to the mean percentage free in those subjects (60.0%). Addition of AAG (2.0 g/l to 50 g/l HSA) did not alter the percentage of theophylline in the unbound form (64.6 ± 0.6% *vs* 65.4 ± 0.6%, $P > 0.05$).

Addition of oleic acid (1.0 mmol/l) to HSA (40 g/l) increased the unbound percentage of theophylline from 65.0% ± 1.2% to 73.8% ± 1.0%, F = 64.24, q = 9.82, $P < 0.001$) and the same concentration of palmitic acid produced a much smaller although still significant increase (to 66.6 ± 1.0%, q = 3.64, $P < 0.05$) (Figure 4).

The effect of pH

The pH of plasma samples immediately before dialysis against phosphate buffer (pH 7.4) was 7.8 ± 0.12 s.d.

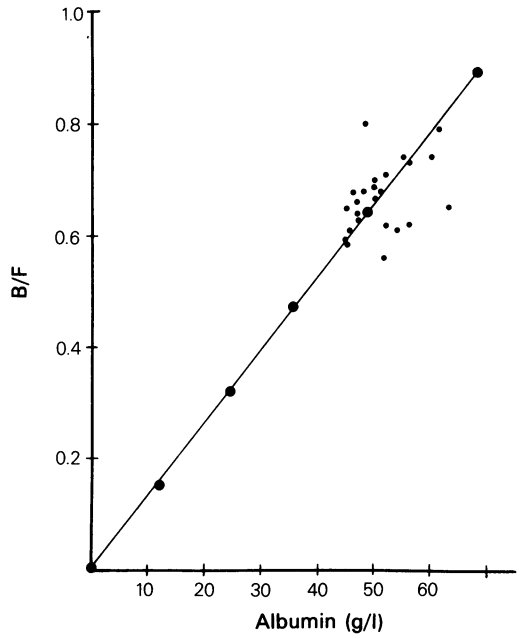

Figure 3 Relationship between the binding ratio of theophylline and albumin concentration in HSA (large closed circles) and in plasma from 24 normal volunteers (small closed circles). For HSA, $r = 0.998$, $n = 6$, $P < 0.001$.

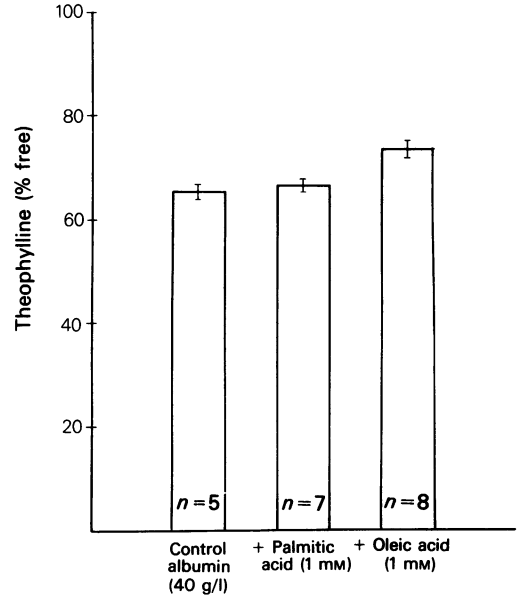

Figure 4 The percentage of theophylline free (unbound) in HSA 40 g/l before ($n = 5$) and after addition of palmitic acid 1 mM ($n = 7$, $P < 0.05$ c/w control) or oleic acid 1 mM ($n = 8$, $P < 0.001$ c/w control).

and after dialysis 7.4–7.45. The relationship between the percentage of theophylline in the unbound form and equilibrium plasma pH is shown in Figure 5. There was a significant negative linear relationship between these variables both for the untreated plasma ($r = 0.977$, $n = 10$, $P < 0.001$) and for the plasma the pH of which had been adjusted with acid or alkali prior to dialysis ($r = 0.995$, $n = 10$, $P < 0.001$). These points were combined when analysis of covariance revealed no significant difference between the regression lines to obtain the equation $y = 230 - 23x$. ($r = 0.981$, $n = 20$, $P < 0.001$, Figure 5).

The effect of theophylline concentration

In two patients the mean ± s.d. percentage of drug unbound between 0.18 and 200 μg/ml was 60.2% ± 2.3% ($n = 9$) and 59.6% ± 1.1% ($n = 10$) respectively and there was no relationship between these levels and equilibrium total plasma theophylline concentration ($r = 0.170$ and -0.392 respectively; $P > 0.05$).

Discussion

Normal subjects

The percentage of drug unbound in plasma in this study was much greater than that observed by many other authors (Koysooko *et al.*, 1974; Aranda *et al.*, 1976; Piafsky *et al.*, 1977; Mangione *et al.*, 1978; Simons *et al.*, 1979; Lesko *et al.*, 1981). We believe that this may relate to methodological differences. All of these above studies except one were performed using ultrafiltration which tends to give higher values for degree of binding, possibly relating to non-specific binding to the membrane (Chignell, 1977). They were also performed at room temperature rather than at 37°C and this will increase the percentage binding of many drugs (Ballard, 1974). Finally the possible effect of pH will be discussed presently. Only three studies employed equilibrium dialysis. In the first study of only eight volunteers the percentage free at pH 7.4 was 55.0%, similar to our results although the temperature was not reported (Vallner *et al.*, 1979). In the study of only seven normal subjects the average percentage free at 37°C and pH 7 was 40%. The reason for this higher apparent degree of binding is unclear since no details of non-specific binding to the membrane or equilibrium pH are given (Koysooko *et al.*, 1974). Finally, Fleetham *et al.* (1981) found that the percentage of drug unbound at 15 μg/ml, 37°C and pH 7.4 was 54.4%, similar to our findings, although they also observed concentration dependent binding of theophylline which we did not. The degree of variability in plasma theophylline binding we observed is in line with several studies (Koysooko *et*

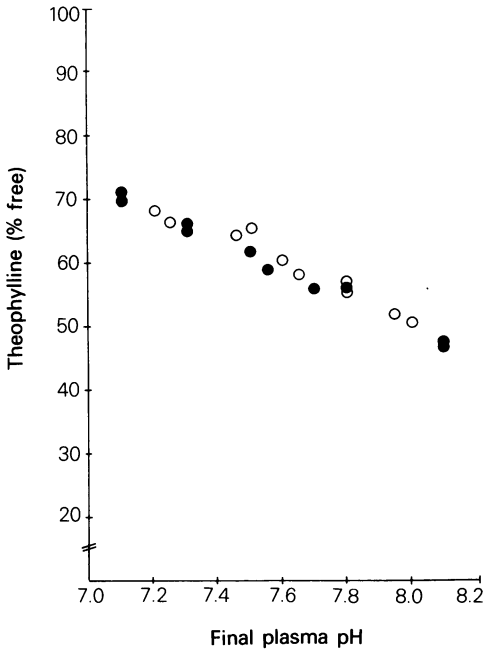

Figure 5 Relationship between the percentage of theophylline free (unbound) in plasma and the plasma pH at equilibrium in normal plasma samples (○) or plasma samples in which plasma pH was adjusted to the desired pH with HCl or NaOH (●). Samples were then dialysed against phosphate buffers of varying pH. ($r = -0.981$, $n = 20$, $P < 0.001$, $y = 233–23x$).

al., 1974; Aranda *et al.*, 1976; Piafsky *et al.*, 1977) although not with that of Simons *et al.* (1978) or Mangione *et al.* (1978) who indicated that the percentage unbound might vary by 50 to 100% even in healthy individuals. The reason for such discrepancies is unclear but may also relate to methodological differences.

The effect of heparin

Heparin has been shown to displace several drugs from binding sites in plasma (Spector & Santos, 1973). This may be related to the high levels of NEFA achieved at the end of equilibrium dialysis rather than the more modest rise in plasma NEFA after heparin when the plasma is measured before dialysis (Brown *et al.*, 1981). Two findings support the hypothesis that the changes seen in binding are in part artefactual. Firstly, there was no relationship between the change in binding and the change in NEFA in the eight subjects studied after heparin. Secondly, the concentration of oleic acid *in vitro* necessary to produce a similar change in binding as produced by heparin was probably four times greater than the probable heparin-induced change in oleic acid (approximately 0.25 mM) associated with a comparable change in binding (oleic acid normally only constitutes 33% of the measurable NEFA).

Whatever the mechanism, it is clear that the results obtained from previous studies should be viewed cautiously if there is any possibility that heparin was administered to the subjects. The small and possibly direct effect of heparin on binding when used as a specimen anticoagulant is unlikely to be important.

The effect of proteins

Albumin appears to be the major binding protein for theophylline. The degree of binding in HSA was very similar to that of plasma at equivalent albumin concentrations. Addition of AAG to and removal of fibrinogen from the sample (serum) did not alter binding in the direction expected. The lack of a significant relationship between theophylline binding and albumin concentration in the 24 volunteers is possibly related to the relatively narrow range of albumin seen in the healthy subjects together with the superimposed effect of other factor(s) on this relationship. The relationship just failed to reach statistical significance.

The effects of NEFA

NEFA are well known to reduce the plasma protein binding of many drugs (Spector & Santos, 1973). The relative effects of the individual NEFAs may vary, however. Oleic acid (C18:1) which constitutes approximately one third of the total plasma NEFA was much more potent in displacing thyroxine from human albumin than palmitic acid (16:1) which constitutes another third of the total plasma NEFA (Tabachnick, 1967). Several other individual NEFAs make up the other one third of the total and are likely to be quantitatively less important. We believe that plasma NEFA is a determinant of theophylline binding for three reasons. Firstly, there was a significant, although weak negative relationship between plasma NEFA and theophylline binding in the 24 healthy subjects. Secondly the individual NEFA (oleic and palmitic acid) when added to plasma, produced a reduction in binding. Thirdly, intravenous heparin reduced binding as well as increasing NEFA although the extent of the binding change which occurred *in vitro* rather than *in vivo* is unknown. It is certainly known that heparin-induced lipolysis continues *in vitro* during dialysis so that NEFA values at equilibrium are two-fold greater than before dialysis (Brown *et al.*, 1981). Although NEFA may thus be a determinant of theophylline binding, the magnitude of their effect in health is likely to be small. Further studies are needed of the importance of NEFA on binding of theophylline in disease states.

The effect of pH

The effect of pH changes on theophylline binding has already been described (Vallner *et al.*, 1979). What has been neglected is the possible effect of pH on the binding when ultrafiltration is employed, as it has been by most groups studying theophylline binding. Certainly we found that the pH of plasma before dialysis was high (7.6–8.1) and would probably remain so during ultrafiltration since no buffer is employed. Thus spuriously high binding might be observed, and could account for the higher binding seen in these studies compared with our own.

The effect of theophylline concentration

It has been suggested that theophylline plasma protein binding could fall with increasing drug concentration (Fleetham *et al.*, 1981). We have been unable to confirm these findings even with much higher drug concentrations than used by the above authors.

One possible explanation for the discrepancy is that the vehicle used to add the theophylline might reduce protein binding, particularly if it contained organic solvents, by protein denaturation. In all our studies, all theophylline solutions, including radiolabelled drug were evaporated to dryness before the plasma HSA or buffer were added. Fleetham *et al.* (1981) do not describe in detail how the drug was added. Our results, however, are in accord with those of Vallner *et al.* (1979) who found no concentration-dependence of theophylline binding, although admittedly between the very narrow range of 5–15 μg/ml.

In conclusion, theophylline appears to bind almost exclusively to albumin unlike the case for many other basic drugs in which AAG is an important binding protein (De Leve & Piafsky, 1981). This may be a major reason why variability in binding between normal individuals is so low since albumin concentration in health varies only slightly whereas AAG can vary more than four-fold. NEFA appear to cause reduced theophylline binding although their levels in health are such that their quantitative effect on theophylline binding is likely to be small. Similarly, the pH of blood in normal subjects varies only very slightly and would therefore be of little importance.

All of these factors are likely to be much more variable in disease, however, and could contribute to greater variability in theophylline binding. Certainly we would recommend that any future studies of theophylline binding in disease should be interpreted cautiously if heparin is given to the patient.

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