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# DETERMINATION OF THEOPHYLLINE BINDING TO HUMAN SERUM PROTEINS BY ISOTACHOPHORESIS

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## SUMMARY

Free theophylline was isolated from human serum by ultrafiltration and analysed in a leading electrolyte of 7.5 m*M* morpholinoethanesulphonic acid with ammediol as a counter ion at pH 8.90 and α-alanine as a terminator. The UV (280 nm) absorbance of the theophylline spike between serine and bicine as spacers was integrated. Binding percentages to human pool serum, human albumin and $\alpha_1$-acid glycoprotein (orosomucoid) were determined at physiological concentrations, and found to be 55, 44 and 12%, respectively. The calibration lines were straight from 0 to 30 mg/l, with a standard deviation of 0.2 mg/l. The detection limit was 1 mg/l. The time of analysis was 12 min at 40 μA in a 0.2 mm I.D. capillary.

## INTRODUCTION

Theophylline is a well known, useful drug for the control of bronchiospasm and the determination of its concentrations in serum is important. Oral or rectal administration of theophylline is the main treatment for chronic bronchospastic disorders and in acute asthmatic attacks the drug is administered intraveneously to relieve bronchospasm.

Progressive improvement of pulmonary function in response to the bronchodilatator effect of theophylline has been shown over the range of 5–20 mg/l. The optimal therapeutic response and theophylline toxicity are generally associated with total serum concentrations above 10 and 20 mg/l, respectively. Severe theophylline toxicity with convulsions and death has been reported. In man theophylline is converted mainly into 1,3-dimethylurate, 1-methylurate and 3-methylxanthine, which are excreted in the urine.

Several methods have been reported for the assay of theophylline in serum[1,2]. Existing methods for the determination of total theophylline include homogeneous enzyme immunoassay (EMIT)[3,4] gas–liquid chromatography (GLC)[3,5–7], high-performance liquid chromatography (HPLC)[3,4,8], thin-layer chromatography (TLC)[9] and isotachophoresis (ITP)[10].

In ITP, the spike method is applied and in our investigations we have studied

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some of the problems associated with this technique. In this respect, it is important to note that theophylline in serum is partly bound to serum protein, mainly to albumin. Most methods determine total theophylline, by denaturing the proteins with, *e.g.*, perchloric acid. The pharmacokinetic effect of a drug is mainly determined by the free concentration, while the amount bound to protein serves as a buffer. The variation in albumin concentration *in vivo* and the possible competitive binding by other drugs make it unlikely that binding percentages can be considered constant. Therefore, the determination of the free drug is more useful.

In this paper, we report an investigation of the binding of theophylline to total proteins in pooled serum, human serum albumin (HSA) and $\alpha_1$-acid glycoprotein ($\alpha_1$-AGP, orosomucoid) by means of capillary ITP using a discrete spacer technique.

## EXPERIMENTAL

### *Materials and equipment*

Theophylline was obtained from ICN-K&K Labs. (Plainview, NY, U.S.A.), morpholinoethanesulphonic acid (MES), bishydroxyethylaminoethanesulphonic acid (BES), bicine and the proteins human serum albumin and $\alpha_1$-acid glycoprotein from Sigma (St. Louis, MO, U.S.A.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.). All chemicals except theophylline were of analytical-reagent grade. Pooled serum was kindly supplied by St. Joseph Ziekenhuis (Eindhoven, The Netherlands) and stored at −20°C until used. Ultrafiltration CF 25 Centriflow filters (molecular weight cut-off 25,000) were purchased from Amicon (Danvers, MA, U.S.A.).

ITP was performed in equipment developed by Everaerts *et al.*[11]. For detection the 280-nm line of an iodine plasma lamp was used. The operational system is specified in Table I.

### *Procedure*

Volumes of 200 µl of the samples were diluted with an equal volume of a solution of the spacers bicine and serine, both at concentrations of 0.5 g/l. After

TABLE I

OPERATIONAL SYSTEM FOR THE ITP DETERMINATION OF THEOPHYLLINE

Capillary, I.D. 0.2 mm; driving current, 40 µA; analysis time, 12 min.

| *Parameter* | *Electrolyte* | |
|---|---|---|
| | *Leading* | *Terminating* |
| Anion | MES | α-Alanine |
| Concentration | 7.5 *M* | 5 m*M* |
| Counter ion | Ammediol* | Barium |
| pH | 8.90 | *ca.* 10 |
| Additives | None** | None |

* Ammediol = 2-amino-2-methyl-1,3-propanediol.

** In the experiments reported here it has been shown not to be necessary to use additives to increase zone sharpness.

vigorous mixing with a vortex mixer the sample was centrifuged for 5 min at 1000 *g* in a CF 25 Centriflow filter. A 1-$\mu$l volume of the filtrate was injected directly. The theophylline spike between the spacers was recorded at 280 nm (see Fig. 1); the transmission signal was converted into absorbance units by a home-made lin–log convertor and integrated by an SP 4000 chromatography data system (Spectra-Physics, Santa Clara, CA, U.S.A.).

Possible sources of error include variations in the relatively small injection volume, evaporation and the fact that dilution may occur during filtration as the filter has to be previously soaked in hot water. Therefore, the zone length of one of the spacers was used as an internal standard.

## RESULTS AND DISCUSSION

Fig. 1 shows the separation of pooled serum, (a) blank and (b) spiked with theophylline. In both experiments bicine and serine were used as spacers. Additional impurities are minimized by choosing a narrow mobility interval between the leading and terminating. It should be noted that uric acid can be determined in the same run, as it migrates zone electrophoretically in the leading electrolyte (MES).

For monitoring the UV signal in HPLC, the use of absorbance units for quantification is most commonly used. In ITP, however, the use of a lin–log convertor is not as widespread, mainly because in ITP most quantitative information is obtained from zone lengths and not by measuring areas. However, for quantification with the steady-state mixed zone (SSMZ)[12] or spike method[10,12,13], the use of absorbance units is unavoidable owing to deviations from to Beer's law, as can be seen from Figs. 2 and 3 (calibration graphs for theophylline in aqueous solution). Area measurement of the transmittance signal (see Fig. 2b) gives a considerable deviation from linearity compared with absorbance areas (see Fig. 2a). Further, the spike area is clearly preferred to the spike height, as can be seen from the spike height calibration graphs in Fig. 3a and b. A plateau value is eventually reached, above which zone length can be used for quantification.

Because the therapeutic total concentrations range between 5 and 20 mg/l, the UV spike area method must be used.

Theophilline calibration graphs were constructed by addition of various con-

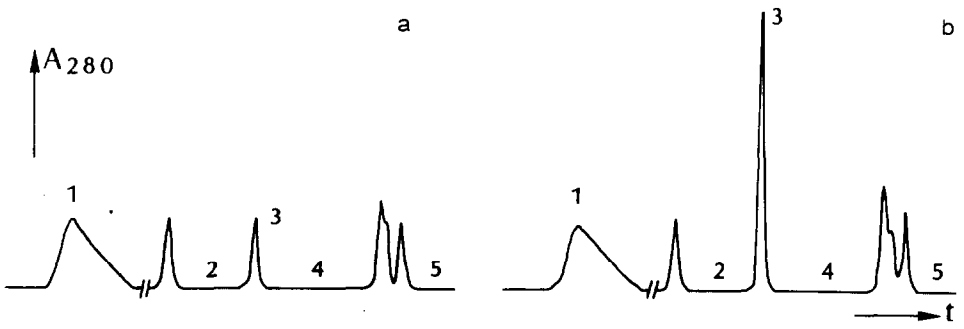


Fig. 1. ITP separation of pooled serum in the operational system of Table I: (a) blank and (b) spiked with 10 mg/l of theophylline. 1 = Uric acid in leading electrolyte (MES); 2 = bicine spacer; 3 = theophylline; 4 = serine spacer; 5 = terminator ($\alpha$-alanine). The time base for uric acid is different.

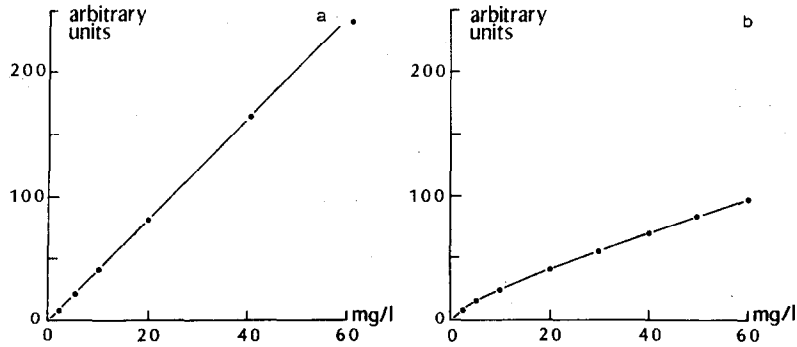


Fig. 2. Spike area calibration graphs for theophylline in water. Comparison of the results of (a) absorbance and (b) transmittance measurements.

centrations to water, pooled serum and standard physiological protein solutions. The results were always corrected with those obtained from a blank run. After addition of the spacers, the samples were equilibrated at room temperature for up to 3 h at 30-min intervals. After ultrafiltration free theophylline was determined, and the equilibration time was found not to be critical. When studying protein–drug binding, care should be taken that the binding equilibrium does not significantly change during the separation of the bound and free fractions. In this respect, the filtration time could be critical. However, experiments have shown that the filtrate theophylline level does not change significantly when filtration is extended from 1 to 5 min. Possible adsorption of theophylline to the filter material was checked by analysing a physiological solution of theophylline before and after filtration. Filter adsorption was found to be negligible.

The calibration graph for theophylline in water was straight from 0 to 30 mg/l (see Fig. 4). Recovery from a physiological solution containing 100 m*M* NaCl, 3 m*M* $KH_2PO_4$, 2.5 m*M* $CaCl_2$ and 1 m*M* $MgSO_4$ was determined and found to be 100%.

A total pooled serum protein binding of 55.1% was calculated from the slope of the calibration graphs in water and pooled serum, respectively. This is in good agreement with the value of 56.4% reported for serum of adults[14]. Other workers[15,16] reported higher values, whereas there seems to be a considerable dependence on pH[17].

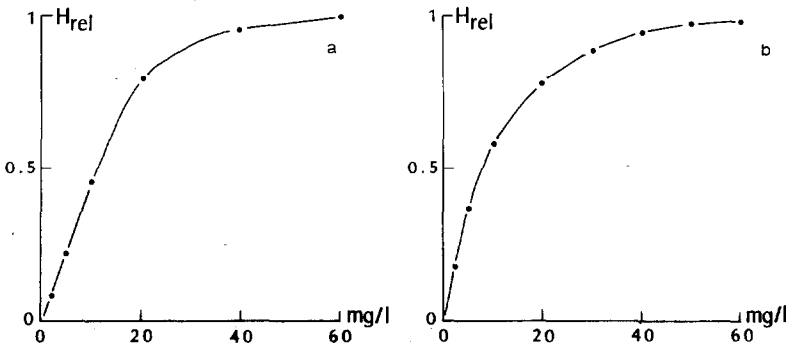


Fig. 3. Relative spike height calibration graphs for theophylline in water. Comparison of the results of (a) absorbance and (b) transmittance measurements. The unit value corresponds to the plateau height.

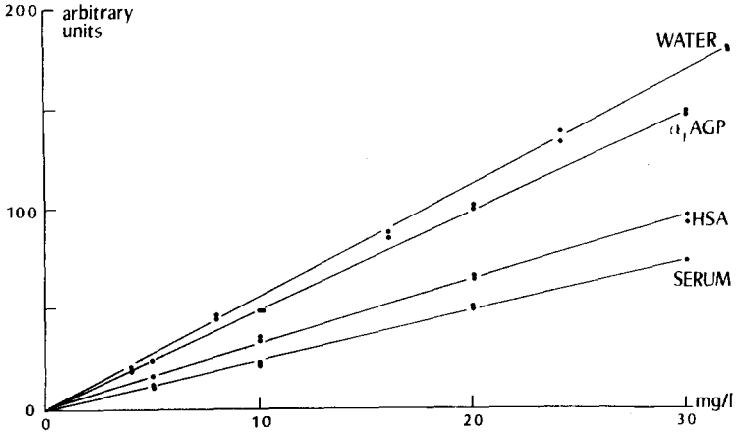


Fig. 4. Calibration graphs (absorbance spike area) for the determination of free theophylline in water (correlation coefficient = 0.999), pooled serum (correlation coefficient = 0.997), human serum albumin (HSA) (correlation coefficient = 0.998) and $\alpha_1$-AGP (correlation coefficient = 0.999). The slopes are 5.92, 2.66, 3.29 and 5.18, respectively.

Albumin is mainly responsible for drug binding in serum, principally because of its high concentration. This was verified with a calibration graph in a physiological albumin solution (see Fig. 4), in which the albumin concentration was equal to that of the pooled serum (40 g/l). The latter was determined by ITP at pH 6.0 with 0.01 *M* chloride as a leading ion, histidine as a counter ion and BES as a terminator. A binding percentage of 44.4% was found for the albumin solution. The remaining 10.7% binding must be attributed to a number of other proteins, present in serum. A strong binding to $\alpha_1$-AGP was assumed. The $\alpha_1$-AGP concentration in the pooled serum was not determined, but a value of 1 g/l was taken, because normal values were reported to range from 0.75 to 1.00 g/l[18]. Theophylline binding in this $\alpha_1$-AGP solution amounted to 12.4% (see Fig. 4).

From the results obtained, it can be concluded that albumin and $\alpha_1$-AGP are mainly responsible for total human serum binding of theophylline to the extents of

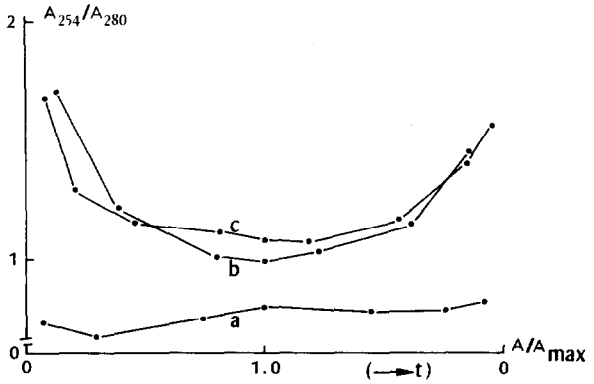


Fig. 5. Ratio of absorbances at 254 and 280 nm determined with the dual-wavelength detector[14] of the theophylline spike in (a) aqueous standard solution containing 15 mg/l, (b) pooled normal serum without theophylline and (c) a patient's serum.

44 and 12%, respectively. It is likely that the binding percentage will depend on the protein concentrations, which are known to vary *in vivo*. A decreasing binding percentage due to saturation of sites is usually encountered at higher drug concentrations, but it has not been found at therapeutic theophylline levels.

The possibility of using ITP for the determination of theophylline in patient's sera, as was suggested by Moberg and Hjalmarsson[10], was investigated in a comparative study with HPLC[13]. The results show a reasonable correlation in the range 1–10 mg/l of free theophylline (correlation coefficient 0.89, $n = 22$), but with a considerable bias (2.4 mg/l in favour of ITP). The bias is caused by the relatively high background signal (Fig. 1a), which with pooled serum can be corrected for, whereas for patients' sera it cannot.

Curiously, in the paper by Moberg and Hjalmarsson[10], the standard addition calibration graph shows a considerable background signal (5 mg/l) as well. The background signal may be decreased with a smaller mobility window between the spacers.

The dual-wavelength detector[19] for ITP was applied to verify the impurity of the background signal by measuring the ratio of the absorptions at 254 and 280 nm. This ratio was fairly constant over the entire theophylline spike range in a standard solution (Fig. 5a), but was not with a pooled serum (Fig. 5b) or a patient's serum (Fig. 5c), where the ratio was higher and by far constant over the theophylline spike. As there is no reason to assume that this background signal will be equal in all patient's sera, it cannot be corrected for as previously suggested[10].

## CONCLUSIONS

It has been shown that theophylline binding to proteins can be determined with the ITP spike technique, using two separate spacers, whereas the relatively high background signal prohibits its use in routine analyses of patient's sera.

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