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Note

New rapid assay of theophylline in plasma by isotachophoresis

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Theophylline (1,3-dimethylxanthine) is a key drug in the treatment of asthma, because of its effectiveness as a bronchodilator. The therapeutic value depends on the concentration in the blood. Levels of 10—20 μg/ml are considered to be therapeutic plasma concentrations. The rate of elimination from the circulation varies markedly among individual patients. Children and smokers eliminate theophylline fast. Levels higher than 20 μg/ml result in unacceptable side-effects. A "standard" dosage will, in many cases, result in overdosage or insufficient therapy, and consequently analysis of the theophylline concentration in plasma is necessary to ensure adequate therapy. For these analyses a rapid quantitative method is necessary which, since the patients are often children, should require only a small volume of plasma.

Existing methods commonly used for theophylline analyses are spectrophotometry, gas—liquid chromatography (GLC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and the enzyme multiplied immunoassay technique (EMIT). The spectrophotometric methods [1—3] require relatively large amounts of plasma (1—3 ml) and have low specificty due to interference by both endogenous and exogenous substances, such as caffeine in coffee and tea and theobromine in chocolate. Gas chromatographic methods [4, 5], which eliminate the interference by such substances and by many other drugs, have been developed. Plasma volumes of 100 μl are sufficient, but the extraction and derivatization steps give an over-

all analysis time of about 30—45 min even though the time for the GLC separation is only a few minutes. Quantitative TLC has been evaluated recently [6, 7], but it is time consuming if not used batch-wise. To obviate the need for derivatizing the theophylline, a number of HPLC procedures have been proposed during the last few years [8—16]. HPLC requires some type of sample preparation, such as extraction, and/or protein precipitation followed by centrifugation to remove solid material. The volumes of plasma needed are about 100 µl. The direct injection of serum onto a guard column has recently been described [17]; the volume of sample then required was only 10 µl, but the column had to be repacked frequently. EMIT requires only simple sample preparation [18]. The analysis time is short, but the reagents are expensive.

Since most of the above procedures are cumbersome and laborious, we investigated the possibility of using isotachophoresis for the analysis of theophylline. The isotachophoretic technique has been described by several authors — Haglund [19], Arlinger [20], and recently in an extensive review by Everaerts et al. [21]. It was shown that theophylline could be separated and readily quantified with this technique without laborious pretreatment of the sample.

EXPERIMENTAL

Isotachophoretic conditions

The isotachophoretic analyses were performed with an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a 23-cm capillary tube. Initially the leading electrolyte used was 5 m*M* HCl adjusted with Tris (Sigma, St. Louis, Mo., U.S.A.) to pH 8.4 to give a broad separation range. Later on, a narrow separation range was obtained with 5 m*M* glycyl-glycine (Fluka, Buchs, Switzerland), adjusted to pH 8.4 with Tris, as leading electrolyte. In both cases 0.2% (w/v) hydroxypropylmethylcellulose (HPMC, Methocel 90 HG, 15,000 cps; Dow Chem. Co., Midland, Mich., U.S.A.) was added to the leading electrolyte to minimize electroendosmosis. The terminating electrolyte was initially 10 m*M* glycine (Sigma) adjusted to pH 9.0 with $Ba(OH)_2$, but was later changed to 5 m*M* L-serine (Sigma) adjusted with $Ba(OH)_2$ to pH 9.5.

The analyses were run at a constant current of 100 µA, and the total analysis time was about 10 min. The transmission at 254 nm was recorded at a chart speed of 6 cm/min. The capillary was thermostatted to 10°.

Standard solutions

Theophylline, theobromine and caffeine were obtained from Sigma.

Standard solutions of theophylline in water were prepared at concentrations of 10, 20, 40, 60 and 80 mg/l. The stock solutions were used for making the calibration curves according to the procedure given below.

Calibration curve of theophylline in plasma

The plasma calibration curve was obtained by mixing 50 µl of plasma with 50 µl of theophylline from each of the five standard solutions and adding 100 µl of 38% (w/v) polyethylene glycol 6000 (PEG). The final plasma concentrations of theophylline were then 2.5, 5, 10, 15, and 20 mg/l. The solution was mixed thoroughly for half a minute. After centrifugation for about 5 min

at 1000 *g*, the clear supernatant was decanted and 2 μl were analysed. Care must be taken not to inject any precipitate, since this will affect the analysis result. The samples were injected by means of a microlitre syringe, equipped with a stop on the needle to ensure injection at a fixed position at the border between the leading and terminating electrolytes.

Plasma samples from patients

When assaying plasma samples from patients, 50 μl of plasma were mixed with 150 μl of PEG. A standardized volume of 4 μl was analysed. Samples containing less than 25 pmoles of theophylline were re-injected at a volume of 8 μl, and samples with more than 125 pmoles were re-run with a volume of 2 μl, to ensure more reliable values.

RESULTS AND DISCUSSION

Capillary isotachophoresis has been described as a useful technique even for quantitative determinations of picomole amounts. It was shown by Arlinger in 1974 [22] that the height of the UV signal of an ATP zone remains constant until the zone length falls below the aperture diameter of the UV cell. For narrower zones, there is a linear relationship between UV peak height and the amount of the compound injected. Svoboda and Vacík [23] and Wielders [24] have discussed the basic theory of the response of the UV detector when analysing very small amounts of sample which give zone lengths of less than the slit width of the detector. It was shown that a linear relationship exists between the height of the UV peak and the amount of sample within a certain interval. The possibilities of the "UV spike" method are demonstrated in Fig. 1, which is a plot of peak height versus amount and concentration of theophylline in plasma. It can be deduced from this standard curve that amounts of theophylline between 25 and 125 pmoles can be accurately determined. UV-absorbing compounds adjacent to the trace component zone of interst may

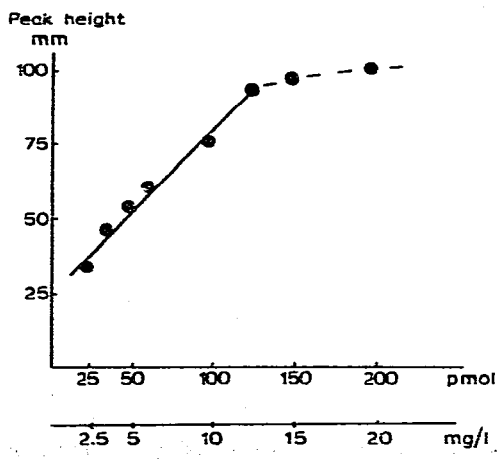

Fig. 1. Plot of peak height versus amount and concentration of theophylline in plasma; 2 μl of each theophylline concentration were injected. Glycyl-glycine was used as leading electrolyte and serine as terminating electrolyte. For further details, see Experimental.

influence the accuracy of measuring the peak height. Therefore, a low and constant level of impurities in the electrolytes and standard solutions is very important. To be able to detect trace amounts of theophylline in a complex mixture such as plasma or serum, it is necessary to remove interfering proteins by a precipitation step. Several precipitating agents were tried. PEG was found to be superior to the others. PEG is easy to handle, not too viscous at 38% w/v, does not increase the analysis time by adding extra ions and precipitates most of the proteins with a mobility similar to that of theophylline.

Fig. 2a illustrates the analysis of 250 pmoles of theophylline. A 50-μl volume of plasma was spiked with 50 μl of 0.2 m*M* theophylline, and 100 μl of PEG were added to precipitate the proteins; after centrifugation 5 μl of the supernatant were injected into the Tachophor. However, as can be seen from the UV record, some UV-absorbing compounds with a mobility similar to that of theophylline appear on either side of the theophylline zone. These UV absorbing compounds were found to influence the accuracy when quantifying the theophylline. Therefore, to minimize the influence of these UV-absorbing compounds, a number of non-UV-absorbing ions with mobilities very close to that of theophylline were tried as discrete spacers.

Fig. 2b illustrates the same experiment as in Fig. 2a, but with 10 nmol of glycyl-glycine added as spacer ion. The glycyl-glycine is shown to have a mobility close to the theophylline, and most of the UV-absorbing compounds are spaced apart from the theophylline zone by it.

In the experiment illustrated in Fig. 2c 10 nmol of serine has been added to

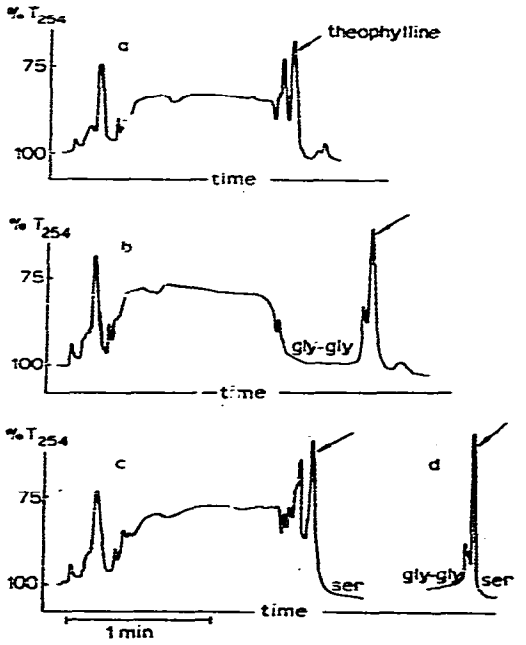

Fig. 2. Isotachophoretic analysis of a mixture of plasma, theophylline and PEG (used to precipitate most of the proteins). The arrow indicates the theophylline peak. In (a) HCl was used as the leading ion and glycine as the terminating ion; in (b) glycyl-glycine was added to the mixture; and in (c) serine was added to the mixture. In (d) glycyl-glycine was used as the leading ion and serine as the terminating ion; the sample mixture was the same as in (a).

the plasma sample. At the pH at which this separation is performed, the serine has a net mobility just below that of theophylline, and the small UV-absorbing peak appearing just after the theophylline peak (see Fig. 2a and b) has now been spaced apart by the serine.

The separation system can obviously be optimized by using glycyl-glycine as leading ion and serine as terminating ion. In this way a highly discriminating system has been developed, which is illustrated in Fig. 2d. In this electrolyte system neither theobromine nor caffeine will interfere, as they lie outside the narrow, selective mobility range. Standard calibration curves (Fig. 1) were made with plasma spiked with theophylline and PEG, using glycyl-glycine as the leading ion and serine as the terminating ion; some typical plasma analyses are shown in Fig. 3. The standard curve had to be checked each time new electrolytes were made up, because of differences in the amount of impurities in the electrolytes.

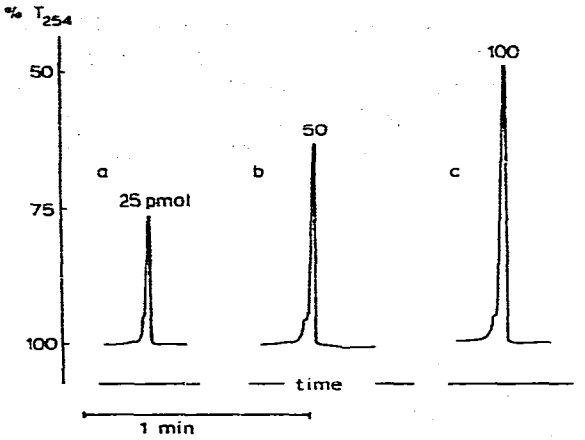

Fig. 3. Typical isotachopherogram used to prepare the calibration curves of a mixture of plasma, theophylline and PEG. Leading electrolyte was 5 m*M* glycyl-glycine and terminating electrolyte was 5 m*M* serine. Analysis time 10 min. For further details, see Experimental.

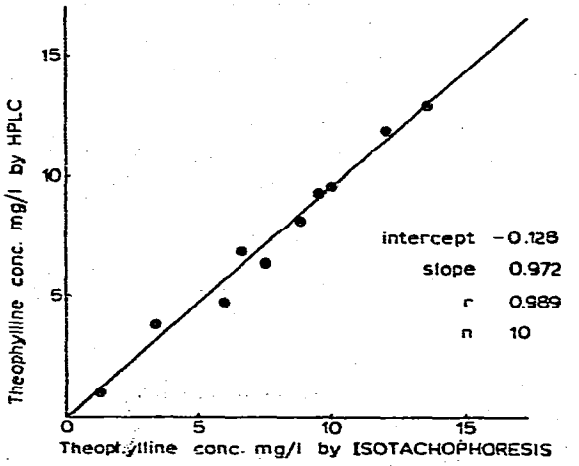

Fig. 4. Correlation of theophylline concentration in ten patient plasma specimens as measured by isotachophoresis and by HPLC.

The precision of the isotachophoresis method has been calculated from a standard calibration curve (see, for example, Fig. 1, where eight different concentrations of theophylline were measured four times each). The standard deviation was ± 2.7 mm. Samples giving peak heights below 30 mm (29% of the full UV-absorbance level of theophylline) ought to be re-run at the double volume to give a more reliable result.

Plasma samples from patients receiving theophylline therapy were analysed and quantitated using the standard curve. Ten plasma samples from patients were run twice and the mean value was compared with the mean value of double runs of the same sample analysed on HPLC according to the method of Jusko and Poliszczuk [13]. The isotachophoresis and HPLC techniques compare very well, as can be seen in Fig. 4.

CONCLUSIONS

Analytical isotachophoresis in capillaries has the unique advantage of combining accurate quantitative information with a short analysis time and high sensitivity. The only pretreatment of the plasma samples which is necessary is a simple precipitation and centrifugation. The versatility of the method makes it possible to select discriminating electrolytes, to prevent interference from related xanthines such as theobromine and caffeine.

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