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RAPID ASSAY FOR THEOPHYLLINE IN CLINICAL SAMPLES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PAMELA J. NAISH and MICHAEL COOKE*

Department of Inorganic Chemistry, The University, Bristol BS8 1TS (Great Britain)

and

ROBIN E. CHAMBERS

The Department of Chemical Pathology, The Royal Infirmary, Bristol BS2 8HW (Great Britain)

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SUMMARY

A fast, sensitive and highly specific method for the determination of theophylline in human serum is reported. Using a C_{18}-bonded reversed-phase column with an acetonitrile—acetate buffer mobile phase theophylline is completely resolved not only from other dietary xanthines and their metabolites but also from co-administered drugs such as paracetamol and phenobarbitone. Use of β-hydroxyethyltheophylline as internal standard allows a within batch precision of 2.0% and a between batch variation of 3.0%. Factors involved in the development of the method and its performance are discussed.

INTRODUCTION

The therapeutic effects of many drugs are dependent on their concentrations in biological fluids rather than their administered dosages. As the rate of metabolism of such drugs may differ widely between individuals the monitoring of drug levels in body fluids can be very important. Theophylline, a bronchodilator used extensively in the treatment of asthma operates over a narrow therapeutic range (10—20 μg ml^{-1} [1—3]) below which it is ineffective and above which toxic side-effects may occur [4, 5].

Plasma rather than saliva or urine has usually been chosen as the biological fluid for analysis of theophylline for three main practical reasons. Firstly, the levels of theophylline in plasma are approximately twice those present in saliva [6]. Secondly, apart from 3-methylxanthine [6, 7], plasma contains negligible

*To whom correspondence should be addressed.

quantities of the theophylline metabolites that are present in urine and therefore the possibility of interference is reduced. Thirdly, no simple correlation exists between the levels of theophylline in urine and plasma. Urine theophylline levels therefore do not provide a reliable indication of the concentration of the drug in the body.

Before the estimation of theophylline in plasma can be performed the plasma proteins, which account for about 7% by mass [8], must be removed. This may be achieved by simple denaturation with trichloroacetic acid [9] or organic solvents such as acetonitrile [10] or by ultrafiltration using a high-molecular-weight filter [6, 11]. Difficulties may occur, however, because the volume of sample available for analysis is often limited, particularly for neonates, where a heel-prick will provide approximately 100 μl of sample.

Methods which have been used to determine theophylline include ultraviolet spectrophotometry [12], radioimmunoassay [13], thin-layer chromatography [14], together with gas [15] and liquid chromatographic techniques [10, 16—23]. The high-performance liquid chromatographic (HPLC) determination of theophylline has usually been performed with a C_{18} reversed-phase bonded to an irregular microparticulate support. Typical column efficiencies ranged from 3000 to 8500 plates metre^{-1} [10, 18—23]. However, many of these methods suffer from one or more serious disadvantages such as lengthy sample preparation [19], poor chromatography (bad peak shape, low column efficiency, incomplete resolution of theophylline from plasma peaks [23]), high retention time [18] or applicability only to macrosamples [11].

The present work describes a rapid, specific method for the determination of theophylline in microsamples of human plasma. Studies of some seventy-five patients over a three-month period [24] suggest that this procedure is not only superior in performance to other gas and liquid chromatographic methods but also is sufficiently reliable to be used routinely in clinical laboratories.

EXPERIMENTAL

Liquid chromatography

The liquid chromatograph consisted of a Waters Assoc. Model 6000 pumping system and a Pye Unicam LC3 variable wavelength UV detector fitted with an 8-μl flow-cell. The detection wavelength was 273 nm and the detector sensitivity was 0.02 a.u.f.s. The 10 cm × 5 mm I.D. column (Shandon Southern Products, Runcorn, Great Britain, integrated column and septum injector system) was slurry packed in methanol containing a trace of acetate buffer, with 5-μm ODS-Hypersil (Shandon Southern Products), a C_{18}-bonded spherical 5-μm packing material. The solvent used to compress the slurry was hexane. The packing pump was of the pneumatic amplifier type (H.S.C.P., Bourne End, Great Britain) and the packing pressure was 6000 p.s.i. The detector output was to a suitable recorder with 10 mV f.s.d. All chromatograms were obtained at ambient temperature. A Hamilton 10-μl syringe with 75 mm length needle was used for injection of sample volumes. Quantitation was by comparison of peak heights with an internal standard. Operating pressure was 1200—1300 p.s.i. with a flow-rate of 1.5 ml min^{-1}.

Reagents and chemicals

The mobile phase was acetate buffer—acetonitrile (92:8). The buffer was prepared by adjusting the pH of a 20 mmol l^{-1} solution of sodium acetate in singly distilled water to 4 with reagent-grade glacial acetic acid. The buffer was prepared weekly and stored at 4° until required. Due to a negative volume of mixing, volumes of acetate buffer and acetonitrile were measured separately and subsequently mixed together. Degassing of the mobile phase was achieved by warming to ca. 40° under reduced pressure (ca. 15 mm Hg) for 10 min. During use solvents were maintained in the degassed state with a slow stream of helium. Care must be taken to ensure that the helium flow does not alter the composition of the acetonitrile—buffer mixture as a small reduction in the acetonitrile content leads to a large increase in retention time (Fig. 1). All mixed solvents were stirred continuously whilst the chromatograph was running.

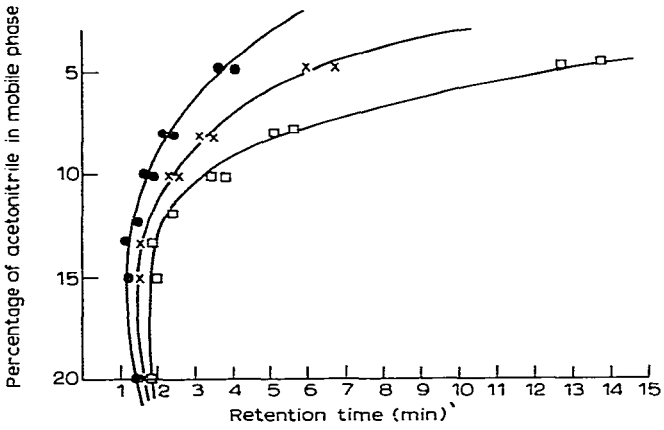

Fig. 1. Variation of retention time with percentage of acetonitrile in the mobile phase. •, Theobromine; x, theophylline; □, caffeine.

Sample collection and storage

Samples were collected from out-patients attending an asthma clinic. Upon receipt in the laboratory the samples were centrifuged and the plasma stored at - 20° until required for analysis.

Sample preparation

A stock solution of 237.5 ml chloroform and 12.5 ml isopropanol (95:5 mixture) together with 1.4 mg of β-hydroxyethyltheophylline as internal standard was prepared and stored at 4° until used. An aliquot of 25 μl of plasma was transferred via a 100-μl syringe into a 10-ml tapered centrifuge tube and 250 μl of the organic extracting solution (see above) were added from a 500-μl syringe. The tube was sealed with a rubber septum, the contents vortexed for one minute to give thorough mixing and centrifuged for one minute. A 200-μl amount of the chloroform layer was removed and evaporated to dryness. The residue was dissolved in 50 μl of mobile phase and vortexed for one minute prior to injection. For a 25-μl sample of plasma in 250 μl of extracting solution recovery of theophylline was 71%.

RESULTS AND DISCUSSION

Chromatographic aspects

The ratio of acetonitrile to acetate buffer was found to be critical to the separation. Slight alteration of the ratio brought about significant variations in retention time (Fig. 1). An acetate buffer—acetonitrile (92:8) mixture was selected for use, this being a compromise between maximum resolution of peaks and minimum analysis time per chromatogram.

Variation in the pH of the mobile phase was also found to be significant to the determination. The maximum difference in retention time between theophylline and dietary xanthines occurred at pH 4.0 (Fig. 2).

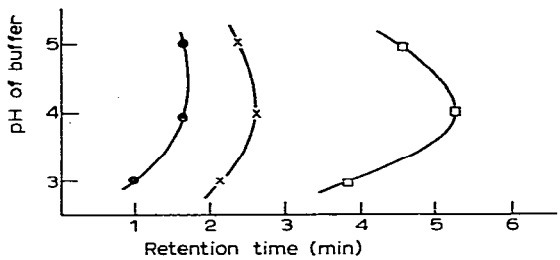

Fig. 2. Variation of retention time with the pH of the acetate buffer in the mobile phase. •, Theobromine; x, theophylline; □, caffeine.

The optimum flow-rate was found to be 1.5 ml min^{-1} which allowed an operating pressure of 1200—1300 p.s.i. At this working pressure regular changes of septum every fifteen to twenty injections were necessary.

The eluate was monitored at 273 nm corresponding to λ_{max} for theophylline (ϵ = 96,700) i.e. the optimum detection wavelength. Detection at 254 nm or 280 nm resulted in greatly reduced sensitivity. As these two wavelengths are commonly available in "fixed-wavelength detectors" it should be recognised that the use of such detectors seriously degrades the performance of the method.

The use of a spherical 5-μm particle packing material gave considerable improvement in performance when compared with other methods [10, 18—23]. Typical column efficiency with this material was 30,000 plates per metre. Whilst this was more than adequate for the resolution of theophylline, theobromine and caffeine (Fig. 3A) such performance is desirable. For example one of the most likely, and often neglected, of possible interferents in the determination of theophylline, is paracetamol, a common analgesic. Indeed paracetamol was found to be present in several of the seventy-five patients studied during this work. The resolution of paracetamol from theophylline is illustrated in Fig. 3B. Other possible interferents include the metabolites of theophylline, 1,3-dimethyluric acid, 1-methyluric acid and 3-methylxanthine.

The retention times of these and other related compounds are given in Table I. The only interfering compound was 1,7-dimethylxanthine, a metabolite of caffeine [25]. As this compound has not been detected in plasma in significant concentrations, interference is unlikely in practice. A second advantage, result-

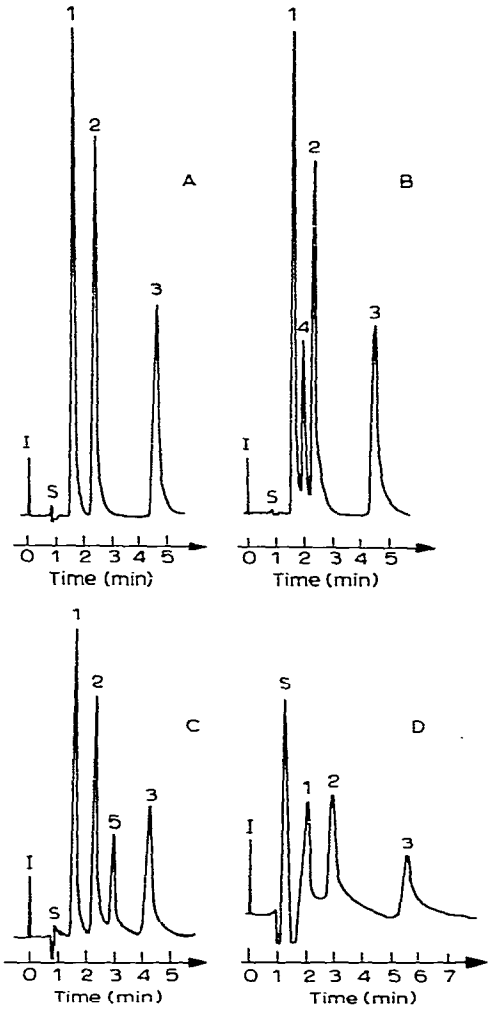

Fig. 3. (A) Optimised separation of theobromine (1), theophylline (2), and caffeine (3). S = solvent front. (B) The resolution of paracetamol (4) from theophylline (2). (C) The position of the internal standard (5) relative to theophylline (2) and caffeine (3). (D) The effect of injecting the sample in 100% acetonitrile. 1 = Theobromine; 2 = theophylline; 3 = caffeine.

ing from the high efficiency of the column was that considerable deterioration in the condition, and thus performance, of the column could be tolerated before column repair became essential. Acceptable results were still obtained when column efficiency had decreased to half of its original value. A daily wash of the column with methanol was found to extend its working lifetime thus reducing routine column maintenance still further.

Internal standard

Substituted xanthines which are not used as drugs, do not occur in dietary items, and are not metabolic products, were obvious choices for the internal standard. Three possible compounds were investigated, β-hydroxyethyltheo-

TABLE I

RETENTION TIMES OF THE VARIOUS COMPOUNDS STUDIED

Compound	Retention time (min)
Theophylline	2.50
Theobromine	1.65
Caffeine	4.65
Hypoxanthine	0.95
3-Methyluric acid	0.95
Xanthine	1.00
1-Methyluric acid	1.15
3-Methylxanthine	1.35
1-Methylxanthine	1.40
1,3-Dimethyluric acid	1.65
1,7-Dimethylxanthine	2.50
Paracetamol	2.15
(Phenobarbitone	29.0)*
β-Hydroxyethyltheophylline (I.S.)	3.20

*Phenobarbitone was only detected as a very small peak at high concentrations (due to a very low absorbance at 273 nm), and thus it can be ignored.

phylline, 8-chlorotheophylline and 7-β-hydroxypropyltheophylline. The retention times of these were 3.20, 5.45 and 5.50 min, respectively. All three showed similar absorbance at 273 nm and chromatographed well giving resolved symmetrical peaks. 8-Chlorotheophylline was rejected because of low solubility in the mobile phase at the 40 $\mu g\ ml^{-1}$ level. β-Hydroxyethyltheophylline, rather than 7-β-hydroxypropyltheophylline, was selected as the best internal standard because it eluted between theophylline and caffeine (Fig. 3C) thus adding no extra time to a chromatographic run. Chromatographic analysis time is thus reduced to 5 min.

Linearity of the assay

Using the extraction procedure detailed above, standard plasma samples with theophylline concentrations of 0, 0.5, 1, 2, 5, 10, 20, 40, 60, 80 and 100 $\mu g\ ml^{-1}$ were analysed. The results obtained over the range 0—40 $\mu g\ ml^{-1}$ were linear (index of determination 0.9977) but a slight upward curve was displayed at higher concentrations. The index of determination was 0.9927 for the range 0—100 $\mu g\ ml^{-1}$. As the therapeutic range (10—20 $\mu g\ ml^{-1}$) is contained within the linear portion of the calibration graph, the departure from linearity at high concentrations is not significant.

Analysis of plasma samples

A set of seventy-five samples from subjects attending out-patient asthma clinics was analysed. All samples had previously been analysed by the gas chromatographic method currently in use [15] at the Bristol Royal Infirmary. Batches of sixteen samples were analysed, consisting of thirteen prepared plasma samples together with two standards of concentrations 10 $\mu g\ ml^{-1}$ and 20 $\mu g\ ml^{-1}$ respectively and one control sample with a concentration near the middle of the therapeutic range. Duplicate injections of each sample were made.

The results obtained by HPLC are compared directly with those produced by gas chromatography in Fig. 4. The within batch coefficient of variation was determined by ten replicate assays of the control sample to be 2.0% (mean value 14.9 $\mu g\ ml^{-1}$). The between batch coefficient of variation was 3.0% (thirteen determinations, mean value 15.1 $\mu g\ ml^{-1}$). The figures may be directly compared with values for the gas chromatographic method [15] of 3.5% within batch and 5.6% between batch.

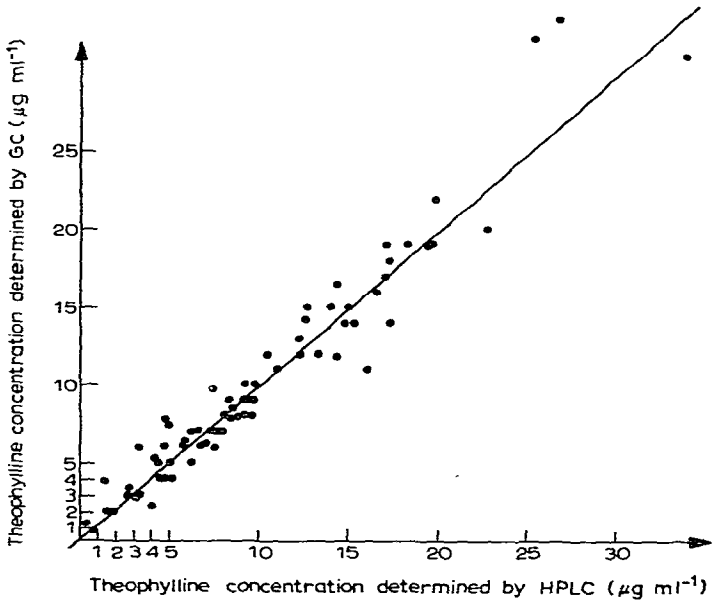

Fig. 4. Correlation of data from HPLC and gas chromatographic analyses. Index of determination, 0.964; slope, 1.011; intercept, + 0.089.

Due to the nature of the assay, many of the normal sources of variation have been eliminated. The only accurate volume measurements required are those of the sample (25 μl) and the 250 μl of extracting solution. The internal standard removed any errors caused by subsequent volume measurements, including injection. Furthermore any reduction in peak height, caused by variable increases in retention time or by band broadening as the column deteriorated, resulted only in a loss of sensitivity. Without an internal standard, this would have drastically affected the peak height response with respect to concentration.

Daily calibration of the method, by using two standards at 10 $\mu g/ml$ and 20 $\mu g/ml$ concentrations, eliminated errors due to slight changes in the response of the assay. These can be caused by such factors as small changes in the extracting solution (chloroform readily evaporates for example).

The speed of the method can be most important for clinical usage, especially in emergency cases, and therefore this procedure was designed to give accurate analyses in the minimum time. The fastest procedure developed was to initially prepare two samples of the batch while the liquid chromatograph stabilised. This preparation took 12 min. These samples were then run with duplicate injections. Since the possible elution of caffeine had to be allowed for, the chro-

matography time per injection was 5 min and thus 10 min per sample. The time per injection could be reduced to 3 min by injection of samples immediately after elution of the internal standard peak. The caffeine peak would then elute near the solvent front of the next injection, without interference with the theophylline peak. Other samples were then prepared during the chromatographic runs (each pair taking 12 min to prepare, while the previous pair takes 20 min to chromatograph). Computation of results was then performed during chromatography of the final pair of samples.

Potential chromatographic difficulties

As the developed method is very simple to operate it is likely that routine clinical determinations will be performed by chromatographically inexperienced personnel. We therefore report three areas where particularly careful observation is necessary.

Contrary to published results [26], the solvent in which the theophylline samples are injected was shown to be critical. Injection in pure acetonitrile produced poor peak shapes (Fig. 3D, compared with 3A). The decrease in peak height resulted in significantly reduced sensitivity. This effect is probably due to the theophylline being present in an un-ionised form when dissolved in pure acetonitrile, but existing in a protonated form when the recommended mobile phase (i.e. pH 4) is used. The step in the method involving evaporation of the chloroform extract to dryness and dissolution in the mobile phase is thus essential.

Injection of the sample at any height above the top of the column packing reduced the column efficiency. The sample should be injected into a layer of glass beads packed on top of the column as close as possible to the packing material without disturbing it. Disturbance of the column causes decreased column efficiencies and tends to block the syringe. For example, injection of the sample at a point ca. 5 mm above the column bed i.e. towards the top of the layer of glass beads was observed to reduce column efficiency from 25,000 to 7,700 plates m^{-1}. The injection of samples as close to the top of the packing as possible is therefore essential.

During use the packing material of the column shrinks slightly producing a void at the top of the column. The reason for this is uncertain, but may be due to further polymerisation of the bonded phase via cross-linking of the silane groups. The consequence, however, is the doubling (Fig. 5B), and in severe cases tripling, of peaks. The initial observation is the development of shoulders on each peak (Fig. 5A). The effect is readily cured by removal of the top few millimetres of dirty packing followed by repacking with fresh material using the chromatographic pump. We have restored the efficiency of the column used in the development of this work to about 30,000 plates m^{-1} five times. No permanent deterioration of the column has occurred.

In an attempt to prevent void development the top of the column was modified (Fig. 6). A gauze, permeable to 5-μm particles was placed on the top of the column packing. The column insert was positioned to hold this gauze in place and was then half filled with packing material as in normal column repair. A second gauze, that retained 5-μm particles, was cut to fit easily inside the column insert on top of the packing material. The remaining space was filled

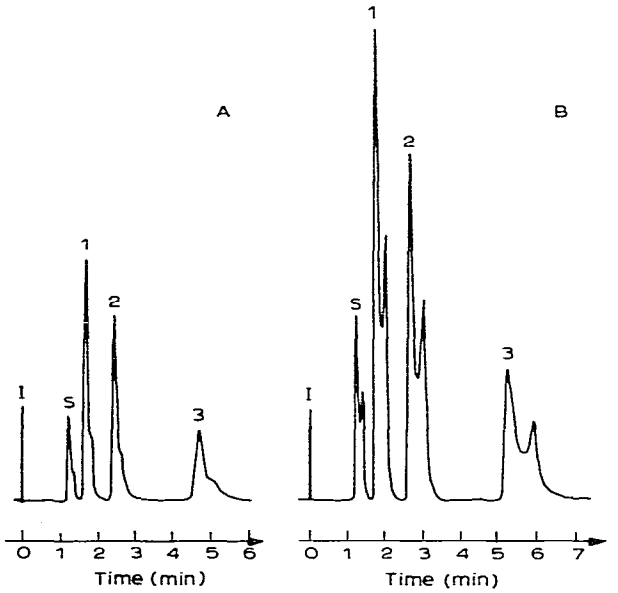

Fig. 5. Degradation of chromatography caused by void formation at the top of the column. For details, see text. 1 = Theobromine; 2 = theophylline; 3 = caffeine; I = point of injection; S = solvent front.

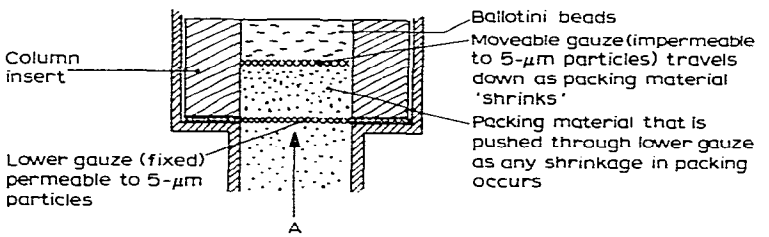

Fig. 6. Moving gauze modification to head of column. Void formation at point A is prevented.

with small glass beads (ballotini). As bedding down occurred the top gauze was forced down by the high pressure preventing the formation of voids at the top of the packing material. Instead the void was generated at the top of the bed of glass beads. Some success has been achieved using this modification as the problem of double peaks has not been observed during the last two hundred injections.

CONCLUSION

An HPLC method has been developed that is entirely suitable for the routine clinical monitoring of theophylline. The high specificity, high sensitivity, applicability to microsamples, good accuracy, precision and speed of analysis make it an attractive procedure when compared with other HPLC methods, gas chromatographic methods and the more conventional techniques of thin-layer chromatography and UV spectrophotometry.

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