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Determination of theophylline in serum and saliva in the presence of caffeine and its metabolites

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ABSTRACT

Because of marked variability in its metabolic clearance and its narrow therapeutic range (10–20 μg/ml) investigation of each patient's clearance of theophylline is desirable. The author reports here a rapid reversed-phase high-performance liquid chromatographic (HPLC) method to determine, within 3 min, the theophylline in serum and saliva in the 0.1–50 μg/ml range. A fast HPLC column, 10 × 4.6 mm, packed with 3-μm spherical ODS packing is used with acetonitrile–methanol–buffer pH 4.7 (4:7:89) to achieve separation of theophylline from paraxanthine and matrix components. Since theophylline is a major pediatric bronchodilator, the feasibility of assay in saliva was investigated as an alternative route for determining the clearance in stressed asthmatic children. Using this method it was found that the ratio of theophylline in simultaneous serum and saliva samples is very consistent over time in the same person (±3.99%), but inter-individually this consistency is reduced ten-fold. Simultaneous serum and saliva samples need be taken only once to obtain the ratio and the kinetics followed further with salivary samples only.

INTRODUCTION

Considerable variability is encountered in the pharmacokinetic profile of theophylline in human populations because of inter-individual differences in its absorption and metabolism [1]. These differences in the metabolic clearance of theophylline and its toxicity at serum concentrations >20 μg/ml [2] make investigation of each patient's clearance of the drug desirable so that dosage can be regulated to maintain a therapeutic blood theophylline level. To achieve this a rapid and accurate method of assaying theophylline in blood is necessary.

Various analytical methods have been reported for the determination of theophylline in body fluids. Direct spectrophotometric methods [3,4] are either lacking in specificity or are time-consuming. In a review of drug level monitoring of theophylline, Kucharczyk and Segelman [5] reported extensively on both immunochemical and high-performance liquid chromatographic (HPLC) methods and find both equally reliable. However, whilst enzyme-multiplied immunoassay

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(EMIT) is rapid and convenient [6] it has lately been shown to be erroneous by up to 400% in the presence of caffeine and its metabolites [7], and fluorescence polarization immunoassay (FPIA) [8], whilst convenient, is too expensive for routine studies. Chromatography is the most reliable approach to the assay, but gas chromatographic procedures require extraction and derivatization which lengthen the assay time considerably [9,10]. HPLC is now commonly used for the assay of theophylline in biological samples [11–20]. However, on investigation it has been found that many of the published methods [11,12,16,17,19,20] allow co-elution of theophylline (1,3-dimethylxanthine) with paraxanthine (1,7-dimethylxanthine), the major metabolite of caffeine (Fig. 1). Any satisfactory method must allow separation of these two components.

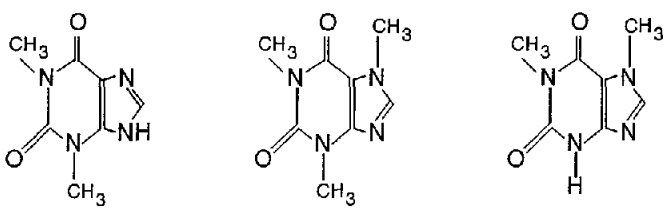

Fig. 1. Structures of caffeine, its major dimethylxanthine metabolite paraxanthine and theophylline.

Jaber *et al.* [21] have recently used an EMIT method to follow salivary theophylline levels for comparison with simultaneous serum theophylline levels. Since theophylline is a commonly used pediatric bronchodilator and a non-invasive method of determining its clearance in an already stressed child is desirable, the author has investigated the assay of theophylline in both serum and saliva in the presence of caffeine and paraxanthine. In addition, the relationship of the simultaneous levels of theophylline in the two body fluids has also been investigated in adult subjects.

EXPERIMENTAL

Reagents

All solvents used were spectroscopic grade from Burdick and Jackson (Muskegon, MI, USA) and all water was purified by the Milli-Q system (Millipore, Milford, MA, USA). The internal standard, sulphapyridine, as well as theophylline, caffeine and paraxanthine were supplied by Sigma (St. Louis, MO, USA). All other reagents were analytical reagent grade. The elution buffer was 0.05 *M* potassium dihydrogenphosphate containing 1 ml/l triethylamine and adjusted to pH 4.7 with orthophosphoric acid.

Internal standard

Sulphapyridine was found during a previous study to exhibit similar polarity to theophylline and thus was satisfactory as an internal standard in the serum clean-up, elution and detection conditions used. A 10 μg/ml solution of sulphapyridine in elution buffer was satisfactory as internal standard for the concentration range investigated.

Sample collection

Theophylline (250 mg) was ingested with 250 ml of water at zero time by informed staff volunteers. Other than prohibiting intake of xanthine-containing beverages for 3 h prior to and during the study, no dietary restrictions were enforced. Blood samples were collected in plain Vacu-test tubes at 0, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7 and 8 h via a cannula with heparin lock in the antecubital vein. After 15 min for complete clotting, each sample was centrifuged and the serum transferred to a clean glass vial. Immediately prior to collecting the blood sample, salivary excretion was stimulated by rinsing the mouth with water and chewing on a plug of Parafilm. Saliva was then collected in a sterile stoppered glass test-tube. All samples were stored at −18°C until assayed, within a few days after collection.

Sample preparation

Serum. Internal standard solution (25 μl) and buffer (25 μl) were added to 50 μl of serum in a centrifuge tube. A further 25 μl of 20% perchloric acid was then added, the tube shaken on the vortex mixer and centrifuged at 2000 *g* for 2 min. The resulting supernatant was injected onto the column via a 10-μl loop injector.

Saliva. The saliva samples were centrifuged at 3000 *g* for 4 min to remove cellular debris and undissolved contaminants. A 50-μl volume of the centrifuged saliva was added to 50 μl of water in a test tube and shaken. A 10-μl volume of this was loaded in forward flush mode into a 10-μl loop connected to a 1-cm pre-column in the loop position on a standard Valco six-port autoinjector valve (Fig. 2). After injection onto the analytical column with 2 ml of mobile phase, the injector valve was returned to the load position and the pre-column and loop backwashed with 2 ml of 50% methanol in buffer followed by 1 ml of water using a gastight flushing syringe. Just prior to loading the next sample, the pre-column clean-up system was forward flushed with 1 ml of mobile phase.

Standard preparation

Serum. Standard (25 μl) in elution buffer (range 0.2–20 μg/ml) was added to 50 μl of drug-free serum in a centrifuge tube along with 25 μl of internal standard solution. A 25-μl volume of 20% perchloric acid was added to precipitate the protein, and the mixture was shaken, centrifuged and injected as for samples.

Saliva. Standard (50 μl) was added to 50 μl of drug-free saliva in a glass test tube and shaken. A 10-μl volume was loaded in the forward flush direction into

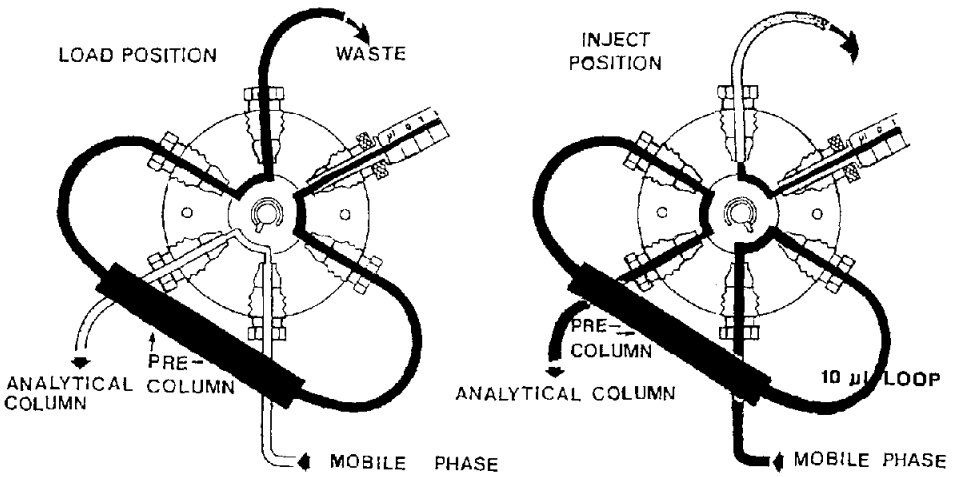

Fig. 2. Valco injector valve showing arrangement of 10-µl loop and pre-column for forward flush on-line end-cutting of saliva samples.

the 10-µl loop plus pre-column system and treated the same way as saliva samples.

Chromatography

HPLC was performed on a Spectra Physics SP 8100 liquid chromatograph with a pneumatically controlled six-port Valco loop injector valve fitted with a 10-µl loop. Separation was achieved on a Perkin-Elmer HS-3 C_{18} 100 mm × 4.6 mm I.D. reversed-phase column. A 10 mm × 4.6 mm I.D. home-made quard column packed with Shandon Hyperspheres 5-µm ODS was inserted in the normal guard column position immediately in front of the analytical column when assaying serum samples and inserted in the loop position (Fig. 2) when assaying saliva. Isocratic elution with acetonitrile–methanol–pH 4.7 potassium phosphate buffer (4:7:89, v/v) was performed at a flow-rate of 2 ml/min and a column temperature of 36°C.

A Spectra Physics SP8440 UV–VIS variable-wavelength detector with a 10-µl flow cell was used at 247 nm for analyte detection. The detector output was monitored simultaneously on a Perkin-Elmer 56 strip-chart recorder and a Spectra Physics SP 4200 integrator. The concentration of theophylline in serum was calculated on the basis of peak-height ratio of theophylline to internal standard from the calibration curves. The concentration in saliva was calculated directly by peak height from the salivary standard calibration curves.

RESULTS

Using the above methods good separation and detectability of theophylline in serum and saliva could be achieved, free of interference from caffeine metabolites

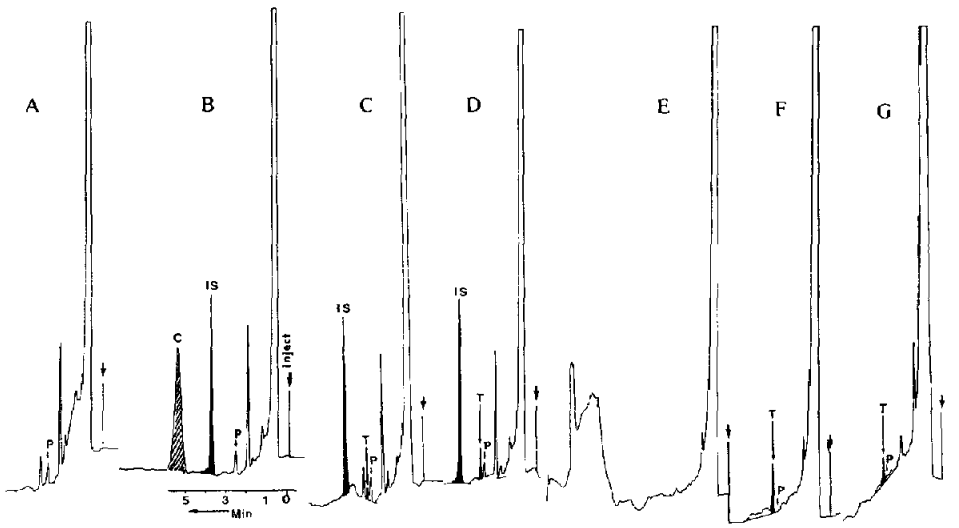

Fig. 3. Chromatograms of serum, saliva and standard samples. (A) Blank serum from coffee consumer; (B) serum 1 h after 100 mg caffeine following a 36-h abstinence from xanthine-containing beverages; (C) 1 μg/ml standard in serum; (D) serum sample 7 h after a 250-mg theophylline slow-release formulation; (E) blank saliva; (F) 5 μg/ml standard in saliva; (G) 7-h saliva sample. Peaks: T = theophylline; P = paraxanthine; C = caffeine; IS = internal standard.

or matrix components, with an elution time of less than 3 min ($k' = 4.03$), as can be seen in the chromatograms (fig. 3). No interfering components were found in the pre-dosing serum or saliva of five volunteers.

The standard response curves were linear: concentration = $-0.064 + 19.7555 \times$ peak-height ratio ($\pm 0.82\%$) for serum theophylline and concentration = $-0.064 + 19.7555 \times$ peak height for saliva theophylline over the standards range used, 0.5–50 μg/ml. Using the criterion of detectability as three times the system noise, the detection limit in serum was 100 ng/ml using a 10-μl loop but could be reduced to 25 ng/ml if a 50-μl loop was used and 10 ng/ml could be distinctly seen, but was less than three times the noise. The detection limit in saliva was 200 ng/ml but this could be reduced to 50 ng/ml if a 50-μl sample was injected. The inter-sample standard deviations were 0.82% for theophylline in serum and 4.3% for theophylline in saliva over six samples of each at 2 μg/ml. The recovery of theophylline from blank serum spiked with 2 μg/ml theophylline was 97.5–101.2%. The internal standard recovery ranged from 92.7 to 95.8% over all the standards and samples.

The pharmacokinetic profile for theophylline in both serum and saliva in five subjects revealed that, when dosing with a rapidly absorbed theophylline formulation, the salivary and serum concentration ratios were consistent in each individual after completion of absorption and distribution. When the theophylline was in a slow-release formulation, the salivary-to-serum concentration ratios

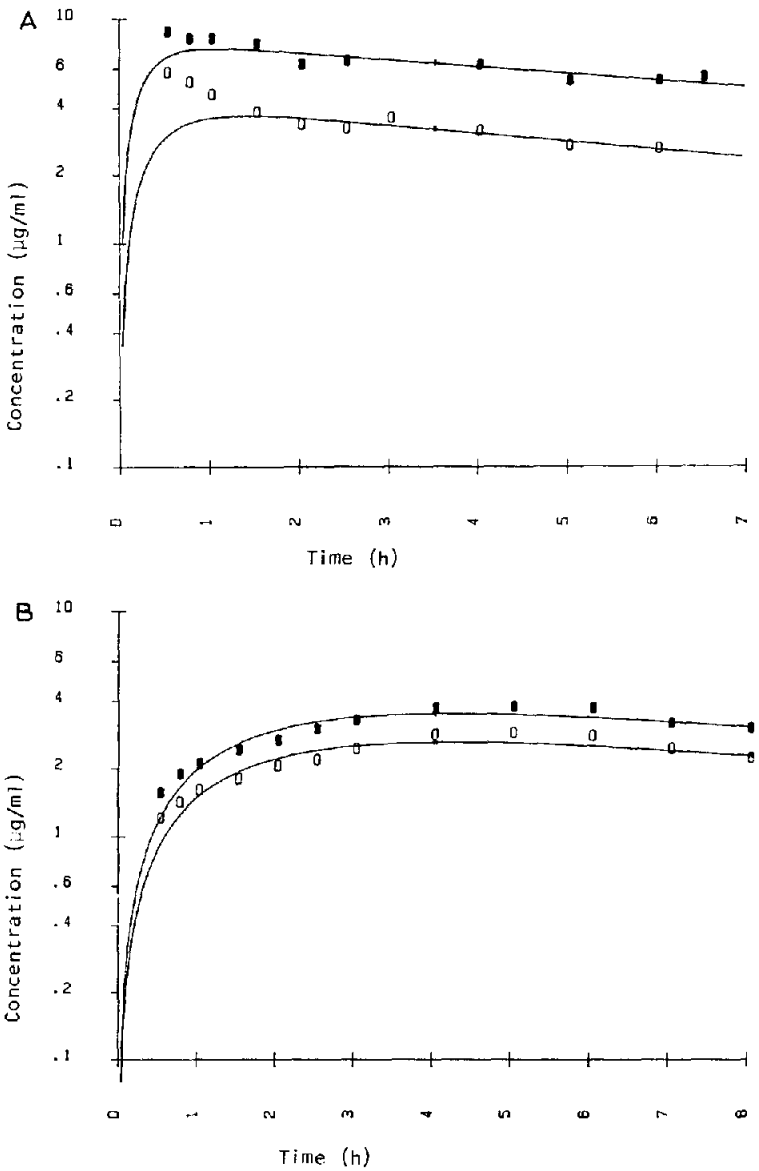

Fig. 4. Theophylline concentration–time curves for serum and saliva. (A) 250 mg theophylline (aminophylline); (B) 250 mg theophylline slow-release formulation (Nuelin SA 250). (●) Serum; (○) saliva.

were consistent from early in the absorption phase. Each subject exhibited his own consistent ratio. The lowest ratio was 0.501 ± 0.011 and the highest was 0.71 ± 0.028 in the five subjects. The pattern of the relationship between serum and saliva theophylline levels is shown graphically in the concentration–time curves (Fig. 4).

DISCUSSION

Trial determinations with some assay methods for theophylline taken from available literature [11,12,16,17,19,20] revealed that paraxanthine, the major metabolite of caffeine, was co-eluting with theophylline from the column. Whilst the rapid assay method developed by Chiou *et al.* [15] for the separation of theophylline and paraxanthine on a short C_{18} column using N,N-dimethylformamide and methanol in phosphate buffer (1:4:95) (v/v) gives good separation, it has the drawback that the high UV–VIS background from the dimethylformamide necessitates detection above 270 nm, resulting in a loss of sensitivity (limit of detection 0.25 μg/ml). Moreover, the dimethylformamide is highly irritant to the skin and mucosa, and the sample clean-up necessary prior to injection is complicated and time-consuming, thus this method was not investigated. The reversed-phase programmed elution method of Matsumoto *et al.* [18] using on-line sample clean-up gives excellent separation of theophylline and paraxanthine and eliminates cumbersome off-line sample clean-up. However, gradient elution is required and the stabilization time necessary after each separation, as well as a run time of 30 min, makes the method impractical for routine assays, although it does show that, with optimization of mobile phase strength, ODS columns can give baseline resolution of paraxanthine and theophylline. Grgurinovich [14] reported a method using octyl (C_8) columns with tetrahydrofuran in buffer at pH 6.5, which surprisingly allowed separation of the two dimethylxanthine isomers within 6 min and thus appeared to be ideal. However, trial with an equivalent octyl column (Spherisorb C_8) did not separate these isomers, and decreasing or increasing the mobile phase strength did not appreciably change the column selectivity or resolution.

Investigation on a 250 mm × 4.6 mm I.D. column with 5-μm ODS packing revealed that optimal selectivity for the dimethylxanthines on end-capped ODS packing occurred with a mobile phase of acetonitrile–methanol–pH 4.6 buffer (4:7:89) at 37°C, but the elution time for theophylline was 16 min at 2 ml/min. High back-pressure prevented any significant increase in flow-rate. Baseline resolution required at least 12 000 plates on-column with the optimized mobile phase. A shorter column with the same packing would allow a shorter retention time and even a higher flow-rate, but then the plate number would be correspondingly reduced. The modern short fast liquid chromatography columns offer a fast elution, low back-pressure and particularly low plate height for the 3-μm double end-capped ODS packings. This type of column was therefore investigated, and a 100 mm × 4.6 mm I.D. Perkin-Elmer HS-3 C_{18} with a 3-μm ODS packing and 11 000 plates on column under optimal assay conditions, was selected. A homemade 10 mm × 4.6 mm I.D. guard column with 5-μm spherical ODS packing preceded the analytical column, and dead-volume in all connections was minimized to maintain the high resolution. These columns answered the requirements of high plate number and the necessary selectivity when isocratically eluted with an acetonitrile–methanol–buffer mobile phase. Their short length and moderate

back-pressure allowed theophylline to elute in less than 3 min at a flow rate of 2 ml/min.

The ability to follow serum theophylline through salivary levels is an attractive alternative to serum sample collection. This is particularly so for pediatric subjects where blood collection only adds further stress to the asthmatic state. With this in mind, the author investigated the determination of theophylline in saliva. Initially saliva samples were prepared like serum samples, but since little or no protein was precipitated, the perchloric acid was excluded from the sample preparation method. However, it was found essential to centrifuge the whole untreated saliva, to get a clean supernatant saliva free from cellular debris, prior to adding the internal standard to the samples. Initially, the assay of the saliva seemed the same as for serum samples except that no protein was precipitated, however, the significance of non-precipitation of protein soon became evident in the form of interfering late-eluting bands as can be seen in Fig. 3E. On-line end-cutting of the samples was investigated by placing the guard column in the injector valve loop position as shown in Fig. 2 and investigating the pre-column washing and mobile phase flow parameters necessary to end-cut the sample.

The end-cutting procedure finally developed also washed away part of the internal standard used, so the effect of the end-cutting on reproducibility in the absence of an internal standard was investigated. This revealed that, if care was exercised in loading the 10-μl sample as accurately as possible, the error at 2 μg/ml was 4.3% when no internal standard was used. This was felt to be acceptable for a rapid assay methodology and so finally neither an internal standard nor protein precipitation was used in the sample preparation.

Trials done in five staff subjects showed a very consistent intra-individual correlation between the serum and salivary levels after absorption and distribution were completed (Fig. 4). No similar consistency was found inter-individually, and the standard deviation between the different intra-subject averages was an order of magnitude larger than the intra-individual deviation. The saliva-to-serum ratio ranges in each individual varied from 0.501 $\pm$ 0.011 to 0.71 $\pm$ 0.028. These results do not concur with the intra-subject ratios found by Boobis and Trembath [22] who found that below 5.5 μg/ml salivary levels were 40% of the plasma level whilst in the therapeutic range (10–20 μg/ml) the salivary levels were approximately 67% of the plasma levels. Since some of these samples were assayed by EMIT and some by gas chromatography it is possible that methodology was the source of the wide range (0.38–1.17) found for the saliva-to-serum ratio. Later work from the same institution [23] on nineteen subjects showed that considerable inter-individual differences in the saliva-to-serum ratio occurred with the closest correlation occurring 6 h after dosing. Most importantly the study [23] demonstrated that the theophylline concentration in saliva is independent of salivary flow-rate.

The results found in the present study are more consistent with those found by Koysooko *et al.* [24] whose results showed a similar constancy of the intra-indi-

vidual saliva-to-serum ratio, although the inter-individual range of each ratio average on seven subjects was narrowed, varying from 0.46 to 0.59 (0.52 ± 0.034). Jaber *et al.* [21] found an inter-subject range of 0.57–0.84 (0.68 ± 0.072) using each subject's ratio average, which result is similar to the range found in the present study.

CONCLUSION

It is thus concluded that the fast HPLC method for the determination of theophylline described above is both rapid and reliable. The ratio of the salivary to serum concentrations is individual, perhaps due to unique idiosyncratic transport and excretion of theophylline in the salivary glands. Either solely salivary or serum values over a 4-h time interval are sufficient to determine the half-life of theophylline, but at least one simultaneous pair of salivary and serum samples is necessary from each individual to obtain the serum-to-salivary ratio for the estimation of serum values from salivary results.

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