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Note

Improved method for the determination of theophylline in plasma and urine using high-performance liquid chromatography

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A variety of analytical methods have been reported for the analysis of theophylline and its metabolites in biological fluids. Gas chromatographic procedures [1–8] involved either multiple solvent extractions or chemical derivatization. A combined gas chromatographic–mass spectrometric procedure [9] was also reported. Some direct spectrophotometric methods [10,11] have also been reported but suffer from the disadvantages of requiring large volumes of biological fluids and being time-consuming to perform.

High-performance liquid chromatography (HPLC) now seems to be the most popular method for the determination of theophylline in biological samples. Many methods have been reported [12–24], and numerous problems have been encountered and not adequately resolved. A review of the analysis of anti-asthmatic drugs was given by Kucharczyk and Segelman [25] where HPLC methods were compared with enzyme-multiplied immunoassay techniques. Excellent correlations between the results of these two assays have been reported by several workers [26–28].

A common problem in the HPLC assay of theophylline is the potential for interference from concomitantly administered drugs. This has been investigated by several workers [26–29] where as many as fourteen commonly prescribed drugs have been screened and have not interfered with theophylline determinations. The common theme in these reports is that the isolation procedures required to extract theophylline from biological matrices in combination with the HPLC conditions employed preclude interference from other drugs. By far the most common source of interference is from theophylline metabolites; the inability to separate theophylline from some of its metabolites in a reasonable time [12,19,20] has been a common problem. The separation of theophylline from

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1,7-dimethylxanthine, the major metabolite of caffeine, remains as the most serious potential for interference in any HPLC assay [12,14,19,20,26]. Several approaches have been tried to resolve theophylline from 1,7-dimethylxanthine chromatographically. Kabra and Marton [27] made extensive modifications to the injector loop, the detector flow-cell, and to the connecting tubing in the HPLC system. Van Aerde et al. [30] used a silica column and normal-phase chromatography, and Bock et al. [28] used a 3-μm C_{18} reversed-phase HPLC column. Baseline resolution of the two components in combination with good peak shape for theophylline, however, has still not been achieved.

In the present procedure theophylline is completely resolved from its metabolites and from caffeine and its metabolites. In particular, baseline resolution of theophylline and 1,7-dimethylxanthine is achieved. Theophylline and its internal standard are separated in less than 5 min. The sample clean-up procedure is simple and rapid for both plasma and urine and gives chromatograms that are essentially free from endogenous interference.

EXPERIMENTAL

Reagents and materials

Dichloromethane and N,N-dimethylformamide were purchased from Mallinckrodt (Paris, KY, U.S.A.). Propan-2-ol and diethyl ether were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and J.T. Baker (Phillipsburg, NJ, U.S.A.), respectively. Theophylline, caffeine and their metabolites were all obtained from Sigma (St. Louis, MO, U.S.A.). The internal standard, β-hydroxyethyltheophylline, was also obtained from Sigma. Blank human plasma was supplied by Sera-Tec Biologicals (North Brunswick, NJ, U.S.A.), and Milli-Q-water was used throughout. Norit A neutral charcoal was supplied by Fisher (Pittsburgh, PA, U.S.A.). All of the other chemicals were either reagent or HPLC grade and were supplied by Fisher.

Instrumentation

A Varian LC-5000 HPLC system equipped with a built-in column heater and a UV-100 variable-wavelength ultraviolet detector were used for this work. The autosampler used was a Waters WISP 710B, and peak areas were measured by electronic integration using a Spectra-Physics 4270 computing integrator.

Chromatographic conditions

An SSI pre-column filter was connected to a Sepralyte C_{18} (5 cm $\times$ 4.6 mm I.D., 3 μm particle size) HPLC column (Analytichem International, Harbor City, CA, U.S.A.). The mobile phase consisted of 0.05 *M* ammonium dihydrogen phosphate and 0.01 *M* phosphoric acid containing N,N-dimethylformamide (1%, v/v) and methanol (4%, v/v). The column was maintained at 50°C, and a flow-rate of 1.5 ml/min was used. The wavelength used for this analysis was 276 nm, and the detector was set at 0.005 a.u.f.s. with a 0.5-s time constant. The integrator was set to an attenuation of 2.

Analysis of plasma

Stock solutions of theophylline and β-hydroxyethyltheophylline, the internal standard, were prepared in water (1 mg/ml). Working theophylline standard solutions were prepared at 400, 200, 100, 50, 25 and 10 μg/ml, and a working internal standard solution was prepared at 200 μg/ml. This produced equivalent plasma concentrations of 40, 20, 10, 5, 2.5 and 1 μg/ml theophylline and 20 μg/ml internal standard. A charcoal suspension was prepared of neutral Norit charcoal (0.5 g) in water (250 ml).

Frozen blank plasma was thawed at room temperature and centrifuged (10 min at 2000 *g*). An aliquot (1 ml) was taken, and working theophylline standard (100 μl) together with working internal standard (50 μl) were added. An aliquot (2 ml) of the stirred charcoal suspension was added, and the total contents vortex-mixed (15 s). The mixture was centrifuged (15 min at 2000 *g*) and the supernatant discarded. An aliquot (5 ml) of an extraction solvent consisting of propan-2-ol–dichloromethane–diethyl ether (10:65:25, v/v/v) was added to the charcoal residue and vortex-mixed (15 s). The mixture was centrifuged (15 min at 2000 *g*), and the organic supernatant removed and evaporated to dryness under dry nitrogen at 37°C. The residue was dissolved in mobile phase (0.5 ml) and an aliquot (10 μl) injected for HPLC analysis.

Patients' plasma samples were assayed by substituting the blank plasma and using water in place of the theophylline working standard.

Analysis of urine

Working theophylline standards were prepared in water at concentrations of 1000, 500, 250, 100, 50, 25 and 10 μg/ml. This produced equivalent urine concentrations of 100, 50, 25, 10, 5, 2.5 and 1 μg/ml theophylline. The working internal standard solution used was 1 mg/ml.

Frozen blank urine was thawed at 37°C in a shaking water-bath. An aliquot (1 ml) was removed, and working theophylline standard solution (100 μl), working internal standard solution (100 μl), phosphate buffer (100 μl, 1 *M*, pH 7.4) and the extraction solvent (5 ml) used in the plasma extraction were added. The tube was vortex-mixed (15 s) and then centrifuged (15 min at 2000 *g*). The organic layer was removed and evaporated. The residue was dissolved in mobile phase (0.5 ml) and an aliquot (10 μl) injected for HPLC analysis.

Patients' urine samples were assayed using the same analysis scheme by substituting the blank urine and using water in place of the theophylline working standard.

RESULTS AND DISCUSSION

A major problem in the analysis of theophylline has been the resolution of theophylline and 1,7-dimethylxanthine, the primary caffeine metabolite, using reversed-phase HPLC. The caffeine metabolite carried through all of the sample clean-up procedures that our laboratory tried, and the problem, therefore, had to be overcome by chromatographic separation from theophylline. The incorpora-

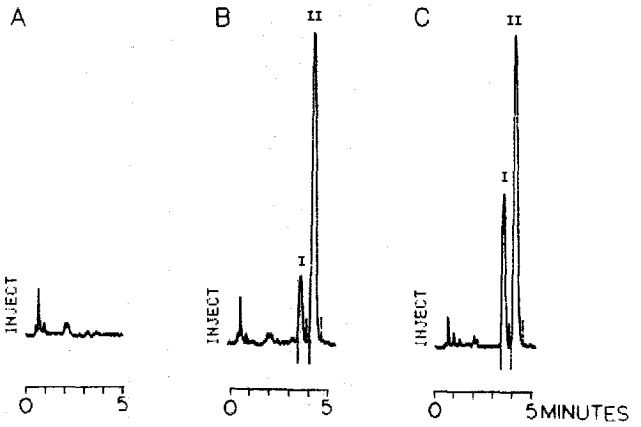

Fig. 1. Typical chromatograms for theophylline in plasma. (A) Blank human plasma; (B) blank plasma containing 1 μg/ml theophylline (I) and 10 μg/ml internal standard (II); (C) patient plasma containing 2.4 μg/ml theophylline and 10 μg/ml internal standard.

tion of dimethylformamide (1%, v/v) into the mobile phase solved the problem and resulted in baseline resolution of theophylline and 1,7-dimethylxanthine.

Clean-up of plasma samples prior to chromatographic analysis involved adsorption of the drug and internal standard onto charcoal followed by desorption with an organic solvent mixture. The solvent was evaporated to dryness and the residue redissolved. The overall recovery rate using this procedure was around 50% for the drug and 70% for the internal standard. The resulting chromatograms were essentially free from endogenous interference, and typical chromatograms are shown in Fig. 1. Theophylline eluted at a retention time of 3.7 min and β-hydroxyethyl theophylline, the internal standard, at 4.3 min. The limit of detection for theophylline in plasma using this procedure was around 0.25 μg/ml (based on a signal-to-noise ratio of 5:1).

Urine samples were extracted directly with the same solvent used for the plasma analysis. This gave adequate clean-up of the urine samples, and the charcoal adsorption step used for plasma analysis was, therefore, unnecessary. The overall recoveries of drug and internal standard were 70 and 60%, respectively. Typical chromatograms from this procedure are shown in Fig. 2 and show very little endogenous interference. The small peak eluting immediately before theophylline in chromatogram C is 1,7-dimethylxanthine, the caffeine metabolite, indicating that this patient had ingested a caffeine-containing product. The baseline resolution of theophylline and 1,7-dimethylxanthine is clearly seen in this chromatogram. The limit of detection for the assay of theophylline in urine was around 1 μg/ml by this method (based on a signal-to-noise ratio of 5:1).

The selectivity of the assay for both plasma and urine samples was confirmed by running pre-dose blank samples. No interfering peaks eluted at the retention times of the drug and internal standard.

Reproducibility of the assay procedures described for plasma and urine was assessed by calculating the inter-day variation for each point on the standard lines. The data are summarized in Table I. For the plasma lines, all values had a

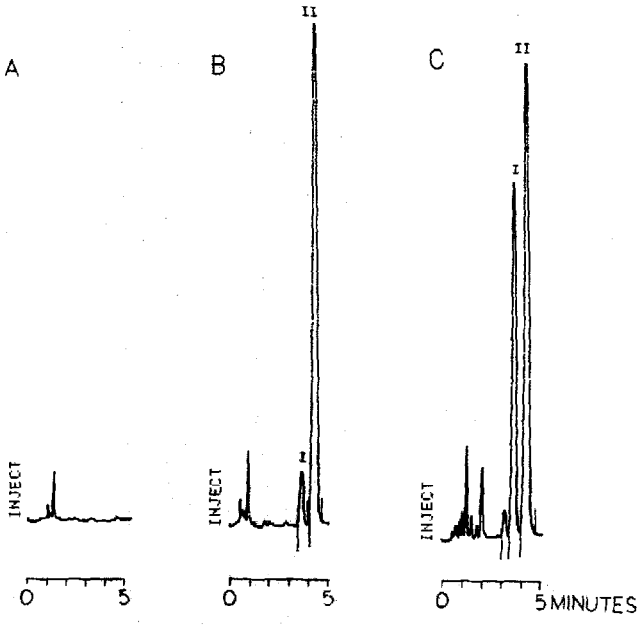

Fig. 2. Typical chromatograms for theophylline in urine. (A) Blank human urine; (B) blank urine containing 5 μg/ml theophylline (I) and 100 μg/ml internal standard (II); (C) patient urine containing 39.2 μg/ml theophylline and 100 μg/ml internal standard.

TABLE I

INTRA-DAY AND INTER-DAY VARIATION OF THEOPHYLLINE IN THE ANALYSIS OF PLASMA AND URINE

Concentration (μg/ml)	Intra-day		Inter-day	
Plasma				
1	0.0390 ± 0.0021	5.4	0.0373 ± 0.0029	7.8
2.5	0.1045 ± 0.0044	4.2	0.1033 ± 0.0064	6.2
5	0.2297 ± 0.0060	2.6	0.2230 ± 0.0075	3.4
10	0.4791 ± 0.0092	1.9	0.4695 ± 0.0096	2.0
20	0.9847 ± 0.0364	3.7	0.9887 ± 0.0328	3.3
Urine				
5	0.0920 ± 0.0075	8.2	0.0825 ± 0.0024	2.9
12.5	0.2209 ± 0.0058	2.6	0.2194 ± 0.0075	3.4
25	0.4427 ± 0.0061	1.4	0.4352 ± 0.0027	0.6
50	0.8875 ± 0.0076	0.9	0.8892 ± 0.0107	1.2
75	1.3545 ± 0.0087	0.6	1.3387 ± 0.0201	1.5
100	1.7984 ± 0.0228	1.3	1.7877 ± 0.0215	1.2

TABLE II

QUALITY-CONTROL DATA FOR THEOPHYLLINE IN PLASMA AND URINE

Sample	Target (μg/ml)	n	Concentration found (mean $\pm$S.D.) (μg/ml)	R.S.D. (%)
Plasma	2.5	22	2.6 ± 0.1246	4.9
	17.5	22	17.9 ± 0.8841	5.0
Urine	12.5	11	12.6 ± 0.3601	2.9
	7.5	11	74.8 ± 1.4379	1.9

relative standard deviation (R.S.D.) of less than 8%, and for the urine standard lines less than 3.3%. The accuracy of the method was checked by preparing quality-control samples in the appropriate biological fluid at low and high points on the standard lines. These samples were frozen and then assayed with patients' samples. Plasma assays were run daily for three weeks and quality-control samples were run on each day of analysis. The overall mean for the low quality-control samples was 2.6 μg/ml (target = 2.5 μg/ml) with a relative standard deviation of 4.9%. The high quality-control sample had a mean of 17.9 μg/ml (target = 17.5 μg/ml) with an R.S.D. of 5%. Urine samples were run for a two-week period, giving corresponding values of 12.6 μg/ml (target = 12.5 μg/ml; R.S.D = 2.9%) and 74.8 μg/ml (target = 75 μg/ml; R.S.D. = 1.9%) for the quality-control samples (Table II).

The linearity of each standard line was confirmed by plotting the drug concentration against ratio of drug/internal standard peak area. Linear regression equations were used to determine the equations of the lines. Correlation coefficients of higher than 0.9998 were consistently achieved. In this particular study the range of the plasma standard line used was 1–20 μg/ml. The method describes the preparation of a standard line up to 40 μg/ml, and linearity is maintained to at least this concentration if required.

In summary, this method overcomes the problem of resolving 1,7-dimethylxanthine from theophylline. The methods of sample preparation for plasma and urine are simple and rapid and produce clean chromatograms. The peak shapes for the drug and internal standard are good and the assay time is less than 5 min. The method has the necessary sensitivity for measuring therapeutic concentrations of theophylline and has been successfully used to assay patients' samples.

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