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TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR QUANTITATION OF THEOPHYLLINE IN PLASMA

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SUMMARY

Two high-performance liquid chromatographic methods are described for the assay of theophylline in plasma. Both allowed the separation of theophylline from the caffeine metabolites, theobromine and 1,7-dimethylxanthine. Method A, using 8-chlorotheophylline as internal stanard, involved back extraction of theophylline from organic extract with 0.1 *M* sodium hydroxide. Method B used generally accepted solvent extraction followed by evaporation and β-hydroxyethyltheophylline as internal standard. High-performance liquid chromatographic analyses were performed on reversed-phase phenyl columns (25 × 0.46 and 25 × 0.41 cm) using 20% methanol in 20 m*M* phosphate buffer at pH 5.6 for Method A and 2% acetonitrile and 8% methanol in 20 m*M* phosphate buffer for Method B. The column effluent was monitored at UV 273 nm.

Standard curves for both Methods A and B were fitted by linear regression ($r > 0.999$) in the concentration range of 0.05—50 μg/ml. Either method was selective, accurate and reproducible over the concentration range 0.08—26 μg/ml. However, compared with Method B, Method A provided significant advantages in terms of simplicity, speed and efficiency.

INTRODUCTION

Theophylline [I; 1,3-dimethylxanthine (Fig. 1)] has been used primarily for the treatment of bronchial asthma, but also apnea of the newborn. Because

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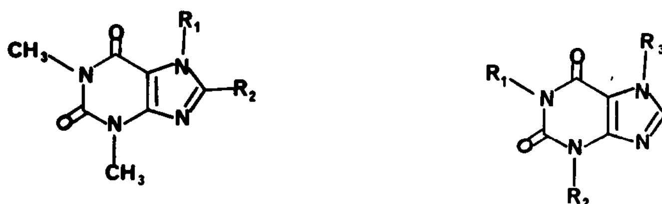

I,	$R_1 = R_2 = H$	Theophylline	IV,	$R_1 = R_2 = R_3 = CH_3$	Caffeine
II,	$R_1 = H$, $R_2 = Cl$		V,	$R_1 = H$, $R_2 = R_3 = CH_3$	Theobromine
III,	$R_1 = CH_2CH_2OH$, $R_2 = H$		VI,	$R_1 = R_3 = CH_3$, $R_2 = H$	1, 7 - Dimethylxanthine

Fig. 1. Chemical structures of theophylline (I), internal standards (II and III) and caffeine metabolites (IV, V and VI).

of the narrow therapeutic range (5—20 μg/ml in plasma) of I [1, 2], it is necessary to monitor its plasma levels by a selective assay method.

A number of high-performance liquid chromatographic (HPLC) methods have been reported for the determination of I in biological fluids. Most analyses by HPLC are developed on an octadecyl reversed-phase column with buffer systems [3—6]. Several methods utilize straight-phase systems [7, 8], ion-pair [9] and ion-exchange [10] chromatography. Of serious concern in the assay of I in plasma is the separation of I from the caffeine (IV) metabolites, theobromine (V) and 1,7-dimethylxanthine (VI) (Fig. 1). Specifically, VI coelutes with I in the most common reversed-phase HPLC system.

Several procedures for the preparation of biological samples have been reported. Extraction with organic solvent followed by evaporation and reconstitution of the residue [4, 6, 11] and dilution of plasma with or without eliminating proteins [3, 5, 10] are generally accepted methods. As multiple samples are analyzed, the first of these methods becomes lengthy while the latter involves special maintenance. Thus, a simple and rapid method for the assay of multiple samples was considered desirable.

Two selective methods for the assay of I in plasma have been developed. Method A is unique because of back extraction with base and has the advantage of avoiding solvent evaporation. Method B is a generally accepted extraction procedure [4, 6, 12] with a newly developed chromatographic system. This report describes two sample preparation procedures, two HPLC methods and the results of the validation study.

EXPERIMENTAL

Materials

Membrane filters (0.45 μm, Rainin Instrument, Woburn, MA, U.S.A.) were used for filtration of the HPLC mobile phase. Disposable extraction columns (Clin-Elut, 1-ml size, Analytichem International, Lawndale, CA, U.S.A.) and disposable polypropylene centrifuge tubes (15 ml, Evergreen Scientific, Los Angeles, CA, U.S.A.) were utilized for extraction of samples. Adjustable microliter pipettes (Pipetman® Model P-200 and P-1000D, Rainin Instrument) were used for the preparation of standards and validation samples.

Theophylline (I), 8-chlorotheophylline (II), and IV were purchased from Aldrich (Milwaukee, WI, U.S.A.). β-Hydroxyethyltheophylline (III), V and VI were obtained from Sigma (St. Louis, MO, U.S.A.). All chemicals used were analytical grade and the chromatographic solvents used were HPLC grade.

Apparatus

A modular high-performance liquid chromatograph was assembled consisting of a pump (Model 45, Waters Assoc., Milford, MA, U.S.A.), an autosample injector (WISP® Model 710B, Waters Assoc.), a variable-wavelength UV spectrophotometer (DuPont, Wilmington, DE, U.S.A.), a recorder (Omniscribe® B-5000 strip chart recorder, Houston Instruments, Austin, TX, U.S.A.), and a power controller (Model 211, Autochrom, Milford, MA, U.S.A.). Stainless-steel columns (25 × 0.46 cm I.D. and 25 × 0.41 cm I.D.) packed with 5-μm Spherisorb® phenyl (Deeside Industrial Estate, Lwyd, U.K.; Hauppauge, NY, U.S.A.) at 8000 p.s.i. (550 bar) were used for all analyses. A laboratory data system (Model 3352, Hewlett-Packard, Avondale, PA, U.S.A.) was used for quantitation and identification of chromatographic peaks. A pH meter (Accumet® Model 610A, Fisher Scientific, Pittsburgh, PA, U.S.A.) was used to adjust the pH of the HPLC mobile phase. A rugged rotator (Model PD-250, Glas-Col Apparatus, Terre Haute, IN, U.S.A.) was used for rotary mixing. A dry heat bath system (SC-3 sample concentrator, Tecam® Dri-Block DB-3, Princeton, NJ, U.S.A.) was used in Method B to evaporate the solvent of organic extracts of plasma samples.

Preparation of standard solutions

A stock standard solution was prepared by dissolving 10 mg of I in 10 ml of deionized water. This stock solution (50 μg per 50 μl) was serially diluted with deionized water to prepare the following standard solutions: 10, 5, 2.5, 1.25, 0.25 and 0.05 μg per 50 μl of I.

Preparation of plasma standards

Duplicate plasma standards were prepared by spiking human control plasma (0.2 ml) with an adequate volume (10—100 μl) of the standard solutions to produce the following concentrations: 50, 25, 12.5, 2.5, 0.5, 0.1 and 0.05 μg/ml. On each day of sample analysis, the duplicate plasma standards were prepared and analyzed concomitantly with the samples.

Preparation of validation samples

Validation samples at six different concentrations (0.08, 0.5, 1.8, 9, 17 and 26 μg/ml) were prepared by diluting a small volume (144—260 μl) of the theophylline standard solutions to 2 ml with control human plasma. For each method, two sets of triplicate samples at each concentration were prepared by pipetting 0.2 ml of each sample into coded tubes. One set of the samples was analyzed upon preparation, and the other set was kept frozen in a laboratory freezer for two weeks prior to analysis.

Extraction procedures

Method A. To 0.2 ml of plasma were added 0.1 ml of a solution of the internal standard (1 mg per 100 ml of II in water) and one drop of 1 *M* hydro-

chloric acid to adjust the mixture to pH 2—3. After vortexing, the samples were extracted with 3 ml of 10% isopropanol in chloroform by rotomixing for 20 min and centrifuged. The upper layers were carefully removed with a pipet and discarded. Approximately 2.8 ml of each of the organic layers was back extracted with 0.2 ml of 0.1 *M* sodium hydroxide by rotomixing for 20 min. After centrifugation, the basic aqueous layers were withdrawn and 20 μl of each were analyzed by HPLC as described in Method A under Chromatographic conditions.

Method B. To 0.2 ml of plasma were added 0.2 ml of a solution of the internal standard (0.5 mg per 100 ml of III in water). The mixture was vortexed and transferred to extraction columns by pipet. The columns were eluted with 4 ml of 10% isopropanol in chloroform. Approximately 3 ml of each of the organic eluents was evaporated to dryness with the aid of a nitrogen stream. Each of the dried residues was dissolved in 0.2 ml of HPLC mobile phase. Aliquots of the reconstituted solutions were analyzed by HPLC as described in Method B under Chromatographic conditions.

Chromatographic conditions

Method A. The column used was 5-μm phenyl, 25 × 0.46 cm I.D. The mobile phase was 20% methanol in 20 m*M* potassium phosphate (monobasic) buffer and the mixture was adjusted to pH 5.6 with 8% phosphoric acid. The flow-rate was 1.5 ml/min and the UV detector was used at 273 nm, the absorbance at 0.02 a.u.f.s. and the pressure used was 2500—3000 p.s.i. (172—206 bar). A typical retention time of the internal standard (II) was 3.8 min and of I was 5.5 min.

Method B. The column used was 5-μm phenyl, 25 × 0.41 cm I.D. The mobile phase was 2% acetonitrile and 8% methanol in 20 m*M* potassium phosphate (monobasic) buffer (pH ca. 5), the pH of the mixture was not adjusted. The flow-rate was 1.8 ml/min. A typical retention time of I was 6.0 min and of the internal standard (III) was 7.1 min. Detector, absorbance and pressure were the same as described in Method A.

Extraction efficiency

The recoveries by either extraction Method A or B were determined by comparing the peak heights of extracted plasma standards (minus any theophylline contribution from the control) with those of unextracted standards. Duplicate unextracted standards were prepared at the same concentrations as plasma standards by diluting aqueous standard and internal standard solutions with water to 0.2 ml total volume.

Quantitation

The peak height ratios of I to the internal standard were obtained from the plasma standards with the aid of a laboratory data system. The ratios were analyzed by linear regression with respect to their concentrations in the plasma standards. The concentrations of I in the validation samples were determined by inverse predication from the linear regression of the standards. The minimum quantifiable level (MQL) was determined by the linear regression of the three lower concentration standards.

Reproducibility

Reproducibility of the methods was evaluated by repeated assay of daily prepared plasma standards in the concentration range 0.05—50 μg/ml.

RESULTS AND DISCUSSION

High-performance liquid chromatography

The primary requirement of the chromatographic system was the separation of I from the other caffeine metabolites (V and VI) in plasma. Typical chromatograms of the extracts of control and spiked plasma by Methods A and B are shown in Figs. 2 and 3, respectively. Complete peak resolution of the three compounds and the internal standard was achieved using either of the two described HPLC systems. The concentration of caffeine (IV) and its xanthine metabolites (I, V and VI) is varied in the plasma obtained from uncontrolled caffeine intake subjects.

A 5-μm phenyl column and methanol—phosphate buffer as mobile phase were used in the HPLC systems for Methods A and B. For Method A, careful pH adjustment of the mobile phase is required to assure baseline separation of I from the internal standard (II) and the potential interferences. The retention time of II was markedly dependent on mobile phase pH. However,

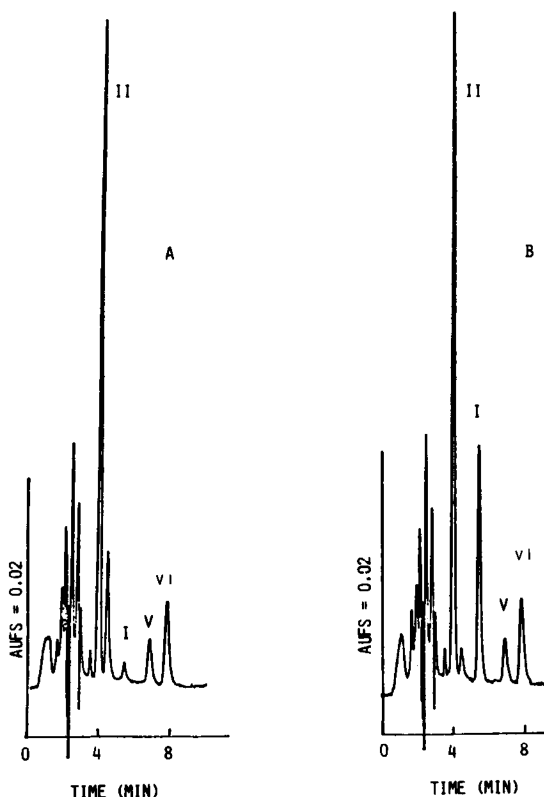

Fig. 2. Chromatograms of plasma extracts by method A. (A) Control plasma from subjects with uncontrolled caffeine intake; (B) 1.8 μg/ml theophylline spiked plasma.

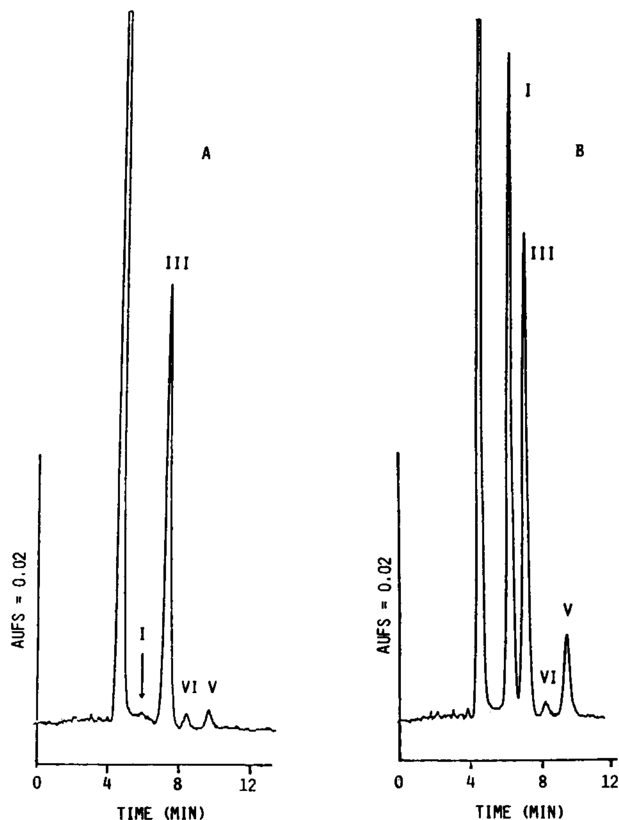

Fig. 3. Chromatograms of plasma extracts by method B. (A) Control plasma from subjects with uncontrolled caffeine intake (small amount of caffeine was detected at t_R ca. 16 min); (B) 9.0 μg/ml theophylline spiked plasma.

for Method B, pH adjustment of the mobile phase was not necessary. A small percentage of acetonitrile in the mobile phase resulted in good resolution of the drug from the interference.

Extraction and recovery

For Methods A and B, 10% isopropanol in chloroform was used as an extracting solvent. For Method A, the organic extract was back extracted with 0.1 *M* sodium hydroxide and for Method B, the organic extract was concentrated with the aid of a nitrogen stream. With either of the described extraction procedures, the overall recovery was about 83% in the concentration range 0.1—50 μg/ml. For internal standards at the concentrations used, the recoveries were about 82% for II and 73% for III.

Compounds I and II in the organic extract were quantitatively extracted with the base, due to the weak acidic hydrogen at the 7-position of their structures. However, because of its neutral character, IV was not extracted with the base (Fig. 4A); therefore, the analysis time for each sample by HPLC could be reduced. When using Method A, the extraction efficiency of I was the same at both pH 3 and pH 7, while the extraction efficiency of II was increased two-fold at pH 3. Thus, for Method A, the plasma was extracted in acidic medium.

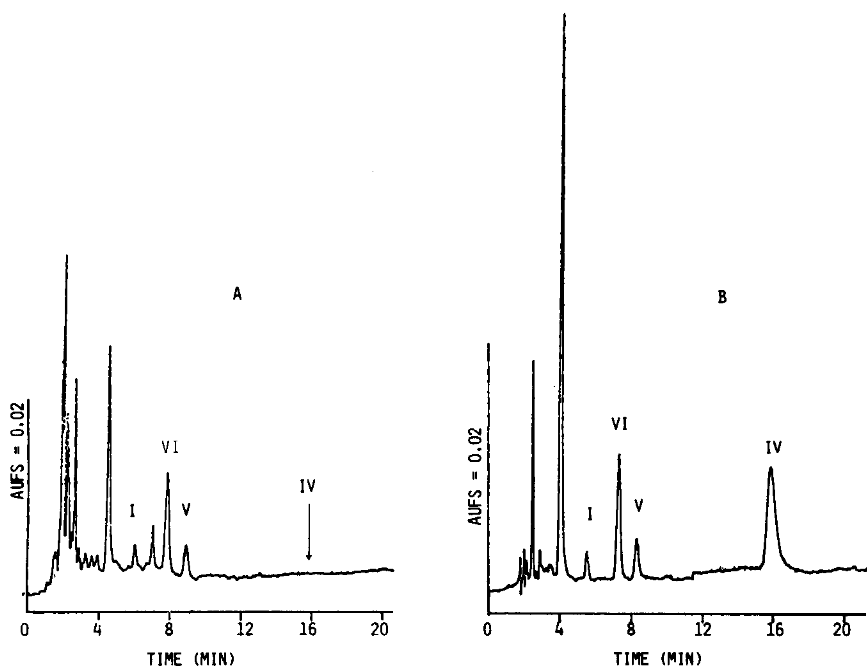

Fig. 4. Chromatograms of the extracts of control plasma from subjects with uncontrolled caffeine intake (Method B chromatographic conditions were used). (A) Extracted by Method A procedure; (B) extracted by Method B procedure.

TABLE I

SUMMARY OF DATA FROM ASSAY OF I IN PLASMA — CONCENTRATION (μg/ml)

Nominal concn.	Found concn. in fresh samples			Found concn. in frozen samples		
	Mean ± S.D. (n = 3)	R.S.D. (%)	Mean percent difference*	Mean ± S.D. (n = 3)	R.S.D. (%)	Mean percent difference
Method A						
0.08	0.07 ± 0.01	14.3	−12.5	0.08 ± 0.01	12.5	0.0
0.5	0.52 ± 0.01	1.9	+4.0	0.51 ± 0.01	2.0	+2.0
1.8	1.82 ± 0.00	0.0	+1.1	1.83 ± 0.03	1.6	+1.7
9.0	9.45 ± 0.04	0.4	+5.0	9.31 ± 0.12	1.3	+3.4
17.0	17.63 ± 0.76	4.3	+3.7	17.75 ± 0.33	1.9	+4.4
26.0	27.08 ± 0.32	1.2	+4.2	26.08 ± 0.49	1.9	+0.3
Method B						
0.08	<MQL**			0.08 ± 0.01	12.5	0.0
0.5	0.48 ± 0.02	4.2	−4.0	0.48 ± 0.01	4.3	−4.0
1.8	1.79 ± 0.07	3.9	−0.6	1.75 ± 0.03	1.7	−2.8
9.0	9.45 ± 0.17	1.8	+5.0	9.15 ± 0.33	3.6	+1.7
17.6	17.06 ± 0.70	0.4	+0.3	17.12 ± 0.42	2.5	+0.7
26.0	26.11 ± 0.31	1.2	+0.4	25.16 ± 0.63	2.5	−3.2

*Mean percent difference = $\frac{\text{mean} - \text{nominal}}{\text{nominal}} \times 100$.

**MQL (minimum quantifiable levels) = 0.07 ± 0.04 μg/ml (n = 3).

Quantitation and assay characteristics

The peak height ratios of I to the internal standard versus concentration in the plasma standards were fitted by linear regression ($r > 0.999$, concentration range 0.05—50 μg/ml). For the quantitation of unknown samples, two standard curves were used: the low standard curve (0–2.5 μg/ml) for concentrations less than 2.5 μg/ml and the full standard curve (0—30 μg/ml) for concentrations between 2.5 and 30 μg/ml. Mean MQL was 0.07 ± 0.05 μg/ml (n = 6) for Method A and 0.07 ± 0.04 μg/ml (n = 2) for Method B.

The precision and accuracy of the methods were evaluated by repeated analyses of fresh and frozen validation samples at each concentration. The validation data for Methods A and B are summarized in Table I. For both Methods A and B, the precision of the assays, expressed as relative standard deviation (R.S.D.), ranged from 0% to 14.3%, and the accuracy defined by the ranges of the mean percent difference from the nominal levels varied from −12.5% to +5% over the concentration range of 0.08—26.0 μg/ml. These results show the equivalence of the two assays and the stability of I to freezing.

Reproducibility, tested by repetitive assay of daily prepared plasma standards, is presented in Table II. Because Method B used a generally accepted procedure, the reproducibility of the method was not elaborated. The narrow range of correlation coefficients, slopes and y-intercepts for low and full standard curves shows inter-day reproducibility of the methods.

TABLE II

STANDARD CURVE SUMMARIES

Standard curve No.	Low standard curve (0—2.5 μg/ml)			Full standard curve (0–50 μg/ml)		
	Slope	y intercept	Correlation coefficient	Slope	y intercept	Correlation coefficient
Method A						
1	0.139	0.016	0.9984	0.124	0.038	0.9997
2	0.156	0.029	0.9996	0.138	0.063	0.9996
3	0.142	0.021	0.9948	0.127	0.046	0.9998
4	0.140	0.021	0.9996	0.129	0.052	0.9997
5	0.141	0.026	0.9984	0.123	0.057	0.9996
6	0.142	0.031	0.9998	0.132	0.073	0.9994
Mean (n = 6)	0.143	0.024	0.9984	0.129	0.055	0.9996
S.D.	0.006	0.005	0.0017	0.005	0.012	0.0001
Relative S.D. (%)	4.2	20.8	0.2	3.9	21.8	0.01
Method B						
1	0.300	0.030	0.9998	0.288	0.055	0.9999
2*	0.151	0.008	0.9982	0.136	0.040	0.9996
Mean (n = 2)			0.9990			0.9998
S.D.			0.0008			0.0002
Relative S.D. (%)			0.1			0.02

*The concentration of the internal standard was one-half of that of standard curve 1. Therefore, means of slopes and y intercepts were not calculated.

CONCLUSION

Two selective methods for the assay of I in plasma have been developed and validated with both fresh and frozen spiked samples. Method A involved back extraction of I in organic extract with 0.1 *M* sodium hydroxide while Method B used generally accepted solvent extraction followed by evaporation. The validation study showed that both methods were equivalently accurate, selective and reproducible. However, compared with Method B, Method A provided significant advantages in terms of speed and efficiency.

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