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MICROASSAY FOR THE SIMULTANEOUS DETERMINATION OF THEOPHYLLINE AND DYPHYLLINE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An improved and simplified high-performance liquid chromatographic method is presented for the simultaneous determination of theophylline and dyphylline in serum. The internal standard, β-hydroxyethyltheophylline (20 μl of a 100 μg/ml solution) is added to 50 μl of serum. Serum proteins are precipitated by the addition of 30 μl of 40% trichloroacetic acid solution. The mobile phase consists of a sodium acetate buffer with 6% acetonitrile. Chromatogram run time is 6 min. The sensitivity limit for both compounds is 0.25 μg/ml. This method is interference-free from the major metabolites of theophylline and other drugs commonly coadministered with theophylline.

INTRODUCTION

Dyphylline [7-(2,3-dihydroxypropyl)theophylline] has pharmacologic properties similar to theophylline and therefore may be used in the treatment of reversible bronchoconstrictive disease. At present, the therapeutic effect of these two drugs appears to be dependent upon their concentration in the blood. There is some evidence that a combined preparation of dyphylline and theophylline may result in less frequent adverse side-effects than an equivalent dose of theophylline alone [1,2].

Although guidelines for monitoring of dyphylline during therapy have not yet been developed, it is expected that a serum therapeutic range of the drug can be established in similar situations as proposed for theophylline [3]. A number of assays have been developed using high-performance liquid chromatography (HPLC) for theophylline [4–15] and dyphylline [16–25]. However, few procedures have been developed to quantitate both drugs simultaneously [13,22,24,25].

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These methods require long and complicated sample preparation and long chromatogram run times.

This prompted us to develop an HPLC method for the quantitation of both theophylline and dyphylline simultaneously in serum which utilizes a small sample volume, has a short chromatogram run time and requires a short and simple sample preparation.

EXPERIMENTAL

Apparatus

A U6K injector and an M45 pump system were used in conjunction with an M440 absorbance detector set at 280 nm (Waters Assoc., Milford, MA, U.S.A.). A C_{18} pre-column cartridge was used in conjunction with a Nova-Pak C_{18} Radial-Pak cartridge (100 mm×5 mm I.D., 4 μm particle packing, Waters Assoc.) for the chromatographic separation.

Reagents and standards

The sources of the compounds used to make the standards and internal standard are as follows: theophylline (Fisher Scientific, King of Prussia, PA, U.S.A.), 7-(2,3-dihydroxypropyl)theophylline (dyphylline, Aldrich, Milwaukee, WI, U.S.A.) and β-hydroxyethyltheophylline (BHET) (Sigma, St. Louis, MO, U.S.A.). Sodium acetate and acetonitrile (Fisher Scientific) were of HPLC grade. Glacial acetic acid and 40% trichloroacetic acid solution were also obtained from Fisher Scientific.

The internal standard stock solution was prepared by diluting 100 mg of BHET in 100 ml of distilled water. This stock solution was further diluted 1:10 with distilled water to yield the desired concentration of 100 μg/ml. Aliquots of the internal standards were stored at −20 °C and remained stable for approximately six months.

Xanthine-free serum was obtained from normal volunteers who had abstained from all foods and beverages containing caffeine and other xanthines for 48 h. The standards were made by dissolving 100 mg of theophylline and 100 mg of dyphylline in 100 ml of xanthine-free serum. This stock solution was further diluted with xanthine-free serum to obtain nine serum standards and four quality controls in the concentrations listed in Table II. A xanthine-free serum sample was used as 0.0 μg/ml. Standards and patient samples were also stored at −20 °C.

Extraction procedure

Blood samples were centrifuged for 5 min at 1380 *g* to separate the serum from the whole blood. The serum was harvested with a Pasteur pipet and transferred to a 12×75 mm labeled test tube. Into a microcentrifuge tube were placed 20 μl of the internal standard solution and 50 μl of the serum sample. Serum proteins were precipitated by the addition of 30 μl of 40% trichloroacetic acid solution added to the serum and internal standard. The mixture was vortexed for 30 s.

After centrifugation of the sample in a microfuge at 15 000 *g* for 4 min, the supernatant was transferred to a second labeled microcentrifuge tube and cen-

trifuged for 1 min. A 15-μl aliquot of this sample was injected directly onto the chromatographic system.

Chromatographic conditions

The mobile phase consisted of 0.01 *M* sodium acetate buffer with 6% acetonitrile. After lowering the pH to 4.0 with glacial acetic acid, the mobile phase was magnetically stirred and aspirated under a vacuum until degassed.

A flow-rate of 2.3 ml/min was used at a pressure of 12 MPa. The system was at ambient temperature. Detector sensitivity was set at 0.2 absorbance units. The chart speed was 4 mm/min. The total run time selected was 6 min. The concentrations of theophylline and dyphylline in the quality controls and patient samples were calculated using the peak-area ratios of theophylline and dyphylline and the use of the line of best fit describing the relationship between peak-area ratio and concentration for the serum standards.

Extraction recovery

The assay recovery of theophylline, dyphylline and the internal standard was assessed at 40, 20 and 5 μg/ml each of theophylline and dyphylline. Five replicates of each of the three standards were extracted and injected. Five injections of each of the three standards prepared in aqueous solutions were directly injected. The assay recovery at each concentration was computed using the following equation: recovery = (peak area extract)/(mean peak area direct injection) $\times$ 100%.

RESULTS

The chromatogram resulting from the injection of an extract of a xanthine-free serum sample obtained from a volunteer who had abstained from all xanthine-containing foods and beverages for 48 h is shown in Fig. 1A. Fig. 1B illustrates the response to injection of 20 μg/ml theophylline, 20 μg/ml dyphylline and 100 μg/ml BHET. The retention times of theophylline, dyphylline and BHET were 3.34, 4.08 and 4.83 min, respectively. Comparison of Fig. 1A and Fig. 1B illustrates the lack of interference from endogenous components of serum with this assay. A chromatogram of a patient serum sample is presented in Fig. 2.

Each standard curve showed excellent linearity (mean $r^2 \geq 0.9996$) over the range of concentrations examined.

The extraction recoveries of theophylline and dyphylline are presented in Table I. The sensitivity limits for theophylline and dyphylline were found to be 0.25 μg/ml based on a signal-to-noise ratio of 2.5. The assay recovery ranged from 97.0 to 99.4%. Recovery of the internal standard was calculated to be 99%.

The inter-day variability of the assay over five consecutive days is presented for the standards and quality controls in Table II. The intra-day variability for the quality controls is presented in Table III. All inter- and intra-day coefficients of variation (C.V.) were less than 4.00%.

Drugs commonly administered with theophylline or dyphylline, as well as the major metabolites of theophylline, were tested for interference using the retention times of the compounds. These compounds included: acetaminophen, aspi-

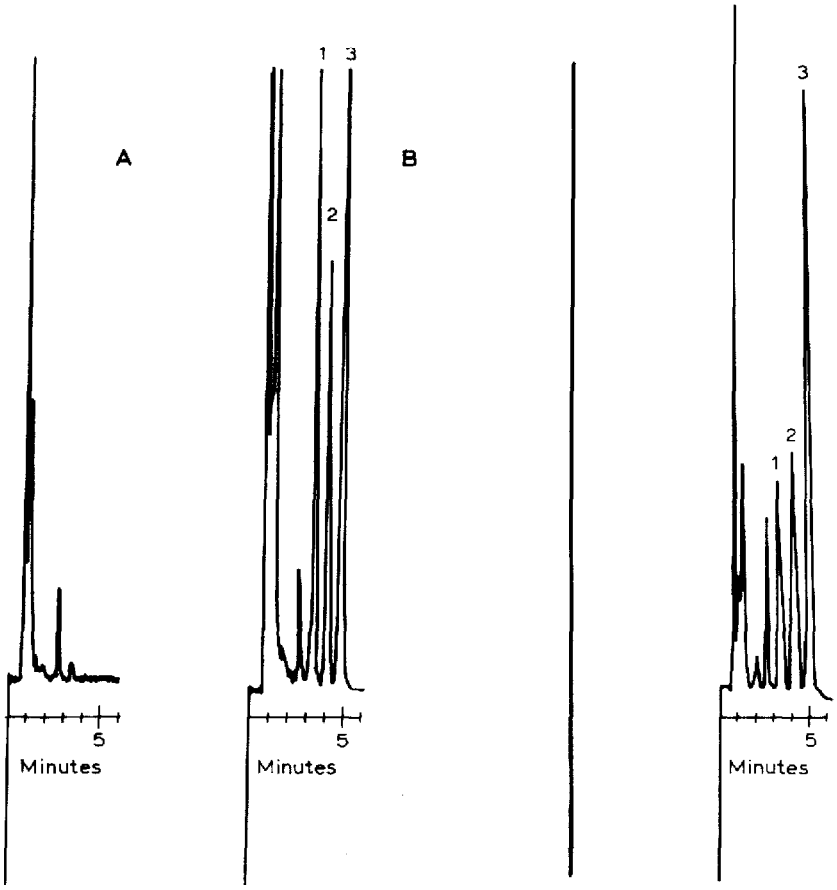

Fig. 1. (A) Chromatogram resulting from the injection of a xanthine-free serum sample obtained from a volunteer who had abstained from all xanthine containing foods and beverages for 48 h. (B) Chromatogram resulting from the injection of standard 4 using the following peak indentifications: 1 = theophylline (20 μg/ml); 2 = dyphylline (20 μg/ml); 3 = BHET (100 μg/ml).

Fig. 2. Chromatogram of serum sample of patient T.R. following dosing with theophylline and dyphylline using the following peak identifications: 1 = theophylline; 2 = dyphylline; 3 = BHET.

TABLE I

EXTRACTION RECOVERY OF THE ASSAY

Concentration (μg/ml)	Recovery (mean ± S.D., $n = 5$) (%)	
	Theophylline	Dyphylline
40.00	98.9 ± 0.8	97.0 ± 1.2
20.00	99.4 ± 0.9	97.3 ± 1.6
5.00	99.0 ± 1.2	97.1 ± 1.0

TABLE II

INTER-DAY VARIABILITY OF THE ASSAY

Concentration added (μg/ml)	Theophylline		Dyphylline	
	Found (mean ± S.D.) (μg/ml)	C.V. (%)	Found (mean ± S.D.) (μg/ml)	C.V. (%)
Standards (n=5)				
60.00	59.96 ± 0.11	0.180	60.02 ± 0.17	0.280
40.00	40.00 ± 0.18	0.450	40.06 ± 0.15	0.370
30.00	30.08 ± 0.09	0.300	29.86 ± 0.41	1.370
20.00	20.05 ± 0.09	0.450	19.94 ± 0.09	0.450
10.00	10.01 ± 0.01	0.100	10.01 ± 0.03	0.300
5.00	5.00 ± 0.10	0.005	5.02 ± 0.02	0.400
1.00	1.00 ± 0.50	0.005	0.99 ± 0.01	1.010
0.50	0.50 ± 0.006	1.200	0.50 ± 0.02	4.000
0.25	0.25 ± 0.007	2.800	0.25 ± 0.01	4.000
0.00	0.00 ± 0.000	0.000	0.00 ± 0.00	0.000
Quality controls (n=25)				
50.00	50.08 ± 0.92	1.84	49.97 ± 0.17	0.34
25.00	24.89 ± 0.31	1.25	25.06 ± 0.15	0.60
7.50	7.51 ± 0.07	0.93	7.53 ± 0.12	1.59
0.75	0.75 ± 0.02	2.67	0.75 ± 0.02	2.67

rin, trimethoprim and sulfamethoxazole (Bactrim), caffeine, 8-chlorotheophylline, 1,3-dimethyluric acid, 1,7-dimethylxanthine, erythromycin, 3-methylxanthine, 1-methyluric acid, prednisolone, prednisone, metaproterenol, terbutaline and chlorpheniramine maleate.

DISCUSSION

Many assays have been developed to quantitate theophylline [4–15] and dyphylline [16–25] in serum using HPLC. However, few procedures have been developed to quantitate both drugs simultaneously [13,22,24,25].

A procedure presented by Maijub and Stafford [16] is able to quantitate both drugs simultaneously. However, this procedure requires silica column deactivation and extraction from plasma with the recovery of dyphylline only being 65%. A method proposed by Valia et al. [22], while very similar to the method proposed herein, requires a large sample volume (1 ml), has a longer chromatogram run time (12 min) and is less sensitive. This method also requires a long sample preparation time (30 min for one sample). Ou and Frawley [25] proposed a method for the quantitation of eight different compounds determined with a single chromatographic assay. This method utilizes organic phase extraction followed by evaporation under a nitrogen stream. Dyphylline was co-eluted with theophylline, which renders this method inadequate for the quantitation of both drugs simultaneously. A method proposed by Wenk et al. [24] requires a large

TABLE III

INTRA-DAY VARIABILITY OF THE ASSAY

Day	Concentration added (μg/ml)	Theophylline ($n=5$)			Dyphylline ($n=5$)		
		Found (mean ± S.D.) (μg/ml)		C.V. (%)	Found (mean ± S.D.) (μg/ml)		C.V. (%)
1	50.00	50.31	1.98	3.94	50.01	0.13	0.26
	25.00	24.94	0.20	0.80	25.06	0.11	0.44
	7.50	7.51	0.08	1.07	7.49	0.07	0.93
	0.75	0.75	0.02	2.67	0.74	0.02	2.70
2	50.00	49.98	0.14	0.28	50.03	0.14	0.28
	25.00	24.89	0.29	1.17	24.98	0.14	0.56
	7.50	7.56	0.15	1.98	7.49	0.02	0.27
	0.75	0.75	0.02	2.67	0.73	0.01	1.37
3	50.00	50.25	0.20	0.40	49.79	0.11	0.22
	25.00	24.46	0.20	0.82	25.12	0.10	0.40
	7.50	7.54	0.09	1.19	7.52	0.07	0.93
	0.75	0.75	0.02	2.70	0.76	0.01	1.32
4	50.00	49.87	0.21	0.42	49.95	0.26	0.52
	25.00	25.04	0.19	0.76	25.18	0.17	0.68
	7.50	7.42	0.54	0.04	7.48	0.08	1.07
	0.75	0.75	0.01	1.33	0.75	0.01	1.33
5	50.00	49.96	0.16	0.32	49.86	0.10	0.20
	25.00	25.12	0.18	0.72	24.96	0.07	0.23
	7.50	7.53	0.14	1.86	7.55	0.07	0.93
	0.75	0.76	0.02	2.63	0.75	0.03	4.00

sample volume (0.5 ml), a long sample preparation and long chromatogram run time (16 min). This method does not give complete separation of theophylline from 1,7-dimethylxanthine.

In summary, a fast and easy HPLC method for the simultaneous determination of theophylline and dyphylline in serum is presented. This method requires a small sample volume, short sample preparation and a short chromatogram run time. No interferences are seen with any major metabolites of theophylline or drugs commonly coadministered with theophylline or dyphylline.

The small sample volume and sensitivity limit of 0.25 μg/ml makes this assay an excellent choice for the performance of pharmacokinetic studies and in the therapeutic monitoring of pediatric patients.

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