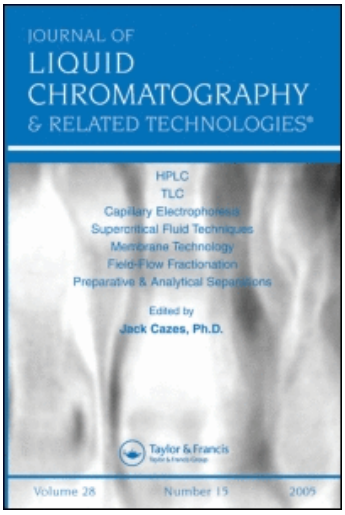

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Steven H. Y. Wongl[a]; Nemat Marzoukl[a]; Suzanne L. McHugh[b]; Elliot Cazesl[a]
[a] Drug Analysis Division Department of Laboratory Medicine, University of Connecticut School of Medicine, Farmington, Connecticut [b] IBM Instruments, Inc., Danbury, Connecticut

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SIMULTANEOUS DETERMINATION OF THEOPHYLLINE AND CAFFEINE BY REVERSED PHASE LIQUID CHROMATOGRAPHY USING PHENYL COLUMN*+

Steven H. Y. Wong[1], Nemat Marzouk[1], Suzanne L. McHugh[2], and Elliot Cazes[1]
[1]*Drug Analysis Division*
Department of Laboratory Medicine
University of Connecticut School of Medicine
Farmington, Connecticut 06032
[2]*IBM Instruments, Inc.*
Danbury, Connecticut 06810

ABSTRACT

Phenyl, C-8, and C-18 columns were evaluated for the simultaneous analysis of theophylline (TH) and caffeine (CA). The phenyl column was chosen for its capability of resolving of 1,7-dimethylxanthine from theophylline. The procedure utilized protein precipitation of either 50 or 100 ul of serum with diluted trichloroacetic acid, followed by analysis with either a manual isocratic or automated gradient elution mode. Chromatographic conditions were: column = phenyl (4.5 x 150 mm with a 4.5 x 50 mm guard column), mobile phase = phosphate or acetate/ACN, and detection wavelength = 280 nm. Peak height or area ratios of TH or CA to the internal standard, beta-hydroxyethyl theophylline (βHET) were linearly correlated to concentration ranges between 2.5 to 20 or 50 mg/L. Precision studies

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+IBM Instruments Liquid Chromatograph has not been certified for clinical applications.

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of "quality control" samples showed that the day-to-day coefficients of variation were less than 9% for both TH and CA. TH plasma concentrations of patients quantitated by this procedure were compared to the clinically accepted assay by FPIA using either polyclonal or monoclonal antibodies. Results were closely correlated. CA concentrations of these "patients" ranged from 0.1 to 5 mg/L. The present study showed that the phenyl column exhibited long-term stability, suitable for clinical application of drug monitoring. Further, the small sample size of 50 ul would be useful for neonatal monitoring of TH and CA.

INTRODUCTION

Theophylline, 1,3 dimethylxanthine, has been used effectively for the treatment of bronchial asthma and apnea in neonates (1-2). Theophylline may enhance the contraction of diaphragm and other respiratory muscles, leading to long-lasting improvement in alleviating diaphragmatic fatigue (3). In children, diet, as an environmental factor, significantly affects theophylline metabolism (4). High protein diets shorten the elimination half-life, while low protein diets increase it. Recent studies showed that caffeine may be preferred to theophylline for the treatment of neonatal apnea (5,6). According to Borg, et al. (5), caffeine is effective due to the potent CNS stimulation, the wide therapeutic index, and the long elimination half-life of about 100 hours, which decreases monitoring frequency. Since caffeine accumulation may be substantial during theophylline therapy, theophylline and caffeine plasma concentrations would be useful in correlating with response. More recently, caffeine was found to be effective for the treatment of asthma (7). With the caffeine dose of 10 mg per kilogram, the mean peak serum concentration is about 14 mg/L, inducing bronchodilator activity, corresponding to a theophylline dose of 5 mg per kilogram, with a mean peak serum of theophylline concentration of 8 mg/L.

In adults, theophylline is demethylated and oxidized to 3 methylxanthine, 1 methyluric acid and 1,3-dimethyluric acid (8). In neonates, theophylline is N-7-methylated to a major metabolite, caffeine, 1,3,7-trimethylxanthine, possibly due to a "not well-developed" cytochrom P-450 system (2,6,7,9,10). Thus, these recent findings indicate the possible need for monitoring of theophylline and/or caffeine, especially for neonates due to their different metabolism.

Clinical monitoring of theophylline, as in the the majority of drugs monitored in clinical laboratories, is mostly performed by automated immunoassays, and less extensively by liquid chromatography (LC) (11). The immunoassays are based on competitive binding with either polyclonal or monoclonal antibodies for enhanced specificity. The popular ones are enzyme multiplied immunoassay techniques (EMIT) by Syva (Palo Alto, CA 94303), fluorescence polarization immunoassay (FPIA) by an autoanalyzer TDx (Abbott Labs, North Chicago, IL 60064), and a dry phase homogeneous immunoassay (ARIS) (Ames, Elkhart, IN 46515). The last is a recently introduced reagent strip technique that can be used to quantitate TH concentrations in an office setting. These techniques are fast - 10-20 minutes - and reliable, but more expensive than the LC assay. Caffeine immunoassay by EMIT had been evaluated (6). Inherent with immunoassays, cross-reactivity can be significant, especially in the monitoring of uremic patient serum. LC assays, however, can often circumvent this problem (6,11-17).

Liquid chromatographic assays of theophylline have been recently reviewed by Christensen and Neims (1). Orcutt, et al., established a micro-method (12). Ou, et al.(17) described a concurrent theophylline and caffeine assay by LC, and later compared it to a EMIT caffeine assay (6). Kabra and Marton (18) quantitated theophylline in 70 seconds. O'Connell and Zurzola (19) described a LC assay of caffeine for bioavailability studies using 130 mg doses, resulting in peak level of 3.6 mg/L in less than 40 minutes. Stavric (20) measured CA and its metabolites in an animal model. The majority of these analyses were performed with reversed phase C-18 or C-8 columns (1,12,17-20).

From our previous experience with tricyclic antidepressants analysis with phenyl column using n-nonylamine/phosphate/ acetonitrile as mobile phase (21), the present study continued to examine the application of the phenyl column in reversed phase mode for assaying theophylline and caffeine with possible adaptation for neonatal monitoring. The assay utilizes a simple preparation by protein precipitation of serum using trichloroacetic acid, followed by analysis with a reversed-phase phenyl column with phosphate or acetate and acetonitrile as mobile phase. The preliminary data were presented elsewhere.*

MATERIALS

Reagents

Acetonitrile and methanol were ultraviolet grade, distilled in glass, purchased from Burdick and Jackson Labs (Muskegon, MI 49442). Trichloroacetic acid and potassium dihydrogen phosphate were "J.T.

Baker-Analyzed" grade from J.T. Baker Chemical Co. (Phillipsburg, NJ 08856). Theophylline, beta-hydroxyethyl theophylline and caffeine were obtained from Sigma Chemical Co. (St. Louis, MO 63178).

Standards

Theophylline (TH), caffeine (CA), and beta-hydroxyethyl theophylline (IS) stock solutions were prepared by dissolving 10 mg each with water in three separate 10 ml volumetric flasks. Aliquots of the TH & CA stock solutions were transferred to five, 50 ml volumetric flasks and mixed with "drug-free" serum to yield the following working standards: 0, 2.5, 5.0, 10.0, 20.0 mg/L. ("Drug-free" serum was checked for non-interference with TH by extracting the chosen serum and analyzing as described later on.) Since CA would most likely be present in small quantities, peak height ratio subtraction was performed as described later in quantitation section. "Quality control" samples, 10 mg/L, were prepared similarly. These samples were frozen until analysis.

Mobile Phase

The mobile phase for the isocratic mode consisted of 0.1M phosphate (pH = 4.0) and ACN (95:5). The phosphate solution was prepared by adding 27.36 gm of potassium dihydrogen phosphate to 2 L of doubled distilled water, adding phosphoric acid to adjust to pH = 4.0. The solution was filtered and kept refrigerated at $4^{o}C$. For analysis, the phosphate and acetonitrile were degassed separately and mixed as described above. The acetate solution for gradient elution was 0.05 M, pH = 5.0, and prepared similarly. At various time intervals, MeOH and/or ACN were mixed with the acetate

according to the program for gradient elution or regeneration as described later on.

Instrumentation

All chromatographic analyses were performed on an LC/9533 Ternary Gradient Liquid Chromatograph equipped with an LC/9523 Variable Wavelength UV Detector (both from IBM Instruments, Inc., Danbury, CT 06810). Detection was at 280 nm. For isocratic analyses, manual injections of 20 uL aliquots were made with a Model 7125 Injector (Rheodyne, Inc., Berkeley, CA 94710) and chromatograms were recorded on a Omniscribe strip chart recorder (Houston Instruments, Austin, TX 78753). For automated gradient analyses, a LC-9505 Automatic Sample Handler and a System 9000 computer with Chromatography Applications Program (CAP) (all from IBM Instruments, Inc.) were coupled to the chromatograph. The columns for both analyses were a Phenyl Mini (4.5 x 50 mm) used as a guard column for a Phenyl-RP (4.5 x 150 mm) (IBM Instruments, Inc.).

Sampling

Patient's blood was collected using either a venipuncture or micro collection device (23).

Extraction

Aliquots (50 ul or 100 ul) of serum standards (0,2.5,5,10, and 20 mg/L), "quality control" samples, and patient serum were added to a series of test tubes. Each tube was treated with an equal volume of I.S. solution and 200 ul of protein precipitation agent - trichloroacetic acid (10%). These sample tubes were vortexed and then centrifuged for 15 minutes. For the isocratic manual method, 20 ul aliquots of the supernatants were injected into the liquid

chromatograph. For the automated gradient elution method, the supernatant was filtered, and 50 ul aliquots were injected.

Chromatographic Parameters

The analysis was carried out at ambient temperature, with a flow rate of 2.0 ml/min. and detection at 280 nm, 0.02 AUFS. The gradient elution program was:

Time in Minutes	%		
	Acetate	MeOH	ACN
0	91	7	2
7	91	7	2
10	85	0	15
13	85	0	15
13.1	91	7	2
16	91	7	2

Column Regeneration was achieved between 13 to 16 minutes.

Quantitation and Statistical Analysis

For the manual isocratic method, theophylline or caffeine to I.S. peak height ratios were plotted against their respective concentrations and linear regression was performed by using the Advance Statistical Analysis of TRS Model III personal computer (Radio Shack, Fort Worth, TX 76111). For the automated gradient method, peak area ratios were correlated with drug concentrations, using the System 9000 computer with CAP. Correlation of patient's TH and CA concentrations by various methods was analyzed by the Advance Statistical Analysis Program.

Fluorescence Polarization Immunoassay

The principle and technical details have been published elsewhere (11). Briefly, using an automated clinical analyzer, TDx,

and commercially available reagents, the patient TH molecules underwent competitive binding with fluorescein labeled TH (tracer) for the polyclonal antibodies. The extent of the polarization of the residual tracer was measured and was automatically computed to yield the patient's TH concentration. This technique did not require sample preparation, and analysis time was about 10 minutes for 20 specimens.

RESULTS

Figures 1-3 show the chromatograms of gradient elution of TH, IS, paraxanthine, theobromine and CA by the phenyl, C-8 and C-18 columns. Elution orders of C-8 and C-18 were similar, but differed from that of the phenyl column. Paraxanthine, 1,7-dimethylxanthine, a metabolite of CA in the neonate, was completely resolved from TH and CA by the phenyl column. Thus, the phenyl column was chosen to be used for the clinical monitoring of TH and CA. Precision studies of the manual isocratic and automated gradient elution modes, outlined in Table 1 and 2, show acceptable means and coefficients of variation. Figures 4 and 5 illustrate that the calibration curves of the manual (between 2.5 to 20 mg/L) and automated method (between 2.5 to 50 mg/L) were linear. The sensitivity, based on S/N of 3, is 0.1 mg/L for both methods. Recoveries, by comparing the peak height of the extracts with those of aliquots of TH and CA, was estimated to be above 95% for both analytes by both methods. Figure 6 shows the isocratic elution chromatograms of extracts of (A) "drug-free" serum, (B) serum with 5 mg/L each of added TH and CA, and (C) a patient serum with 4 mg/L of TH and 0.7 mg/L of CA. Elution volumes of TH, IS and CA were 8,10 and 24 ml, respectively. Total analysis

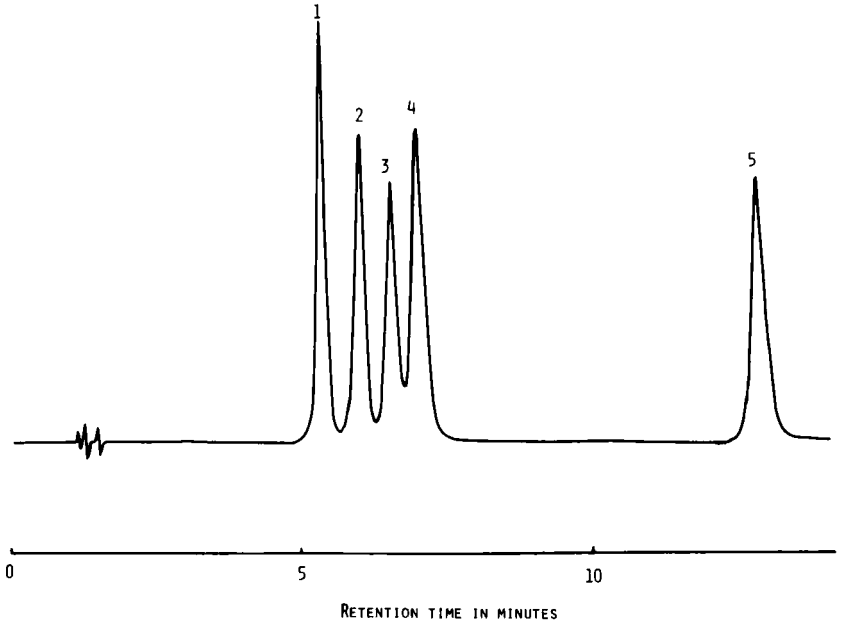

Figure 1 - Chromatogram of gradient elution of xanthine standards on a reversed-phase phenyl column (Peaks identification: 1. TH, 2. I.S., 3. 1,7-dimethylxanthine, 4. theobromine, and 5. CA)

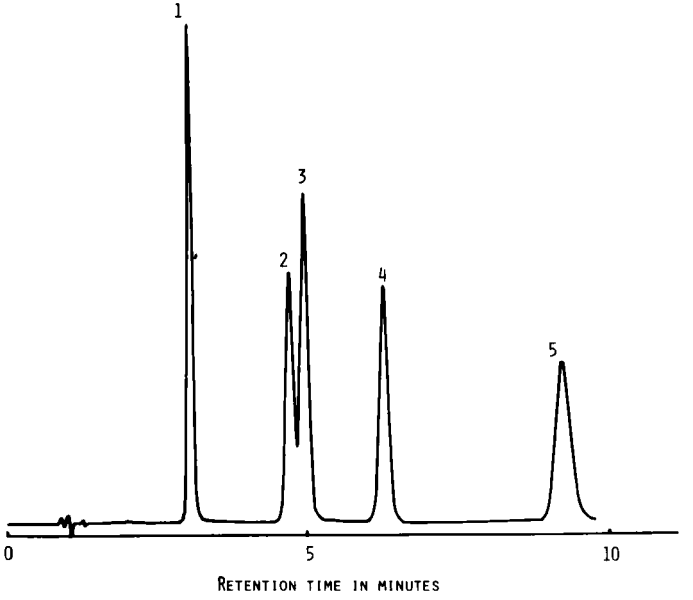

Figure 2 - Chromatogram of gradient elution of xanthine standards on a C-8 column. (Peaks identification: 1. theobromine, 2. 1,7-dimethylxanthine, 3. TH., 4. I.S., and 5. CA)

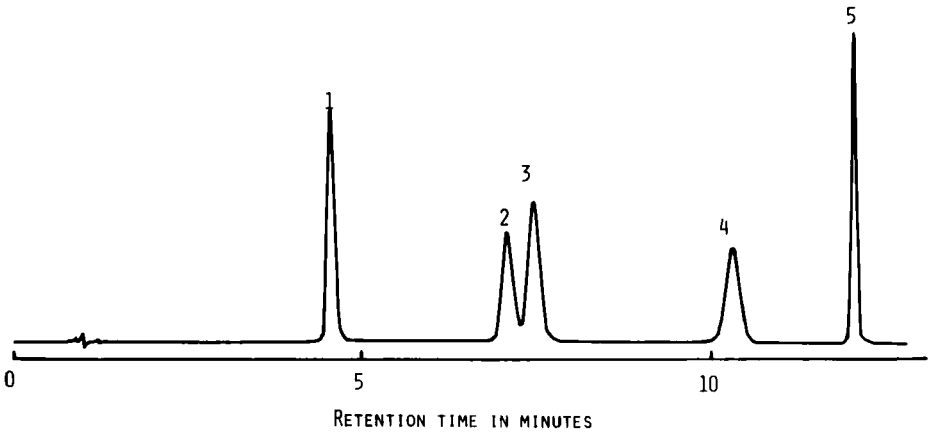

Figure 3 - Chromatogram of gradient elution of xanthine standards on a C-18 column (Peaks identification: 1. theobromine, 2. 1,7-dimethylxanthine, 3. TH, 4. I.S., and 5. CA)

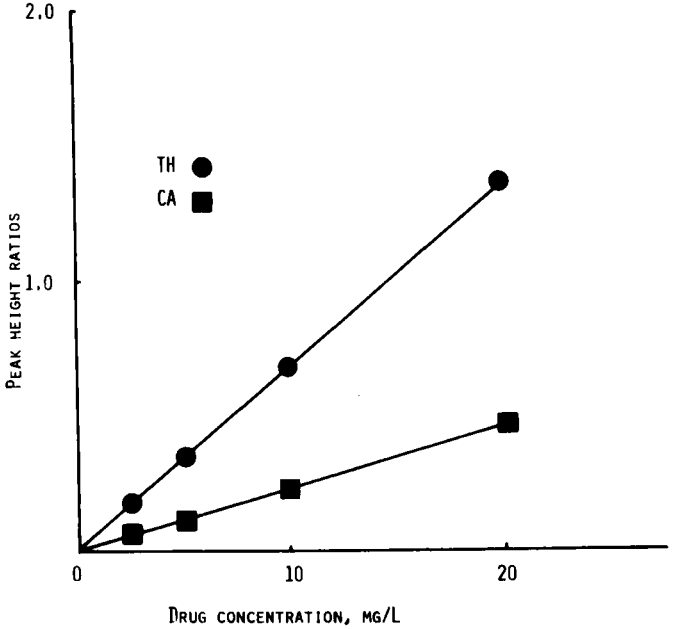

Figure 4 - Calibration curves of theophylline and caffeine using the manual isocratic method (TH: r = 0.9999, Y = 0.0677X + 0.0090, and CA: r = 0.9999, Y = 0.0233 X -0.0020)

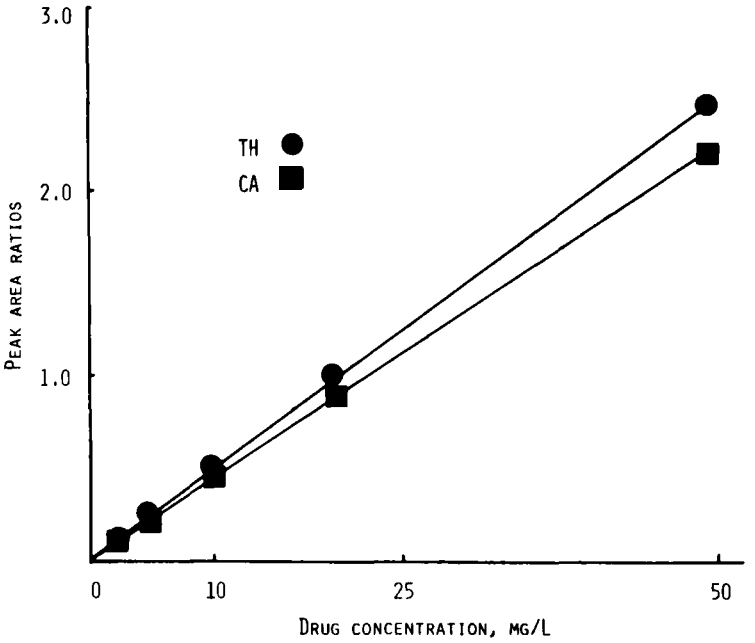

Figure 5 - Calibration curves of theophylline and caffeine using the automated gradient elution method (TH: r = 0.9998, Y = 0.0493X -0.0173, and CA: r = 0.9999, Y = 0.0433X -0.0020)

TABLE 1

Precision and Accuracy of the Manual Isocratic Method

	Within			Day-to-day		
	Mean	% CV	n	Mean	%CV	n
Theophylline, mg/L	10.8	1.7	6	10.1	7.8	35
Caffeine, mg/L	11.1	2.0	6	10.3	8.8	35

TABLE 2

Precision and Accuracy of the Automated Gradient Elution Method

	Within			Day-to-day		
	Mean	% CV	n	Mean	% CV	n
Theophylline, mg/L	9.99	1.13	6	9.84	2.29	34
Caffeine, mg/L	10.22	1.61	6	9.92	3.14	34

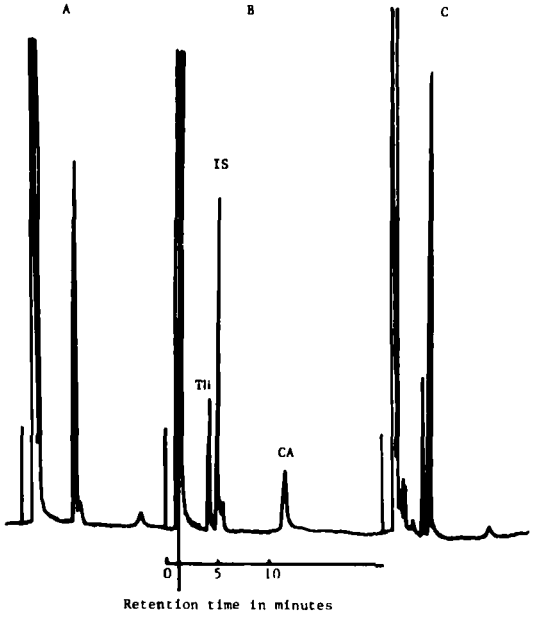

Figure 6 - Chromatograms of extracts of (A) "drug-free" serum with added I.S., (B) "drug-free" serum standard with added TH and CA (5 mg/L), and (C) a patient serum with 4 mg/L of TH and 0.9 mg/L of CA

TABLE 3

Linear Regression Analyses of Theophylline Methods Comparison

	r	Slope	Int.	n
MAN-AUTO	0.983	1.006	-0.244	23
MAN-FPIA	0.979	1.093	-0.162	24
AUTO-FPIA	0.994	1.086	0.127	23

MAN = Manual isocratic LC method
AUTO = Automated gradient elution LC method
FPIA = Fluorescence Polarization Immunoassay by TDx

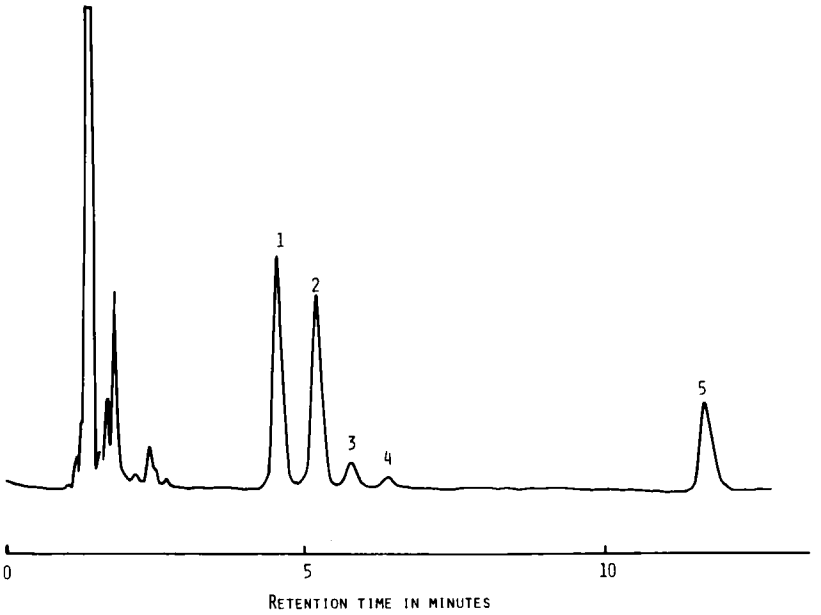

Figure 7 - Chromatogram of a patient serum extract showing TH (7.6 mg/L) (1), IS (2), 1,7-dimethylxanthine (3), theobromine (4) and CA (3.8 mg/L) (5)

time was about 13 minutes. Figure 7 is a gradient elution chromatogram of a patient serum extract. Elution volumes of TH, IS and CA were 9,11 and 23 ml, respectively. Peaks 3 and 4 could possibly be 1,7-dimethylxanthine and theobromine, respectively.

Using both LC methods, and the FPIA, patient's TH were compared as shown by Table 3. Patients' CA concentrations, estimated by the manual LC method, showed substantial amounts of CA, ranging from 0.1 to 5 mg/L. By minimizing the sample size, 50 ul was used for automated analyses of 9 patients. Table 4 outlines the comparison data and regression analysis of these procedures. Results from a

TABLE 4

Comparison of Automated Methods Using 50 and 100 ul Serum and FPIA

I. Patient Data

Sample No.	FPIA	Automated Method 50 ul	Automated Method 100 ul
	Theophylline concentration, mg/L		
T-7164	9	7.9	7.6
T-7165	24	23.4	21.7
T-7166	32	32.4	31.4
T-7167	20	19.1	17.8
T-7168	6	5.5	4.8
T-7199	5	4.5	4.3
T-7280	0.6	<0.5	<0.5
T-7308	4	5.2	4.6
T-7309	7	7.1	8.4

II. Linear Regression Analysis

	50 ul vs FPIA	100 ul vs FPIA	50 ul vs 100 ul
SLOPE	1.002	1.047	0.951
CORR.	0.998	0.993	0.998

TABLE 5

Capacity Factors, k', of Some Common Drugs in Assay for Theophylline and Caffeine

Drug	k'	Drug	k'
1-Methyluric acid	0.6	Meperidine	11.0
Acetaminophen	1.0	Amitriptyline	a
Cimetidine	1.0	Amoxapine	a
1-Methylxanthine	1.2	Desipramine	a
1,7-dimethyluric acid	1.4	Diazepam	a
3-methylxanthine	1.8	Doxepin	a
Theophylline(TH)	3.4	Flurazepam	a
Phenobarbital	3.4	Imipramine	a
B-hydroxyethyl Theophylline (IS)	4.2	7-OH-amoxapine	a
		8-OH-amoxapine	a
1,7-dimethylxanthine	4.4	Nortriptyline	a
Pentobarbital	5.4	n-desmethyl doxepin	a
Secobarbital	7.8	Phenytoin	a
Codeine	9.8		
Caffeine(CA)	11.0		

a = k' >11.0

drug interference study are presented in Table 5, which shows that phenobarbital co-eluted with TH and meperidine with CA under the isocratic conditions.

DISCUSSION

In studying the applicability of various functional groups of reversed-phase packing, the phenyl column offered unique selectivity in combination with the n-nonylamine mobile phase for the analysis of tricyclic antidepressants (21). Previously, Reece, Zacist and Barrow (22) demonstrated a reversed-phase analyses of tricyclic using phenyl column in combination with fluorescence detection. The present study is a continuation of our exploration of the clinical application of phenyl columns.

As shown in the elution order of xanthines by C-8 and C-18, and phenyl columns, the phenyl column was chosen specifically for its capability to resolve TH and 1,7-dimethylxanthine, a caffeine metabolite, in both the isocratic (Table 5) and gradient modes (Fig.7). The resolution of TH from this metabolite is of interest because in heavy coffee drinkers, their serum concentrations of 1,7-dimethylxanthine can occasionally reach as high as 6.9 mg/L (25). As reviewed by Christensen and Neims, resolution of TH from 1,7-dimethylxanthine was not achieved in most of the published TH LC assays using C-18 columns (1,17). Thus, it could be desirable to resolve TH and CA from the interference by this metabolite.

From the precision and recovery studies, the present methods - using both the isocratic and gradient approaches, afforded acceptable means CVs, and recoveries. The day-to-day CVs of the automated gradient elution methods for both TH and CA, were smaller

than those of the manual isocratic procedure. As indicated earlier, the gradient mode would resolve TH and CA from 1,7-dimethylxanthine and theobromine (Fig. 7), then this procedure might be preferred for pharmacokinetic studies involving CA and metabolites. The manual method was chosen for its simplicity in clinical analysis. In addition, the results were compared to a clinically acceptable assay, FPIA of theophylline.

The comparison data show acceptable correlation between the LC and polyclonal FPIA methods for "normal", non-uremic patients. No attempt was made to perform a similar comparison with uremic patient serum since metabolite interference has previously been established for the polyclonal antibody FPIA of TH (13-16). Recently, however, a monoclonal antibody FPIA technique (Theophylline II) was evaluated in our laboratory and the results from uremic patient serum correlated closely with those of subsequent isocratic LC analyses (16). Thus, the clinical efficacy of the present LC assay is even further substantiated.

In comparison to recently published LC assays of TH and CA, the present procedure utilized about 24 mL of mobile phase and total analysis time of 13 minutes: (1) Ou's procedure (17), mobile phase volume = 24 ml, time = 7 min., and temperature = ambient (2), Kabra's procedure (18), mobile phase volume = 5.2 ml, time = 70 sec., and temperature = 50°C. The present procedure differed from Ou's in its ability to resolve 1.7-dimethylxanthine, and from Kabra's in its ability to quantitate both TH and CA. In retrospect, Kabra's procedure may be readily used to quantitate CA (t_R = 1.72

minutes). However, the procedure would require adaptation of the chromatograph for high speed LC and elevated temperature.

Comparing the current method to FPIA procedures, several advantages would be evident. As noted previously, metabolites such as 1,7-dimethyluric acid interfere with some immunoassays of TH in serum of uremic patients (13-16). The present interference study shows that all metabolites elute before or after TH, CA and IS. The present procedure resolves 1,7-dimethylxanthine which has exhibited approximately 40% cross-reactivity in CA immunoassays. Another consideration is sample size. For neonatal monitoring, 50 ul of serum is sufficient for the simultaneous monitoring of both TH and CA.

In choosing the column for drug analysis, a major factor is column stability. From our present experience, the total number of injections on the phenyl columns was about 800 for the manual method and 400 for the automated method without noticeable change of retention times or peak symmetry. The sample preparation technique - protein precipitation and sample filtration - contributed to the stability. Typical start-up time was about 20 minutes. The interference by phenobarbital and merperidine, however, might limit the procedure. Thus, the procedure should be used with the prior knowledge that the patients were not co-medicated with either phenobarbital or meperidine.

In conclusion, the present study shows that the phenyl column can be readily used for simultaneous quantitation of TH and CA. The procedure provides a practical alternative to straight chain functional packing for therapeutic drug monitoring and toxicology.

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