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Rapid Determination of Theophylline in Serum by Electron-Capture GLC

SY-RONG SUN

Received July 13, 1978, from the *Pharmaceutical Products Division, Abbott Laboratories, North Chicago, IL 60064.* Accepted for publication September 11, 1978.

Abstract □ A rapid and sensitive GLC procedure was developed for the determination of theophylline in serum. After extraction from serum with ethyl acetate, theophylline and the internal standard were derivatized with pentafluorobenzyl bromide under alkaline conditions. The derivatives were quantitated by electron-capture detection. The method has a sensitivity of 0.1 μg/ml with a 0.1-ml serum sample.

Keyphrases □ Theophylline—electron-capture GLC determination of serum levels □ GLC, electron capture—analysis, theophylline in serum □ Relaxants, smooth muscle—electron-capture GLC analysis of theophylline in serum

Methods for the determination of theophylline in biological fluids are based on spectrophotometry (1–3), GLC (4–8), TLC (9), and high-pressure liquid chromatography (10–14). Recently, an electron-capture GLC procedure was reported (15) that involves the extraction of theophylline and the internal standard, theobromine, into methylene chloride by elution through a cellulose column. The compounds are then back-extracted into an alkaline aqueous phase and derivatized with pentafluorobenzyl bromide by extractive alkylation. The derivatives are separated from excess reagent by solvent extraction and finally determined by electron-capture GLC. This method is tedious, and the use of theobromine as an internal standard is not a good choice because it is present in beverages such as tea and cocoa and is a metabolite of caffeine (16–18).

The present, rapid method requires only 0.1-ml serum sample. It involves one-step extraction of the serum sample with ethyl acetate, followed by derivatization with pentafluorobenzyl bromide under alkaline conditions at 100°. Excess reagent is removed by evaporation. A chemical isomer of theophylline, which is not present in beverages and is not a metabolite of caffeine, is used as the internal standard. The sensitivity of the assay is 0.1 μg/ml with a 0.1-ml serum sample.

EXPERIMENTAL

Reagents and Materials—Theophylline[1], the internal standard (5,7-dimethyl-2*H*-pyrazolo[3,4-*d*]pyrimidine-4,6-(5*H*,7*H*)-dione, I)[2], and pentafluorobenzyl bromide[3] were used as supplied. The other chemicals were analytical reagent grade.

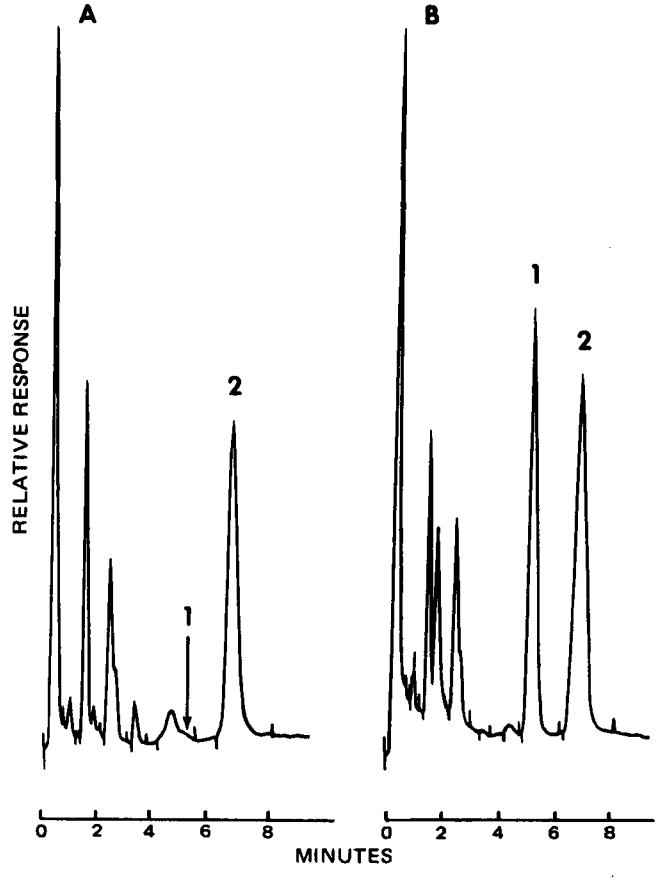

Figure 1—*GLC tracings of extracted serum samples. Key: A, serum blank containing the internal standard; B, serum standard containing the internal standard and theophylline at 5 μg/ml serum; 1, theophylline; and 2, internal standard.*

[1] Theophylline USP ($C_7H_8N_4O_2 \cdot H_2O$).

[2] Nippons Shinyaku Co., Kyoto, Japan.
[3] Pentafluorobenzyl bromide, Pierce Chemical Co., Rockford, Ill.

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Table I—Assay Reproducibility

Serum Theophylline Concentration, μg/ml		
Theoretical	Observed Mean[a] ± *SD*	*RSD*, %
0.10	0.115 ± 0.026	22.2
0.25	0.189 ± 0.029	15.3
0.50	0.489 ± 0.027	5.5
1.00	1.034 ± 0.070	6.8
2.50	2.567 ± 0.219	8.5
5.00	4.942 ± 0.386	7.8
10.00	10.001 ± 0.345	3.4

[a] Mean of three determinations at each concentration.

Table II—Assay Sensitivity

Serum Theophylline Concentration, μg/ml	Peak Area Ratio, Theophylline/Internal Standard
0.25	0.039
	0.046
	0.046
0.50	0.090
	0.084
	0.083
1.0	0.162
	0.174
	0.154
2.5	0.423
	0.393
	0.356
5.0	0.705
	0.836
	0.783

Least-Squares Linear Regression Calculation (19)

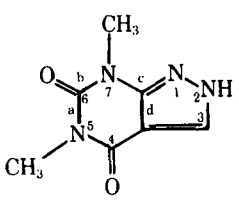
Number of points	15
Slope	0.1540
y-Intercept ± 95% confidence limit	0.00551 ± 0.01466
Correlation coefficient	0.996
Calculated sensitivity[a], μg/ml	0.095 μg/ml

[a] The calculated sensitivity was the *x* value (in concentration) corresponding to the upper part of the 95% confidence interval of the *y* intercept (in peak area ratio).

Apparatus—A refrigerated centrifuge[4] was used. Sample solutions were mixed[5] in the test tubes. Pentafluorobenzyl derivative formation at high temperature was carried out on a heating block[6].

GLC—A reporting gas chromatograph[7] equipped with a computing integrator[8] and a ^{63}Ni constant current electron-capture detector[9] was used. The 1.21-m × 4-mm (i.d.) glass column was packed with 5% OV-225[10] on 80–100-mesh Gas Chrom Q[10]. The column was conditioned at 250° for 16 hr with a carrier gas [argon–methane[11] (95:5)] at 20 ml/min before connection to the detector. The carrier gas cylinder was fitted with an oxygen trap filter[12].

The operating conditions were: column oven temperature, 250°; electron-capture detector temperature, 320°; injection port temperature, 270°; and carrier gas flow rate, 45 ml/min. Under these conditions, the pentafluorobenzyl derivatives for theophylline and the internal standard had retention times of 5.3 and 7.1 min, respectively (Fig. 1).



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[4] Model RC-3, Sorvall, Newton, Conn.
[5] Vortex Genie model K-550-GT mixer, Scientific Industries, Springfield, Mass.
[6] Dri-block DB-3, Techne, Princeton, N.J.
[7] Hewlett-Packard model 5830A.
[8] Hewlett-Packard model 18850A.
[9] Hewlett-Packard model 18803A.
[10] Applied Science Laboratories, State College, Pa.
[11] Matheson Gas Products, Elk Grove Village, Ill.
[12] Altech Associates, Arlington Heights, Ill.

Table III—Assay Accuracy

Serum Theophylline Concentration, μg/ml		
Theoretical	Observed	Difference
10.00	9.60	−0.40
5.00	4.85	−0.15
2.50	2.76	0.26
1.00	1.09	0.09
0.50	0.36	−0.14
0.25	0.16	−0.09
0.10	0.14	0.04
0	0	0
7.50	6.92	−0.58
3.75	3.66	−0.09
1.75	1.79	0.04
0.75	0.79	0.04
0.38	0.52	0.14
6.25	6.26	0.01
3.0	2.76	−0.24

Preparation of Standards—A 200-μg/ml stock solution of theophylline was prepared by dissolving 22.0 mg of theophylline in a 100-ml volumetric flask with distilled water and bringing the mixture to volume. A working serum theophylline standard at 10 μg/ml was prepared by adding 5.0 ml of the stock solution to a 100-ml volumetric flask and bringing the mixture to volume with drug-free serum. Drug-free serum was obtained from volunteers that did not drink coffee, tea, or cocoa. Other serum standards were prepared from serial dilutions (with serum) of the 10-μg/ml serum standard.

The internal standard stock solution (100 μg/ml) was prepared by dissolving 20.0 mg of the internal standard in a 200-ml volumetric flask with methanol and bringing the mixture to volume. A working internal standard solution (3 μg/ml) was prepared by diluting 3 ml of the internal standard stock solution to 100 ml with distilled water.

Assay—To 0.1 ml of serum in a 15-ml conical test tube were added 0.1 ml of 0.1 *N* HCl, 0.1 ml of the internal standard solution, and 1 ml of ethyl acetate. The tube was vortexed for 15 sec and centrifuged at 3000 rpm for 3 min at 10°. Then 0.7 ml of the organic phase was transferred into a 15-ml screw-capped conical test tube and evaporated to dryness at 50° with filtered air.

To the dry residue were added 0.1 ml of sodium carbonate solution (250 μg/ml of water) and 0.1 ml of pentafluorobenzyl bromide in alcohol (2 mg/ml). The mixture was capped, vortexed for 15 sec, reacted at 100° for 20 min on the heating block, and cooled to room temperature. Then 0.5 ml of water and 1.3 ml of benzene were added, followed by vortexing for 15 sec.

One milliliter of the benzene extract was separated and evaporated to dryness at 50° with filtered air. The clear residue was reconstituted with 1.5 ml of benzene by vortexing for 15 sec, and 5 μl of the solution was injected into the GLC column by the automatic sampler[13].

Calculations—The peak area ratios of theophylline to the internal standard were plotted against known standards of theophylline expressed as micrograms per milliliter of serum. The curve was linear in the 0.25–5.0-μg/ml region. Values for unknown serum theophylline concentrations were obtained from the calibration curve.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of an extract from 0.1 ml of serum spiked with theophylline. Peak shapes were symmetrical. Theophylline and the internal standard were resolved from coextractive peaks.

The reproducibility of the assay is shown in Table I. Relative standard deviations ranged from 3.4 to 8.5% for serum concentrations from 0.5 to 10.0 μg/ml. Larger deviations were observed at lower serum concentrations. The calculated sensitivity of the assay was 0.095 μg/ml (Table II).

The accuracy of this assay was tested by analyzing 15 unknown spiked serum samples under blind conditions (Table III). The assay is specific for theophylline; the retention times for pentafluorobenzyl derivatives of theophylline, the internal standard, and theobromine were 5.31, 7.09, and 4.74 min, respectively.

Recoveries averaged 104.0 ± 3.1% (mean ± *SD*) for triplicate serum theophylline samples at 10 μg/ml. This value was calculated by comparing the peak area ratios between the extracted serum sample and the water sample.

This method is specific and sensitive and can be used to conduct single

[13] Hewlett-Packard model 7671A.

oral dose theophylline bioavailability studies in human adults and pediatric patients.

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Sensitive GLC Assay for Pemoline in Biological Fluids Using Nitrogen-Specific Detection

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Received May 31, 1978, from the *Pharmaceutical Products Division, Abbott Laboratories, North Chicago, IL 60064.* Accepted for publication September 9, 1978.

Abstract □ Extractive alkylation was used to determine intact pemoline in serum and urine. Pemoline was extracted into methylene chloride as an ion-pair with tetrapentylammonium hydroxide under alkaline conditions. Evaporation of the solvent at 70° in the presence of methyl iodide yielded the *N,N*-dimethylpemoline derivative. GLC analysis was performed on a 5% FFAP column with nitrogen-specific detection. Sensitivity was 0.05 μg/ml with 1 ml of urine or serum. Calibration curves were linear to at least 4 μg/ml with serum and 15 μg/ml with urine. Precision was excellent with a pooled relative standard deviation of ±7.5% for serum samples in a 0.1–4-μg/ml range.

Keyphrases □ Pemoline—GLC assay using nitrogen-specific detection, biological fluids □ GLC—analysis, pemoline in biological fluids □ Stimulants—pemoline, GLC assay in biological fluids

Several methods to determine pemoline (2-amino-5-phenyl-2-oxazolin-4-one) in biological fluids have been published. In one method (1), pemoline is hydrolyzed to mandelic acid with subsequent oxidation to benzaldehyde. The benzaldehyde is then determined spectrophotometrically. Benzaldehyde generated from pemoline has also been determined by GLC (2). Large sample volumes are required, and the method is tedious and nonspecific. High-pressure liquid chromatography has been applied to urine samples and pharmaceutical preparations (3). However, this method and a TLC method (4) lack the sensitivity for plasma samples.

A GLC method (5) using flame-ionization detection involves hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione with subsequent methylation using diazomethane. Extensive cleanup procedures are needed with blood samples, and 60% of the final extract is injected into the chromatograph to achieve sensitivity. A similar approach was recently taken by Libeer and Schepens (6).

A recent GLC method using electron-capture detection determined 5-phenyl-2,4-oxazolidinedione without methylation (7). The method is sensitive but requires careful column preparation and preservation to minimize tailing and to maximize resolution from coextractives.

This paper describes the application of extractive alkylation (8, 9) and nitrogen-specific detection for the analysis of pemoline in biological fluids.

Table I—Precision of Serum and Urine Pemoline Assay

Pemoline Concentration, μg/ml	Peak Area Ratio[a]	*RSD*, %
	Serum	
0	0	—
0.05	0.015 ± 0.0006	4.0
0.10	0.029 ± 0.0040	13.8
0.25	0.097 ± 0.0038	3.9
0.50	0.17 ± 0.023	13.5
1.0	0.33 ± 0.011	3.3
2.0	0.68 ± 0.013	1.9
3.0	1.0 ± 0.025	2.5
4.0	1.4 ± 0.076	5.4
	Urine	
0	0	—
1.0	0.25 ± 0.0050	2.0
5.0	1.4 ± 0.047	3.4
15.0	4.4 ± 0.095	2.2

[a] Mean ± *SD* of triplicate standards.

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