ping m, methylenes and methines), 3.59 (s, 3H, vinyl methoxy), 3.64 and 3.66 (2s, 3H total, carbomethoxy), 3.72 (d, 1H, J = about 10 Hz, benzylic proton on C-4), 5.00 (s, 1H, vinyl), 5.82 (s and an indistinct overlapping signal, 2H plus 1H, methylenedioxy and proton on C-3), and 6.64 and 6.67 (2s, 1H each, aromatic). Its mass spectrum showed significant ions at m/e 499 (M^+, 40), 469 (12), 314 (19), 298 (100), 266 (17), 185 (36), 173 (29), 146 (71), and 114 (78); high-resolution M^+: calc. for $C_{28}H_{37}NO_7$, 499.257; found, 499.257.

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GLC Determination of Theophylline in Biological Fluids

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Abstract □ A specific GLC procedure for the determination of theophylline in plasma and saliva is described. The method selectively measures theophylline in the presence of its metabolites and other xanthines. The GLC method is sufficiently sensitive to detect 1 μg theophylline/ml plasma or saliva. Analysis for theophylline in plasma and saliva samples obtained from patients showed a saliva–plasma ratio of approximately 0.5.

Keyphrases □ Theophylline—GLC determination in biological fluids in presence of metabolites and other xanthines □ Xanthine derivatives—GLC determination of theophylline in biological fluids in presence of metabolites and other xanthines □ GLC—determination, theophylline in biological fluids

A specific and sensitive analytical method for the quantitative determination of a drug in blood and urine is needed to follow the kinetics of the substance in the body and to control the method of administration to secure optimal effects. Theophylline (1,3-dimethylxanthine) is a widely used xanthine for its antiasthmatic and cardiovascular actions. The method of Truitt *et al.* (1) for the determination of theophylline was based upon spectrophotometric measurement of the azo dye developed by coupling it with Fast Blue 2B. The method of Plummer (2) involved converting theophylline into a copper complex, mixing with potassium iodide solution, and titrating the liberated iodine with thiosulfate. Both these methods are tedious and require large quantities of blood. A UV spectrophotometric method (3–5) is more frequently used for the determination of theophylline in plasma. The sensitivity of this method is good but is subject to interference from other xanthines present in plasma, namely caffeine, theobromine, and theophylline metabolites. In addition, endogenous compounds such as xanthine, hypoxanthine, and uric acid contribute to the absorbance blank. Barbiturates, if present, also interfere in the absorption measurements of theophylline. The presence of these compounds results in an overestimation of the theophylline concentration in the sample. Gupta and Lundberg (6) described a differential spectrophotometric method for determination of theophylline in the presence of barbiturates.

A fast, sensitive, and selective GLC method for the determination of theophylline in plasma and saliva is reported here. It can be used in the presence of theophylline metabolites, caffeine, theobromine, or any barbiturates. If needed, the modified plasma extraction procedure can be used for spectrophotometric analysis.

EXPERIMENTAL

Materials—Analytical grade solvents and chemicals were used without further purification.

Apparatus—A chromatograph[1] equipped with a flame-ionization detector and a 1.83-m × 0.31-cm (6-ft × 0.125-in.) glass column packed with 3% OV-17 on Gas Chrom Q was used. The following conditions of the chromatograph were satisfactory for the separation of theophylline and its metabolites and the internal standard from the extracted components of plasma: injector port temperature, 265°; column temperature, 190°; detector block temperature, 280°; and nitrogen flow, 35 ml/min. Hydrogen and air flow were optimized for maximum response. The retention times

[1] Varian Aerograph model 1200.

Table I—Spectral Properties of Xanthines and Uric Acids[a] and Their Concentration after Theophylline Administration

	UV Absorption, 10 μg/ml			Normal Serum Concentration, μg/ml	Serum Level with Theophylline Therapy, μg/ml
	λ_{max}	A_{280}	ϵ_{280}		
Theophylline	274	0.42	7,600	—	5–20
3-Methylxanthine	271	0.48	8,000	—	3–4
1-Methyluric acid	284	0.55	10,100	—	—
1,3-Dimethyluric acid	285	0.48	9,400	—	—
Xanthine	266	0.37	5,600	1	6
Hypoxanthine	249	0.10	1,400	1.4	7–8.7
Uric acid	284	0.66	11,100	38–70	70–81

[a] Reference 8.

for theophylline and thiobarbital (internal standard) under these conditions were 3.6 and 3.0 min, respectively (Fig. 1).

A recording spectrophotometer[2] was used for UV absorption measurements.

Procedure—A 1-ml sample of plasma or saliva was pipeted into a nitric acid-washed test tube and extracted with 10 ml of organic solvent mixture [ether–dichloromethane–isopropanol (6:4:1)]. The tube was centrifuged for 10 min at 2500 rpm and cooled for 30 sec in a dry ice–acetone bath. The upper organic layer was decanted into another tube containing 2 ml of 1 *N* sodium hydroxide. The contents were mixed well, centrifuged, and cooled, and the upper organic layer was discarded. For UV determination of theophylline, the alkaline solution was used to scan the absorption from 310 to 260 nm. The alkaline solution was acidified with 0.4 ml of 8 *M* phosphoric acid (pH ~5), mixed with 1 ml of saturated solution of sodium sulfate, and extracted twice with 10 ml of the organic solvent mixture (by repeating mixing, centrifuging, and cooling). The internal standard, 30 μg of thiobarbital (or 5 μg of fluoranthene; see *Results and Discussion*), was added and the extract was evaporated to dryness under nitrogen at 40°. The residue was dissolved in 25 μl of tetrapropylammonium hydroxide. Duplicate 3-μl aliquots of the resulting solution were injected in the chromatograph, and the average peak height ratio of theophylline to the internal standard was calculated. The concentration of theophylline in the sample was then calculated from the standard curve.

Standard Calibration Curves—Normal plasma was spiked with known amounts of theophylline (0, 1, 2, 5, 10, 20, and 30 μg/ml) and extracted as described in the procedure for UV determination. To define the standard curve for UV measurements, the difference in absorbance at 275 and 310 nm was plotted against the respective concentration of theophylline. The spiked plasma samples were processed for GLC determination. To define the standard curve for GLC measurements, the average ratio of the height of the theophylline peak to the height of the internal standard peak was plotted against the theophylline concentration (Fig. 2). These standard curves represent an average of at least six samples at each of six data points. The average extraction efficiency in the organic solvent system was 85 ± 2% at each concentration.

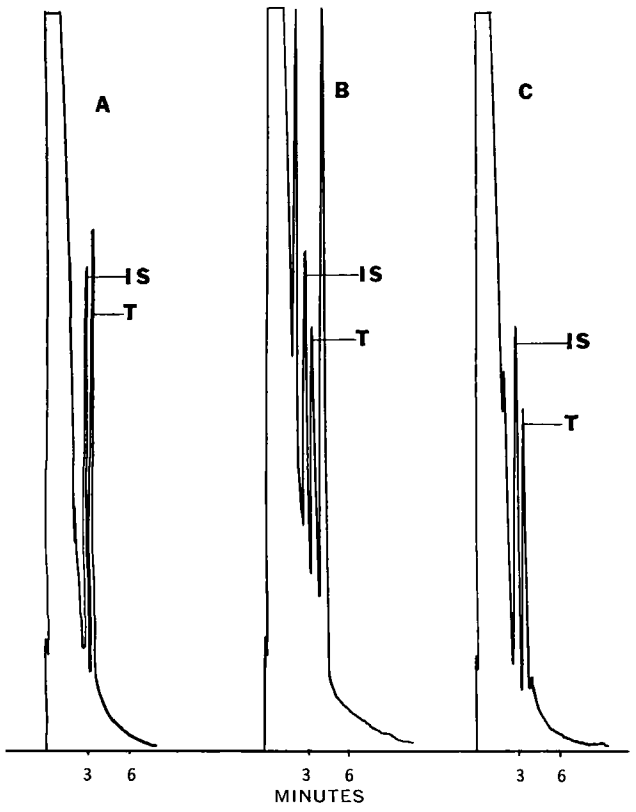

Figure 1—*GLC curves for theophylline (T) and thiobarbital (IS) in unextracted (A), extracted plasma (B), and extracted saliva (C) samples.*

RESULTS AND DISCUSSION

Theophylline is metabolized *in vivo* to 3-methylxanthine (13%), 1,3-dimethyluric acid (35%), and 1-methyluric acid (19%) (7). Administration of theophylline apparently results in increased plasma levels of xanthine, hypoxanthine, and uric acid (Table I)[3]. The UV absorption curves for theophylline and other xanthines are almost superimposable. Uric acids and hypoxanthine have significant absorption at 280 nm (Table I). Therefore, extraction of any of the xanthines, uric acids, or barbiturates with theophylline will

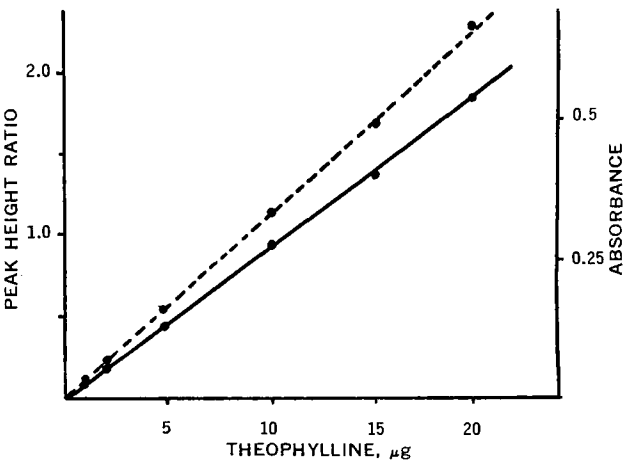

Figure 2—*GLC and UV standard curves for theophylline in unextracted (- - -) and extracted (—) plasma samples.*

[2] Cary model 15.

[3] Dr. Miles Weinberger, National Jewish Hospital and Research Center, Denver, Colo., personal communication.

Table II—Extraction Efficiency for Theophylline and Its Metabolites

Compound	Percentage Extracted in 10 ml of: Ether–Dichloromethane–Isopropanol (6:4:1)	Percentage Extracted in 10 ml of: Chloroform–Isopropanol (19:1)
Theophylline (1–20 μg/ml)	85[a]	85[a]
3-Methylxanthine (2.5–5 μg/ml)	40	40
1,3-Dimethyluric acid (2.5–5 μg/ml)	6	10
1-Methyluric acid (2.5–5 μg/ml)	10	33

[a] Range: 83–87%.

result in overestimation of the theophylline concentration by UV spectrophotometric determination.

The most commonly used assay procedure involves extraction of plasma with a chloroform–isopropanol mixture (19:1), reextraction of theophylline in base, and UV analysis of the alkaline solution (3). When the plasma is extracted with this solvent system, a thick gel forms and makes quantitative transfer of the underlying organic layer difficult. However, a solvent system consisting of ether–dichloromethane–isopropanol (6:4:1) is less dense than plasma and also does not form a gel. So, after extraction and centrifugation, the lower aqueous plasma layer can be frozen in a dry ice–acetone bath and the upper organic layer can be easily decanted. Both solvent systems are equally efficient in extracting theophylline (85 ± 2%) (Table II). However, other interfering components (as already discussed) will also be extracted from the plasma and will result in overestimation of theophylline. In the UV spectrophotometric method, one generally subtracts a constant blank value. In reality, this blank may vary, depending on the endogenous substances present in the plasma and on the length of time theophylline has been administered to the patient. Therefore, a selective and sensitive GLC method was developed for the determination of theophylline.

Theophylline, when converted by tetrapropylammonium hydroxide within the injector port to its propyl derivative, results in a symmetrical peak with a retention time of 3.6 min, distinct from any other xanthines, theophylline metabolites, and barbiturates (Fig. 1). The peak height is proportional to the amount of theophylline. The standard curve is linear in the range studied. The standard curves for extracted and unextracted plasma were determined to define the extraction efficiency (Fig. 2). The GLC method is sufficiently sensitive to detect 1 μg theophylline/ml plasma or saliva[4]. The metabolites of theophylline, namely 3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid, when injected in tetrapropylammonium hydroxide are converted to their propyl derivatives, with retention times of 4.5, 5.5, and 6.1 min, respectively.

Theobromine, when injected in tetrapropylammonium hydroxide solution, is converted into its propyl derivative, with a retention time of 3.3 min. However, when caffeine is injected in aqueous tetrapropylammonium hydroxide solution, it is completely destroyed under the temperature and aqueous alkaline condition in the injection port. If injected in nonaqueous solvent, it is stable and has a retention time of 3.2 min. Barbiturates, if present, have a different retention time from theophylline. Thus, the selective and sensitive GLC method developed eliminates the potential sources of error in the detection of theophylline.

Thiobarbital used as an internal standard has one disadvantage. It is unstable to base and cannot be stored for more than 30 min at ambient temperature. Fluoranthene (5 μg) proved to be a satisfactory alternative internal standard[5]. When injected in the GLC with theophylline, fluoranthene has a retention time of 4.8 min.

[4] Use of an alkali flame-ionization detector using the same column conditions as described for the flame-ionization detector with hydrogen and air flow of 36 and 225 ml/min, respectively, allows one to detect theophylline levels as low as 0.1 μg/ml of plasma or saliva.

[5] Thiobarbital remains the best internal standard for the alkali flame-ionization detector.

Table III—Comparison of Plasma and Saliva Concentrations of Theophylline by UV and GLC Methods

Subject	Sample Source	Micrograms per Milliliter: UV	Micrograms per Milliliter: GLC	Saliva–Plasma Ratio, GLC Method
GC	Plasma	18.2	16.5	
PA	Plasma	5	5	
TA	Plasma	1.5	1.5	
AS	Plasma	17.0	8.2	
MK	Saliva	6.6	6.0	0.500
	Plasma	15.0	12.0	
NA	Saliva	12.0	6.25	0.521
	Plasma	27.0	12.0	
NC	Saliva	5.2	2.10	0.442
	Plasma	6.6	4.75	
JE	Saliva	11.0	10.0	0.513
	Plasma	27.0	19.5	

Plasma and saliva samples obtained from patients being treated with theophylline were analyzed by both UV spectrophotometric and GLC methods (Table III). In some instances, the UV method indicated a significantly higher theophylline concentration than did the GLC method. This may be due to interference from xanthines other than theophylline or from theophylline metabolites. All kinetic studies of theophylline reported in the literature apparently involve the drug determination by the UV method. Because of the potential error involved in the determination of theophylline concentration by the UV method, the kinetic parameters determined may be incorrect.

The saliva–plasma ratio of theophylline found in the four patients studied was about 0.5, thus suggesting that salivary measurements may be used to estimate plasma concentrations of theophylline. The salivary levels reflect the plasma water concentration and are, therefore, directly proportional to blood (or plasma) levels. Routine monitoring of theophylline levels in saliva rather than in plasma would preclude the inconvenience of venipuncture and be particularly suitable for use in children. A detailed kinetic study using the GLC method for plasma and saliva measurements is being conducted. Attempts will also be made to quantitate theophylline metabolites.

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