Gas Chromatographic–Mass Spectrometric Quantitation of Theophylline and Its Metabolites in Biological Fluids

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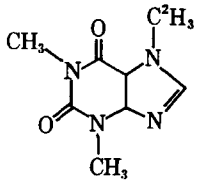
Abstract □ In premature infants, theophylline is converted to caffeine, and the biological half-life is prolonged. To assess the metabolic alterations of theophylline during development of premature infants, a sensitive and simple method was developed which quantitated all theophylline metabolites in plasma, urine, and red blood cells. Theophylline and its metabolites in the sample were converted to the *N*-propyl derivative using *n*-propyl iodide in dimethylformamide with potassium carbonate catalysis and were analyzed under isothermal conditions on a gas chromatograph–mass spectrometer with a 3% methylsilicone–phenylsilicone column. Deuterated caffeine (caffeine-d_3) was used as the internal standard. A selected ion-monitoring technique, together with 70-eV electron impact ionization mode, was used. The ion current ratios between caffeine-d_3 (*m/z* 197) and caffeine (*m/z* 194), theophylline (*m/z* 222), 3-methylxanthine (*m/z* 250), 1,3-dimethyluric acid (*m/z* 280), and 1-methyluric acid (*m/z* 308) were monitored. The total analysis time was 12 min with a detection limit ranging from 500 pg to 10 ng, depending on the metabolites. With this sensitivity, sample sizes of 50–100 μl of plasma and 0.5 ml of urine were sufficient for the analysis of all theophylline metabolites. The coefficient of variation of this method was <5% for the analysis of biological samples.

Keyphrases □ GC–mass spectrometry—quantitation of theophylline and its metabolites in biological fluids, infants □ Theophylline—GC–mass spectrometric quantitation in biological fluids, infants □ Biological fluids—GC–mass spectrometric quantitation of theophylline and its metabolites, infants □ Caffeine—metabolite of theophylline in infants, GC–mass spectrometric quantitation in biological fluids

Theophylline has been used widely for the treatment of apnea in premature infants (1). Due to the narrow margin between effective and toxic doses, the plasma level of this drug during treatment is usually monitored to ensure safety. Over the past several years, a number of analytical methods have been developed specifically for this purpose (2). These include GC methods (3) and the newer high-performance liquid chromatographic (HPLC) methods (2). However, none of these methods are capable of measuring theophylline and metabolites in a microsample from a pediatric population.

The metabolism of drugs in premature infants is different from that of adults. The differences generally are limited to the pharmacokinetic parameters, such as elimination half-life, volume of distribution, and renal clearance. A recent report stating that in premature infants theophylline is methylated to caffeine (4) points out an important change in the concept of drug metabolism in premature infants. Not only the kinetic aspect of drug metabolism must be compared between premature infants and adults, but the quantitation of metabolic pathways also must be studied.



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To investigate the metabolism of theophylline in premature infants, a simple and sensitive GC–MS method was developed. This method is capable of measuring theophylline, theobromine, caffeine, 3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid in as little as 50 μl of plasma and 0.5 ml of urine.

EXPERIMENTAL

Reagents—Caffeine[1], theophylline[1], theobromine (3,7-dimethylxanthine)[2], 3-methylxanthine[2], 1,3-dimethyluric acid[2], and 1-methyluric acid[3] were used as standards. Anhydrous potassium carbonate was of commercial reagent grade. Dimethylformamide[4] and 1-iodopropane[5] were purified by distillation.

7-[$^2H_3$]Methyl-1,3-dimethylxanthine (Caffeine-d_3) (I)—Powdered anhydrous potassium carbonate (20 mg) and 5 drops of iodomethane-d_3 (99 + atom % D)[4] were added to a solution of theophylline (15.8 mg) in 0.25 ml of dimethylformamide. The mixture was heated with occasional swirling at 70° for 15 min. Additional iodomethane-d_3 (1 drop) was then added to the mixture and the reaction was continued for an additional 15 min. After cooling, the reaction mixture was diluted with 4 ml of water and then extracted with methylene chloride (1 ml × 2). The methylene chloride extract was evaporated to dryness to obtain caffeine-d_3 (5.4 mg). GC analysis of this product showed a single peak with no side products. The 70-eV mass spectrum had the following fragments: *m/z* (percent of base peak), 197(100), 112(45), 85(20), 70(14), 67(11), and 58(17). The isotopic purity of this compound was: d_0, 0.09%; d_1, 5.51%; d_2, 6.99%; and d_3, 87.41%.

Internal Standard Solution—The caffeine-d_3 internal standard was dissolved in water to a final concentration of 2.14 μg/20 μl (for urine analysis) and 0.215 μg/20 μl (for plasma analysis).

Standard Solution of Theophylline and Its Metabolites—Caffeine (4.22 mg), theophylline (33.35 mg), 3-methylxanthine (4.40 mg), 1,3-dimethyluric acid (7.98 mg), and 1-methyluric acid (4.43 mg) were suspended in 3 ml of water. To this stirred mixture, a solution of 0.5 *N* NaOH was added drop-wise to convert the turbid suspension into a clear solution. Enough water then was added to make a final volume of 5 ml.

This stock solution then was diluted with water to a final concentration of caffeine in the following order: 1.68 μg/20 μl, 168 ng/20 μl, 16.8 ng/20 μl, and 1.68 ng/20 μl.

Standard Curve—In a glass disposable tube (12 × 75 mm), the standard metabolite solutions (20 μl, containing caffeine and other metabolites in the range of 1.68 μg to 1.68 ng) and 20 μl of internal standard solution (containing caffeine-d_3, 2.14 μg) were evaporated to dryness in a stream of air. Powdered potassium carbonate (0.5 mg), dimethylformamide (0.25 ml), and 1-iodopropane (50 μl) were then added to the dried residue. The mixture was heated at 70° in an evaporator[6] for 15 min. Vacuum was then applied to this unit to evaporate all the solvent and reagents (~15 min). The dried tube was then capped and kept at −20° until ready for analysis.

Similarly, standard curves in urine, plasma, and red blood cells were constructed by spiking blank samples with standard solutions. The spiked samples were then analyzed as described for each individual biological fluid, except in the case of red blood cells. For the standard curve in red blood cells, the spiked cells were not washed with normal saline. The standard curves in biological samples were identical to those in water,

[1] Calbiochem, San Diego, Calif.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Adams Chemical Co., Round Lake, Ill.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Eastman Kodak Co., Rochester, N.Y.
[6] Vortex-Evaporator, Buchler Instruments, Fort Lee, N.J.

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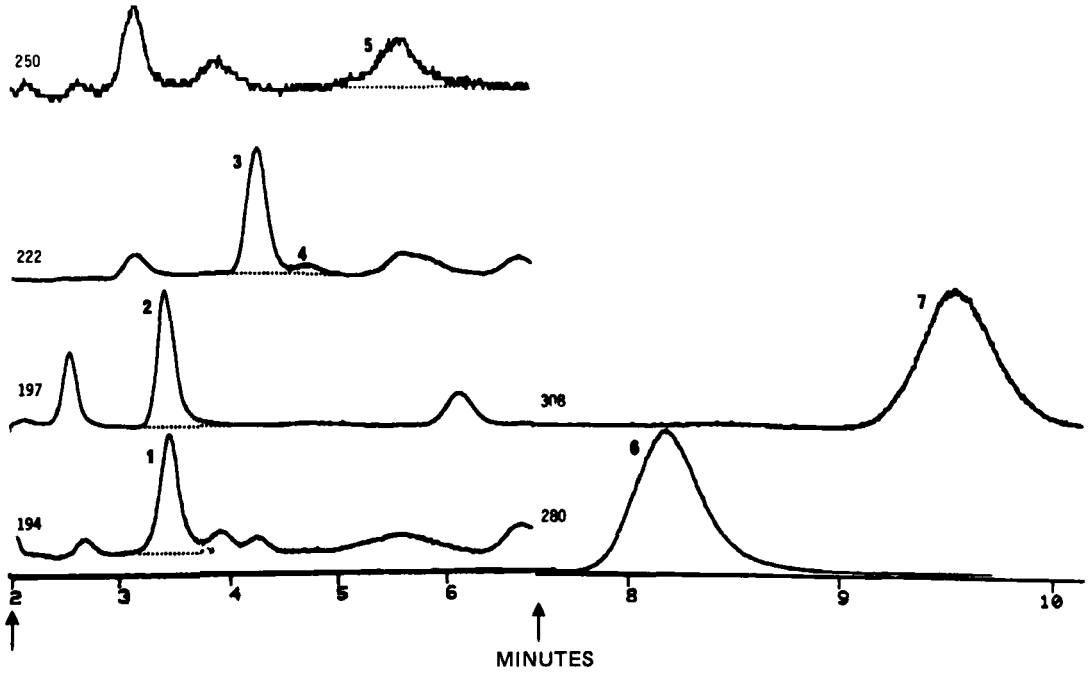

Figure 1—*A representative selected ion-monitoring chromatogram of theophylline metabolites in the urine of premature infants. The metabolites are: (1) caffeine; (2) caffeine-d_3; (3) theophylline; (4) theobromine; (5) 3-methylxanthine; (6) 1,3-dimethyluric acid; (7) 1-methyluric acid. The number in the beginning of each chromatogram indicates the ion (m/z) monitored. The arrows indicate the switching of ion sets monitored.*

with the exception of 3-methylxanthine in urine, in which the slope was lower than that in water.

Analysis of Urine Samples—In a disposable tube (12 × 75 mm), urine (0.5 ml) was mixed with 20 μl of the internal standard solution (2.14 μg of caffeine-d_3). It was then evaporated to dryness at 40° under vacuum in an evaporator (~15 min). Powdered potassium carbonate (5 mg), dimethylformamide (0.25 ml), and 1-iodopropane (50 μl) were added to the residue. The procedure for the standard curve was then followed.

Analysis of Plasma Samples—Plasma (50–100 μl) was mixed with 20 μl of internal standard solution (0.214 μg of caffeine-d_3). The plasma proteins were then precipitated by the addition of methanol (1 ml). After mixing and keeping in an ice bath for 10 min, the mixture was centrifuged and the supernatant was decanted into another disposable tube. It was evaporated in a stream of air, then to dryness at 40° in vacuum in an evaporator. The dried residue was then mixed with powdered potassium carbonate (5 mg), dimethylformamide (0.25 ml), and 1-iodopropane (50 μl). The procedure for the standard curve was then followed.

Analysis of Red Blood Cells—After separating the plasma, the red blood cells were washed twice with normal saline. An aliquot (100 μl) of this cell sample was then mixed with the internal standard solution (0.214 μg of caffeine-d_3 in 20 μl of water), and methanol was added (1 ml) to hemolize the blood cells and to precipitate the proteins. After centrifuging, the supernatant was transferred to another tube and the plasma procedure commenced.

Gas Chromatography—A conventional gas chromatograph[7] was used. It was equipped with a flame ionization detector. The glass column (0.3 cm × 1.83 m) was packed with 50% phenyl silicone and 50% methyl silicone on treated white diatomaceous earth[8]. Nitrogen was used as a carrier gas at 30 ml/min. The column was maintained at 200°. The injection port temperature was 250° and the detector was 350°.

Gas Chromatography–Mass Spectrometry—A computerized GC–MS[9] was used. The GC condition was the same as described in the previous section except that helium was used as carrier gas instead of nitrogen. A glass jet separator, maintained at 250° was used between the GC and the MS ion source, which was maintained at 200°. The analyzer region was kept at 200°. Electron-impact ionization at 70 eV was used for the study. The derivatized sample was dissolved in 50 μl of methylene chloride. After mixing well, the mixture was allowed to stand until the undissolved solid settled at the bottom. An aliquot (1–3 μl) of the supernatant was injected onto the GS–MS. The selected ion-monitoring software provided by the instrument manufacturer was used for the analysis. From 2.5 to 7.5 min, the instrument was focused alternately on *m/z* 194, 197, 222, and 250 with a dwelling time of 100 msec on each. After 7.5 min, the ion fragments *m/z* 280 and 308 were monitored with a dwelling time of 100 msec on each. The total analysis time was 12 min. The standard curves were constructed by plotting the area ratio of each individual ion (caffeine-194, theophylline-222, 3-methylxanthine-250, 1,3-dimethyluric acid-280, and 1-methyluric acid-308) with internal standard (caffeine-d_3-197), against the amounts of each individual metabolite. Regression analysis of the standard curve and data reduction of samples were done with a calculator[10].

Table I—Theophylline and Metabolites in Urine, Plasma, and Red Blood Cells of a Premature Infant

Samples	Metabolites[a], μg/ml					
	I	II	III	IV	V	VI
Urine	2.20 (8.7%)[b]	12.10 (51.5%)	1.70 (7.2%)	0.38 (1.8%)	5.58 (21.8%)	2.14 (9.0%)
Plasma	2.41 (25.5%)	5.70 (64.8%)	0.4 (4.6%)	0.08 (1.0%)	0.3 (3.1%)	0.1 (1.1%)
Red Blood Cells	0.54 (28.7%)	1.00 (57.4%)	0.1 (5.7%)	0.08 (5.0%)	0.05 (2.6%)	0.01 (2.6%)

[a] Metabolites: I, caffeine; II, theophylline; III, theobromine; IV, 3-methylxanthine; V, 1,3-dimethyluric acid; VI, 1-methyluric acid. [b] Values in parentheses are the percentage of total metabolites.

RESULTS AND DISCUSSION

Theophylline is metabolized extensively to 3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid in human adults (5, 6). The current observation that theophylline is also methylated to another pharmacologically active xanthine, caffeine, in premature infants (4, 7, 8) makes it necessary to study the metabolism of theophylline in this population.

There are several published methods capable of measuring theophylline and its metabolites (3, 6, 9). The older method of column chromatography and UV spectrophotometry is too tedious to be useful in the assay of large numbers of samples. The HPLC method described previously (5) lacks the sensitivity required in the analysis of pediatric samples. The preliminary report of another HPLC method (9) has the required sensitivity and simplicity. However, no specificity and reproducibility of the method was reported. To achieve the specificity and sensitivity

[7] Model 5840A, Hewlett-Packard Co., Avondale, Pa.
[8] 3% OV-17 on gas chrom Q, 100/120 #, Applied Science, State College, Pa.
[9] Model 5985A gas chromatograph-mass spectrometer with a Model-7906 disc drive, Hewlett-Packard Co., Palo Alto, Calif.
[10] Model 9830A calculator, Hewlett-Packard Co., Loveland, Col.

Table II—Precision of GC–MS Analysis of Theophylline and Metabolites [a]

Analysis	Metabolites,[b] μg/ml				
	I	II	IV	V	VI
1	0.91	2.97	0.011	1.15	0.60
2	0.91	2.81	0.017	1.14	0.57
3	0.93	2.87	0.014	1.15	0.61
4	0.94	2.83	0.011	1.17	0.61
5	0.91	2.80	0.011	1.23	0.66
Mean ± *SD*	0.92 ± 0.01	2.86 ± 0.07	0.013 ± 0.003	1.17 ± 0.04	0.61 ± 0.03
CV,[c] %	1.1	2.4	23.1	3.4	4.9

[a] Urine sample from a premature infant maintained on aminophylline. [b] Metabolites: See Table I. [c] Coefficient of variation.

required in the analysis of pediatric samples, a GC–MS method with selected ion monitoring technique was chosen. The high specificity obtained by this technique can also reduce the time required for sample purification and, thus, facilitate the analysis of large numbers of samples.

Extraction of Theophylline and Metabolites from Biological Fluids—A number of solvent systems have been used to extract theophylline or its metabolites from biological fluids (3, 9, 10). The extracted metabolites are then analyzed with GC, HPLC, or GC–MS. For less specific quantitative methods, solvent extraction is a necessary step in the procedure to eliminate the possible interfering substances. These solvent-extraction procedures can be grouped into three categories. The most primitive one is the simple solvent extraction with methylene chloride or chloroform (3, 11). A second approach has been the use of a more polar solvent mixture in conjunction with the saturation of biological fluids with salt (9). The third approach is the extractive alkylation procedure with alkyl iodide and hydrophobic quaternary ammonium ion (10). For the analysis of the true composition of metabolites in a sample, the extraction steps should not discriminate among different compounds and distort the results. Caffeine, theophylline, methylxanthines, and methyluric acids are compounds with widely varying polarity and solvent solubility. The simple solvent extraction procedure is not expected to extract any significant amounts of methylxanthines and methyluric acids. In numerous attempts in this laboratory, extractive alkylation with a high concentration of tetrabutylammonium ion in aqueous solution and iodopropane in chloroform failed to recover polar metabolites such as 3-methylxanthine and monomethyluric acids. The recovery experiments using a modified procedure (9) which involved saturating the aqueous standard solution with ammonium sulfate and extracting with a chloroform–isopropyl alcohol mixture, gave the following results (compared with direct derivatization): caffeine, 104 ± 1%; theophylline, 23 ± 3%; 3-methylxanthine, 6 ± 3%; 1,3-dimethyluric acid, 9 ± 3%; and 1-methyluric acid, 5 ± 2% (mean ± *SD*, $N = 3$). The lower recovery obtained in these experiments compared with those reported previously (9) probably resulted from the smaller amount of extraction solvent used (20% of the original solvent volume but extracted twice). Nevertheless, these data did indicate a differential extraction of metabolites, even with the most strenuous extraction procedure, *i.e.*, salt saturation and polar solvents. To overcome the difficulty of low and variable recovery of different metabolites, no solvent extraction was used in the procedure described in this report. The urine samples were dried directly and then derivatized, while the plasma samples were deproteinized with methanol and prepared as the urine samples.

Derivatization with Iodopropane and Sodium Carbonate in Dimethylformamide—For GC analysis, theophylline and metabolites are usually derivatized into *N*-alkylated derivatives such as butyl or pentyl derivatives. The advantage of using the *N*-propyl derivative over other alkylated derivatives has been discussed previously (12). The fastest and most convenient derivatization procedure was found to be the use of iodopropane in a polar solvent, such as dimethylformamide, and a basic catalyst, such as potassium carbonate. This reaction was rapid and was completed within 5 min at 70°. When the reaction mixture was analyzed at different reaction times, the intensity of all the derivatized metabolites did not change with heating from 5 min to 1 hr. This indicated not only that the derivatization procedure was completed within 5 min, but also that the derivatives were stable in the reaction mixture for at least 1 hr. After the reaction, the reagents were evaporated in vacuum. The residue was extracted with methylene chloride (50 μl) before analysis with GC–MS. For lower concentration determinations, larger volumes of methylene chloride can be used to extract the solid residue. The combined extract is then evaporated and reconstituted with a smaller volume of solvent. Water cannot be used to wash the methylene chloride solution of the derivatized sample, since this procedure results in differential extraction. Caffeine has higher water solubility than any other *N*-propylated xanthines and uric acids. Because caffeine-d_3 was used as the internal standard in the procedure, the disproportional removal of caffeine from methylene chloride extract resulted in a falsely higher result for theophylline, 3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid.

Gas Chromatography–Mass Spectrometry—The ideal internal standards for the GC–MS method are the stable isotope-labeled analogues of each metabolite. However, such a task would be impractical for extensively metabolized drugs such as theophylline. Therefore, caffeine-d_3 (I) was chosen as an internal standard for all metabolites. Caffeine-d_3, can be synthesized easily from theophylline and iodomethane-d_3 in 1 hr. It is obtained in high isotopic purity ($do = 0.09\%$).

The *N*-propyl derivatives of theophylline metabolites were well resolved on a 3% mixed phase of phenyl silicone and methyl silicone column in a period of 12 min. Furthermore, their 70-eV electron-impact mass spectra showed prominent molecular ions (12). Therefore, the molecular ions were monitored using a selected ion-monitoring technique. The ion current ratio of the metabolite's molecular ion with that of internal standard (m/z 197) was plotted against the amount of metabolites. The standard curves obtained were linear over a range of 1 ng to 2 μg. However, the detection limit can be lowered further to 500 pg for caffeine and theophylline. The detection limit of other metabolites (3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid) were in the range of 1–20 ng. The lower sensitivity of this procedure toward monomethylxanthine and uric acid metabolites was due to the fact that the derivatives of these metabolites gave molecular ions with lower abundances as a result of more extensive fragmentation.

A typical selected ion chromatogram of urinary theophylline metabolites of premature infants is shown in Fig. 1. The metabolite patterns of theophylline in plasma, urine, and red blood cells taken at the same

Table III—Recovery of GC–MS Procedure for Theophylline and Metabolites in Urine

Samples	Metabolites, μg[a]				
	I	II	IV	V	VI
Urine[b]	0.93	3.50	0.05	1.86	1.48
Urine + Standard 1[c]	1.12	5.30	0.19	2.11	1.54
	(102%)	(110%)	(86%)	(97%)	(93%)
Urine + Standard 2[d]	2.65	16.65	0.98	4.78	3.24
	(102%)	(101%)	(55%)	(95%)	(100%)
Urine + Standard 2[d]	2.65	16.65	0.95	4.78	3.28
	(102%)	(101%)	(54%)	(95%)	(101%)
Recovery Mean ± *SD*	102 ± 0%	104 ± 5%	65 ± 18%	96 ± 1%	98 ± 4%

[a] Metabolite designations (see Table I). [b] Urine (0.5 ml) of a premature infant on theophylline treatment. [c] Standard 1 = caffeine (0.17 μg), theophylline (1.33 μg), 3-methylxanthine (0.17 μg), 1,3-dimethyluric acid (0.32 μg), 1-methyluric acid (0.18 μg). [d] Standard 2 = caffeine (1.68 μg), theophylline (13.34 μg), 3-methylxanthine (1.72 μg), 1,3-dimethyluric acid (3.19 μg), 1-methyluric acid (1.77 μg).

Table IV—Recovery of GC–MS Procedure for Theophylline and Metabolites in Plasma and Red Blood Cells

Samples	Metabolites, μg[a]				
	I	II	IV	V	VI
Plasma Blank[b]	0	0	0.02	0.01	0.01
Plasma + Standard 1[c]	0.35	2.16	0.38	0.60	0.37
	(95%)	(97%)	(103%)	(102%)	(100%)
Plasma + Standard 1[c]	0.36	2.21	0.38	0.57	0.37
	(97%)	(99%)	(103%)	(97%)	(100%)
Plasma + Standard 2[d]	1.74	11.26	1.74	2.77	1.67
	(94%)	(101%)	(98%)	(95%)	(92%)
Cell Blank[e]	0	0	0	0	0
Cells + Standard 1[c]	0.40	2.06	0.30	0.60	0.32
	(108%)	(92%)	(86%)	(103%)	(89%)
Cells + Standard 3[f]	0.07	0.44	0.06	0.11	0.07
	(100%)	(98%)	(86%)	(92%)	(100%)
Average Recovery: Mean ± *SD*	99 ± 6%	97 ± 3%	95 ± 9%	98 ± 5%	96 ± 5%

[a] Metabolite designations (see Table I). [b] Plasma (100 μl) of an adult man without coffee or tea intake. [c] Standard 1 = caffeine (0.37 μg), theophylline (2.23 μg), 3-methylxanthine (0.35 μg), 1,3-dimethyluric acid (0.58 μg), 1-methyluric acid (0.36 μg). [d] Standard 2 = caffeine (1.85 μg), theophylline (11.15 μg), 3-methylxanthine (1.75 μg), 1,3-dimethyluric acid (2.91 μg), 1-methyluric acid (1.80 μg). [e] Washed red blood cells (100 μl). [f] Standard 3 = caffeine (0.07 μg), theophylline (0.45 μg), 3-methylxanthine (0.07 μg), 1,3-dimethyluric acid (0.12 μ), 1-methyluric acid (0.07 μg).

time interval are shown in Table I. The details of this study have been reported elsewhere (12, 13).

Using this sensitive and specific method, it was shown for the first time that some of the 3-methylxanthine and 1-methyluric acid had a possible endogenous origin. In addition, the oxidation pathways of theophylline were quantified and shown to be important. Substantial amounts of theophylline are converted to 1,3-dimethyluric acid in premature infants, while the metabolic capability of converting theophylline to 3-methylxanthine and 1-methyluric acid is relatively undeveloped. The unusual metabolic conversion of theophylline to caffeine in premature infants is also confirmed.

Precision and Recovery of the Method—The precision of this method in analyzing theophylline metabolites is shown in Table II. The analysis of caffeine had the lowest coefficient of variation (*CV*) partly due to the fact that the stable isotope-labeled internal standard of caffeine was used in the procedure. This internal standard compensated for the variation due to incomplete recovery and instrumental instability. With the exception of 3-methylxanthine, the coefficients of variation of analyses are all within 5%. The high variation on 3-methylxanthine analysis was due to the fact that its concentration in the urine of premature infants was low as compared with other metabolites.

The recovery of theophylline metabolites was studied by spiking urine, plasma, and red blood cell samples with solutions of known amounts of metabolites. The samples were analyzed before and after spiking. The results are shown in Tables III and IV. With the exception of 3-methylxanthine, the recovery of all metabolites was essentially quantitative. The lower urinary recovery of 3-methylxanthine at a higher concentration is probably due to incomplete alkylation under experimental conditions. As discussed before, all theophylline metabolites from aqueous solutions were alkylated quantitatively within 5 min of the reaction time. The slower reaction time of 3-methylxanthine in urine is unclear at the present time. It could be partly due to the buffer action of urinary electrolytes, which reduce the effectiveness of catalysis by potassium carbonate. This hypothesis was supported by the fact that recovery of 3-methylxanthine was reduced further without affecting the recovery of all other metabolites when the amount of potassium carbonate was lowered.

Attempts were made to improve the recovery of 3-methylxanthine by increasing the amount of potassium carbonate and *n*-propyl iodide or by increasing the reaction time. The recovery of urinary 3-methylxanthine did improve with these experiments. However, quantitative recovery was never obtained. Because increasing the reagents and the reaction time increases the possibility of interfering peaks without correcting the problem of incomplete recovery of 3-methylxanthine, this modification of the procedure was abandoned.

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