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# SULFONIUM SALTS AS DERIVATIZING AGENTS

# 2[a] DETERMINATION OF THEOPHYLLINE IN PLASMA BY AUTOMATED GAS CHROMATOGRAPHY

BELLE L LEE, PEYTON JACOB, III* and NEAL L BENOWITZ

*Division of Clinical Pharmacology of the Department of Medicine, University of California, San Francisco General Hospital Medical Center, San Francisco, CA 94110 (U S A )* and Langley Porter Psychiatric Institute, University of California, San Francisco, San Francisco, CA 94143 (U S A )*

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## SUMMARY

An automated gas chromatographic method for determination of theophylline in plasma is described A novel feature of this assay is the use of triethylsulfonium hydroxide as an on-column alkylating agent A simple extraction procedure together with automated sample injection and data processing has facilitated processing of large numbers of samples generated in pharmacokinetic studies The between-run coefficient of variation was 2 2% at 5 μg/ml ($n=5$) and 3 9% at 15 μg/ml ($n=6$) Within-run coefficient of variation was 2 4% at 5 μg/ml ($n=9$) and 3 3% at 15 μg/ml ($n=6$) Accuracy was 105% at a concentration of 5 μg/ml and 102% at 15 μg/ml

## INTRODUCTION

Theophylline is an important drug commonly used in the treatment of asthma and chronic obstructive pulmonary disease Since theophylline has a narrow therapeutic index and the rate of clearance varies considerably among individuals [1], plasma level monitoring is necessary to determine the optimum dosing regimen. A variety of methods for the determination of theophylline in biologic specimens have been reported in the literature Methods include ultraviolet spectrophotometry [2], enzyme immunoassay [3–5], liquid chro-

[a]For Part 1, see ref 30

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matography [6–15], gas chromatography (GC) using a nitrogen-selective detector [16–18], flame ionization detection [19–22] and combined gas chromatography–mass spectrometry (GC–MS) [23–25]

In pharmacokinetic studies of theophylline in humans, a method of quantitation suitable for large numbers of samples and sensitive enough to measure low concentrations in small plasma samples several hours after a single dose was needed. This paper describes a novel procedure for GC determination of theophylline in plasma using on-column alkylation with triethylsulfonium hydroxide The method is sensitive enough to measure subtherapeutic concentrations of theophylline in 100-$\mu$l plasma samples, and the simplicity of the extraction procedure and automation of GC allows rapid processing of large numbers of samples

## EXPERIMENTAL

### *Chemicals and reagents*

Theophylline and metabolites were purchased from Sigma (St Louis, MO, U S.A.) and 3-isobutyl-1-methylxanthine (internal standard) was from Aldrich (Milwaukee, WI, U.S A ) Potassium phosphate monobasic from Mallinckrodt (St Louis, MO, U S A ) and silver oxide from General Chemical (New York, NY, U S A ) were reagent grade. Isoamyl alcohol was Baker analyzed reagent, methanol and methyl-*tert* -butyl ether were HPLC grade from Fisher Scientific (Fairlawn, NJ, U S A ) and isopropanol was HPLC grade from Burdick and Jackson (Muskegon, MI, U S A )

### *Synthesis of triethylsulfonium hydroxide*

A 1-g amount (4 mmol) of triethylsulfonium iodide [26] was dissolved in 8 ml of methanol, and 0 5 g (2 mmol) of silver oxide was then added The solution was stirred until the black silver oxide was converted to a greyish precipitate of silver iodide (about 1 h) The salt was centrifuged, and the solution was pipetted into a clean culture tube and stored in a freezer a 4 °C The solution was diluted to a concentration of 0 1 *M* with isoamyl alcohol prior to carrying out analyses The reagent was found to be stable for periods of up to three months stored at 4 °C

### *Extraction procedure*

To 100-$\mu$l aliquots of plasma samples, standards and controls were added the internal standard, 3-isobutyl-1-methylxanthine (100 $\mu$l of 10 $\mu$g/ml), and 0 5 ml of 1 *M* potassium phosphate monobasic Each was extracted with 2 5 ml of methyl-*tert* -butyl ether–isopropyl alcohol (80 20, v/v) in a 100 mm×13 mm screw-top glass culture tube The tubes were mixed for 3 min using a multitube vortex mixer After centrifuging for 10 min at 700 *g* to break emulsions, the tubes were placed in a dry ice–acetone bath for 3 min to freeze the aqueous layers The organic layers were poured into clean tubes and the extracts were

evaporated to dryness with a gentle current of nitrogen, heating in a water bath at 60°C. The extracts were reconstituted with 0.5 ml of the derivatizing agent, 0 1 *M* triethylsulfonium hydroxide in isoamyl alcohol The tubes were mixed briefly and the extracts were poured into autosampler vials and analyzed by GC or GC–MS The extracts were stable for up to 24 h at room temperature, shown by reinjecting standards and controls at the end of a run

*Gas chromatography*

GC analyses were performed using a Hewlett-Packard Model 5880A instrument equipped with nitrogen–phosphorus detectors, a Model 7672 automatic sampler, split–splitless capillary inlet system and Level IV computing integrator A 25 m×0.32 mm I.D fused-silica capillary column, coated with cross-linked 5% phenylmethylsilicone (0 52 μm film thickness), was attached to a glass injection port liner containing a small plug of glass wool The carrier gas (helium) flow-rate was 2 ml/min, detector make-up gas (helium) flow-rate was 25 ml/min and the detector air and hydrogen flow-rates were 50 and 4 ml/min, respectively The injection port temperature was 250°C, the detector temperature 300°C, and the oven temperature was programmed from 90 to 275°C at 25°C/min A 1-μl aliquot of extract was injected in the splitless mode, with the injection port purge time set at 0 5 min

*Calibration procedure*

Stock solutions (1 mg/ml) of theophylline were prepared in methanol–water (50·50, v/v) and stored at 4°C. These solutions were diluted to appropriate concentrations, 2–20 μg/ml theophylline, with human plasma from a local blood bank which had been shown to be free of theophylline Standard curves were constructed by plotting the peak-height ratio of theophylline and internal standard versus theophylline concentration A standard in the middle of the expected concentration range (10 μg/ml) was reinjected and the computing integrator was calibrated using the internal standard method For plasma theophylline, standards of 2, 5, 10 and 20 μg/ml were analyzed for each set of samples A batch of controls prepared by spiking plasma with 5 and 15 μg/ml theophylline as well as blank plasma were included in each run This was used to determine the day-to-day and within-run precision

*Gas chromatography–mass spectrometry*

A Hewlett-Packard 5890A gas chromatograph with a capillary direct interface to a Hewlett-Packard 5970B quadrupole mass-selective detector was used for the GC–MS analyses A 12 m×0 21 mm I D fused-silica capillary column coated with cross-linked 5% phenylmethylsilicone, 0 33 μm film thickness, was used with helium carrier gas at a flow-rate of 1 ml/min Injections of 1 μl were made in the splitless mode at an injection port temperature of 250°C After a 1-min hold, the column oven temperature was programmed from 70 to 145°C

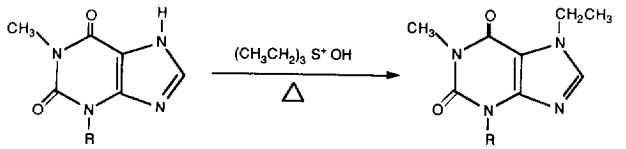
at 60°C/min and then from 145°C to 230°C at 20°C/min The mass spectrometer was calibrated with perfluorotributylamine at masses of 69, 219 and 502 using the Hewlett-Packard software Autotune program. Ionization was carried out by electron impact at 70 eV Spectra for the ethyl derivatives of theophylline, theobromine and paraxanthine standards were obtained scanning the mass range of 35–250 a.m u at a scan rate of 400 a m u./s Quantitative analyses were carried out by selected-ion monitoring (SIM) of *m/z* 95 and 208 for the theophylline derivative and *m/z* 250 for the internal standard, in two time-programmed groups, at a mass peak width of 0.9 a m u and a dwell time of 75 ms MS data was stored and processed with a Hewlett-Packard 59970 Chem Station The data system was used to construct and integrate the ion chromatograms Theophylline concentrations were determined using standard curves obtained by linear regression of peak-area ratios of theophylline (*m/z* 95 or 208)/internal standard (*m/z* 250).

## RESULTS AND DISCUSSION

On-column alkylation has been used to convert various weak acids to derivatives which have better chromatographic properties than the parent compounds. Generally, this has involved formation of an ion pair with a tetraalkylammonium cation, followed by injection into a gas chromatograph, which results in formation of the alkylated derivative in the heated injection port In this way, carboxylic acids, phenols and a variety of compounds possessing acidic N–H bonds, such as barbiturates and many anticonvulsants, have been converted to alkyl derivatives [27] An advantage of this technique is simplicity, since a separate derivatization step is not required. Unfortunately, in some cases the utility is limited by formation of reagent-derived by-products which may interfere with the analysis, such as formation of dimethylaniline from phenyltrimethylammonium hydroxide, by incomplete derivatization or by decomposition induced by the strongly basic derivatizing agent [27,28]

It occurred to us that trialkylsulfonium salts might have advantages over the more commonly used tetraalkylammonium salts The reagent by-products, dialkyl sulfides, are less polar and more volatile than the tertiary amines formed by thermal decomposition of tetraalkylammonium hydroxides In addition, the sulfonium salts are more potent alkylating agents, and lower injection port temperatures can be employed, which may increase yields and prevent side-reactions [29] Whereas the tetraalkylammonium salts have been used primarily for methylation, we have found that the more reactive sulfonium salts can be used to efficiently transfer higher alkyl groups [30] In the present study, both theophylline and the internal standard, 3-isobutyl-1-methylxanthine, were readily converted to the corresponding 7-ethyl derivatives at an injection port temperature of 250°C (Fig 1).

The method described in this paper was designed for determination of large




Theophylline, R = $CH_3$
Internal Standard, R = $CH_2CH(CH_3)_2$

Fig 1 Conversion of theophylline and the internal standard, 3-isobutyl-1-methylxanthine, to 7-ethyl derivatives

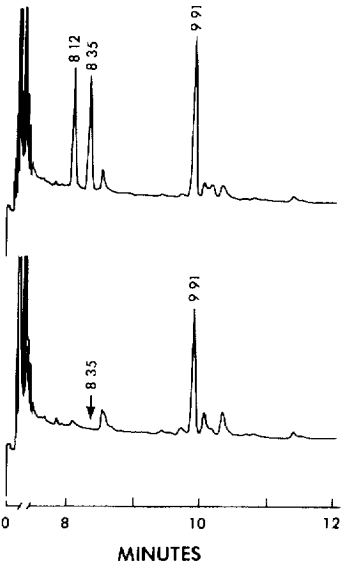



Fig 2 Chromatogram of a blank (lower trace) and a subject's (upper trace) plasma Retention times are theophylline, 8 35 min, 3-isobutyl-1-methylxanthine, 9 91 min, caffeine, 8 12 min

numbers of samples generated in pharmacokinetic studies The one-step extraction procedure is convenient for batch processing of large numbers of samples, and with an automatic sampler up to 100 samples can be analyzed in a 24-h period The ethyl derivatives of both theophylline and the internal standard gave sharp, symmetrical peaks on a fused-silica, 25 m × 0.32 mm I D., 5% phenylmethylsilicone capillary column (Fig 2) The major metabolites of caffeine and theophylline, 1,7-dimethylxanthine (paraxanthine), 3,7-dimethylxanthine (theobromine), 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1,3,7-trimethyluric acid, 1,3-dimethyluric acid, 1,7-dimethyluric acid, 3,7-dimethyluric acid, 1-methyluric acid, 3-methyluric acid and 7-methyluric acid were tested for interference Caffeine, the uric acid derivatives and monomethylxanthine derivatives were well separated from theophylline and the internal standard The dimethylxanthines theobromine and paraxanthine were not completely resolved from theophylline and could cause some inter-

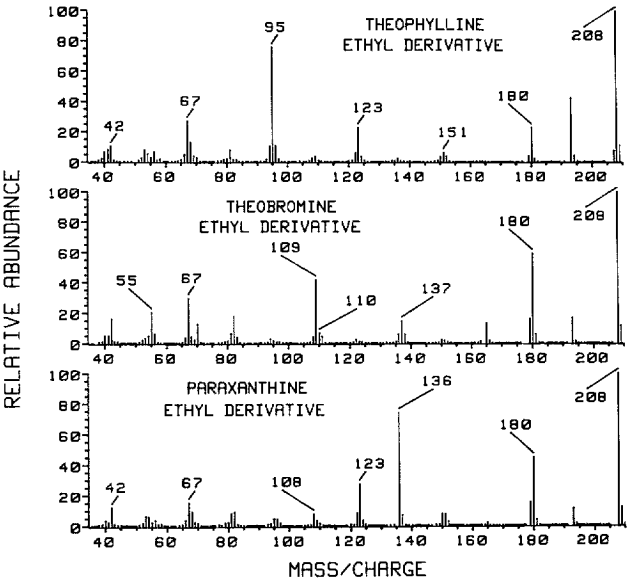



Fig 3 Mass spectra of the ethyl derivatives of theophylline, paraxanthine and theobromine

TABLE I

CONCENTRATION OF THEOPHYLLINE IN PLASMA OF FOURTEEN PATIENTS UNDERGOING THEOPHYLLINE THERAPY DETERMINED BY GC, HPLC AND GC–MS

| Sample | Concentration ($\mu$g/ml) | | | |
|---|---|---|---|---|
| | GC | HPLC[a] | GC–MS | |
| | | | *m/z* 208 | *m/z* 95 |
| T18980 | 11 5 | 13 4 | 12 1 | 11 2 |
| T19004 | 13 5 | 11 8 | 13 0 | 10 3 |
| T19807 | 8 9 | 8 8 | 8 4 | 8 3 |
| T19954 | 13 3 | 14 5 | 13 8 | 12 4 |
| W14931 | 18 2 | 20 5 | 19 4 | 17 6 |
| W14969 | 17 1 | 20 8 | 18 6 | 16 9 |
| W16076 | 20 6 | 23 2 | 22 1 | 19 2 |
| H12042 | 20 4 | 24 0 | 21 5 | 19 9 |
| H11848 | 17 4 | 21 3 | 18 0 | 17 2 |
| W21061 | 23 6 | 27 1 | 24 6 | 21 7 |
| W21405 | 11 9 | 12 1 | 11 1 | 10 6 |
| W19436 | 20 2 | 20 3 | 20 4 | 18 0 |
| H17807 | 20 6 | 23 8 | 20 9 | 19 4 |
| H17817 | 7 6 | 8 1 | 7 8 | 7 8 |
| Mean | 16 1 | 17 8 | 16 6 | 15 0 |
| Correlation with GC ($r^2$) | – | 0 951 | 0 988 | 0 965 |

[a]The HPLC determination was a modification of the method of Tang et al [9]

ference if present in high concentrations The sensitivity of the assay is sufficient for determination of subtherapeutic concentrations (0.5 μg/ml) and accurate determination of terminal half-life

Fig 2 shows a chromatogram of a blank and a subject's plasma Retention times are 8 35 min for theophylline and 9 91 min for the internal standard Caffeine, which was found in most specimens, had a retention time of 8 12 min Drug concentration and peak-height ratios were linearly related in the range 0 5–20 μg/ml. The between-run coefficient of variation was 2 2% at 5 μg/ml ($n=5$) and 3 9% at 15 μg/ml ($n=6$) Within-run coefficient of variation was 2 4% at 5 μg/ml ($n=9$) and 3 3% at 15 μg/ml ($n=6$) Accuracy, determined from the mean values obtained for spiked plasma controls, was 105% at a concentration of 5 μg/ml and 102% at 15 μg/ml.

Since the majority of published methods for theophylline determination utilize high-performance liquid chromatography (HPLC), it was felt that a comparison of results obtained by the method described in this paper with results obtained by HPLC would be worthwhile Plasma samples obtained from fourteen patients undergoing theophylline treatment were analyzed by GC and by the HPLC method of Tang et al [9] As a further test of the specificity of the method, the same samples were also analyzed by GC–MS with SIM of two

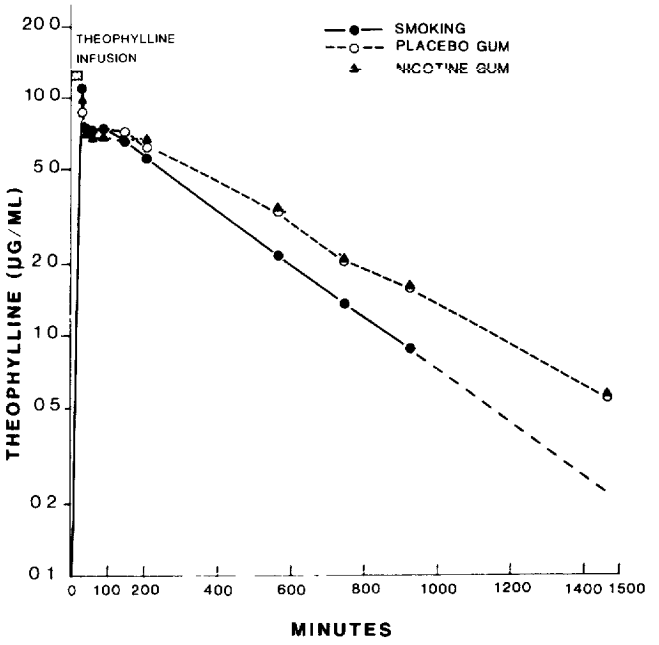



Fig 4 Semilogarithmic plot of plasma concentrations versus time in one subject who received 0 2 mg/kg theophylline per min intravenously for 30 min while smoking, chewing placebo gum and chewing nicotine gum

major ions (Fig. 3) produced by electron impact ionization of theophylline ethyl derivative: the molecular ion $m/z$ 208 and a major fragment $m/z$ 95. An advantage to monitoring the $m/z$ 95 fragment is that a fragment of this mass has low abundance in the mass spectra of the caffeine metabolites paraxanthine and theobromine (Fig. 3). Consequently, potential interference caused by the presence of these substances, which may not be completely resolved chromatographically, can be largely eliminated by basing the analysis on the $m/z$ 95 ion chromatogram. The results, presented in Table I, indicate a good correlation of our GC method with HPLC and GC–MS.

The assay has been used to study the pharmacokinetics of theophylline and the potential interactions of nicotine gum and theophylline in smokers [1]. Plasma theophylline concentration–time curves for one subject studied while smoking, chewing nicotine gum, and chewing placebo gum are shown in Fig. 4. Concentrations were readily determined for 24 h following an intravenous infusion at 0.2 mg/kg per min for 30 min.

## CONCLUSION

Advantages of the method described in this paper include the simplicity of the extraction procedure, a short chromatographic run time, good sensitivity and precision for small plasma samples, and automated sample injection and data processing. The method also demonstrates the utility of sulfonium salts as derivatizing agents for GC.

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