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MICRO METHOD FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF SERUM THEOPHYLLINE UTILIZING AN ORGANIC NITROGEN SENSITIVE DETECTOR*

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SUMMARY

A gas chromatographic micro method utilizing an organic nitrogen sentitive detector for the determination of serum theophylline is described. The method incorporates 3-isobutyl-1-methylxanthine as the internal standard and involves extraction and off-column derivatization of theophylline and the internal standard to their pentyl derivatives. Using 50 µl of serum, concentrations of 1 µg/ml in serum can easily be measured. The method is linear up to 50 µg/ml and the precision of the method is 3.4% in the therapeutic range. No interferences from endogenous compounds or from drugs commonly co-administered with theophylline have been encountered.

INTRODUCTION

Theophylline, 1,3-dimethylxanthine, is a bronchodilator extensively used in the treatment of asthmatics, many of whom are pediatric patients [1]. The serum therapeutic level of theophylline has been demonstrated to be 10—20 µg/ml [2]. At higher serum concentrations, theophylline toxicity is expressed by a number of symptoms, including nausea, irritibility and convulsions; overdoses have also proven fatal [3—5]. The need for an accurate determination of serum theophylline levels is, therefore, well established. Existing spectrophotometric [6—8] and gas chromatographic methods using a flame ionization detector require fairly large amounts of serum (1—3 ml) to achieve the desired sensitivity and precision, an impracticality in pediatric cases. Also in particular

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with the spectrophotometric procedures, there is the possibility of interferences from caffeine, theobromine and other xanthines as well as barbiturates that may be co-administered.

Recently, the obvious need for a sensitive, specific assay for theophylline has produced a relatively large number of new assays utilizing gas chromatography [9—12] or high-pressure liquid chromatography [13—16].

Our gas chromatographic method utilizes an organic nitrogen-sensitive detector which has greatly enhanced sensitivity for organic nitrogen- and phosphorus-containing materials but has a decreased sensitivity as compared with a flame ionization detector for simple carbon containing materials [17].

This detector has both the sensitivity needed to determine theophylline levels in a micro sample, as well as the selectivity necessary to simplify the extraction procedure since it has decreased sensitivity to non-nitrogen- and phosphorus-containing species.

The method involves a single extraction and derivatization procedure which allows relatively fast, accurate and precise determination of serum theophylline levels. To permit easy determination by gas chromatography, the volatilities of theophylline and the internal standard, 3-isobutyl-1-methylxanthine, are enhanced by pentylation [18]. A standard curve is constructed by plotting the ratio of peak heights of derivatized theophylline to derivatized internal standard against theophylline concentration; serum unknowns are determined by reading their values off the standard curve.

EXPERIMENTAL

Reagents

Trimethylanilinium hydroxide (TMAH), 25% in methanol was prepared from trimethylphenylanilinium iodide (Eastman, Rochester, N.Y., U.S.A.) as described by Skinner et al. [19].

Theophylline was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). A stock of 500 μg/ml was prepared by dissolving 100 mg of theophylline in 200 ml of deionized water. Working standards were prepared by diluting the stock standard with deionized water to give 1, 2.5, 5, 10, 15, 25, 35 and 50 μg of theophylline per ml. N, N-Dimethylacetamide, spectro grade and the internal standard, 3-isobutyl-1-methylxanthine (>99%) were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). The internal standard solution was prepared by dissolving 3-isobutyl-1-methylxanthine in 0.1 molar acetate buffer, pH 4.8. 1-Iodopentane was purchased from Eastman. All other solvents were of reagent grade.

Gas chromatography

A Perkin-Elmer dual column gas chromatograph, Model 3920, equipped with a phosphorus/nitrogen detector was used in all experiments. A glass 6 ft. × 2 mm I.D. 3% OV-17 on Gas-Chrom Q (100—120 mesh) (Applied Science Labs., State College, Pa., U.S.A.) column was found most suitable. The following gas chromatographic parameters were used: column temperature, 240° isothermal; detector temperature, 250°; injector temperature, 250°; helium flow-rate, 40 ml/min; amplifier range of 1 and electrometer attenuation setting at ×16;

recorder chart speed, 10 mm/min. The detector had a hydrogen flow of 1.5 ml/min, an air flow of 100 ml/min and course and fine current settings, to heat the bead, were 3 and 550, respectively. Under these conditions, the retention times were 1.5 min for theophylline and 1.9 min for the internal standard.

Procedure

Pipet 50 µl of serum or standard, 100 µl of acetate buffer containing the internal standard and 6 ml of dichloromethane into a Kimax 13 × 100 mm culture tube with PTFE-lined cap. Extract for 5 min on a wrist action shaker. Centrifuge for 2 min at 1000 *g* and remove the upper aqueous layer by aspiration and decant the dichloromethane layer into a clean, dry 15-ml conical centrifuge tube with PTFE-lined cap. Evaporate the dichloromethane to dryness by passing a stream of air into the tube placed in a 50° water-bath. Dissolve the dried samples in 50 µl of N,N-dimethylacetamide, add 10 µl TMAH and vortex for 1 sec to mix. Then add 10 µl 1-iodopentane, cap the tube, vortex again and incubate at room temperature for 10 min. Stop the reaction by adding 0.5 ml of cyclohexane—dichloromethane (95:5) to each tube and vortex 5 sec; a precipitate will form. Centrifuge at 1000 *g* for 2 min and transfer the cyclohexane—dichloromethane layer to a clean dry Kimax 13 × 100 mm culture tube with a glass disposable transfer pipette. Evaporate the organic solvent to dryness by passing a stream of air into the tube in a 50° water-bath. Redissolve each sample by adding 50 µl of methanol quickly capping each tube, and vortexing. Inject 1 µl of this methanol mixture into the gas chromatograph. The serum theophylline concentration is determined from a standard curve established by plotting the peak height ratios of standard to internal standard against the theophylline standard concentration.

RESULTS AND DISCUSSION

Fig. 1A illustrates a chromatogram of a serum free of theophylline without the internal standard added. Fig. 1B is the same serum spiked with theophylline and the internal standard added. Fig. 1C is the same serum with caffeine, theophylline, theobromine, internal standard and phenobarbital added. This figure illustrates the separation of common endogenous compounds which will be coextracted from serum with theophylline and the internal standard.

A typical standard curve is shown in Fig. 2. The linearity of the procedure was found to be good in the range of 0—50 µg/ml. The accuracy of the method was evaluated by recovery studies. Different amounts of theophylline were added to a theophylline free serum and the recovery calculated. Table I shows the results of the recovery study. The average recovery was 101% with a range of 97 to 106%.

The precision of the method was checked by analyzing 18 replicates of a serum sample from an asthmatic patient on chronic theophylline therapy. The mean serum concentration was found to be 14.7 µg/ml, with a standard deviation of 0.5 µg/ml and a coefficient of variation of 3.4%.

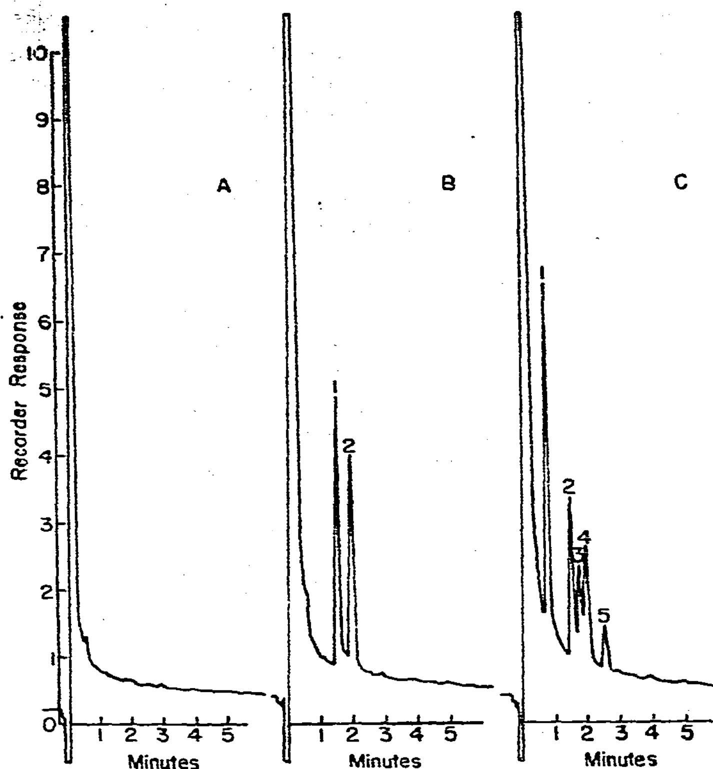

Fig. 1. Gas chromatograms for: (A) serum blank; (B) serum with theobromine (3), inter- internal standard (2); (C) serum with caffeine (1), theophylline (2), theobromide (3), internal standard (4), and phenobarbital (5) added.

TABLE I

RECOVERY OF ADDED THEOPHYLLINE FROM SERUM

Theophylline (μg/ml)		Recovery (%)
Added	Found	
5.0	5.3	106
15.0	15.0	100
25.0	25.4	102
35.0	34.0	97

Drug interferences were studied. Medications containing ephedrine, pseudo-ephedrine, phenobarbital, glyceryl gauiacolate, triprolidine, ampicilline, phenoxy-methylpenicillin, chlorpheniramine maleate and brompheniramine maleate, as well as pure theobromine, and caffeine were dissolved in water. Extraction, derivatization and gas chromatographic determination were performed as described. None of these compounds was found to interfere with the determination of theophylline.

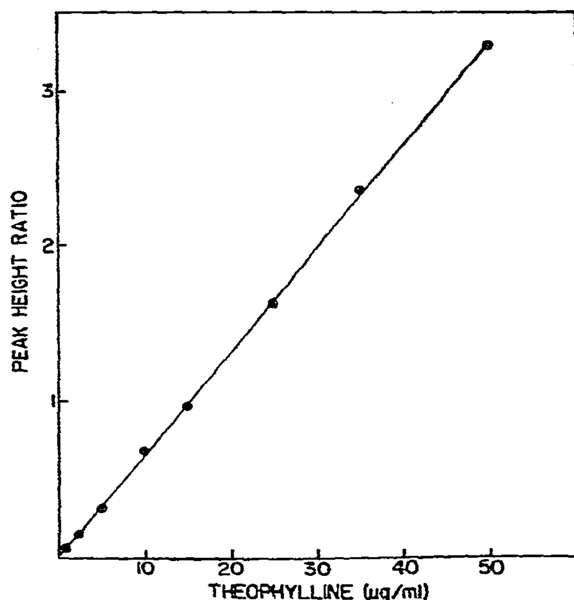

Fig. 2. Standard curve for theophylline.

Urinary metabolites of theophylline include 3-methylxanthine, 1-methyluric acid, and 1,3-dimethyluric acid [20]. In serum, however, only 3-methylxanthine has been detected in significant amounts [13]. Neither 3-methylxanthine nor uric acid was extracted under the above conditions.

With the micro method presented here, plasma or serum samples from patients receiving theophylline can be assayed rapidly and with good accuracy and precision.

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