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IMPROVED THEOPHYLLINE SERUM ANALYSIS BY AN APPROPRIATE INTERNAL STANDARD FOR GAS CHROMATOGRAPHY

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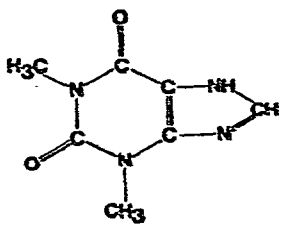
SUMMARY

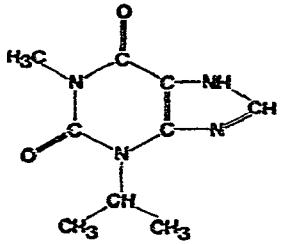
A gas chromatographic method is presented for the measurement of theophylline in serum. To serum samples containing the drug the internal standard, 3-isobutyl-1-methylxanthine, was added and the serum was saturated with ammonium carbonate. A mixture of isopropanol–chloroform (5:95) was used for extraction. The organic phase was filtered and evaporated. The residue, dissolved in chloroform, was extracted with sodium hydroxide. The basic solution was acidified, washed with hexane and re-extracted with isopropanol–chloroform. The organic phase was dried with anhydrous sodium sulphate before evaporation. The sample was derivatized with *n*-Butyl-8 reagent. 85% of theophylline was recovered by extraction. 3-Isobutyl-1-methylxanthine was recovered with the same efficiency as theophylline and gas chromatographed well. The standard curve was linear between 1.0 and 50.0 μg/ml theophylline. No interference was encountered from normal serum constituents or methylxanthines such as caffeine, theobromine, or 3-methylxanthine. Serum samples were stored in the refrigerator for two weeks without significant loss of drug. The precision and accuracy of the method were good.

INTRODUCTION

Maximizing the clinical usefulness of drugs often requires the measurement of serum concentrations[1]. This is particularly true for theophylline (1,3-dimethylxanthine), since both the therapeutic response and the toxic side effects are related to serum concentration rather than dosage[2].



THEOPHYLLINE



3-ISOBUTYL-1-METHYLXANTHINE

The classical spectrophotometric assay by Schack and Waxler[3] is insufficiently specific. Caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) from coffee, tea, chocolate and cocoa and barbiturates such as phenobarbital, frequently administered to patients with asthma, are also measured as theophylline.

The introduction of gas chromatographic (GC) techniques has led to improved selectivity of theophylline analysis. The formation of a butylated derivative[4–6] has eliminated many of the previously interfering compounds. However, GC procedures still possess certain shortcomings. One such inadequacy is the lack of a suitable internal standard[4,6–9] *i.e.*, a chemical which can be recovered from plasma with the same efficiency as theophylline and gas chromatographed with theophylline.

This paper improves on existing GC methods by incorporating an appropriate internal standard, *viz.* 3-isobutyl-1-methylxanthine. The sensitivity, selectivity, linearity, precision and accuracy of this assay are demonstrated. Serum stability is shown. Application of the technique to routine patient serum analysis has been successful.

MATERIALS AND METHODS

Reagents

Theophylline, caffeine and theobromine were purchased from Sigma (St. Louis, Mo., U.S.A.). 3-Isobutyl-1-methylxanthine and 3-methylxanthine were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). *n*-Butyl-8 concentrate was bought from Pierce (Rockford, Ill., U.S.A.). All solvents employed in the assay were distilled before use.

Stock solutions

Theophylline and 3-isobutyl-1-methylxanthine were dissolved in methanol to produce a 1000 μg/ml solution. Solutions were stable indefinitely.

Apparatus

A Packard (Downers Grove, Ill., U.S.A.) Model 417 Becker gas chromatograph with a flame ionization detector was used for all determinations. A Reacti-Therm heating module, used in the preparation of samples for GC, was purchased from Pierce.

Instrumental and chromatographic conditions

The chromatographic column was 10% (w/w) SE-30 on Chromosorb W-HP, 100–120 mesh (Chromatographic Specialties, Brockville, Ontario, Canada). The column material was packed in a glass column, 5 ft. × 0.25 in. O.D., previously treated with 5% dimethyldichlorosilane (Pierce) in toluene overnight. The column was held in the oven with graphite ferrules, "Graphloks" (Supelco, Bellefonte, Pa., U.S.A.), and normal Swagelok fittings. Microsep F-138 septums (Hamilton, Whittier, Calif., U.S.A.) were used.

The operating conditions were as follows: oven temperature, 200°; injector port temperature, 300°; detector temperature, 250°. The nitrogen and hydrogen flow-rates were 100 ml/min and the air flow-rate was about 1 l/min.

Column preparation and conditioning

The preparation of the column packing was modified after the method of Gehrke and Lakings[10]. The desired amount of SE-30 was dissolved in excess chloroform and added to a round-bottomed flask containing Chromosorb W-HP covered with chloroform. The flask was attached to a rotary evaporator and mixed for 15 min. The chloroform was evaporated under vacuum with heating to 60°. The column packing remained under high vacuum for an additional 30 min.

A glass column, the detector end plugged with ¼-in. glass-wool, was filled to the level where the column would enter the injector heater. A second plug of glass-wool was inserted there. The remaining space was filled with uncoated Chromosorb W-HP and a third plug held it in place. This procedure permitted on-column injection of the sample with no apparent bleeding of SE-30 from the injector port.

The column was conditioned by heating at 300° for 2 h under no-flow conditions followed by 24–48 h at 250° with a nitrogen flow-rate of 40 ml/min.

Solvent extraction of theophylline from serum

(1) A 2-ml volume of serum was placed in a 15-ml centrifuge tube, and a 30-μl aliquot of 3-isobutyl-1-methylxanthine stock solution was added. The sample was saturated with ammonium carbonate.

(2) The serum was extracted with a 5-ml volume of isopropanol–chloroform (5:95) by horizontal shaking for 5 min followed by centrifugation for 5 min. The organic layer was filtered through Whatman No. 1 filter paper into a 15-ml test tube and evaporated to dryness in a water-bath at 60° under dry nitrogen. Second and third extractions with subsequent evaporations were carried out.

(3) The residue was dissolved in 1 ml of chloroform and extracted with 1 ml of 0.5 *N* sodium hydroxide by gentle shaking on a vortex mixer and centrifugation for 5 min. Any emulsion formation was broken with warming in a water-bath prior to centrifugation. The aqueous layer was transferred to a 15-ml test tube. The extraction was repeated.

(4) The aqueous solution, acidified with 0.5 ml of 2 *N* hydrochloric acid, was washed with a 5-ml aliquot of hexane by shaking on a vortex mixer. The sample was centrifuged and the hexane layer was discarded. A second hexane wash was performed.

(5) A 5-ml aliquot of isopropanol–chloroform (5:95) was added and the sample was shaken on a vortex mixer. After a 5-min centrifugation the organic layer was transferred to a test tube and dried with 0.5 g of anhydrous sodium sulfate. Extraction of the aqueous layer was performed a second and third time. Subsequent extracts were dried, combined, and evaporated in a 15-ml centrifuge tube. The residue was washed to the bottom of the test tube with 0.5 ml of absolute ethanol and evaporated.

(6) The residue was dissolved in 25–40 μl of acetone stored over anhydrous sodium sulfate. A 25-μl aliquot of *n*-Butyl-8 concentrate was added and the sample was sealed and warmed for 5 min at 70° in a reacti-therm heating module to insure solution. 2–3 μl of sample were injected into the gas chromatograph.

RESULTS

Linearity of response and sensitivity

Fifteen serum samples spiked with theophylline concentrations ranging from

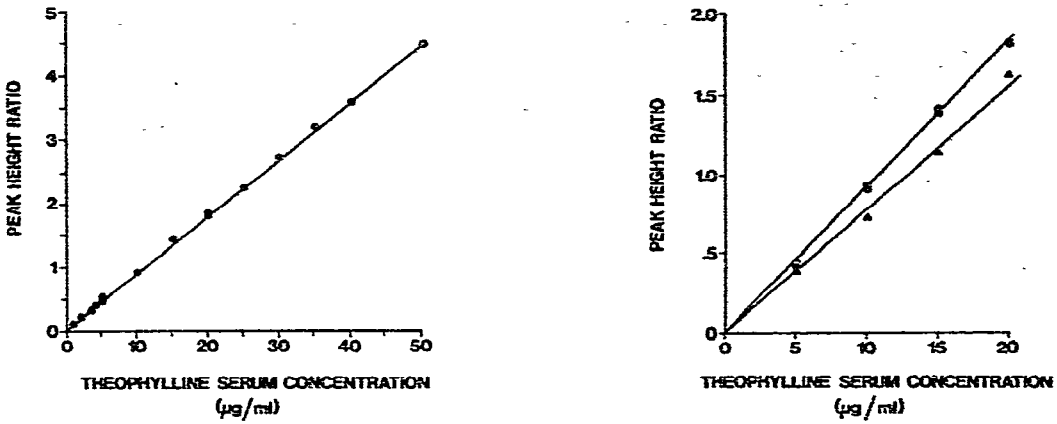


Fig. 1. Standard curve for the quantitative analysis of theophylline in serum.

Fig. 2. Determination of the absolute recovery of theophylline and the relative recovery of internal standard. (□) Theophylline and internal standard were chromatographed directly. (▲) Theophylline was extracted from serum prior to the addition of internal standard (●). Both theophylline and internal standard were extracted from serum.

1.0–50.0 μg/ml were assayed. The standard curve is shown in Fig. 1. The curve was linear over this range. The assay was fully sensitive to 1.0 μg/ml.

Recovery of theophylline and internal standard

Recovery of theophylline from serum is shown in Fig. 2. Recoveries were determined over the range of 5–20 μg/ml. Theophylline extracted from serum was compared to theophylline that was directly chromatographed. In both instances the internal standard was added just before GC. The average recovery of theophylline over the concentration range was 85%.

The recovery of the internal standard, 3-isobutyl-1-methylxanthine, relative to theophylline, is presented in Fig. 2. Both theophylline at 5–20 μg/ml and the internal standard at 15 μg/ml were added to the serum. The standard curve so constructed was compared to the standard curve when theophylline and the internal standard were gas chromatographed directly. The two lines were found to superimpose. Therefore, the internal standard was recovered with the same efficiency as theophylline.

Interfering substances

Caffeine, theobromine or 3-methylxanthine when gas chromatographed did not interfere with either theophylline or the internal standard. The presence of other agents which might invalidate the assay by imposing on the internal standard was also tested. Twenty patient samples were reanalyzed in the absence of internal standard. (Fig. 3). In all samples no peak was detectable in the region where 3-isobutyl-1-methylxanthine would have been chromatographed.

Precision and accuracy

The precision and accuracy of the method were determined by assaying six 2-ml serum samples to which 15.0 μg/ml of theophylline had been added. The mean value was 14.8 ± 0.3 (S.E.) μg/ml.

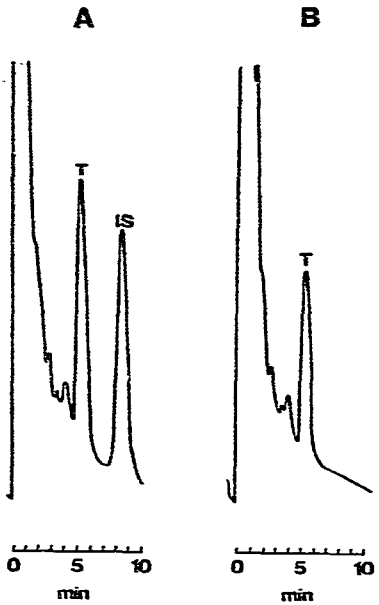


Fig. 3. GC tracing of theophylline (T) at 12.5 μg/ml and internal standard (IS) analyzed from a patient's serum. (A) Internal standard was added to the serum. (B) Internal standard was not added to the serum.

Stability

Six 2-ml serum samples at 15.0 μg/ml were stored at 5° for two weeks. The mean value was 15.1 $\pm$ 0.2 (S.E.) μg/ml.

DISCUSSION

This paper presents a new analytical procedure for measuring theophylline in serum. Good theophylline recovery (85%) by solvent extraction was achieved. The method had the desirable characteristics of good sensitivity (1.0 μg/ml), selectivity, precision, and accuracy.

This method improves on pre-existing methodologies by the incorporation of a suitable internal standard. The chemical, 3-isobutyl-1-methylxanthine, proved to be an excellent internal standard. Over a four-fold concentration range it was recovered from plasma with the same efficiency as theophylline. At isothermal operation it gas chromatographed well, appearing as a single symmetrical peak soon after theophylline.

The solvent extraction technique has certain improvements. The saturation of plasma with ammonium carbonate rather than acidification as used by others[3,8,9] prior to solvent extraction was beneficial. The ammonium carbonate buffers the plasma to a weakly alkaline pH, prevents emulsion formation even after vigorous shaking, creates a "salting-out" effect, and being volatile does not produce a residue. The consequences of this were good theophylline recovery and reduced extraction of extraneous material.

Theophylline does not gas chromatograph well unless it has been derivatized. The use of butylating agents has overcome this problem. It also enables separation of theophylline from caffeine, theobromine, and phenobarbital[4–6], and thereby eliminates several sources of error. However, caffeine, theobromine, and theophylline are metabolized to 1-methylxanthine, 7-methylxanthine, and 3-methylxanthine[11]. During derivatization these compounds might form a compound similar enough to the internal standard to possess like retention times. If the metabolites were present in sufficient quantities artificially low values of theophylline would be calculated. However, the metabolite of theophylline, 3-methylxanthine, when gas chromatographed, did not interfere. The other agents, 1-methylxanthine and 7-methylxanthine were not obtainable in pure form; however, results with patient sera indicate that interference with internal standard peak was never present. Twenty patients on unrestricted diets and with serum theophylline concentrations ranging from 1–62 μg/ml had their sera re-analyzed without the internal standard. At no time was a peak detectable in the region where the internal standard would have been eluted. At present, over fifty patient sera have been analyzed and no interfering substances have been detected.

The formation of a butylated derivative of theophylline with *n*-Butyl-8 is solvent and temperature dependent. Methanol when used as a solvent along with *n*-Butyl-8 produced incomplete derivatization. Acetone proved to be better but derivatization was still incomplete unless acetone was previously dried with anhydrous sodium sulfate. As a rule acetone is stored over anhydrous sodium sulfate.

Although Arbin and Edlund[4] reported that butylation of theophylline occurred instantaneously on mixing, our experience has not been the same. Samples exposed to the butylating solution and then evaporated to dryness did not show any derivative formation after injection into the gas chromatograph. Derivatization only occurred if the butylating solution was injected directly. The derivatives appeared to be formed on-column.

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