Micro-Scale Extractive Alkylation Procedure for the Gas-Chromatographic Measurement of Theophylline in Serum and Saliva, with Use of a Nitrogen Detector

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In this 10-min gas-chromatographic assay for theophylline, a 25-μl portion of serum or saliva sample, 100 μl of extraction reagent, and 1.0 ml of solvent were mixed, the solvent separated and evaporated, and an aliquot of the reconstituted extract injected into the gas chromatograph. At a concentration of 11 mg/liter, within-run precision (CV) was 3.6% and run-to-run 2.9%. At 22 mg/liter, within-run precision was 2.8%. Concentration and instrument response are linearly related between 2 and 40 mg/liter. The limit of detection was 0.4 mg/liter. Results correlated well (r = 0.98) with those by another micro-scale gas-chromatographic method. Analytical recovery was estimated to exceed 90%, and no interferences from other xanthines or related drugs were observed.

Most gas-chromatographic methods for theophylline have two disadvantages: poor extraction of the drug from serum, requiring large solvent/serum ratios (1), and the tedious derivatization and clean-up steps required before a nitrogen detector can be used (2, 3). The extractive alkylation technique (4, 5) overcomes these disadvantages. The following theophylline method, based on this technique, is simpler and faster than other published methods.

Materials and Methods
Reagents and Standards

Theophylline was a gift from Mallinckrodt Inc., St. Louis, Mo. 63042. 3-Isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, Wis. 53233; tetrahexylammonium hydrogen sulfate from Regis Chemical Co., Morton Grove, Ill. 60053. Methylene chloride, methanol, n-hexane, and 1-isodopentane were ACS or Reagent Grade. The extraction reagent was prepared by dissolving 90 mg of tetrahexylammonium hydrogen sulfate in 20 ml of 0.1 mol/liter aqueous sodium hydroxide. This solution was freshly prepared every 4 weeks and stored at 4 °C. Each morning a portion was removed for that day’s work, kept at room temperature for 8–10 h, and any that was left over was discarded. The solvent was a mixture of 200 ml of methylene chloride, 2.0 ml of 1-isodopentane, and 0.20 ml of a 1 g/liter solution of 3-isobutyl-1-methylxanthine (the internal standard) in methanol. This solution was stable for at least two months at 4 °C. The theophylline standard was prepared by adding 4.9 ml of fresh drug-free serum to 0.100 ml of stock theophylline solution, 1 g/liter in methanol. After the mixture was stirred at room temperature for 15 min, 0.10-ml aliquots were stored in well-stoppered tubes at −20 °C. A well-mixed pool of patients’ sera that had been submitted to the laboratory for theophylline analysis was divided into 0.10-ml aliquots, stored in well-stoppered tubes at −20 °C, and used as a control. The standard and control were stable for at least two months at −20 °C.

Gas Chromatography

The analyses were performed on a Perkin-Elmer Model 3920B gas chromatograph equipped with a nitrogen detector. The glass column, 180 × 0.2 cm (i.d.), was packed with 3% OV-17 on 100/120 Gas Chrom Q. The chromatographic conditions were: oven 240 °C, injector 250 °C, detector 280 °C, helium carrier gas flow 20 ml/min, hydrogen flow 5.5 ml/min, air flow 95 ml/min, potentiometer setting 450, attenuation 32 × 10.

Procedure

The standard and control tubes were thawed, centrifuged, and vortex-mixed thoroughly before use. Eppendorf pipets were used in the “reverse” mode (6) to deliver 25 μl of serum (standard, control, patient) and 100 μl of the extraction reagent to the bottom of 16 × 100 mm disposable culture tubes. A volumetric pipet was used to deliver 1.0 ml of the solvent to each tube. The contents of the tubes were vortex-mixed for 15 s, then centrifuged at 900 × g for 60 s. The tubes were “rolled” in a nearly horizontal position so that the lower solvent layer would break through the aqueous layer and then the solvent was decanted into a disposable 12 × 75 mm culture tube. Occasionally a droplet of the aqueous layer would spill into the smaller tube; in this case the smaller tube was “rolled” as before and the solvent decanted into a second 12 × 75 tube. The solvent was evaporated in a 45 °C water bath under a gentle flow of air. The extract was reconstituted by adding 25 μl of n-hexane to the 12 × 75 tube, stopping the tube, and vortex-mixing for 5 s. Rolling the tube in a horizontal position while vortexing removed the material deposited on the walls of the tube. A 1-μl aliquot of the extract was injected into the gas chromatograph. Concentrations were calculated by comparing the theophylline/internal standard peak-height ratios of the unknowns to that of the standard. Typical chromatograms of a serum blank, a patient’s serum, and a standard are shown in Figure 1.

Results and Discussion

Within-run and run-to-run reproducibility was assessed by use of pooled serum samples submitted to the laboratory for theophylline analyses. Eight replicate analyses within the same run produced a mean and standard deviation of 11.14 ± 0.40 mg/liter (CV, 3.6%). For 16 samples of the same pool, run at least once a day during an 11-day period, the mean and standard deviation were 11.34 ± 0.33 mg/liter (CV, 2.9%). One patient’s serum, analyzed eight times within the same run,
gave a mean and standard deviation of 22.16 ± 0.62 mg/liter (CV, 2.8%).

The linearity was tested with prepared mixtures of drug-free serum and a stock theophylline solution. Four mixtures containing 2.0, 12.5, 20, and 40 mg of theophylline per liter were analyzed in duplicate, and the theophylline/internal standard peak height ratios were plotted vs. concentrations. Linear regression analysis of the data resulted in a line with a slope of 0.4085, y-intercept of +0.02, and correlation coefficient of 0.9996.

I did a correlation study, using 11 serum samples that had been submitted to the laboratory for theophylline analysis, comparing results by the method of Lowry et al. (3) with results by the present method. The slope by linear regression analysis was 0.967, the y-intercept was +0.8, and the correlation coefficient was 0.976.

Sera from 21 hospital patients selected at random were analyzed by the present method, and the largest background peak found corresponded to 0.13 mg of theophylline per liter. Accordingly, the detection limit for the present method was set at 0.40 mg/liter.

Analytical recovery was estimated by analyzing 20-μl aliquots of serum containing theophylline by both the present method and another method in which a nitrogen detector is used (2). The absolute theophylline peak heights of the two methods under the same gas-chromatographic conditions were compared. Using the reported value of 82% recovery for the comparison method, the recovery of the present method was calculated to be 98% (i.e., assuming that the derivatization reaction in the comparison method was 100% complete).

Saliva and blood samples were collected from a patient 2.5 h after ingestion of 400 mg of theophylline (two Elixophylline capsules). A saliva-based standard was prepared in the same manner as the serum standard. The saliva sample and the corresponding serum sample were analyzed for theophylline by the present method. The saliva/serum concentration ratio was 5.7/9.4 = 0.57, which compares well with the ratios found in other studies (2, 7).

When I attempted to use the flame ionization detector with the present method, a large peak, apparently endogenous to serum, appeared close to the internal standard peak and could not be resolved from it.

Possible interfering substances, including xanthines and drugs taken concurrently with theophylline, were tested by the present method. None interfered with the theophylline or internal standard peak.

In summary, for those laboratories in which a gas chromatograph with a nitrogen detector is available, the present method is faster (10 min for a single sample, 21 min for standard, control and patient samples) and simpler (only one reagent and a solvent solution are used) than other published methods, while retaining excellent accuracy and precision. The extractive alkylation technique exemplified by the present procedure holds promise of being the basis for simple procedures for barbiturates, anti-convulsants, and other drugs having similar weak acid-type chemical structures.

References


