The specificity of ethanol methodologies is of concern in both clinical and forensic applications. Some current methods have significant cross reactivity with other alcohol compounds, such as isopropanol, which are occasionally encountered concurrently with ethanol in biological fluids. Our evaluation of the REA method indicated no cross reactivity toward methanol, isopropanol, or acetone at concentrations that might be expected in toxic situations. We did not determine the assay’s cross reactivity toward other alcohol compounds; however, the incidence of such volatiles in biological fluids is extremely rare.

The REA assay for ethanol requires no instrument modification of the TDx analyzer. However, because ethanol reagent is retained in the probe after analysis, the manufacturer recommends an automated wash protocol before subsequent use of the analyzer for other assays.

We conclude from our evaluation of the REA ethanol method that the assay gives reproducible and accurate results that compare well with those obtained with a commonly used GLC method and the Du Pont acc. The REA method on the TDx analyzer is generally easier and faster than GLC and, unlike the Du Pont acc, allows automated analysis of whole blood without pretreatment. We found the TDx REA assay for ethanol a useful method for quantifying ethanol in blood, serum, and urine in clinical and forensic specimens.

We acknowledge support from Abbott Diagnostics, who provided the resources to perform this evaluation. We also thank Dr. David Yost, Research Biochemist, Abbott Laboratories, for his assistance and time, and Dr. Brian Pape, New England Pathology Services, for review of this manuscript and for many helpful discussions.

References


Particle-Enhanced Turbidimetric Inhibition Immunoassay for Theophylline Evaluated with the Du Pont ac

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We evaluated the Du Pont Particle-Enhanced Turbidimetric Inhibition Immunoassay (PETINIA) for theophylline. The imprecision (CV) of the assay was <4.7% between-run and <3.6% within-run for theophylline concentrations between 5 and 30 mg/L. Standard curves for the assay were linear for theophylline concentrations from 0 to 46 mg/L and were stable throughout the study (i.e., for at least three months). The monoclonal antibody against theophylline used in this assay increases specificity; of the possibly interfering drugs, metabolites, and anticoagulants tested, only 1,3-dimethyluric acid and EDTA showed measurable effects. Bilirubin (<300 mg/L), hemoglobin (<6 g/L), or lipemia (triglycerides <6 g/L) does affect the quality of the assay. Analytical recovery of theophylline added to serum (5 to 40 mg/L) averaged 98% (range 93% to 112%). Comparison of results for patients’ sera by the PETINIA method with those by enzyme immunoassay (EMIT) and by “high-performance” liquid chromatography yielded slopes and intercepts not significantly different from 1.0 and 0.0, respectively, and correlation coefficients ranging from 0.986 to 0.995.

Additional Keyphrases: enzyme immunoassay, liquid chromatography compared • monitoring therapy • asthma • apnea in neonates • turbidimetry • PETINIA

Theophylline (1,3-dimethylxanthine) is a widely used drug. Its pharmacological properties include relaxation of bronchial smooth muscle and stimulation of the central nervous system, actions important for treating asthma and neonatal apnea, respectively. For treating asthma, theophylline concentrations between 10 and 20 mg/L in serum are effective (1). Lower concentrations (5–10 mg/L) will reduce the frequency of neonatal apnea (2); however, in neonates this therapeutic range is complicated by theophylline being metabolized to caffeine (1,3,7-trimethylxanthine), which is active in the same way (3). As theophylline concentrations increase above the therapeutic range, adverse effects be-
come more frequent and severe and can result in life-
threatening cardiac arrhythmias or seizures.

Theophylline concentrations in serum may be measured by (e.g.) homogeneous enzyme immunoassay (4), fluores-
cence polarization immunoassay (5), "high-performance"
liquid chromatography (HPLC) (6–8), substrate-labeled flu-
orescence immunoassay (9), nephelometric inhibition im-
unoassay (10), and radioimmunoassay (11).

The present assay, the Particle-Enhanced Turbidimetric
Inhibition Immunoassay (PETTINA) for theophylline, was evaluated at three medical centers (12–14). It can be used with Du
Pont’s Automated Clinical Analyzers (aca) II, III, or IV.

Materials and Methods
The participating medical centers are designated Lab A, Lab B, and Lab C.

Reagents
aca THEO assay. This assay for theophylline (aca THEO) is a
nonisotopic competitive-binding immunoassay based on the
inhibition of agglutination of latex particles, coated with
covalently-bound theophylline, by a monoclonal antibody
against theophylline; the degree of inhibition of the latex-
theophylline particle agglutination is proportional to the
amount of theophylline in the patient’s sample or the
theophylline (Fig. 1). The rate of agglutination is monitored
at 340 nm by measuring the change in turbidity after the
particle-bound theophylline, antibody, and patient’s sample
are mixed. The reagents for this assay, which in addition to
particle-bound theophylline and antibody include surfac-
tants, polyethylene glycol, and protein-denaturing agents,
are supplied in a plastic pack designed to be used with the
aca discrete analyzer. Serum-based calibrators and aca THEO
reagent packs were furnished by the Du Pont Co.

Control materials. Serum-based quality-control materials
containing theophylline were "Stratus TDM Controls I and
III" (Dade Division, American Hospital Supply Corp., Mi-
ami, FL 33152) and "TheraChem Anticonvulsant/Theophyl-
line Control, Low and High" (Fisher Scientific Co., Orange-
burg, NY 10962).

A. Sample with low theophylline concentration

B. Sample with high theophylline concentration

Fig. 1. Principle of the present technique
A low concentration of theophylline in the sample increases latex-particle
agglutination with the antibody and increases turbidity; a high concentration
of theophylline results in decreased agglutination and turbidity

Procedures

Calculations. The relation between the rate of change of
turbidity during an assay and the theophylline concentra-
tion can be converted to a linear expression by using the
following logit function:

\[
\text{Theophylline concn, mg/L} = \frac{C_1}{C_0 - C_1} f(x) - 0.5
\]

Constants C0 and C1 are related to the slope and intercept of
the standard curve (milliabsorbance units vs concn. of
theophylline calibrator) and are calculated for each new lot
of reagent by the user; C2 and C3 are lot-specific lineariza-
tion constants, determined by the supplier and included in
the product insert; mA is the rate of change of turbidity in
milliabsorbance units per 17.0 s. The aca III and IV calcu-
late the theophylline concentration with the above function.
For use with the aca II, Du Pont supplies a special graph
paper incorporating the above logit function; the rate of
theophylline change (mA) is plotted against the corre-
passing concentrations of the theophylline calibrators, and values
for patients’ samples are determined by interpolation from
this standard curve.

In this evaluation, Lab A used the aca III, Lab B the aca II
and III, and Lab C the aca II. The aca THEO determinations
at each evaluation site were obtained from a calibration
curve derived at the start of the study from the average of
five replicate measurements of five concentrations of theo-
ephylline (2, 6, 10, 21, and 40 mg/L). At the conclusion of
this study the Du Pont Co. recalculated all theophylline mea-
surements at each institution, using three replicates of the
2, 10, and 40 mg/L concentrations; their results were
identical to those obtained from the standard curves based
on five theophylline concentrations. The product insert now
recommends calibration based on only these three calibra-
tors, assayed in triplicate.

EMIT assays. Reagents for use in the Enzyme-Multiplied
Immunoassay Technique (EMIT) were purchased from Syva
Co., Palo Alto, CA 94303. EMIT assays were performed at
Labs A and B with the Syva Lab 6000 system according to
the manufacturer’s instructions. Lab C performed the EMIT
assays on a Roche Cobas-Bio centrifugal analyzer, using
the method of Ou et al. (12).

HPLC assays. Lab A used the HPLC method of Butrimo-
vitz and Raisys (6), with the following modifications: the
mobile phase was tetrahydrofuran/methanol/sodium acetate
buffer (10 mmol/L, pH 5.0, 1/7/92 by vol), and the
RCl-100 system included a column of 5-μm Radial-Pak C18 (both
from Waters Associates, Milford, MA 01757). With these
modifications theophylline could be separated from the
caffeine metabolite, paraxanthine, 1,7-dimethylxanthine.

At a flow rate of 5 mL/min the retention times for para-
xanthine, theophylline, and 1,7-dimethylxanthine were
measured to be 5.2, 5.8, and 6.7 min, respectively.

Lab B used the HPLC assay of Sample et al. (7), with a
Model ALC 200 system with a column of μ-Bondapak C18
(both from Waters Associates). Theophylline was extracted
from serum into an equivolumetric mixture of chloroform and
isopropanol, which contained 1,7-dimethylxanthine as
the internal standard. The organic (lower) layer was
removed after centrifugation. the solvent was evaporated, and the residue, dissolved in methanol, was injected into the HPLC system. Between-run precision (CV) for this assay was 6.6% and 5.1% for control samples containing 10 and 30 mg/L of theophylline, respectively.

Lab C performed the HPLC assays by the method of Ou and Frawley (8). Between-run precision (CV) for this procedure was 3% for a control sample containing 13.7 mg of theophylline per liter.

Evaluation of Analytical Variables

**Calibration curve stability.** During the course of this study (two months at Lab A, three months at Labs B and C), the calibration curve in each laboratory was validated weekly by assaying the five calibrators in triplicate. Linear regression statistics were calculated from the average assayed value of each calibrator vs its assigned value; acceptable limits for this comparison were slope = 1.00 ± 0.05 and intercept = ±0.8 mg/L. None of the laboratories needed to recalibrate the assay during the study.

*Linearity.* Linearity of the standard curve for the *aca theo* assay was confirmed over the range 2 to 40 mg/L by assaying dilutions, in triplicate, of a patient's specimen containing about 40 mg of theophylline per liter. At each evaluation site this high-concentration theophylline specimen was diluted with a drug-free serum pool, the dilution ratios (high-concentration sample: drug-free serum) were 1:0.0, 0.9:1.1, 0.8:0.2, 0.7:0.3, 0.6:0.4, 0.5:0.5, 0.4:0.6, 0.3:0.7, 0.2:0.8, 0.1:0.9. Lab A did not use the 0.3:0.7 and 0.5:0.5 dilutions but included a 0.05:0.95 sample.

*Precision.* Precision both within-run and between-run was determined at several theophylline concentrations between 5 and 30 mg/L. Over a 20-day period, duplicate samples of the commercial serum-based controls and pooled patients' sera containing theophylline were assayed by the *aca theo* method and by the comparison methods. Within-run and between-run precision were calculated from these results with an ANOVA technique (13).

*Analytical recovery.* In each laboratory four different drug-free serum pools were split into four parts, which were supplemented with theophylline to give final concentrations of 5, 10, 20, or 40 mg/L. Each sample was assayed in triplicate by the *aca theo* method, and analytical recovery of theophylline was calculated.

Correlation Studies

At each evaluation site, results by the *aca theo* method were compared with those by the EMIT assay and by HPLC. Patients' theophylline samples were split and analyzed in duplicate by the *aca theo* and EMIT methods, and the results were averaged; single aliquots of the same samples were analyzed by HPLC. Results were compared statistically by linear-regression analysis.

Potentially Interfering Substances

Pooled serum containing final concentrations of bilirubin of 170 mg/L (Lab A) and 300 mg/L (Lab B), or of hemoglobin (from lysed erythrocytes) of 6.0 g/L (Lab A) and 5.5 g/L (Lab B) were used. Turbid specimens were pooled lipemic serum (Lab A, triglyceride concentration = 35 g/L) and (Lab B) serum supplemented with 6 g of "Intralipid" (Cutter Laboratories, Emeryville, CA 94608) per liter. Each of these pools was serially diluted with serum containing very low concentrations of the three endogenous substances to yield six concentrations of each potentially interfering substance. Each of these samples was then supplemented with theophylline (10 and 40 mg/L) and assayed by the *aca theo* method.

Effects of some commonly administered drugs were determined by assaying commercial serum controls containing the respective drug at concentrations exceeding its therapeutic range. The effects of several chemically related xanthine and uric acid derivatives (caffeine, theobromine, paraxanthine, 3-methylxanthine, 1-methyluric acid, and 1,3-dimethyluric acid) were determined by assaying a serum pool containing 10 mg of theophylline per liter, to which an equal concentration of the potentially interfering substance was added. The cross reactivity of these compounds was calculated as the increase in apparent theophylline concentration when the interfering substance was present.

The effects of heparin (lithium and sodium salts) and EDTA anticoagulants and of the serum separator materials in "Corvac Tubes" (Monoject Div., Sherwood Medical, St. Louis, MO 63103), "SST Tubes" (Becton-Dickinson Laboratory Div., Rutherford, NJ 07070), and "SureSep Tubes" (General Diagnostics, Morris Plains, NJ 07950) were also evaluated. The effect of freezing and thawing of samples was determined by measuring theophylline concentrations in samples before and after four freeze–thaw cycles.

**Results and Discussion**

Table 1 summarizes results of the linearity study. The *aca theo* assay showed a linear response over a wide range of concentrations (0–46 mg/L), in agreement with the product insert. This encompasses the concentrations found in the vast majority of clinical specimens: in only one of 348 clinical samples we studied theophylline exceeded 40 mg/L (43.3 mg/L). Samples with concentrations exceeding 40 mg/L may validly be diluted with drug-free serum or water and re-assayed.

Table 2 shows results for within-run precision of the *aca theo* and EMIT methods. The very small within-run CV of the *aca theo* method (<3.6%) obviates the need to assay samples in duplicate. In general, this CV was less than for the EMIT method. The mean, SD, and CV data were calculated from 20 paired-sample measurements assayed on 20 separate days. The between-run precision results (Table 2) were obtained by the ANOVA technique (13) from the same 20 paired measurements used to determine the within-run precision; again the imprecision was less than for the EMIT Lab 6000 system. In addition, the HPLC method used by Lab A showed higher CVs (5.1% and 4.2% for the Stratus I and III control samples, respectively) than the *aca theo* method.

Table 3 gives the analytical recovery of theophylline added to drug-free serum, with the mean for all laboratories at each concentration indicated at the bottom. Recoveries over the concentration range of 5 to 40 mg of theophylline per liter averaged 98% (range 93% to 112%).

The good correlation between the *aca theo* method and the EMIT methods are shown in the upper panel of Figure 2. No significantly discrepant values (>30% difference) were noted with any of the EMIT procedures. In the lower panel of Figure 2 the *aca theo* method is compared with three
different reversed-phase HPLC procedures. Again, the linear-regression statistics indicate no significant differences between results by the ace THEO and the HPLC procedures.

Figure 3 illustrates the lack of significant interference by hemoglobin (<6.0 g/L), bilirubin (<300 mg/L), or moderate lipemia (triglycerides <6.0 g/L). Grossly lipemic specimens (triglycerides >6.0 g/L) showed a proportional increase in apparent theophylline concentration. For example, in the presence of 35 g of triglycerides per liter the 10 and 40 mg/L theophylline specimens showed apparent concentrations of 20.6 and 66.2 mg/L, respectively. Removal of the lipid particles by ultracentrifugation (about 150 000 x g) left a clear serum layer, analysis of which by the ace THEO method (in triplicate) gave mean values of 11.5 and 44.8 mg/L, respectively.

Table 4 lists drugs and uric acid and xanthine derivatives for which we found no significant interfering effects on the
**Table 4. Drugs and Metabolites Showing No Significant Cross Reactivity in the aca Theo Assay**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>1-Methylxanthine</td>
</tr>
<tr>
<td>N-Acetylprocainamide</td>
<td>3-Methylxanthine</td>
</tr>
<tr>
<td>Amikacin</td>
<td>7-Methylxanthine</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Metoprolol</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Paraxanthine</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Phenoxytin</td>
</tr>
<tr>
<td>8-Chlorotheophylline</td>
<td>Phenytin</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Prednisone</td>
</tr>
<tr>
<td>Disopyramide</td>
<td>Primidone</td>
</tr>
<tr>
<td>Dyphylline</td>
<td>Quinidine</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>Theobromine</td>
</tr>
<tr>
<td>3-Iso-butyl-1-methylxanthine</td>
<td>Tobramycin</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>1,3,7-Trimethyluric acid</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Uric acid</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Valproic acid</td>
</tr>
<tr>
<td>1-Methyluric acid</td>
<td>Xanthine</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of hemoglobin (A), bilirubin (B), and lipemia (C), on the aca Theo assay, as determined by Lab A (▲) and Lab B (●).

Either serum or heparinized plasma may be used in the assay, but use of EDTA results in a false increase in apparent theophylline concentration. This procedure requires only 40 µL of serum. It is unaffected by high concentrations of bilirubin, hemoglobin, and triglycerides (lipemia). These features make the method attractive for pediatric use.

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**References**