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Interference in the Proposed Selected Method for Determination of Theophylline by Liquid Chromatography

To the Editor:

The proposed Selected Method for determination of theophylline by "high-pressure" liquid chromatography (HPLC) (1) prompts us to point out that the application of this method may lead to strongly misleading results, potentially dangerous to many asthmatic patients.

Firstly, we believe that the design of the method is not always in agreement with what can be considered generally accepted practice in bioanalysis. For example the term "internal standard" is misleading because this compound is not added directly to the biological sample in as small an aqueous volume as possible (2), but is dissolved in the extraction solvent. Such an "internal standard" may not correct for losses of substances that are difficult to extract from biological matrices.

The term "analytical recovery" is also misleading in that it is expressed as relative recovery (drug-supplemented serum vs drug-supplemented water taken through the entire procedure). However, no data are given on absolute recoveries (drug-supplemented serum taken through the entire procedure vs drug-supplemented water taken through the HPLC-detector), which are essential for judging the accuracy of the method. Moreover, the precision of the method at various serum concentrations within the therapeutic range is not specified.

To facilitate inter-laboratory comparisons, it would have been much more practical to express retention behavior of the various compounds as relative retention times or as k' values, rather than absolute retention times.

Another criticism concerns the question of interference by other drugs. The author has restricted himself to parent compounds only, neglecting the possibility that metabolites may cause interference as well.

In Table 1 of Broussard's paper several drugs are listed that are not eluted from the column within 20 min. It is likely, however, that metabolites—which are usually more polar—will be eluted from the reversed-phase column more rapidly than the parent compounds. On the other hand, compounds eluting after 20 min may interfere with the next sample injected.

Unfortunately, some more modern potential interferences have not been investigated. We know from our own experience and from the literature (e.g., 3) that interference may be caused by such drugs as ampicillin, cephalospors, and sulfonamides (e.g., trisulfapyrimidine and sulfamethoxazole), which are often used as co-medication with theophylline in asthmatics. Acetazolamide, often reported to interfere with theophylline determination by HPLC (4, 5), is also not listed in the Table.

However, our major concern regards the potential interference of 1,7-dimethylxanthine (paraxanthine), a major metabolite of caffeine and present in significant amounts in serum of all coffee or tea drinkers and other caffeine users. In many HPLC systems, the retention time for this compound is similar to that for theophylline. Values equivalent to as much as 3.5 mg of theophylline per liter of serum have been reported after normal coffee and tea intake (6–8).

When we decided to check the potential interference of 1,7-dimethylxanthine, following the proposed analytical conditions as closely as possible, we found that 1,7-dimethylxanthine has exactly the same relative retention time as theophylline.

To determine the absolute analytical recovery of 1,7-dimethylxanthine, we added it to xanthine-free calf serum at a concentration of 10.0 mg/L. The results (mean 102.0%, SD 5.5%, n = 7), showed that the extraction of this compound in the proposed method is complete.

The absolute recovery for theophylline determined this way, also at a concentration of 10 mg/L, was 84.3% (SD 3.0%, n = 7), which is significantly lower than the relative figures reported by Broussard.

Table 1 lists the k' values, the α-values (for the separation factor vs the internal standard) and the numbers for the recoveries for theophylline, 1,7-dimethylxanthine, and caffeine.

In serum from a healthy volunteer who did not take any theophylline but who frequently drinks coffee and tea (as many as eight cups daily), we found in a sample taken at 2230 hours (1 h after the last cup of coffee) a peak identified by mass-spectrometric analysis as 1,7-dimethylxanthine. Its concentration was 6.9 mg/L, which could erroneously be interpreted as 8.1 mg of theophylline per liter.

Given a desired therapeutic theophylline concentration in serum of 10–20 mg/L, the error due to this interference would be on the order of 30%.

Obviously, using the proposed Selected Method thus may easily result in dose adjustments leading to undertreatment and potential danger for the patient.

Methods that avoid chromatographic interference of 1,7-dimethylxanthine are presented in references 9–15.

References

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The author of the method responds:

To the Editor:

The comments of Dr. Jonkman and colleagues are welcome and appreciated. Although the 8-chlorotheophylline is not added to the sample in as small an aqueous volume as possible, the purpose of an internal standard is accomplished by having the 8-chlorotheophylline in the extraction solvent.

The question of analytical recovery vs absolute recovery is somewhat controversial. Unless the concept of analytical recovery is used, one is faced with the choice of either using only those procedures that yield 100% absolute recovery or using a correction factor to compensate for incomplete recoveries. When performing an assay in which an aqueous standard is taken through the procedure, it is sufficient to show that the analytical recovery is acceptable in order to verify the accuracy of the method.

We confirmed that 1,7-dimethylxan- thine has the same retention time as theophylline, but did not obtain interference of the magnitude reported by Jonkman and colleagues. When the experiment was repeated on the serum of a healthy volunteer who was not taking theophylline, but who drinks at least 10 cups of coffee daily, a peak was obtained which gave an apparent theophylline concentration of 2.0 mg/L (as compared to the 8.1 mg/L obtained by Jonkman and colleagues).

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Gamma-Glutamyltransferase in Saliva

To the Editor:

Gamma-glutamyltransferase (GGT; EC 2.3.2.2), an enzyme of cellular membranes, is found in many tissues and fluids of the body. We have investigated and presented here the normal values for GGT in human saliva from females and males of different ages, salivary GGT activity before and after meals or exercise, and the effects of storage at various temperatures on enzymic activity.

The healthy volunteers we studied were 50 females, ages 2–84 years (mean ± SEM, 38.02 ± 3.35 years) and 50 males, ages 7–80 years (mean ± SEM, 39.22 ± 2.97 years). In 25 subjects, saliva and blood samples were taken after overnight fasting. Likewise, in 25 cases GGT activity was assayed before a meal and 5 min after. No subjects were taking any drugs or alcohol, and informed consent was obtained from each of them for this investigation.

Specimens representing mixed saliva were collected during about 45 s of unstimulated excretion. About 0.5 mL of collected saliva was centrifuged at 1300 × g for 10 min. (Sometimes the supernatant liquid was not clear and we repeated the centrifugation at the same speed.) We measured enzyme activity in the supernates immediately after centrifugation or after storage at −20 °C for no longer than one week. GGT activity in serum and saliva was determined spectrophotometrically (Boehringer Mannheim Corp.; Monotest, γGT new), according to Szasz (1), at 30 °C. The within-assay CV was 3.2% (n = 15) for a mean salivary GGT activity of 6.64 U/L; the between-assay CV was 6.2% (n = 15) for a mean activity of 4.03 U/L. The day-to-day CV for salivary GGT activity was 22% in a subject studied over a one-month period, with a salivary mean activity of 5.58 U/L.

The GGT values (mean ± SEM) for the healthy unmedicated males and females were 5.44 ± 0.24 and 4.67 ± 0.22 U/L, respectively (significantly different, p < 0.02, by Student’s t-test). Salivary GGT activity before and after meals was 4.47 ± 0.41 and 4.60 ± 0.36 U/L, respectively (difference not significant by paired Student’s t-test).

Figure 1 shows the values obtained for different age groups. There was no correlation between age and saliva GGT activity in either females or males (r = 0.111 and 0.070, respectively). When GGT activity was determined in serum and saliva from 25 healthy humans, the values obtained were 16.28 ± 1.79 and 5.34 ± 0.35 U/L, respectively, with no correlation between the two sets of values (r = 0.246). Saliva was sampled from five young, healthy subjects before a 2-h tennis match and 5 min and 4 h after the match. We saw no significant differences in salivary GGT activity after this exercise (mean ± SEM: 4.52 ± 0.75, 5.66 ± 0.8, and 4.02 ± 0.69 U/L, respectively).

To test thermal stability, we kept six samples at −20 °C and then at room temperature (approximately 20 °C). In the latter case, GGT activity decreased 51.2 ± 4.9% after seven days (p < 0.006); after 30 days only 12.59 ± 4.22% of the enzyme activity could be measured (p < 0.001) (Figure 2). Samples stored at −20 °C were thawed and tested at 1, 2, 3, 4, 8, 10, 16, 24, and 52 weeks without significant loss in activity; the specimens were re-frozen after each testing.

Values for salivary GGT obtained in the presence of glycylglycine (80 μmol/L, pH 7.8), glutamine (80 μmol/L, pH 7.8), or a mixture of serine plus betaine (3 and 7 μmol/L, respectively, pH 9.0) were 900%, 320%, and 60%, respec-