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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THEOPHYLLINE IN PLASMA AND SALIVA IN THE PRESENCE OF CAFFEINE AND ITS METABOLITES AND COMPARISONS WITH THREE OTHER ASSAYS

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SUMMARY

A new ion-pairing reversed-phase high-performance liquid chromatographic assay for theophylline is described which allows the separation of theophylline from 1,7-dimethylxanthine — a metabolite of caffeine which interferes with most theophylline assay procedures. Levels of 1,7-dimethylxanthine equivalent to 3 mg/l theophylline were seen in individuals not taking theophylline but who drank three to four cups of coffee per day. This compound was not seen in individuals abstaining from xanthine-containing foods and beverages.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is a powerful, smooth muscle relaxant used primarily in the treatment of chronic obstructive pulmonary disease where dilation of the bronchi and pulmonary vasculature are warranted [1]. The pharmacological activity of theophylline has been shown to be highly correlated with its concentration in plasma [2]. However, because of the large inter-individual variation in the clearance of the drug, dosages must be individualized in order to optimize therapy [3]. Sensitive and specific assays for theophylline are therefore central to successful therapy with the drug. In this context, older spectrophotometric assays of the drug [4] involving a single or double extraction as the basis for selectivity have been superseded by high-performance liquid chromatographic (HPLC) and immunochemical assays for

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routine clinical determination [5,6]. While interference from other xanthines, e.g. caffeine (1,3,7-trimethylxanthine) and theobromine, have been eliminated with these more sophisticated techniques, a metabolite of caffeine (paraxanthine, 1,7-dimethylxanthine, a chemical isomer of theophylline) has been shown to interfere with theophylline determinations in assays using traditional reversed-phase chromatographic separations [5,7]. Thus the potential for interference with the measurement of theophylline may exist in patients consuming caffeine-containing beverages or foods.

In a previous report [8] we have described an ion-pairing HPLC assay for theophylline and its metabolites in urine which was capable of separating theophylline from paraxanthine. However, this procedure required an ion-pairing liquid—liquid extraction step followed by ion-pairing gradient elution HPLC and was rather complicated and time consuming for the routine application to the measurement of theophylline in plasma and saliva. Consequently, we have made extensive modifications to the above procedure to make it suitable for the routine determination of theophylline in plasma and saliva in the presence of paraxanthine (and other caffeine metabolites) and to assess the magnitude and frequency of this interference. In addition, we report the results of a blind investigation in which plasma samples containing theophylline in the presence and absence of paraxanthine were sent to three other laboratories in order to assess the significance of the interference under the conditions used by each laboratory.

MATERIALS AND METHODS

Apparatus

A Model 8500 high pressure solvent delivery system/automatic sample injector (Varian, Palo Alto, CA, U.S.A.) was used in conjunction with a Hitachi 155-30 variable-wavelength UV detector set at 274 nm (Altex, Berkeley, CA, U.S.A.) and a Hewlett-Packard integrator Model 3380A (Hewlett-Packard, Avondale, PA, U.S.A.). A slurry packed LiChrosorb RP-2 (4.5 cm × 2.0 mm I.D.) precolumn was used in conjunction with an Ultrasphere ODS 5-μm (15 cm × 4.6 mm I.D.) analytical column (Altex) for achieving separation and quantitation.

Reagents and standards

Sources of the xanthine and uric acid derivatives were as follows: caffeine and uric acid (Eastman-Kodak, Rochester, NY, U.S.A.), theophylline and β-hydroxyethyl theophylline (Sigma, St. Louis, MO, U.S.A.), 1-methylxanthine, 3-methylxanthine, 1,7-dimethylxanthine, 1-methyluric acid and 1,3-dimethyluric acid (Adams Chemical, Round Lake, IL, U.S.A.). Tetrabutylammonium hydrogen sulphate was obtained from Aldrich (Milwaukee, WI, U.S.A.) and sodium acetate was of analytical grade. Acetonitrile and methanol were of HPLC grade.

The mobile phase consisted of 0.01 mol/l sodium acetate and 0.005 mol/l tetrabutylammonium hydrogen sulphate with the pH adjusted to 4.75 with either 0.1 mol/l sodium hydroxide or 0.1 mol/l acetic acid. Methanol was added (12.5%, v/v) and the mobile phase was filtered through a Millipore HA

(0.5 μm) solvent filtering system (Millipore, Bedford, MA, U.S.A.).

The internal standard solution was prepared by dissolving 2.5 mg/l β-hydroxyethyltheophylline in acetonitrile. Standard solutions of theophylline were prepared by dissolving theophylline in acetonitrile (100 mg/l). Blank (caffeine-free) plasma and saliva were obtained from a normal volunteer who had abstained from caffeine-containing foods and beverages for at least 72 h.

Procedure

Plasma. Plasma samples (0.5 ml) were transferred to a 7-ml glass tube and the proteins precipitated by the addition of 5 μg of internal standard in 2 ml of acetonitrile and vortexed for 15 sec. After centrifugation (3000 *g*, 10 min) the supernatant was transferred to another 7-ml glass tube and placed in a Buchler vortex evaporator thermostated to 50°C (Fort Lee, NJ, U.S.A.) for approximately 20 min at which time the removal of the acetonitrile was evidenced by an abrupt change in the vacuum chamber pressure. A 20-μl quantity of the remaining aqueous sample was injected directly onto the chromatographic system. Standard curves of theophylline in plasma were prepared by pipetting an appropriate volume of theophylline standard solution into a 7-ml glass tube, evaporation to dryness under a stream of nitrogen, addition of 0.5 ml blank plasma and vortexing thoroughly (15 sec). The samples were then processed as described above.

Saliva. Saliva samples were thoroughly vortexed and approximately 1.5 ml were transferred to a polypropylene Eppendorf microcentrifuge tube and centrifuged (5 min at 15,000 *g*). A 20-μl quantity of the supernatant was injected directly onto the chromatographic system. Standard curves of theophylline in saliva were made by pipetting an appropriate volume of the theophylline standard solution into 7-ml glass tubes, evaporation under a stream of nitrogen, addition of 1.5 ml of caffeine-free saliva and thoroughly vortexing. The samples were then processed as described above.

Chromatographic conditions

A flow-rate of 1.5 ml/min was used at ambient temperature generating a back pressure of approximately 2500 p.s.i. (170 bar). A sample run time of 12 min was selected in order to routinely estimate caffeine levels in outpatient samples. However, the theophylline and internal standard peaks were invariably eluted within 6—7 min.

Comparison with other assays

In order to estimate the frequency with which interference by paraxanthine occurs in routine clinical laboratory theophylline assays, duplicate spiked samples of plasma containing 0, 1, 3, 6, 10 and 18 mg/l theophylline in the presence and absence of 3 mg/l paraxanthine were distributed to three cooperating clinical laboratories routinely assaying theophylline in plasma; one using the EMIT enzyme immunoassay technique and two using HPLC assays, the details of which are as follows.

Laboratory 1. Precipitate 100 μl plasma with 100 μl acetonitrile containing β-hydroxyethyltheophylline (15 mg/l). Centrifuge and inject 20 μl supernatant onto analytical system: mobile phase, 10% acetonitrile in 0.01 mol/l

phosphate buffer, pH 4.5; flow-rate, 2 ml/min; room temperature, Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} column, 30 cm × 3.9 mm I.D.

Laboratory 2. Add 100 μl 0.1 mol/l phosphate buffer, pH 5.0 containing β-hydroxyethyltheophylline (15 mg/l) to 100 μl of plasma. Extract with 200 μl diethyl ether, centrifuge, evaporate supernatant to dryness and make up into 20 μl acetonitrile and inject 10 μl onto analytical system: mobile phase, 10% acetonitrile in 0.01 mol/l phosphate buffer, pH 5.0; flow-rate, 2 ml/min; 50°C; Altex Ultrasphere ODS column 15 cm × 4.6 mm I.D.

Laboratory 3. Used the EMIT enzyme immunoassay analysis technique as described by the manufacturer (Syva, Palo Alto, CA, U.S.A.).

RESULTS

The result of the analysis of a standard solution of theophylline (1,3-MX), β-hydroxyethyltheophylline (IS), caffeine (1,3,7-MX), and 3-methylxanthine (3-MX), 1-methylxanthine (1-MX), paraxanthine (1,7-MX), 1 methyluric acid (1-MU) and 1,3-dimethyluric acid (1,3-MU) is shown in Fig. 1A. The chromatogram from a blank sample of plasma obtained from a volunteer abstaining from caffeine-containing foods and beverages is shown in Fig. 1B. Fig. 1C shows the chromatogram from a patient administered 200 mg aminophylline q.i.d. who had been drinking three to four cups of coffee during the same day. Note the absence of theophylline and other interfering xanthine metabolites in the blank plasma sample but their abundance in significant

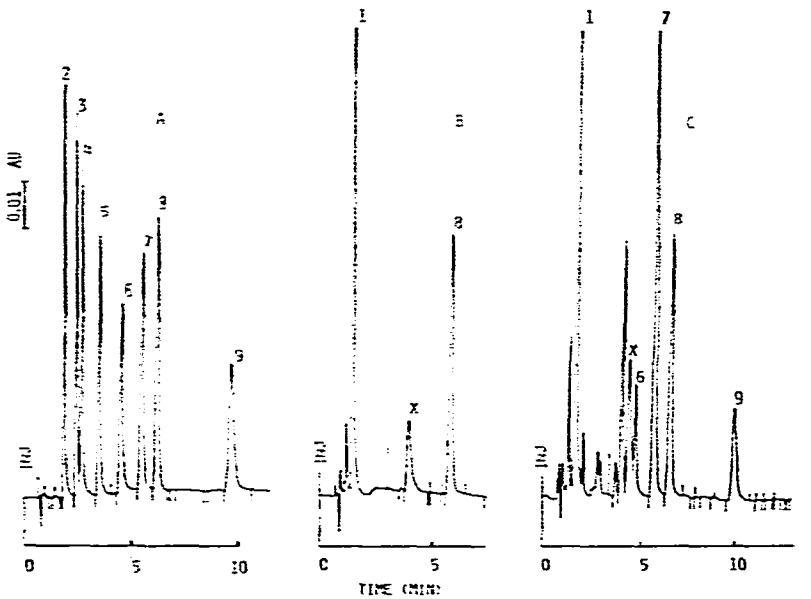

Fig. 1. Chromatograms of: A, standard of methylxanthines and methyluric acids; B, blank plasma; C, plasma from a patient taking theophylline and drinking coffee. Peaks: (1) uric acid, (2) 3-MX, (3) 1-MX, (4) 1-MU, (5) 1,3-MU, (6) 1,7-MX, (7) 1,3-MX (theophylline), (8) IS (β-hydroxyethyltheophylline), (9) caffeine, each at 8.0 mg/l. X is an unknown peak present in all plasma samples and the large peak preceeding X in C was unique to this patient, not corresponding to any of the standards 1—9.

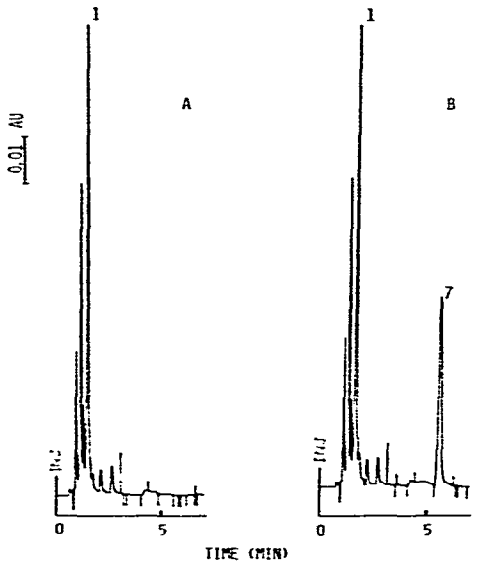

Fig. 2. Chromatograms of: A, blank saliva sample, B, saliva from a patient taking theophylline. Peaks as in Fig. 1.

concentrations in the patient sample. Fig. 2A shows the chromatogram from blank saliva and Fig. 2B, the chromatogram of saliva obtained from a patient administered 400 mg aminophylline q.i.d.

The following drugs were injected onto the chromatographic system at equivalent therapeutic concentrations (where known) and were found not to interfere with either theophylline or the internal standard: propranolol, acebutolol, quinidine, chlorthiazide, hydrochlorthiazide, hydrallazine, acetazolamide, naproxen, ketoprofen, phenobarbital, phenytoin, salicylic acid and procainamide.

Peak area ratio of theophylline to the internal standard was related to plasma theophylline concentration and was found to be linear within the range of 0.5—30.0 mg/l ($r^2 > 0.999$). Table I shows the interday reproducibility coefficient of variation (C.V.) of the theophylline internal standard

TABLE I

REPRODUCIBILITY OF PEAK HEIGHT RATIO VS. CONCENTRATION FOR THEOPHYLLINE IN PLASMA

Within-day C.V. was 1.0% ($n = 8$).

Concentration (mg/ml)	Mean peak height ratio	Interday C.V. (%)
0	0	—
1	0.1402	2.6
3	0.4244	1.8
6	0.8464	1.1
10	1.4227	0.76
18	2.5390	1.70

TABLE II

REPRODUCIBILITY OF PEAK AREA VS. CONCENTRATION FOR THEOPHYLLINE IN PLASMA

Within-day C.V. was 2.5% (n = 9).

Concentration (mg/ml)	Mean area	Interday C.V.* (%)
9	0	—
1	5.6393	10.25
2	11.2190	8.1
4	22.7000	4.85
8	45.3400	5.06

*See text for explanation of large coefficients of variation.

peak height ratio at several different concentrations (n = 8). The within-day reproducibility (C.V.) of 12 mg/l control sample was 1.0% (n = 5).

Absolute peak area of theophylline was related to salivary concentration and was found to be linear over the range of 0.5—20 mg/l (r^2 = 0.998). Table II shows the inter-day reproducibility (C.V.) of the absolute peak area at several different concentrations (n = 9). The higher C.V. value reflects inter-day variation of the slope of the relationship of peak area vs. theophylline concentration, probably due to minor variations in solvent composition, pH, temperature. The within-day reproducibility (C.V.) of a 4 mg/l control sample was 2.5% (n = 5).

The average plasma concentrations of caffeine and paraxanthine in seven volunteers drinking three to four cups of coffee between the hours of 8 a.m. and noon were 9.0 mg/l and 3.0 mg/l, respectively, while that of theophylline was 0.5 mg/l. Fig. 3 shows a chromatogram from a volunteer (K.T.M.) who

Fig. 3. Chromatogram of plasma from a volunteer drinking coffee. Peaks as in Fig. 1.

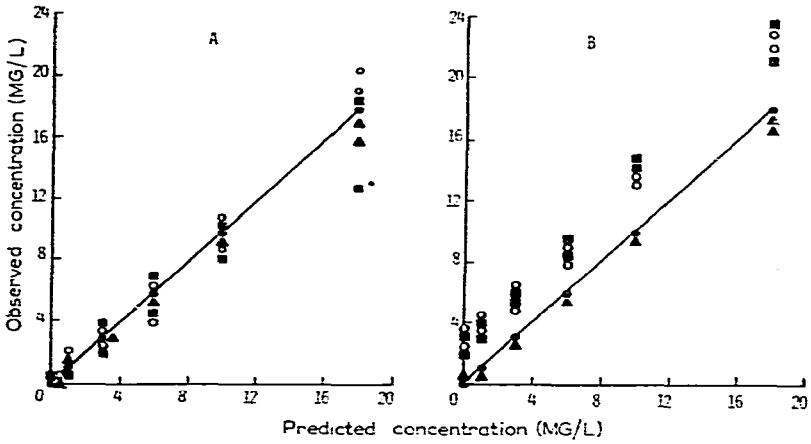

Fig. 4. Relationship between measured and spiked theophylline in plasma for four different assays. A, in the absence of paraxanthine; B, in presence of paraxanthine. The point marked with an asterisk (Laboratory 2) was not included in statistical analyses. •, This assay; ○, Laboratory 1, ■, Laboratory 2; ▲, Laboratory 3.

had drunk four cups of coffee between the hours of 8:00 a.m. and 10:00 a.m. The sample was drawn at 3:00 p.m. Note the presence of caffeine (approx. 6.9 mg/l), theophylline (approx. 0.7 mg/l) and paraxanthine (approx. 2.0 mg/l) indicating metabolic conversion of caffeine to paraxanthine and, to a much lesser extent, to theophylline.

Fig. 4A and Table III show the relationship between the concentration of theophylline reported for the four assays and that with which the samples were actually spiked in the absence of paraxanthine. All assays produced similar results although the variability in the measurements was greater from the three clinical laboratories. The intercepts were not significantly different from zero at the 0.05 level. The variability in the assays as assessed by the residual sums of squares (R.S.S.) and r^2 was much lower in the present HPLC assay than that used by Laboratories 1 and 2. The variability in the EMIT assay used by Laboratory 3 lay between these two extremes. Fig. 4B and Table IV show the relationship between the concentration of theophylline reported for the four assays and the actual spiked concentration in the presence of paraxanthine. The intercept values from Laboratories 1 and 2 were signifi-

TABLE III

SUMMARY OF STATISTICS OF DATA OBTAINED FROM DIFFERENT LABORATORIES FOR THE MEASUREMENT OF THEOPHYLLINE IN THE ABSENCE OF PARAXANTHINE

d.f. = Degrees of freedom.

	R.S.S.	d.f.	r^2	Slope	Intercept*
This assay	0.0487	10	0.9999	0.9864	—0.03
Laboratory 1	4.9448	10	0.9908	1.0770	—0.32
Laboratory 2	5.8778	9	0.9805	0.9753	0.24
Laboratory 3	1.4906	10	0.9961	0.9081	0.10

*Intercepts not different from zero at $P = 0.05$.

TABLE IV

SUMMARY OF STATISTICS OF DATA OBTAINED FROM DIFFERENT LABORATORIES FOR THE MEASUREMENT OF THEOPHYLLINE IN THE PRESENCE OF PARAXANTHINE

	R.S.S.	d.f.	r^2	Slope	Intercept*
This assay	0.1060	10	0.998	0.9631	—0.00
Laboratory 1	4.6315	10	0.9917	1.0967	2.30
Laboratory 2	4.7778	10	0.9917	1.1167	3.39
Laboratory 3	1.0340	10	0.9975	0.9405	0.03

*Intercepts from Laboratories 2 and 3 were significantly different from zero at $P = 0.05$.

cantly different from zero at the 0.05 level but those from the assay described herein and from Laboratory 3 were not. The variability in the assays, as assessed again by the R.S.S. and r^2 showed that the data from Laboratories 1 and 2 were the most variable. The variability from the described assay herein is the least variable and the variability from the EMIT assay is intermediate. Thus, both of the clinical laboratories using standard reversed-phase HPLC methods failed to distinguish between theophylline and paraxanthine and reported spuriously high values. The reversed-phase ion-pair assay and the EMIT assay, however, are specific for theophylline in these circumstances.

DISCUSSION

The accurate and specific measurement of theophylline in plasma is a necessary part of dosage individualization in patients with obstructive lung diseases. The therapeutic range of theophylline in plasma seems to be between approximately 5 and 20 mg/l although some workers have reported beneficial effects from a concentration as low as 2 mg/l [9]. While the metabolites of theophylline do not appear to accumulate in the plasma of individuals taking theophylline [10], they do appear to be present in the plasma of individuals who consume large amounts of caffeine-containing foods or beverages. This is undoubtedly due to the larger amounts formed from the large quantity of caffeine intake. One caffeine metabolite in particular, paraxanthine, has been shown to interfere with standard reversed-phase HPLC assay [5,7] of theophylline although the magnitude of this interference was not previously known. Our study has shown that the presence of this compound in plasma after drinking a typical number of cups of coffee may result in an apparent plasma theophylline concentration of approximately 3 mg/l although this is expected to be highly variable depending on the individuals, on the nature of caffeine intake, time of sampling, etc. The major difference between the assay described herein and those in which the interference occurs is the presence of the ion-pairing agent tetrabutylammonium which appears to preferentially increase the retention time of theophylline relative to paraxanthine. The reasons for this increase in retention time are not clear. The inclusion of the ion pair in the solvent mixture was originally decided upon in order to increase the retention times of the polar and poorly retained uric acid metabolites of theophylline which, at pH 4.75, would exist in an equilibrium form between the negatively

charged urate ion and the neutral uric acid species. Ion-pair formation between the urate ion and the relatively bulky, non polar tetrabutylammonium ion would therefore increase the retention of these compounds [8]. However, it was also noticed that the retention of theophylline was enhanced to the extent that excellent resolution of theophylline and paraxanthine was attained. Paradoxically, theobromine (the 3,7-dimethylxanthine isomer) had an exceedingly short retention time, appearing between 3-methylxanthine and 1-methylxanthine on the chromatogram. Again, the reason for this unusual chromatographic behavior is unknown.

The ion-pairing system described was exceedingly sensitive to the presence of acetonitrile in the sample. Thus the plasma sample workup by Laboratory 1 resulting in an injection sample of acetonitrile—water (50:50, v/v) produced an unacceptable loss of resolution in the ion-pairing analytical system. The complete or almost complete removal of acetonitrile from the precipitated plasma sample was therefore essential.

The use of a protective precolumn in the system resulted in an analytical column life in excess of 4000 samples although the life of the precolumn was only 500—1000 samples. This may be due to the effect of remaining soluble proteins in the sample or due to the solubility enhancing effect of ion-pairing solvent systems on silicates which may accelerate degeneration of low coverage bonded phase packing materials [11]. Only the exhaustively silylated high coverage bonded phase packing materials have resisted this apparent detrimental effect of the ion-pairing solvent system reported herein. Other packing materials, in particular C_{18} μBondapack (10 μm) and C_{18} Spherisorb (5 μm), resulted in unacceptably short column life as evidenced by a rapid and irreversible loss in retention and resolution.

A comparison of the four theophylline assays is instructive in the light of their intended application. The two clinical laboratory assays utilize slightly shorter sample workup procedures and slightly shorter analysis time although the latter difference is marginal. As the additional step of removing acetonitrile from the sample is necessary in order to perform the assay reported herein, selectivity and accuracy are reduced somewhat by the clinical laboratory procedures in the interests of speed. Although increasing the flow-rate of the ion-pair assay to 2.0 ml/min results in an analysis time of less than 5 min with no loss of resolution between theophylline and the internal standard, this action limited the ability to detect the metabolites of theophylline and caffeine in plasma and, therefore, for routine purposes, was not taken. The EMIT enzyme immunoassay system is apparently specific for theophylline, as would be expected from its immunochemical basis. This assay has been criticized when used in pharmacokinetic studies for its slight, but systematic bias [12], although this was not immediately apparent from the data presented in the present study. However, this is probably not as critical when a single, clinical sample is required as the need for selectivity and accuracy in the concentration ranges typically encountered.

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