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Short communication

Threshold level for theophylline in doping analysis

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Abstract

HPLC in the reversed-phase mode is used to assay methylxanthines including theobromine, paraxanthine, theophylline and caffeine in urine. The calibration graphs show good linearity in the concentration range 0–10 μg/ml. The limit for accurate quantitation of theophylline was 0.25 μg/ml. Between 6 and 20% of the parent drug is recovered in urine (0–12 h) after the oral administration of sustained release preparations containing 150 and 250 mg theophylline to four volunteers. Theophylline levels above 0.25 μg/ml were found in 1539 out of 3885 urine samples collected from athletes during unannounced doping control in Flanders. Statistical evaluation of the results gives a far outside value [75th percentile+(3× interquartile range)] of 2.25 μg/ml. The ratio theophylline paraxanthine (TP/PX) as an indicator for the non-dietary intake of theophylline seems to be more reliable. The far outside ratio was 0.20. To ensure with the greatest possible degree of certainty that no false-positive result is reported, decision limits of 5 μg/ml and 0.50, for theophylline and the ratio TP/PX respectively, are proposed.

Keywords: Theophylline

1. Introduction

Theophylline is a widely used bronchodilating agent with a narrow serum therapeutic range. Similarly to the other methylxanthine derivatives caffeine and theobromine, theophylline relaxes smooth bronchial muscle, stimulates the central nervous system, stimulates cardiac muscle and produces diuresis. Although caffeine has been considered the most potent of the methylxanthines, theophylline produces more profound and potentially more dangerous CNS stimulation than does caffeine. Theophylline is on the list of forbidden doping substances as issued by the Flemish Government [1]. As theophylline is also a metabolite of caffeine [2–4], a threshold level is needed for doping analysis purposes.

In 1995, in a preliminary study [5] urinary levels of caffeine and some metabolites including theobromine, theophylline and paraxanthine were measured in a sedentary population ($n=200$) and in athletes ($n=545$). Based on these values and the levels found during some excretion studies, a preliminary theophylline threshold of 5 μg/ml was proposed. In addition the ratio theophylline paraxanthine (TP/PX), independent of urinary specific gravity, appeared to be a reliable indicator for the intake of theophylline.

In this work the complete results of the excretion studies are presented and a larger athletic population ($n=3885$) was used to define threshold levels for theophylline.

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2. Experimental

2.1. Reagents and standards

Caffeine, theobromine and theophylline were gifts from Merck (Darmstadt, Germany). Paraxanthine and β-OH-ethyltheophylline were purchased from Sigma (St. Louis, MO, USA). Dichloromethane, methanol, ammonia, sodium chloride and ammonium chloride were all analytical grade.

Ammonia buffer (pH 9.5) was prepared by the addition of ammonia to a saturated ammonium chloride solution.

HPLC grade tetrahydrofurane was from Merck and aqueous HPLC solvent was prepared using water obtained from a Milli-Q water purification system from Millipore (Brussels, Belgium).

2.2. Analytical procedure

To 1 ml of urine in a 15-ml screw capped glass tube were added 100–120 mg of sodium chloride, 50 μl of I.S. (β-OH-ethyltheophylline 100 μg/ml) and 100 μl of ammonia buffer. Extraction was performed by rolling with 5 ml CH_2Cl_2–MeOH (9:1) for 10 min. After centrifugation for 5 min at 150 *g* the aqueous (upper) phase was aspirated and the organic phase transferred to a clean glass tube and evaporated under nitrogen at 40 °C. The residue was dissolved in 200 μl mobile phase and 20 μl of the solution was injected.

A standard curve was constructed by analysing aqueous solutions (range 0–10 μg/ml) in quadruplicate for each concentration.

Each batch of routine samples was preceded by a QC sample (1:1 diluted horse urine) containing theobromine (5 μg/ml), paraxanthine (5 μg/ml), theophylline (2.5 μg/ml) and caffeine (5 μg/ml).

2.3. Accuracy

The between-run accuracy from QC samples was measured during 10 days.

2.4. Chromatographic conditions

The chromatographic assembly consisted of a Model P4000 liquid chromatograph (TSP, Fremont, CA, USA), a Model AS 3000 autosampler (TSP) and a Spectra Focus forward optical scanning detector (TSP) set at 275 nm. PC1000 Spectacle software (TSP) and an IBM 466 DX2/S were used to generate chromatographic data. Peak heights were used for quantitation.

The column was a Hypersil 5 ODS, 100×3 mm I.D., 5 μm (Chrompack, Antwerp, Belgium) with an appropriate precolumn (10×2 mm I.D., 40 μm, C_{18}). The loop volume was 20 μl. The mobile phase was tetrahydrofuran–water (1:100, v/v) at a flow-rate of 1.0 ml/min.

2.5. Human study

Four healthy male subjects who had given their informed consent participated in the excretion study. Subjects 1 and 2 regularly consumed caffeine containing soft drinks. Subject 4 occasionally drank tea, while Subject 3 drank at least ten cups of coffee/day.

All subjects ingested one tablet of the sustained release preparation THEO-2 (Galephar, Brussels, Belgium) containing 150 mg theophylline after a light breakfast. Two weeks later one tablet of the sustained release preparation THEOLAIR L.A. 250 (Riker Benelux, Diegem, Belgium) containing 250 mg theophylline was taken by the same volunteers.

Subjects were allowed caffeine-containing beverages ad libitum.

Total urine was collected in capped bottles before (0 h) and 2,4,6,9 and 12 h after the administration of theophylline. After 24, 30, 48, 60 and 72 h a volume of urine was also delivered. Pre-administration urine (8 volumes during the day preceding the experiment) was also taken. All samples were analysed in duplicate.

From October 1993 till December 1995 all urine samples collected for doping analysis (n=3885) were controlled for the presence of theophylline, theobromine, paraxanthine and caffeine.

2.6. Statistical evaluation

A nonparametric form of analysis was used to evaluate the data from the 1539 urine samples with theophylline levels higher than 0.25 μg/ml. Assay results were ranked numerically, the interquartile

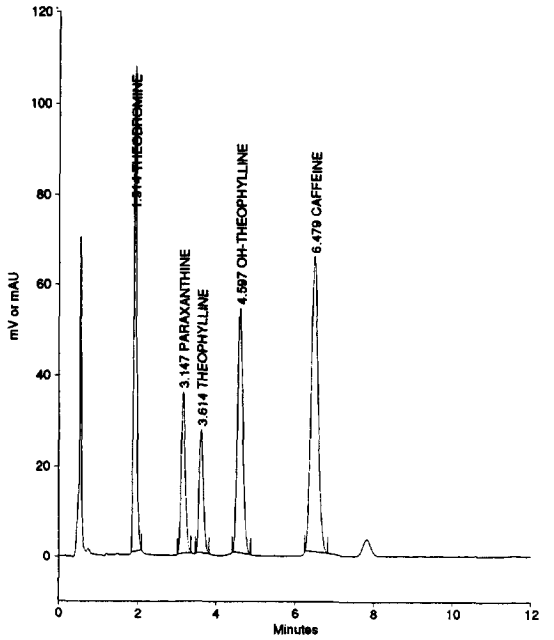

Fig. 1. Chromatogram of a QC sample containing theobromine, theophylline, paraxanthine and caffeine. Internal standard β-OH-ethyltheophylline (for concentrations, see Section 2.2).

range (IQR) and the far outside value defined as {(3×IQR)+Q3}, where Q3 is the 75th percentile, were calculated. The software package Statview (Albacus concepts, Berkeley, CA, USA) was used.

3. Results and discussion

A representative chromatogram obtained from a QC sample is shown in Fig. 1. Theobromine, paraxanthine, theophylline, caffeine and the I.S. were well separated. The calibration graphs showed good linearity in the 0–10 μg/ml concentration range. Correlation coefficients were 0.9997, 0.9995, 0.9996 and 0.9992 for theobromine, paraxanthine, theophylline and caffeine, respectively. The limit for accurate quantitation of theophylline ($S/N=3$) was 0.25 μg/ml. The accuracy of the method was demonstrated by analysing QC samples on 10 different days. The results were 4.90±0.44, 4.79±0.34, 2.55±0.23 and 4.77±0.34 μg/ml for theobromine, paraxanthine, theophylline and caffeine, respectively.

The concentrations of theophylline after the intake of THEO-2 are given in Table 1. From these results,

Table 1
Theophylline urinary concentrations C (μg/ml), TP/PX values and % dose excreted after 12 h (THEO-2, 150 mg theophylline)

Time (h)	Subject 1		Subject 2		Subject 3		Subject 4	
	C	TP/PX	C	TP/PX	C	TP/PX	C	TP/PX
0	0.00	0.00	0.00	0.00	0.43	0.14	0.15	0.09
1	[a]		0.50	0.20	0.68	0.30	0.36	0.25
2	0.70	0.56	1.84	0.75	1.77	0.99	0.94	0.93
3	[a]		4.16	1.45	3.09	2.01	1.26	1.39
4	3.70	3.01	2.68	2.14	5.24	3.48	1.32	2.05
6	8.95	4.57	5.77	2.36	6.05	4.84	2.78	2.92
9	7.77	4.86	8.18	2.53	6.31	3.25	3.31	4.04
12	4.50	5.00	7.99	2.67	2.86	3.21	4.35	6.21
24	7.56	1.84	5.58	2.55	1.99	3.65	4.89	2.31
30	9.05	1.58	n.d.	–	1.18	0.22	3.21	1.99
36	5.02	1.50	3.96	0.82	0.53	0.15	2.36	1.35
48	2.02	1.31	1.49	0.56	0.36	0.18	1.41	0.76
60	n.d.		0.00	–	0.40	0.06	0.00	–
72	0.98	0.11	n.d.	–	n.d.	–	0.39	0.28
% Dose (12 h)	8.8		21.1		9.1		12.9	

[a] = No urine available.
n.d. = Not determined.

it seemed that theophylline concentrations started to rise 2 h after the intake and remained increased for at least 24 h. A wide interindividual variation in time to peak concentration was found. Maximum concentration values ranged from 5 to 9 μg/ml.

Similar individual differences in theophylline concentrations were also noticed after the ingestion of 250 mg tablets (Table 2). Peak concentrations were obtained after 4–6 h, somewhat earlier than with THEO 2. Increased theophylline levels were found during at least 24 h. Approximately between 6 and 20% of the administered dose was excreted as the parent drug in 12 h.

A chromatogram obtained from a routine doping sample is shown in Fig. 2. Suspected theophylline cases are controlled for specificity by evaluating the UV spectrum and peak purity. The presence of theophylline is confirmed by gas chromatography–mass spectrometry (GC–MS).

Fig. 3 shows the distribution of theophylline concentrations in athletes' urine specimens. Assay values range from 0 to 52 μg/ml. Only 17 of the data points had values >5 μg/ml, three of which were >20 μg/ml. Theophylline levels above 0.25 μg/ml were found in only 1539 out of 3885 samples.

For drug control in sports, distinction should be made with the greatest degree of certainty possible between dietary theophylline and theophylline medication. Therefore, only theophylline-containing urine

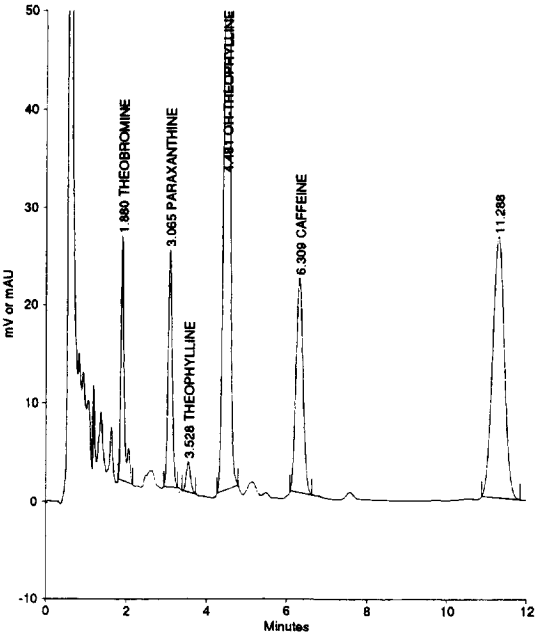

Fig. 2. Chromatogram of a routine sample. Measured concentrations were: theobromine, 0.7 μg/ml; paraxanthine, 1.6 μg/ml; theophylline, <0.25 μg/ml; and caffeine, 1.0 μg/ml.

Table 2
Theophylline urinary concentration C (μg/ml), TP/PX values and % dose excreted after 12 h (THEOLAIR, 250 mg theophylline)

Time (h)	Subject 1		Subject 2		Subject 3		Subject 4	
	C	TP/PX	C	TP/PX	C	TP/PX	C	TP/PX
0	0.00	0.00	0.00	0.00	0.36	0.12	0.00	0.00
1	[a]		3.76	1.38	1.70	0.70	1.80	0.56
2	5.95	3.07	13.47	4.85	4.75	1.59	4.38	1.45
3	[a]		29.00	10.47	5.04	1.68	4.46	1.69
4	8.25	9.82	33.93	10.86	5.81	2.03	9.75	1.93
6	49.14	14.50	21.01	10.43	7.94	2.45	8.93	2.85
9	35.18	8.20	24.25	8.58	6.76	1.72	9.14	1.93
12	4.53	5.09	22.19	6.93	4.05	1.69	9.57	1.92
24	10.20	0.87	11.85	3.65	4.51	1.51	5.90	1.12
30	9.46	0.60	8.54	2.44	2.82	0.20	2.23	0.33
36	2.15	0.48	4.19	1.59	0.74	0.17	1.62	0.25
48	2.48	0.20	1.80	0.71	0.64	0.12	0.46	0.16
60	n.d.		0.00		0.49	0.10	0.00	0.00
72	n.d.		n.d.		0.42	0.08	0.00	0.00
% Dose (12 h)	5.7		9.6		19.9		20.2	

[a] = No urine available.
n.d. = Not determined.

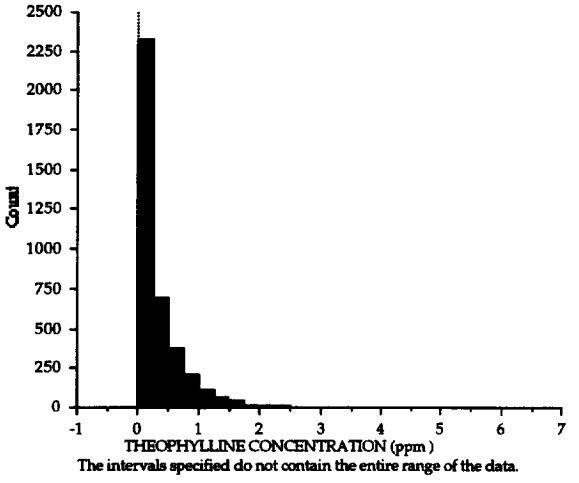

Fig. 3. Distribution of theophylline concentrations in 3885 urine samples, from athletes at unannounced doping control (October 1993–December 1995).

specimens were used for statistical evaluation. As a result the distribution was nongaussian. The IQR was calculated (Q3−Q1) as 0.47. The far outside value, defined as {(3×IQR)+Q3} was 2.25 μg/ml. Taking into account that nearly 60% of the 3885 samples did not contain theophylline or had a level below the quantitative detection limit, a decision level of 5 μg/ml will certainly ensure that no false positive results are reported.

When this level was applied to the urine samples from the 150 mg administration study (Table 1) only 11 specimens would be positive. Moreover, none of the samples collected from Subject 4 had a value >5 μg/ml. After the intake of 250 mg, 22 samples should have tested positive, generally for periods between 4–30 h after administration (Table 2). However, only three samples from Subject 3 had values distinctly exceeding 5 μg/ml.

Caffeine is predominantly metabolised [6] by demethylation to paraxanthine (72%), theobromine (20%) and theophylline (8%). The renal elimination of these dimethylxanthines is dependent on urinary flow [7–9]. Although the caffeine urinary metabolite pattern can be expected to vary, the habitual coffee intake does not affect the metabolite pattern of caffeine [7]. Therefore the TP/PX ratio should be a reliable marker of the intake of non-dietary theophylline [5].

A number of drugs coadministered with theophylline have been shown to interfere with theophylline pharmacokinetics [10]. Those interactions might be caused by other drugs influencing the liver function. However, Birkett et al. [11] and Lelo et al. [6] indicated that a common group of cytochrome P-450 isoenzymes was involved in the metabolism of dimethylxanthines. Therefore the ratio TP/PX should not be substantially influenced by the intake of other drugs.

The distribution of the TP/PX values for those samples with theophylline levels >0.25 μg/ml is presented in Fig. 4. The distribution was non gaussian. The median was 0.074, 17 samples had TP/PX values between 0.25 and 0.50; 16 urine specimen had ratios >0.50, the highest value being 20.

Transformations of the data ($\sqrt{x}$, $\sqrt[3]{x}$, log x) did not yield useful results. The IQR was 0.035 and the far outside value 0.2. A decision level of 0.5 for TP/PX is therefore proposed. Applying this threshold value to the urine samples after the 150 mg study (Table 1), should result in positive tests for all samples from 2 to 48 h (Subject 3 from 2 to 24 h) after administration.

When the absolute theophylline value (5 ppm) was applied to the urine samples of Subject 4, no samples should test positive. However, the application of the

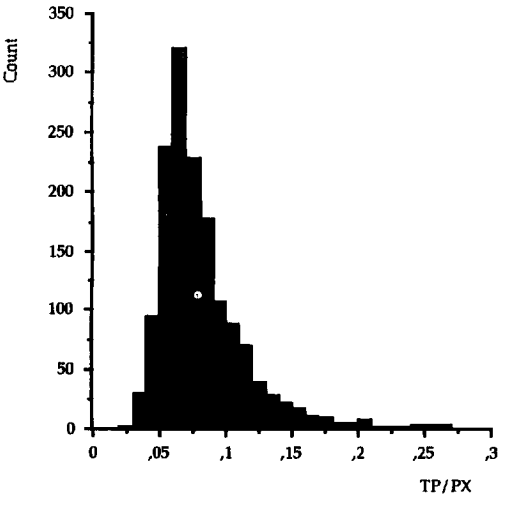

Fig. 4. Distribution of TP/PX in urine samples with theophylline levels >0.25 μg/ml (unannounced doping control October 1993–December 1995).

TP/PX ratio resulted in positive samples from 2 till 48 h.

This ratio seems to be more reliable than the theophylline concentration threshold, as e.g. in Subject 3 (250 mg administration) approximately the same detection times were found as in the other subjects when the TP/PX decision level was applied.

As the TP/PX ratio is not dependent on the specific gravity of the urine and results in longer detection times, it should be preferred as a threshold value for the non dietary intake of theophylline. In those athletes refraining from drinking coffee (no paraxanthine) an absolute concentration of 5 ppm is proposed as the decision limit.

Acknowledgments

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