Simultaneous Determination of Caffeine, Theobromine, and Theophylline by High-Performance Liquid Chromatography

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Abstract

This work relates the development of an analytical methodology to simultaneously determine three methylxanthines (caffeine, theobromine, and theophylline) in beverages and urine samples based on reversed-phase high-performance liquid chromatography. Separation is made with a Bondesil C18 column using methanol–water–acetic acid or ethanol–water–acetic acid (20:75:5, v/v/v) as the mobile phase at 0.7 mL/min. Identification is made by absorbance detection at 273 nm. Under optimized conditions, the detection limit of the HPLC method is 0.1 pg/mL for all three methylxanthines. This method is applied to urine and to 25 different beverage samples, which included coffee, tea, chocolate, and coconut water. The concentration ranges determined in the beverages and urine are: < 0.1 pg/mL to 350 µg/mL and 3.21 µg/mL to 71.2 µg/mL for caffeine; < 0.1pg/mL to 32 µg mL and < 0.1 pg/mL to 13.2 µg/mL for theobromine; < 0.1 pg/mL to 47 µg/mL and < 0.1 pg/mL to 66.3 µg/mL for theophylline. The method proposed in this study is rapid and suitable for the simultaneous quantitation of methylxanthines in beverages and human urine samples and requires no extraction step or derivatization.

Introduction

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) are alkaloids naturally present in green and black tea, coffee, and cocoa. Caffeine is added to soft drinks as a flavoring agent (1), and from dietary sources is the most frequently and widely consumed central-nervous-system stimulant today (2,3). Nevertheless, it was classified as a drug of abuse by the International Olympic Committee (IOC) when present in urine at concentration levels of > 12 µg/mL (2,4,5,6). The high levels of caffeine consumption have been implicated in various disorders including the increase of gastric-acid secretion, kidney malfunction, heart disease (cardiac arrhythmia), and disturbances of the central nervous system such as seizures and delirium. However, caffeine is utilized as a coadjuvant in many pharmaceutical formulations (7).

Theobromine and theophylline are active ingredients of bronchodilator drugs that are used in the treatment of acute and chronic asthma. The IOC does not consider theobromine and theophylline illicit substances in humans, though their administration to racing animals is prohibited (5,7).

There is an increasing interest in developing analytical methodologies for the determination of caffeine, theobromine, and theophylline in food products, biological fluids, and the quality control of decaffeinated beverages or chocolate, as well as in pharmaceutical manufacturing processes. Several chromatographic methods have been proposed for the determination of these methylxanthines in a variety of matrices (4,5,6). However, only a few of them permit the complete separation of the three compounds (8), require sample pretreatment before the determination step, or do not show very low detection limits. In this study, an analytical methodology was developed to simultaneously determine caffeine, theobromine, and theophylline in beverages, chocolate, and human urine based on reversed-phase high-performance liquid chromatography (HPLC) with UV detection, which permits the direct injection of beverage samples without any pretreatment or derivatization, except for a filtration step. This procedure allows for the determination of the compounds in less than 12 min with a detection limit of 0.1 pg/mL.

Experimental

Reagents and standards

Methanol, ethanol (HPLC grade, 99.9%), and acetic acid were obtained from Merck (Darmstadt, Germany) and filtered in a 0.45-µm membrane. Caffeine (Carlo Erba, Milan, Italy) was puri-

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fied by sublimation. Purity was established by gas chromatography. Theophylline was obtained by extraction from 100 mg medicinal Talofiline (Novartis-Sandoz, Switzerland) and purified by recrystalization. Theobromine (Sigma Chemical Co., St. Louis, MO) was also purified by recrystalization. The purities of the theophylline and theobromine were verified by comparing their melting points with the literature. Purified water was obtained by distillation and filtration through an E-pure Alltech system (Deerfield, IL).

Urine samples were obtained from volunteers of the local community.

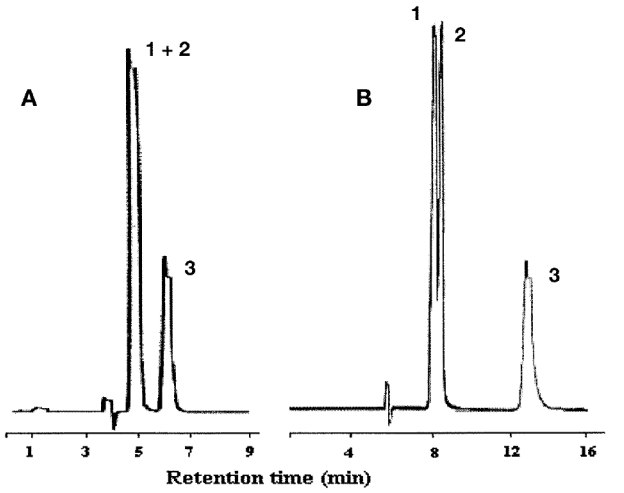

Figure 1. Chromatograms obtained for methylxanthine standards: (A) mobile phase methanol–water (40:60, v/v) (pH = 6.14) and (B) methanol–water–acetic acid (20:79:1, v/v/v) (pH = 3.12). Numbers 1, 2, and 3 represent theobromine, theophylline, and caffeine, respectively.

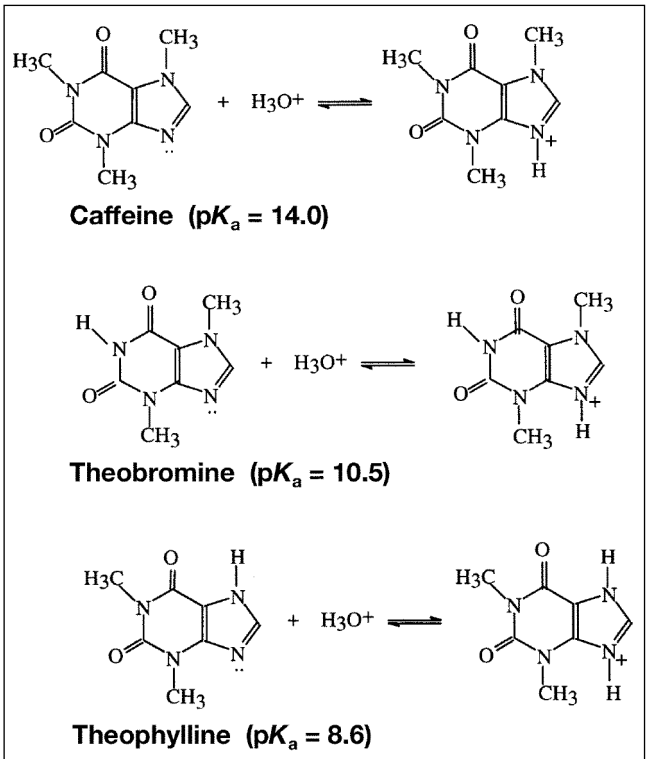

Figure 2. Methylxanthines protonated.

Preparation of methylxanthine stock solutions

Stock solutions of caffeine, theobromine, and theophylline were prepared by dissolving 40 mg of each in 200 mL of ethanol–water (50:50, v/v) or methanol–water (50:50, v/v) and filtered through a 0.45-µm membrane filter. They were stored at 40°C in dark-glass flasks.

Preparation of methylxanthine calibration solutions

The standard solutions were prepared by dilution in the water of the methylxanthine mixture stock solutions within the concentration range of 0.25 to 60 µg/mL. They were stored in the dark-glass flasks at 4°C. In these conditions they remained stable for 60 days.

Sample preparation

All of the sample solutions were filtered using Whatman 41 filter paper (twice, double filter). The consistency of the coffee sample was 150 mL hot water per 4 g coffee, which was approximately 1 cup (variable amounts were used to reproduce normal conditions of use). The soluble coffee was 150 mL hot water per 1 g of coffee, and was also approximately 1 cup (variable amounts were used to reproduce normal conditions of use). The tea was brewed with the tea-bag (2 g) and 150 mL boiling water for 3 min. The cocoa consisted of 0.2 g chocolate per 20 mL water and was cleaned from grease by Soxhlet extraction with hexane. Coconut water was filtered, and urine was centrifuged and stored at 40°C until the analysis.

Compounds separation

The separation was made across a Bondesil C18 5-µm column

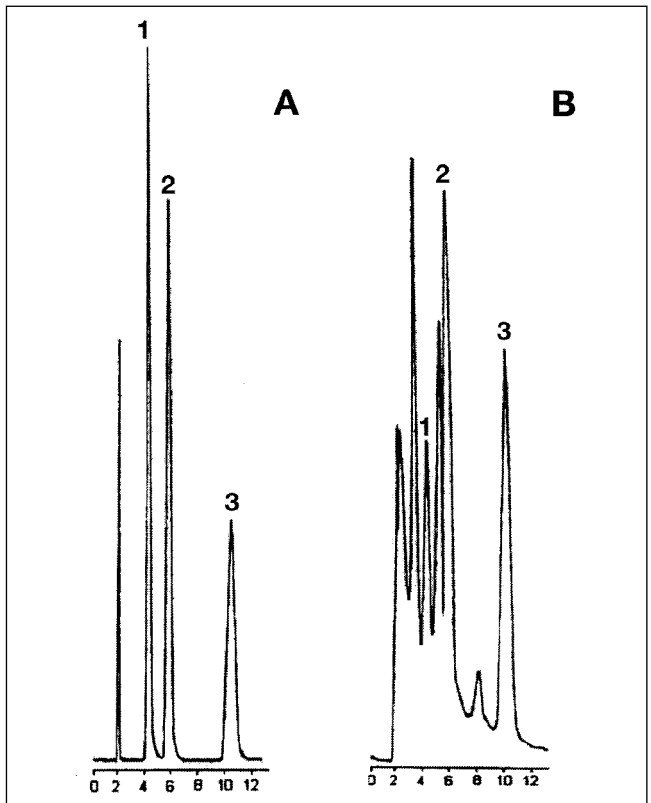

Figure 3. Chromatograms obtained for (A) methylxanthine standards and (B) a coffee sample with methanol–water–acetic acid (20:75:5, v/v/v). Numbers 1, 2, and 3 represent theobromine, theophylline, and caffeine, respectively.

(4.0-mm × 15-cm) (Varian, Walnut Creek, CA) using methanol–water–acetic acid or ethanol–water–acetic acid (20:75:5, v/v/v) as the mobile phase at a flow rate of 0.7 mL/min with a Varian liquid chromatograph Model 2510 equipped with a Rheodyne (Cotati, CA) injector with a 20-µL sample loop.

Compound detection was made by absorbance (Varian UV–vis detector Model 2550) at 273 nm (AUFS = 0.04).

Results and Discussion

The detection limit that an analytical procedure may achieve greatly depends on the reagent blank quality. The purification of caffeine by sublimation and of theophylline and theobromine by recrystalization resulted in a very low blank level for all methylxanthines, thus resulting in a detection limit of the HPLC method (signal-to-noise ratio = 3 based on peak height) of 0.1 pg/mL using the detector in the scale of 0.005 AUFS for caffeine, theophylline, and theobromine.

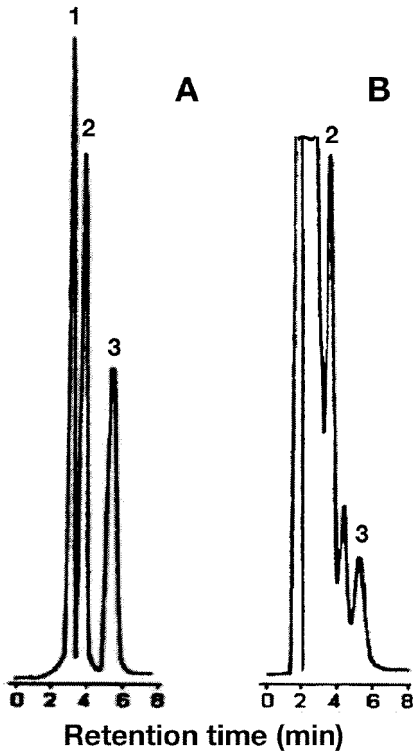

Figure 4. Chromatograms obtained for (A) methylxanthine standards and (B) a urine sample with ethanol–water–acetic acid (20:75:5, v/v/v). Numbers 1, 2, and 3 represent theobromine, theophylline, and caffeine, respectively.

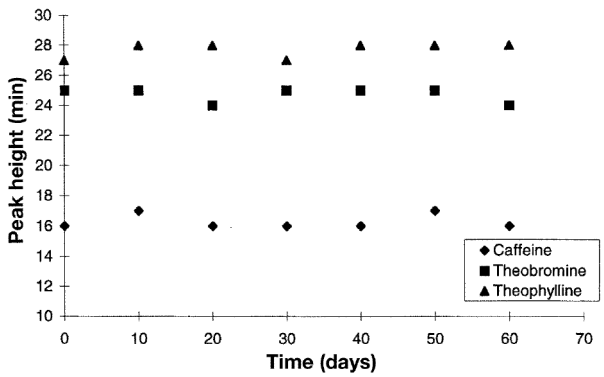

Figure 5. Study of the methylxanthines' stability.

Most of the methods described in the literature for methylxanthine analysis use mobile phases containing only methanol–water (apparent pH = 6.14) (8), which allows caffeine quantitation but does not separate theophylline from theobromine (Figure 1). Methylxanthines can suffer protonation, which results in ionic species stabilized by resonant and inductive effects (Figure 2). If the pH decreased below 4, the xanthines became protonated and the interaction with C18 reverse-phase columns increased. Because of this, acetic acid was added to the mobile phase (apparent pH < 3) in order to increase the acidity, which resulted in good separation conditions (Figures 1 and 3). The HPLC determination was completed in approximately 12 min when the mobile phase methanol–water–acetic acid (20:75:5, v/v/v) was used (Figure 3) or 6 min when the mobile phase ethanol–water–

Table I. Calibration Curves of HPLC for the Determination of Caffeine, Theophylline, and Theobromine

Theobromine	Theophylline	Caffeine
$y^* = 1.3057x^\dagger + 10.8$ $r^\ddagger = 0.9996$	$y = 0.8057x + 8.4667$ r = 0.9994	$y = 1.7286x + 10.667$ r = 0.9998

* *y*, peak height.
† *x*, concentration (µg/mL).
‡ r, correlation coefficient.

Table II. Methylxanthine Mean Concentrations Determined in Several Food Samples

Beverage	Caffeine (µg/mL)	Theobromine (µg/mL)	Theophylline (µg/mL)
Chocolate			
Dietetic	4.00	17.0	$< 10^{-7}$
With milk	5.00	21.0	$< 10^{-7}$
Coffee			
A	350	17.0	$< 10^{-7}$
B (decaffeinated)	26.0	13.0	47.0
C (soluble)	122	12.0	15.0
D	67.0	$< 10^{-7}$	$< 10^{-7}$
Tea			
Fruits	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
Balm (mint)	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
"Espinheira santa"	11.0	9.00	9.00
"Boldo"	$< 10^{-7}$	12.0	$< 10^{-7}$
Mate	62.0	32.0	21.0
"Carqueja"	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
Black	217	12.0	$< 10^{-7}$
Black with cinnamon	17.0	$< 10^{-7}$	$< 10^{-7}$
Camomile	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
Coconut water	0.26	$< 10^{-7}$	8.87

Table III. Methylxanthine Mean Concentrations Determined in Several Samples of Human Urine

Beverage	Caffeine (µg/mL)	Theobromine (µg/mL)	Theophylline (µg/mL)
1*	71.2	10^{-7}	10^{-7}
2*	40.1	10^{-7}	10^{-7}
3†	3.21	13.2	10^{-7}
4‡	13.3	10^{-7}	66.3
5†	2.67	1.81	2.83
6†	4.90	10^{-7}	1.50

* Smokers, high consumption of coffee.
† Nonsmokers.
‡ Asthma carrier, use of 100 mg Talofilina (of the 20 analyzed samples 14 presented methylxanthine concentrations below the detection limit).

acetic acid (20:75:5, v/v/v) was used (Figure 4).

The stability of the standards and samples containing methylxanthines is one of the fundamental factors for the warranting of exact results (8). Because of this, a study of the stability of the methylxanthine standards was conducted for 60 days. The standards were prepared in methanol or ethanol and stored in dark-glass flasks at 4°C. They showed good stability (Figure 5) and could be used safely for at least two months.

Different concentrations of caffeine, theophylline, and theobromine solutions, in the concentration range of 10 to 60 µg/mL (n = 6 points), were injected into the HPLC system, and the peak heights obtained were plotted against concentration. The calibration curves showed good linearity, as is shown in Table I.

A precision study in the determination of caffeine, theophylline, and theobromine in seven different aliquots provided a relative standard deviation of 0.44%, 1.02%, and 0.64%, respectively, in peak-height variation.

Table II shows the results obtained in the caffeine, theobromine, and theophylline analysis in food samples. The methylxanthine-determined concentrations were in the range of < 0.1 pg/mL to 350 mg/mL for caffeine, < 0.1 pg/mL to 32 mg/mL for theobromine, and < 0.1 pg/mL to 47 mg/mL for theophylline. The methylxanthine concentrations (determined by the 20 analyzed urine samples) were < 0.1 pg/mL to 71.2 mg/mL for caffeine; < 0.1 pg/mL to 13.2 mg/mL for theobromine, and < 0.1 pg/mL to 66.3 mg/mL for theophylline. The largest values of caffeine concentrations were observed in the urine collected from two different smokers and consumers of great amounts of coffee. The highest value for theophylline concentration was observed in the urine of an asthma carrier who was being medicated with the bronchodilator 100 mg Talofilina (Table III).

Conclusion

The proposed method was shown to be appropriate for the separation and simultaneous quantitation of caffeine, theobromine, and theophylline in samples of food products and human urine, which presented high sensibility and quickness. The analyzed samples needed no pretreatment or derivatization. They needed only filtration and if necessary a suitable dilution.

Methanol is internationally one of the main solvents employed in liquid chromatography. Its substitution for ethanol in mobile phases used in HPLC systems results in lower costs and toxicity. In this study, it shows the same effectiveness in the separation of methylxanthines.

The application of the method for urine analysis allows for doping control in sporting competitions because caffeine is considered an illicit drug for humans by the IOC, and the theobromine and theophylline are prohibited in tests for animals. The method can also be applied in the control of formulations of medications because the three methylxanthines in this study are generally used as therapeutic agents, and, in food products, mainly because there exists suspicions that caffeine causes teratogenic activity.

Acknowledgments

This work was supported by the National Research Council of Brazil (CNPq) and SEPLANTEC/CADCT-BA. The authors thank Prof. Dr. Pedro Afonso de Paula Pereira for his useful discussions.

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Manuscript accepted September 5, 2001.