Quantification of caffeine from urine in Spanish triathletes

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Cuantificación de cafeína en orina de triatletas españoles

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Resumen

Introducción: La ingesta de cafeína sigue siendo común entre los atletas con el objetivo de mejorar su rendimiento deportivo, aunque desde el 1 de enero de 2004, la cafeína ha sido eliminado de la lista de sustancias prohibidas de la Agencia Mundial Antidopaje, aunque está consensuado no sobrepasar ciertos límites (12 µg mL^{-1}) por los efectos secundarios producidos por esta. El uso de una rápida y fácil extracción en fase sólida para la cafeína seguido de cromatografía líquida es útil para evaluar la presencia en muestras de orina.
Propósito: Investigar la presencia de cafeína urinaria de triatletas españoles usando una nueva extracción en fase sólida rápida y fácil seguido de cromatografía líquida acoplada a detección de ultravioleta (CL-UV).
Métodos: Las muestras urinarias se extrajeron con un cartucho Sep-Pak de fase extractiva C18 seguido por CL-UV siendo utilizada la β-hidroxiteofilina como estándar interno. Se estudió veintiséis triatletas españoles de nivel competitivo de edades comprendidas entre 24,4 y 25,9 años. Las muestras urinarias se recogieron después de la competición.
Resultados: El método de extracción optimizado tiene un límite de detección una recuperación de 0,05 µg mL^{-1} y 92,8%, respectivamente. Se detectó la presencia de cafeína urinaria en un 80,8% de los triatletas estudiados con un rango de 0,06 a 1,99 µg mL^{-1}. El porcentaje de muestras urinarias por debajo del LD (expresado como muestras negativas) fue de un 19,2%.
Conclusiones: El método optimizado es útil para el análisis de cafeína urinaria proporcionando una adecuada relación coste beneficio para el análisis rutinario de este compuesto; las características del método son su adecuada robustez, precisión y manejo de la muestra mínima. Su aplicación en muestras reales ha demostrado la presencia de cafeína en orina, pero ninguno de los triatletas superaba el punto de corte establecido por la Agencia Mundial Antidopaje (12 µg mL^{-1}).

Palabras clave:
Cafeína. Orina.
Triatletas. CL-UV.
Extracción en fase sólida.

Summary

Introduction: The ingestion of caffeine is also common in a specific group of people as are athletes, in order to improve athletic performance although from January 1st 2004, caffeine has been removed from the World Anti Doping Agency list of prohibited substances.The use of rapid and easy solid-phase extraction for caffeine extraction followed by liquid chromatography is useful to evaluate the presence in urine samples.
Purpose: To investigate the presence of caffeine in urine samples from Spanish competitive triathletes using a new easy and fast caffeine extraction method followed HPLC-UV detection.
Methods: Samples were extracted from urine in a reversed-phase C18 Sep-Pak Classic cartridge followed by HPLC coupled in the UV detector. β-hydroxyteofiline was used as internal standard. Twenty-six Spanish elite men triathletes with age 24.4–25.9 were studied. Urine samples were collected after competition.
Results: The extraction method followed HPLC-UV detection has a limit of detection and recovery of 0.05 µg mL^{-1} and 92.8%, respectively. Twenty-six urine samples from triahtletes were analyzed after competitions detecting in 80.8% values of caffeine that ranged from 0.06 to 1.99 µg mL^{-1}. The percentage of urine samples below the LOD (reflected as negative samples) was 19.2%.
Conclusions: The optimized method is useful for the analysis of caffeine in human urine which provided an adequate cost-benefits ratio for routine analysis of this compound; its valuable features are sufficient sample throughput, robustness, precision and minimal sample handling. Its application in reals samples demonstrated the presence of caffeine in urine but any studied triathletes showed values above the former WADA cut-off (12 µg mL^{-1}).

Key words:
Caffeine. Urine.
Triathletes. HPLC-UV.
Solid-phase extraction.

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Introduction

Caffeine is an alkaloid naturally present in plants used world-wide in many kind of beverages as a stimulator of the central nervous system and cardiac stimulant[1]. The ingestion of caffeine is also common in a specific group of people as are athletes, in order to improve athletic performance[2-4]. However, one should not forget that the consumption of high concentration of caffeine, 150 to 200 milligrams per kilogram of body mass, can cause some undesirable effects such as cardiac arrhythmia, excitement, nausea and gastric acidity[5]. As a result of the reported abuse of high doses the International Olympic Committee (IOC) had classified caffeine as a drug of abuse when it was present in human urine with concentrations higher than 12 μg mL^{-1}. Nevertheless, from January 1st 2004 on, caffeine has been removed from the World Anti Doping Agency[6] list of prohibited substances with the caveat that the doping laboratories keep on monitoring caffeine levels[7]. In triathletes, among other sports, the use of caffeine is reflected in the literature[3,8-11].

Caffeine was reported to be absorbed into plasma and excreted in urine following oral dose in humans. Different analytical methods have been used for the determination of caffeine when administered in biological matrices. The first method was from Axelrod and Reichenthal[12] involving extraction with an organic solvent, re-extraction into aqueous acid and measurement of the ultraviolet absorbance. Other techniques has been used such as high- performance liquid chromatography-UV/DAD, high- performance liquid chromatography-mass spectrometry, gas chromatography, gas chromatography–mass spectrometry, micellar electrokinetic chromatography (MEKC), electrophoresis with paper chromatography, radioimmunoassay, spectrophotometric analysis, thin-layer chromatography (TLC) plates and capillary electrophoresis[13-16]. In biological samples, interesting compounds are often present at low concentrations and may be accompanied by excessive amounts of high molecular-weight material. Consequently, clean-up have to be carried out prior to analysis in order to avoid destruction of analytical column. In the past, liquid-phase extraction was common; however solid-phase extraction (SPE) has been progressively replacing the liquid–liquid extraction, because it is more easy and solvent saving which leads to reduction in pollution and offering wide sorbent selection[17].

The present work reports the analysis of caffeine from urine in twenty-six Spanish elite men using a rapid and easy solid-phase for caffeine extraction followed by HPLC-UV detection.

Materials and methods

Subjects and samples

Twenty-six elite men triathletes were recruited in Spain. They were fully informed about all procedures and risks of the investigation before their written informed consent was obtained. The study was approved by the Ethics Committee of the University of Valencia (Spain). The average age of the participants was 25.0 years [95% confidence interval (CI), 24.4–25.9 years] and were non-smokers and free from medication. All samples corresponded to the urine were collected after competition, acidified to pH 3.5 and stored frozen at -20 °C.

Materials and reagents

Caffeine and β-hydroxyteofiline, as internal standard, were supplied by Sigma-Aldrich (Madrid, Spain). Methanol and tetrahydrofurane (THF) for HPLC grade were purchased from Merck (Darmstadt, Germany). Sep Pak C18 cartridges (Waters-Millipore, Milford, MA, USA). Deionized water used in the HPLC mobile phase was obtained by a Milli-Q purified system (Millipore Ibérica, Barcelona, Spain). A stock solution of caffeine and β-hydroxyteofiline was prepared in both in methanol at the concentration of 100 μg mL^{-1}. All stock solutions were stored at 4ºC.

Sample preparation

Urine samples were homogenized with a Sonicator and caffeine, containing the internal standard, was extracted from urine in a reversed-phase C18 Sep-Pak Classic cartridge. The cartridge was conditioned prior to use with 1 mL of methanol, followed by 5 mL of distilled water. 100 μL of pH 9.5 ammonium buffer and 50 μL of internal standard were added to 2 mL of urine. The mixture was slowly loaded onto the Sep-Pak C18 (flow-rate 2 ml min^{-1}). The cartridge was rinsed with 10 mL of distilled water, 5 mL of a mixture of water with 0.5% THF and 1 mL of solvent B with 1% of pH 9.5 ammonium buffer in order to remove matrix interferences. The adsorbed fraction containing caffeine and the internal standard was eluted with 1 mL solvent B: methanol (40:60 v/v) and directed injected in the HPLC-UV system.

Chromatographic procedure

Chromatographic analysis was performed using a Jasco (Madrid, Spain) LC system PU-2089 Plus equipped with Quaternary Gradient Pump, a Rheodyne model 7725i injector (20 μL loop), and a UV detector L-7400 LaChrom from Merck. Caffeine and the internal standard were eluted with a Phenomenex (Madrid, Spain) Luna C18 (250 mm x 4.6 mm I.D., 5 μm particle size) analytical column, preceded by a security guard cartridge C18 (4 mm x 2 mm I.D.). The mobile phase included solvent A, methanol, and solvent, B, methanol:THF:water (10:0.5:89.5). The flow rate was 0.8 mL/min. It was monitored at 278 nm, where the absorbance of caffeine and internal standard were maximum. Separations were performed under isocratic conditions with 10% of solvent A (90% solvent B) during 20 min. For the column wash step, solvent A increased up to 40% during 2 minutes and return to the analytical conditions. Peak areas were measured and the ratio with the peak area of the internal standard was used for all calculations. The results of the analysis were expressed as μg $mL.^{-1}$ urine.

Results

Validation procedure

Calibration curve was obtained with human caffeine-free urine spiked (Figure 1B) with known amounts of the stock solution of caffeine and internal standard: 15, 10, 5, 2.5, 1.25, 0.65, 0.1 and 0.05 μg mL^{-1}. The between-days coefficient of variation (at 1.25 μg mL-1) was 7%. Calibration curves were constructed by plotting the peak area ratio of caffeine to β-hydroxyteofiline against the amount of standard concentrations.

Figure 1. Chromatograms from blank (A), spiked (B) and positive (C) urines from triathletes.

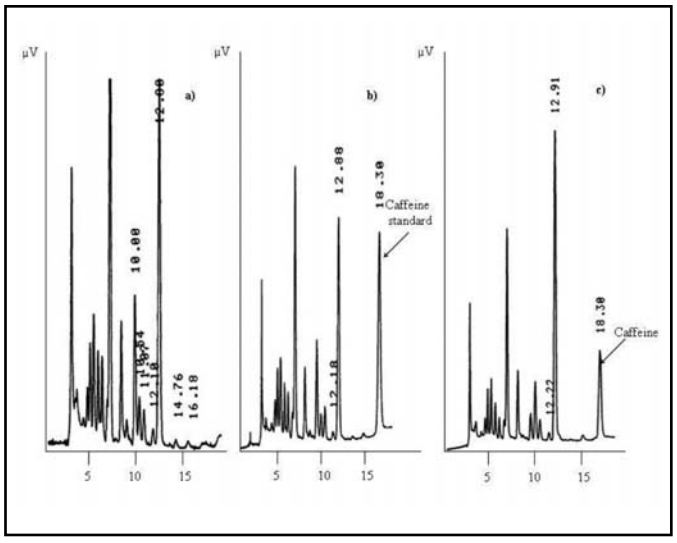
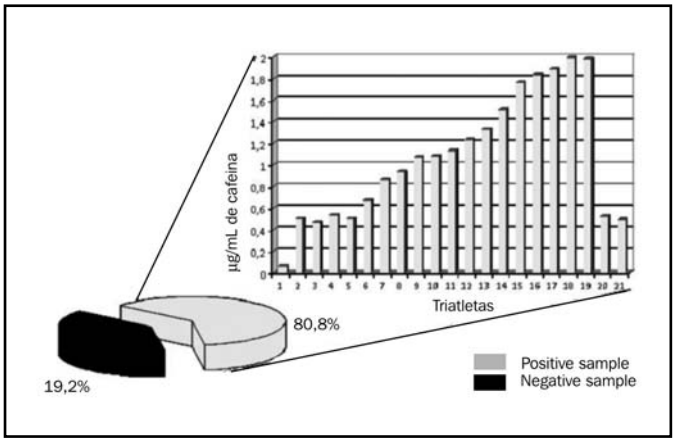


Figure 2. Caffeine in urine from studied triathletes.



Correlation coefficients and the equations describing the calibration curves were determined by linear regression analysis. The recovery of caffeine (92.8%) and β-hydroxyteofiline (96.1%) was measured by comparing peak-area of extracted quality control with the peak-area area from direct injection of an aqueous solution containing the same amount of caffeine (n = 6). Limit of detection (LOD; 0.05 µg mL^{-1}) and limit of quantification (LOQ; 0.1 µg mL^{-1}) were calculated by injection of freshly prepared extracts from aqueous standard solutions of caffeine as well as in caffeine-free urine spiked diluted to known concentrations to a final response equal to three and ten times, respectively of the signal-to-noise ratio.

Application of analytical procedure to triathletes

Figure 2 shows the distribution of all urine analyzed in relation to the caffeine concentration measured. The percentage of urine samples below the LOD (reflected as negative samples) was 19.2% (Figure 1A), the remaining 80.8% of the urine samples (Figure 1C) contained caffeine in a concentration higher than 0.05 µg mL-1. The range of urinary caffeine concentrations was from 0.06 to 1.99 µg mL^{-1}.

Discussion

Validation procedure

Our study reported an easy and fast solid-phase extraction method of caffeine from urine with subsequent HPLC/UV determination. The chromatogram obtained following solid phase extraction and HPLC analysis shows no interference from urine components, indicating an efficient clean up method used and selectivity of the method. Most of the solid-phase extraction and liquid-liquid extraction methods in the literature include an evaporation step[13-16]. Compared to these methods, in our case the evaporation step is not necessary for the caffeine determination, thus we achieve a time reduction for sample preparation. Moreover, some liquid-liquid extraction methods yielded limits of detection higher[16] than those obtained with solid-phase extraction. On the other hand, our LOD (0.05 µg mL-1) is lower than the GC-MS analytical procedure use by Del Coso *et al.*[8].

Application of analytical procedure to triathletes

The wide range of caffeine concentrations determined may be due to complex caffeine metabolism with significant differences between species as well as between individuals in different clinical situations[18]. Others works had also found wide caffeine concentrations ranges in urine samples, i.e. in the study of Conway *et al.*[9] they found caffeine concentrations ranging from 0.16 to 2.35 µg mL^{-1} in the urine of triathletes and cyclists after a single caffeine dose of 6 mg Kg^{-1}. Del Coso *et al.*[8] carried out a study in urine samples for doping control from several sports and they had a proportion with caffeine concentrations above the former WADA cut-off of 0.6%. Furthemore, they reflected that the sports with the highest concentration of caffeine were triathlon (3.3 ± 2.2 µg mL^{-1}) and older competitors (>30 years) had higher levels of caffeine in their urine than younger competitors (<20 years). A survey of 140 competitors at the 2005 Ironman Triathlon World Championships revealed only 72% of the athletes were aware of this. 89% of athletes indicated they planned on using caffeine either before or during competition[9]. On the other hand, one out of twenty-six (4%) triathletes residing in the Western Cape region took supplements daily of caffeine[11]. According to Del Coso *et al.*[8], we think that the use of caffeine is related to an attempt to increase performance, mainly in endurance sports as is the triathlon.

Conclusion

In conclusion, a high percentage in our study had the presence of this compound in urine but any studied triathletes showed values above the former WADA cut-off (12 µg mL^{-1}) being applied a sensitive and selective method for the analysis of caffeine in human urine which provided an adequate cost-benefits ratio for routine analysis of this

compound; its valuable features are sufficient sample throughput, robustness, precision and minimal sample handling.

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