# Concomitant Quantification of Caffeine, Cotinine and N-methyl Uric Acid in Urine

Applications for athlete monitoring and pharmacological screening

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Simultaneous determination of caffeine, cotinine, and 1N-methyluric acid in urine has numerous applications in determining patterns of use by athletes, according to the World Anti-Doping Agency (WADA) which includes these substances on the Monitoring List. The method can provide information on the enzymeinducing activity on CYP1A2 of polycyclic aromatic hydrocarbons from cigarette smoke. Urine samples from 30 people (15 smokers and 15 non-smokers) were sampled 6 hours after having consumed a beverage with a total caffeine content of 200 mg. A HPLC with UV detection method was used which allowed concomitant determination of the three analytes. The results obtained show different values of caffeine urine concentrations ( $6.47 \pm 3.63 \mu$ M/L in smokers vs  $10.09 \pm 5.68$  in non-smokers, p < 0.05) and a higher elimination of N1-methyluric acid by over 50% in smokers (identifiable due to the presence of cotinine, the main metabolite of nicotine).

Keywords: caffeine, cotinine, smoking, CYP1A2-mediated metabolism, sport monitoring

Caffeine intake is common in the general population by the use of stimulating drinks and it is also present in numerous dietary supplements for athletes based on their ergogenic and lipolytic properties [1]. Caffeine is also widely used as the main ingredient in energy drinks on the market [2]. Caffeine was included until 2004 on the WADA (World Anti-Doping Agency) list of forbidden substances (with a threshold of 12  $\mu$ g/mL in urine, i.e. about 61.8  $\mu$ mol/ L) lately included in the monitoring list (in 2017 also) to highlight the clichés of abusive use in sports. The current *in competition only* monitoring list includes among other substances caffeine and nicotine [3]. Exposure to nicotine is caused almost exclusively by smoking, but cigarette smoke also contains polycyclic aromatic hydrocarbons; these are potent inductors of CYP1A2 isoforms of chromosomal oxidases involved in the hepatic metabolism of many drugs, including caffeine. CYP1A2 is a highly inducible enzyme and polycyclic aromatic hydrocarbons in cigarette smoke are involved in carcinogenesis (bioactivation of procarcinogens such as benzo[a]pyrene),

in the induction process the transactivation of AHR (aromatic hydrocarbon receptor) occurs at nuclear level [4].

The first step in caffeine metabolism occurs almost exclusively at hepatic level by 3-N-demethylation to paraxanthine (1,7-dimethylxanthine), an exclusive CYP1A2-dependent pathway; paraxanthine is the quantitative dominant metabolite in phase I (> 80% of the total); the rest of the metabolites are considered secondary from the quantitative point of view, the other isoforms (CYP3A4, CYP2C8, CYP2C9) [5-7] being involved in their biosynthesis; each type of metabolic reaction is CYP1A2 dependent, but the importance of quantitative catalytic action in each metabolic reaction is different (in the following order 3-N-demethylation > C-8-hydroxylation > 7-N-demethylation > 1-N-demethylation). In phase II reactions, paraxanthine undergoes a 7N-demethylation process (CYP1A2-dependent), then is converted to 1Nmethyluric acid by xanthine oxidase, only 3% of the ingested caffeine dose being eliminated with urine as such [5, 6] figure 1.

CYP3A4 (33%)



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Secondarily, paraxanthine may be a substrate of NAT2 (N-acetyltransferase-2) and CYP2A6, but these are minor metabolic pathways, which are of no quantitative importance (therefore the FDA recommends the clinical use of caffeine as a nontoxic substrate, easily accessible for investigating the catalytic action of CYP1A2).

Chronic smoking by repeated systemic exposure to polycyclic aromatic hydrocarbons decreases plasma and urinary concentrations and consequently the therapeutic efficacy of many drugs metabolised via CYP1A2 (caffeine, theophylline, clozapine, olanzapine, verapamil [4]), but also substances prohibited to athletes such as aromatase inhibitors (exemestane) [8].

Nicotine is primarily metabolised via the CYP2A6 isoform to cotinine - the major metabolite, the CYP1A2 enzyme pathway being minor (5-10% of the total) [9]. The presence of cotinine in the urine may be a smoking marker (also taking into account a longer half-life than nicotine) that indirectly reflects exposure to polycyclic aromatic hydrocarbons, carcinogenic and CYP1A2 inducers [10] since cotinine and nicotine do not influence the metabolism of caffeine [11].

# **Experimental part**

The main purpose of the paper was to develop an analytical method that would allow co-determination of caffeine and the main metabolite (1N-methyluric acid) as well as cotinine in the urine. The method can be used to monitor athletes (according to the WADA list) but also to clarify smoker / non-smoking status in patients with apparent resistance to CYP1A2 substrate drugs (theophylline-treated asthma, psychotic patients treated with clozapine or olanzapine) when they deny being smokers. Also, in the absence of exposure to cigarette smoke, the method can provide preliminary information on the catalytic activity of CYP1A2 in order to select patients for costly pharmacogenetic tests (CYP1A2 phenotyping that requires determination of caffeine and all its metabolites or genotyping to identify the alleles involved in the expression of the CYP1A2 gene, which is an inaccessible method in current routine practice).

The study was approved by the Research Ethics Committee of UMF Targu Mures (47 / 21.03.2016) and all participants signed the informed consent.

#### Study subjects

The study included 30 healthy volunteers, 15 smokers and coffee consumers, and 15 and non-smoking coffee consumers.

# Study protocol

Six of the 15 non-smokers were asked to provide a *blank* urine sample. They did not consume coffee or caffeine-containing products (tea, beverages, or caffeine-containing foods) 48 h prior to sampling (considering the caffeine half-life of about 6 h).

On the day of the study, all subjects were administered 400 mg of caffeine citrate (corresponding to 200 mg of caffeine according to Romanian pharmacopoeia *Coffeini*  *et acidum citricum* FRX) [12] in 150 mL of sugarsweetened solution (similar to an energy drink used by athletes). Urine samples were subsequently collected from each participant.

Sampling was performed 6 h after consumption of the energy drink prepared as described above and administered after *a jeun* urination; participants were advised to drink 500 mL of water (2 x 250 mL water at 2 and 4 h respectively) to limit variability due to diuresis following intake of different liquids.

Urine samples were stored until the day of HPLC analysis in the freezer at -20°C. Sampling is described schematically in figure 2.

For the clarification of the smoker / non-smoker status, regardless of self-reported smoking, a urinary cutt-off value of 550 ng/mL was considered for active smokers, according to data published by *Zieliñska-Danch W et al.* [13]

#### Standards and reagents

Standards were purchased as follows: cotinine, caffeine and 1N-methyl uric acid from Sigma-Aldrich, Germany and were of >98.00% purity. The solvents (methanol and acetonitrile) were HPLC grade and were purchased from Merck, Germany.

# Preparation of stock solutions

Solutions of 1 mg / mL of caffeine and cotinine were prepared by weighing an appropriate amount and dissolving in methanol. 1N-methyl uric acid was dissolved in 0.1 M methanolic NaOH. Stock solutions were prepared by diluting with methanol.

#### HPLC system

Merck-Hitachi equipped with a binary pump (L-7100), DAD detector (L-7455), autosampler (L-7200), column oven (L-735) and degasor (L-7612).

# Chromatographic conditions

The compounds were separated on a 5  $\mu$ m RP 18 XTerra column (250 x 4 mm) by gradient elution. The mobile phase solvents were: solvent A, a mixture of water: acetic acid – 99.9:0.1, solvent B was acetonitrile and solvent C was methanol. A good resolution was obtained by using the following gradient: 0-15 min: 92:4:4 to 60:0:40 (liniar gradient), as described in 2007 by Begas E et al. [14]

The flow rate was 1.70 mL/min. Detection was performed over the range of 201-400 nm. The HPLC method with DAD detector allows the simultaneous determination of the compounds of interest at different wavelengths so caffeine and cotinine were analysed at 210 nm and N1 methyluric acid at 280 nm.

#### Extraction of urine compounds

1 mL of urine was stirred to extract the compounds of interest with 2 mL of isopropyl alcohol: chloroform (15:85 v/v) for 20 min at vortex. The upper aqueous phase was removed, and the organic phase, after drying over  $Na_2SO_4$ , was concentrated by evaporation to dryness and



Fig. 2. The collection of urine samples



Fig. 3. Sample processing

suspended in 200  $\mu L$  of methanol. The extraction scheme can be seen in figure 3.

# Statistical analysis

The results are presented as mean  $\pm$  SD. For the analysis of the differences between the samples, the t-student test for unpaired values was used, with the statistical significance threshold set to p <0.05. Calculations were performed using the GraphPad Prism 5 software for Windows.

# **Results and discussions**

In the chromatographic conditions described, the separation of caffeine and N1-methyluric acid was achieved.

Five different samples were obtained from non-smokers who agreed not to consume coffee, chocolate, green tea, or other foods with potential caffeine content.

Median calibration curve (n = 5) for caffeine and N1methyluric acid was prepared by extraction after spiking a blank sample of urine with known concentrations of caffeine and N1-methyl uric acid, respectively, and had 5 concentration levels / compound (0.4-10  $\mu$ g / mL caffeine and 3.5-50  $\mu$ g / mL N<sup>1</sup>- metil uric acid). The calibration curve was ASC = f (concentration) and was ASC = 0.0109 ( $\pm$  0.0054) c + 0.0456 ( $\pm$  0.0071) for caffeine and AUC = 0.0234 ( $\pm$  0.0049) c + 0.0567 ( $\pm$  0.0073) for N1-methyluric acid. The coefficient of determination was >0.99 for both substances.

In the urine samples of the smokers, the peak of cotinine was also present at a retention time of 3.95 min, which can be seen in figure 4.

Determination of extraction efficiency was performed by spiking a blank urine sample with known concentrations of caffeine and N1-methyl uric acid in the middle of the calibration curve concentration range (3 µg/mL caffeine and 30 µg/mL acid N1-methyluric) and the calculation for the two compounds of the percentage ratio between the added concentration and the calculated one. The calculated efficiency was 87.23%. HPLC method proved good linearity on the determination interval. Lower limit of quantification (LLQ) for caffeine was 0.15 µg / mL and 1 µg / mL for N1 methyluric acid. Coefficients of variation (CV%) were  $\pm 15\%$  from the theoretical value at all concentration levels, acceptable for bioanalytical determinations.

For non-smokers, cotinine concentrations were well below the threshold of 550 ng / mL. All smokers in the study were correctly identified using this threshold value of cotinine.

At the administered dose, all caffeine concentrations in the urine were lower than 5 mg/mL (25.74  $\mu$ M / L), well below the limit of 12 mg/mL (61.7  $\mu$ M / L) imposed by WADA (prior to 2004).

Concentrations of N1-methyl uric acid and caffeine are shown in table 1.

There are significant differences between caffeine and N1-methyluric acid in the urine of smokers and nonsmokers (smokers with significantly lower caffeine levels and higher 1N-methyluric acid). This fact is also reflected by the mean molar ratio (N1-methyl uric acid/caffeine) significantly different (p < 0.01) for the two categories of subjects. This report has Gaussian distribution in both population categories but with different distribution frequency (fig. 5).

Caffeine can be used for the phenotyping of CYP1A2 in the blood (caffeine / paraxanthine ratio) or urine (molar ratio of 1-methylxanthine + 1N-methyluric + 5acetylamino-6-formylamino-3-methyluracil / 1, 7dimethylurate) [6, 15, 16], but smoking significantly alters the results obtained by increasing CYP1A2 expression figure 6.

Therefore, for the phenotyping of CYP1A2, among the exclusion criteria, it is essential to clarify the smoking status, which is incompatible with this method of CYP1A2



 Table 1

 URINE CONCENTRATIONS OF ANALYTES

	Smokers		Non-smokers	
Compound	$Mean \pm SD \; (\mu M/L)$	Confidence interval	$Mean \pm SD \; (\mu M/L)$	Confidence interval
		95%		95%
N1-methyluric	85.90 ± 47.71*	59.48-112.3	55.31 ± 28.96	39.27-71.34
acid				
Caffeine	6.47 ± 3.63*	4.46-8.48	10.09 ± 5.68	6.95-13.23
Average molar	17.14 ± 11.23**	10.92 - 23.35	6.40 ± 4.01	4.18 - 8.63
ratio (caffeine /				
N1- methyluric				
acid)				

N1- methyluric acid/caffeine ratio



Fig. 5. Distribution freevency of N1-methyluric acid/caffeine ratio in smokers and non-smokers



enzymatic activity determination. Methods used for highlighting abusive use in sport (monitoring caffeine use in athletes, identification of doping in racing horses) focus only on the quantitative determination of caffeine and metabolites in urine (highlighting threshold values, before 2004 this was 12 mg/mL in humans), without determining the relative ratio between the compounds and without performing determinations of the type of metaboliser (fast, intermediate, slow) or the presence of enzyme inducers such as cigarette smoke [7].

CYP1A2 genotyping is a very expensive method, justified only in specific clinical situations - as a genetic marker for rapid response to aromatase inhibitor-treated patients in breast cancer [17]. Moreover, genetic variability of CYP1A2 may differentiate athletes for whom caffeine has an ergogenic effect more important than in the general athlete population [18]. Smoking modifies the proportion and amount of metabolites for substances hepatically transformed via CYP1A2, that is why smokers require higher doses of medication to achieve plasma therapeutic level (theophylline, some antipsychotics) [19]; on the contrary, when metabolites are responsible for the

Fig. 6. The main enzymes (both microsomal and unmicrosomal) involved in caffeine metabolism (CYP1A2, CYP2A6, xanthine oxidase, NAT2 (N-acetyltransferase-2)

therapeutic effect, the effectiveness of the medication can be increased in smokers, e.g. inhibition of platelet functions by clopidogrel. [20].

Monitoring of urinary elimination of caffeine and nicotine required by WADA may be influenced by chronic smoking by decreasing the amount of caffeine eliminated as such, not metabolised in urine. Chronic smoking is harmful to health, in addition to cardiovascular and lung risk, smoking is described as an endocrine disruption [21]. It also affects long term sports performance; however, there are many cases where smoking has a high prevalence (especially in professional football players). There are several available studies concerning overcoming the limit imposed by WADA for caffeine (prior to 2004) in various sports, but the results can be influenced not only by the ingestion of ergogenic supplements with caffeine but also by diuresis, genetic polymorphism and smoking. However, data from literature present the results obtained from 20,686 urine samples from athletes (collected between 2004-2008). Thus, a very small proportion of samples exceeding the threshold of 12  $\mu g$  / mL (0.6%) was shown, with the vast majority of positive samples having a caffeine content of less than 5  $\mu g$  / mL [22].

The amount of caffeine ingested by athletes varies very widely, also considering its high therapeutic index: low doses (about 3 mg / kg body weight, about 200 mg, equivalent to 2 cups of coffee), moderate (5-6 mg / kg body weight) or large (6-13 mg / kg body weight) [1]. However, the current Romanian Pharmacopoeia limits caffeine doses as follows - the maximum dose for one administration - 500 mg, for 24 hours - 1500 mg [FRX]. The influence of smoking may also be manifested for CYP1A2-intensively metabolised compounds, such as exemestane (aromatase inhibitor, doping substance only in males) [8]; also, some doping compounds (anastrozole, also an aromatase inhibitor) may increase the effect of caffeine administered concomitantly by CYP1A2 inhibition [23].

# Conclusions

Simultaneous determination of caffeine and cotinine in urine enables monitoring the use of ergogenic products containing caffeine concomitantly establishing the athlete's smoking / non-smoking status. Smoking significantly alters the amount of caffeine eliminated in unchanged form in the urine as well as the proportion of its CYP1A2 metabolites. The method can also be used to emphasize the enzymatic induction responsible for lowering the therapeutic efficacy of other CYP1A2 isoform substrates (antipsychotics, theophylline) in conditions of unmodified posology if the patient denies his / her smoking status.

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