AFRICAN JOURNAL OF MEDICINE and medical sciences

VOLUME 30, NUMBERS 1 & 2, MARCH AND JUNE 2001

EDITOR:
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ASSISTANT EDITOR:
A. O. UWAIFO

ISSN 1116 — 4077

Urinary recovery of caffeine and its metabolites in healthy African children

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Summary

Consumption of caffeine containing products is very popular in African children, particularly during ill health in the belief that caffeine promotes good health. This study aims to define the metabolism of caffeine, which takes place in the liver in a group of healthy Nigerian children. About 100mg of caffeine was ingested after an overnight fast. Urine was collected before caffeine ingestion and over 12-hour periods for 36 hours in 13 healthy Nigerian children. The percentage of caffeine and metabolites recovered in urine was determined by high performance liquid chromatography.

The total urinary caffeine and metabolites recovered over the 36-hour sampling period was 63.6%, with only 0.4% of the caffeine dose ingested recovered as unchanged caffeine during the same period. Insignificant amounts of 3,7-dimethyluric acid (0.2%), 3-methyluric acid (0.3%) and 1,3,7-dimethyluric acid (0.4) were recovered in the 36hour urine sample. This study also found that the N3-demethylation pathway was the principal pathway of caffeine metabolism accounting for 83.3% of the total metabolites recovered while C8-hydroxylation accounted for only 0.6% of metabolites recovered. The pattern of urinary metabolites recovered suggested that N3-demethylation is the principal pathway of caffeine metabolism in healthy African children and that small amounts of unchanged caffeine, as well as 3,7-dimethyluric acid, 3-methyluric acid and 1,3,7-dimethyluric acid were recovered during the sampling period.

Keywords: *Urinary recovery, caffeine metabolism, African children*

Résumé

La consomation des produits contenant de la caffeine et popuaire chez les enfants Africain, particulierement pendant les etats maladif, cur il porte à croire que la caffeine contiue a un boin état de santé. Cette etude avait pour but de definier le metabolism de la caffeine au niveau du foie d'un metabolism de la cafine, au niveau du foie d'un groupe d'enfants Nigerian bien portant. Cent grammes de caffeine avaent été ingesté après une ruiit à Jeune. Les urines avaient été ingestion de la caffeine, et pendant une periode allant de 12 à 36 heures après ingestion chez un groupe de 13 enfants Nigerians en parfait etat de sante. Le pourcentage de caffeine dans les urines avaient été determine pas chromatographie liquide à haute performance.

Le total des metabolites de la caffeine recuperé pendant une periode de 36 heures etait de 63.6% avec sentement 0.4% de la dose de caffeine ingesté non metabolic. Des quanties non-significative de acide 3-7 dimethylurique (0.2%), acide 3-methylutique (0.3%) et acide 1-3-7 dimethylurique (0.4%) avaient été recupere pendant les 36 heures de collection de specimen urinaire. Cette etude avaient aussirovre que le cycle metabolique de la demethylation N3 etait le cycle metabolique principal de la caffeine et etait responseble du metabolisme de 83.3% de la totalite des metabolites recuperes alorsque l'hydroxylation C8 avaient été responsable de 0.6% sentement des metabolites recuperés. Le mode des metabolites urinaries recupere suggere que la methylation N3 est le metabolism principal de la caffeine chez enfants Africains en bonne sante. Deplus, une quantite insignificative de caffeine non-metabolise, de meme que l'acide 3-7 dimethylurique, 3-methylunque et l'acide 1-3-7 dimethylurique avait été recuperce pendant la periode d'echantillonage.

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Introduction

Caffeine, which is consumed in the form of coffee, tea, soft drinks as well as prescription and non-prescription drugs, is probably the most widely consumed pharmacologically active agent in the world [1,2] with an estimated daily *per-capita* consumption varies between 200mg to 450mg [2,3]. Caffeine consumption is popular in Nigeria both in adults and children, in good health and disease states. The popularity of caffeine which has been classified as a multi-purpose, Generally Recognised As Safe substance (GRAS) [4] is probably due to its anti-soporific, diuretic and stimulant properties [3].

Ingested caffeine is almost completely absorbed within 30-120 minutes [5] and the absorbed caffeine is metabolised in the liver by a group of cytochrome P450 enzymes, which are responsible for the principal metabolites of caffeine metabolism by way of N-1, N-3, and N-7 demethylation [2,6-8]. Other enzymes involved in caffeine metabolism include N-acetyltransferase and xanthine oxidase [2,6-8]. These groups of enzymes are responsible for the hepatic metabolism of caffeine into 14 different metabolites comprising trimethylxanthine, dimethylxanthines and monomethylxanthines as well as trimethyluric acid, dimethyluric acids and monomethyluric acids which are then excreted in urine (Figure 1) [6-8].

Considerable variability in caffeine metabolism and urinary recovery have been reported and this variability has been attributed to differences in ethnic origin [9], dietary

differences [10,11], the presence or absence of diseases, [12,13] and differences in renal excretion of metabolites [14]. Total urinary recoveries of caffeine and metabolites ranged form 44% to 55% in 24 hours urine samples [7,8] with maximum recovery of caffeine occurring between 2 and 4 hours post caffeine ingestion while maximum recoveries of 1-methylxanthine [1X], 7-methylxanthine [7X] and 1,7-dimethyluric acid [17U] occurring 6-8 hours after caffeine administration [8].

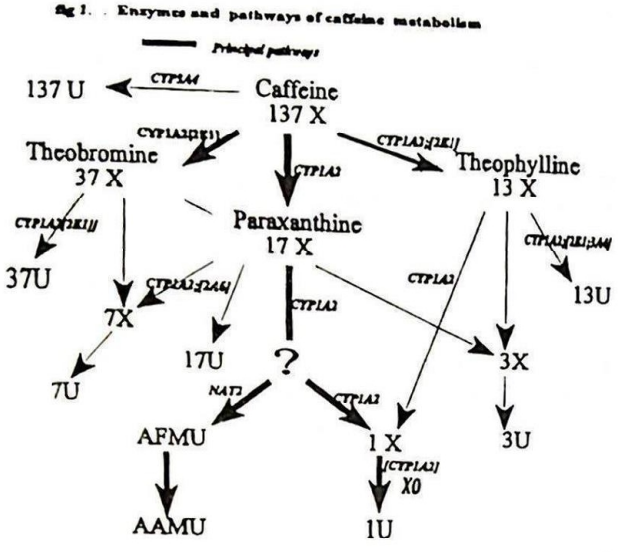

Fig. 1: The enzymes and metabolite pathways of caffeine after ingestion

137U	1,3,7 trimethyluric acid	37U	3,7 dimethyluric acid	13U	
1,3	dimethyluric acid	17U	1,7 dimethyluric acid		
7U	7 methyluric acid	1U	1 methyluric acid		
3U	3 methyluric acid	1X	1 methylxanthine		
3X	3 methylxanthine	7X	7 methylxanthine		
AFMU	5-acetylamino-6-formylamino-3-methyluracil	CYP	Cytochrome P450		
AAMU	5-acetylamino-6-amino-3-methyluracil	XO	Xanthine oxidase		
NAT2	N-acetyltransferase				

The aim of this study is to define the metabolism of caffeine, which is an indirect measure of liver function, in a group of healthy Nigerian children and to quantify the amount of urinary recovery of caffeine and its metabolites in these children.

Subject materials and methods

Informed consent to participate in the study was obtained from each volunteer as well as parent/caregiver after the joint Ethical Committee of the University College Hospital/University of Ibadan, Ibadan, Nigeria, granted institutional approval.

Healthy volunteers

This group consisted of 13 healthy children, comprising of 9 males and 4 females with a mean age ± SEM of 8.83±0.45 years and mean weight ±SEM of 22.81±1.97 kg. The volunteers had abstained from caffeine containing beverages and kolanuts in the preceding two weeks and during the conduct of this study.

Exclusion criteria

All subjects with hepatic, renal or concomitant medications were excluded from the study.

Chemicals

Caffeine and its metabolites were obtained from Sigma (United Kingdom). The 5-acetylamino, 6-formylamino-3-methyluracil [AFMU] was kindly supplied by Professor Pons of Department de Pharmacologie Perinatale et Pediatrique, Hopital Saint Vincent de Paul, Paris, France. Ammonium sulphate, 4-nitrophenol and HPLC grade chloroform were supplied by BDH [United Kingdom]. Fisons Scientific Equipment [United Kingdom] supplied HPLC grade methanol.

Instrumentation

An applied chromatographic system by Waters Associates (Harrow, UK), comprising twin headed reciprocating pumps (Model 510), Waters Intelligent Sample Processor (Models 710B and 712) and Lambda-Max Ultraviolet detectors (Models 481 and 486) were used. For separation of compounds, 30cm x 3.9mm i.d. and 15cm x 4.6mm i.d. RP18 Stainless steel columns were used (HPLC Technologies, United Kingdom).

Conduct of study

All subjects fasted overnight (from last meal at night) and at 7am were given 100mg of caffeine dissolved in 150ml of water with food intake allowed 2hours after caffeine ingestion. Urine samples were collected at commencement of study and all urine samples voided during 12hour intervals were collected and measured. Urine sampling continued for the next 36 hours after caffeine administration. Ten mililitre aliquots of urine samples were buffered with IM HCl to pH 3.5. The buffered urine was frozen at –20°C until transferred in dry ice to Southampton, United Kingdom for analysis.

Analytical methods

Urine samples were thawed by placing storage tubes under cold running water. Caffeine and metabolites were extracted by the modified method of Grant *et al.*, [7]. To a 200µl of urine was added a saturating amount of ammonium sulphate (approximately 120mg) with 50µl (5µg) of 4-nitrophenol added as internal standard. The mixture was vortexed for 30 seconds and then extracted with 6ml of chloroform: isopropanol (85.15v/v) followed by centrifugation at 2500rpm for 5minutes. The organic phase was removed and evaporated to dryness at 40°C under a stream of oxygen free nitrogen.

The residue was reconstituted in 200µl of mobile phase consisting of 0.13M ammonium acetate and 0.02M sodium acetate, which has been buffered to pH 3.8 or 6.2 and consisted of 95% buffer and 5% methanol and this also served as elution solvent. The reconstituted extract of urine was analysed isocratically by automatic injection of 15µl of samples by a Waters Intelligent Sample Processor (WISP) through a Lichrosorb 5µm RP18 30cm x 3.9mm i.d. stainless steel. The stainless steel column was maintained at 44°C in a water bath.

The mobile phase was pumped at 1.1ml/min, which generated a backpressure of 2600 – 3000psi. The retention times in minutes of eluate peaks were 3-methyluric acid (3U)

4,7, 7-methyluric acid (7U) 5,9, 7-methylxanthine (7X) 6.6, 1-methyluric acid (1U) 6.7, 3-methyluric acid (17U) 15.6, paraxanthine (17X) 21.0, theophylline (13X) 22.4, 1,3,7-trimethyluric acid (137U) 26.0, 4-nitrophenol 28.1 and caffeine (137X) 59.5 minutes. Because of co-elution, and to achieve separation of 7X and 1U as well as 37U and 3X, the same mobile phase was buffered to pH 6.2 with resultant separation achieved with the retention times of 4.9, 6.3, 9.0 and 9.2 minutes for IU, 37U, 7X and 3X respectively. The eluate peaks were detected at 285nm with an ultraviolet Waters 486 detector.

With regards to AFMU (5 acetylamino- 6-formylamino-3 methyluracil), urine was centrifuged for 10minutes at 3000rpm after buffering to pH 3.5 with IM HCl. To 200µl of supernatant was added 200µl of IM NaOH and mixture allowed to stand for 30 minutes in order hydrolyse AFMU to AAMU after 200µl of IM HCL was added. The mixture was then made up to 2ml with mobile phase comprising of 0.05M sodium acetate and 0.05M ammonium acetate and methanol (98:2v/v). The mixture was then centrifuged at 10,000rpm for 10 minutes at 20°C. A 15µl of supernatant was then injected on to a 25cm x 4.6mm i.d. 5µm ODS column using a WISP. The AAMU (5, acetylamino-6-amino-3 methyluracil), was eluted using a gradient system comprising two Waters 6000A pumps injecting Solvent A comprising 0.05M sodium acetate and 0.05M ammonium acetate/methanol (98.2v/v) and Solvent B comprising of 20% A + 80% methanol through an automated gradient controller. The AAMU peak was detected at 4.5 minutes at a wavelength of 264nm using a Waters 486 detector.

Calibration curves were made with known amounts of caffeine and metabolites in blank urine samples and calibration curves were linear up to 20µg/ml for 1-methyluric acid and 1-methylxanthine. For the other compounds, calibration curves were constructed up to 10µg/ml, while for AAMU, calibration curves were linear up to 100µg/ml.

Results

Caffeine and its metabolites excreted and recovered during the sampling period were expressed as molar percentages of the 100mg of caffeine ingested by each volunteer. The results are quantified 12 hourly as mean ± SEM.

Metabolite recoveries

Total urinary recovery

Over the 36 hour sampling period, 63.13% of the ingested dose of caffeine was recovered in the urine of these children.

Caffeine (137X). Only 0.44% of the ingested dose of caffeine was recovered unchanged in the urine of healthy volunteers throughout the 36 hour sampling period, with maximally detectable amounts of caffeine during the first 12 hour sampling period (Table 1). Similar amounts of 1,3,7 trimethyluric acid (137U) were recovered in the urine samples over the 3 different 12hour sampling periods.

Dimethylxanthines and dimethyluric acids. Paraxanthine (17X) [5.2%] was the largest dimethylaxanthine recovered in the urine over the total duration of sampling and recovery was similar throughout the sampling period (Table 1).

Table 1: Urinary caffeine and metabolites after a single oral dose of 100mg of caffeine in 13 healthy Nigerian children: (Data expressed as mean±SEM and percentage molar fractions of dose)

Sampling times	0-12h	12-24h	24-36h
Metabolites			
137X	0.29±0.10	0.08±0.06	0.07±0.07
137U	0.23±0.09	0.12±0.05	0.03±0.01
13X	0.17±0.13	0.10±0.04	0.15±0.09
13U	0.16±0.11	0.09±0.05	0.33±0.21
17X	3.25±0.85	1.07±0.35	1.85±0.29
17U	0.66±0.31	1.77±1.09	1.31±0.97
37X	3.07±0.96	1.33±0.68	0.14±0.09
37U	0.05±0.05	0.11±0.08	0.03±0.03
7X	1.82±0.40	0.34±0.11	1.27±0.87
7U	0.19±0.13	0.20±0.12	0.02±0.02
3X	0.30±0.19	0.21±0.16	0.02±0.02
3U	0.31±0.17	0.02±0.02	0.01±0.01
1X	4.83±2.72	2.70±1.35	1.49±0.64
1U	5.50±2.7	5.18±3.05	0.99±0.61
AAMU	13..27±5.8	5.25±2.16	4.19±4.09

Monomethylxanthines and monomethyluric acids. Insignificant amounts of 3-methylxanthine (3X) 0.5% and 3-methyluric acid (3U) 0.3% were recovered in the urine of these children, whereas 9.0% and 11.7% of the ingested dose of caffeine were recovered as 1-methylxanthine (1X) and 1-methyluric acid (1U) respectively during the 36hour sampling period (Table 1). AAMU was the largest metabolite of caffeine metabolism recovered in the urine of the children studied (Table I).

Metabolite pathways

N3-demethylation accounted for most of the pathway of caffeine metabolism with 83.3% of the total demethylation in the children studied. N7-demethylation pathway accounted for 3% of the total demethylation in the subjects while only 0.65% of the total caffeine metabolic pathway is 8-hydroxylated into 137U. N1-demethylation accounted for 13.7% of caffeine metabolism in these healthy children.

Discussion

The metabolism of caffeine is complex but it is efficiently eliminated by hepatic metabolism into dimethylxanthines, dimethyluric acids, monomethylxanthines, monomethyluric acids and AFMU. Caffeine metabolism is affected by several factors, which include smoking [10], diseases such as malaria [12] and hepatic cirrhosis [13] which have been shown to alter the metabolic clearance of caffeine. The study demonstrated that the total urinary recovery of caffeine and metabolites was 63.6% of an ingested dose compared with almost 100% recovery in healthy adult Oriental volunteers [14] but higher than the recoveries in healthy Caucasians [8,15].

The differences in urinary recovery may not be attributable to developmental immaturity of the organ systems involved in caffeine elimination as the enzymes responsible for total demethylation, N3- and N7-demethylation reach adult values by approximately 4 months postnatal age [16]. Similarly,

renal excretory and gastrointestinal maturity approach adult values by 8 months of age and all our volunteers were much older for these factors to be relevant to our findings. The consumption of broccoli and charbroiled foods have been shown to induce enzyme metabolism of caffeine [10], while naringenin may inhibit caffeine metabolism. The ingestion of these items may account for the variable rate of formation or recovery in caffeine metabolites [10,11], however, these food items are not consumed by Nigerians, though no data exists on dietary influence on drug metabolising capacity in Nigerians.

Other factors affecting urinary recoveries of caffeine and metabolites include acetylation status [17] such that fast acetylators excrete more AFMU than slow acetylators, while slow acetylators excrete more 1-methylxanthine and 1-methyluric acid in urine. Previous studies using isoniazid and sulphadiazine as probes for NAT2 activity found that 52-55% of Nigerians are fast acetylators [18,19]. This would suggest that both fast and slow acetylators may be fairly represented in this study.

Demethylation by the 1N- and 7N- pathways accounted for small fractions of the total caffeine metabolites. These demethylation pathways have been shown in Caucasians to be well developed by the age of one year [16,20]. The maturity of these enzyme pathways responsible for demethylation may not account for these values in the African children studied, as the mean age was more than 8 years.

This study confirmed the wide inter-individual variability in caffeine metabolism reported by many authors [9,10, 21,22] which has been attributed to smoking habits [10], and race [9] as the subjects studied are pre-pubertal and the roles of diet/xenobiotics was not evaluated in this study.

Conclusion

This study demonstrated less than 70% of an ingested dose of caffeine in healthy Nigerian children studied was recovered and that 3N-demethylation is the principal pathway of caffeine metabolism in Nigerian children.

Acknowledgements

O. O. Akinyinka wishes to acknowledge the assistance of the European Community for the sponsorship and Dr. A.G. Falade for sample collection. The authors are grateful to Professor Pons of Department de Pharmacologie Perinatale et Pediatrique, Hospital Saint Vincent de Paul, Paris, France for the supply of 5-acetylamino, 6 formylamino-3-methyluracil AFMU).

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