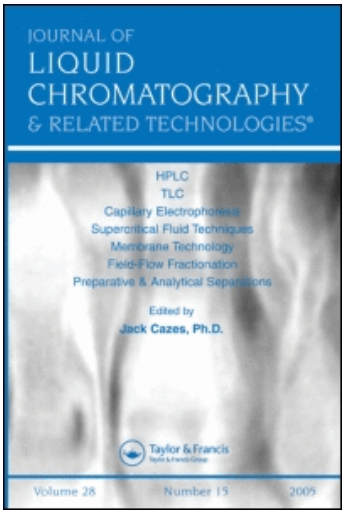

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DETERMINATION OF THE CAFFEINE METABOLITE AFMU IN HUMAN URINE BY COLUMN SWITCHING HPLC

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ABSTRACT

A column switching HPLC procedure is described for the quantification of the caffeine metabolite AFMU (5-acetylamino-6-formylamino-3-methyluracil) from other metabolites in human urine. The procedure is simple, easy to use and allows the reliable quantification of AFMU without interference from other metabolites.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is widely used in the human diet. It can be studied as a probe drug for the assessment of variability in biotransformation capacity. In the 1980's, examining caffeine metabolism to determine genetic acetylator phenotype and genotype of human population groups was common practice (1-4). Caffeine is particularly well suited as a test drug for many reasons such as availability, ease of administration and safety. Physiological

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reasons for studying caffeine metabolism include biotransformation by the liver, minimal renal elimination (5), two or more enzymes are involved in the degradation mechanism, there is low binding to protein in the serum, rapid and complete gastrointestinal absorption, and the metabolites are evenly distributed throughout the body water.

To date, high performance liquid chromatography is the method of choice for the determination of caffeine and its metabolites, mono- and dimethylxanthines, uric acids and 5-acetylamino-6-formylamino-3-methyluracil (AFMU). Grant et al. (6) developed a procedure for the extraction and subsequent HPLC separation and quantification of dimethylated xanthines, ureates and AFMU. Other HPLC procedures have been published, a selected number is listed here (7,8).

Tang et al. (9) were the first to isolate and characterize AFMU from human urine after administration of caffeine. Grant et al. (11) subsequently demonstrated that the urinary excretion of this compound was both bimodally distributed and interethnically variable and that the polymorphic liver N-acetyltransferase enzyme was involved in its formation in man. Their original analysis of population variability in caffeine metabolite excretion (12) has therefore been expanded to take into account the acetylator phenotype of all subjects as determined by their level of AFMU production (6).

Lorenzo and Reidenberg (10) reported in 1989 that AFMU degrades under physiological conditions, i.e. at the pH and temperature of urine in the bladder. This observation has been previously reported by Tang et al. (2) as a potential source of error. Tang et al. (9) also reported that there is chemical evidence that AFMU could be readily converted to AAMU (5-acetylamino-6-amino-3-methyluracil).

Later, Tang et al. (2) decided, to convert AFMU to the more stable AAMU to determine populations' phenotyping. The main disadvantage in doing so is the reduced numeric difference between the critical values characterizing slow and rapid acetylators in the measurement of the metabolic ratio AAMU/(AAMU+1X+1U) compared with AFMU/1X ratio, where 1X and 1U are 1-methylxanthine and 1-methyluric acid, respectively.

In this study we report a column switching high performance liquid chromatography procedure for the quantitative determination of AFMU in urine, which is simple, easy to use and resolves AFMU from AAMU and the other components in urine.

EXPERIMENTAL

Materials

Ammonium sulfate and glacial acetic acid were purchased from Aldrich Chemical Company (Milwaukee, WI), hydrochloric acid was obtained from Fisher Scientific (Fairlawn, NJ). The organic solvents methanol, chloroform, isopropanol and acetonitrile were distilled in glass (Burdick & Jackson, Muskegon, MI). Mobile phases were prepared in distilled/deionized water, filtered and degassed. AFMU was purchased from B.K. Tang, University of Toronto, Canada. AAMU was prepared from AFMU by the procedure of Tang et al. (9).

Apparatus

The chromatograph was a Hewlett-Packard Model 1090M equipped with a column switching valve, an automatic injection system, and a diode array detector. Column I was a 100 x 4.6 mm I.D., 5 µm Hypersil ODS column (Hewlett-Packard), and column II was a 300 x 7.8 mm I.D., Bio-Gel SEC 20-XL (BioRad) (see schematic, Figure 1).

Sample Clean-up

The procedure used for the extraction of caffeine metabolites from urine was that published by Grant et al. (6) and is summarized below:

> The pH of the urine samples was adjusted to pH 3.5 using 1N HCl. A 0.2 ml aliquot of urine and a saturating amount of ammonium sulfate (approximately 120 mg) were added to a 15 ml centrifuge tube and vortexed for 30 seconds. 6 ml of 3:1 chloroform/isopropanol was

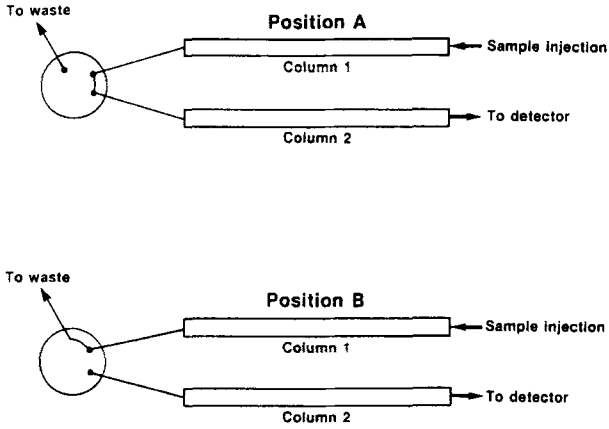

FIGURE 1. A schematic of the column switching procedure used in this study. See text for details.

added to the centrifuge tube and the tube was shaken vigorously for 2 minutes. The extraction mixture was then centrifuged for 7 minutes at 2500 rpm. The organic layer was removed and dried under nitrogen gas at 30-33°C. The sample was reconstituted with 0.2 ml of 0.1% acetic acid.

Separation and Quantitation

A flow rate of 1 ml/minute was maintained throughout the separation. All mobile phase component changes were by a linear gradient. The columns were equilibrated in position A (figure 1) with 3% acetonitrile, 97% 0.1% acetic acid (0.1% HAc). At time 0.01 minutes after injection, the column position switched to B to allow the components traveling with the solvent front to pass through to waste. At time 2.0 minutes, the column position switched to A to allow the AFMU peak to load onto column II. Between time 3.0 to 3.1, the mobile phase was changed to 100% 0.01% HAc. These conditions were held until 20.0 minutes when the column position switched to B. Eliminating the organic component from the

mobile phase after the AFMU peak elutes off of column I prevents other components from eluting from column I onto column II, essentially "freezing" the components on column I while the AFMU passes through column II to the detector. Between 20 and 21 minutes, the mobile phase changed from 100% ACN and column I was washed to waste until time 30 minutes. The mobile phase returned to the initial conditions (3% ACN, 97% 0.1% HAc) between 30 and 31 minutes and the column switched back to position A to equilibrate for the next injection. Each injection was 15 µl. The UV absorbance was monitored at 280 nm until run time 20 minutes.

RESULTS AND DISCUSSION

AFMU is one of the most important metabolites in the measure of the phenotype ratio. Without a reliable determination of AFMU, one cannot use the equation AFMU/(1X + 1U) to characterize metabolism. The methyl xanthines and methyl uracils can all be quantitated using the reverse phase HPLC method of Tang et al. (6), however, AFMU cannot be resolved sufficiently from other caffeine metabolites in urine to allow reliable, reproducible quantitation. The method described herein offers a way to quantitate AFMU in urine.

The chromatogram in Figure 2 shows standard AFMU as eluted from the C_{18} and gel filtration columns in series. Column switching was not employed. Note the presence of AAMU as an impurity in the standard. When the column switching program is utilized (see figure 3) the AAMU peak is not observed. This demonstrates that a small "window" of the eluants from column I is passed through column II to the detector. All of the peaks contained in the sample which elute from column I prior to the AFMU pass through to waste. After the AFMU peak is "loaded" onto column II, the mobile phase is quickly changed to 100% aqueous, essentially "freezing" the remaining non-polar components in the sample on the C_{18} column while the AFMU, and the components which co-elute with AFMU from column I, pass through the gel column to the detector. After the AFMU peak has

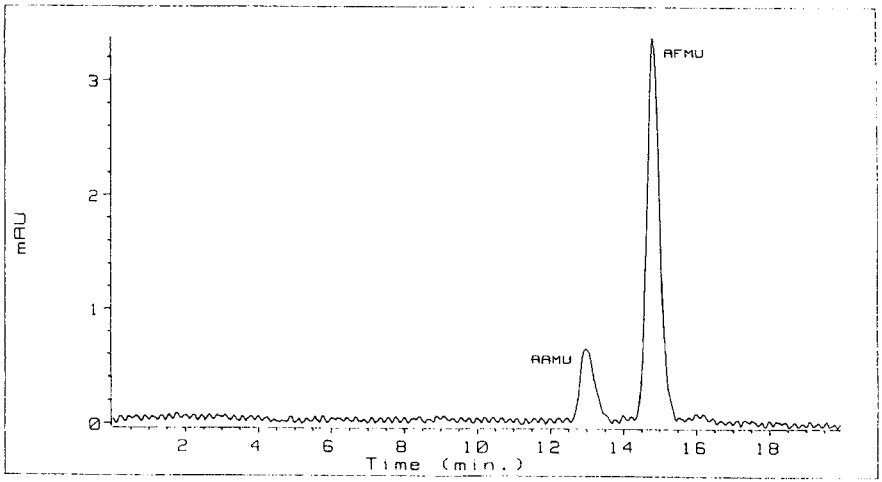

FIGURE 2. A chromatogram of AFMU standard as eluted from the C_{18} and gel filtration columns in series: column I is a 100 x 4.6 mm, 5 µm hypersil ODS (Hewlett-Packard); column II is 300 x 7.8 mm Biogel SEC 20-XL (BioRad); detection was carried out at 280 nm at a flow rate of 1 ml/min. The mobile phase is described in the Experimental Section.

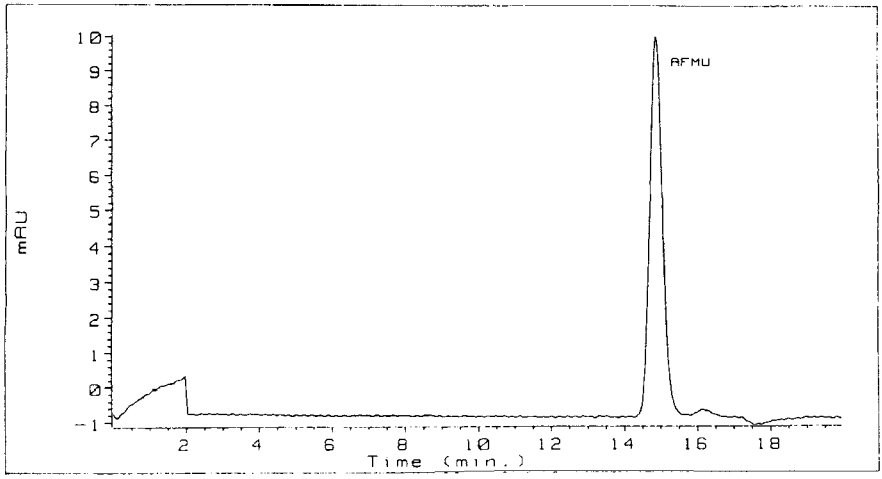

FIGURE 3. Same as Figure 2 using column switching and a step gradient. See text for details.

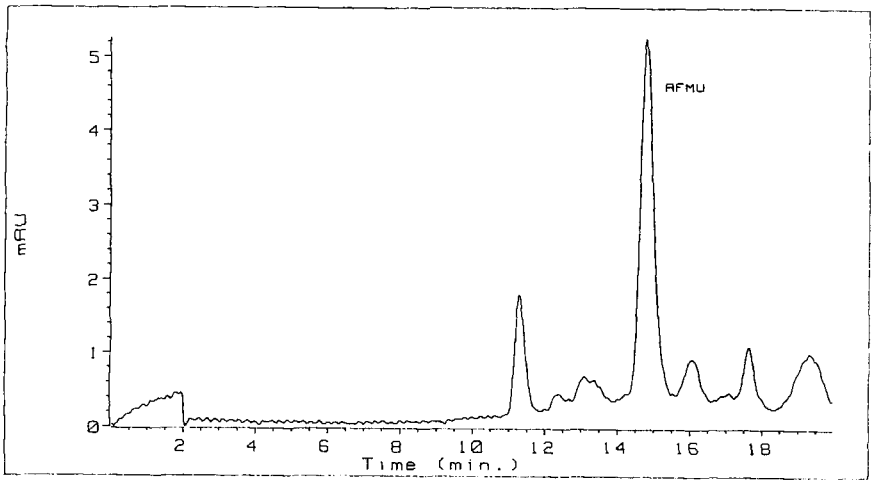

FIGURE 4. Chromatogram of AFMU from urine extract using column switching. Experimental details are in the text.

been detected, the mobile phase path is switched back to position B and the non-polar components of the sample are washed from the C_{18} column to waste. Figure 4 shows a typical chromatogram of a urine sample which has been extracted as described in the experimental section. The AFMU has been resolved from the other components in the sample.

The advantages of this procedure are two-fold. The AFMU is separated from the remaining caffeine metabolites and other components in urine. Also, the sample extract may be used to determine the other caffeine metabolites by the reverse phase HPLC method (6).

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