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Note

Determination of acetylated caffeine metabolites by high-performance exclusion chromatography

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Although caffeine is the most widely used drug, its metabolism is not fully understood [1, 2]. Recently, two acetyluracil metabolites have been identified, i e. 5-acetylamino-6-amino-3-methyluracil (AAMU) [3–5] and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) [6], in man. It has been established that the molar portion of AFMU excreted in urine by different subjects coincided with their acetylation status [7]. Thus, a simple non-invasive method was developed for such a purpose [8]. However, because of the instability of AFMU, urine sampling and storage must be properly controlled [9] Thus, erroneous results of AFMU determinations in basic urines must be expected, and applicability of this method in various clinical settings was limited

AFMU deformylates quantitatively to the stable AAMU Hence, we conjectured that the level of AAMU, which represents total acetyluracil after conversion of AFMU, may improve the estimation of acetylation capacity This paper describes a simple and precise high-performance liquid chromatographic (HPLC) procedure for AAMU and 1-methylxanthine (1X) by exclusion chromatogaphy

EXPERIMENTAL

Materials

Caffeine was obtained from Sigma (St. Louis, MO, U S A), 1X from Pfaltz & Bauer (Stanford, CT, U.S A.) and benzyloxyurea from ICN Pharmaceuticals (Life Science Group, Plainview, NY, U.S A) AFMU was isolated from urine as described earlier [6].

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Apparatus

The HLPC system used for the assay consisted of a Model 590 solvent delivery system coupled with a WISP autosampler/injector and a Model 440 fixed-wavelength UV detector and/or a Model 481 variable-wavelength detector (Waters Scientific) A Bio-Gel TSK-20 (10 μm particle size, 300 × 7 5 mm I D) gel chromatography column (Bio-Rad, Mississauga, Canada) was used

Procedure

To 50 μl of urine were added 50 μl of 0 1 *M* sodium hydroxide After 10 min at room temperature, 50 μl of 0.1 *M* hydrochloric acid were added to neutralize the excess sodium hydroxide. Then 0 1 ml of the internal standard solution containing 0.1 mg of benzyloxyurea was added An aliquot of 20 μl of this mixture was injected into the TSK column, eluted with 0.1% acetic acid at a flow-rate of 0 8 ml/min, and monitored by UV aborbance at 254 nm.

Calibration curve

Known amounts of AFMU and 1X in the range 0 2—5.0 and 0 5–2 0 μg, respectively, were added to 50 μl of blank urine and then processed as described above

RESULTS AND DISCUSSION

Initially, the concentration of AAMU in urine was measured by high-performance anion-exchange chromatography [10, 11] However, the required time of 40 h per sample is too long for routine usage. All attempts failed to determine AAMU in urine by normal-phase or reversed-phase chromatography under various conditions The difficulties can be summarized by stating that AAMU was not retained by reversed-phase and was retained too much by normal-phase columns, it co-eluted with many polar substances of urine Ion-pair agents failed to improve separation Finally, the application of exclusion chro-

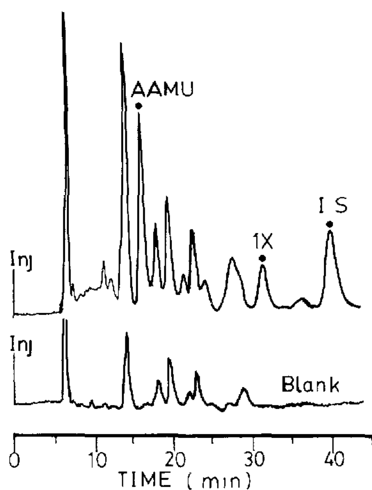

Fig 1 HPLC profiles of a blank urine and a urine sample 6 h after ingestion of 300 mg of caffeine The retention times are as follows AAMU, 16 2 min, 1X, 32 0 min, I S (internal standard), 40 8 min

matography, which is rather rare for the quantitation of drugs and metabolites, produced clean separation of AAMU and 1X (the other major caffeine metabolite in urine)

This method requires a small amount of urine with minimum sample preparation. Complete conversion of AFMU to AAMU can be achieved under mild basic conditions (pH 10) for 10 min. Prolonged reaction times of more than 30 min produce substances that may interfere with determinations of 1X and the internal standard.

Fig. 1 shows the HPLC chromatograms of a blank urine and a urine 6 h after ingestion of caffeine. The retention times of AAMU and 1X were 16 2 and 32.0 min, respectively. Other metabolites of caffeine with retention times of 24.5, 28.0 and 35.0 min were not identified and they did not interfere with AAMU and 1X determination The total run time is 45 min.

The calibration curves of AAMU and 1X passed through the origin and were linear in the rangs studied, with a slope of 0 97 per μg of AFMU and 0 45 per μg of 1X (correlation coefficients of 0.999 for each compound). Centrifugation of urine containing visible precipates is not recommended because of loss of 1X which could be as much as 50%. Such urine samples should first be shaken well and then treated as normal urine The precipitate will be dissolved after base and acid treatment as described under *Procedure*

This HPLC procedure was calibrated by addition of known amounts of AFMU and 1X to blank urine The mean recovery of AFMU and 1X was 99 2%, with a standard deviation of 2.0% (n = 5, at 1.0 μg per sample). On a day-to-day basis, the coefficient of variation in peak-height ratios of AAMU and 1X to the internal standard was 2.4 and 4 4%, respectively. The detection limit for AAMU and 1X is estimated to be 0.02 μg. Maximum detection sensitivity could be obtained at 265 nm, with a detection limit of 0 01 μg for

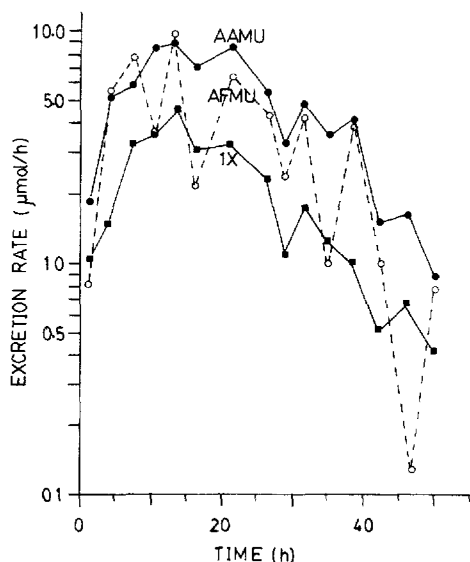

Fig 2 Urinary rate of excretion of AAMU, AFMU and 1X from a healthy subject after ingestion of 300 mg of caffeine AAMU and 1X were determined by the method described above AFMU levels were determined by a previously published HPLC method [8]

compound. For routine usage, a UV monitor at 254 nm was sufficient. After 20–30 samples, the column should be washed with 50% methanol at a flow-rate of 0.8 ml/min for 2 h.

Application

Fig. 2 shows the urinary rate of excretion of AAMU, 1X and AFMU after an oral dose of caffeine. The levels of AFMU in this illustration were measured by a previously published HPLC method [8]. The AAMU curve is almost superimposable on the AFMU curve, except for the sudden dips of the latter. The rate of excretion of AAMU parallels that of 1X, this suggests parallel pathways for their formation. There were no detectable amounts of AAMU 72 h after caffeine intake in any tested subject, a fact which suggests that caffeine is the only source of AAMU.

This method is used currently to measure acetyluracils in adults, children and in animals in order to determine acetylation capacity.

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