ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 693–700

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Stability of 5-acetamido-6-formylamino-3-methyluracil in buffers and urine

Pierre Wong *, Gérald Villeneuve, Vicky Tessier, Kris Banerjee, Hinko Nedev, Bertrand J. Jean-Claude, Brian Leyland-Jones

Department of Oncology, McGill University, McIntyre Medical Science Building, Suite 701, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada H3G 1Y6

Received 2 May 2001; received in revised form 16 October 2001; accepted 23 October 2001

Abstract

The caffeine metabolite 5-acetamido-6-formylamino-3-methyluracil (AFMU) and its product of spontaneous deformylation 5-acetamido-6-amino-3-methyluracil (AAMU) were synthesized. Their ultraviolet absorption spectra differed significantly from each other and wavelengths of absorption maximum and molar extinction coefficients varied with pH. The changes of the absorption spectrum parameters of AFMU and AAMU with pH indicated that they ionized with pK_a of 5.7 and 8.3, respectively. The spontaneous deformylation of AFMU in solutions of different pH and urine were investigated spectrophotometrically and by high-performance liquid chromatography. The data showed the following: (a) AFMU transformed uniquely to AAMU; (b) deformylation obeyed first-order kinetics under the different conditions tested; (c) the half-life of AFMU varied between 7.8 and 36 h between pH 9.0 and 2.0 at 24 °C, with a maximum of 150 h at pH 3.0; (d) AFMU deformylated below pH 2.0 and above pH 10.0 with a half-life of less than 4.6 h; (e) half-lives of AFMU in urine were 57 and 12.5 h at 24 and 37 °C, respectively, comparable to those in buffers at equivalent pH and temperature. The results are discussed in relation to the mechanism of deformylation and the use of caffeine as a probe drug for NAT2 phenotyping.

Keywords: 5-Acetamido-6-amino-3-methyluracil; 5-Acetamido-6-formylamino-3-methyluracil; Caffeine; ELISA; HPLC; NAT2 phenotyping; Urine

1. Introduction

N-acetyltransferase 2 (NAT2) is a phase II metabolic enzyme of xenobiotics which catalyzes *N*-acetylation of arylamines. The enzyme is polymorphic: slow and fast acetylators can be distinguished in a given population. The NAT2 polymorphism is clinically of interest as a result of its association with adverse drug effects, cancer susceptibility and findings that progression of certain diseases can alter NAT2 phenotype [1–5].

NAT2 phenotype can be determined using caffeine as a probe drug [6–9]. Toward this end, a urine sample is collected 4–5 h after an intake of

* Corresponding author. Tel.: +1-514-398-5940; fax: +1-514-398-5111.
E-mail address: pwong@med.mcgill.ca (P. Wong).

0731-7085/02/$ - see front matter
PII: S0731-7085(01)00656-2

caffeine (either a tablet or a cup of coffee). After extraction of caffeine metabolites and their separation by either high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), the ratio of 5-acetamido-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1X) is measured. However, the use of caffeine as probe drug for NAT2 phenotyping has been questioned owing to the intrinsic instability of AFMU which deformylates to 5-acetamido-6-amino-3-methyluracil (AAMU) (Fig. 1) [10]. Currently, there is little quantitative data on the stability of AFMU in aqueous solutions [10,11]. For our studies on NAT2 phenotyping by enzyme linked immunosorbent assay (ELISA) a quantitative knowledge of the stability of AFMU was necessary since AFMU is transformed to AAMU before the ELISA and there was an absence of correlation between amounts of AFMU determined by HPLC and amounts of AAMU determined by ELISA [12]. This article reports ultraviolet absorption spectra of AFMU and AAMU as a function of pH and kinetics data on the deformylation of AFMU in buffers and in urine as a function of pH and temperature. A preliminary report of this work has been presented [13].

2. Experimental

2.1. Chemical reagents

Acetonitrile, HPLC grade, was from Fisher Scientific Ltd (Nepean, ON, Canada); acetic anhydride, HEPES and trizma base were from Sigma–Aldrich (St. Louis, MO); formic acid, 98–100% pure, was from A&C American Chemicals Ltd. (Montreal, Que., Canada); silica gel 60 (230–400 mesh; E. Merck) was from Silicycle Inc. (Quebec, Que., Canada); water was distilled and filtered through a Millipore Milli Q-water system; other reagents used were of ACS grade.

2.2. Syntheses

The product AAMU was synthesized as previously described and was recrystallized from water [14,15]. The caffeine metabolite AFMU was synthesized by a modified procedure of Tang et al. [11]. Two hundred and fifty milligrams of AAMU was heated at 50 °C for 12 h in 19.84 ml (150 mmol) of a 1:1 mole ratio of acetic acid anhydride and formic acid. After evaporation of the solvents, the residue was re-heated with a fresh solution of a 1:1 mole ratio of acetic acid anhydride and formic acid for another 12 h. The product AFMU was separated from the diacetylated 5,6-diamino-3-methyluracil by flash chromatography on a silica gel column (20×150 mm) using a chloroform–methanol solution (9:1, v/v) as the eluent. Between 8 and 10 mg of AFMU was obtained. The AFMU preparation was composed of greater than 95% of AFMU and less than 5% of AAMU, as determined by peak area measurements after HPLC: the purity of AFMU could not be precisely determined since AAMU peak is asymmetric, but it is no lower than 95% since the peak area of an asymmetric peak would be the same or greater than that of a symmetric peak.

2.3. Spectrophotometric determinations

Spectrophotometric determinations of AFMU and AAMU solutions were carried out with a Shimadzu spectrophotometer UV160U model.

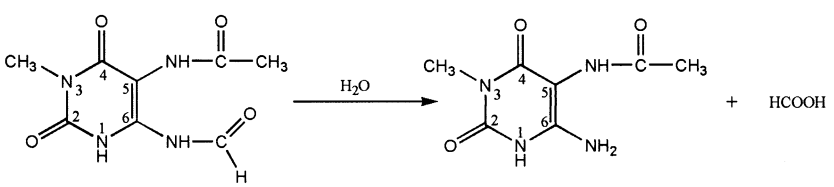

Fig. 1. Reaction of deformylation of AFMU into AAMU.

The reference and sample cell holders were either at 24 °C or heated at 37 °C with a water bath and circulating pump. The temperature of the solution was measured with a VWR traceable thermometer.

2.4. Determination of AFMU concentrations

The exact AFMU concentrations of stock solutions were determined as follows. To the reference and sample cells 1 ml of a NaOH solution (pH 12.0; 0.01 N) was pipetted. After adjusting the absorbance to zero at 266 nm, 50 μl of water and 50 μl of an AFMU solution were pipetted in the reference and sample cells, respectively. The change of absorbance was recorded until a constant value was reached, when all AFMU had been transformed into AAMU (20–25 min). Using a molar extinction coefficient of 1.47×10^4 M^{-1} cm^{-1} for AAMU, the AFMU concentration was calculated.

2.5. Absorption spectra and pK_a determinations of AFMU and AAMU

Absorption spectra of AFMU were recorded between 320 and 240 nm at 24 °C in water and solutions of HCl (pH 2.0; 0.01 N) and sodium carbonate (pH 9.5; 0.05 M). The AFMU concentration in the different solutions was 1.69×10^{-5} M. Absorption spectra of AAMU were recorded between 320 and 240 nm at 24 °C in water and solutions of HCl (pH 2.0; 0.01 N) and NaOH (pH 12.0; 0.01 N). The AAMU concentration in the different solutions was 4.9×10^{-5} M. The pK_a of AFMU and of AAMU were determined as follows. The change of the wavelength of absorption maximum of AFMU and the change of absorbance at the wavelength of absorption maximum of AAMU were measured at different pH with these solutions: HCl (pH 2.0; 0.01 N); sodium acetate (pH 4.4–5.4; 0.05 M); sodium phosphate (pH 5.6–8.0; 0.05 M); Tris–HCl (pH 7.8–8.5; 0.05 M); sodium carbonate (pH 8.5–11.0; 0.05 M), NaOH (pH 12.0; 0.01 N). The pK_a values were derived from the theoretical curves fitting the data points (Graphpad Prism software).

2.6. Stability of AFMU

The deformylation of AFMU in solutions of different pH was followed spectrophotometrically at 300 nm. To a 1-ml cell with a tightly fitting teflon stopper 1 ml of the solution was pipetted. After equilibration at the desired temperature, 10 μl of a stock solution of AFMU (8×10^{-3} M in water) or 50 μl of a solution of AFMU (1.75×10^{-3} M in the same solution as in the cell) was added. After mixing, the transformation of AFMU into AAMU was monitored by recording the decrease of the absorbance at 300 nm either continuously or at intervals of 5, 10, 20 or 30 min, depending on the rate of the transformation. The stability of AFMU was studied in the following solutions: HCl (pH 1.0–3.0; 0.1–001 N); sodium acetate (pH 3.5–5.0; 0.05 M); citric acid–disodium phosphate (pH 5.0; 0.025–0.05 M); sodium phosphate (pH 6.0–7.5; 0.05 M); NaCl–KCl–disodium phosphate–potassium dihydrogen phosphate (PBS) (pH 7.4; 0.137–0.00268–0.01–0.00176 M); HEPES (pH 8.0; 0.05 M); Tris–HCl (pH 8.0; 0.05 M); sodium carbonate (pH 9.0–11.0; 0.05 M); NaOH (pH 12–12.7; 0.01–0.5 N). The stability of AFMU in urine samples was studied by HPLC as follows. Three to five millliliters of urine, which were collected 4 h after caffeine uptake, was pipetted into a 15-ml glass conical tube with a screw cap, and the tube was left standing at 24 or 37 °C. As a function of time, caffeine metabolites were extracted from 200 μl aliquots according to a method described previously [7] and were separated by HPLC. The peak area ratio of AFMU to 1X was taken as indicative of the extent of deformylation of AFMU to AAMU.

2.7. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was performed using a Spectra-Physics pump (P100) model, a Rheodyne 7123 syringe loading sample injector model with a 20-μl loop, a Spectra-Physics detector (UV 100) model and a Spectra-Physics DataJet integrator model. Caffeine metabolites were separated on a Luna C18 (2) 5 μ column (250 × 4.6 mm) (Phenomenex;

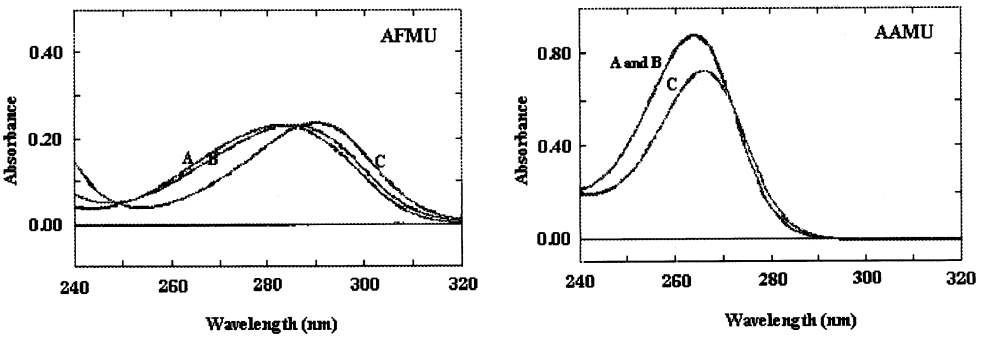

Fig. 2. Ultraviolet absorption spectra of AFMU and AAMU in water and solutions of different pH at 24 °C. Absorption spectra A, B and C of AFMU were obtained in a HCl solution (pH 2.0; 0.01 N), water and a sodium carbonate solution (pH 9.5; 0.05 M), respectively. AFMU concentration was 1.69×10^{-5} M. Absorption spectra A, B and C of AAMU were obtained in water and solutions of 0.01 N HCl (pH 2.0; 0.01 N) and NaOH (pH 12.0; 0.01 N), respectively. AAMU concentration was 4.9×10^{-5} M.

Torrance, CA). Samples of 20 μl were injected. The column was eluted isocratically at a flow rate of 1 ml min^{-1} with an acetonitrile (5%), acetic acid (0.05%) solution (v/v) [8]. The eluate was monitored at 280 nm. Retention times of AAMU, AFMU and 1X were 3.8, 5.7 and 11.5 min, respectively.

3. Results and discussion

The deformylation of AFMU into AAMU can conceivably be followed by HPLC since AAMU and AFMU have different retention times, 3.8 and 5.7 min, respectively. However, this method has the disadvantage to be discontinuous and that amount of AAMU can not be reliably measured because of the asymmetry of its peak. Moreover, an internal standard would be required for comparisons of the data. Therefore, we have investigated the possibility that the deformylation could be monitored by absorbance spectrophotometry since AFMU and AAMU absorb ultraviolet light and that this method would allow the deformylation to be continuously followed. Toward this end, the absorption spectra of AFMU and AAMU as a function of pH were determined. The ultraviolet absorption spectrum of AFMU in water was characterized by a single broad maximum at the wavelength of 284.7 nm, which was the same as that previously reported in water [11]. However, the wavelength of absorption maximum (λ_{max}) varied with pH (Figs. 2 and 3). Above pH 6.8, it was 289–290 nm, but below pH 6.8, it gradually shifted to a value of 282–283 nm until pH 5.0 and subsequently remained constant with a pH decrease to pH 1.0, the lowest pH value tested. The molar extinction coefficient (ε_{max}) of AFMU did not vary significantly between pH 2.0 and 9.5 and a value of 1.33×10^4 M^{-1} cm^{-1} was obtained in water. The observations on the absorption spectra of AAMU in water and in solutions of different pH were basically the same as those reported previously [16]. They can be summarized as follows. The absorption spectra of AAMU in water and in a HCl solution (pH 2.0; 0.01 N) were identical and characterized by a single narrow maximum at the wavelength of 264 nm (Fig. 2). The value of λ_{max} remained constant until pH 7.0. Above pH 7.0, it shifted gradually toward longer wavelength with increasing pH until a constant value of 266 nm was reached at pH 9.5. Moreover, ε_{max} was constant between pH 2.0 and 7.0, but above pH 7.0, it decreased gradually until a constant value was reached at pH 11.0 (Figs. 2 and 3). Values of ε_{max} of AAMU below pH 7.0 and at pH 11.0 were 1.80×10^4 and 1.47×10^4 M^{-1} cm^{-1}, respectively. The sigmoid curves of the change of λ_{max} of AFMU and of the change of absorbance at λ_{max} of AAMU as a function of pH indicated that AFMU and AAMU ionized with pK_a of 5.7 and 8.3, respectively (Fig. 3).

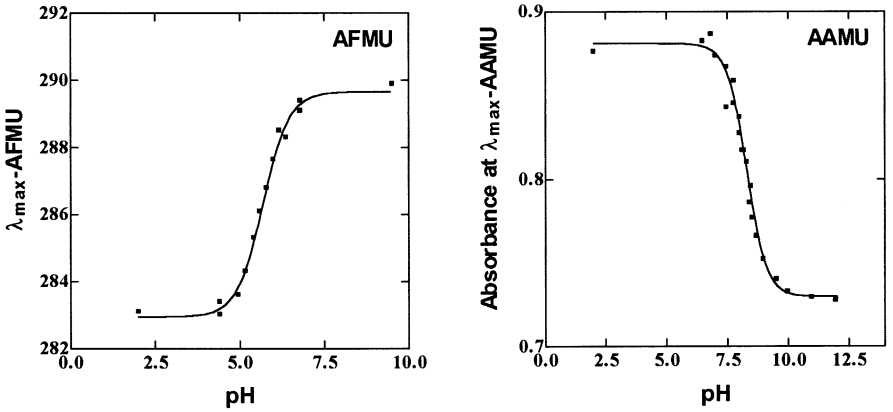

Fig. 3. Change of the wavelength maximum of AFMU and change of the absorbance at the wavelength of absorption maximum of AAMU as a function of pH at 24 °C. Solid curves were derived by analysis of the data points on the basis that the changes with pH correspond to ionizations of the nitrogen atoms no 1 of AFMU and AAMU with pK_a of 5.7 and 8.3, respectively.

The characteristic features of the absorption spectra of AAMU and AFMU indicated that the spontaneous deformylation of AFMU could be monitored spectrophotometrically. An initial experiment performed was monitoring the change of absorption spectrum of AFMU as a function of time in a NaOH solution (pH 12.0; 0.01 N) at 24 °C, a condition known to rapidly deformylate AFMU [11]. The absorption spectra obtained confirmed unequivocally that AFMU decomposes solely to AAMU as the different absorption spectra were characterized by the presence of isobestic points at 248.9 and 275.5 nm and that the final absorption spectrum was identical to that of AAMU (Fig. 4). We next investigated the stability of AFMU in solutions of different pH at 24 °C by measuring the change of absorbance with time at 300 nm, a wavelength where AAMU does not have any significant absorbance. The results are presented in Table 1 and Figs. 5 and 6. They can be summarized as follows. (a) The deformylation of AFMU to AAMU under the conditions tested obeyed first-order kinetics at the AFMU concentration generally used of 8.33×10^{-5} M (Fig. 5). (b) At pH 3.0, which is close to the pH of standard urine storage conditions (pH 3.5), AFMU is maximally stable and deformylates with a $t_{1/2}$ of 150 h. (b) At pH 1.0, AFMU deformylates relatively rapidly with a $t_{1/2}$ of 4.6 h. (c) Between pH 2.0 and 3.0, AFMU is quite stable with a $t_{1/2}$ greater than 36 h. (c) Between pH 3.0 and 8.0, AFMU is also stable with a $t_{1/2}$ greater than 27 h. (d) Above pH 8.0, AFMU begins to deformylate more rapidly with a $t_{1/2}$ lower than 7.8 h.

The kinetics data of AFMU deformylation confirm that pH 3.0 is the most suitable pH for storing urine and caffeine metabolite extract solutions. In addition, they demonstrate that AFMU is relatively stable over the pH range of 2.0–8.0 at 24 °C, indicating that deformylation of AFMU is negligible over a relatively long period of time

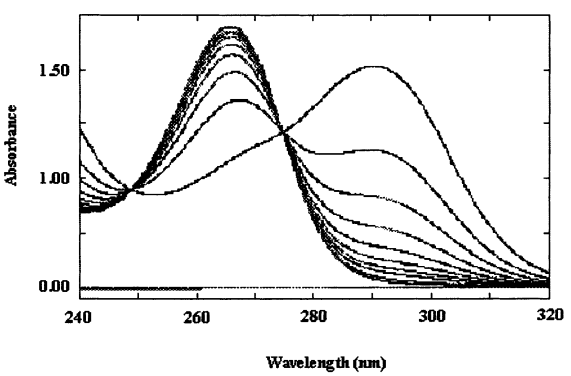

Fig. 4. Change of the ultraviolet absorption spectrum as a function of time of AFMU in a NaOH solution (pH 12.0; 0.01 N) at 24 °C. Absorption spectra were recorded after 25, 120, 210, 300, 390, 480, 570, 660, 900 and 1320 s. The AFMU concentration was 1.63×10^{-4} M.

Table 1
Rate constants and half-lives of deformylation of AFMU into AAMU in solutions of different pH at 24 °C

Solution	pH	k (h^{-1}) $\times 10^2$	$t_{1/2}$ (h)
HCl (0.1 N)	1.0	15.1	4.6
HCl (0.01 N)	2.0	19.2 ± 0.13 (2)[a]	36.1 ± 2.3 (2)
HCl (0.001 N)	3.0	0.46 ± 0.01 (2)	150.6 ± 3.3 (2)
Sodium acetate (0.05 M)	3.5	0.61	113
Sodium acetate (0.05 M)	4.0	0.63	109.9
Sodium acetate (0.05 M)	5.0	0.95 ± 0.07 (2)	72.9 ± 5.0 (2)
Citric acid–disodium phosphate (0.025–0.05 M)	5.0	0.95	72.8
Sodium phosphate (0.05 M)	6.0	1.40 ± 0.13 (2)	49.5 ± 4.2 (2)
Sodium phosphate (0.05 M)	6.5	1.57	44.1
PBS	7.4	1.48 ± 0.07 (2)	46.8 ± 2.1 (2)
Sodium phosphate (0.05 M)	7.5	1.30	53.2
HEPES (0.05 M)	8.0	2.09	33.1
Tris–HCl (0.05 M)	8.0	2.50	27.7
Sodium carbonate (0.05 M)	9.0	8.88	7.8
Sodium carbonate (0.05 M)	10.0	57.2	1.21
Sodium carbonate (0.05 M)	11.0	468.8	0.148
NaOH (0.01 N)	12.0	770	0.09
NaOH (0.05 N)	12.7	2310	0.03

[a] The number in parentheses refers to the number of determinations.

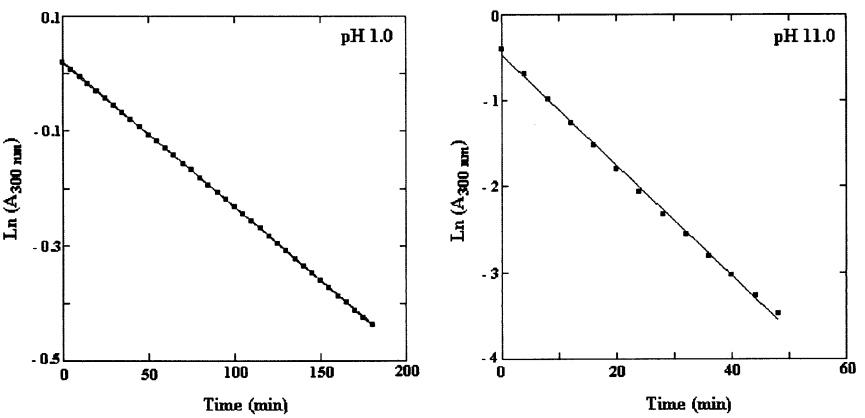

Fig. 5. Semi-logarithmic plots of the change of the absorbance at 300 nm as a function of time of AFMU solutions at pH 1.0 and 11.0 at 24 °C. Linear curves were obtained at pH 1.0 and 11.0 as well as at other pH and in urine, indicating that deformylation obeyed first-order kinetics. Half-lives of deformylation were 4.6 h and 8.9 min at pH 1.0 and 11.0 at 24 °C, respectively.

under urine sample storage conditions (pH 3.5 and −20 °C) and in various solutions used at different stages for NAT2 phenotyping by HPLC or CE. This conclusion was confirmed by the high stability of AFMU under those storage conditions and in these various solutions (Table 2) [7,8]. The observations indicating that the deformylation is pH-dependent and that the $t_{1/2}$ of deformylation decreases below and above pH 3.0 suggest that the deformylation is mediated by hydrogen and hydroxide ions and that the mechanism is predominantly dependent on the hydrogen ion below pH 3.0, but is predominantly dependent on the hydroxide ion above pH 3.0.

It was essential to investigate the deformylation of AFMU in urine since it is collected 4–5 h after

caffeine intake and that it can conceivably contain substances, absent in buffers, which catalyze the deformylation. However, the deformylation of AFMU could not be examined spectrophotometrically in urine due to the strong absorbance of the urine itself. To circumvent this difficulty, the deformylation of AFMU was directly analyzed in urine (pH 6.74) by HPLC after collecting urine 4 h after caffeine ingestion and using 1X present in the urine as the internal standard. The results showed the following. The deformylation obeyed first-order kinetics in urine (pH 6.74) with half-lives of 57 and 12.5 h at 24 and 37 °C, respectively. Half-life values were comparable to those observed in buffers at equivalent pH and temperatures: half-lives of 44 and 12.7 h were obtained in a sodium phosphate buffer (0.05 M; pH 6.5) at 24 and 37 °C, respectively. The following can be concluded based on these results. (a) Urine does not contain substances catalyzing the deformylation of AFMU. (b) The fraction of AFMU degraded over the 4–5 h period prior to collection of urine is significant but not extensive since the $t_{1/2}$ of AFMU in urine at 37 °C indicates no more than 15–16 and 18–20% of AFMU is deformylated after the 4 and 5 h, respectively. Thus, amounts of AFMU determined by HPLC may reflect adequately amounts of AFMU produced in the body if it is also considered that AFMU is produced over a period of time 4–5 h in the body. However, it is suggested that the collection of urine at no more than 2–3 h after caffeine intake, particurlaly in view that urine pH may be higher than normal in pathological states: it was observed that 30% of AFMU was deformylated after 4 h at pH 8.1 and 37 °C ($t_{1/2}$, 7.7 h), a pH that can be observed in pathological states.

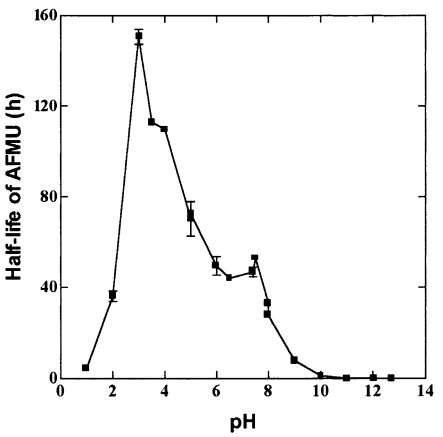

Fig. 6. Deformylation half-life of AFMU as a function of pH at 24 °C. Half-life values were taken from Table 1.

Table 2
Rate constants and half-lives of deformylation AFMU under storage conditions for urine and in different solutions used at various stages for NAT2 phenotyping by HPLC

Solution	pH	k (h^{-1}) $\times 10^3$	$t_{1/2}$ (h)
Urine (−20 °C)	3.5	ND[a]	ND[a]
0.045% acetic acid, 120 mg/200 μl $(NH_4)_2SO_4$	3.4	9.5	72.7
95% chloroform, 5% isopropanol	–	2.2	346.5
0.045% acetic acid	3.4	2.8	247.5
5% acetonitrile, 0.05% acetic acid	3.4	2.7	256.7
9% methanol, 0.045% acetic acid	3.4	2.8	247
Methanol[b]	–	87.7	0.79

[a] ND, no deformylation of AFMU was observed after 5 months of storage of urine at pH 3.5 and −20 °C.

[b] The rate constant and half-life of deformylation of AFMU in methanol were determined since it has been reported previously to be unstable in this solvent [11].

In conclusion, the stability of AFMU in buffers and urine at different pH and temperature was investigated since the use of caffeine as a probe drug for NAT2 phenotyping has been questioned, owing to the intrinsic instability of AFMU which deformylates to AAMU. Moreover, a quantitative knowledge of its stability under different conditions was necessary for our studies of NAT2 phenotyping by ELISA since AFMU is transformed to AAMU before the ELISA and that there was an absence of correlation between amounts of AFMU determined by HPLC and amounts of AAMU determined by ELISA. The results indicated that AFMU is a relatively stable compound and thus caffeine can safely be used as a probe drug for NAT2 phenotyping provided that urine is collected 2–3 h after caffeine intake.

References

[1] D.A. Evans, *N*-acetyltransferase, Pharmacol. Ther. 42 (1989) 157–234.

[2] N.P. Lang, M.A. Butler, J. Massengill, M. Lawson, R.C. Stotts, M. Hauer-Jensen, F.F. Kadlubar, Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase the risk for colorectal cancer or polyps, Cancer Epidemiol. Biom. Prev. 3 (1994) 675–682.

[3] C.B. Ambrosone, J.L. Freudenheim, S. Graham, J.R. Marshall, J.E. Vena, J.R. Brasure, A.M. Michalek, R. Laughlin, T. Nemoto, K.A. Gillenwater, P.G. Shields, Cigarette smoking, *N*-acetyltransferase 2 genetic polymorphisms, and breast cancer risk, J. Am. Med. Assoc. 276 (1996) 1494–1501.

[4] I. Cascorbi, J. Brockmoller, P.M. Mrozikiewicz, S. Bauer, R. Loddenkemper, I. Roots, Homozygous rapid arylamine *N*-acetyltransferase (NAT2) genotype as a susceptibility factor for lung cancer, Cancer Res. 56 (1996) 3961–3966.

[5] W.M. O'Neil, B.M. Gilfix, A. Digirolamo, C.M. Tsoukas, I.W. Wainer, N-acetylation among HIV-positive patients and patients with AIDS: when is fast, fast and slow, slow?, Clin. Pharmacol. Ther. 62 (1997) 261–271.

[6] D.M. Grant, B.K. Tang, W. Kalow, A simple test for acetylator phenotype using caffeine, Br. J. Clin. Pharmacol. 17 (1984) 459–464.

[7] M.A. Butler, N.P. Lang, J.F. Young, N.E. Caporaso, P. Vineis, R.B. Hayes, C.H. Teitel, J.P. Massengill, M.F. Lawsen, F.F. Kadlubar, Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites, Pharmacogenetics 2 (1992) 116–127.

[8] D.K. Lloyd, K. Fried, I.W. Wainer, Determination of *N*-acetylator phenotype using caffeine as a probe compound: a comparison of high-performance liquid chromatography and capillary electrophoresis methods, J. Chromatogr. 578 (1992) 283–291.

[9] D. Meacher, R.E. Rasmussen, D.B. Menzel, Analysis of NAT and CYP1A2 phenotypes and NAT2* genotype by capillary electrophoresis, Biomarkers 3 (1998) 205–218.

[10] B. Lorenzo, M.M. Reidenberg, Potential artifacts in the use of caffeine to determine acetylation phenotype, Br. J. Clin. Pharmacol. 28 (1989) 207–208.

[11] B.K. Tang, D.M. Grant, W. Kalow, Isolation and identification of 5-acetylamino-6-formylamino-3-methyluracil as a major metabolite of caffeine in man, Drug Metab. Dispos. 11 (1983) 218–220.

[12] P. Wong, K. Banarjee, J. Massengill, S. Nowell, N. Lang, B. Leyland-Jones, Validity of an ELISA for *N*-acetyltransferase-2 (NAT2) phenotyping, J. Immunol. Methods 25 (2001) 1–9.

[13] P. Wong, G. Villeneuve, V. Tessier, H. Nedev, K. Banarjee, B.J. Claude, B. Leyland-Jones, NAT2 phenotyping: characterization of AFMU stability, Proc. Am. Assoc. Cancer Res. 41 (2000) Abst. 225, 25.

[14] W. Pfleiderer, Über die darstellung von 4,5-diaminopyrimiden, Chem. Ber. 90 (1957) 2272–2276.

[15] P. Wong, B. Leyland-Jones, I.W. Wainer, A competitive antigen enzyme linked immunosorbent linked assay for the determination of *N*-acetyltransferase (NAT2) phenotypes, J. Pharm. Biomed. Anal. 13 (1995) 1079–1086.

[16] K. Fink, W.S. Adams, W. Pfleiderer, A new urinary pyrimidine, 5-acetamido-6-amino-3-methyluracil. Isolation, identification and synthesis, J. Biol. Chem. 239 (1964) 4250–4256.