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A competitive enzyme linked immunosorbent assay for the determination of *N*-acetyltransferase (NAT2) phenotypes

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Abstract

The ratio of 5-acetylamino-6-amino-3-methyluracil (AAMU) to 1-methylxanthine (1X) in urine samples after caffeine ingestion can be used to indicate human *N*-acetyltransferase (NAT2) phenotypes. In previous studies, this ratio has been determined by LC or capillary electrophoresis. The possibility that this ratio could be determined by competitive antigen enzyme linked immunosorbent assays (ELISAs) has been investigated. Polyclonal antibodies were raised in rabbits against synthetic derivatives of AAMU and 1X, and competitive antigen ELISAs were developed after isolation of the IgGs by ion-exchange chromatography. The competitive antigen ELISA correctly identified previously determined NAT2 phenotypes and gave the expected distribution of slow and fast N-acetylators within a group of 48 individuals.

Keywords: 5-Acetylamino-6-amino-3-methyluracil; Caffeine metabolites; Immunoassay; 1-Methylxanthine; *N*-acetylator phenotyping; Pharmacokinetics

1. Introduction

Individuals are genetically polymorphic in their rate of enzymatic N-acetylation of drugs via the *N*-acetyltransferase (NAT2) pathway [1–3]. Two major metabolic phenotypes can be distinguished: fast and slow N-acetylators. Knowledge of this phenotype is useful because of its association with adverse drug effects, diverse toxicities and predisposition to disease. For example, N-acetylation polymorphism has been linked to a detoxification pathway of some environmental carcinogenic arylamines and their is a higher frequency of bladder cancers among chemical dye workers who are slow N-acetylators [4].

A number of probe drugs can be used to determine the NAT2 phenotype [1–3]. Of these, caffeine is the most frequently utilized probe because it is widely consumed and relatively safe [5–7]. In studies involving this probe, the phenotype has generally been determined from ratios of the caffeine metabolites 5-acetylamino-6-amino-3-methyluracil (AAMU) or 5-acetylamino-6-for-mylamino-3-methyluracil(AFMU) and 1-methyl-xanthine (1X). In these studies, the subjects are given an oral dose of a caffeine-containing substance, and the urinary concentrations of the target metabolites determined by LC [1,3,5–12] or capillary electrophoresis (CE) [13,14].

The number of clinical protocols requiring the determination of NAT2 phenotypes is rapidly increasing and this group has sought to develop an enzyme linked immunosorbent assay (ELISA) for use in these studies. ELISAs

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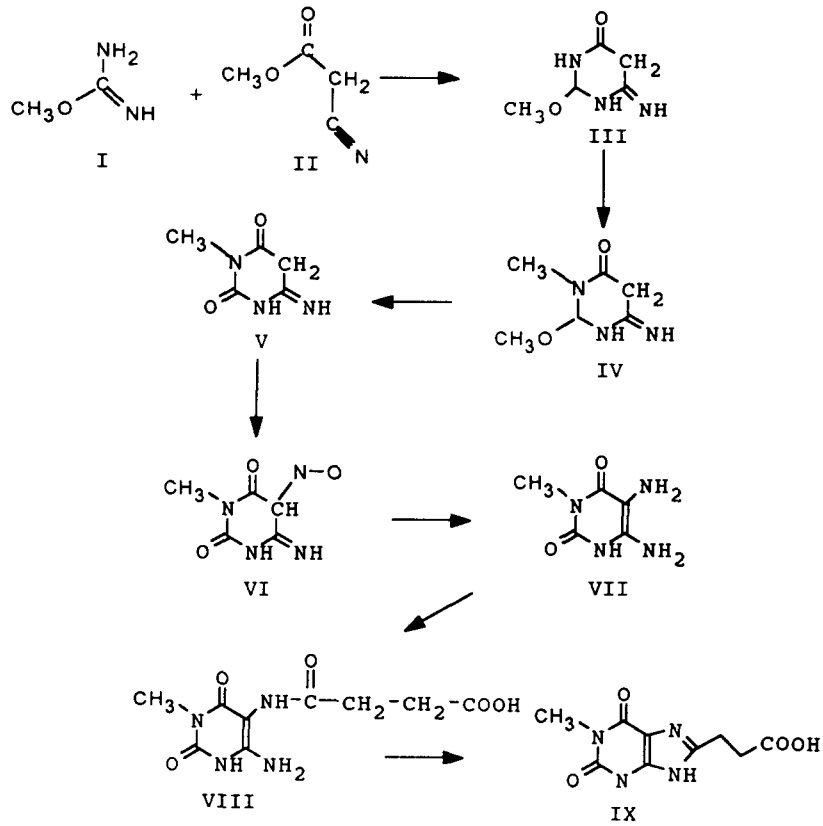

Fig. 1. Synthetic route for the production of AAMU and 1X derivatives.

have been successfully applied in the determination of low amounts of drugs and other antigenic components in plasma and urine samples [15,16], involve no extraction steps and are simple to carry out. This article reports the development of an ELISA based on antibodies against AAMU and 1X which can be used to determine NAT2 human phenotypes.

2. Materials and methods

2.1. Chemicals and reagents

Cyanomethylester, isochloroformate, dimethylsulfate, sodium methoxide, 95% pure, and tributyl-amine were purchased from Aldrich, (Milwaukee, WI, USA); horse radish peroxidase was purchased from Boehringer Manheim (Montreal, Que., Canada); corning easy wash polystyrene microtiter plates were bought from Canlab (Montreal, Que., Canada); *o*-methylisourea hydrochloride was obtained from Lancaster Laboratories (Windham, NH, USA); alkaline phosphatase conjugated to goat anti-rabbit IgGs was from Pierce Chemical Co. (Rockford, IL, USA); bovine serum albumin fraction V initial fractionation by cold alcohol precipitation (BSA), complete and incomplete Freund's adjuvants, diethanolamine, *p*-nitrophenol phosphate disodium salt, *o*-phenylenediamine hydrochloride; porcine skin gelatin, rabbit serum albumin (RSA). Tween 20 and ligands used for testing the antibody cross reactivities were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Whatman DE52 diethylaminoethyl-cellulose was obtained from Chromatographic Specialities Inc. (Brockville, Ont., Canada). Dioxane was obtained from A & C American Chemicals, Ltd. (Montreal, Que., Canada) and was refluxed over calcium hydride for 4 h and distilled before use. Other reagents used were of analytical grade.

2.2. Synthetic procedures

The synthetic route for the production of AAMU-hemisuccinate (VIII) and 1-methylxanthine-8-propionic acid (IX) is presented in Fig. 1. Compounds III–VI were synthesized according to the procedure of Pfleiderer [17] and compound VII was synthesized following the procedure described by Lespagnol et al. [18].

Synthesis of AAMU-hemisuccinate (VIII)

VII (0.30 g) was dissolved in water (5 ml) and succinic anhydride (0.33 g) was added to the resulting solution. The mixture was stirred until the succinic anhydride dissolved. During this process, the pH of the solution was maintained between 8 and 9 with a 3 N NaOH solution. The reaction was completed when all the succinic anhydride had dissolved and the pH remained above 8. The hemisuccinate was precipitated by acidification to pH 0.5 with a 12 N HCl solution. The precipitate was collected by filtration on Whatman No. 1 paper, washed with water and dried.

Synthesis of the 1-methylxanthine-8-propionic acid (IX)

This product was synthesized according to a modified procedure of Lespagnol et al. [18]. VIII (0.2 g) was dissolved in 2–3 ml of a 15% NaOH solution. The resulting solution was stirred at 100 °C until all of the solvent had evaporated and then maintained at this temperature for an additional 5 min. The resulting solid was allowed to cool to room temperature, dissolved in 10 ml water and IX precipitated by acidification to pH 2.8 with 12 N HCl. After cooling at 4 °C for 2.5 h, the precipitate was collected by filtration on a Whatman No. 1 paper and dried. It was recrystallized from water ethanol (20:80, v/v), using charcoal to decolorize the solution.

Other syntheses

AAMU was synthesized from compound VII according to the procedure of Fink et al. [19]. 5-Acetylamino-6-aminouracil and 5-acetamino-6-amino-1,3-dimethyluracil were synthesized by the same procedure as that of compound VII, using acetic anhydride.

2.3. NMR spectroscopy

^{1}H and ^{13}C NMR spectra of compounds VIII and IX were obtained using a 500 mHz spectrometer (Varian XL 500 mHz, Varian Analytical Instruments, San Fernando, CA, USA) using deuterated dimethyl sulfoxide as solvent.

2.4. Conjugation of the haptens to bovine serum albumin (BSA) and rabbit serum albumin (RSA)

The AAMU-hemisuccinate (VIII) and the 1-methylxanthine derivative (IX) were conjugated to BSA and RSA according to the mixed anhydride method of Erlanger et al. [20]. After conjugation, the free hapten was separated from the conjugate by dialyzing against 1 l water for 3 days at room temperature; water was changed twice a day. The conjugates were stored as 1-ml aliquots at −20 °C.

2.5. Coupling of haptens to horse radish peroxidase

The AAMU and IX derivatives (VIII and IX) were conjugated to horse radish peroxidase by the procedure of Fickling et al. [15]. After conjugation, the free derivatives were separated from the conjugate by gel filtration on a 20 × 1.6 cm Sephadex G25 fine column equilibrated with a sodium phosphate buffer (100 mM, pH 7.0). The conjugate was stored at 4 °C in a 0.02% thiomersal solution.

2.6. Antibody production

Four mature female New Zealand White rabbits (Charles River Canada, St. Constant, Que., Canada) were used for antibody production. The protocol employed in this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care.

An isotonic saline solution (0.6 ml) containing 240 μg of BSA-conjugated antigen was emulsified with 0.6 ml of a complete Freund's adjuvant. 0.5 ml of the emulsion (100 μg of antigen) was injected per rabbit intramuscularly or subcutaneously. Rabbits were subsequently boosted at intervals of three weeks with 50 μg of antigen emulsified in incomplete Freund's adjuvant. Blood was collected by venipuncture of the ear 10–14 days after boosting. Antisera were stored at 4 °C in the presence of 0.01% sodium azide.

2.7. Antiserum titers

The wells of a microtiter plate were coated with 10 μg ml^{-1} of rabbit serum albumin-AAMU (or IX) conjugate in carbonate buffer (100 mM, pH 9.6) for 1 h at 37 °C (100 μl per well). They were then washed three times with 100 μl TPBS (phosphate buffer saline containing 0.05% Tween 20) and unoccupied sites were blocked by an incubation with 100 μl of TPBS

containing 0.05% gelatin for 1 h at 37 °C. The wells were washed three times with 100 μl TPBS and 100 μl of antiserum diluted in TPBS was added. After 1 h at 37 °C, the wells were washed three times with TPBS, and 100 μl of goat anti-rabbit IgGs-alkaline phosphatase conjugate diluted with PBS containing 1% BSA was added. After 1 h at 37 °C, the wells were washed three times with TPBS and three times with water. To the wells were added 100 μl of a solution containing $MgCl_2$ (0.5 mM) and *p*-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min at room temperature, the absorbency was read at 405 nm with a microtiter plate reader. The antibody titer is defined as the dilution required to change the absorbance by one unit (1 au).

2.8. Isolation of rabbit IgGs

The DE52-cellulose resin was washed three times with sodium phosphate buffer (500 mM, pH 7.50), the fines were removed and the resin was equilibrated with a sodium phosphate buffer (10 mM, pH 7.50). The resin was packed in 1.6 ± 50 cm column and eluted with the 200–300 ml equilibrating buffer before use. To antiserum obtained from 50 ml of blood (30–32 ml) was added dropwise 25–27 ml 100% saturated ammonium sulfate solution with a pasteur pipette. The suspension was left at room temperature for 3 h and centrifuged for 30 min at 2560*g* at 20 °C. The pellet was dissolved with 15 ml sodium phosphate buffer (10 mM, pH 7.50) and dialyzed against 1 l of the buffer for two days at room temperature with the buffer changed twice per day. The dialyzed solution was centrifuged at 2560*g* for 10 min at 20 °C to remove precipitate formed during dialysis. The supernatant was applied to the ion-exchange column. Fractions of 7 ml were collected. After application, the column was eluted with the equilibrating buffer until the absorbance at 280 nm became less than 0.05 au. It was then eluted with the equilibrating buffer containing 50 mM NaCl. Fractions having absorbances greater than 0.2 at 280 nm were saved and stored at 4 °C.

2.9. Double immunodiffusion in agar plate

An 0.8% agar gel in PBS was prepared in a 60×15 mm petri dish. Rabbit serum albumin (100 μl of 1 mg ml^{-1}) conjugated either to AAMU or 1X was pipetted in the center well, and 100 μl of rabbit antiserum was pipetted in peripheral wells. The immunodiffusion was carried out in a humidified chamber at 37 °C overnight and the gel was inspected visually.

2.10. Competitive antigen ELISA

Buffers and water without additives were filtered through millipore filters, kept for 1 week, and BSA, antibodies, Tween 20 and horse radish conjugates were added to these buffers and water just prior to use. Urine samples were usually collected 4 h after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at −80 °C. They were diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and were subsequently diluted with water to give concentrations of AAMU and 1X no higher than 3×10^{-6} M in the ELISA. All the pipettings were done with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well or last row, 100 μl of a carbonate buffer (100 mM, pH 9.6) containing 2.5 μg ml^{-1} antibodies was pipetted. After 90 min at room temperature, the wells were washed three times with 100 μl of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.5% Tween 20.

After the initial wash, unoccupied sites were blocked by incubation for 90 min at room temperature with 100 μl TPB containing 3% BSA. The wells were washed four times with 100 μl TPB. This was followed by additions of 50 μl of 12 μg ml^{-1} AAMU-HRP or 1X-HRP conjugate in 2x TPB containing 2% BSA, and 50 μl of either water, standard (13 standards; AAMU or 1X, 2×10^{-4}–2×10^{-8} M) or sample in duplicate. The microplate was gently shaken with an orbital shaker at room temperature for 3–4 h. The wells were washed three times with 100 μl with TPB containing 1% BSA and three times with 100 μl water containing 0.05% Tween 20. To the washed plate was added 150 μl of a substrate buffer composed of citric acid (38 mM) and sodium phosphate dibasic buffer (67 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% *o*-phenylenediamine hydrochloride. After 20–25 min at room temperature with shaking, the reaction was stopped with 50 μl of 2.5 M HCl. After shaking the plate for 3 min, the absorbances were read with a microtiter plate reader at 490 nm.

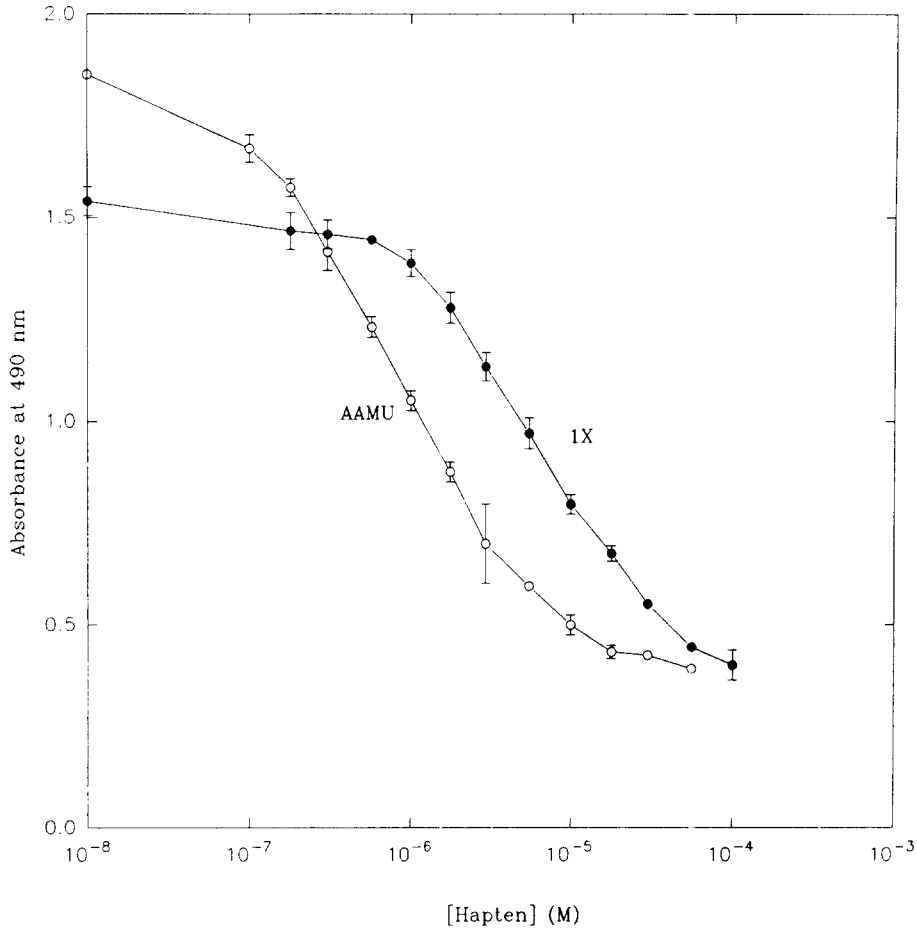

Fig. 2. Absorbance competitive antigen ELISA curves of AAMU-Ab and 1X-Ab. Each curve represents triplicate determinations in a single run. The error bars represent standard deviations. For data points with no error bars, the error bars are less than the size of the symbol.

2.11. Protein determination

Protein determination was done by the procedure of Lowry et al. [21].

3. Results and discussion

For antibody production against AAMU and 1X, derivatives of these compounds were synthesized according to Fig. 1 (VIII and IX), and structures were confirmed by ^{1}H and ^{13}C NMR spectral analyses (data not shown). The AAMU and 1X derivatives were then conjugated to BSA by the mixed anhydride method, and the conjugates injected into four rabbits. Each rabbit produced antibody titers of 30 000–100 000 as determined by ELISA. This was also indicated by strong precipitin lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to RSA. On this basis, a competitive antigen ELISA was developed using a procedure similar to that developed by Fickling et al. for caffeine [15].

The competitive antigen ELISA curves of AAMU-Ab and 1X-Ab obtained by duplicate determinations are presented in Fig. 2. The experimental conditions were: background was less than 0.10 au; the practical limits of detection of AAMU and 1X were 2×10^{-7} M and 2×10^{-6} M, respectively, concentrations 500 and 50 times lower than those in urine samples from previous phenotyping studies [8]; the intra-assay and inter-assay coefficients of variation of AAMU and 1X were 15–20% over the concentration range of 0.01–0.05 mM.

A variety of conditions for the ELISA were tested and a number of noteworthy observations were made: gelatin, which was used in the competitive antigen ELISA determination of

Table 1
Cross reactivity of AAMU-Ab and 1X-Ab towards different caffeine metabolites and structural analogs

Compound	% Cross-reaction [a]	
	AAMU-Ab	1X-Ab
Xanthine	0	0
Hypoxanthine	0	0
Methylxanthine (1X)	0	100
3-Methylxanthine	0	0
7-Methylxanthine	0	0
8-methylxanthine	0	0
1,3-Dimethylxanthine (theophylline)	0	0.2
1,7-Dimethylxanthine (paraxanthine)	0	0.5
3,7-Dimethylxanthine (theobromine)	0	0
1,3,7-Trimethylxanthine (caffeine)	0	0
Uric acid	0	0
1-Methyluric acid	0	11
1,7-Dimethyluric acid	0	0
Guanine	0	0
Uracil	0	0
5-Acetamino-6-amino-uracil	0.6	0
5-Acetamino-6-amino-3-methyluracil (AAMU)	100	0
5-Acetamino-6-amino-1,3-dimethyluracil	0	0

[a] "0" indicates either an absence of inhibition or an inhibition no higher than 40% at the highest compound concentration tested in the ELISA (5×10^{-3} M); concentrations of 5-acetamino-6-amino-1-methyluracil (AAMU) and 1-methylxanthine (1X) required for 50% inhibition in the competitive antigen ELISA were 1.5×10^{-6} M and 10^{-5} M, respectively.

caffeine in plasma [15], could not be used in our ELISA owing to excessive background absorbency which varied between 0.5 and 1.0 au; in the absence of Tween 20, absorbency changes per 15 min decreased by a factor of at least 3, and calibration curves were generally erratic; absorbency coefficients of variation of samples increased by a factor of 3–4 when the conjugates and haptens were added to the wells as a mixture instead of individually.

The cross reactivities of AAMU-Ab and 1X-Ab were tested using a wide variety of caffeine metabolites and structural analogs, and the results are presented in Table 1. AAMU-Ab appeared highly specific for binding AAMU, while 1X-Ab appeared relatively specific for binding 1X. However, a 11% cross reactivity was observed with 1-methyluric acid (1U), a major caffeine metabolite.

The relatively high level of cross reactivity of 1U is, however, unlikely to interfere significantly in the determination of 1X and the assignment of NAT2 phenotypes, since the ratio of 1U:1X is no greater than 2.5:1 in 97% of the population [5,7]. This is confirmed by measurements of apparent concentrations 1X when the ratio 1U:1X was varied between 0 and 8.0 at the fixed 1X concentration of 3×10^{-6} M (Table 2). The maximum effect on the determination of 1X concentration occurred at a 1U:1X ratio of 6.0, where an apparent 50% increase in 1X concentration was observed. At 1U:1X ratios of 2.5 and 3.0, the apparent increases were 22% and 32%, respectively.

The ability of this ELISA to accurately determine NAT2 phenotypes was assessed by cross-validating the method using urine samples from 30 subjects previously studied using a CE method [13]. The NAT2 phenotypes agreed in 29 of the 30 individuals. In the CE method,

Table 2
Effect of the ratio 1U:1X on the determination of 1X concentration by ELISA at the fixed 1X concentration of 3×10^{-6} M

1U:1X ratio	$[1X] \times 10^6$ (M)
0.0	3.00
0.50	2.75
1.00	3.25
1.50	3.25
2.00	3.60
2.50	3.65
3.00	3.95
4.00	4.20
5.00	4.30
6.00	4.50
8.00	4.30

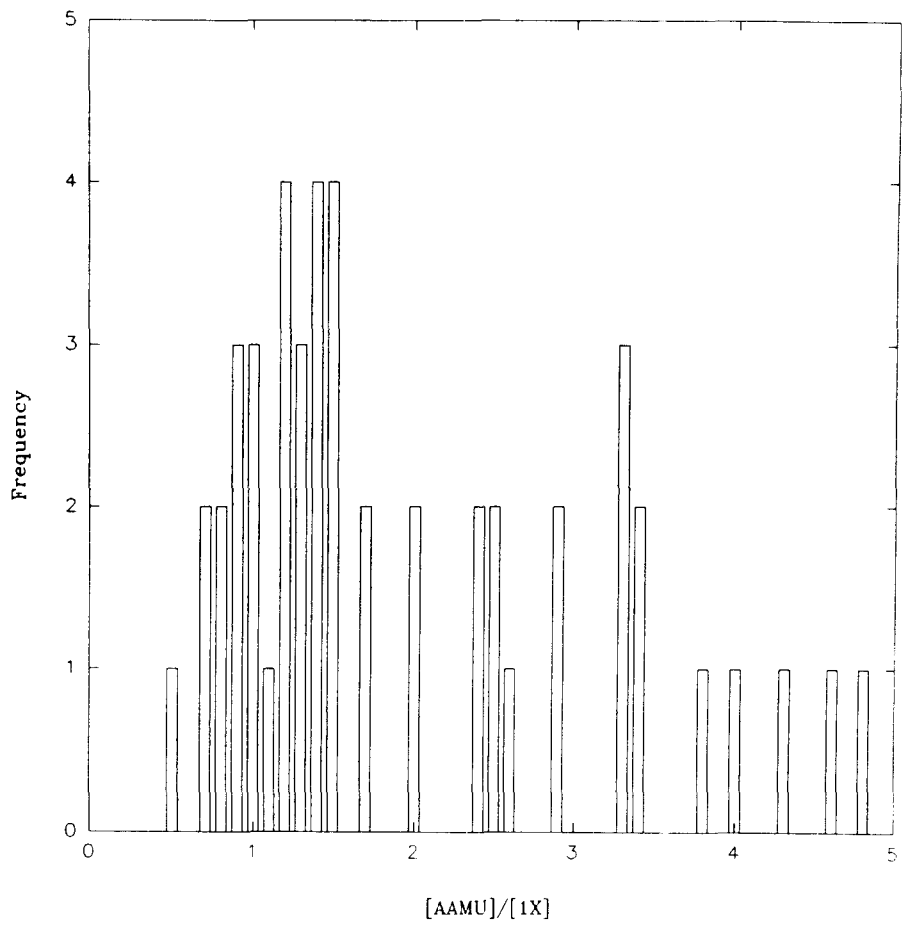

Fig. 3. Histogram of molar ratio of AAMU/1X. This histogram was obtained with a group of 48 individuals. Assuming an antimode of 1.80, 60.4% and 39.6% of the individuals were slow and fast acetylators, respectively, values similar to those previously reported [3].

the phenotype was determined using AFMU/1X peak height ratios rather than the AAMU/1X molar ratios used in the ELISA. When the molar ratios determined by ELISA and the peak height ratios determined by CE were correlated by regression analysis, the calculated regression equation was $y = 0.48 + 0.87x$, with a correlation coefficient (r) of 0.84. Taking into account that these two ratios are not exactly equal and that Kalow and Tang have pointed out that using AFMU rather than AAMU can lead to a misclassification of NAT2 phenoptyes [1], there is a remarkable agreement between the two methods.

4. Conclusions

The ELISA described in this report has been used to determine NAT2 phenotypes (the frequencies of distribution of slow and fast acetylators, for a group of 48 individuals) using caffieine as the test drug. The method is relatively simple to carry out and can be applied for analyzing multiple urine samples. Assuming that the molar AMMU/1X ratio antimode is 1.80 [5,8], the test population contained 60.4% slow acetylators and 39.6% fast acetylators (Fig. 3). This is consistent with previously reported distributions [1–3,7,8].

Acknowledgments

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References

[1] W. Kalow and B.K. Tang, Clin. Pharmacol. Ther., 53 (1993) 503–514.

[2] D.M. Grant, K. Morike, M. Eichelbaum and U.A. Meyer, J. Clin. Invest., 85 (1990) 968–970.

[3] U.A. Meyer, Proc. Natl. Acad. Sci. USA, 91 (1994) 1983–1984.

[4] D.W.J. Clark, Drugs, 29 (1985) 342–375.

[5] J.A. Carrillo and J. Benitez, Clin. Pharmacol. Ther., 55 (1994) 293–303.

[6] W. Kalow and B.K. Tang, Clin. Pharmacol. Ther., 49 (1991) 508–518.

[7] B.K. Tang, D. Kadar, L. Qian, J. Iriah, J. Yip and W. Kalow, Clin. Pharmacol. Ther., 49 (1991) 648–657.

[8] A.J. Kilbane, L.K. Stilbart, M. Manis, I.Z. Beitins and W.W. Weber, Clin. Pharmacol. Ther., 47 (1990) 470–477.

[9] B.K. Tang, T. Zubovits and W. Kalow, J. Chromatogr., 375 (1986) 170–173.

[10] M.-P. Gascon, T. Leeman and P. Dayer, Schweiz. Med. Wschr., 117 (1987) 197–1976.

[11] A. El-Yazigi, K. Chaleby and C.R. Martin, J. Clin. Pharmacol., 29 (1989) 246–250.

[12] M. Hildebrand and W. Seifert, Eur. J. Clin. Pharmacol., 37 (1989) 525–526.

[13] D. Lloyd, K. Fried and I.W. Wainer, J. Chromatogr., 578 (1992) 283–291.

[14] R. Guo and W. Thormann, Electrophoresis, 14 (1993) 547–553.

[15] S.A. Fickling, S.M. Hampton, D. Teale, B.A. Middleton and V. Marks, J. Immun. Methods, 129 (1990) 159–164.

[16] P. Tijssen, Practice and theory of enzyme immunoassays, in R.H. Burdon and R.W. Knippenberg (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 15, Elsevier, Amsterdam, 1985.

[17] W. Pfleiderer, Chem. Ber., 90 (1957) 2272–2276.

[18] A. Lespagnol, M. Debaert and N. Minard-Vaillant, Chim. Ther., 5 (1970) 321–326.

[19] K. Fink, W.S. Adams and W. Pfleiderer, J. Biol. Chem., 249 (1964) 4250–4256.

[20] B.F. Erlanger, F. Borek, S.M. Beiser and S. Lieberman, J. Biol. Chem., 228 (1957) 713–727.

[21] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265–275.