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Short communication

Determination of *p*-aminosalicylic acid and its *N*-acetylated metabolite in human urine by capillary zone electrophoresis as a measure of in vivo *N*-acetyltransferase 1 activity

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Abstract

A capillary zone electrophoresis method has been developed for the determination of *p*-aminosalicylic acid (PAS) and its metabolite, *N*-acetyl-*p*-aminosalicylic acid (*N*-acetyl-PAS), in urine. A linear relationship was observed between time-normalized peak area and the concentration of the parent and metabolite with correlation coefficients greater than 0.9990. The method could be applied to the determination of PAS and *N*-acetyl-PAS in human urine without any sample pretreatment. A good separation of the analytes is achieved in a run time of 12 min (15 min total, including capillary wash). Using PAS as a probe for *N*-acetyltransferase 1 activity, 20 healthy volunteers were phenotyped after oral administration of a 1 g dose. The preliminary results seem to indicate a bimodal distribution of *N*-acetyl-PAS/PAS molar ratios. ©1997 Elsevier Science B.V.

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1. Introduction

N-Acetyltransferase (NAT; EC 2.3.1.5) is an enzyme important in the biotransformations of many xenobiotic compounds. The acetyl group from acetyl CoA is transferred to an arylamine substrate via a two step substituted enzyme reaction mechanism [1]. An interesting aspect of NAT enzymes is that they are generally subdivided into two classes, monomorphic (NAT1) or polymorphic (NAT2) acetylators, depending on the substrate in question. At the genetic level NAT1 has recently been shown to be polymorphic in the polyadenylation signal [2] and there is some preliminary phenotypic data supporting the incidence of NAT1 polymorphism [3].

Examples of NAT2 substrates include: sulfamethazine, caffeine, dapsone and procainamide. To date, most pharmacological research has focused on NAT2 because of the implications of its polymorphism. In addition, NAT2 phenotypes can be readily assessed by a non-invasive test [4–6]. In this approach the urinary concentrations of two caffeine metabolites, 1-methylxanthine (1X) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU), are determined by HPLC [4], CE [5,6] or ELISA [7]. The AFMU/1X ratio reflects the NAT2 phenotype.

Among the substrates metabolized by NAT1 are

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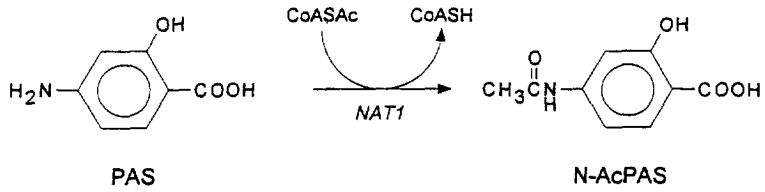

Fig. 1. Chemical structures for PAS and N-acetyl-PAS. CoASAc: acetyl coenzyme A, CoASH: coenzyme A.

p-aminosalicylic acid (PAS) and sulphamethoxazole (SMX), an antimicrobial drug frequently used to treat HIV infected patients. SMX hypersensitivity is common in people infected with HIV [8]. This hypersensitivity has been correlated with a slow NAT2 phenotype, despite the fact that it is actually a NAT1 substrate [9]. This correlation may be due to the availability of acetyl CoA, which is a required cofactor for both enzymes. There is increasing interest in NAT1 phenotyping because of its role in the metabolism of SMX, the possibility that it may be polymorphic and its implication in the activation of certain procarcinogens [10]. Hence, there is a need for a quick and simple analysis of NAT1 phenotype.

PAS is a commonly used tuberculostatic agent which is also a substrate for NAT1. PAS has been used as an in vivo probe of NAT1 activity. In these studies, post-dose urine levels of PAS and its *N*-acetylated metabolite (*N*-acetyl-PAS) were measured [3,11]. The *N*-acetyl-PAS/PAS ratios were used for phenotypic assignment. Fig. 1 shows the structures of PAS and *N*-acetyl-PAS and the metabolic pathway to convert one to the other.

Previous studies involving the measurement of in vivo NAT1 activity used high performance liquid chromatography as the method of detecting the two analytes [3,11]. This paper describes a capillary zone electrophoresis method which provides an excellent separation of the analytes in a period of under 12 min. The specifications of the method and the results obtained for the NAT1 phenotyping of a small sample population are shown.

2. Experimental

2.1. Chemicals

Sodium monohydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) was purchased from J.T. Baker (Phillipsburg, NJ, USA), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) was obtained from Fisher (Fair Lawn, NJ, USA) and PAS was purchased from ICN Biomedicals (Aurora, OH, USA). *N*-Acetyl-PAS was synthesized according to the method described below.

2.2. Synthesis and characterization of 2-hydroxy-4-acetylamino benzoic acid (N-acetyl-PAS)

p-Aminosalicylic acid (5.0 g, 0.33 mol) was dissolved in 2 *M* hydrochloric acid (100 ml) and stirred with a solution of sodium acetate (50 g in 250 ml water) at 0°C. Acetic anhydride (58 ml) was added to the mixture which was stirred overnight and allowed to warm to room temperature as the ice gradually melted [12]. The brown precipitate formed was filtered, washed with distilled water (50 ml), air-dried and then dissolved in the minimum amount of 0.1 *M* sodium hydroxide. The solution was stirred overnight at room temperature, after which concentrated hydrochloric acid was used to adjust the pH of the solution to 2 [13]. The solution was then extracted with ethyl acetate (2×100 ml), and the extracts were dried over anhydrous sodium sulphate, filtered and concentrated to dryness under reduced pressure on a rotary evaporator. Hexane was then used to wash the solid residue from the flask and filtered to give 2-hydroxy-4-acetylamino benzoic acid. IR (KBr disk): 3357, 2875, 1683, 1604, 1388, 790 cm^{-1}; MS: *m/z* 196 (M^+); ^1H NMR (400 MHz, DMSO): δ 2.1 (s, 3H), 7.0 (dd, 1H, J=9 Hz, J=2 Hz), 7.4 (d, 1H, J=2 Hz), 7.7 (d, 1H, J=9 Hz), 10.2 (s, 1H), 11.3 (broad s, 1H), 13.6 (broad s, 1H).

There was a shoulder on the δ 2.1 NMR signal consistent with up to 5% contamination of the diacetyl-PAS derivative. It was subsequently noticed that the peak shape of the *N*-acetyl-PAS was non-symmetrical. Injections of smaller amounts of the metabolite revealed a small shoulder, equivalent to about 4% of the peak area.

2.3. Apparatus

The CZE separation was carried out using a SpectraPHORESIS 1000 instrument (Thermoseparation Products, Mississauga, Ontario, Canada). The separation capillary was 44 cm long (36.5 cm to detector), with an internal diameter of 50 μm (Polymicro Technologies, Phoenix, AZ, USA). The separation buffer for analysis of urine samples was prepared by mixing 75 m*M* solutions of mono- and dibasic sodium phosphate to give a pH of 7.0. The temperature of the capillary oven was set to 30°C. A separation potential of 13.8 kV was used giving a current flow of approximately 70 μA. No sample preparation was required for fresh urine; thawed samples occasionally required centrifugation. Hydrodynamic injections of 1 s were employed throughout by applying a vacuum of 10.3 kPa to the cathode end. Detection was carried out by on-line UV absorbance monitoring, with a detector rise time of 1 s, at 254 nm for the first 5 min to detect the electroosmotic flow (EOF) and then at 305 nm for the remainder of the run to detect the analytes. Both *N*-acetyl-PAS and PAS migrated within 12 min under these conditions. To obtain reproducible results the capillary was conditioned between each run with 0.1 *M* sodium hydroxide (1 min), distilled water (1 min), followed by separation buffer (2 min). In addition, the capillary was washed at the start of each day for 5 min with each of the following: 1 *M* hydrochloric acid (30°C), 1 *M* sodium hydroxide (60°C), 0.1 *M* sodium hydroxide (60°C) and distilled water (30°C).

2.4. Preparation of standards

Calibration curves were prepared by spiking blank urine with varying amounts of stock solutions of PAS and *N*-acetyl-PAS. The stock solutions were prepared by dissolution of PAS and *N*-acetyl-PAS in urine to give concentrations of 0.8 mg ml^{-1} and 6 mg ml^{-1}, respectively. Two molar equivalents of sodium hydroxide were added to aid with the dissolution of *N*-acetyl-PAS. The range of concentrations in the standard curves was 0–800 μg ml^{-1} for PAS and 0–4000 μg ml^{-1} for *N*-acetyl-PAS. The *N*-acetyl-PAS curves were adjusted to account for the 4% impurity in the standard.

2.5. Experimental subjects

Twenty healthy volunteers (9 male and 11 female; age 18–55) participated in this study. Each subject gave informed consent in accordance with the guidelines of the Montreal General Hospital Ethics Committee. Among the subjects were 18 Caucasians and 2 Asians. The molar ratios of *N*-acetyl-PAS/PAS obtained from the Asian volunteers were not significantly different from the others and therefore they were not distinguished in the results. The volunteers were asked to provide a blank urine sample before ingesting 1 g of the sodium salt of PAS (Nemasol Sodium, ICN Canada, Montreal, Quebec, Canada). Urine samples were collected over the 0–2 h post-dose period, in accordance with previous studies by Cribb et al. [11], and samples were frozen at −20°C until analysis.

2.6. Data analysis

Probit analysis consisted of plotting the log of *N*-acetyl-PAS/PAS vs. its probit (difference, in standard deviations, from the mean+k, where k was an integer yielding a positive probit). Curves with equations of the form $y=ae^{bx}$ were tested for goodness of fit to discern the antimode [14]. The antimode was defined by the point at which the curves crossed such that there was complete segregation between the 2 test groups.

3. Results and discussion

3.1. Sample electropherograms

Using the experimental conditions described, the extent of in vivo *N*-acetylation of PAS by NAT1 was determined. Fig. 2A shows a typical electropherogram of blank urine. No significant interferences are seen with urine directly injected onto the capillary. The electropherogram in Fig. 2B is representative of a 0–2 h urine sample after the subject ingested 1 g of PAS. Fig. 2C shows a different sample of pre-dose urine spiked with 100 and 1200 μg ml^{-1} of PAS and *N*-acetyl-PAS, respectively. It was discovered that aspirin (or a metabolite thereof) acted as an interferent for this method since it migrated very close to the

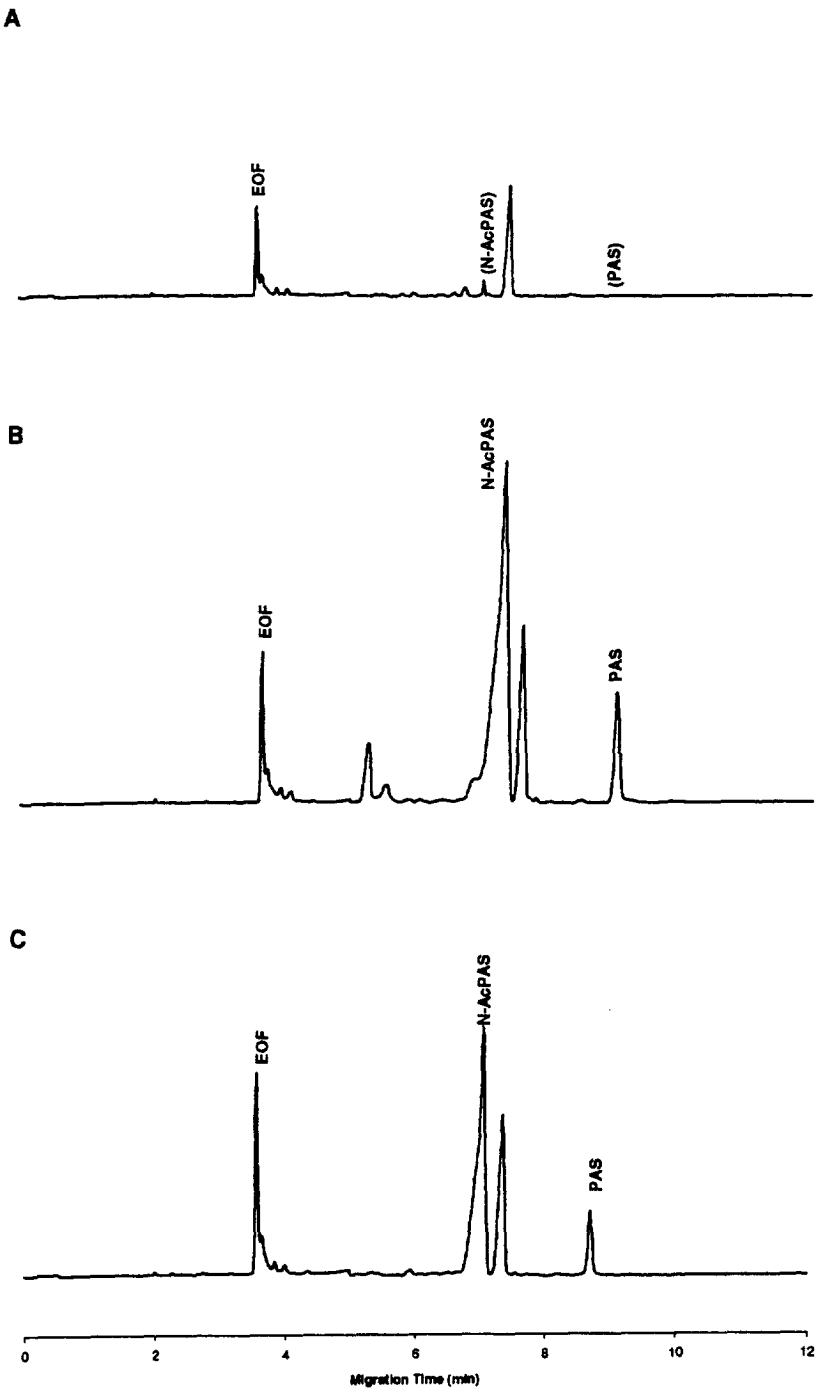

Fig. 2. CZE electropherograms of a patient's urine (A) before ingesting 1 g of PAS, (B) 0–2 h after PAS was taken. (C) Blank urine spiked with 100 and 1200 μg ml^{-1} of PAS and N-acetyl-PAS, respectively.

N-acetyl-PAS peak. Any subjects taking aspirin daily were excluded since the aspirin peak was not completely resolved in the 0–2 h electropherogram. No other potential interferents have been investigated.

3.2. Linearity

Calibration curves were made by plotting the peak area divided by the migration time against the concentrations of PAS and *N*-acetyl-PAS added to drug-free urine. Regression analysis of the least-squares line for the data shows a good straight line fit over the full concentration range for PAS and *N*-acetyl-PAS (R=0.9999 and 0.9990, respectively). The equation for the line for PAS was $Y=52.7(9)X+74(156)$, where Y represents the time-normalized peak area and X is the analyte concentration in μg ml^{-1}. For *N*-acetyl-PAS the equation for the calibration curve was $Y=36.5(9)X+1927(810)$. The intercept reflects the small interference seen in the blank (Fig. 2A). Each set of standards was run in duplicate as were the patient samples.

3.3. Precision of the method

The results of the intra- and inter-day variability studies at high and low concentrations of PAS (200 and 20 μg ml^{-1}) and *N*-acetyl-PAS (4000 and 200 μg ml^{-1}) are summarized in Table 1. The inter- and intra- day coefficients of variation were 2.9% or less.

3.4. Determination of phenotype

The phenotype determinations were made by measuring the molar ratios of *N*-acetyl-PAS to PAS. All subjects had molar ratios less than 20, except one which had a higher *N*-acetyl-PAS/PAS ratio of 32. The histogram of the results obtained from the twenty healthy volunteers is shown in Fig. 3. From this data, although preliminary, it appears a bimodal distribution occurs which would support the notion of NAT1 polymorphism. The antimode, calculated from probit analysis, was 10.2.

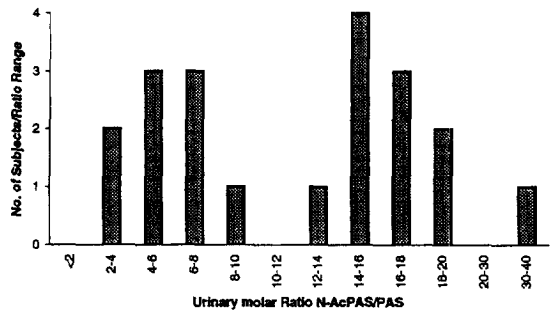

Fig. 3. Histogram of the distribution of NAT1 phenotypes among 18 normal individuals.

4. Conclusions

This investigation has shown that it is possible to phenotype patients for NAT1 activity in urine by CE with on-capillary UV absorbance at 305 nm using a sodium phosphate buffer (75 m*M*, pH 7.0). This method provides an approach for the determination of PAS and *N*-acetyl-PAS in human urine without any sample pretreatment. The speed, simplicity and non-invasive nature of the method would make it suitable for large-scale determination of NAT1 phenotypes.

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Table 1
Intra-day and Inter-day precision

Reproducibility (%C.V.)	PAS		*N*-Acetyl-PAS	
	Low concentration (20 μg ml^{-1})	High concentration (200 μg ml^{-1})	Low concentration (200 μg ml^{-1})	High concentration (4000 μg ml^{-1})
Intra-day (n=5)	1.3	2.9	2.1	2.0
Inter-day (n=3)	1.8	2.2	2.9	2.0

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