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Note

Ion-pair high-performance liquid chromatographic determination of isoniazid and acetylisoniazid in plasma and urine

Application for acetylator phenotyping

JAN-OLOF SVENSSON*, ARMEN MUCHTAR* and ÖRJAN ERICSSON

Department of Clinical Pharmacology, Huddinge Hospital, S-141 86 Huddinge (Sweden)

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Isoniazid can be used for the determination of acetylator phenotype either by measuring the plasma half-life of the drug or by determining the ratio of the metabolite, acetylisoniazid, to the parent drug [1]. Plasma concentrations of isoniazid have been measured spectrophotometrically [2] and the metabolic ratio has been determined in urine colorimetrically [3], in serum and urine with fluorometry [1], and in serum, plasma and urine with high-performance liquid chromatography (HPLC) [4—6]. In our laboratory we have previously used a spectrophotometric method [2] to determine the plasma half-life. This method is rather time-consuming, the sensitivity is in some instances inadequate, and it requires several blood samples from the patient. We have now developed a new method, based on ion-pair HPLC, with high sensitivity and simple purification and derivatization steps. It enables determination of acetylator phenotype with a single blood or urine sample, by measurement of the ratio of acetylisoniazid to isoniazid. The method is currently used both for phenotyping and for determination of isoniazid plasma concentrations.

EXPERIMENTAL

Materials

Isoniazid (INH) and isonicotinic acid were obtained from Ferrosan (Malmö,

*Present address: Department of Pharmacology, University of Indonesia, School of Medicine, Salemba 6, Jakarta, Indonesia.

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Sweden). Acetylisoniazid (AINH) solution was made from INH according to the method of Eidus et al. [3]. Isonicotinuric acid was synthesized from INH according to the method used by Rohrlich [7] for preparation of nicotinuric acid, and identified by mass spectrometry. Centrifree ultrafilters were obtained from Amicon (Danvers, MA, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). 1-Dodecyl sulphate (sodium salt) was chromatography-grade (Beckman, Berkeley, CA, U.S.A.). Acetonitrile was HPLC-grade. All other chemicals were analytical reagents. The water used was deionized.

Apparatus

The chromatographic equipment consisted of a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Model 7120 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 10-μl loop, an Ultrasphere ion-pair 150 × 4.6 mm I.D. reversed-phase column (5-μm particles; Beckman) and a Spectromonitor III variable-wavelength UV detector (Laboratory Data Control).

Chromatographic conditions

The eluent was a 10 m*M* sodium dihydrogen phosphate buffer (pH 3.0, adjusted with phosphoric acid) containing 1 m*M* dodecyl sulphate and 25% acetonitrile. The flow-rate was 1.5 ml/min. The temperature was ambient, and the detector wavelength was set at 270 nm.

Sample preparation

Plasma. The centrifree ultrafilters were filled with ca. 1 ml of plasma, and centrifuged for 30 min at 2600 *g* in a 35° rotor angle centrifuge. Then 100 μl of the ultrafiltrate were mixed with 10 μl of 25% phosphoric acid and 1 μl of propionic anhydride. After 10 min reaction time, 10 μl of the mixture were injected on to the column.

Urine. A 1.0-ml volume of urine was mixed with 3.0 ml of 0.1 *M* hydrochloric acid and 50 μl of propionic anhydride. After 10 min reaction time the mixture was passed through a Sep-Pak C_{18} cartridge (pretreated with 5 ml of methanol and 5 ml of water) at a flow-rate of 10—15 ml/min. The last millilitre of the eluate was collected, and 10 μl were injected on to the column.

Standard curves

Standard curves were obtained by analysis of plasma and urine spiked with INH and AINH. Peak areas (peak height multiplied by peak width at half height) were measured. The standard concentration ranges for INH were 3—150 μmol/l in plasma, and 12 μmol/l to 12 mmol/l in urine. The standard concentration ranges for AINH were: plasma, 2.85—142.5 μmol/l, and urine, 11.4 μmol/l to 11.4 mmol/l. We found linear relationships between peak areas and concentrations.

RESULTS AND DISCUSSION

Owing to severe tailing it was found necessary to derivatize INH before chro-

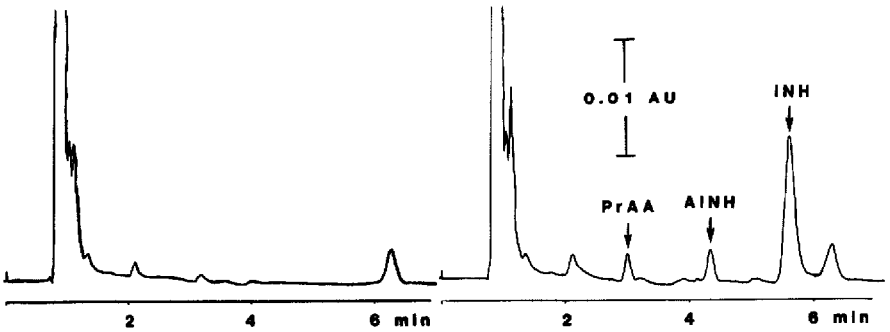

Fig. 1. Chromatograms from plasma before and 4 h after dosing. The peaks correspond to propionic anhydride (PrAA), 11.9 μmol/l acetylisoniazid (AINH), and 101 μmol/l (propionyl) isoniazid (INH).

matography. Preparation of the propionyl derivative, PrINH, considerably improved the peak shape and the sensitivity of the method.

Ultrafiltration of the plasma samples proved to give sufficient purification. INH has no protein binding [8]. Recovery in the ultrafiltrate is 100% for both INH and AINH. Ultrafiltration gives lower protein content (more than 99.9% are removed) than other deproteinization techniques, and there is no protein denaturation causing liberation of protein-bound substances. Phosphoric acid is added to the ultrafiltrate to give a suitable pH for chromatography.

The propionylation is complete after 10 min and the concentrations of AINH and PrINH are stable for at least 6 h. Unchanged propionic anhydride gives a peak in the chromatogram (Fig. 1). However, within 30 min the anhydride is completely hydrolysed and the peak disappears.

In urine, ca. two thirds of the INH is present in the form of acid-labile hydrazones [1, 9]. Eidus et al. [3] hydrolysed these hydrazones by adding 0.5 ml of 0.5 *M* hydrochloric acid to 1.0 ml of urine, and keeping it at room temperature for 15 min. We found that hydrolysis and derivatization could be done simultaneously, giving the same result as with pre-hydrolysis. The propionylation is complete after 10 min and the concentrations of AINH and PrINH are stable for at least 6 h. After propionylation of INH, the solution was cleaned by running it through a Sep-Pak C_{18} cartridge. As both AINH and PrINH are charged at this pH (ca. 1.0), they are very water-soluble and virtually unretained on the Sep-Pak. In the eluate from the Sep-Pak the concentrations of the two compounds have reached the concentrations in the incoming solution after 2.5 ml.

The detector wavelength was set at 270 nm, where both AINH and PrINH show absorption maxima.

The coefficients of variation were: for INH in plasma, 1.5% at 60 μmol/l (n = 11); for AINH in plasma, 0.7% at 57 μmol/l (n = 13); for INH in urine, 1.0% at 385 μmol/l (n = 5); and for AINH in urine, 1.1% at 2.19 mmol/l (n = 5). The minimum detectable concentrations were: for INH and AINH in plasma, ca. 1 μmol/l; in urine, ca. 4 μmol/l.

Thirty patients without severe renal disease have been tested for acetylation phenotype, both with the new HPLC method and with the spectrophotometric method [2]. Blood samples were drawn before the dose of INH (ca. 10 mg per

kg body weight, range 5—18.1 mg/kg), and after 2, 4, 6 and 8 h. A blank urine sample was taken before the dose, when the patient emptied the bladder, and then urine was collected 0—4 h after the dose.

Chromatograms from plasma before, and 4 h after a dose of INH are shown in Fig. 1. Chromatograms from urine collected before dosing and 0—4 h after dosing, are shown in Fig. 2. The INH metabolites isonicotinic acid and isonicotinuric acid gave peaks in the chromatograms from urine, whereas their concentrations in plasma were normally below the detection limit. No interfering peaks from concomitant drug therapy or endogenous compounds have been observed in plasma or urine.

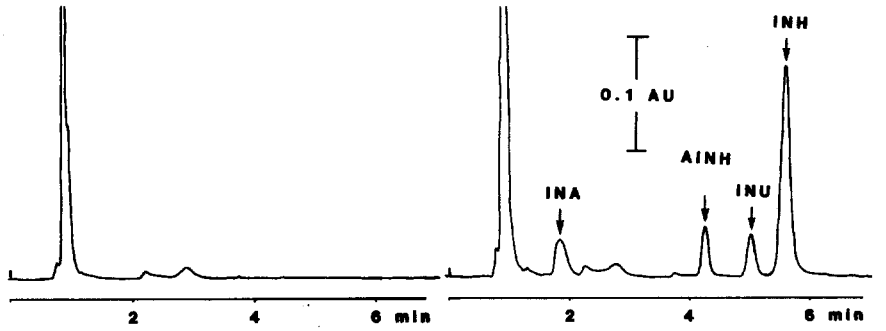

Fig. 2. Chromatograms from urine sampled before dosing and collected 0—4 h after dosing. The peaks correspond to isonicotinic acid (INA), 640 μmol/l acetylisoniazid (AINH), isonicotinuric acid (INU) and 4.33 mmol (propionyl) isoniazid (INH).

The INH concentrations in plasma varied from less than 1 to 160 μmol/l. The spectrophotometric and HPLC methods correlated well ($y = 1.01x - 0.63$). When the HPLC method was used, twenty-one patients were classified as slow acetylators ($t_{½} > 2.1$ h) and nine patients were classified as rapid acetylators ($t_{½} < 2.1$ h). The spectrophotometric method gave the same result, except that for two rapid acetylators the half-life could not be accurately determined because of low INH concentrations at 6 and 8 h (Table I). The AINH concentrations in plasma varied between 4.6 and 73 μmol/l.

Calculation of the ratio of AINH to INH in plasma at 2, 4, 6 or 8 h gave the same discrimination between the phenotypes as determination of the INH plasma half-life (Table I).

TABLE I

COMPARISON OF DIFFERENT METHODS FOR PHENOTYPING

Twenty-one patients were classified as slow acetylators and nine as rapid acetylators.

Phenotype	$t_{½}$ (h)		AINH/INH ratio				
	Spectr.	HPLC	Plasma				Urine
			2 h	4 h	6 h	8 h	0—4 h
Slow acetylators	2.5—5.4	2.4—5.8	0.058—0.22	0.10— 0.39	0.16— 0.58	0.22— 0.79	0.093—0.47
Rapid acetylators	1.2—2.0*	0.9—1.7	0.56 —2.0	1.7 —10.0	3.5 —19.5	5.7 —25.4**	0.94 —3.4

*Seven patients ($t_{½}$ was not measurable in two patients).
**Three patients (INH concentrations were below the detection limit in six patients).

In urine the INH concentrations varied between 185 μmol/l and 12 mmol/l and the AINH concentrations varied between 114 μmol/l and 9.3 mmol/l. Phenotyping by calculation of the ratio of AINH to INH in urine gave the same result as that determined from INH plasma half-life (Table I).

CONCLUSION

A method for simultaneous determination of isoniazid and acetylisoniazid in plasma and urine is described. It involves simple purification steps, with ultrafiltration of plasma, and removal of interfering matter in urine with a Sep-Pak C_{18} cartridge. Isoniazid is derivatized with propionic anhydride to propionylisoniazid. The compounds are separated on a reversed-phase HPLC column with ion-pair formation, using 1-dodecylsulphate as counter ion, and quantitated with a UV detector. The method has been used for determination of acetylator phenotype.

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