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Note

Hydrophilic ion-pair reversed-phase high-performance liquid chromatography for the simultaneous assay of isoniazid and acetylisoniazid in serum: a microscale procedure

M.A. MOULIN, F. ALBESSARD, J. LACOTTE and R. CAMSONNE*

Department of Pharmacology, University Hospital Center, 14033 Caen Cedex (France)

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Since acetyl isoniazid (AINH) generates stoichiometrically acetyl hydrazine which is a potent hepatotoxin [1], the simultaneous determination of serum isoniazid (INH) and AINH is of value in acetylator phenotyping and drug monitoring. Gas chromatographic assays for these compounds via their silylated [2] or hydrazone [3] derivatives as well as ion-pair high-performance liquid chromatographic (HPLC) assay [4] are tedious because of the large sample (3 ml) and reagent volumes involved. In this paper, a sensitive (0.5 mg/l) micro method for the simultaneous determination of serum INH and AINH using 0.5 ml of sample and hydrophilic ion-pair reversed-phase HPLC is described. Electron-impact (EI) and chemical-ionization (CI) mass spectrometry of the peaks of interest (INH, AINH) are used in assessing the selectivity of this assay. The total assay time (extraction and chromatography) is shortened from 90 to 30 min.

EXPERIMENTAL

Instrumentation

HPLC was performed on a model ALC/GPL 244 liquid chromatograph (Waters Assoc., Paris, France) equipped with a Model 440 UV (254 nm) detector and a column 30 cm × 3.9 mm packed with μBondapak C_{18} (10 μm particle size) (Waters Assoc.). Five per cent of methanol in 95% of 0.1 *M* KH_2PO_4 (pH 6.9) were used (degassed) as the optimal isocratic mobile phase with a flow-rate of 2 ml/min.

The mass spectrometry was performed on a Jeol 300 D EI—CI magnetic mass spectrometer using a Jama 2000 computer system (Jeol Europe, Rueil Malmaison, France) by direct probe insertion. In the ion source of the mass

spectrometer, AINH and INH volatilized at about 85°C and 110°C, respectively. In the CI mode, methane was used as reactant gas at a pressure of 1 bar.

Chemicals

Pure INH was obtained from Roche (Neuilly/Seine, France). AINH was from Sigma (St. Louis, MO, U.S.A.) and nicotinic amide (NA) from Lemat et Boinot (Paris, France). Other chemicals were of analytical reagent grade from E. Merck (Darmstadt, G.F.R.).

Assay procedure

To 500 μl of blank human serum spiked with 0.25, 0.5, 1.0, 2.5, 3.5 μg of INH or AINH, both in the form of freshly prepared aqueous solution (10 mg/l), or to 500 μl of serum sample, in a 10-ml screw-capped glass tube were added 2.5 μg of NA (aqueous solution of 100 mg/l) as internal standard (IS), 150 μl of 0.1 *M* sodium hydroxide and 0.5 g of solid ammonium sulfate in three small portions, followed by gentle shaking. The mixture was shaken for 5 min with 3 ml of chloroform and centrifuged at 520 *g* for 5 min. To 2—2.5 ml of organic phase in a 4-ml tapered tube were added 200 μl of 0.05 *M* sulfuric acid. The mixture was shaken for 5 min then centrifuged; 30 μl of clear supernatant were injected into the chromatograph.

In the selectivity study, eluted INH and AINH peaks from the HPLC of samples from patients given INH + ethambutol + rifampin were collected, extracted as previously described, and 100 μl of the chloroform phase were transferred into the capillary quartz tube then evaporated for direct insertion into the mass spectrometer.

RESULTS AND DISCUSSION

Extraction

Due to their polar nature, INH, AINH and NA were extracted on the basis of ion-suppression according to the satisfactory method of Saxena et al. [4] using sodium hydroxide and solid ammonium sulfate, which also precipitate plasma proteins, except with the use of chloroform alone as organic solvent. Once extracted on the day of sampling, the extract can be stored at 4°C before chromatography without significant loss for two weeks. Under these conditions recoveries and coefficients of variation (CV) at concentration levels of 0.5—7.0 mg/l INH and AINH were 101 ± 4% and 98 ± 5%, respectively. Otherwise, only 75 ± 18% recovery of INH was obtained if the serum samples were kept for longer than 24 h, even at 4°C, before the extraction step, while the recovery of AINH remained unchanged. The reason for this erratic loss is not clear. Indeed, if INH can be generated from its labile hydrazone as previously quoted [5], the serum INH after storage should be rather higher.

Chromatograms and the results of a patient sample

In the chromatographic step, 0.1 *M* phosphate was used as a hydrophilic counter-ion in an optimalized pH 6.9 condition. All peaks were well resolved within 7 min (Fig. 1). Retention times (t_R) were 3.3 ± 0.2, 3.9 ± 0.3, 5.8 ± 0.3

min for AINH (I), INH (II) and NA (III), respectively. HPLC elution profiles of AINH and INH in the serum of a patient 2 h (Fig. 1a) and 4.5 h (Fig. 1b) after a 5 mg/kg oral dose of INH are shown for comparison. The large "lump" after the elution of III in fig. 1b was regularly present and is due to serum components; the t_R of this, as in the chromatogram of blank serum (not shown), was 19.8 ± 0.3 min. The data for this example are summarized in Table I.

The elimination half-life of INH from serum was 2.8 h. Depending upon both phenotype and physiopathological factors, these data vary largely from one patient to another and suggest a measure of individual kinetic parameters of INH and their use in the calculation of dose regimen.

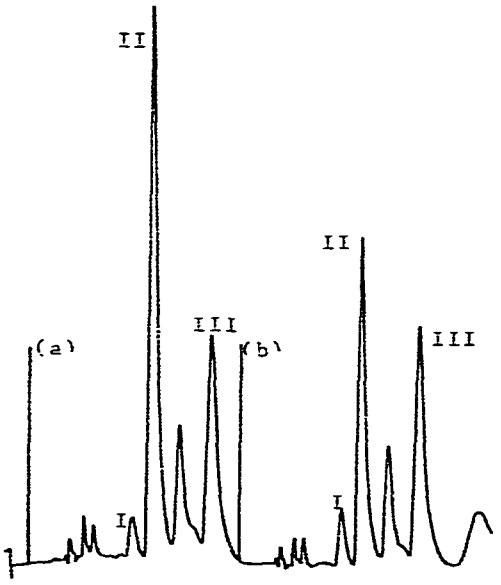

Fig. 1. Elution profiles of AINH (I) and INH (II) from patient serum at 2 h (a) and 4.5 h (b) after an oral dose of INH, using NA (III) as internal standard.

TABLE I

SERUM AINH AND INH CONCENTRATIONS IN A PATIENT AFTER AN ORAL DOSE OF INH

See text for experimental details.

Sampling time after dosing (h)	INH (mg/l)	AINH (mg/l)	AINH/INH ratio
2	7.4	1.4	0.189
4.5	4.0	2.0	0.50

Standard curve

The calibration curves [i.e. peak height ratios of INH/IS and AINH/IS versus appropriate amounts (see assay procedure) of drug and AINH in the therapeutic concentration range (1–3 mg/l) of INH in serum] were linear: for INH = $0.693x - 0.04$ ($r = 1$), and for AINH = $0.226x + 0.04$ ($r = 0.977$). Minimal detectable amounts calculated as greater than two standard deviations from zero dose of INH and AINH were 0.3 ± 0.15 and 0.25 ± 0.1 mg/l. The coefficient of variation for within-run and between-run of INH and AINH were in the same range, 6 ± 0.5% and 8 ± 0.7%, respectively.

Mass spectra

Typical mass spectra of peaks I and II obtained from one patient exceptionally treated with the three first-line anti-tuberculosis drugs INH + ethambutol + rifampin (in which serum AINH must be closely monitored because of intrinsic hepatotoxicity and microsomal enzymatic induction of rifampin) are shown in Fig. 2. The EI mass spectrum of peak I (Fig. 2a)

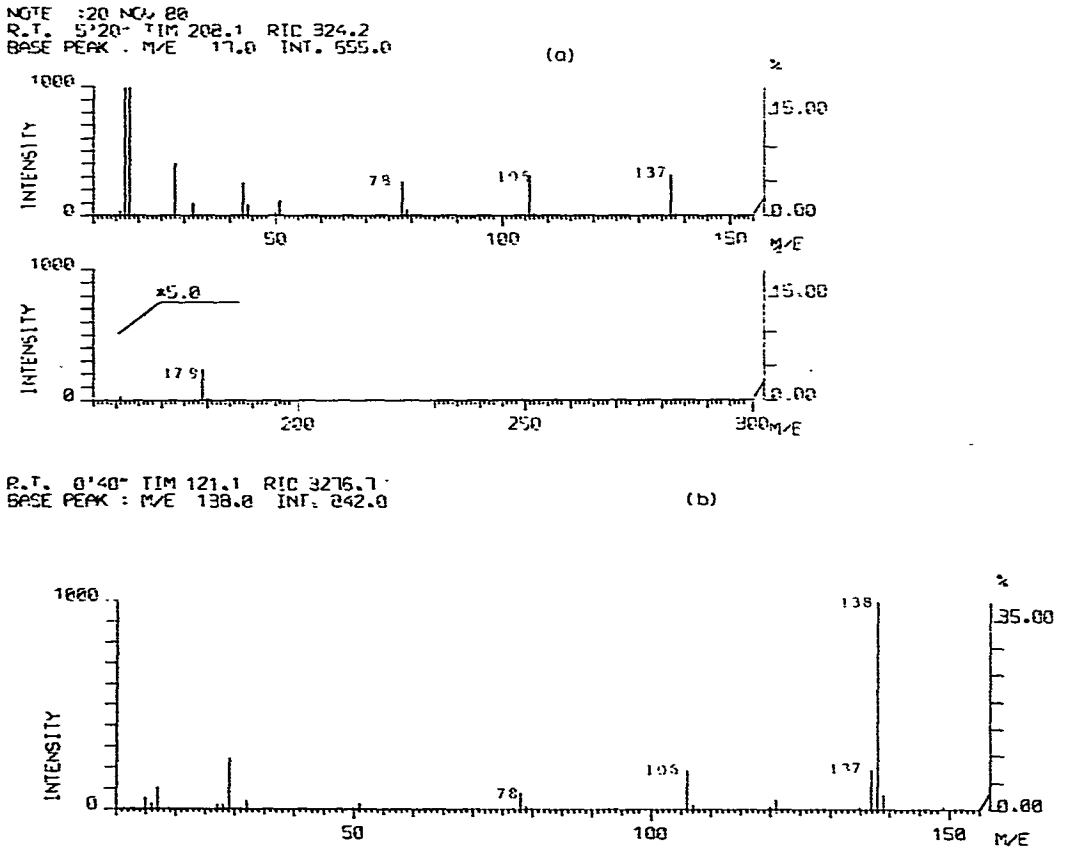

Fig. 2. Typical EI mass spectrum of peak I (a) and methane CI mass spectrum of peak II (b) eluted and extracted from the serum of a patient given INH + ethambutol + rifampin. The spectra are consistent with those of pure AINH and INH, respectively.

containing a molecular ion (M^+) at *m/e* 179 and ions 137 (INH), 106, 78, 51 and 43, was consistent with pure AINH. The identification was easier with CI because of minimal sample ion fragmentations [6] as shown for peak II (Fig. 2b). This methane CI mass spectrum presented a quasi-molecular ion (M+1) at *m/e* 138 (base peak) and 137, 106 and 78 as prominent ions; this was consistent with pure INH. The typical ion M + 29 due to methane CI was small but visible. This indicated lack of intereference in the assay by ethambutol, rifampin, their metabolites, and serum components.

This convenient and rapid assay has been tested for eight months in our laboratory and is of great interest in the routine profiling of serum INH and AINH at therapeutic levels of INH for patients with a high risk of developing hepatitis [7]. Most interesting is the fact that this method might easily be suitable if needed to quantitate INH and AINH in urine.

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