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Short communication

Therapeutic isoniazid monitoring using a simple high-performance liquid chromatographic method with ultraviolet detection

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Abstract

Simultaneous measurement of isoniazid and its main acetylated metabolite acetylisoniazid in human plasma is realized by high-performance liquid chromatography. The technique used is evaluated by a factorial design of validation that proved to be convenient for routine drug monitoring. Plasma samples are deproteinized by trichloroacetic acid and then the analytes are separated on a μBondapak C_{18} column (Waters). Nicotinamide is used as an internal standard. The mobile phase is 0.05 *M* ammonium acetate buffer (pH 6)–acetonitrile (99:1, v/v). The detection is by ultraviolet absorbance at 275 nm. The validation, using the factorial design allows one to: (a) test the systematic factors of bias (linearity and matrix effect); (b) estimate the relative standard deviations (RSDs) related to extraction, measure and sessions assay. The linearity is confirmed to be within a range of 0.5 to 8 μg/ml of isoniazid and 1 to 16 μg/ml of acetylisoniazid. This method shows a good repeatability for both extraction and measurement (RSD INH=3.54% and 3.32%; RSD Ac.INH=0.00% and 5.97%), as well as a good intermediate precision (RSD INH=7.96%; RSD Ac.INH=15.86%). The method is also selective in cases of polytherapy as many drugs are associated (rifampicin, ethambutol, pyrazinamide, streptomycin). The matrix effect (plasma vs. water) is negligible for INH (3%), but statistically significant for Ac.INH (11%). The application of this validation design gave us the possibility to set up an easy and suitable method for INH therapeutic monitoring. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Isoniazid (INH, isonicotinic acid hydrazide) is the drug most widely used in the treatment of tuberculosis. Isoniazid is mainly metabolized to acetylisoniazid (Ac.INH) by *N*-acetyltransferase type 2 in the liver. Ac.INH is devoid of antituberculosis activity. The rate of INH acetylation is controlled by

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genetic polymorphism, thus all populations studied show a bimodal distribution of the acetylation capacity and individuals can be classified as either slow or fast acetylators [1–9].

The acetylation polymorphism is associated with a large interindividual variability in both plasma concentration and half life of INH. Clinical consequences are more important for slow acetylators who are prone to develop major side effects such as peripheral neuropathy and systemic lupus erythematosus, whereas fast acetylators may undergo therapeutic failure. Published data suggest that the therapeutic plasma concentration of INH should range from 1 to 2 μg/ml 3 h after the oral administration to be considered efficient [10]. The method of Vivien et al. [10] used for the determination of the therapeutic dosage required to obtain the efficient INH concentration is based on the linear correlation between the plasma concentration of INH 3 h after the administration of the drug and the dose.

It appears from these findings that the measurement of INH and Ac.INH plasma concentrations is necessary for the determination of the acetylator phenotype and INH dosing adjustment. This allows to one restore therapeutic efficiency and minimize the side effect risks. Moreover, measuring Ac.INH plasma levels allows to one ascertain that the most excessive fast acetylators have taken INH medication.

Several methods have been described to measure the INH and its acetylated metabolite (Ac.INH) in plasma, urine and serum. Non chromatographic methods, such as colorimetry, spectrophotometry, and spectrofluorimetry, lack specificity [11–13]. Gas chromatography methods involve long times and high cost of analysis [14].

Several high-performance liquid chromatography (HPLC) methods using ultraviolet or fluorimetric detection have been proposed to allow simultaneous measurement of INH and Ac.INH in biological fluids. These methods always require either sample purification with organic solvent [8,15], derivatization after sample purification [16–21] or liquid–solid extraction through an in-line column [22].

In this study we describe a HPLC method for the simultaneous measurement of INH and its acetylated metabolite (Ac.INH) in plasma based on trichloroacetic deproteinization samples. Quality evaluation involved factorial validation design which was developed in previous studies, particularly on methotrexate, itraconazole, fluconazole and clozapine [23–26]. The validation results showed that this method is selective and sensitive enough to measure the relatively low concentrations of INH and Ac.INH in plasma. The factorial design allowed us to check some main validation parameters [27,28] including linearity, matrix effect, repeatability and intersession precision.

2. Experimental

2.1. Reagents

Isoniazid and its main acetylated metabolite (Ac.INH) were provided by F. Hofman-La Roche (Bâle, Switzerland). Nicotinamide used as chromatographic internal standard (I.S.), was obtained from Merck (Darmstadt, Germany). Human plasma was provided by the Transfusion Center in Rabat, Morocco.

Methanol and water were LiChrosolv grade and acetonitrile was HPLC grade. They were provided, along with trichloroacetic acid, by Merck. Glacial acetic acid was from BDH (Poole, UK).

The buffers consisted of 0.05 and 0.5 *M* acetic acid in water adjusted to pH 6 and 8.2, respectively, using ammonia. Stock solutions of analytes were prepared at a concentration of 1 g/l in methanol and stored at +4°C. The working solution contains 100 μg/ml of INH and 200 μg/ml of Ac.INH. From the last solution five calibration standards of both INH and Ac.INH were prepared in water or in human plasma using a serial dilution with geometric steps of 2 (INH: 0.5–1–2–4 and 8 μg/ml; Ac.INH: 1–2–4–8 and 16 μg/ml).

2.2. Apparatus

The HPLC system was composed of a pump Model L-6200, a variable-wavelength UV–Vis spectrophotometric detector Model L-4200, a chromatointegrator Model D-2500 and a Rheodyne loop injector (50 μl), all from Merck–Hitachi (Tokyo, Japan). The column was a μBondapak C_{18}

(300×3.9 mm) from Waters (Laboratory Instruments Network, Villers-les-Nancy, France).

2.3. Chromatographic conditions

The isocratic mobile phase was a mixture of ammonium acetate buffer (0.05 *M*, pH 6)–acetonitrile (99:1, v/v). The elution was done at room temperature with a flow-rate of 1.2 ml/min (pressure=90 bar). The analysis was performed at 275 nm with a range of 0.02 AUFS.

2.4. Sample processing

To 500 μl samples of plasma or aqueous calibration solutions, we added 250 μl of the deproteinization solution which consisted of 15 μg/ml nicotinamide in 10% trichloroacetic acid. The mixture was vigorously vortex-mixed for 1 min and then centrifuged for 10 min at 3000 *g*. The trichloroacetic supernatant was withdrawn and then was half diluted with the ammonium acetate buffer (0.5 *M*, pH 8.20) in order to neutralize the excess trichloroacetic acid.

2.5. Validation design and analysis of variance

The considered validation design illustrated by Fig. 1 was previously described as a mixed factorial and nested experimental design [23–26,29]. It involved three crossed factors which are drug concentrations (fixed, five levels), sample matrix (fixed, two levels: water and plasma) and assay session repetition (random, three levels). According to the nested intra-cell factor, the calibration standards were in duplicate and each of them was measured twice, in order to estimate separately the variance from sample processing and from chromatographic measurement.

The details of analysis of variance (ANOVA)

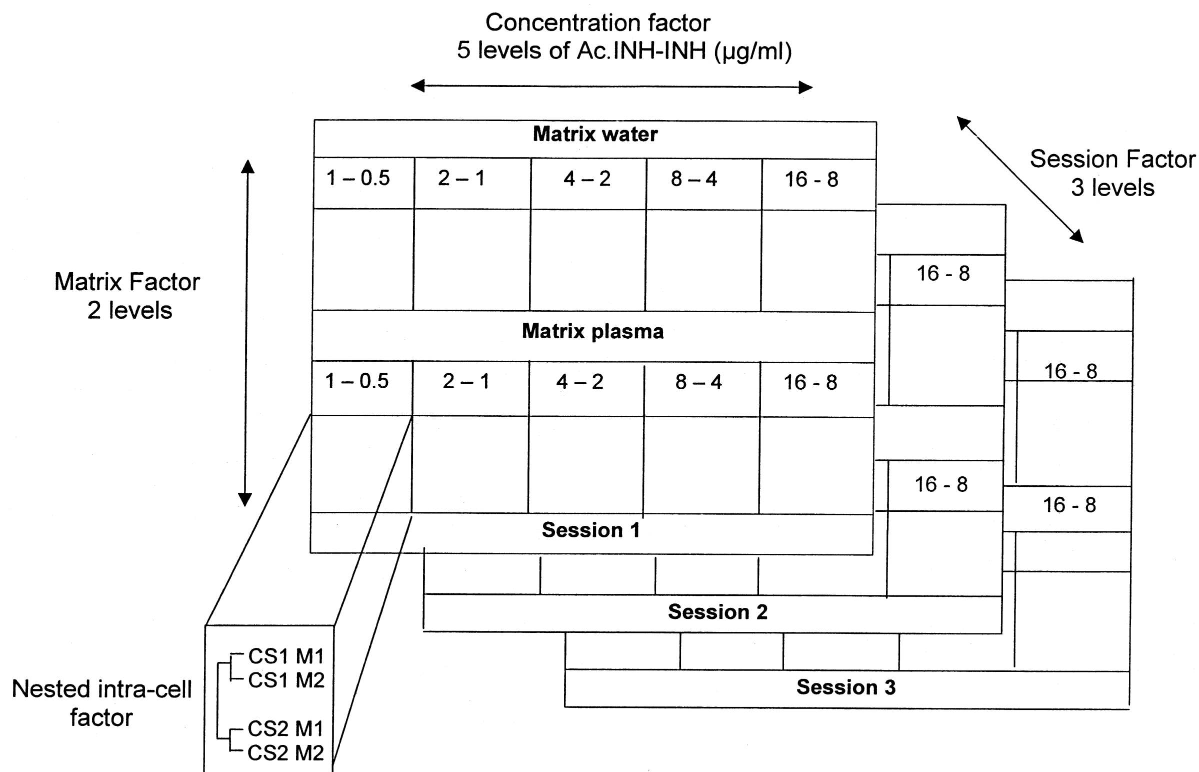

Fig. 1. Structure of the validation design. The insert shows the nested intra-cell factor as explained in the text. CS: Calibration standard. M: Measure.

calculation and statistical tests used in validation design are reported in previous reviews [23–26,29]. Briefly, ANOVA was performed on napierian logarithmic transforms of concentrations and of peak height relative to the I.S. The precision, linearity and matrix effect of the method were tested for significance by Snedecor's F-test, and the deviation of slope from 1 (analytical non-linearity) by a Student's t-test.

3. Results and discussion

3.1. Validation results

3.1.1. Selectivity

Fig. 2I and II shows chromatograms obtained from water and plasma samples spiked with INH and Ac.INH at the lowest and highest concentrations. Fig. 2III shows a typical chromatogram of blank plasma. Fig. 2IV shows chromatogram of plasma sample from a tuberculosis patient taking INH and pyrazinamide.

INH, Ac.INH and nicotinamide are, respectively, well separated at 7.8, 9.7 and 13.9 min. The small percentage of acetonitrile (1%) in the mobile phase offered an optimum separation and avoids a potential interference between the peak of Ac.INH and the solvent front peak. No interfering endogenous substance was observed at the retention times of these compounds, and none of the following drugs of comedication interfered with INH, Ac.INH or nicotinamide: rifampicin, pyrazinamide, ethambutol and streptomycin. The retention time of pyrazinamide was 11.8 min and did not hinder the INH and Ac.INH determination.

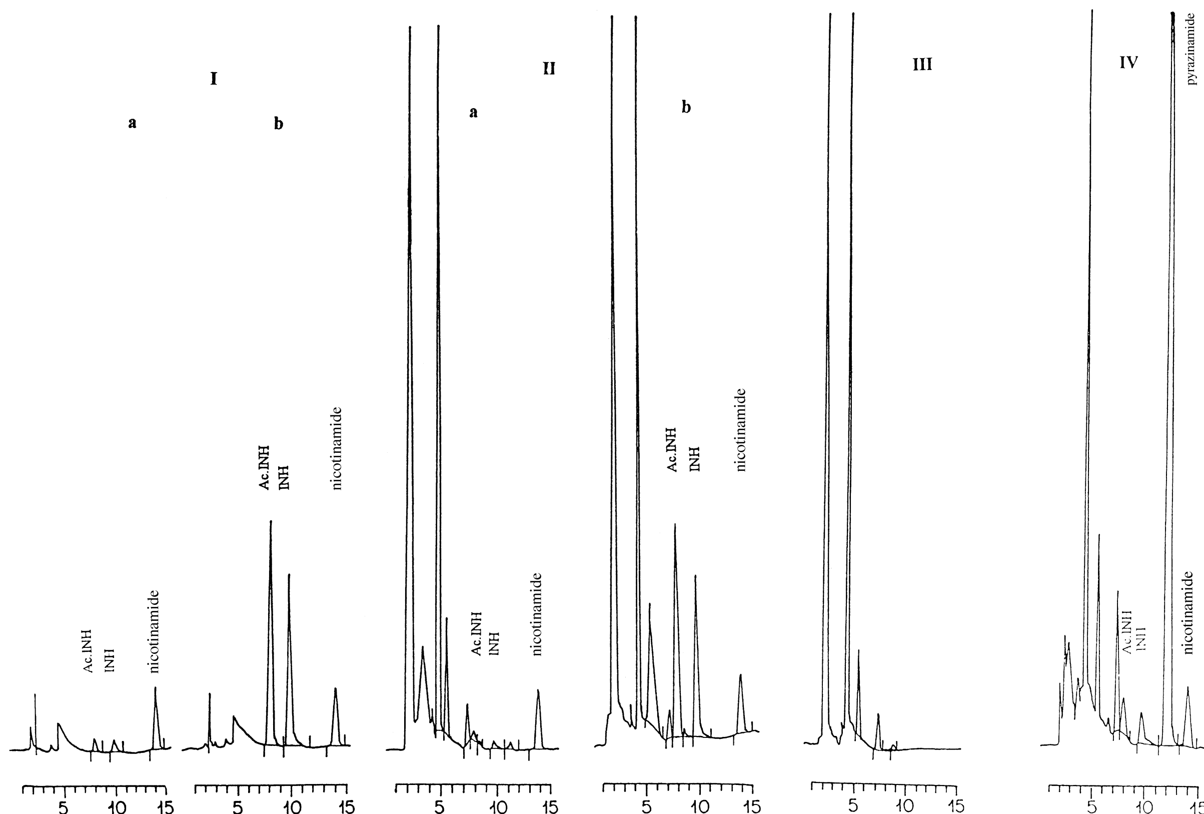

Fig. 2. Chromatograms of water (I) and plasma (II) spiked with 1 μg/ml of Ac.INH and 0.5 μg/ml of INH (a), 16 μg/ml of Ac.INH and 8 μg/ml of INH (b); blank plasma (III); plasma sample from patient (No. 4) administered 250 mg INH/day and 500 mg pyrazinamide/day.

Table 1
Main validation characteristics of the HPLC assay of INH and Ac.INH

Validation characteristics	Isoniazid				Acetylisoniazid			
		RSD (CV, %)	test	P		RSD (CV, %)	test	P
Analytical linearity								
Regression slope	1.03				1.07			
Slope test			t=7.60	+++			t=6.08	+++
Curvature test			F=1.04	NS			F=9.84	+
Sigmoidicity test			F=0.25	NS			F=0.03	NS
Matrix effect								
Plasma/water (%)	97.80				89.65			
Intermatrix test			F=3.00	NS			F=36.67	+++
Within-session precision								
RSD (%) measure		3.32				5.97		
RSD (%) sample processing		3.54	F=3.26	+++		0.00	F=0.98	NS
Between-session RSD (%) (measure+sample processing)		7.96	F=3.27	NS		15.86	F=7.11	++

Plasma/water (%): percent ratio of measures from plasma and from water sample. P: Significance level of t-tests (probability of slope being different from 1) and F-test. +++: $P<0.001$; ++: $0.001<P<0.01$; +: $0.01<P<0.05$; NS: $0.05<P$.

3.1.2. Linearity

The results summarized in Table 1 show that the bilogarithmic regression slope of INH was significantly greater than 1 by less than 5% ($P<0.001$). Neither the curvature nor the sigmoidicity were significant. So the peak height relative to I.S. measurements may be considered proportional to the concentrations, which agrees with a linear model without intercept.

The regression slope of Ac.INH was significantly greater than 1 by less than 10% ($P<0.001$). The curvature was significant ($0.01<P<0.025$) but the sigmoidicity was not (Table 1). These results indicate that the response function is linear with an intercept.

3.1.3. Matrix effect

The percent ratios of plasma vs. water concentration were 97.80% for INH and 89.65% for Ac.INH (Table 1). The effect of plasma components on sample processing was then negligible for INH (3%; $0.1<P<0.25$) but significantly important for Ac.INH (11%; $P<0.0005$). This can be ascribed to retention of Ac.INH on the protein precipitate during the trichloroacetic acid deproteinization. Ac.INH, more lipophilic than INH, appears to be much more retained. Consequently, simultaneous measurement of INH and Ac.INH require calibration in plasma. Calibration in water would give an 11% underestimation of Ac.INH plasma concentrations in the patients.

3.1.4. Within-session repeatability

The overall variation coefficients of INH and Ac.INH were, respectively, 3.32 and 5.97% for the measurement, 3.54 and 0.00% for the sample processing (Table 1). The variability introduced by trichloroacetic acid deproteinization, as a method of sample processing, was significant for INH ($P<0.0005$) but not for Ac.INH ($P>0.1$).

These results show a good repeatability of the assay in both sample processing and chromatographic measurement.

3.1.5. Intersession precision

The between-session coefficients of variation were 7.96% for INH and 15.85% for Ac.INH (Table 1), indicating that the intersession precision was relatively better for INH than for Ac.INH.

3.2. Application to patients data

The analytical method described earlier was used for the simultaneous determination of plasma concentrations of INH and its acetylated metabolite in 13 tuberculosis patients. Blood samples were taken 3 h after the administration of a single oral dose of INH (D=5.2±0.3 mg/kg body mass) to fasting subjects.

The plasma concentrations were considerably different, ranging from 0.84 to 5.34 μg/ml of INH (C_3INH) and from 1.08 to 7.41 μg/ml of Ac.INH (C_3Ac.NH) (Table 2). Only six patients had an INH concentration within the efficient range of 1 to 2 μg/ml. For the seven other patients, we adjusted the INH dose using the inactivation index of INH I_3= (C_3INH+0.6)/D (Vivien's method) [8–10]. The administered dose had to be decreased in six patients (mean±SD: 2.34±0.51 mg/kg) and increased in one subject (8 mg/kg).

The measurement of C_3INH and C_3Ac.INH also allowed calculation of the metabolic ratio Rm= C_3Ac.INH/C_3INH, which has been widely used for the determination of the acetylator phenotype [1–9]. The calculated Rm suggest different acetylator phenotype between patients (Table 2). Nevertheless, the lack of antimode that characterizes the Moroccan population limits the classification of the individuals as slow or fast acetylators. The extrapolation from known population data was difficult because of ethnic variation. Studies in Mediterranean populations showed that the slow acetylator phenotype was predominant. The proportions obtained were 83% in Egypt [30], 66.3% in Greece [31], 65.4% in Span [32] and 22.8% in Tunis [33]. Few data were available before this date in Morocco. A further study on acetylator phenotyping will be necessary to explore the metabolic acetylation in this population.

4. Conclusion

The HPLC method proposed here proved to be convenient for the simultaneous measurement of INH and its acetylated metabolite Ac.INH. The validation design allows one to test the systematic factor of bias, particularly linearity and matrix effect, and to estimate the RSD related to extraction, measure and sessions assay. The results obtained showed a good linearity as well as a good within and between session precision for both INH and Ac.INH. Although the matrix effect is negligible for INH, the calibration must be done in plasma in order to give a reliable estimation of analytes particularly Ac.INH in the patients.

The deproteinization with trichloroacetic acid gives an easy, rapid and convenient separation of analytes from plasma sample. Previously published method based on the trichloroacetic acid deproteinization usually included a derivatization step, and the

Table 2
Plasma concentrations of INH and Ac.INH, inactivation index (I_3) and ratio of Ac.INH to INH (Rm) at 3 h after INH administration to 13 tuberculosis patients

Patient No.	INH dose (mg/kg of body mass)	Concentration (μg/ml)		Inactivation index of INH (I_3)	Rm
		C_3INH	*C_3Ac.INH*		
1	5.45	0.84	6.25	0.26	7.44
2	5.45	1.11	2.63	0.31	2.37
3	5.17	1.64	4.83	0.43	2.94
4	5.38	1.70	2.92	0.46	1.72
5	5	1.83	4.11	0.45	2.24
6	5.43	1.91	4.41	0.50	2.31
7	5.68	2.07	4.33	0.49	2.09
8	4.76	3.11	1.08	0.65	0.35
9	4.90	3.36	7.41	0.83	2.20
10	5.38	4.25	1.55	0.99	0.36
11	5	4.26	1.77	0.90	0.41
12	4.86	4.33	1.82	0.99	0.42
13	4.86	5.34	1.58	1.22	0.29

measurement of Ac.INH was done after conversion to INH by hydrolysis [17,18]. This method is sufficiently robust for therapeutic INH monitoring, and could be used to explore the interindividual variability of INH acetylation in the Moroccan population.

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