Solid-Phase Extraction and HPTLC Determination of Isoniazid and Acetylisoniazid in Serum. Comparison with HPLC

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Abstract

A new solid-phase extraction (SPE) and quantitative determination of isoniazid (INH) and its acetyl metabolite (AcINH) in serum by high-performance thin-layer chromatography (HPTLC) is presented. Alkalized serum samples with nicotinamide as an internal standard are applied to an SPE cartridge containing a new SPE sorbent, [poly (divinylbenzene-co-*N*-vinylpyrrolidone)]. A simple procedure of conditioning, washing, and eluting steps is described. After evaporation of the eluates to dryness and reconstitution, one-dimensional HPTLC is performed on silica gel plates with ethyl acetate–methanol (70:30) as a mobile phase. Quantitation is done by densitometry. Convenient validation parameters are obtained (linearity, limits of detection and quantitation, precision, and accuracy) for INH and AcINH. The method is compared with a high-performance liquid chromatographic (HPLC) technique developed in the laboratory, and satisfactory correlation is found between data from the two techniques. The HPTLC method is sensitive and specific and is used to quantitate INH and AcINH in patient blood serum, and the results are compared with those obtained by HPLC.

Introduction

Isoniazid (INH) (Figure 1) is administrated with rifampicine, ethambutol, and pyrazinamide in tuberculosis treatment. It is metabolized by hepatic *N*-acetyltransferase to acetylisoniazid (AcINH), and the ratio AcINH–INH defines phenotypes into slow and rapid acetylors (1,2). Secondary effects of INH and its metabolites are detailed in the literature, depending of the acetylor phenotype (3,4). The therapeutic effectiveness and side effects of INH must then be related to its seric concentration. A number of analytical methods are described in the literature including bromometry (5), nonaqueous titrimetry (6), spectrophotometry (7–9), chemiluminescence (10), fluorimetry (11–13), colorimetry (14,15), gas chromatography (GC) (16), and GC–mass spectrometry (17). Since their introduction, high-performance liquid chromatography (HPLC) methods have become more used (18–26). We developed a high-performance thin-layer chromatography (HPTLC) of INH and AcINH with liquid–liquid extraction (27). In this study, a new solid-phase extraction (SPE) combined with HPTLC is used to determine INH and its acetyl metabolite (AcINH) in serum. The results were compared with an HPLC technique.

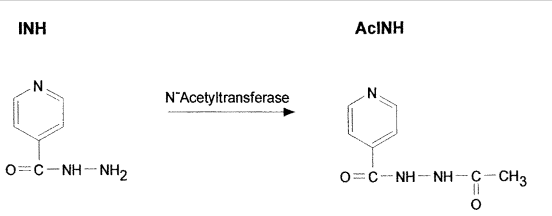

Figure 1. Biotransformation of INH to AcINH.

Experimental

Reagents

All solvents (spectroscopic grade) used were from Fluka (Buchs, Switzerland). INH and nicotinamide used as internal standard were from Hoffmann-LaRoche (Basel, Switzerland). AcINH was prepared and identified as described previously (23).

Apparatus

A Waters chromatograph with a 600E pump (Milford, MA), 7625i Rheodyne injector with 20-µL sample loop (Rohnert Park, CA), and a Waters diode array detector 991 were used. Separations were carried out on a Novapak C_{18} (150 × 4.6 mm) from Waters, preceded with a C_{18} homemade guard column (2 × 0.4 cm). The mobile phase was: 0.1M phosphate buffer (pH 7)–methanol (97.5:2.5, v/v). The flow rate was fixed to 1 mL/min. Data were collected with a Millenium 32 program (Waters). Quantitation was made at 254 nm.

Thin-layer chromatography (TLC) was performed on 10- × 10-cm HPTLC plates precoated with silicagel 60 (layer thickness, 200 µm) (Merck ref. 5631). Samples were applied to the plate by means of a Camag microapplicator (Muttenz, Switzerland), 8 mm from the lower edge of the plate and 10 mm apart, starting 10 mm from the side of the plate; the distance between zones was 10 mm. Development was carried out in a linear developing chamber (Camag) and quantitated by densitometry with a TLC/HPTLC scanner (Camag). The running distance was fixed to 8 cm, and the temperature was ambient. The measurement wavelength was 254 nm, the slit dimension 1 × 0.1 mm, and resolution 0.1 mm. Peak areas were used for quantitation. Plates were developed with ethyl acetate–methanol (70:30, v/v).

Extraction of INH and AcINH from serum

SPE of the samples was carried on a poly(divinylbenzene-co-*N*-vinylpyrrolidone) cartridge (Oasis HLB, 60 mg, Waters). First, conditioning was made by flushing the cartridge with 1 mL methanol, 1 mL acetonitrile, and 1 mL 0.05M phosphate buffer (pH 4.5). Then, 0.5 mL serum containing nicotinamide (5 mg/L) was applied by allowing it to pass through the bed with minimal suction, the cartridge washed with 1 mL 0.05M phosphate buffer (pH, 4.5), and suctioned dry. Elution was made with 1 mL methanol–acetonitrile (1:1, v/v). The sample was then evaporated to dryness under nitrogen at 30°C and reconstituted with 100 µL methanol. One microliter was plotted to the HPTLC plate, and 20 µL was injected into the HPLC chromatograph.

Calibration

Calibration was accomplished using the following procedure. Ten 0.5-mL of blank sera was used, to which various INH and AcINH concentrations (0.5–10 mg/L) and a constant nicotinamide concentration (5 mg/L) were added. The samples were deproteinized, extracted, and analyzed as described previously (the Extraction of INH and AcINH from serum subsection).

Limits of detection and quantitation

The detection limit (LOD) was estimated as the drug amount in serum that corresponded to four times the baseline noise. The limit of quantitation (LOQ) was determined as the lowest concentration of the calibration curve.

Precision and accuracy

For spiked serum with different amounts of isoniazide, acetylisoniazide, and internal standard, the interrun precision of the selected method was estimated by calculating the coefficient of variation (CV) of the concentrations measured at different days (n = 6); the intrarun precision was determined the same way on the same day (n = 10). The accuracy was calculated as %bias.

Table I. HPTLC and HPLC Parameters of INH and AcINH*

		HPTLC		HPLC	
		Nicotinamide (R_f = 0.6)		Nicotinamide (k' = 2.71)	
		INH	AcINH	INH	AcINH
Plotted or injected volume (mL)		1	1	20	20
Retention factor		0.35	0.5		
Capacity factor				1.60	2.40
Calibration curve	slope	0.170	0.151	0.113	0.101
	y intercept	0.018	0.013	0.009	0.010
	correlation coefficient	0.990	0.991	0.990	0.991
	applied quantities (ng)	0.5–10	0.5–10	10–200	10–200
	concentration range (mg/L)	0.5–10	0.5–10	0.5–10	0.5–10
LOD	applied quantity (ng)	0.15	0.2	0.4	0.5
	concentration (mg/L)	0.15	0.2	0.02	0.025

* See experimental conditions in the text.

Table II. Precision, Accuracy, and Recovery of INH and AcINH in Spiked Serum

	Concentration (µg/mL) (n = 3)	Recovery (%)	Bias (%)		RSD (%)	
			Within run	Between run	Within run	Between run
INH	0.5	87.2 ± 4.9	94.1	94.3	6.4	6.7
	2	94.7 ± 3.0	94.5	95.9	4.3	4.3
	10	96.1 ± 4.0	95.9	97.0	4.1	4.8
AcINH	0.25	87.2 ± 4.9	95.2	94.8	6.8	6.4
	1	88.3 ± 4.0	93.3	94.9	5.2	5.2
	5	93.7 ± 4.1	94.9	98.0	5.0	4.6

Selectivity and specificity

To evaluate possible endogenous interferences, five blank sera were analyzed with the proposed procedure.

Results and Discussion

Calibration graph

Retention factors of isoniazide, acetylisoniazide, and nicotinamide are given in Table I. Calibration curves were obtained by least-squares linear regression analysis of the peak area ratio y of analyte–internal standard versus analyte concentration x. The method was linear in the range 0.5–10 mg/L for INH and AcINH; their corresponding equations are given in Table I.

Recovery

The recoveries of INH and AcINH from spiked serum samples were calculated by comparing peak areas at low, medium, and high concentration levels with those obtained from the analysis of corresponding standard dilutions in methanol plotted directly to the HPTLC plate. The corresponding values are shown in Table I, which indicates a satisfying recovery of INH and AcINH in the concentration range studied.

Precision and accuracy

Table II summarizes the within- and between-run precision and accuracy for the determination of INH and AcINH in spiked serum, as described in

the Experimental section. Within- and between-run percent relative standard deviations (%RSD) (precision) by all the methods at low concentration were less than 7% and 5% at high concentration. The accuracy of the method expressed as %bias for within run, and between run were less than 93% at low and high concentrations.

Selectivity and specificity

Using the HPTLC chromatographic conditions, the other antituberculosis drugs did not interfere. Rifampicine and pyrazinamide retention factors were, respectively, 1 and 0.86. No endogenous or extraneous peaks were observed interfering with the assay of isoniazide.

LOD and LOQ

LOD at s/n = 4 and the LOQ, the lowest concentration of the calibration curve, are given in Table I.

Application of method

Several serum samples were analyzed by HPTLC and HPLC.

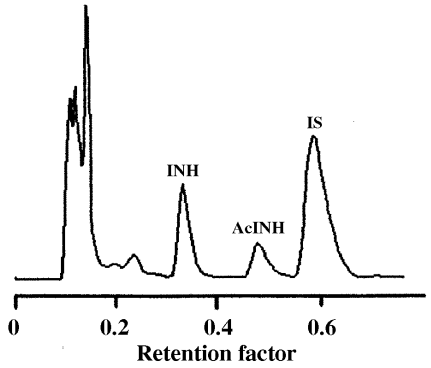

Figure 2. Typical HPTLC chromatograms of serum: INH, AcINH, and nicotinamide.

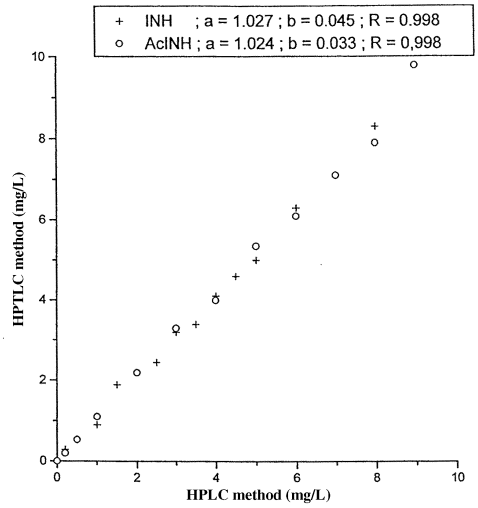

Figure 3. Correlation between HPLC and HPTLC results of INH and AcINH: a = slope, b = *y* intercept, and *r* = correlation coefficient.

Typical chromatograms are shown in Figure 2. The data presented in Figure 3 are indicative of a satisfactory correlation between the results obtained from the two methods over the concentration range studied. The equations of the straight lines are given in Figure 3.

Conclusion

The method described in this report has proved useful for the analysis of isoniazid and its seric metabolite AcINH in the concentration range 0.5–10 mg/L. It includes an SPE of the drug and its metabolite and their HPTLC on silica. The method was fully validated, accurate, and compared with the HPLC method. The HPTLC assay is more attractive because of its speed (approximately 30 min/10 samples) and simplicity. It can be used for routine analysis in the clinical laboratory to find precise acetylor phenotype by the ratio AcINH–INH or to determine INH seric concentration.

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