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SIMULTANEOUS HPLC DETERMINATION OF ISONIAZID AND ACETYLISONIAZID IN PLASMA

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SUMMARY

A rapid and simple high-performance liquid chromatographic method has been developed for simultaneous determination of isoniazid (INH) and acetylisoniazid (AcINH) in microsamples of plasma. Plasma samples were deproteinated by addition of trichloroacetic acid, and the drug and its metabolite were then separated by reversed-phase HPLC on an octadecylsilane-bonded silica column. The mobile phase was a gradient prepared from an aqueous solution of 1-hexanesulfonic acid sodium salt (pH 3) and acetonitrile. The effluent was monitored by UV detection at 290 nm. Calibration plots were linear in the range 0.5 to 15.0 μg mL^{-1} and the limit of quantification was 0.5 μg mL^{-1} for both drugs. The lower limits of detection for INH and AcINH were 0.24 and 0.12 μg mL^{-1}, respectively. Results from analysis of quality-control samples at concentrations of 0.8, 5, and 13 μg mL^{-1} were indicative of good repeatability and precision. Recovery from plasma was 64% for INH and 55% for AcINH. INH was more stable than AcINH in plasma at –80°C.

INTRODUCTION

Pulmonary tuberculosis is an important public health concern in Mexico [1]. Isoniazid, rifampicin, pyrazinamide, and etambutol, have been classified as first-line drugs for treatment of the disease [2]. Isoniazid (INH, isonicotinyl hydrazine) is the drug most widely used for treatment of tuberculosis. It is mainly metabolized in the liver, where acetylisoniazid (AcINH) is formed by the action of *N*-acetyltransferase [3].

Several high-performance liquid chromatography (HPLC) methods with UV, fluorimetric, or mass spectrometric detection have been used for simultaneous measurement of INH and AcINH in biological fluids. Although

single-ion monitoring is one of the most specific methods available, it requires access to a mass spectrometer. One example is an LC–MS method used for quantification of INH and etambutol in human plasma [4]. Many procedures require either sample purification with organic solvent [5–7], derivatization after sample purification [8–12], or liquid–solid extraction by use of an in-line column [13–15]. Most of these methods are laborious or require use of 0.5 to 2 mL sample [11–14,16,17].

In this paper we describe an HPLC method for the simultaneous measurement of INH and its acetylated metabolite (AcINH) in microsamples of plasma that have been deproteinated with trichloroacetic acid [16, 18]. INH and its metabolite were separated on a reversed-phase column by ion-pairing (1-hexanesulfonic acid, sodium salt) with gradient elution and UV detection.

EXPERIMENTAL

Reagents

Isoniazid (INH) was provided by Laboratorio Valdecasas (México, D.F.) and acetylisoniazid (AcINH) was synthesized by the reaction of INH with acetic anhydride by the method described by Olson et al. [19].

Trichloroacetic acid, phosphoric acid, acetic anhydride, and glacial acetic acid were all of analytical-reagent grade (Merck, Germany). 1-Hexanesulfonic acid sodium salt was analytical grade (Sigma–Aldrich). Acetonitrile and water were HPLC grade (Merck).

Sample Preparation

Samples of heparinized plasma or aqueous calibration solutions (100 μL) were deproteinated with 40 μL 15% (*w*/*v*) trichloroacetic acid in 1.5-mL polypropylene Eppendorf tubes. The mixture was vigorously vortex mixed for 2 min then centrifuged for 10 min at 3000*g*. The trichloroacetic acid supernatant (pH 1) was then withdrawn and evaporated to dryness for 10 min at ambient temperature under a gentle nitrogen stream. The dry residue was dissolved in 100 μL of an aqueous solution of 1-hexanesulfonic acid sodium salt (20 mM, pH 3 adjusted with phosphoric acid). This solution (20 μL) was injected for HPLC analysis.

Chromatography

HPLC was performed with a Waters Associates (Milford, MA, USA) 1525 multisolvent delivery system, a Rheodyne 7725 injector with

20-μL loop, and a Waters 2487 variable-wavelength UV–visible detector. Compounds were separated on a 150 mm × 3 mm, 3.5-μm particle, Waters X-terra RP_{18} column protected by a 20 mm × 2 mm, 3.5-μm particle, Waters Guard Pak X-terra RP_{18} precolumn.

The mobile phase was a binary linear gradient prepared from an aqueous solution of 1-hexanesulfonic acid sodium salt solution (20 mM, pH 3, adjusted with phosphoric acid) and acetonitrile. The proportion of the components was initially 90:10 for 2 min. This was changed to 50:50 over 3 min, maintained for 1.5 min. Finally the proportion was changed to 90:10 over 1.5 min and maintained for 4.5 min for re-equilibration. The flow rate was 0.4 mL min^{-1} and detection was performed at 290 nm. Detector output was acquired and processed by Waters Breeze V3.2 software.

Calibration

Calibration plots were constructed by analysis of appropriate working solutions of INH and AcINH prepared in water and in blank heparinized plasma. Concentrations of 0.0, 0.5, 1.0, 3.0, 7.0, 11.0, and 15.0 μg mL^{-1} were used for both drugs, and the samples were treated as described above for samples.

Validation procedures

The method was validated in accordance with published guidelines [20].

Within-run precision and standard deviation as a measure of accuracy were examined by supplementing blank plasma with appropriate amounts of each of the two compounds to yield quality-control (QC) samples containing 0.8, 5, and 13 μg mL^{-1}. The QC samples were divided into equal portions, and each was analyzed (n = 10) as a separate sample by use of the procedure described above. To avoid the risk of possible drug degradation during storage, QC samples were prepared each day from the stock solutions prepared on the first day of the study.

Inter-assay reproducibility was determined on three separate occasions by replicate analysis of each of the QC samples. Results were regarded as satisfactory if they were within ±15% of the actual value, except for the limit of quantification, for which 20% was regarded as satisfactory.

Specificity was assessed in the presence of rifampicin, pyrazinamide, and etambutol.

To determine long-term freezer stability of AcINH and INH in plasma, QC solutions were analyzed in triplicate after storage of the solutions

at −80°C for 1, 2, and 30 days. The drug was regarded as stable if more than 90% was intact at the end of the study period.

Data Analysis

Student's *t*-test was used to examine differences between sets of results. Differences were regarded as significant if $P < 0.05$.

RESULTS AND DISCUSSION

Extraction of INH and AcINH from plasma was achieved by a simple deproteination with trichloroacetic acid; this results in easy, rapid, and convenient separation of the analytes [16,18]. The chromatograms obtained under the assay conditions used were clean, despite injection of the sample on to the column without pre-purification.

Ion pair chromatography with 1-hexanesulfonic acid sodium salt in the mobile phase results in retention of INH, a polar molecule of low molecular weight, on the column by the formation of a complex. It is important the proportion of 1-hexanesulfonic acid sodium salt in the mobile phase is relatively high at the beginning of the chromatographic run (gradient starts with 90% of this phase). Under these conditions the hydrocarbon chain of the ion pair interacts with the octadecylsilane chains of the stationary phase and the complex is retained long enough to be chromatographically separated.

This HPLC method enabled rapid simultaneous measurement of INH and its acetylated metabolite AcINH in plasma samples. Use of the gradient described resulted in sharp and symmetrical peaks.

Total analysis time, including sample pretreatment and rapid elution, was less than 15 min.

Results from Validation

Selectivity

We demonstrated the absence of interfering endogenous compounds in blank plasma; a representative chromatogram is shown in Fig. 1a. Addition of the two compounds to plasma samples showed they were well separated; no interfering peaks were observed in blank plasma samples. Retention times were 4.5 and 7.4 min for INH and AcINH (Fig. 1b). In therapeutic treatment, INH is often co-administered with the other antituberculosis drugs rifampicin, pyrazinamide, and etambutol. When samples

were spiked with these drugs no interference was observed in the chromatogram (Fig. 1c). Similar results were obtained from analysis of plasma samples from patients treated with 300 mg INH, 600 mg rifampicin, 2000 mg pyrazinamide, and 1200 mg etambutol (Fig. 1d).

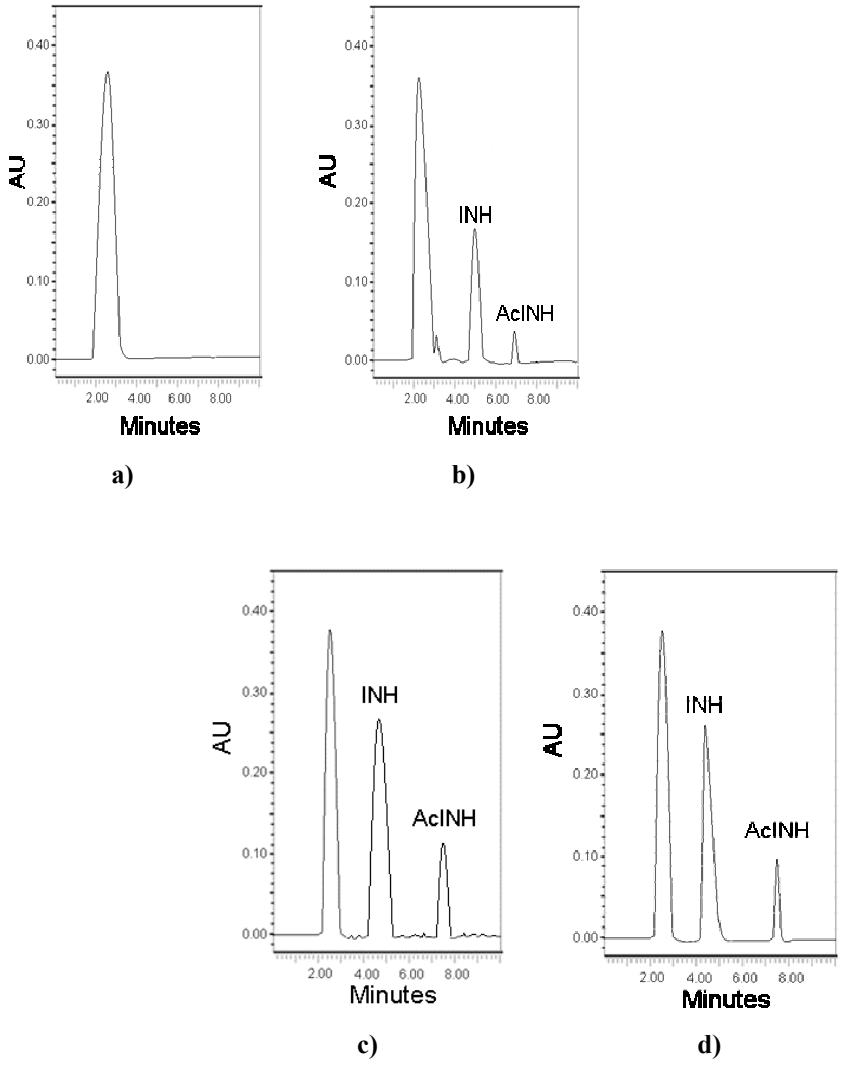

Fig. 1

Chromatograms obtained from (a) blank plasma, (b) plasma spiked with 7 µg mL^{-1} INH and 7 µg mL^{-1} AcINH, (c) plasma spiked with INH, rifampicin, pyrazinamide, and etambutol, and (d) plasma from a patient given 300 mg INH, 600 mg rifampicin, 2000 mg pyrazinamide, and 1200 mg etambutol

Linearity

When average peak area was plotted against the INH concentration and its metabolite in plasma the plots were linear in the range 0.5 to 15.0 μg mL^{-1}. Typical calibration plots for plasma extracts had good correlation coefficients (0.9998 for INH and 0.9905 for AcINH; $n = 6$ calibration points).

Limits of Quantification and Detection

The limit of quantification, defined as the lowest concentration that could be measured with accuracy and precision, i.e. within ±20% of the actual value [20], was 0.5 μg mL^{-1}. The lower limits of detection of INH and AcINH (three times the baseline noise) were 0.24 and 0.12 μg mL^{-1}, respectively.

Intra-Day Repeatability

Assay performance was evaluated as intra-day accuracy and precision, determined by replicate analysis of QC samples. The results obtained are listed in Table I.

Table I

Within-run precision and accuracy of the HPLC method

Actual value (μg mL^{-1})	INH			AcINH		
	0.8	5	13	0.8	5	13
Mean concentration found (μg mL^{-1})	0.9	4.5	12.7	0.7	4.6	12.5
Number of replicates	10	10	10	10	10	10
Standard deviation (*SD*)	0.04	0.3	0.6	0.02	0.06	0.2
CV (%)[a]	4.5	7.0	5.0	2.9	4.3	0.9
Accuracy (%)[b]	10.4	−9.8	−2.2	−4.3	−6.9	−3.7

[a] $CV = (SD/\text{Mean}) \times 100\%$

[b] $[\{(\text{Amount found}) - (\text{amount added})\}/(\text{amount added})] \times 100\%$

These results show the repeatability of the assay, including both sample processing and chromatographic measurement, is good. Small deviations from perfect accuracy were observed (i.e. 10.4% at most).

Inter-Assay Precision

As is apparent from Table II, inter-assay coefficients of variation

determined from experiments performed on three days ($n = 6$) were <5%, this is indicative of good assay precision.

Table II

Reproducibility and accuracy of the method

Actual value (μg mL^{-1})	INH			AcINH		
	0.8	5	13	0.8	5	13
Mean concentration found (μg mL^{-1})	0.84	5.0	13.1	0.8	4.7	12.8
Number of replicates	6	6	6	6	6	6
Standard deviation (*SD*)	0.03	0.2	0.4	0.02	0.06	0.2
CV (%)[a]	3.3	4.6	3.3	3.2	1.2	1.7
Accuracy (%)[b]	4.5	0.8	1.2	−3.5	−6.6	−1.8

[a] $CV = (SD/\text{Mean}) \times 100\%$

[b] $[\{(\text{Amount found}) - (\text{amount added})\}/(\text{amount added})] \times 100\%$

Recovery

Recovery was determined by dividing the peak area obtained from analysis of each of the two compounds added to plasma by that observed for the same amount of each compound injected directly into the chromatograph. Recovery of INH and AcINH from plasma was 64 and 55%, respectively; these values were constant in the concentration range studied and are higher than those obtained in other studies [21,22]. Acetylisoniazid was partially retained (11%) on the protein precipitate when trichloroacetic acid was used for deproteination [16]. This partially explains the low recovery of acetylisoniazid.

Stability

Experiments conducted in our laboratory showed that QC solutions of INH in plasma were stable for at least 30 days at −80°C; the amount of the initial concentration remaining after this time was 98.35 ± 2.07%. In contrast, loss of AcINH in these plasma samples was substantial after storage at the same temperature for 1 day (a decrease to 77.16 ± 3.46% of the initial concentration). These results are given in Table III.

These results suggest that determination of both INH and AcINH in patient plasma should be performed as soon as possible after sampling and that chromatography must be performed immediately after sample preparation.

Table III

Stability of INH and AcINH in plasma samples at −80°C

Actual value (μg mL^{-1})	INH			AcINH		
	0.8	5	13	0.8	5	13
Mean initial concentration (μg mL^{-1})	0.866	4.861	13.487	0.757	4.655	12.055
CV (%)[a]	2.16	4.1	0.26	2.24	6.30	0.29
Number of replicates	6	6	6	6	6	6
Mean final concentration (μg mL^{-1})	0.858[b]	4.860[b]	12.95[b]	0.556[c]	3.62[c]	9.68[c]
Recovery (%)[d]	99.07	99.97	96.01	73.44	77.76	80.29
CV (%)[a]	2.18	4.1	0.26	9.2	1.58	0.61
Number of replicates	6	6	6	6	6	6

[a]$CV = (SD/\text{Mean}) \times 100\%$
[b]Data obtained after 30 days
[c]Data obtained after 1 day
[d][(Initial concentration)/(Final concentration)] × 100%

CONCLUSION

The sample volume required is only 100 μL, compared with 500 μL to 2 mL for other methods, and minimum sample handling is required. The reagents used in the method are inexpensive and readily available, and the procedure does not involve any critical experimental conditions.

This HPLC method may be suitable for quantification of INH and AcINH in small samples of plasma.

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