ELSEVIER

Journal of Chromatography B, 670 (1995) 259–266

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic analysis of the antituberculosis drugs aconiazide and isoniazid

Eugene B. Hansen Jr.[a,*], Kenneth L. Dooley[b], Harold C. Thompson Jr.[a]

[a]*Department of Health and Human Services, Food and Drug Administration, Division of Chemistry, National Center for Toxicological Research, Jefferson, AR 72079, USA*

[b]*Department of Health and Human Services, Food and Drug Administration, Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA*

First received 12 October 1994; revised manuscript received 30 March 1995; accepted 11 April 1995

Abstract

Reversed-phase HPLC methods are described for determining the stability and concentration certification of the antituberculosis prodrug aconiazide (ACON) in aqueous dosing solution and for assessing the concentrations of ACON and isoniazid (INH) in plasma from ACON-treated male and female Fischer-344 rats. ACON was analyzed in plasma by direct injection; it was separated on a 250 × 4.6 mm I.D. 5 μm C_{18} column using a 40% aqueous methanol mobile phase containing 5 g/l ammonium formate, and detected at 313 nm. INH was determined in the plasma of treated rats after a two-step precipitation of plasma proteins; it was separated on a 250 mm × 4.6 mm I.D. 5 μm CN column, eluted with 5% aqueous isopropanol containing 5 g/l ammonium formate, and detected with an electrochemical detector at +0.8 V. These methods allow a simple, rapid, and reliable determination of ACON and INH in plasma down to 0.1 μg/ml.

1. Introduction

Since its introduction in 1952, isoniazid (INH) has been the most commonly used antibiotic for the treatment and prevention of tuberculosis. INH can cause a variety of severe side effects, most of which have been thought to be associated with certain INH metabolites [1–4]. For these reasons INH has been extensively studied and numerous chromatographic methods for the determination of INH in biological samples have been reported [5–10].

Aconiazide (ACON), the isonicotinylhydrazone of 2-formylphenoxyacetic acid, was synthesized as one of several antituberculosis isonicotinylhydrazones [11]. ACON appears to be less toxic than INH and equally effective against *M. tuberculosis* [12,13]. There has been recent interest in using ACON as an INH prodrug and the pharmacokinetics and bioavailability of INH after ingestion of ACON in humans have been reported [14]. However, little is known about the toxicities of ACON and consequently, we have performed animal toxicological studies using Fischer-344 rats [15,16].

Prior to the initiation of animal tests, analytical procedures were required for determining the stability of ACON in the dosage form and for the determination of ACON and INH in the plasma of treated animals. None of the existing pro-

* Corresponding author.

SSDI 0378-4347(95)00176-X

cedures reviewed for the analysis of INH met our needs for analyzing INH in plasma from ACON-dosed animals, and although a pharmacokinetic study by Peloquin et al. [14] reported a HPLC analysis for ACON in human serum, a description of the procedure was not reported.

This paper describes HPLC methodologies developed to determine ACON concentrations and stability in aqueous dosing solutions and to determine unbound ACON and INH concentrations in plasma from Fischer-344 rats treated with ACON.

2. Experimental

2.1. Chemicals and reagents

All solvents were HPLC grade and all other chemicals were reagent grade and used as purchased. Deionized water was prepared for chromatography using a Milli-Q Water Purification System (Waters Associates, Milford, MA, USA).

Aconiazide (4-pyridinecarboxylic acid, ((2-(carboxymethyoxy) phenyl)methylene)hydrazide) (ACON), and isoniazid (4-pyridine carboxylic acid hydrazide) (INH) were obtained from Raylo Chemicals (Edmonton, Canada). Chemical characterizations for positive identification and purity assessment of both compounds were performed using mass spectrometry, ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy, and high-performance liquid chromatography (HPLC). Results from these analyses were consistent with the proposed chemical structures for each compound and a purity of 98% and 99% was indicated for ACON and INH, respectively.

Stock ACON solution (1 mg/ml)

A 100-mg amount of ACON was dissolved in 100 ml of 1% aqueous $NaHCO_3$. The solution was prepared daily as needed.

Stock INH solution (1 mg/ml)

A 100-mg amount of INH was dissolved in 100 ml water and was prepared daily as needed.

2.2. Differential pulse voltammetry

Differential pulse voltammetry measurements were carried out using a Model BAS-100A electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN, USA) equipped with a Ag/AgCl reference electrode, a steel wire auxiliary electrode, and a glassy carbon working electrode. Each compound was dissolved in 100 m*M* aqueous ammonium formate at a concentration of 1.0 mg/ml and the solutions were bubbled with nitrogen gas prior to measurements. The glassy carbon electrode surface was renewed before each measurement by using a polishing pad and a few drops of alumina slurry. Differential pulse voltammetry analysis was conducted using a scan range of +0.4 V to +1.2 V, a scan rate of 5 mV/s, and a pulse amplitude of 50 mV.

2.3. HPLC apparatus and chromatographic conditions

HPLC was conducted with a Model 100A high-pressure pump (Beckman Instruments/Altex Scientific Operations, Berkeley, CA, USA), a Model 7125 injector (Rheodyne, Cotati, CA, USA), and a Model 4270 computing integrator (Spectra-Physics, San Jose, CA, USA).

ACON was analyzed with a 250 × 4.6 mm I.D. 5 μm C_{18} HPLC column (Supelco, Bellefonte, PA, USA) that was eluted with a mobile phase consisting of 40% aqueous methanol containing 5 g/l ammonium formate. The mobile phase flow-rate was 1.0 ml/min and the eluate detected using a Model 160 UV–visible detector (Beckman) adjusted to either 254 (dosing solution analysis) or 313 nm (plasma analysis).

INH was analyzed with a 250 × 4.6 mm I.D. 5 μm LC-CN column (Supelco) using a mobile phase of 5% aqueous isopropanol containing 5 g/l ammonium formate. The flow-rate was 1.0 ml/min and the peaks were detected with a Model 5100A electrochemical detector (ESA, Bedford, MA, USA) equipped with a Model 5011 analytical cell and a Model 5020 guard cell. The guard cell was set at +1.0 V and was placed between pump and injector. The high-sensitivity analytical cell was operated in the positive polari-

ty (oxidative) mode with the first detector set at +0.6 V, the second detector set at +0.8 V, and the gain set at 1.

2.4. Plasma collection

The dosage groups consisted of a vehicle control (water) and ACON at 100, 200, and 400 mg/kg body weight. The animals were treated daily. One hour after dosing a rat was anesthetized by exposure to CO_2 and blood collected from the retro-orbital sinus into EDTA-containing tubes. Following centrifugation, the plasma was decanted and stored at −70°C until HPLC analysis.

2.5. Determination of ACON in plasma

Frozen rat plasma was brought to room temperature and mixed thoroughly by vortex-mixing. Typically, a 5-μl aliquot of the raw plasma was injected into the HPLC system. Depending on the concentration of unbound ACON in the plasma, a maximum injection volume of 50 μl was used. The eluate was detected by UV absorbance at 313 nm. The amount of ACON in the plasma (μg/ml) was determined by an external standard method that compared plasma ACON peak areas to an aqueous ACON standard solution whose concentration was similar to that determined in the plasma.

2.6. Determination of INH in plasma

INH in rat plasma was analyzed by vigorously mixing a 250-μl aliquot of plasma with 150 μl of 10% aqueous zinc sulfate (w/v) in a 13-mm disposable culture tube. The tube was centrifuged at 1000 *g* for 1 min and a 250-μl aliquot of the supernatant was transferred to a new tube and 100 μl of methanol added. The sample was mixed and centrifuged and 5 μl of the clear supernatant was injected into the HPLC. The eluate was monitored with the electrochemical detector at +0.8 V. The amount of INH in the plasma was determined by comparison of its peak area to the peak area obtained from an aqueous INH standard. The values were corrected for the appropriate dilutions and procedure recovery and calculated as μg/ml plasma.

2.7. Stability of ACON dosing solution

Triplicate dosing solutions of ACON were prepared at concentrations of 75 and 150 mg/ml (free acid equivalent) by dissolving the ACON in an equimolar amount of aqueous NaOH (3.1% w/v) and adjusting the solution to pH 7 by the dropwise addition of either dilute NaOH or HCl. The solutions were passed through 0.45-μm Nylon-66 filters and stored in 125-ml borosilicate glass Erlenmeyer flasks fitted with Teflon-lined screw caps. The flasks were allowed to stand on the laboratory bench top, under fluorescent lighting, and at ambient temperature. Aliquots of 1 ml were diluted to 100 ml with 1% (w/v) aqueous $NaHCO_3$ and analyzed by HPLC. The ACON in the HPLC eluate was detected by UV absorbance at 254 nm and the amount of ACON determined by comparison of peak areas to those obtained from freshly prepared 1 mg/ml ACON standard solutions. Aliquots were analyzed initially and at intervals of 3, 6, 8 and 10 days.

3. Results and discussion

3.1. Electrochemical oxidation of ACON and INH

Differential pulse voltammetry measurements were performed to determine the applicability of on-line electrochemical detection of ACON and INH in the oxidative mode. Fig. 1A shows a differential pulse voltammogram for the oxidation of ACON between +0.4 and +1.2 V, which confirms an optimal oxidative potential of +0.9 V for ACON detection. Although electrochemical detection of ACON was not utilized in this study, these results are reported to provide reference data for future studies with ACON that may require the enhanced sensitivity of electrochemical detection.

The differential pulse voltammogram of INH (Fig. 1B) shows an oxidative response beginning

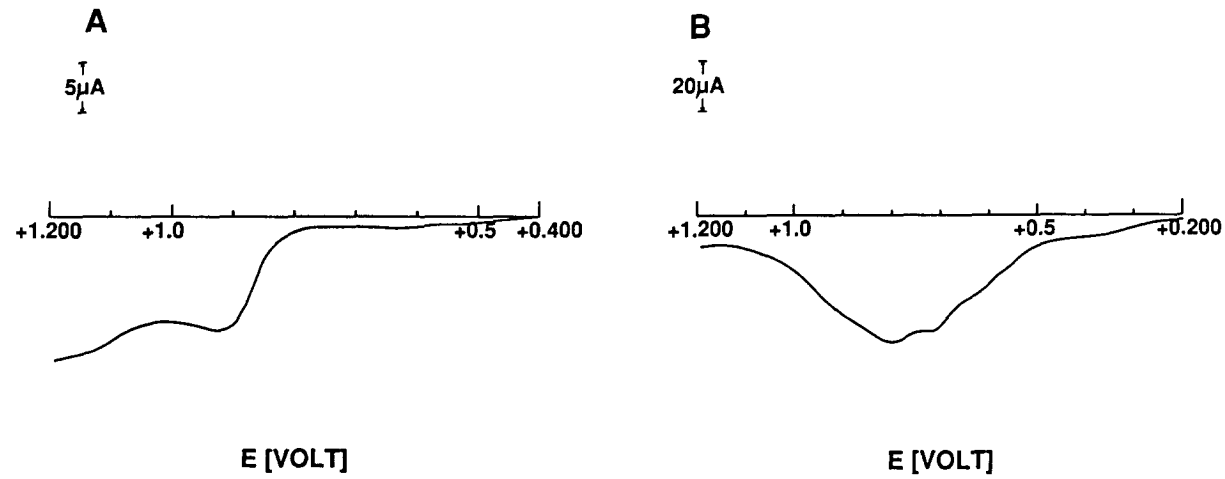

Fig. 1. Differential pulse voltammograms of (A) ACON and (B) INH.

at +0.6 V and a maximum response at +0.8 V. This response is in good agreement with that previously used for the HPLC electrochemical detection of INH [10].

These data demonstrate the potential utility of on-line oxidative electrochemical detection for the HPLC analysis of ACON and INH.

3.2. HPLC analysis

ACON

Fig. 2 shows the HPLC-UV (254 nm) chromatogram obtained from a 1 mg/ml ACON standard solution that was 10 days old. The major peak seen at 6 min is due to ACON. The minor peaks seen at approximately 2 and 3 min are INH and 2-formylphenoxyacetic acid (2-FPAA), respectively, showing that ACON undergoes some hydrolysis when dissolved at a concentration of 1 mg/ml (structures shown in Fig. 3). Positive identification of these compounds was confirmed by HPLC–thermospray mass spectrometric analysis. The retention times and thermospray mass spectra obtained were identical to those obtained from authentic compounds. The rate of hydrolysis at ambient temperature occurring in the 1 mg/ml solutions was determined by measuring the recovery of ACON in freshly prepared solutions and after 7 h. Initially, solutions ($n = 5$) showed only one HPLC peak

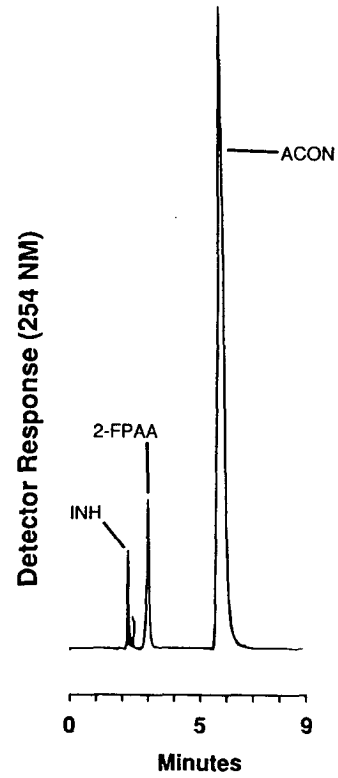

Fig. 2. HPLC–UV (254 nm) chromatogram obtained from a 1 mg/ml ACON standard solution that was 10 days old.

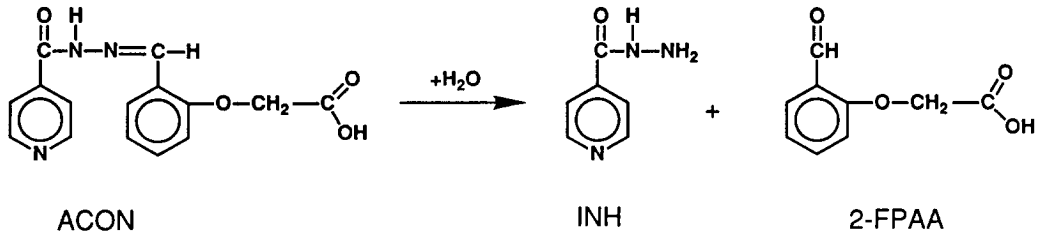

Fig. 3. Hydrolysis of ACON in aqueous solution.

due to ACON. After 7 h at ambient conditions, 97.3 ± 1.5% of the initial ACON remained and two additional HPLC peaks were easily detected at 254 nm. The basic pH of the $NaHCO_3$ solution did not seem to affect the rate of hydrolysis since analysis of 1 mg/ml ACON solutions at pH 7 gave similar results. The purpose of showing these data is to demonstrate that a standard solution of ACON in 0.1% $NaHCO_3$ may be used for quantitation up to 7 h with acceptable variations in concentration (< 3%). The hydrolysis of ACON was not observed in the aqueous dosing solutions prepared at 75 and 150 mg/ml (vide infra).

Because no suitable sample preparation procedure for the HPLC analysis of ACON in plasma was found, raw plasma samples were injected into the HPLC system and an unbound ACON detected in the eluate at 313 nm. Fig. 4 shows HPLC chromatograms obtained from 5-μl injections of a 1 μg/ml ACON standard aqueous solution (Fig. 4A), plasma obtained 1 h after treatment of a control rat with water (Fig. 4B), and rat plasma obtained 1 h after treatment with 200 mg/kg ACON (Fig. 4C). The peak in Fig. 4C is consistent with a concentration of ACON in plasma of 0.6 μg/ml. Using the described conditions ACON was fully resolved from endogenous plasma components, yielded a sharp and symmetrical peak with a total chromatographic time less than 7.5 min, and the minimum detection limit was 0.1 μg/ml. Fig. 2 shows that the retention time for INH using this HPLC system is about 2.3 min. Unfortunately, Fig. 4C clearly shows that the concurrent analysis of low levels of INH in plasma using these conditions was not possible since a large amount of polar components from the plasma coeluted with INH.

INH

Electrochemical detection of INH was utilized because absorbance detection did not provide the sensitivity needed after the plasma samples were diluted to precipitate the proteins and unbound ACON and would have required an extra concentration step in the procedure. Simple dilutions of the sample prior to HPLC analysis was much more cost-efficient in terms of supplies and time requirements. Fig. 5 shows chromatograms obtained from the analysis for INH in the rat plasma samples described in Fig. 4. Fig. 5A is

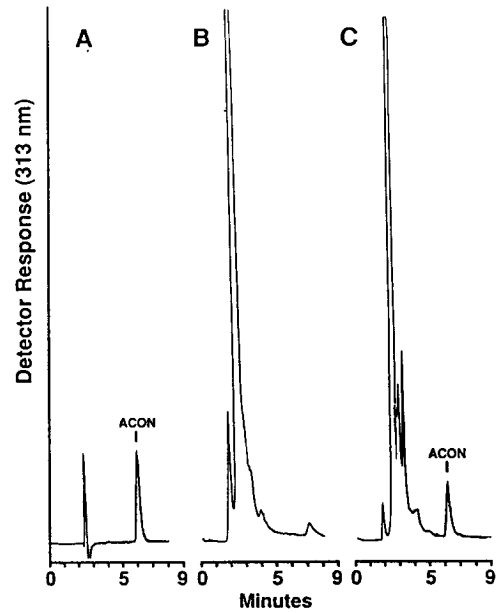

Fig. 4. Representative chromatograms from 5-μl injections of a 1 μg/ml ACON standard solution (A), plasma obtained 1 h after treatment of a control rat with water (B), and rat plasma obtained 1 h after treatment with 200 mg/kg ACON (C); (C) represents analysis of ACON in plasma at 0.6 μg/ml.

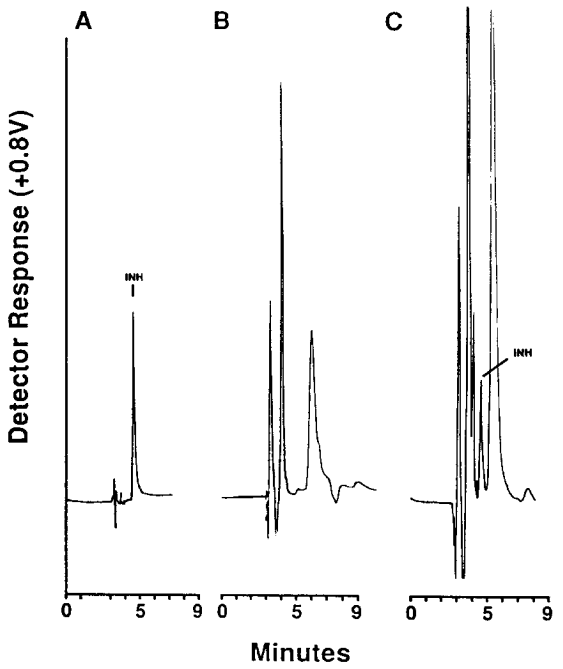

Fig. 5. Representative chromatograms showing the analysis for INH in rat plasma from ACON-treated rats. (A) Chromatogram from a 5-μl injection of a 1 μg/ml INH aqueous standard solution. (B) Chromatogram obtained from the analysis of the water-treated control rat. (C) Chromatogram obtained from analysis of a rat treated with 200 mg/kg, representing the analysis of INH at 1.9 μg/ml plasma.

the chromatogram from a 5-μl injection of a 1 μg/ml INH standard solution, Fig. 5B is the water-treated control rat, and Fig. 5C is the rat sample treated with 200 mg/kg, which indicates an INH concentration of 1.9 μg/ml plasma. The retention time for INH was 4.4 min, the chromatographic run time required 8 min to clear late eluting plasma components, and the minimum detection limit was 0.1 μg/ml.

3.3. Recovery, linearity, and precision

ACON

Recovery of ACON from rat plasma was determined by comparing peak areas obtained from aqueous standard solutions and spiked plasma. Recovery determinations of ACON from spiked plasma were not consistent. We found the drug to be strongly bound to plasma components, most probably proteins, in a very inconsistent manner immediately after being spiked into plasma. Preliminary investigations of sample preparation procedures including liquid–liquid extraction, solid-phase extraction, and plasma protein precipitation gave unacceptable results at spiked levels as high as 10 μg/ml.

For direct injection analysis, triplicate plasma aliquots (1 ml) were spiked with ACON at 10 μg/ml, thoroughly vortex-mixed, and 5 μl injected. The recoveries were $87.4 \pm 3.2\%$; however, further assays of plasma spiked at 50, 5, and 1 μg/ml on a different day showed ACON recoveries of 50.2, 13.8, and 0%, respectively, with the intra-assay coefficient of variation (C.V.) for the 5 μg/ml spike being 7.6%. Although drug-free plasma spiked with ACON at 1 μg/ml gave 0% recovery, plasma from treated rats whose equilibrated unbound ACON plasma levels were ≤ 1 μg/ml, were readily detected using the direct injection method. These data suggest that comparing recovery values from ACON-spiked plasma with those from the direct injection analysis of plasma from ACON-treated rats is not appropriate. The use of an internal standard was considered but not used because this approach would not be expected to affect the recovery of ACON when spiked at ≤ 1 μg/ml because it is converted to a different form, i.e. protein bound, that cannot be detected. For the purpose of our toxicological study, we did not use a spike recovery correction for plasma samples analyzed for ACON because the steady-state free ACON plasma concentrations were considered most important.

Peloquin et al. [14] report a recovery of 100% and a detection limit of 0.05 μg/ml for the HPLC determination of ACON in human serum. However, the methodology used is not described, and the study reports that ACON was not detected in the serum samples assayed. These findings are not surprising since precipitation of proteins in our ACON-spiked plasma samples resulted in drastic or complete loss of ACON, presumably because of the facile binding of ACON noted above.

Calibration curves were obtained for aqueous ACON solutions by plotting the detector response (peak area) versus the amount of ACON

(μg) injected. For the absorbance detector at 313 nm triplicate 20-μl injections containing 0.001, 0.002, 0.004, 0.008, 0.016, and 0.020 μg ACON were used for standardization and linear regression analysis. The calibration equation was $y = 3173x + 1444$, the correlation coefficient (r^2) was 0.9997, and the coefficient of variation (C.V., %) at the mid-point of the curve (0.008 μg) was 0.92%. For detection at 254 nm the amount of ACON injected ranged from 0.22 to 12.7 μg ($n = 11$). The response for this range was linear ($r^2 = 0.9993$) and the calibration equation was $y = 321x - 159$. Although not used for quantitation during this study, a standard curve for the detection of ACON by electrochemical detection at +0.9 V was obtained and is reported; the amount of ACON injected ranged from 0.01 to 0.38 μg ($n = 5$), the calibration equation was $y = 3023x + 1392$, and $r^2 = 0.9999$.

INH

Recovery of INH was determined from analysis of 1-ml aliquots of INH-spiked plasma. Volumes of 250 μl were taken through the two-step precipitation procedure and the HPLC peak areas compared to those from aqueous INH standard solutions. The INH recoveries and their inter-assay coefficient of variation (C.V., %) at four levels (2–10 μg/ml) are shown in Table 1.

Calibration curves were obtained for INH in aqueous solution using electrochemical detection at +0.8 V. Triplicate 50-μl injections containing 0.0025, 0.005, 0.01, 0.02, 0.04, and 0.05 μg INH were made. The calibration equation was $y = 14340x - 13013$, the correlation coefficient (r^2) was 0.9999, and the coefficient of variation (C.V., %) at the mid-point of the curve (0.02 μg) was 1.4%.

3.4. Stability of ACON in aqueous dosing solution

ACON solutions assayed for stability were detected at 254 nm instead of 313 nm because the products of ACON hydrolysis, INH and 2-FPAA, are not readily detected using the higher wavelength and detection of low concentrations of these compounds was desired. Results from the stability study of ACON dosing solutions at 75 and 150 mg/ml are shown in Table 2. These assays showed that aqueous ACON at 75 and 150 mg/ml and pH 7, maintained under ambient lighting and temperature in sealed borosilicate glass flasks, is stable for a minimum of 10 days. These data, obtained at ambient temperatures and storage on the laboratory bench top were important for our study because animals were dosed daily up to 182 consecutive days by gavage, using an automated system. These data show that special storage considerations of the dosing solution, i.e. temperature and light exposure, were not necessary and that seven aliquots of dosing solution, each to be used on a daily basis,

Table 1
Recoveries of INH from Fischer-344 rat plasma

Spiked plasma concentration (μg/ml)	Recovery[a] (%)	n[b]	Inter-assay C.V. (%)
2	73.9 ± 8.3	13	11.2
3	79.9 ± 2.84	3	3.55
5	81.4 ± 2.5	2	3.07
10	76.5 ± 16.0	6	20.9

[a] Mean and standard deviation of the percent recovery of INH-spiked Fischer-344 rat plasma samples assayed according to the method described in Section 2.

[b] Number of spiked plasma samples assayed at the concentration level specified. The results were generated from assays performed over an eight-month period.

Table 2
Stability of ACON in aqueous dosing solution[a]

Sampling interval (days)	ACON recovered from aqueous dosing solution[b] (%)	
	75 mg/ml	150 mg/ml
1	99.2 ± 1.5	97.8 ± 2.2
3	98.0 ± 1.1	99.9 ± 1.1
6	98.9 ± 0.97	99.7 ± 2.5
8	99.8 ± 1.3	98.9 ± 1.4
10	107 ± 1.0	99.3 ± 1.0

[a] In sealed glass containers, under fluorescent lighting, and at ambient temperature.

[b] Mean and standard deviation of triplicate samples.

could be prepared weekly and placed within easy access of the automated pipette without compromising the integrity of the dosing solution which is a considerable time-saving step.

3.5. Application

These assays were used to determine the levels (μg/ml) of unbound ACON and INH in Fischer-344 rat plasma samples from a fourteen-day, repeat dose, toxicity evaluation and dose range finding study with ACON [14], and a six-month toxicity comparison of ACON and INH [15]. These studies generated a sample set of over 400 plasma samples for ACON and INH analysis. Using the HPLC methodologies reported here we found that 12 plasma samples per day could easily be assayed for both drugs. In addition, the minimal sample preparation used did not adversely affect the stability of the HPLC columns throughout the course of the entire study.

4. Conclusion

The objectives of this study were to develop simple and rapid analytical procedures, employing HPLC, for determining the concentration and stability of ACON in aqueous dosing solutions and for determining ACON and INH in the plasma of treated animals. The HPLC systems described for the analysis of ACON and INH provide adequate retention and resolution from endogenous compounds found in Fischer-344 rat plasma. The facile binding of ACON to plasma components was the likely cause for inconsistent recovery determinations from spiked samples. However, this did not affect, in any way, the collection of data relevant to the toxicology study. This is the first report to extensively describe HPLC methodologies for the study of ACON disposition and provides a basis for future investigations of alternative human antituberculosis drugs.

References

[1] R.A. Garibaldi, R.E. Drusin, S. Ferebee and M.B. Gregg, Am. Rev. Respir. Dis., 106 (1972) 357.
[2] R.A. Alford, in G.L. Mandell, R.G. Douglas and J.E. Bennett (Editors), Principles and Practice of Infectious Diseases, Churchill Livingstone, New York, NY, 3rd ed., 1990, p. 350.
[3] AHFS, Isoniazid, in G.K. McEvory (Editor), AHFS Drug Information 1990, American Society of Hospital Pharmacists, Bethesda, MD, 1990, p. 344.
[4] J.A. Timbrell, J.R. Mitchell, W.R. Snodgrass and S.D. Nelson, J. Pharmacol. Exp. Ther., 213 (1980) 364.
[5] M.R. Holdiness, J. Chromatogr., 340 (1985) 321.
[6] R.N. Gupta and L. Maelly, J. Chromatogr., 425 (1988) 441.
[7] K. Haruichi, K. Hiroake, F. Koichi, Y. Nobutsugu and N. Takashi, Ther. Drug Monitor., 13 (1991) 428.
[8] A. Defilippi, G. Piancone, R.C. Laia, S. Balla and G.P. Tibaldi, J. Chromatogr., 656 (1994) 466.
[9] A. Walubo, P. Smith and P.I. Folb, J. Chromatogr., 658 (1994) 391.
[10] A. El-Yazigi and D.A. Raines, Pharm. Res., 9 (1992) 812.
[11] A. Zubrys and C.O. Siebenmann, Can. J. Chem., 33 (1955)
[12] C.O. Siebenmann and A. Zybrys, Antibiotics Annual, 1954–1955, Medical Encyclopedia, New York, NY, p. 442.12.
[13] H.R. Held and S. Landi, J Pharm. Sci., 69 (1980) 1284.
[14] C.A. Peloquin, G.T. James, L.D. Craig, M. Kim, E.A. McCarthy, D. Ikle and M.D. Iseman, Pharmacotherapy, 14 (1994) 415.
[15] K.L. Dooley, E.B. Hansen, Jr., W.G. Sheldon and F.A. Beland, J. Am. Coll. Toxicol., 12 (1993) 329.
[16] F.A. Beland, K.L. Dooley, E.B. Hansen, Jr. and W.G. Sheldon, J. Am. Coll. Toxicol., (1995) in press.