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Journal of Chromatography B, 705 (1998) 323–329

JOURNAL OF
CHROMATOGRAPHY B

Sensitive liquid chromatographic technique to measure isoniazid in alveolar cells, bronchoalveolar lavage and plasma in HIV-infected patients

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Received 24 March 1997; received in revised form 13 October 1997; accepted 15 October 1997

Abstract

The need to monitor the effectiveness of antimicrobial drugs in treating opportunistic infections such as tuberculosis in HIV-infected patients requires the development of sensitive assays. A suitable HPLC method was developed to measure the concentration of isoniazid (INH) in plasma 1 h after a standard 300 mg dose and to detect the low levels typically found in alveolar cells obtained by bronchoalveolar lavage of subjects maintained on a standard regimen of the drug. Following extraction with a chloroform–butanol mixture, the INH was back-extracted into dilute acid which was subsequently analyzed by HPLC using a CN reversed-phase column and an acetonitrile–isopropanol based mobile phase. Another HPLC method was developed using direct injection and a polymer based column to measure minute concentrations of INH in the cell-free lavage. In both systems, detection of the drug was accomplished with a sealed coulometric detector (+0.6 V) capable of giving a consistent daily response without adjustment. When saline, cellular extracts and plasma from untreated subjects were spiked with various amounts of INH and analyzed, the lowest level of quantitation was 10, 25 and 100 ng/ml, respectively. Calibration curves showed good linearity when spiked concentrations were compared to peak areas (r=0.991, 0.993 and 0.998, respectively). Alveolar cell extracts and cell-free bronchoalveolar fluid from HIV-positive patients maintained on a standard INH regimen had detectable levels of INH 4 h after a 300 mg oral dose. The plasma INH at 1 h had a range of 0.3–7.1 μg/ml (n=50). Precision studies with plasma spiked at 0.1, 0.5, 1.0 and 5.0 μg/ml revealed within-run coefficients of variation (C.V.s) of 8.9, 7.2, 4.2 and 4.9%, respectively and analytical recoveries of 97, 108, 108 and 98%, respectively. The day-to-day C.V.s for the plasma method were 7.6, 4.9 and 3.8% at concentrations of 0.5, 1.0 and 3.0 μg/ml, respectively. The results suggest that this rugged HPLC technique can quantitate INH in 1 h plasma with good precision and can be used to estimate the very low INH concentrations found in alveolar cells and cell-free lavage recovered from patients undergoing anti-tuberculosis therapy. © 1998 Elsevier Science B.V.

Keywords: Isoniazid

1. Introduction

The current resurgence of tuberculosis, particularly among immunocompromised individuals [1] has resulted in an increased use of the well established antibiotic, isoniazid (INH), whose chemical structure is:

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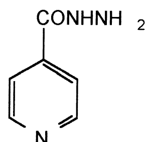
PII S0378-4347(97)00510-0



The emergence of drug resistant mycobacteria [2] and the discovery that the progression of tuberculosis is accelerated in HIV-positive patients [3], suggests that in-depth studies of the transport and localization of INH might be beneficial. Since the mycobacteria are known to survive within macrophages [4], other workers have studied the ability of these cells to accumulate INH in vitro [5].

An in vivo study of INH uptake by pulmonary macrophages when given orally in a standard dose to volunteer subjects would therefore be a reasonable approach to assess the intrapulmonary pharmacokinetics of the drug in patients at risk for developing tuberculosis. Previous publications from this institution showed the feasibility of assessing the intrapulmonary pharmacokinetics of antibiotics using bronchoscopy, bronchoalveolar lavage and a sensitive high-performance liquid chromatography (HPLC) drug detection system [6,7]. Since an earlier report by Guillaumont et al. [8] suggested the feasibility of detecting INH in pulmonary macrophages, we decided to use these techniques to investigate the intrapulmonary concentrations of the drug in HIV-positive and negative volunteers following a standard oral dose. To obtain pure extracts suitable for HPLC, we utilized the extraction technique proposed by Hutchings et al. [9]. A rugged in-line coulometric detector was used to give a sensitive and specific measurement of INH in plasma, bronchoalveolar cell extracts and cell-free fluid. Since this work was begun, Hansen et al. [10] have published a method for detecting INH in rat plasma using a similar strategy.

2. Experimental

2.1. Drug administration

Written, informed consent was obtained from all subjects prior to the oral administration of 300 mg INH/day for five days. Blood samples were taken 1 h after the last dose, rapidly centrifuged at ambient temperature and the EDTA–plasma stored at −20°C. HPLC analysis was performed within three days. A bronchoalveolar lavage was performed at 4 h as follows: four 50-ml aliquots of sterile saline (0.9 g/dl) were separately instilled into the right middle lobe and aspirated. After discarding the first aspirate, the remaining aspirates were pooled and centrifuged for 5 min at 400 *g* and 4°C. To minimize possible enzymatic degradation, the cell-free supernatant (CFS) was quickly separated and stored at −70°C pending analysis. The cellular pellet was resuspended in 2 ml of 5% (v/v) acetonitrile and sonicated for 2 min at 4°C using a Fisher 550. The sonicate was centrifuged at 3000 *g* (4°C) for 15 min and the supernatant stored at −70°C pending analysis (BAL sonicate). When the CFS and BAL sonicates were subsequently thawed, they were subjected to 3000 *g* centrifugation at ambient temperature, decanted and promptly analyzed.

2.2. Sample and standard extraction

The plasma and BAL sonicates were extracted by a modification of procedure used by Hutchings et al. [9] as follows: 500 μl of plasma or BAL sonicate was mixed with 200 μl of 20 g/dl sodium chloride and 5 ml chloroform–butanol (70:30, v/v). After vortexing briefly and mixing on a rotator for 10 min, the samples were centrifuged for 10 min at 3000 *g* and ambient temperature. The upper aqueous phase was discarded by aspiration and the INH back-extracted into 500 μl of 30 m*M* phosphoric acid. After mixing and centrifuging as above, the upper layer was partially neutralized with 10 μl of 4 *M* KOH and mixed with 10 μl of working internal standard (I.S.) before transfer to suitable vials for prompt HPLC analysis.

2.3. Calibration

Stock 1 mg/ml standard solutions of INH and I.S. (diphenylcarbazide) were formulated in 50% (v/v) methanol, stored at −20°C and appropriately diluted with saline to make fresh working standards when needed for spiking (see Section 3). Standard curves were prepared by spiking 1 ml of blank plasma or resuspended BAL sonicates, obtained from healthy

volunteers not taking INH, with various aliquots of working standard, up to a maximum of 50 μl (the volume was kept to 1 ml by the prior removal of the appropriate amount of matrix). Controls were formulated in a similar manner to the standards for the within-run precision study, but because of the instability of INH in plasma during long term storage at −20°C [11], saline spiked controls (0.5, 1.0 and 3 μg/ml) were used for day-to-day monitoring.

One ml aliquots of saline, spiked with appropriate volumes of working INH standard, were used to prepare the CFS calibration curve and controls.

2.4. HPLC

A Waters WISP autosampler (Waters, Milford, MA, USA) was used in conjunction with a Phenomenex (Torrance, CA, USA) CN reversed-phase column (250×4.6 mm, 5 μm, ambient temperature) for the analysis of the plasma and BAL sonicate extracts. The mobile phase consisted of 70% 50 m*M* KH_2PO_4, 30% acetonitrile–isopropanol (4:1, v/v) flowing at 0.8 ml/min. Peak detection was accomplished with an ESA (Chelmsford, MA, USA) electrochemical detector set at +0.6 V. Twenty-five μl aliquots of the CFS obtained from the pulmonary lavage were directly injected into the HPLC instrument which was equipped with a Polymer Labs. (Amherst, MA, USA) polystyrene–divinylbenzene column (250×4.6 mm, 5 μm, ambient temperature). A mobile phase consisting of 5% acetonitrile and 15 m*M* ammonium hydroxide, flowing at 0.5 ml/min was used.

2.5. Data analysis

Peak areas were monitored and the linear calibration curves created without weighting for both HPLC systems using E Z Chrom software (Scientific Software, San Ramon, CA, USA). For nongraphical presentation, the calibration curves were reduced to a linear, least-squares equation ($y=a+bx$) using a TI-85 graphics calculator (Texas Instruments, Dallas, TX, USA). The ratio of the peak areas (INH/I.S.) was used as the x-variable parameter in the linear equation for the plasma/BAL sonicate standard curves and the INH peak area only was used for the saline-based CFS linear equation. The standard deviation (S.D.) of the residuals ($S_{y/x}$), used here to indicate deviation from the least-squares line, was calculated for each calibration curve using these equations and a standard formula $S_{y/x}=\sqrt{\Sigma(\Delta y)^2/n-2}$, where y is the INH concentration and n is the number of data points on the curve.

2.6. Materials

The solvents and salts used to prepare the mobile phases and extraction solutions were purchased from Fisher Scientific (Santa Clara, CA, USA). The I.S. and INH standard were from Sigma (St Louis, MO, USA). The INH 300 mg capsules given to the subjects were from Barr Labs. (Pomona, NY, USA).

3. Results

Fig. 1a and Fig. 1b show typical chromatograms obtained when 15 μl of blank and spiked plasma (0.5 μg/ml) extracts were analyzed for INH as described. The INH (retention time, t_R: 4.7 min) and I.S. (t_R: 8.3 min) peaks were clearly separated from each other and from potentially interfering compounds. A comparison with a pure 50 μg/ml INH standard in mobile phase revealed that the extraction procedure had a recovery of 76%. When plasma was extracted and analyzed without the addition of I.S., no peaks were obtained in the region of the chromatogram where I.S. usually appears (8.3 min). Fig. 1c is a chromatogram from the 1 h plasma of an INH-treated subject showing the presence of INH at 4.7 min.

Fig. 2a and Fig. 2b show typical chromatograms for blank and spiked (50 ng/ml) BAL sonicate. Fig. 2c depicts the chromatogram for the BAL extract obtained from a subject who was maintained on the five day INH regimen. The t_R values of the INH and I.S. were similar to that obtained with plasma extracts. Although Figs. 1 and 2 only show the chromatogram up to 14 and 17 min, respectively, each analysis was run for 30 min, revealing no further peaks.

Fig. 3a and Fig. 3b show the chromatograms obtained with blank and spiked saline (50 ng/ml), respectively. In this direct injection HPLC system, the INH peak appeared at 14.6 min. Since no I.S.

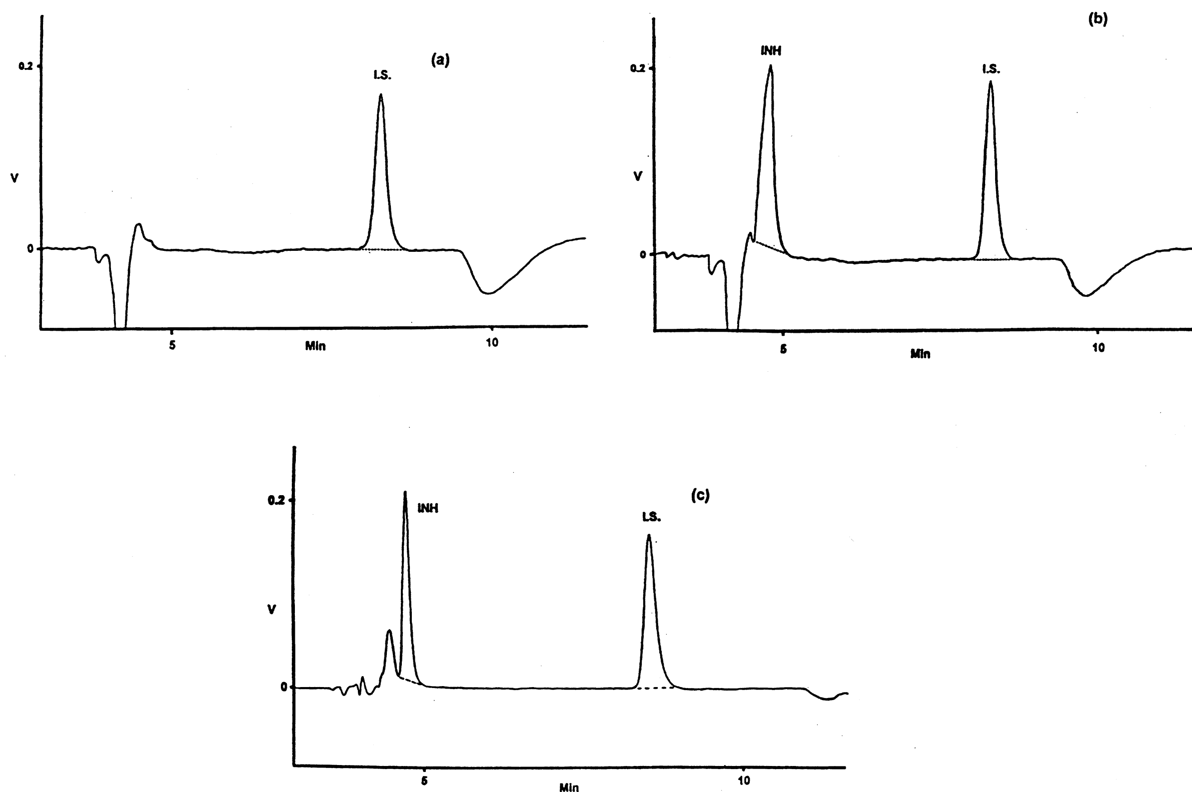


Fig. 1. Typical plasma INH chromatograms: (a) blank plasma, (b) plasma containing 0.5 μg/ml of INH and (c) plasma from a subject 1 h post 300 mg INH.

was used for these analyses, duplicate injections were performed. CFS from INH-treated subjects gave chromatograms similar to that depicted in Fig. 3b, but with varying peak sizes corresponding to the INH peak at 14.6 min (chromatogram not shown). As seen in Fig. 3a, the direct injection method gave a clean baseline in the INH region (14.6 min) when saline alone was used.

A linear calibration curve prepared using eight 1-ml aliquots of pooled blank plasma spiked with varying volumes of INH working standard in the range 0.1 to 10 μg/ml had the following regression characteristics: μg/ml = 0.03 + 0.37·area ratio. S.D. of the residuals ($S_{y/x}$) = 0.25, and correlation coefficient (r) = 0.998. Similarly, a calibration curve prepared with five 1-ml aliquots of BAL extract obtained from an INH-naive subject in the range 25 to 100 ng/ml had the regression equation: ng/ml = −0.84 + 80.0·area ratio, $S_{y/x}$ = 0.42, and r = 0.993. The CFS calibration curve was prepared by spiking five 1-ml aliquots of saline with INH in the range 10–100 ng/ml and analyzing in duplicate by HPLC using the direct injection technique. The regression equation was: ng/ml = $-7.73 + 9.2 \cdot 10^{-5}$·peak area, $S_{y/x}$ = 5.7, r = 0.991.

Table 1 shows the performance characteristics of the method when plasma, BAL sonicate and saline samples were spiked with appropriate volumes of working INH and repeatedly analyzed as described. The results shown are the mean ± 1S.D. Although an unacceptable C.V. of 15.7% was found at a plasma concentration of 50 ng/ml, when concentrations of 0.1, 0.5, 1.0 and 5.0 μg/l were repeatedly analyzed, C.V.s of 8.9, 7.2, 4.4 and 4.9% and recoveries of 97, 108, 107.8 and 98.4% were obtained, suggesting good within-run precision at these plasma concentrations (see Table 1). When the inter-day precision of the plasma INH assay was monitored on 12

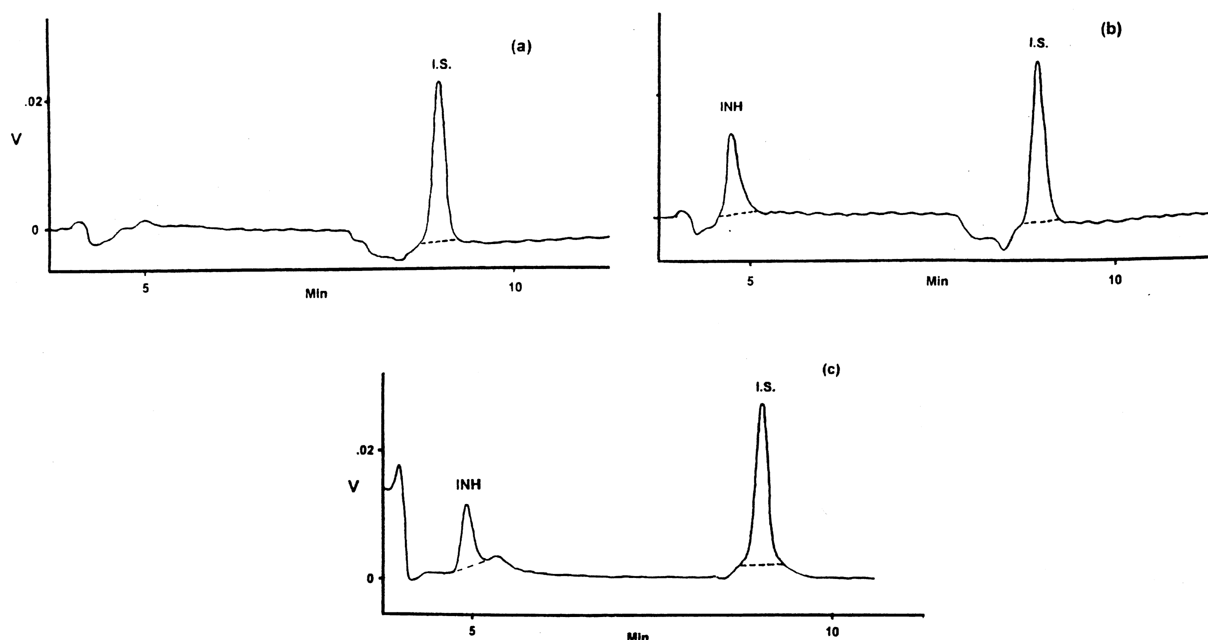


Fig. 2. Typical INH chromatograms from BAL sonicates: (a) blank, (b) BAL sonicate containing 50 ng/ml of INH and (c) BAL sonicate from a subject maintained on a standard INH regimen.

separate days using saline spiked controls, C.V.s of 7.6 (0.5 μg/ml), 4.9 (1.0 μg/ml) and 3.8% (3.0 μg/ml) were obtained, suggesting that the method has good reproducibility. Table 1 also shows the precision of the method for the assay of INH in BAL sonicates spiked at 10 and 50 ng/ml. The C.V.s suggest that the method could be used reliably to detect INH above 50 ng/ml in this matrix. However, at 10 ng/ml an unacceptable C.V. was obtained (18.5%) and the analytical recovery increased to 146%. When saline was spiked with INH and the direct injection technique used, acceptable precision and recovery was obtained at 10 and 50 ng/ml, indicating that the drug could be detected as low as 10 ng/ml in the cell-free saline lavage.

Table 2 lists the results obtained when INH was analyzed in various fluids obtained from subjects 4 h following the oral administration of 300 mg of the

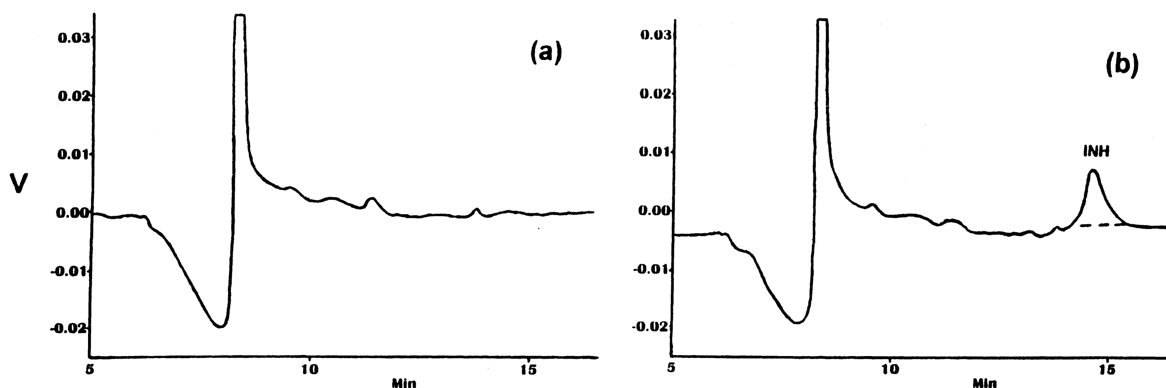


Fig. 3. Typical saline INH chromatograms: (a) blank, (b) saline containing 50 ng/ml of INH.

Table 1
Precision data for INH analysis in plasma, BAL sonicate and saline

Theoretical INH concentration (ng/ml)	Analyzed INH concentration (ng/ml)	Analytical recovery (%)	n	C.V. (%)
Plasma				
50	49±7.7	98	10	15.7
100	97±8.6	97	11	8.9
500	540±39	108	16	7.2
1000	1078±48	107.8	16	4.4
5000	4921±241	98.4	11	4.9
BAL sonicate				
10	14.6±2.7	146	15	18.5
50	54.8±6.7	109.6	15	12.2
Saline				
10	11.3±1.2	113	17	11.0
50	51.2±3.1	102.4	17	6.0

drug and the performance of bronchopulmonary lavage. The results shown are the mean±1S.D. In the 15 patients studied, no significant difference in plasma INH concentration was found between HIV-positive male subjects and healthy male controls when the unpaired *t*-test was applied ($p>0.05$). Detectable amounts of INH were found in the CFS and BAL extracts from the pulmonary lavages, but there was no significant difference in the two groups of patients in this preliminary study. Interestingly, the results suggest that healthy female subjects might have higher INH concentrations than healthy males in each compartment, although a more extensive study is needed to show a statistically significant difference.

Table 2
Isoniazid concentrations in pulmonary CFS, BAL sonicate and plasma of HIV-positive and negative patients

Gender	HIV status	n	CFS (ng/ml)	BAL (ng/ml)	Plasma (μg/ml)
M	+	5	26.8±14.3	38.6±25.2	2.7±2.6
M	−	4	22.5±3.7	34.5±31.3	1.9±0.8
F	−	6	34.0±8.7	36.3±8.7	4.0±2.8

4. Discussion

The purpose of this study was to demonstrate that the performance characteristics of our HPLC methods were adequate for the routine assay of plasma INH 1 h post oral administration of the drug and for assessing the minute concentrations of INH present in alveolar cells and cell-free fluid obtained by bronchoalveolar lavage from subjects maintained on a five day standard regimen of the drug.

The back extraction technique, first published by Hutchings et al. [9] utilizes the imino and hydrazine groups to alternately depolarize and polarize the INH molecule resulting in a clear aqueous extract from plasma or cellular sonicate. The cyano reversed-phase column retains the basic INH, allowing acidic and neutral compounds to pass through faster and resulting in a remarkably clean chromatogram. Enhanced specificity is achieved by using an electrochemical detector which is only responsive to a small subclass of compounds which are electrochemically active. Furthermore, the reliability of the analytical cell and the consistent detector response is a significant factor in the reproducibility and ruggedness of the overall assay.

Because diphenylcarbazide is not recovered with the back-extraction technique, we had to add the I.S.

to the final extract instead of to the original sample. Although iproniazid has been used successfully as an I.S. by Hutchings et al. [9], the compound was not well separated from INH in our isocratic system. Since INH in plasma is known to be unstable when stored at −20°C [11], we minimized this problem by extracting the samples within two days of receipt.

Saline based controls were used for day-to-day assay monitoring since we found that INH did not deteriorate when stored in saline for up to three months. In pharmacokinetic analysis of 73 different subjects, many of whom were taking multiple medications related to the treatment of AIDS, we did not encounter any interference in plasma INH monitoring using this technique (Lee et al., in preparation).

The performance characteristics of the plasma assay suggest that 100 ng/ml is a conservative lower limit of sensitivity. Routine monitoring of patients on a standard regimen of INH with sampling close to the T_{max} (1 h) should not be a problem since the lowest level found in this study was 300 ng/ml. Because the BAL sonicate contains much less protein than plasma, a lower level of sensitivity can be achieved (25 ng/ml). On the other hand, since the supernatant obtained by centrifuging the bronchoalveolar lavage was free from protein contamination, we could utilize the direct injection technique to analyze the minute amount of INH typically present in this compartment. Because the extraction step with organic solvents was eliminated, a lower level of sensitivity was achieved. While the precision and linearity studies indicated that INH levels as low as 10 ng/ml could be detected in CFS, duplicate or triplicate injections should be used with peak averaging.

The plasma INH concentrations found in this study at 1 h varied from 0.3 to 7.1 μg/ml for all subjects studied. This finding is comparable to a range of 1–2 μg/ml found by Sadeg et al. [12] and a range of 2.6–5.5 μg/ml reported by Seth et al. [13]. There was no significant difference in the mean INH concentrations between the three patient groups in this preliminary study. The analytical results from the cellular and supernatant fractions of the alveolar lavage show that this method can be successfully used to detect the very low INH concentrations typically present in these compartments. The feasibility of using this HPLC technique to study the intracellular pharmacokinetics of INH migration into pulmonary macrophages in HIV infection is also indicated. More extensive studies are being considered which will explore differences in INH availability at the cellular level in AIDS patients.

Acknowledgements

The authors wish to acknowledge the laboratory assistance of Ms Farzaneh Raoufi, B.S. whose diligence was invaluable in performing these analysis. The role of the clinical staff in the Department of Epidemiology and in the General Clinical Research Center is also gratefully acknowledged. Supported by a grant from the Division of Research Resources, US Public Health Service (5 MO1 RR-00079) and a grant from the NIH (AI 36054).

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