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Quantification of isoniazid and acetylisoniazid in rat plasma and alveolar macrophages by liquid chromatography–tandem mass spectrometry with on-line extraction

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Abstract

To evaluate if pulmonary delivery of microparticles loaded with a prodrug of isoniazid (INH), isoniazid methanesulfonate (INHMS), can target alveolar macrophages (AM) and reduce metabolism of INH, an HPLC–MS/MS assay with automated online extraction for quantification of INH and its metabolite acetylisoniazid (AcINH) in plasma and AMs was developed and validated. Reproducibility in rat plasma and homogenate of a rat AM cell line, NR8383, for INH and AcINH showed excellent precision and accuracy with calibration curves exhibiting linearity within a range of 1–250 ng/ml of INH and 0.05–50 ng/ml of AcINH ($r^2 > 0.99$). The validated methods were successfully applied to pharmacokinetic study of INHMS-loaded microparticles in rats, demonstrating efficient targeting of AMs and reduction of INH metabolism.

Keywords: Acetylated isoniazid; Isoniazid; LC/LC–MS/MS; Microparticles; PLA; Pharmacokinetics; Tuberculosis

1. Introduction

Tuberculosis (TB) is a communicable infectious disease caused by inhalation of micro-droplets containing tubercle bacilli, *Mycobacterium tuberculosis* (*M. tuberculosis*) [1]. Although modern chemotherapy has been used to treat TB for over 50 years, TB is still the leading cause of death in the world from a single infectious disease and is projected to remain one of the world's top 10 causes of adult mortality in the year 2020 [2,3]. A reason for the unsuccessful TB control is the patients' low level of adherence to the prescribed therapy, which is partly due to the frequent dosing [4] and significant side effects [5] of anti-TB medications.

To minimize toxicity and improve patients' compliance, biodegradable polymeric microparticles have been intensely explored as carriers for sustained and/or targeted delivery of anti-TB drugs [6–18]. Interestingly, despite intense research in this area, there has been no report examining the effects of delivering these microparticles directly to the AM on metabolism of anti-TB drugs. To address this issue, sensitive and efficient methods capable of determining very small amount of INH and its metabolite in limited AM samples collected from either human or animals are needed.

Several simple color tests are available for simple qualitative analysis of INH [19–21], but results from these tests must be interpreted with caution as many other drugs give similar reaction. More advanced HPLC analytical techniques were later

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developed for qualitative and quantitative analysis of INH and AcINH in biological specimens. These assays used UV [22–25], fluorimetric [26,27], or electrochemical [28–30] methods for detection of INH and its metabolites. Although more specific, the sensitivity of these methods was not very good. Under the reported chromatographic conditions, the methods were only sensitive enough to determine INH and its metabolites in the mg/L range. This can pose potential problems for intracellular detection of INH and/or its metabolites, which may be present in AMs at extremely low levels.

Because of its specificity and sensitivity, tandem MS coupled with chromatography is a rapidly growing method for analysis of drugs in different matrices. Tandem MS coupled with GC [31], LC [32–34], hydrophilic interaction chromatography [35] have recently been reported for quantification of INH. Although very sensitive, none of the reported methods was designed to analyze metabolism of INH. A GC–MS method was reported in a much earlier study for determination of INH and its metabolites [36]. Despite its modest sensitivity (in the range of 0.01–2 μg/ml), the method required deuterated internal standards, tedious extraction and double-derivatization procedures. In this study, we reported the development and validation of a simple but highly sensitive LC–MS/MS method for the quantification of INH, and its metabolite, AcINH, in rat plasma and homogenate of a rat AM cell line, NR8383, and applied these methods to the pharmacokinetic study of INH-loaded microparticles in rats. Our data indicated that (A) the analytical methods are accurate and in compliance with the acceptance criteria as set forth in current guidance documents issued by the US Food and Drug Administration, and (B) microparticles prepared by PCA can efficiently target INH to AMs and reduce INH metabolism.

2. Experimental

2.1. Materials

INH and the internal standard iproniazid (IPN, Fig. 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). IPN was of chemical reagent grade with ∼100% purity. AcINH (Fig. 1) was synthesized in-house using the method previously described by Fox and Gibas [37]. An ionizable prodrug of INH, isoniazid methanesulfonate (INHMS), was synthesized from INH using our previously described method [38]. Microparticles were prepared from poly(L-lactide) (PLA, MW = 137,000, inherent viscosity = 1.00 dl/g) that was purchased from Birmingham Polymers, Inc. (Birmingham, AL, USA). Methanol and water were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was of reagent grade and was purchased from Fisher Scientific. Rat plasma was obtained from Harlan Bioproducts for Science (Indianapolis, IN, USA). All other chemicals were obtained from Fisher Scientific.

2.2. NR8383 cell culture

NR8383 (ATCC CRL-2192, Rockville, MD), a rat AM cell line that exhibits many characteristics of AMs, was cultured in F-12K medium (Gibco) supplemented with 15% fetal calf serum and 1% antibiotics as previously described [39,40].

2.3. Instrumentation

The samples were analyzed using an LC/LC–MS/MS system. The two HPLC systems consisted of the following components: HPLC I: G1312A binary pump, G1322A degasser and G1329 autosampler equipped with a G1330 thermostat; HPLC II: G1312A binary pump, G1316A column thermostat (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) and the two HPLC systems were connected via a six-port column switching valve (see Fig. 2). The MS/MS system was an Applied Biosystems/Sciex API4000 (Applied Biosystems, Foster City, CA, USA).

2.4. Preparation of working solutions and samples

2.4.1. Standard and stock solutions

Stock solutions of INH, AcINH, and IPN were prepared at a concentration of 10 mg/ml by three independent weightings and dissolving each compound in HPLC-grade water containing 0.1% formic acid. These solutions were stored at −80 °C between uses. Working calibrator and quality control sample solutions were freshly made from the frozen stock solutions by appropriate dilution using HPLC-grade water containing 0.1% formic acid.

2.4.2. Rat plasma and alveolar homogenate samples

Calibration curves and quality control samples were prepared by spiking INH or AcINH working solutions to blank rat plasma (INH: 1, 2.5, 5, 10, 25, 50, 100, 250 ng/ml; AcINH: 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 ng/ml) or homogenates of NR8383 cells (INH: 2.5, 10, 25, 50, 100 ng/ml; AcINH: 0.25, 0.5, 1,

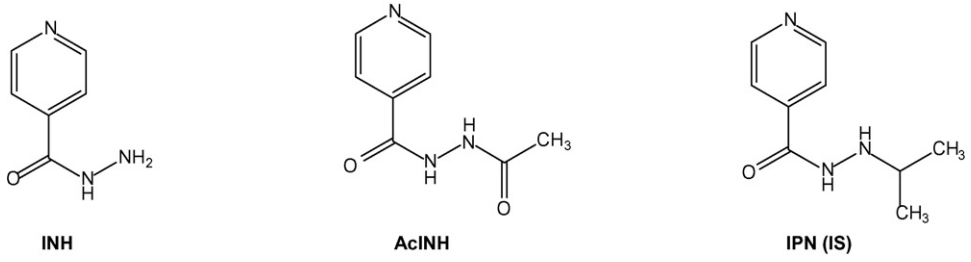

Fig. 1. Chemical structures of INH, AcINH and the internal standard IPN.

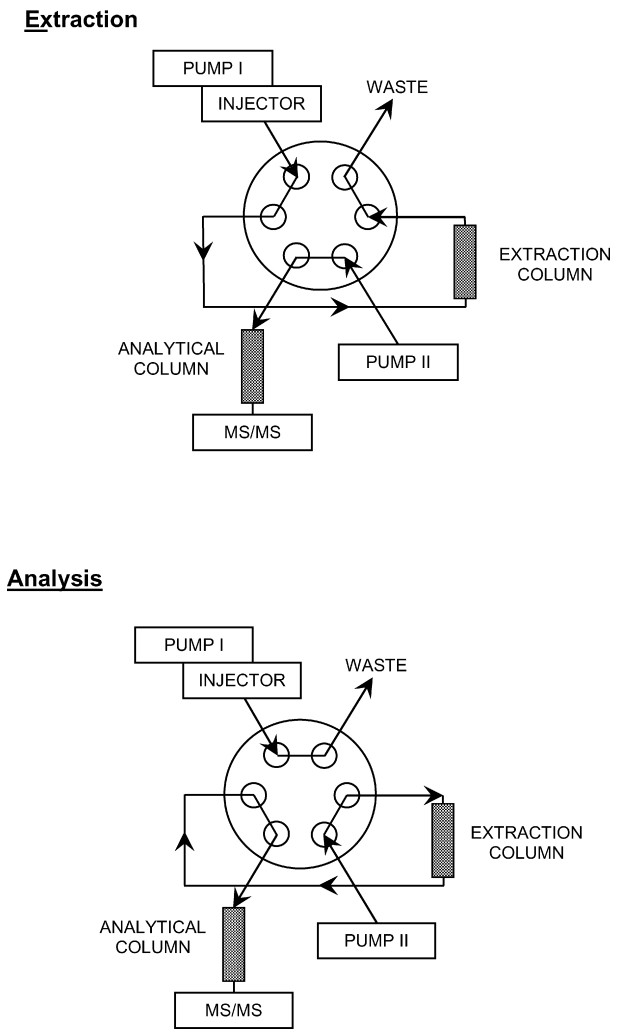

Fig. 2. Connection of the column-switching valve. HPLC I: pump I, injector, extraction column; HPLC II: pump II, analytical column, tandem mass spectrometer (MS/MS).

2.5, 5, 10, 25 ng/mL). NR8383 homogenates were obtained by subjecting harvested NR8383 cells suspended in HPLC-grade water containing 0.1% formic acid to sonication on ice. To allow distribution of drugs in plasma and AM homogenates, samples were incubated on ice for 10 min. Then 100 μl aliquots were transferred into 1.5 ml conical polypropylene tubes with snap-on lids and analyzed immediately.

2.5. Extraction procedure and tandem mass spectrometry

To 100 μl of plasma or AM homogenate sample, 20 μl of the internal standard IPN working solution was added. The plasma and AM homogenate were deproteinized with 400 μl of acetonitrile, and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to a fresh conical polypropylene tube and was evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 1 ml of water containing 0.1% formic acid and centrifuged at 10,000 rpm at 4 °C for 30 min prior to injection into the LC/LC–MS/MS system.

Samples (100 μl) were loaded onto an extraction column (4.6 mm × 12.5 mm, 5 μm particle size, Eclipse XDB-C8, Agilent) and washed with 0.1% formic acid at a flow rate of 1 ml/min. The temperature of the extraction column was set to 30 °C. After 0.14 min, the switching valve was activated (Fig. 2) and the analytes were backflushed onto the analytical column (3 mm × 300 mm, 3 μm particle size, Aquasil C_{18}, Thermo Hypersil-Keystone, Bellefonte, PA, USA). The mobile phase consisted of methanol and 0.1% formic acid in water. The analytes were eluted with the following gradient program (Table 1): 0–6 min, 8% methanol; 6–9 min, 8–98% methanol; 9–16 min, 98% methanol; 16–18 min, 98–8% methanol; hereafter, the column was re-equilibrated to starting conditions. The flow rate was 0.2 ml/min. The analytical column was maintained at 30 °C.

The Applied Biosystems/Sciex API4000 mass spectrometer fitted with a turbo ionspray interface source was used as detector. Positive ions were monitored. The turbospray gas was nitrogen at 8 l/min at 350 °C. The flow rates of curtain, nebulizing and collision gases were set to 30 and 12, respectively. For quantification, the MS/MS was operated in the multiple reaction monitoring (MRM) mode to monitor the internal standard, INH, and AcINH, with the dwell time set at 500 ms for each mass transition. The system was controlled and data were collected and processed using Analyst Software (version 1.3.1., Applied Biosystems).

2.6. Method validation

2.6.1. Acceptance criteria

The assay was considered acceptable if precision (% coefficient of variance or % C.V.) at each concentration was less than 15% for intra- and inter-day variability. The accuracy compared to the nominal value should be within ± 15% for intra- and inter-day comparison. The calibration curve should have a correlation coefficient r of 0.99 or better.

2.6.2. Calibration curve

Six samples of each concentration were measured. Calibration curves were obtained by plotting the peak area ratios of analyte to internal standard versus the nominal analyte concentrations. Linearity of the calibration curves was assessed using regression analysis implemented in the Analyst software without weighting.

2.6.3. Lower limit of quantitation (LLOQ)

The LLOQ was determined as the lowest quantity consistently achieving an intra-day accuracy ≤±20% of the nominal concentration, an intra-day precision ≤20%.

2.6.4. Intra- and inter-day precision and accuracy

Intra-day precision and accuracy were determined by analysis of quality control samples ($n = 6$) extracted in one batch. For the determination of inter-day precisions and accuracies six replicates per concentration were extracted and analyzed on three different days (total: $n = 18$/concentration level and matrix). Precisions are reported as coefficient of variance in % and accuracies in % of the nominal concentration.

Table 1
Time program for solvent delivery pumps and valve switching

Time	HPLC I	HPLC II	Column Switching
0.00 min	0% methanol, 1 mL/min	8% methanol, 0.2 mL/min	Valve in extraction position
0.14 min	0% methanol, 1 mL/min		Valve switches to analysis position
1.00 min	100% methanol, 1 mL/min		
2.00 min			Valve switches back to extrraction position
3.00 min	100% methanol, 1 mL/min		
3.20 min	0% methanol, 0.01 mL/min		
6.00 min		8% methanol, 0.2 mL/min	
9.00 min		98% methanol, 0.2 mL/min	
16.00 min		98% methanol, 0.2 mL/min	
16.50 min		8% methanol, 0.2 mL/min	
20.00 min	0% methanol, 0.01 mL/min		
22.00 min	0% methanol, 1 mL/min	8% methanol, 0.2 mL/min	

2.6.5. Recovery

Recoveries were calculated from the quality control samples ($n = 3$). The extraction efficiency was determined by comparison of the mass spectrometer responses of the extracted samples with the responses after injection of respective amounts of internal standard or standard solutions of INH and AcINH directly on the analytical column, bypassing the extracting column.

2.6.6. Matrix interferences and carry-over effects

The lack of matrix interferences was established by analysis of blank plasma or AM samples ($n = 6$). The lack of carry-over effects was examined by alternately analyzing blank plasma or AM samples ($n = 6$) and plasma or AM samples containing concentrations of INH and AcINH at the upper limit of quantification (ULOQ) ($n = 6$). Ion suppression by the ion pair reagents and the matrices were assessed following the protocol described by Müller et al. [39]. In brief: Analysis of ion suppression was set up by continuously infusion of each analyte solution (10 μg/ml) separately by a syringe pump (KD Scientific, Holliston, MA) at a flow rate of 20 μl/min via a PEEK T-connector into the elute from the LC column. The effect of injecting blank extracted samples ($n = 10$ for each matrix, from 10 different animals or AM preparations) on the continuous signal produced by post-column infusion of the analytes was assessed.

2.6.7. Stability

The stability of INH and AcINH in rat plasma following 6, 12 and 48 h at room temperature, 4 °C, −20 °C or −80 °C was tested with quality control samples ($n = 3$) at two concentration levels (INH: 1 and 10 ng/ml; AcINH: 0.25 and 2.5 ng/ml). The stability was evaluated by the difference from the mean of initial concentrations, and expressed as the percentage remaining (%).

2.7. Application for pharmacokinetic study

2.7.1. Microparticle formation

PLA microparticles laden with INHMS were prepared and characterized using our previously described procedure [41]. Briefly, the resulting drug/polymer particles were nearly spherical in shape, with particle sizes ranging between 1 and 3 μm in diameter that are optimal for deep lung penetration and targeting of AMs. In general, a loading factor in the range of ∼6–30% of the total mass of the particles was obtained. The loading factor is defined as the actual weight% drug found in the microparticles.

2.7.2. Animal study

In order to assess the ability of the assay to simultaneously measure INH and its acetylated metabolite (AcINH) and to confirm that microparticles prepared with PLA and loaded with INHMS can indeed target AMs and reduce the metabolism of INH to AcINH, a pharmacokinetic study was conducted in male Sprague-Dawley rats (250–350 g each). Briefly, plasma and AMs were collected after oral administration of INH by gavage or INHMS-loaded microparticles by intra-tracheal instillation. Details regarding the procedures of drug administration and plasma/AM samples collection were recently reported elsewhere [41].

The animal study was approved by the local Institutional Animal Care and Use Committee (IACUC). Animals received humane care following current guidelines such as the "Guide

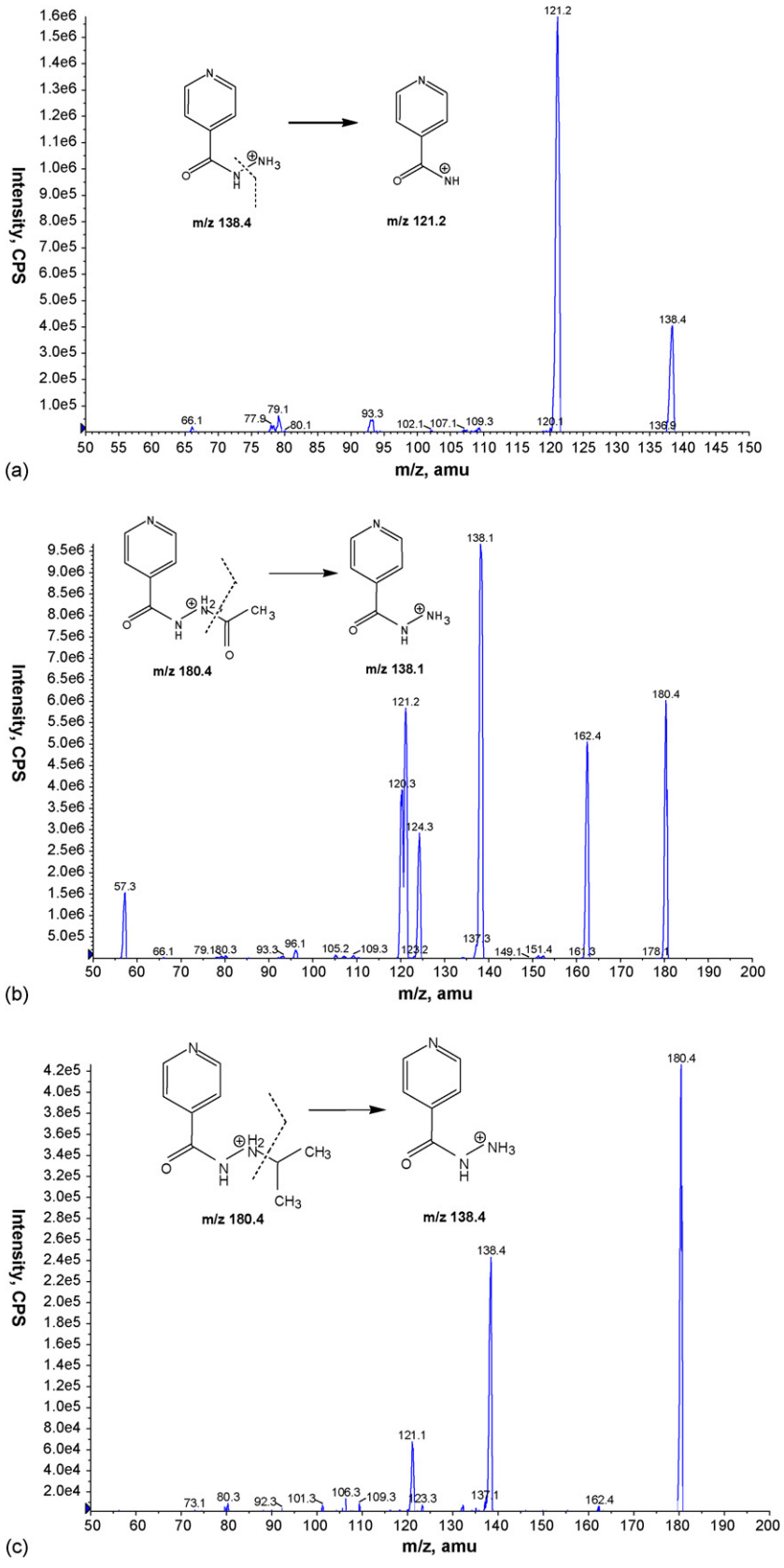

Fig. 3. Representative product ion mass spectra of (a) INH, (b) AcINH and (c) the internal standard IPN.

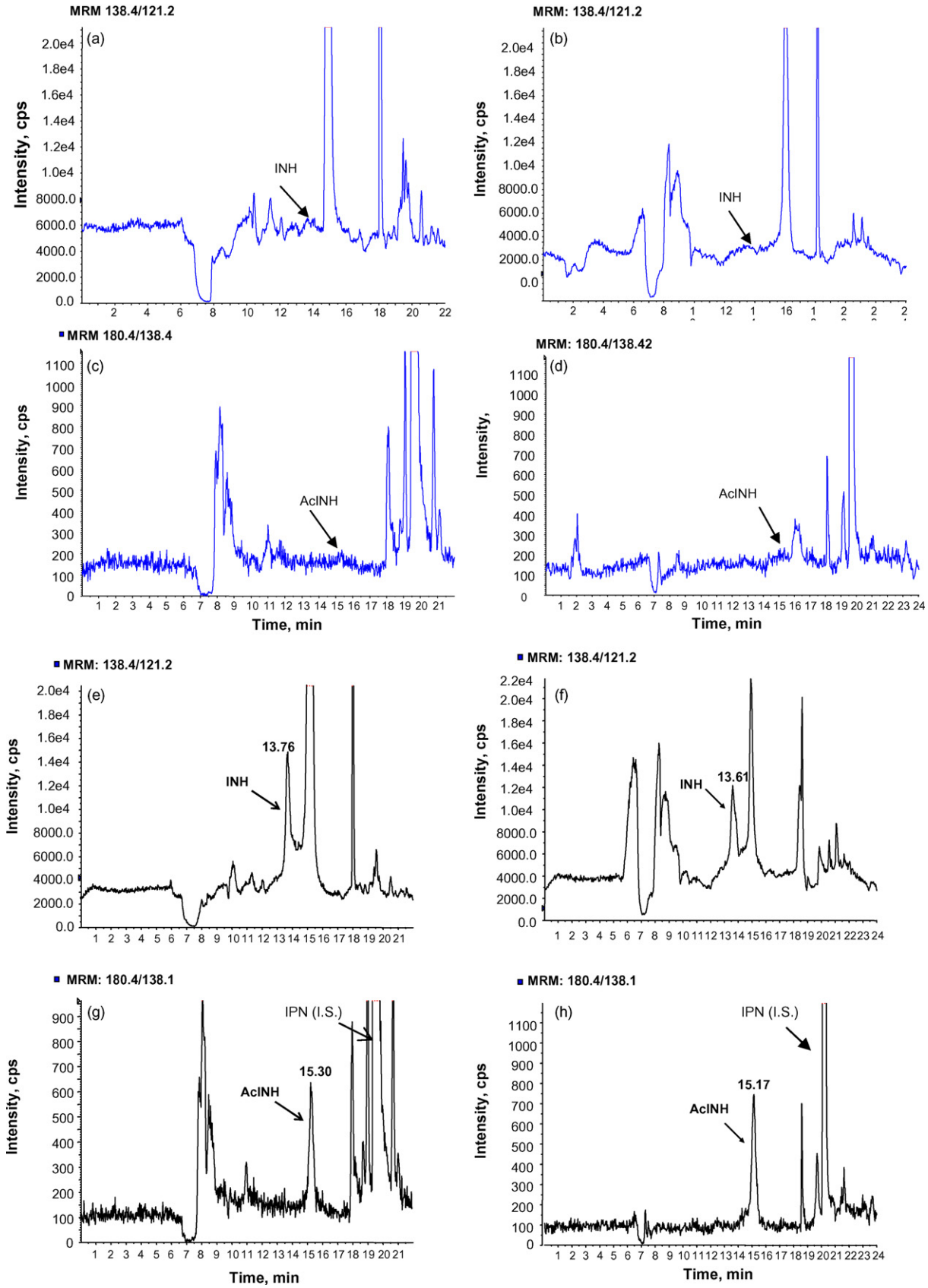

Fig. 4. Representative blank ion chromatograms and ion chromatograms at the lower limit of quantitation (LLOQ). (a) Monitoring $m/z = 138 \rightarrow 121$ (INH) after extraction of blank rat plasma; (b) monitoring $m/z = 138 \rightarrow 121$ (INH) after extraction of rat alveolar macrophages; (c) monitoring $m/z = 180 \rightarrow 138$ (AcINH) after extraction of blank rat plasma; (d) monitoring $m/z = 180 \rightarrow 138$ (AcINH) after extraction of blank rat alveolar macrophages; (e) INH (1 ng/ml) in rat plasma; (f) INH (2.5 ng/ml) in rat alveolar macrophages; (g) AcINH (0.1 ng/ml) in rat plasma; (h) AcINH (0.05 ng/ml) in rat alveolar macrophages.

for the Care and Use of Laboratory Animals" prepared by the National Institute of Sciences and published by the National Institutes of Health (NIH publication No. 80–123, revised 1985).

3. Results

3.1. High performance liquid chromatographic separation

INH and AcINH are hydrophilic compounds that are easily eluted from a normal reversed phase C_{18} column. Therefore, to chromatically resolve these compounds, we used a highly aqueous mobile phase and an Aquasil C_{18} column that provides appropriate retention and selectivity for polar compounds (Table 1).

3.2. Mass spectrometric detection

Secondary amino groups are present in both the structures of the internal standard and the analytes (Fig. 1). Therefore, the positive ion mode was used for MS detection of these compounds. Fig. 3 shows the mass spectra of the proton adduct $(M+H)^+$ peaks of INH at *m/z* 138 and AcINH and IPN at *m/z* 180. The following precursor → product ion transition of INH (*m/z* 138.4 → 121.2) and AcINH and IPN (*m/z* 180.4 → 138.1) were monitored. Although the same ion transition of *m/z* 180.4 → 138.1 was used for monitoring both AcINH and IPN, detection of these analytes was not a problem due to the difference in their HPLC retention times (Fig. 3). Representative MRM ion chromatograms of rat plasma or an AM homogenate sample spiked with INH, AcINH and the internal standard are shown in Fig. 4.

3.3. Validation

The LLOQ of INH was 1 ng/ml in plasma and 2.5 ng/ml in AM homogenate. The LLOQ of AcINH was 0.1 ng/ml in plasma and 0.05 ng/ml in AM homogenate. The upper limit of quantification of INH was 250 ng/ml both in plasma and in AM homogenate. The upper limit of quantification for AcINH was 50 ng/ml in plasma and 10 ng/ml in AM homogenate. Calibration curves were linear with correlation coefficients $r^2 > 0.99$ for both INH and AcINH.

Intra-day, inter-day precisions and accuracies were within the pre-defined acceptance limits (Table 2). No matrix interferences (Fig. 4) or carry-over effects were detected. The absolute recoveries of INH after protein precipitation were above 80% from both plasma and AM homogenate. The absolute recoveries of AcINH and of INH were ≥56% from plasma and ≥68% from AM homogenate (Table 2). Due to the high sensitivity for those analytes and accuracies and precisions within the acceptance limits, the relatively low absolute recoveries were not considered a problem.

INH has been shown to be stable in macrophage cultures [42] but unstable in blood even when blood samples were stored at −20 °C [43–45]. Because there is not much information available on the stability of this drug and its metabolite in plasma, we conducted stability study of INH and AcINH in plasma. Degradation of INH in plasma was observed as early as 6 h after storage at −20 °C (Fig. 5). However, no significant loss of INH was detected when the plasma samples were stored at −80 °C for up to 48 h (Fig. 6). Based on this information, all plasma samples in the current study were stored at −80 °C and analyzed within 2 days upon their collection from animals. In contrast, AcINH showed good stability in plasma and no significant degradation of AcINH was detected even after the plasma samples were kept at room temperature for 48 h (Fig. 6).

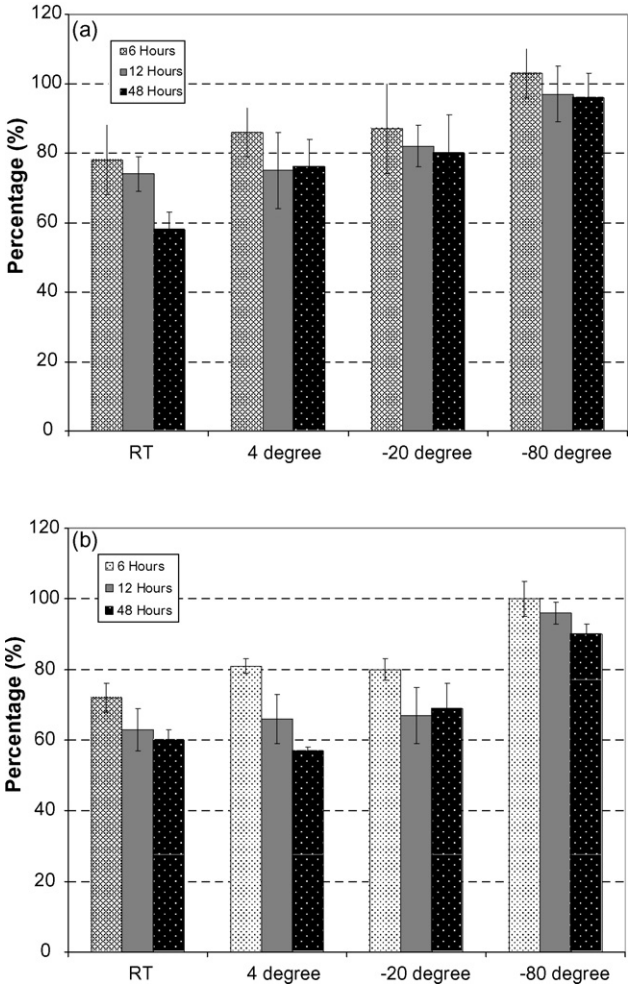

Fig. 5. Stability of INH in rat plasma: (a) INH = 1 ng/ml; (b) INH = 2.5 ng/ml. Data are presented as means ± standard deviations ($n = 3$).

3.4. Ion suppression effects of hydrophobic ion pair agent or the matrices on INH and AcINH

Because ion pairing agents are known for their ion suppression effect [46] and were employed for efficient incorporation of INHMS (prodrug of INH) into polymeric microparticles in the present study [41], spiked plasma and AM homogenate samples supplemented with HIP were used to verify the ion suppression effect of THA. Chromatograms of plasma and AM homogenate samples spiked with the hydrophobic ion pairing agent THA were not significantly different from controls that lacked THA,

Table 2
Summary of the calibration curve parameters, intra- and inter-assay precision and accuracy, and recovery of INH and AcINH in rat plasma and alveolar macrophage homogenate

	INH		AcINH	
	Plasma	Alveolar macrophage homogenate	Plasma	Alveolar macrophage homogenate
Lower limit of detection (ng/ml)	0.25	1	0.025	0.025
Linearity range (ng/ml)	1–250	2.5–250	0.1–50	0.05–10
Regression analysis	$y = 0.124x + 0.00283$ ($r = 0.9992$, $n = 5$)	$y = 0.685x + 3.4e{-}006$ ($r = 0.9979$, $n = 5$)	$y = 0.0211x + 0.0356$ ($r = 0.9986$, $n = 5$)	$y = 1.15x + 0.0347$ ($r = 0.9988$, $n = 5$)
Intra-day accuracy ($n = 6$)	1 ng/ml −2.6% 10 ng/ml −3.9% 250 ng/ml +2.0%	2.5 ng/ml −9.6% 25 ng/ml −5.6% 250 ng/ml 0%	0.1 ng/ml +8.0% 2.5 ng/ml +13.0% 50 ng/ml −0.5%	0.05 ng/ml +4.0% 0.5 ng/ml −2.9% 10 ng/ml +1.0%
Intra-day precision ($n = 6$)	1 ng/ml 9.1% 10 ng/ml 12.0% 250 ng/ml 1.3%	2.5 ng/ml 3.6% 25 ng/ml 7.9% 250 ng/ml 6.3%	0.1 ng/ml 7.9% 2.5 ng/ml 6.1% 50 ng/ml 6.3%	0.05 ng/ml 3.2% 0.5 ng/ml 3.0% 10 ng/ml 3.1%
Inter-day accuracy (3 days, $n = 18$)	1 ng/ml −3.1% 10 ng/ml +3.0% 250 ng/ml 0%	2.5 ng/ml −6.6% 25 ng/ml −8.7% 250 ng/ml −0.7%	0.1 ng/ml +4.0% 2.5 ng/ml +6.0% 50 ng/ml −0.7%	0.05 ng/ml +13.0% 0.5 ng/ml −5.9% 10 ng/ml +1.4%
Inter-day precision (3 days, $n = 18$)	1 ng/ml 7.3% 10 ng/ml 13.4% 250 ng/ml 7.1%	2.5 ng/ml 6.6% 25 ng/ml 8.7% 250 ng/ml 8.7%	0.1 ng/ml 7.6% 2.5 ng/ml 9.7% 50 ng/ml 5.0%	0.05 ng/ml 8.0% 0.5 ng/ml 11.0% 10 ng/ml 4.8%
Recovery	≥83%	≥81%	≥56%	≥68%

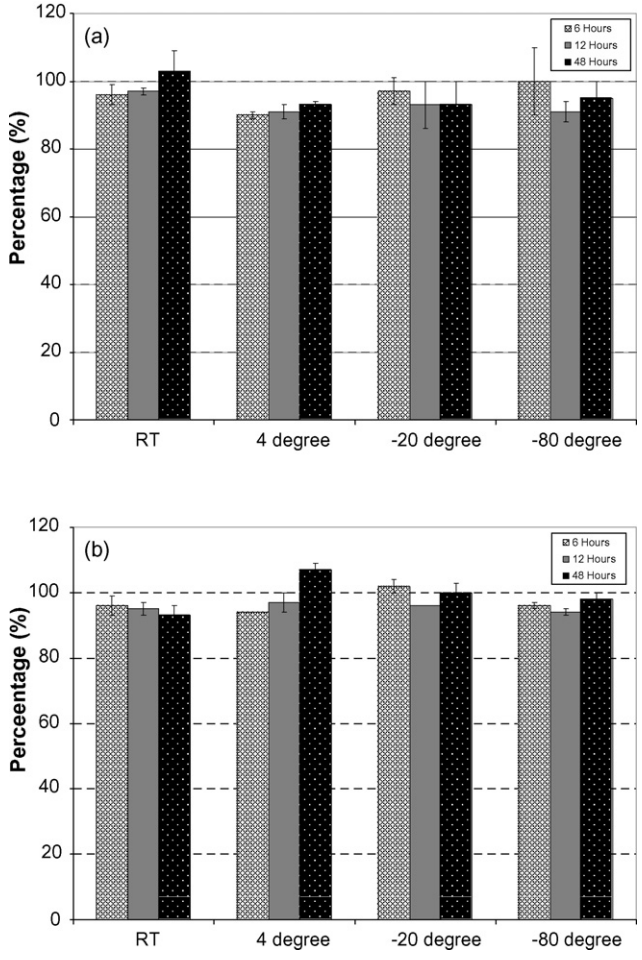

Fig. 6. Stability of AcINH in rat plasma: (a) AcINH = 0.1 ng/ml; (b) AcINH = 0.05 ng/ml. Data are presented as means ± standard deviations ($n = 3$).

suggesting that intensity of the ion signals was not suppressed by THA. The matrices during the retention time of the injection peaks, but did not cause ion suppression during elution of the analyte peaks or the internal standard. A representative ion suppression experiment is shown in Fig. 7.

3.5. Method application

After the validation, the analytical method was successfully used for the simultaneous determination of INH and its acetylated metabolite, AcINH, in rat plasma and lavaged AMs. Our published data indicate that only PLA microparticles loaded with INHMS provided sustained and targeted delivery of INH to AMs and that this method of delivery led to substantial reduction in the blood levels of AcINH [41]. Fig. 8 shows the representative ion chromatograms of INH and AcINH in plasma or AM homogenates obtained from Sprague Dawley rats administered either INH solutions by gavage (Fig. 8a) or INHMS in PLA microparticles by intra-tracheal instillations (Fig. 8b, intra-tracheal dose was 1.5% of the oral dose). It can be seen that when AcINH was present at high concentration, as in the case of rats given orally administered INH (Fig. 8a), a significant AcINH peak was observed. Because INH and AcINH were baseline resolved in the chromatogram, detection of INH in a matrix containing high amounts of AcINH was possible.

4. Discussion

The purpose of this study was to develop an analytical assay for a pharmacokinetic study in rats to confirm that drug-laden microparticles can target INH to AMs and, thereby, reduce the

production of AcINH, a major and potential toxic metabolite of INH. Due to the limited number of AMs that can be collected from each animal (<3 millions), a highly sensitive assay was therefore required for detection of the relatively low concentrations of INH and AcINH to be encountered in small numbers of AMs.

Because of their versatility, HPLC techniques with ultraviolet [27,42,47,48] or electrochemical [49] or fluorimetric detectors [50] have been employed by various investigators for detection of INH and/or its acetylated metabolites. These HPLC-based methods often rely on elaborate extraction schemes and/or chemical derivatization after sample purification to impart favorable elution and detection characteristics to INH. And in the case of acetylated INH, an inherent disadvantage of the chemical derivatization method is that AcINH must first be converted to INH before derivatization can occur [27]. Besides adding time to the analysis time, this method precludes simultaneous detection of INH and AcINH. Two research groups have recently reported a simple HPLC method with UV detection for simultaneous measurement of INH and its main acetylated metabolite AcINH in human plasma or urine matrixes [51,52]. The methods used either trichloroacetic acid deproteinization followed by separation on a μBondapak C_{18} column [51] or multi-steps centrifugation followed by filtration [52] to purify samples prior to HPLC analyses. Although a much more straightforward approach than the chemical derivatization approach, the methods suffered from sensitivity problems. The reported LLOQs of both methods showed sensitivities ranging from 0.125 to 8 μg/ml for INH and 0.125 to 16 μg/ml for AcINH, which was too insensitive for the purported study.

Due to its high selectivity and ability to handle small sample volumes with low concentrations of analytes and conclusively identify the analytes, HPLC coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC–MS/MS) is a frequently used analytical platform in the bioanalytical field. Despite these positive attributes, no assay has been reported before for simultaneous determination of INH and AcINH using the combination of LC and tandem MS. We report here a validated LC/LC–MS/MS method that is capable of simultaneous detection of INH and AcINH in both plasma and AM matrixes. Our analytical platform uses fast semi-automated column-switching techniques for on-line sample preparation. This approach significantly cuts down on the analysis time since the samples

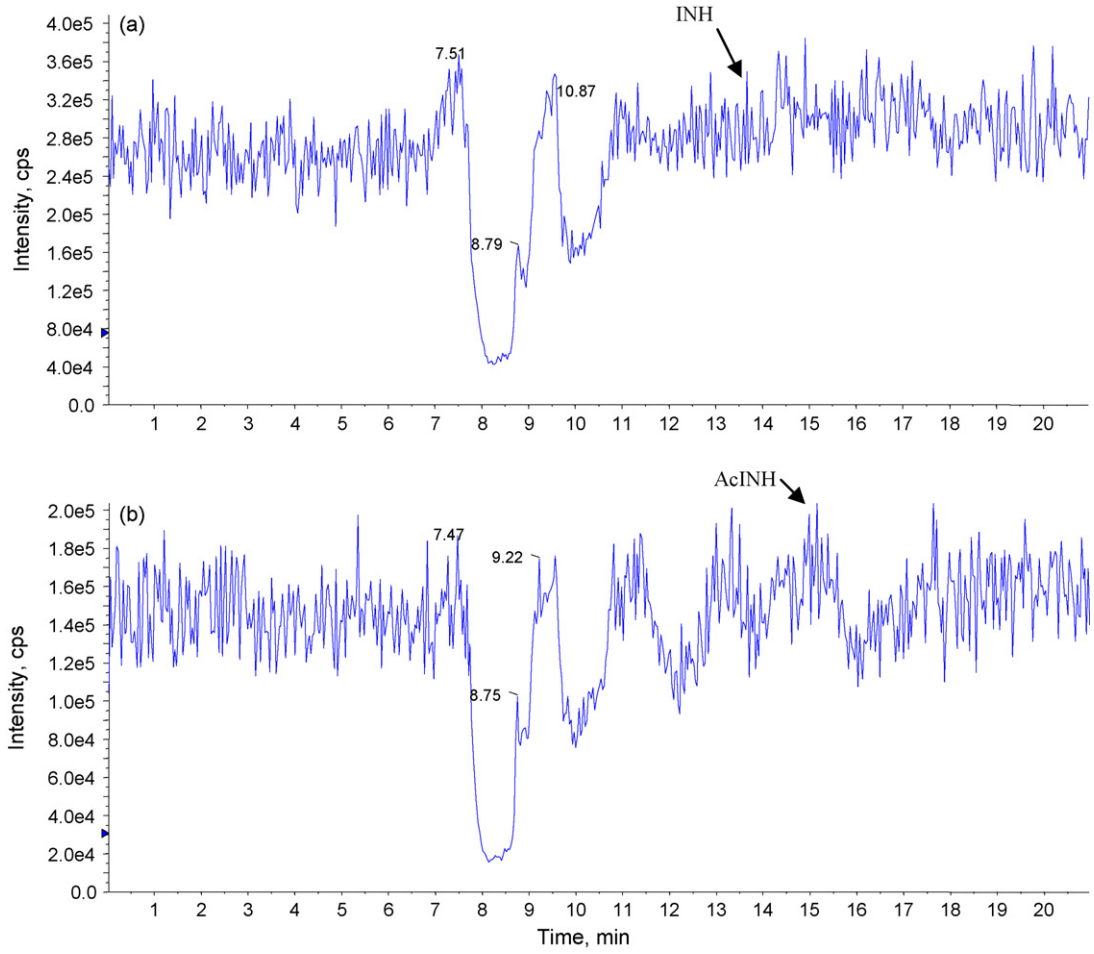

Fig. 7. Representative ion suppression experiment. Ion suppression was tested for each of the analytes and matrices collected from different animals and cell cultures with $n = 10$ blank matrix preparations. Matrix-induced ion suppression was detected at the retention time of the injection peak but not during elution of the analytes (retention times marked by arrows). During the shown experiment 100 μl extracted blank plasma was injected while INH (a) or AcINH (b) was continuously infused post-column using a syringe pump (20 μl/min of a 10 μg/ml water solution).

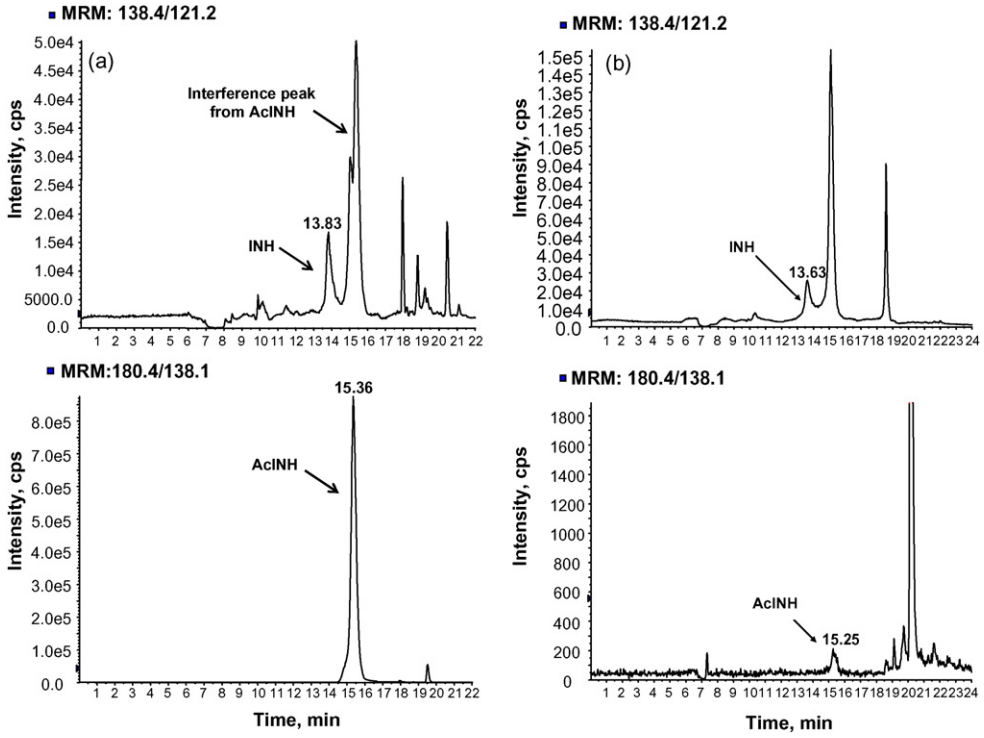

Fig. 8. Representative ion chromatograms of (a) a plasma sample collected from a rat 4 h after oral administration of 2 mg of INH (b) an alveolar macrophage sample collected from a rat 1 h after intra-tracheal instillation of 4 mg of INHMS-loaded PLA particles (containing 0.056 mg INHMS or 0.030 mg INH). The concentrations measured in (a) were 1.5 ng/ml INH and the AcINH concentration was above the upper limit of quantitation (=50 ng/ml). This sample was diluted 1:10 and re-analyzed. The concentration was found to be 186 ng/ml. The concentrations measured in (b) were 4.4 ng/ml INH and the AcINH concentration was below the lower limit of quantitation (=0.05 ng/ml).

containing both INH and AcINH can be run in the same batch and in the same run.

INH, a pivotal agent in the treatment of TB, is mainly metabolized to AcINH by genetically polymorphic arylamine *N*-acetyltransferase type 2 (NAT2), a hepatic phase II drug-metabolizing enzyme [53]. The acetylation polymorphism is associated with a larger inter-individual variation in both plasma concentration and half-life of INH [53,54]. Most existing studies indicate that not only INH concentrations but also efficacy [55] and toxicity [56–58] are linked to the activity of NAT2 enzyme. For instance "slow acetylators" are more likely to develop adverse reactions such as peripheral neuritis, hepatic toxicity, and systematic lupus erythematosus-like syndrome. In contrast, "fast" or "rapid acetylators" may undergo therapeutic failure and require taking larger doses of INH. Thus, measurement of INH and AcINH plasma concentration is necessary for the determination of the acetylator phenotypes and INH dosing adjustment. Although originally developed as a complementary assay to support our pharmacokinetic study of our newly developed drug delivery system, our LC/LC–MS/MS assay, due to its user-friendly attribute, can easily be adapted to the pharmacokinetic study of "rapid acetylators" or "slow acetylators" according to the patients' *N*-acetylation capacity.

5. Conclusion

A sensitive and accurate LC/LC–MS/MS assay for quantification of INH and AcINH in rat plasma and AM was developed and validated following the regulatory standards. This method provides a number of analytical advantages including its robustness, reliability, reproducibility, specificity and without time-consuming sample preparation procedures. In summary, the assay met all pre-defined performance acceptance criteria and was successfully used in our laboratory to confirm microparticles prepared with PCA and loaded with a prodrug of INH can target INH to AM and cut down on the production of a potentially toxic acetylated metabolite of INH.

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