Journal of Chromatography B, 878 (2010) 2286–2291

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Simultaneous determination of isoniazid, rifampicin, levofloxacin in mouse tissues and plasma by high performance liquid chromatography–tandem mass spectrometry

Ping-Fei Fang[a], Hua-Lin Cai[a,b], Huan-De Li[a,*], Rong-Hua Zhu[a], Qin-You Tan[a,b], Wei Gao[a], Ping Xu[a], Yi-Ping Liu[a], Wen-Yuan Zhang[a], Yong-Chang Chen[a], Feng Zhang[a]

[a] *Clinical Pharmacy and Pharmacology Research Institute, Second Xiangya Hospital, Central South University, Changsha 410011, China*
[b] *School of Pharmaceutical Sciences, Central South University, Changsha 410013, China*

ARTICLE INFO

Article history:
Received 18 May 2010
Accepted 25 June 2010
Available online 3 July 2010

Keywords:
Mass spectrometry
Tissue
Plasma
Isoniazid
Rifampicin
Levofloxacin

ABSTRACT

A rapid and selective high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for simultaneous determination of isoniazid (INH), rifampicin (RFP) and levofloxacin (LVX) in mouse tissues and plasma has been developed and validated, using gatifloxacin as the internal standard (I.S.). The compounds and I.S. were extracted from tissue homogenate and plasma by a protein precipitation procedure with methanol. The HPLC separation of the analytes was performed on a Welch materials C4 column (250 mm × 4.6 mm, 5.0 μm, USA) at 25 °C, using a gradient elution program with the initial mobile phase constituting of 0.05% formic acid and methanol (93:7, v/v) at a flow-rate of 1.0 ml/min. For all the three analytes, the recoveries varied between 83.3% and 98.8% in tissues and between 75.5% and 90.8% in plasma, the accuracies ranged from 91.7% to 112.0% in tissues and from 94.6% to 108.8% in plasma, and the intra- and inter-day precisions were less than 13.3% in tissues and less than 8.2% in plsama. Calibration ranges for INH were 0.11–5.42 μg/g in tissues and 0.18–9.04 μg/ml in plasma, for RFP were 0.12–1200 μg/g in tissues and 4.0–200 μg/ml in plasma, and for LVX were 0.13–26.2 μg/g in tissues and 0.09–4.53 μg/ml in plasma. The lower limits of quantification (LLOQs) for INH, RFP and LVX in mouse tissues were 0.11, 0.12 and 0.13 μg/g and for those in mouse plasma were 18.1, 20.0 and 21.8 ng/ml, respectively. The limits of detection (LODs) for INH, RFP and LVX in mouse tissues were 0.04, 0.05 and 0.05 μg/g and for those in mouse plasma were 5.5, 6.0 and 6.6 ng/ml, respectively. The established method was successfully applied to simultaneous determination of isoniazid, rifampicin and levofloxacin in mouse plasma and different mouse tissues.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The clinical treatment of tuberculosis commonly comprises combinatorial regimens of three or four drugs to prevent resistance and the treatment consisting of isoniazid (INH), rifampicin (RFP) and levofloxacin (LVX) is widely used [1]. Although a standardized short course of therapy is effective to the majority of tuberculosis patients, it has been shown that among them a small group of patients with tuberculosis respond poorly to the therapy [2–4]. It is well known that P-glycoprotein (P-gp) expressed in a large range of normal tissues as the drug efflux pump may add potential to the development of drug resistance as well in HIV [5] and cancer therapies [6], therefore this may also be possible in tuberculosis therapy [7]. Our group undertook a study with the purpose of investigating whether P-gp is involved during anti-tuberculosis treatment. In order to evaluate active transports of the anti-tuberculosis drugs into or outside the respective tissue or organ, we intended to develop an analytical method to test the drug concentrations in various tissues and plasma for comparison purposes. Over the years, many quantification methods for the individual determination of the three anti-tuberculosis drugs in biological matrices have been reported, but the very different polarities of the three drugs lead to a great difficulty in analyzing them by a single-run chromatographic separation. However, there have been a few reported assay methods for simultaneous quantification of two of the three drugs in different biological matrices recently [8–12]. A C18 column (250 mm × 4.6 mm or 50 mm × 2.0 mm) was used to separate compounds in most of these methods. However, the separations carried out on C18 columns result in unsatisfactory peak shape [11] and long retention time of the analytes so a more desirable kind of separation column should be chosen for

* Corresponding author. Tel.: +86 731 85292095; fax: +86 731 84436720.
E-mail addresses: fangpingfei@163.com, lihuande1953@126.com, chinesepharm@yeah.net (H.-D. Li).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.06.038

the analysis. Although two studies on analysis of anti-tuberculosis drugs in tissues have been published to date [12,13], they require large amounts of sample and rather complex clean-up procedures, which is not practical for high-throughput analysis of a lot of samples due the time of extraction. As far as we know, no method that can simultaneously measure the three drugs in biological matrices has been developed. The aim of this study was to develop and to validate a rapid, selective and low-cost HPLC–MS/MS method for high-throughput and simultaneous determination of INH, LVX and RFP in mouse plasma and different tissues including brain, lung, liver, kidney and small intestine.

2. Experimental

2.1. Equipments, materials and reagents

The chromatography was performed on an ACQUITYTM UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven enabling temperature control of analytical column. Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro™ API mass spectrometer (Waters Corp.) with an electrospray ionization (ESI) interface. INH (99.5% purity), RFP (99.0% purity), LVX (97.3% purity) and internal standard (I.S.) gatifloxacin (98.0% purity) were purchased from National Institute for The Control of Pharmaceutical and Biological Products (Beijing, China). The structures of these compounds are presented in Fig. 1. Methanol (HPLC grade) was purchased from Caledon Laboratories Ltd. (Georgetown, Canada). Formic acid (AR grade) was purchased from Chemical Reagent Factory of Hunan (Changsha, Hunan, China). Distilled water was prepared by a laboratory purification system, and filtered through 0.22 μm membrane filter before use. Kunming mice (male and female; aged 2 months; weight 18–20 g) were obtained from the Experimental Animal Center of the Second Xiangya Hospital of Central South University, and housed under specific pathogen-free (SPF) conditions.

2.2. Preparation of stock solutions, calibration standard and quality control samples

The primary stock solutions were prepared in methanol (452 μg/ml for INH, 2000 μg/ml for RFP, 272 μg/ml for LVX and 96 μg/ml for I.S.). Working solutions were obtained by sequentially diluting the stock solutions with methanol. All the standard solutions were stored at −20 °C. Calibration curves were prepared in blank mouse tissue homogenate mixture (brain, liver, lung, kidney and small intestine). Appropriate volumes of working solutions were added to tissue homogenate mixture or plasma to yield seven-point calibration curves for the analytes. The concentrations of the standards are shown in Table 1. Quality control (QC) samples that were run in each assay were prepared at three concentration levels which are bolded in Table 1.

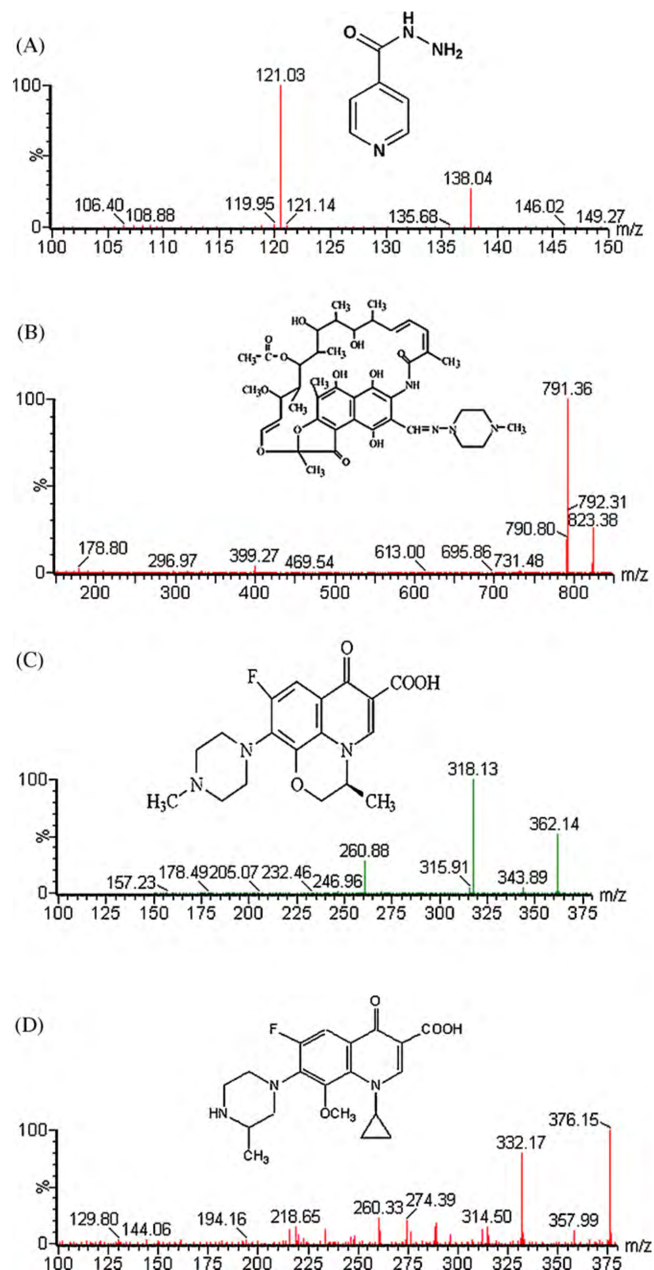

Fig. 1. MS/MS fragmentations and chemical structures of the analytes and I.S.: (A) INH; (B) RFP; (C) LVX; and (D) I.S. (gatifloxacin).

Table 1
The preparation of calibration curve, its equation and correlation coefficient for each analyte in mouse tissue and plasma. (The three concentration levels of quality control samples are bolded.).

Sample	Analyte	Point on the calibration curve							Calibration equation	Correlation coefficient
		1	2	3	4	5	6	7		
Tissue	INH (μg/g)	0.11	**0.22**	0.43	**1.08**	2.17	**4.34**	5.42	$y = 0.8651x + 0.0098$	0.9997
	RFP (low, μg/g)	0.12	**0.30**	0.75	**2.40**	6.00	**15.0**	24.0	$y = 0.5745x - 0.0096$	0.9996
	RFP (high, μg/g)	24.0	**48.0**	96.0	**240**	480	**960**	1200	$y = 0.4422x - 0.0010$	0.9990
	LVX (μg/g)	0.13	**0.33**	0.98	**2.62**	6.54	**16.4**	26.2	$y = 1.6391x + 0.0774$	0.9990
Plasma	INH (μg/ml)	0.18	**0.36**	0.72	**1.81**	3.62	**7.24**	9.04	$y = 2.9994x + 0.0205$	0.9998
	RFP (μg/ml)	4.00	**8.00**	16.0	**40.0**	80.0	**160**	200	$y = 4.5168x - 0.0061$	0.9998
	LVX (μg/ml)	0.09	**0.18**	0.36	**0.91**	1.81	**3.62**	4.53	$y = 9.9073x + 0.0877$	0.9982

2.3. Sample preparation

For the single tissue homogenate preparation, tissue (0.1 g brain, lung, liver, kidney or small intestine) was precisely weighed and placed into a homogenizer, gradually added with 600 μl of 10% methanol and homogenized for 5 min. The drug-free tissue homogenates obtained from the five different tissues were mixed together in equal volumes to produce the blank tissue homogenate mixture for the method validation. A volume of 150 μl of the homogenate (single or mixed) was transferred into a tube containing 600 μl methanol and 50 μl of I.S. (0.96 μg/ml gatifloxacin). After 1 min of vortex-mixing, the mixture was centrifuged at 10,000 rpm for 5 min at room temperature (25 °C). Finally, a total volume of 20 μl of the supernatant was aspirated for analysis by the autosampler.

For the plasma sample preparation, mouse plasma (150 μl) was placed into a tube containing 600 μl methanol and 50 μl of I.S. (0.96 μg/ml gatifloxacin). After 1 min of vortex-mixing, the mixture was centrifuged at 10,000 rpm for 5 min at room temperature (20 °C). Then, 150 μl of the supernatant was transferred to a 96-well microplate and 20 μl of the solution were injected for analysis.

2.4. Chromatographic conditions

Chromatographic separation of the analytes was performed on a C4 column (250 mm × 4.6 mm, 5.0 μm, Welch materials, USA) with a column temperature at 25 °C. The flow-rate was 1.0 ml/min, and the post-column splitting ratio was 3:1. The mobile phase was a gradient of a mixture of 0.05% formic acid in water (solvent A) and methanol (solvent B). The gradient profile used began with a isocratic elution of (A:B) 93:7 v/v for 4.5 min, followed by a gradual linear decrease of A to (A:B) 88:12 v/v until 9 min and then a steep linear increase of B to (A:B)10:90 v/v until 13 min. Finally, the mobile phase was reset to (A:B) 93:7 v/v at 13 min and stayed constant for 3.5 min (including a 1.5 min autosampling interval before each run) to equilibrate for the next injection.

2.5. MS/MS detection conditions

The ESI source was operated in positive mode and the final optimized conditions were as follows: capillary voltage, 3.50 kV; cone voltage, 21 V for INH, 30 V for LVX, 26 V for RFP and 30 V for I.S.; extractor voltage, 3 V; collision energy, 13 eV for INH, 18 eV for LVX, 18 eV for RFP and 18 eV for I.S.; source temperature, 120 °C; desolvation temperature, 400 °C; cone gas flow, 50 l/h; desolvation gas flow, 750 l/h and collision gas (argon) flow 0.16 ml/min. Quantification was achieved under multiple reaction monitoring (MRM) mode using the following transitions: INH m/z 138.0–121.0, RFP m/z 823.4–791.4, LVX m/z 362.1–318.1 and gatifloxacin (I.S.) m/z 376.2–332.2.

2.6. Validation of the method

The method validation procedure was carried out according to FDA guidance for bioanalytical method validation [14]. Calibration was performed by a least-squares linear regression of the peak area ratios of the drugs to the I.S. versus the respective standard concentration. The accuracy calculated at the same three concentrations of QC samples was defined as the ratio of mean computed value to the true value expressed as percentage. Recovery was determined by comparison of pre-extraction spiked QC samples with post-extraction spiked blank mouse plasma or tissue samples. Precision assays were carried out 5 times using three different concentrations on the same day and over 5 different days. The stabilities of the analytes in mouse sample under various storage conditions were evaluated as follows: QC samples were subjected to processed samples kept at 4 °C in autosampler for 12 h, to long-term (30 days) storage conditions (−70 °C), and to four freeze–thaw cycles stability studies. Freezing was performed at −70 °C for 24 h and thawed at ambient temperature. All the stability studies were conducted QC samples at three concentration levels with 5 determinations for each. The lower limit of quantification (LLOQ) was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio of 10, with the acceptable accuracy of ±15% and a precision below 15%. The limit of detection was defined as the lowest concentration level resulting in a peak area with a signal-to-noise ratio of 3. The matrix effects, i.e., ion suppression or enhancement, were measured as described by Matuszewski et al. [15]. Briefly, the analytes at three concentration levels were spiked into 600 μl supernatant obtained by deproteinizing 150 μl blank tissue homogenate mixture or plasma with 600 μl methanol, mixed for 30 s and analyzed. The corresponding peak areas (A) were compared with those of the standard solutions diluted with 600 μl methanol directly (B). The ratio (A/B × 100%) was used to evaluate the matrix effect.

2.7. Application of the method

We determined the peak concentration ranges of each drug in plasma and various tissues obtained from mice which were intraperitoneally administered once daily with converted clinical standard oral dosages of INH (45 mg/kg), RFP (67.5 mg/kg) and LVX (45 mg/kg), alone or in combination for 1day or for 10 days. 3 h after the last administration, samples were then taken and stored at −80 °C until analysis. Comparisons of the drug concentrations in different tissues and plasma between day 1 and day 10 were made using paired two-tailed Student t-tests. Comparisons between drug concentrations at day 10 obtained from monotherapy, two-drug combination therapy and three-drug combination therapy were performed with one-way ANOVA. Difference were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Optimization of sample preparation procedure

Due to the instability of INH and RFP in aqueous solutions, sample handling of the reported methods usually requires an addition of antioxidant to prevent oxidation during the following extraction, concentration and reconstitution steps [10,11], which was rather complex, labor- and time-consuming. Moreover, in our preliminary experiments it was found that the antioxidant (ascorbic acid) added to samples would result in MS/MS signal suppression of the analytes. Since the drug concentrations in mouse samples were fairly high in the present study, we conducted the analyses by directly injecting supernatants of the samples after methanol deproteinization without adding any antioxidant, which was rapid, simple and reliable, although a part of the sensitivity of the method was lost due to the lack of concentration step.

Since the combined blank tissue homogenate contains all the information of each single tissue, it was prepared and used in the method validation for the sake of convenience. The calibration curves we established using the blank mixed tissue homogenate are fit for the determination of the three drugs in each single tissue. To prove their versatilities, we prepared single drug-containing tissue homogenate at the same three concentration levels of QC samples ($n = 5$). The drug concentrations of INH, RFP and LVX in the five different tissue homogenates were calculated by their respective versatile calibration curves and tested for accuracies, intra- and inter-day precisions. The accuracies ranged from 90.3% to 113.4%. The intra- and inter-day precisions were below 13.5%.

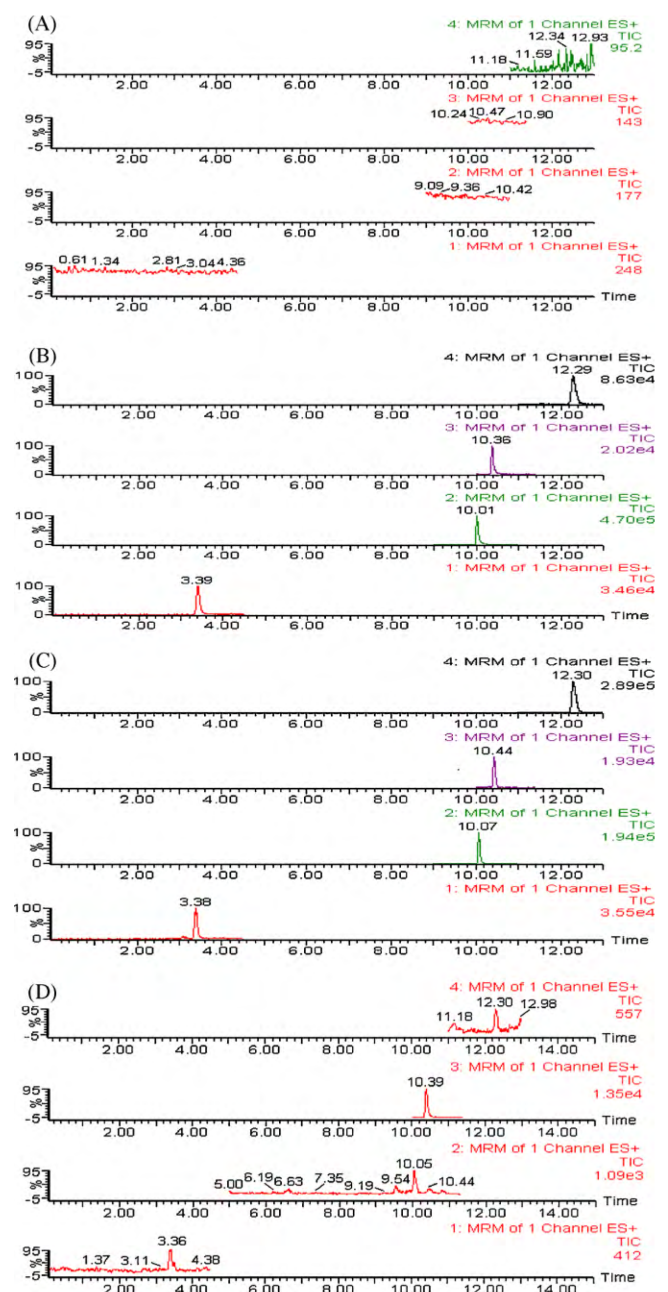

Fig. 2. Selective ion chromatograms (bottom-up order: Channel 1: INH m/z 138.0–121.0, collision energy 13 eV; Channel 2: LVX m/z 362.1–318.1, collision energy 18 eV; Channel 3: I.S. m/z 376.2–332.2, collision energy 18 eV; Channel 4: RFP m/z 823.4–791.4, collision energy 18 eV). (A) Blank mixed mouse tissue homogenate; (B) mixed mouse tissue homogenate spiked with standards and I.S. (1.08 μg/g for INH, 2.40 μg/g for RFP and 2.62 μg/g for LVX); (C) post-dose mouse tissue sample, the represented tissue is small intestine; (D) mouse tissue homogenate at LLOQ for the analytes and I.S. (INH at 0.11 μg/g, RFP at 0.12 μg/g and LVX at 0.13 μg/g).

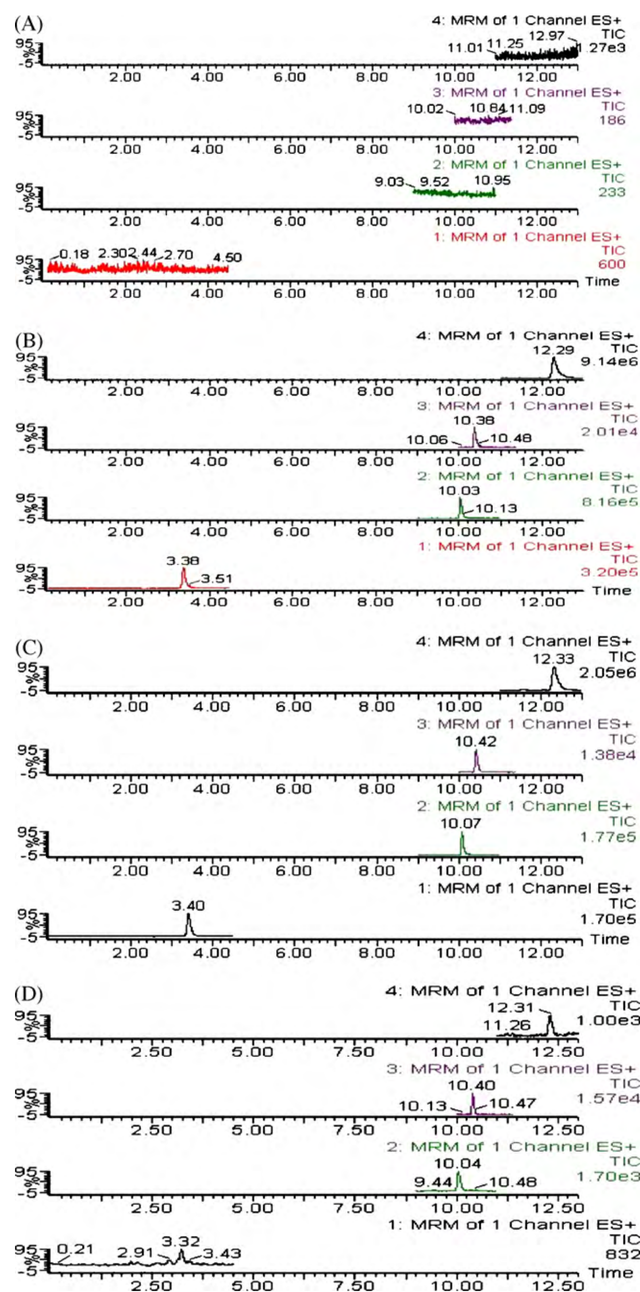

Fig. 3. Selective ion chromatograms (bottom-up order: Channel 1: INH, Channel 2: LVX, Channel 3: I.S., Channel 4: RFP). (A) Blank mouse plasma; (B) mouse plasma spiked with standards and I.S. (1.81 μg/ml for INH, 40 μg/ml for RFP and 0.91 μg/ml for LVX); (C) post-dose mouse plasma sample; (D) mouse plasma at LLOQ for the analytes and I.S. (INH at 18.1 ng/ml, RFP at 20.0 ng/ml and LVX at 21.8 ng/ml).

3.2. Optimization of MS and separation conditions

During MS tuning, it was found that INH, RFP and LVX were better detected in the positive ion mode. The product ion spectrum of the $[M+H]^+$ ion of INH showed a major fragment ion at m/z 121.0 (Fig. 1A) due to loss of neutral NH_3. In the cases of LVX and gatifloxacin, major fragment ions were observed at m/z 318.1 (Fig. 1C) and m/z 332.2 (Fig. 1D), due to loss of CO_2, and for RFP the major fragment ion was at m/z 791.4 (Fig. 1B) due to loss of neutral CH_3OH, respectively. It is known that the (C4) butyl bonded phase gives shorter analysis times of non-polar compounds without significantly affecting the polar ones, while still maintaining high resolution when compared to longer chain bonded chemistries. So when it came to the analysis of mixtures with a wide range of polarity, we selected a C4 column which seemed to be more suitable than C18 column. The devised method is the first time to allow the three analytes being successfully analyzed in a single chromatographic separation within 15 min. The chromatograms of blank, spiked, represented mouse tissue and LLOQ samples are shown in Fig. 2A–D. The chromatograms of blank, spiked and real mouse plasma samples and LLOQ samples are shown in Fig. 3 A–D. Lower initial concentrations of organic phase (7–12%) led to the elution of high polar compounds, i.e., INH at 3.4 min, a consequent rapid gradient change to higher concentration of the organic component (90%) was used to elute medium and low

Table 2
Precisions, accuracies, recoveries and stabilities for INH, RFP and LVX in mixed mouse tissue homogenate.

Analyte	Added concentration (μg/g)	Precision R.S.D. (%)		Accuracy (%) mean ± S.D.	Recovery (%) mean ± S.D.	Stability R.S.D. (%, $n = 5$)		
		Intra-day	Inter-day			Processed sample at 4 °C (12 h)	Long-term storage at −70 °C (30 days)	Four freeze–thaw cycles
	0.22	7.3	4.4	99.7 ± 7.3	96.5 ± 7.5	1.6	1.6	3.3
INH	1.08	5.2	6.1	101.1 ± 5.2	95.5 ± 6.4	1.2	3.7	7.7
	4.34	4.4	5	100.8 ± 4.4	98.8 ± 3.8	3.6	1.1	5.4
	0.30	6.5	8.1	97.2 ± 6.5	83.3 ± 8.8	3.8	2.2	6.5
PFP (Low)	2.40	6.5	12.5	106.3 ± 6.5	84.2 ± 5.0	4.1	3.6	2.4
	15.0	3.5	13.3	109 ± 3.5	87.6 ± 6.5	3.3	1.4	4.4
	48.0	6.3	8.8	91.7 ± 6.3	84.3 ± 8.5	2.3	2.7	7.5
PFP (High)	240	5.5	9.1	98.7 ± 5.5	87.4 ± 6.9	1.7	5.1	2.4
	960	6.8	7.8	102.5 ± 6.8	89.2 ± 5.6	2.1	3.1	4.7
	0.33	2.1	4	111.9 ± 2.1	95.4 ± 5.3	1.5	1.3	1.7
LVX	2.62	2.3	2.8	112.0 ± 2.3	95.5 ± 5.5	2.3	1.3	5.3
	16.4	4.3	2.8	105.5 ± 4.3	92.6 ± 7.6	2.7	4.7	3.4

polar compounds, i.e., LVX at 10.0 min, I.S. at 10.4 min and RFP at 12.3 min.

3.3. Method validation

The good linear seven-point calibration curves were described by the equations summarized in Table 1 with their correlation coefficients all above 0.999 in mouse tissue and above 0.99 in mouse plasma. The accuracies, intra- and inter-day precisions and stabilities for the three analytes in mouse tissue homogenate and plasma are shown in Tables 2 and 3. All the RSD were below 13.3% ($n = 5$). The LLOQs for INH, RFP and LVX were 0.11, 0.12 and 0.13 μg/g in mouse tissues and were 18.1, 20.0 and 21.8 ng/ml in mouse plasma, respectively. Traditionally, the lowest concentration of the standard calibration curve is used to define the LLOQ value because researchers usually want to quantify the analyte as low as possible. However, in our study, since the drug concentrations were relatively low in the tested mouse tissue while very much high in the tested mouse plasma (as shown in Table 4), the lowest concentrations in calibration of the three analytes were exactly the LLOQ values for tissue, which is consistent. But for mouse plasma, we had to establish the calibration with their starting concentrations much higher than the LLOQs in order to cover the quite high concentration ranges of the analytes, which is therefore no longer identical. The LODs for INH, RFP and LVX in mouse tissues were 0.04, 0.05 and 0.05 μg/g and for those in mouse plasma were 5.5, 6.0 and 6.6 ng/ml, respectively. The mean matrix effects (and standard deviations) were 94.6 ± 4.1% for INH, 99.3 ± 6.1% for RFP and 106.7 ± 5.0% for LVX in mouse tissues and were 93.6 ± 3.6% for INH, 99.4 ± 5.7% for RFP and 94.1 ± 6.0% for LVX in mouse plasma. The ion suppression/enhancement and low variability of each analyte was negligible and the quantifications of them were not affected by the matrix effect.

3.4. Application of the method

The data obtained from mouse tissues and plasma are summarized in Table 4. For INH concentrations, there are significant differences between day 1 and day 10 in all tested types of sample. At day 10, there are significant differences of INH concentrations between any two of the three regimens in mouse lung, liver and kidney tissues. For RFP concentrations, there are significant differences between day 1 and day 10 in all tested types of sample except small intestine. At day 10, there are significant differences of RFP concentrations between any two of the three therapies in mouse brain, lung and liver tissues. There are significant differences of LVX concentrations in all tested types of sample between day 1 and day 10. At day 10, significant differences of LVX concentrations were found between any two of the three regimens in brain, lung, liver and kidney. These results suggest that the concentrations of anti-tuberculosis drugs in organs and plasma can be influenced by different therapeutic durations and regimens. However, additional

Table 3
Precisions, accuracies, recoveries, and stabilities for INH, RFP and LVX in mouse plasma.

Analyte	Added concentration (μg/ml)	Precision R.S.D. (%)		Accuracy (%) mean ± S.D.	Recovery (%) mean ± S.D.	Stability R.S.D. (%, $n = 5$)		
		Intra-day	Inter-day			Processed sample at 4 °C (12 h)	Long-term storage at −70 °C (30 days)	Four freeze–thaw cycles
INH	0.36	2.4	3.7	106.0 ± 2.4	75.8 ± 5.2	1.2	2.4	3.7
	1.81	6.0	7.3	103.2 ± 6.0	75.5 ± 9.7	4	3.5	7.3
	7.24	3.7	8.0	106.0 ± 3.7	77.0 ± 3.6	1.6	1.7	5.2
	8.0	2.7	6.1	98.2 ± 2.7	82.3 ± 4.2	2.6	2.7	7.1
RFP	40.0	4.0	8.2	94.6 ± 4.0	83.4 ± 7.9	1.5	3.1	6.4
	160	3.6	5.8	108.8 ± 3.6	85.1 ± 5.0	2.2	3.6	9.8
	0.18	6.6	5.7	102.6 ± 6.6	83.9 ± 9.8	1.8	1.6	1.7
LVX	0.91	4.0	5.0	106.1 ± 4.0	90.8 ± 8.3	1.3	3.4	4.3
	3.62	4.7	3.7	99.5 ± 4.7	85.9 ± 4.0	3.7	4.7	2.8

Table 4
Distributions of RFP, INH and LVX in tissues and plasma after intraperitoneal administration in mouse. (Mean ± S.D.).

Analyte	Regimen	Time point	Concentrations (n = 12, μg/g in tissues and μg/ml in plasma)					
			Brain	Lung	Liver	Kidney	Small intestine	Plasma
INH	INH alone	1 day	1.30 ± 0.12	3.86 ± 0.28	1.01 ± 0.10	1.85 ± 0.25	2.21 ± 0.17	1.38 ± 0.14
		10 days	0.67 ± 0.09	2.09 ± 0.16	0.54 ± 0.05	1.35 ± 0.11	2.30 ± 0.16	1.60 ± 0.18
	INH + RFP	1 day	1.17 ± 0.11	3.68 ± 0.50	0.91 ± 0.08	1.88 ± 0.24	2.17 ± 0.21	1.30 ± 0.17
		10 days	0.35 ± 0.05	0.87 ± 0.11	0.37 ± 0.05	0.97 ± 0.14	2.40 ± 0.30	1.58 ± 0.21
	INH + RFP + LVX	1 day	1.25 ± 0.10	3.74 ± 0.25	0.96 ± 0.06	1.91 ± 0.16	2.25 ± 0.29	1.38 ± 0.12
		10 days	0.62 ± 0.06	1.12 ± 0.10	0.46 ± 0.05	1.15 ± 0.09	2.25 ± 0.16	1.51 ± 0.17
RFP	RFP alone	1 day	1.13 ± 0.09	44.54 ± 1.53	201.99 ± 9.21	6.49 ± 0.59	117.58 ± 9.13	34.04 ± 1.84
		10 days	0.86 ± 0.07	6.66 ± 0.38	114.65 ± 5.25	6.32 ± 0.75	117.77 ± 4.61	96.85 ± 3.72
	RFP + INH	1 day	1.03 ± 0.11	46.49 ± 3.41	182.55 ± 11.94	5.87 ± 0.47	111.10 ± 9.25	33.32 ± 1.63
		10 days	0.66 ± 0.12	5.61 ± 0.27	104.01 ± 12.82	6.18 ± 0.71	133.78 ± 11.25	92.07 ± 3.43
	RFP + INH + LVX	1 day	1.37 ± 0.12	49.14 ± 2.10	199.09 ± 11.14	6.72 ± 0.51	122.37 ± 5.75	31.36 ± 1.61
		10 days	0.98 ± 0.06	9.75 ± 0.99	131.46 ± 6.78	7.55 ± 0.38	113.30 ± 5.07	99.62 ± 2.46
LVX	LVX alone	1 day	0.71 ± 0.07	4.64 ± 0.48	4.95 ± 0.50	7.79 ± 0.87	10.95 ± 1.18	1.03 ± 0.15
		10 days	0.59 ± 0.05	2.92 ± 0.28	4.35 ± 0.24	4.86 ± 0.56	10.74 ± 1.28	1.37 ± 0.12
	LVX + RFP	1 day	0.73 ± 0.07	4.85 ± 0.56	5.20 ± 0.48	7.94 ± 0.75	10.66 ± 0.95	1.02 ± 0.13
		10 days	0.37 ± 0.04	1.32 ± 0.05	3.66 ± 0.32	3.79 ± 0.44	9.41 ± 0.45	1.31 ± 0.14
	LVX + INH + RFP	1 day	0.68 ± 0.06	4.69 ± 0.51	5.05 ± 0.59	7.67 ± 0.69	10.55 ± 0.88	1.00 ± 0.14
		10 days	0.31 ± 0.05	0.76 ± 0.06	2.95 ± 0.22	2.77 ± 0.22	9.48 ± 0.52	1.24 ± 0.14

studies will be needed to correlate the concentrations of these drugs in tissues and plasma with in vivo effects of P-gp on development of anti-tuberculosis drug resistance.

4. Conclusion

We describe an HPLC/MS/MS technique that was developed to simultaneously measure the concentrations of three anti-tuberculosis drugs in mouse tissues and plasma. The devised method allows for the simple, rapid and low-cost quantification of INH, RFP and LVX, and offers a means of high-throughput and simultaneous monitoring of anti-tuberculosis drugs. The greatest advantage of the present method is the shortening of the analysis time of the compounds and the simplifying of the preparation of the sample.

References

[1] H.M. Blumberg, W.J. Burman, R.E. Chaisson, C.L. Daley, S.C. Etkind, L.N. Friedman, P. Fujiwara, M. Grzemska, P.C. Hopewell, M.D. Iseman, R.M. Jasmer, V. Koppaka, R.I. Menzies, R.J. O'Brien, R.R. Reves, L.B. Reichman, P.M. Simone, J.R. Starke, A.A. Vernon, Am. J. Respir. Crit. Care. Med. 167 (2003) 603.

[2] B.I. Vishnevskii, O.A. Manicheva, E.B. Vishnevskaia, N.N. Mel'nikova, T.F. Otten, Probl. Tuberk. Bolezn. Legk. 11 (2006) 18.

[3] Z.M. Fuentes, J.A. Caminero, Arch. Bronconeumol. 42 (2006) 194.

[4] J. Pereira, Rev. Port. Pneumol. 10 (2004) 97.

[5] M. Huisman, J. Smit, A. Schinkel, Aids 14 (2000) 237.

[6] H. Thomas, H. Coley, Cancer Control 10 (2003) 159.

[7] S. Gollapudi, M. Reddy, P. Gangadharam, T. Tsuruo, S. Gupta, Biochem. Biophys. Res. Commun. 199 (1994) 1181.

[8] P.J. Smith, J. van Dyk, A. Fredericks, Int. J. Tuberc. Lung. Dis. 3 (1999) S325.

[9] A. Walubo, P. Smith, P.I. Folb, J. Chromatogr. B 658 (1994) 391.

[10] B.D. Glass, S. Agatonovic-Kustrin, Y.J. Chen, M.H. Wisch, J. Chromatogr. Sci. 45 (2007) 38.

[11] S.H. Song, S.H. Jun, K.U. Park, Y. Yoon, J.H. Lee, J.Q. Kim, J. Song, Rapid. Commun. Mass. Spectrom. 21 (2007) 1331.

[12] D. Bao, T.T. Truong, P.J. Renick, M.E. Pulse, W.J. Weiss, J. Pharm. Biomed. Anal. 46 (2008) 723.

[13] I. Calleja, M.J. Blanco-Príeto, N. Ruz, M.J. Renedo, M. Dios-Viéitez, J. Chromatogr A 1031 (2004) 289.

[14] Guidance for Industry Bioanalytical Method Validation from FDA, US Department of Health and Human Services, Food and Drug Administration, CDER, Rockville, USA, 2001.

[15] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.