Research Article
ISSN: 0976-1209

Available online through
http://jprsolutions.info

A new, simple and rapid method for simultaneous determination of ethambutol and isoniazid in dried blood spots by LC-MS/MS and its application to pharmacokinetic study.

P Pavan Kumar[a,*], T E G K Murthy[b]
[a]Krishna University, Machilipatnam, Andhra Pradesh 521001, India.
[b]Bapatla College of Pharmacy, Bapatla, Guntur District, Andhra Pradesh 522101, India.

Received on: 17-12-2013; Revised on: 29-12-2013; Accepted on:31-01-2014

ABSTRACT

Background: Ethambutol (EMB) and isoniazid (INH) are first-line anti tubercular drugs. The combination formulation is used for the continuation phase of tuberculosis. A new, simple and rapid method for simultaneous determination of ethambutol and isoniazid in dried blood spots (DBS) was developed and validated using LC-MS/MS. This method was successfully applied to pharmacokinetic study following oral administration of tablet of fixed dose combination containing ethambutol hydrochloride 800 mg and isoniazid 300 mg in human volunteers. **Methods:** Whole blood samples were spotted, dried and a 3 mm punch was extracted with methanol. The separation was performed on kromasil C18 column (150×4.6mm i.d., 5µm particle size) using a mobile phase composed of 0.1% formic acid in water and methanol (35:65 v/v) at a flow rate of 0.8 mL/min. The total run time was 3.5 min with retention times of 1.34 min for EMB, 1.90 for INH and 2.44 for IS. **Results:** The method was linear over the concentration range of 50-4000 ng/mL for EMB and 100-5500 ng/mL for INH with all correlation coefficients greater than 0.9943 and 0.9941 for EMB and INH respectively. The intra-day accuracy of the method was ranged from 95.5% to 103.4% and the inter-day accuracy was between 97.8% and 103.8% with the precision less than 8.4 and 7.5% respectively. The stability of both the analytes was evaluated under various conditions. **Conclusion:** The method described here was validated in accordance with "Guidance for industry: Bioanalytical method validation, USFDA (2001)" and the method was successfully applied to the pharmacokinetic study after oral administration. DBS technique has been demonstrated to produce similar PK profiles as that of plasma quantitation methods, supporting the use of DBS as an alternative for plasma pharmacokinetic studies.

Keywords: Tandem Mass Spectrometry, Tuberculosis, Validation, Ethambutol, Isoniazid

1. INTRODUCTION

Tuberculosis (TB) is the most common infectious disease, causing millions of deaths annually. In 2011, number of deaths due to TB was 1.1 million, as reported by world health organization.[1] The treatment of TB is comprised of combinatorial regimens of first-line anti-tubercular drugs (Ethambutol, Isoniazid, Pyrizinamide and Rifmapicin), of which, fixed dose combination of isoniazid and ethambutol is commonly used in continuation phase.

Over the years, many conventional methods for the quantification of first-line anti-TB drugs have been reported.[2-7] Recently few liquid chromatography tandem mass spectrometry methods have been developed for one or more of first-line anti tubercular drugs in plasma.[8-14]

***Corresponding author.**
P Pavan Kumar
Krishna University, Machilipatnam, Andhra Pradesh 521001, India.

Plasma processing is tedious. Blood collection for harvesting plasma typically requires ≥5 mL of blood, which can cumulate to large volumes when conducting pharmacokinetic studies. In recent years, DBS sampling technique raised for application in TK studies, PK studies and therapeutic drug monitoring studies.[15-23] DBS offers several advantages over conventional plasma sampling such as: DBS typically requires the collection of a few drops of blood onto filter paper (usually less than 50 µL), many analytes are stable within DBS samples at room temperature for several weeks to months, storage is simplified and shipment of samples from clinical sites to analytical laboratories unlike plasma does not require extra- special transport conditions and samples in a DBS format also present less of a biohazard risk compared with liquid plasma or blood and can enhance the stability of some compounds.

In this study, we have developed and validated a simple and rapid

method for simultaneous determination of ethambutol and isoniazid in DBS and was successfully applied to pharmacokinetic study.

2. EXPERIMENTAL

2.1. Chemicals

Ethambutol, isoniazid and nicorandil (internal standard, IS) were purchased from Clearsynth Labs Pvt. Ltd., Mumbai, India. Formic acid (HPLC grade) was purchased from Sigma. Methanol (HPLC grade) was obtained from J.T Baker, USA. Milli-Q water was collected from milli-Q system. Whatman DMPK-C cards were obtained from Whatman (GE Healthcare). Human blood with K_3EDTA anticoagulant was procured from Navjeen Blood Bank, Hyderabad.

2.2. Instrumentation

An AB Sciex API3000 with Shimadzu LC-20AD as front end was used to analyze the samples. The isocratic mobile phase consisted of 0.1% formic acid in water and methanol in the ratio of 35:65 v/v with a flow rate of 0.8 mL/min was used to separate both the analytes and internal standard on a Kromasil, C18, 150 x 4.6 mm, 5 µ analytical column. The analytical column compartment was maintained at 40^0C. A mixture of water and acetonitrile in the ratio of 50:50 v/v was used as autosampler rinsing solution. The AB Sciex API3000 mass spectrometer was operated in multiple reaction monitoring (MRM) using ESI+ mode. The MRM [precursor→product] transitions (m/z) were: EMB (205.20→116.10), INH (138.10→121.10) and IS (212.10→136.10). Nitrogen was used as collision activated dissociation (CAD) and curtain gas. The following source dependent parameters were optimized through flow injection analysis (FIA) and were found to give the best sensitivities: the source temperature was set to 400 ^{0}C and the ion spray voltage was set at 5000V.

2.3. Preparation of stock solutions, standard calibrators and quality control samples

The individual standard stock solutions (1.0 mg/mL) of EMB and INH were prepared in HPLC grade methanol. These stock solutions were used to prepare combined working standard solution of EMB/INH in 50% methanol in water. Twenty five microliters of working solutions were spiked to 475 µL of whole blood to give final concentrations of EMB/INH for standard calibrators (50/100, 100/200, 250/350, 500/700, 1000/1400, 2000/2800, 3000/4200 and 4000/5500 ng/mL). Quality control samples with following concentrations were prepared at 4 different levels: 50/100, 150/300, 1700/2000 and 3500/5000 ng/mL. The internal standard stock solution (0.5 mg/mL) was prepared in methanol and spiking solution of concentration 5 µg/mL was prepared in methanol. All the solutions were stored at 4°C. DBS samples were prepared by spotting 25 µL of whole blood contained either standard or QC onto Whatman DMPK-C cards. After spotting, the cards were allowed to dry for at least 2 hours. Once dried, cards were placed in plastic bags with desiccant and stored at room temperature and -20°C to assess the storage condition.

2.4. Sample Preparation

A 3 mm diameter disk was punched out from the blood spots for extraction and placed into a 2 mL micro-centrifuge tube. An aliquot of 300 µL of methanol containing 5 µg/mL of internal standard was added to each tube. Samples were vortexed for 10 min and centrifuged for 5 min at 14000 rpm. Supernatants were then transferred to auto sampler vials for LC-MS/MS analysis.

2.5. Method Validation

The assay was validated using whole blood (DBS) fortified with the analytes. Validation parameters including selectivity, accuracy, precision, linearity, lower limit of quantification (LLOQ), matrix effect, recovery, dilution integrity and stabilities were tested. The performance of assay was demonstrated if inter-day and intra-day accuracy (% deviation from nominal) and precision (%CV) were within 15% at all QC levels, except the lower limit of quantification (LLOQ) where within 20% was allowed. The accuracy compared with the nominal value had to be within ±15% for both inter-day and intra-day variability, except at LLOQ (±20%). The calibration curve was to have correlation coefficient (r^2) = 0.99.

2.6. Pharmacokinetic Study

This validated method was used to investigate the pharmacokinetic parameters of EMB and INH after oral administration of tablet of fixed dose combination containing ethambutol hydrochloride 800 mg and isoniazid 300 mg in human volunteers. Pharmacokinetic parameters were calculated using non-compartmental analysis performed in Phoenix 1.3 (Pharsight Corporation, North Carolina, USA).

3. RESULTS AND DISCUSSION

3.1. Optimization of LC-MS/MS Conditions

During MS tuning, positive and negative ESI modes were tested. Positive ESI mode gave good sensitivity and signal-to-noise ratio for both the analytes and internal standard. A series of experiments with different LC columns, mobile phase compositions were tested to obtain good sensitivity, peak shape and high analytical throughput for both analytes. The chemical structures of EMB, INH and Nicorandil (IS) along with their MS/MS spectrum are shown in Fig. 1. The representative chromatograms of DBS samples spiked with EMB, INH and their corresponding blank samples are shown in Fig. 2. Blank and Blank with IS samples were free from interference and signal-to-noise ratio of the LLOQ samples for both EMB and INH were more than five.

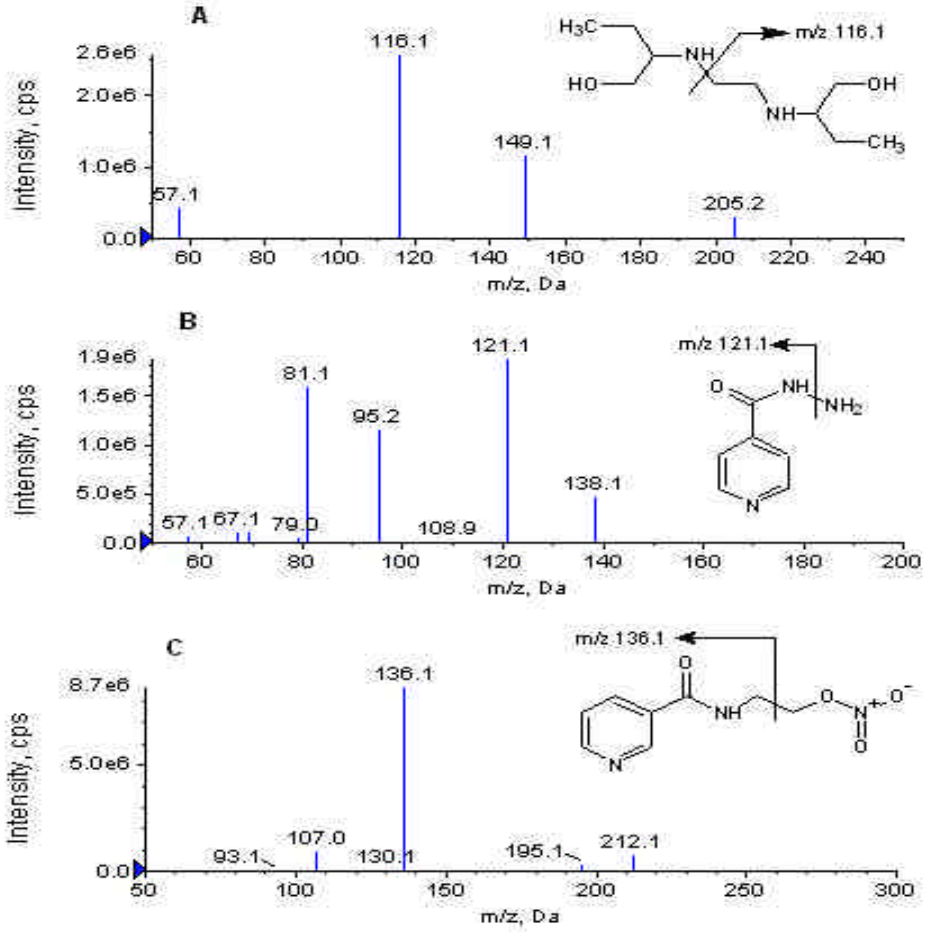

Fig. 1. Chemical structures and MS/MS spectra of EMB (A), INH (B) and Nicorandil (IS) (C)

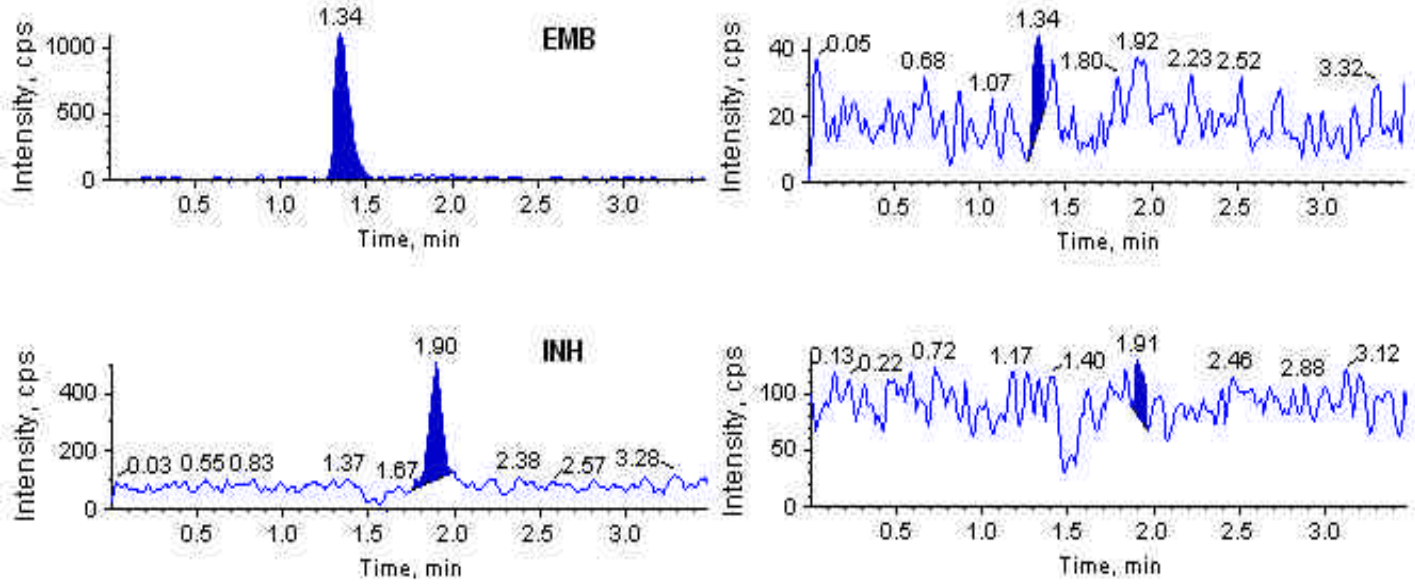

Fig. 2. Representative chromatograms of DBS samples spiked with EMB, INH and their corresponding blank samples.

3.2. Method Validation

3.2.1. Selectivity

The selectivity was evaluated using six different human blood lots. These samples were processed as per the method described earlier. No significant interference was observed at the respective retention times of EMB, INH and IS (<20% of LLOQ for analytes and <5% for IS).

3.2.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were plotted as peak area ratios of ethambutol/IS and isoniazid/IS versus their corresponding concentrations. The lower limit of quantification (LLOQ) was determined as the lowest concentration of calibration curve consistently achieving accuracy better than ±20% of nominal concentration and imprecision ≤20%. The calibration curves showed good linearity over the concentration range of 50-4000 ng/mL for EMB and 100-5500 ng/mL for INH.

3.2.3. Precision and accuracy

Precision and accuracy of this method were evaluated by calculating intra-day and inter-day variations at four QC concentrations (50/100, 150/300, 1700/2000 and 3500/5000 ng/mL) in six replicates. As summarized in Table 1, the intra-day accuracy of the method was ranged from 95.5% to 103.4% and the inter-day accuracy was between 97.8% and 103.8% with the precision less than 8.4 and 7.5% respectively. These results indicate that the method possessed good precision and accuracy.

3.2.4. Matrix effect

Matrix effect (ion suppression or enhancement) was assessed by comparing mean analyte peak areas of extracted samples to the non-extracted solutions. The variability in the peak areas of analytes expressed as %CV in fortified post-extracted samples into six different DBS extracts was considered as measure of the relative matrix effect.[24] The variability expressed as %CV was between 5.2% and 7.2% for both low and high QC levels investigated. The matrix effect evaluations were within acceptance criteria.

3.2.5. Recovery

To determine recovery 25 µL spots were made and allowed to dry. The entire spot was then extracted as per the procedure mentioned earlier. Recovery for ethambutol, isoniazid and nicorandil (IS) were calculated for DBS in six replicates at three concentrations (150/300, 1700/2000 and 3500/5000 ng/mL). Resulting peak areas of ethambutol, isoniazid and IS after extraction from DBS were compared with the peak areas of blank samples fortified after extraction with non-extracted standards. The mean extraction recoveries of EMB and INH from DBS were 90.54±0.47% and 75.24±2.4%. Results of recovery meet the acceptance criteria for bioanalytical assays.

3.2.6. Dilution integrity

An approach called internal standard-tracked dilution was used to evaluate dilution integrity of DBS samples.[25] Dilution integrity experiment was performed with concentration of twice the ULQ for ethambutol and isoniazid (8000/11000 ng/mL) in triplicate. The mean of these individual determinations was 7561.0 and 10679.3 ng/mL for EMB and INH, represents 94.5, 97.1% of nominal concentrations with a standard deviation (SD) of 200.8 and 173.7 respectively.

3.2.7. Stability

The stability of EMB and INH were evaluated by exposing to different conditions (autosampler, storage stability at room temperature and -20°C), at two QC concentration levels (low and high) in six replicates. The stability QC sample concentrations were back-calculated against freshly extracted calibration curve and compared with those obtained from freshly prepared QC samples. The results revealed that EMB and INH were stable in DBS for at least 7 days at room temperature and 22 days at -20°C and 51 h in autosampler. All the stability experiments met the acceptance criteria of the regulatory guidance.[26] The results were tabulated in Table 2.

Table 1. Summary of precision and accuracy results of ethambutol and isoniazid in DBS samples.

Analyte name	Nominal concentration (ng/mL)	Intra-day (n=6)			Inter-day (n=30)		
		Calculated concentration (mean±SD, ng/mL)	Precision (%CV)	Accuracy (%)	Calculated concentration (mean±SD, ng/mL)	Precision (%CV)	Accuracy (%)
EMB	50.0	47.8±3.0	6.3	95.5	48.9±2.7	5.6	97.8
	150.0	153.4±8.2	5.4	102.2	154.4±8.5	5.5	102.9
	1700.0	1758.4±109.0	6.2	103.4	1762.0±103.5	5.9	103.6
	3500.0	3604.2±255.7	7.1	103.0	3544.0±221.6	6.3	101.3
INH	100.0	99.3±5.5	5.6	99.3	102.0±7.6	7.5	102.0
	300.0	296.5±17.2	5.8	98.9	304.9±20.3	6.6	101.6
	2000.0	2018.6±147.5	7.3	100.9	2075.0±148.5	7.2	103.8
	5000.0	5008±418.8	8.4	100.2	5022.7±292.8	5.8	100.5

Table 2. Stability data of ethambutol and isoniazid.

Stability Experiment	Nominal Concentration (ng/mL) EMB/INH	Ethambutol			Isoniazid		
		Calculated Concentration (n=6, mean±SD, ng/mL)	Precision (n=6, %CV)	Accuracy (%)	Calculated Concentration (n=6, mean±SD, ng/mL)	Precision (n=6, %CV)	Accuracy (%)
Autosampler stability (51 h)	150/300	154.8±7.2	4.7	103.1	298.6±17.5	5.9	99.3
	3500/5000	3633.4±203.9	5.6	103.7	4833.2±562.2	11.6	96.4
Storage stability (at room temperature for 7 days)	150/300	148.9±15.8	10.6	99.1	296.7±21.8	7.4	98.6
	3500/5000	3415.8±323.6	9.5	97.5	4724.5±382.9	8.1	94.2
Storage stability (at -20°C for 22 days)	150/300	151.7±6.5	4.3	101.0	297.0±23.2	7.8	98.7
	3500/5000	3455.9±206.4	6.0	98.6	4601.5±104.9	2.3	91.8

3.2.8. Pharmacokinetic Study

The validated method was successfully used to analyze ethambutol and isoniazid concentrations in DBS samples of human volunteers under fasting conditions after oral administration of a tablet containing ethambutol hydrochloride/isoniazid 800/300 mg. There were a total of 25 blood collection time points including the predose sample. The blood samples were collected and spotted onto filter paper cards at 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 24.00 and 48.00 hours post dose. The maximum DBS concentration (C_{max}), time at which the concentration reached the maximum (T_{max}), area under DBS concentration-time curve (AUC_{0-t}, AUC_{0-Inf}) for EMB and INH were calculated. The detailed pharmacokinetic parameters are shown in Table 3.

Table 3. Mean pharmacokinetic parameters of ethambutol, isoniazid.

Pharmacokinetic parameter	Ethambutol (mean±SD, n=6)	Isoniazid (mean±SD, n=6)
C_{max} (ng/mL)	2.5±0.8	4.9±1.9
T_{max} (h)	2.9±0.9	1.5±0.7
AUC_{0-t} (ng h/mL)	12.3±2.4	22.1±9.6
AUC_{0-Inf} (ng h/mL)	13.0±3.0	22.8±9.8

4. CONCLUSIONS

The DBS technique has been successfully applied to quantification of ethambutol and isoniazid. All the validation parameters such as selectivity, precision and accuracy, linearity, matrix effect, recovery and stabilities have been successfully evaluated. In addition, the technique has been demonstrated to produce similar PK profiles as that of plasma quantitation methods,[27-28] supporting the use of DBS as an alternative for plasma pharmacokinetic studies.

ACKNOWLEDGEMENTS

Authors thank the ARL (I) Pvt. Ltd., Mumbai, India for the acceptance to carry out this research work.

REFERENCES

1. Global Health Observatory Health Repository, WHO. http://apps.who.int/gho/data/view.main.57016ALL?lang=en. Accessed 4 January 2013.
2. Breda M, Marrari P, Pianezzola E, Strolin BM. Determination of ethambutol in human plasma and urine by high-performance liquid chromatography with fluorescence detection. *J Chromatogr A*. 1996;729:301–307.
3. Khuhawar MY, Rind FMA. Liquid chromatographic determination of isoniazid, pyrazinamide and rifampicin from pharmaceutical preparations and blood. *J Chromatogr B*. 2002;766:357–363.
4. Moussa LA, Khassouani CE, Soulaymani R, Jana M, Cassanas G, Alric R, Hue B. Therapeutic isoniazid monitoring using a simple high-performance liquid chromatographic method with ul-traviolet detection. *J Chromatogr B*. 2002;766:181–187.
5. Guermouche S, Guermouche MH. Solid-phase extraction and HPTLC determination of isoniazid and acetylisoniazid in serum. Comparison with HPLC. *J Chromatogr Sci*. 2004;42:250–253.
6. Allanson AL, Cotton MM, Tettey JNA, Boyter AC, Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. *J Pharm Biomed Anal*. 2007;44:963–969.
7. Zhou Z, Chen L, Liu P, Shen M, Zou F. Simultaneous determination of isoniazid, pyrazinamide, rifampicin and acetylisoniazid in human plasma by high-performance liquid chromatography. *Anal Sci*. 2010;26:1133–1138.
8. Chen XY, Song B, Jiang HJ, Yu K, Zhong DF. A liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of isoniazid and ethambutol in human plasma. *Rapid Commun Mass Spectrom*. 2005;19:2591–2596.
9. Granados O, Meza G. A direct HPLC method to estimate streptomycin and its putative ototoxic derivative, streptidine,

in blood serum: application to streptomycin-treated humans. *J Pharm Biomed Anal*. 2007;43:625–630.

10. Song SH, Jun SH, Park KU, Yoon Y, Lee JH, Kim JQ, Song J. Simultaneous determination of first-line anti-tuberculosis drugs and their major metabolic ratios by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2007;21:1331–1338.
11. Wang A, Zhang W, Sun J, Liu JF, Sang YS, Gao S, Hee ZG. HPLC-MS analysis of isoniazid in dog plasma. *Chromatographia*. 2007;66:741–745.
12. Gong Z, Basir Y, Chu D, McCort-Tipton M. A rapid and robust liquid chromatography/tandem mass spectrometry method for simultaneous analysis of anti-tuberculosis drugs-ethambutol and pyrazinamide in human plasma. *J Chromatogr B*. 2009;877:1698–1704.
13. Huang L, Marzan F, Jayewardene AL, Lizak PS, Li X, Aweeka FT. Development and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for determination of isoniazid in human plasma. *J Chromatogr B*. 2009;877:285–290.
14. Zhou Z, Wu X, Wei Q, Liu Y, Liu P, Ma A, Zou F. Development and validation of a hydrophilic interaction liquid chromatography–tandem mass spectrometry method for the simultaneous determination of five first-line antituberculosis drugs in plasma. *Anal Bioanal Chem*. 2013;405:6323–6335.
15. AbuRuz S, Millership J, McElnay J. Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *J Chromatogr B*. 2006;832:202-207.
16. Barfield M, Spooner N, Lad R, Parry S, Fowles S. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. *J Chromatogr B*. 2008;870:32-37.
17. Abu-Rabie P, Spooner N. Direct quantitative bioanalysis of drugs in dried blood spot samples using a thin-layer chromatography mass spectrometer interface. *Anal Chem*. 2009;81:10275-10284.
18. Abbott R, Smeraglia J, White S, Luedtke S, Brunet L, Thomas E, Globig S, Timmerman P. Conference report: connecting strategies on dried blood spots. *Bioanalysis*. 2010;2:1809-1816.
19. Bowen CL, Hemberger MD, Kehler JR, Evans CA. Utility of dried blood spot sampling and storage for increased stability of photosensitive compounds. *Bioanalysis*. 2010;2:1823-1828.
20. Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis*. 2010;2:1791-1796.
21. Barfield M, Wheller R. Use of Dried Plasma Spots in the Determination of Pharmacokinetics in Clinical Studies: Validation of a Quantitative Bioanalytical Method. *Anal Chem*. 2011;83:118-124.
22. Keevil BG. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. *Clin Biochem*. 2011;44:110-118.
23. Kole PL, Majithia R, Singh TRR, Garland MJ, Migalska K, Donnelly RF, McElnay J. Dried blood spot assay for estimation of metronidazole concentrations in rats and its application in single animal drug pharmacokinetic study. *J Chromatogr B*. 2011;879:1713-1716.
24. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Anal Chem*. 2003;75:3019–3030.
25. Liu G, Snapp HM, Ji QC. Internal standard tracked dilution to overcome challenges in dried blood spots and robotic sample preparation for liquid chromatography/tandem mass spectrometry assays. *Rapid Commun Mass Spectrom*. 2011;25:1250–1256.
26. US Food and Drug Administration, Guidance for industry. 2001. Bioanalytical method validation.
27. Charles AP, Amy EB, George SJ, Roger WJ, James MC, David EN. Pharmacokinetics of Ethambutol under Fasting Conditions, with Food, and with Antacids. *Antimicrob Agents Chemother*. 1999;43:568–572.
28. Charles AP, Namdar R, Dodge AA, Nix DE. Pharmacokinetics of isoniazid under fasting conditions, with food, and with antacids. *Int J Tuberc Lung Dis*. 1999;3:703-710.

Source of support: Nil, Conflict of interest: None Declared