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**A UPLC-MS-MS method for the simultaneous quantification of first line antituberculars in plasma and in peripheral blood mononuclear cells.**

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**Running Title:** First line antitubercular quantification in plasma and PBMCs

**Keywords:** PBMCs, ethambutol, isoniazid, pyrazinamide, rifampicin, therapeutic drug monitoring, TDM, HPLC, determination

## Synopsis

**Objectives** Tuberculosis is currently the second cause of death among patients affected with infectious diseases. Quantification of drug levels in plasma and in cells where *Mycobacterium tuberculosis* persists and growths may be useful in understanding the appropriateness of dosage regimens. We report a new and fully validated chromatographic method to quantify first line antituberculars in plasma and in peripheral blood mononuclear cells (PBMCs). The method was used for plasma and cell quantification of antituberculars in patients in treatment with standard oral therapy.

## Methods

Ethambutol, isoniazid, pyrazinamide, and rifampicin were extracted from plasma and PBMCs using two separated and optimized procedures; analysis was performed using an UPLC coupled with mass-mass detector system (UPLC-MS-MS). Antitubercular levels in patients were assayed at the end of dosing interval ( $C_{\text{trough}}$ ) and two hours post dose ( $C_{\text{max}}$ ).

## Results

The method resulted accurate and precise, recovery and matrix effect resulted reproducible. While rifampicin intracellular concentrations were similar to plasma values (median intra-PBMCs  $C_{\text{max}}$  7503 ng/mL versus median plasma  $C_{\text{max}}$  6505 ng/mL), isoniazid and pyrazinamide were lower (median intra-PBMCs  $C_{\text{max}}$  12 ng/mL versus median plasma  $C_{\text{max}}$  3258 ng/mL for isoniazid and 2364 ng/mL versus median plasma  $C_{\text{max}}$  26988 ng/mL for pyrazinamide); ethambutol intracellular concentrations were significantly higher than plasma values (median intra-PBMCs  $C_{\text{max}}$  73334 ng/mL versus median plasma  $C_{\text{max}}$  2244 ng/mL).

## Conclusions

The method was suitable for both therapeutic drug monitoring and for pharmacokinetic analysis. Should the clinical usefulness of measuring antitubercular drugs intracellular concentrations be confirmed, this method could be useful to enhance the clinical application of intra-PBMC evaluation.

## **Introduction**

In 2012 it was estimated that 8.6 million people worldwide developed tuberculosis (TB) and 1.3 million died with it<sup>1</sup>; TB is considered the second cause of death among infectious diseases after HIV infection. Suboptimal concentrations (mostly in the plasma compartment) have been associated with delayed mycobacteria clearance, relapse and selection of drug resistant strains.<sup>2-9</sup>

Macrophages play an important role in the control of *Mycobacterium tuberculosis* growth, spread, and granuloma formation<sup>10</sup> and immunodeficient patients are at higher risk of developing disseminated and extrapulmonary infections. As antibacterial activity is concentration-dependent, antitubercular drugs must reach appropriate levels at the site of action, i.e. inside infected macrophages.<sup>4</sup> However since resident macrophages are usually derived from circulating monocytes and since they are far more easily accessed, it may be relevant to measure antitubercular drugs in peripheral blood mononuclear cells (PBMCs).

We aimed to develop and validate a new chromatographic method to quantify ethambutol, isoniazid, pyrazinamide, and rifampicin in plasma and in PBMCs.

## **Materials and methods**

*Chemicals and reagents:* Ethambutol, isoniazid, pyrazinamide, rifampicin, thymidine, and 6,7-Dimethyl-2,3-di(2-pyridyl)quinoxaline (QX) were purchased from Sigma Aldrich (St.Louis, MO, USA). Acetonitrile HPLC grade and methanol HPLC grade were purchased from VWR (Radnor, PA, USA). Formic acid was obtained by Sigma Aldrich. HPLC grade water was obtained by Milli-DI system coupled with a Synergy 185 system by Millipore (Billerica, MA, USA). Blank PBMCs were taken from buffy coat of healthy donors, kindly supplied by the Blood Bank of the Maria Vittoria Hospital (Turin, Italy).

*Equipment:* The chromatographic system used was an Acquity™ Ultraperformance Liquid Chromatography system (UPLC) (Waters, Milford, MA, USA) coupled with a Quadrupole Detector (TQD). An Acquity™ UPLC HSS T3 1.8 µm (2.1x150 mm) column (Waters, Milford, MA, USA), protected by a ACQUITY UPLC column In-Line Filter (Waters, Milford, MA, USA) was used. MS/MS settings, values of cone voltage, collision energy, and mass transitions are reported in Table S1.

*Stock solutions, standards, and quality controls:* Stock solutions of ethambutol, isoniazid, and rifampicin were made in methanol 100%, pyrazinamide stock solution was prepared in water:methanol (50:50; v/v), stock solution of thymidine was done in water 100%, stock solution of QX was prepared in water:methanol (10:90; v/v).

Internal standard working solution (IS) was made by diluting thymidine and QX in water:methanol (50:50; v/v) at the concentration of 20 mg/L and 1 mg/mL, respectively. Calibration ranges and QCs levels are reported in Table S2.

*Extraction procedure from plasma:* 200  $\mu$ L of STD, QCs, and patients samples were added to 50  $\mu$ L of IS and 400  $\mu$ L of acetonitrile. After centrifugation at 20000 x g for 10 min at 4°C, supernatant was diluted 1:10 with water before injection in UPLC system.

*Extraction procedure from PBMCs:* STDs, QCs, and samples of patients consisting of 1 mL of PBMCs at the concentration of  $10 \times 10^6$  cells/mL were spiked with 50  $\mu$ L of IS solution. Each sample was vortexed for 15 seconds and then sonicated in a water bath for 15 minutes at room temperature. Samples were centrifugated at 20000 x g for 10 min at 4°C, and supernatant was transferred to glass tubes. Supernatant was evaporated in a vacuum-centrifuge at 60°C, reconstituted with 300  $\mu$ L of water:acetonitrile (95:5;v/v) and injected in UPLC system.

*Recruiting of patients, sampling, and calculation of cell associated drug concentrations:* Clinical samples were collected after obtaining written informed consent, according to local Ethics Committee indications (ASLTO2, protocol "TB INTRA"). The method was tested on patients who attended the Amedeo di Savoia Hospital (Turin). All subjects were over the age of 18 years and received standard oral dosages, given on an empty stomach, of ethambutol (20 mg/kg/day), isoniazid (5 mg/kg/day), pyrazinamide (20 mg/kg/day), and rifampicin (10 mg/kg/day). Samples were obtained after four weeks of treatment. Venous blood samples were collected at the end of the dosing

interval ( $C_{\text{trough}}$ ) and two hours post dose ( $C_{\text{max}}$ ). They were centrifuged ( $900 \times g$ , 15 min at  $4^{\circ}\text{C}$ ) to separate the plasma. PBMCs were isolated from blood (28 mL) using BD Vacutainer® CPT™ tubes. Tubes were centrifuged at  $800 \times g$ , 15 min at  $20^{\circ}\text{C}$ . PBMCs were then washed 2 times in ice cold NaCl 0.9% solution. Cell number and mean cellular volume (MCV) were determined by an automated cell counter (Z2™ COULTER COUNTER®, Beckman Coulter, Brea, CA, USA). Plasma samples and the resulting pellet of PBMCs, dissolved with 800  $\mu\text{l}$  of solution water:methanol (30:70; v/v), were stored at  $-80^{\circ}\text{C}$ . The time taken from blood sampling to PBMC storage was less than 1 h. PBMCs associated concentrations of antituberculars, expressed in ng/mL, were obtained using the following formula: antitubercular amount (ng) / number of PBMCs  $\times$  MCV (fL)  $\times 10^{-12}$ .<sup>11-15</sup>

## Results

### *Validation of the assay*

The assay was validated in accordance with FDA guidelines. {FDA, 2013 #16}

Representative chromatograms are reported in Figure S1.

Absence of interference from endogenous and exogenous compounds (antibacterials, antiretrovirals) was confirmed by the analysis of six different blank plasma and PBMC samples.

A quadratic forced through the zero calibration curve (mean  $r^2 > 0.99$ ) was used for all drugs assayed for both plasma and PBMCs. Intra and inter-day accuracy (percentage error) and precision (percentage relative standard deviation, R.S.D%) was lower than 15% (n=5, Table 1). The lower limit of quantification, LLOQ, was considered the lowest point of the calibration curve (Table S2). The LOD for the plasma and PBMC method was 58 ng/mL and 2.93 ng/mL for ethambutol, 58 ng/mL and 0.391 ng/mL for isoniazid, 68 ng/mL and 0.391 ng/mL for pyrazinamide, and 117 ng/mL and 0.976 ng/mL for rifampicin.

Recovery was evaluated by calculating the ratio among peak area of extracted and un-extracted QCs (n=5).<sup>16</sup> Matrix effect was assayed by calculating the ratio among peak area of un-extracted QCs and standards of the analytes present in the reconstitution solvent [water and acetonitrile (95:5, v/v)].<sup>16</sup> Results are reported in Table S3.

Antituberculars were found to be stable (degradation lower than 20%) in stock solutions and in plasma at -80°C for one month and after three cycles of freezing at -80°C and thawing at room temperature (over 1 h).

### *Absence of antitubercular loss during the washing procedure of PBMCs.*

To assess if the washing procedure of PBMCs leads to drug loss, 40 mL of washing supernatant were collected and quantified as reported above. We found that antitubercular levels in the washing supernatant was lower than the LOD and we can conclude that no drug loss happens during the washing procedure of PBMCs.



*Analysis of antitubercular concentrations in patients.*

In Figure 1, plasma and PBMCs associated concentrations obtained from 15 subjects are reported. As already reported in literature <sup>2</sup>, isoniazid and rifampicin had undetectable or very low plasma  $C_{\text{trough}}$  levels and ethambutol and pyrazinamide had low  $C_{\text{trough}}$  levels. Plasma  $C_{\text{max}}$  were within the target therapeutic range for the four drugs quantified. <sup>2</sup> Isoniazid, pyrazinamide, and rifampicin had undetectable or very low intracellular  $C_{\text{trough}}$  levels. Isoniazid and pyrazinamide seemed not to accumulate in PBMCs, rifampicin accumulates around 1-fold within PBMCs and ethambutol accumulates around 30-fold.

## Discussion

We developed and validated a new chromatographic method to simultaneously quantify first line antituberculars in plasma and PBMCs. To date, only one study reported an assay to simultaneously quantify first-line antitubercular drugs plasma concentrations using HPLC-MS/MS.<sup>17</sup> Our method of extraction from plasma, compared to the previous published method, has the advantage of a faster extraction procedure using a single precipitation step with acetonitrile and direct injection in UPLC. The method described by Song et al.<sup>17</sup> involves two steps of deproteinization and a run time of 4 minutes. The longer time of chromatographic run used in our method (6 minutes) allows to better separate analytes reducing potential interferences from endogenous and exogenous compounds and reducing matrix effect.

The only method published on antitubercular associated PBMC concentrations was described by Hartkoorn et al.<sup>18</sup> and it only quantifies rifampicin. In our method calibration curves and QCs used for the intracellular quantification of antituberculars were prepared in PBMCs and recovery and matrix effect were evaluated using PBMCs differently from what observed in the above cited method<sup>18</sup>. Furthermore we measured MCV (median MCV = 272 fL) instead of assuming a fixed MCV of 400 fL; this may be more accurate when estimating intracellular concentrations reducing the probability of under or overestimating drug levels in PBMCs.<sup>11, 13-15, 19</sup> Rifampicin accumulation inside PBMCs was lower to what was previously observed (intracellular to plasma ratios of 1 versus 1.8 in the work from Hartkoorn et al.<sup>18</sup>), but further studies are needed to confirm these findings.

A high interpatient variability was observed both in plasma and intracellular concentrations thus supporting the use of therapeutic drug monitoring for optimizing the antitubercular drug pharmacokinetic profile. The reported intra-PBMC concentrations warrant further investigation in order to study the potential concentration-dependant efficacy or toxicity in patients treated for tuberculosis. This newly developed method that allows for rapid and simultaneous quantification of

first-line plasma and intra-PBMC antitubercular drug concentrations may be a useful tool for tailoring antitubercular regimens in selected patients.

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### **Transparency declarations**

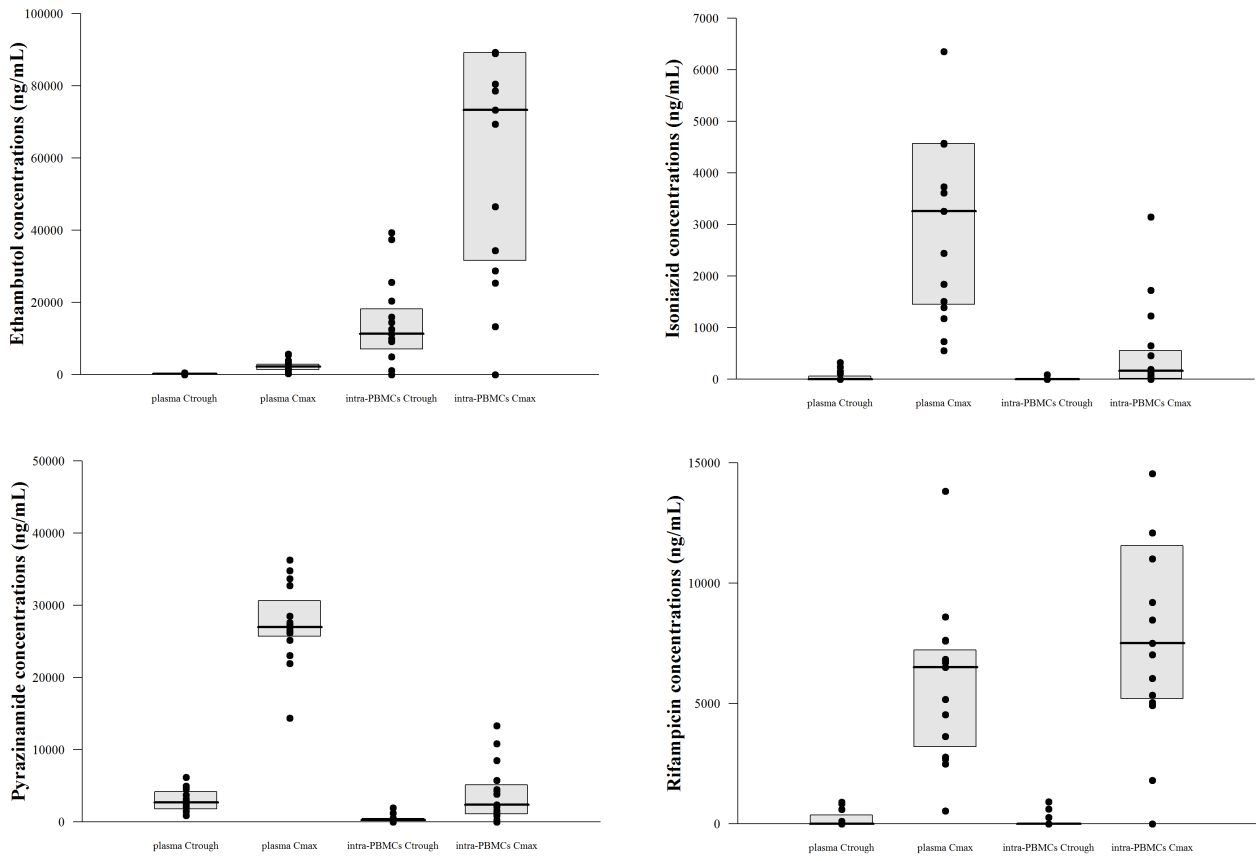
None to declare.

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	Mean accuracy (% error)				Mean precision (R.S.D.%)			
	EMB	INH	PZA	RFP	EMB	INH	PZA	RFP
Method of extraction from plasma								
Intra-day								
QC H	7.81	7.91	6.09	7.21	10.15	7.06	5.09	9.72
QC M	7.96	7.87	4.65	6.05	6.96	12.74	6.08	6.25
QC L	3.28	3.39	8.00	11.37	2.46	4.59	8.00	3.09
Inter-day								
QC H	6.88	8.18	10.41	7.43	12.28	9.31	8.77	7.71
QC M	4.54	7.03	8.33	8.22	10.62	9.90	8.33	9.05
QC L	6.78	9.48	4.82	8.77	12.92	13.63	6.97	12.65
Method of extraction from PBMC's								
Intra-day								
QC H	5.54	7.44	9.65	8.00	13.04	11.84	6.63	10.50
QC M	4.04	4.16	10.17	7.41	4.72	6.09	2.98	8.77
QC L	7.36	6.08	8.25	7.21	8.51	11.99	8.81	13.12
Inter-day								
QC H	8.20	5.07	8.67	5.69	12.19	11.48	13.34	9.81
QC M	7.17	4.87	6.88	4.97	10.40	7.07	9.87	6.48
QC L	12.66	9.98	7.87	7.48	14.22	12.60	9.44	9.13

**Table 1.** Average values of intra and inter-day accuracy and precision values obtained from the method of extraction from plasma and from PBMCs. R.S.D, relative standard deviation; QC, quality control; H, high; M, medium; L, low; EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RFP, rifampicin.



**Figure 1.** Plasma and PBMCs associated concentrations of the four antituberculars obtained from 15 subjects.

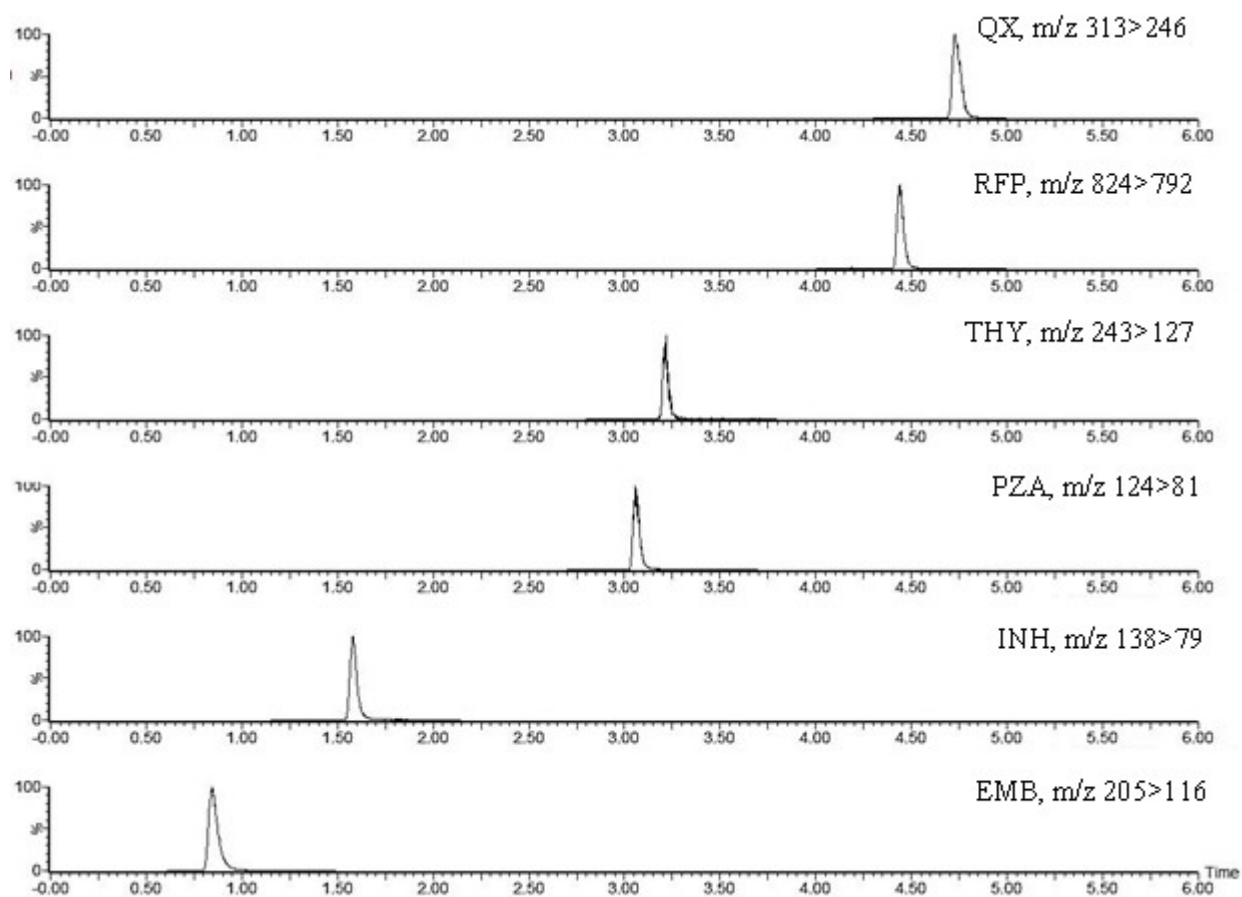
	Cone voltage (V)	Collision Energy (eV)	MRM transition ( <i>m/z</i> )
EMB	25	16	205 → 116
INH	32	24	138 → 79
PZA	25	15	124 → 81
RFP	35	16	824 → 792
QX	58	40	313 → 246
THY	14	14	243 → 127

**Table S1.** MS/MS conditions and drug MRM transitions. The mass spectrometer was settled in the positive ion mode, with a capillary voltage of 3.5 kV, a source temperature of 150°C, and a desolvation temperature of 500°C. The nitrogen gas flow was 800 L/h and 50 L/h for desolvation and cone, respectively. MS/MS, mass mass; MRM, multiple reaction monitoring. EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RFP, rifampicin, THY, thymidine; QX, dimethyl-2,3-di(2-pyridyl)quinoxaline.

Standard and quality control levels used for quantification in plasma				
	EMB (ng/mL)	INH (ng/mL)	PZA (ng/mL)	RFP (ng/mL)
STD 1	117	117	547	469
STD 8	15000	15000	70000	60000
QC H	12000	12000	60000	50000
QC M	4000	4000	20000	16000
QC L	300	300	1500	1500
Standard and quality control levels used for quantification in PBMCs				
	EMB (ng/10x10 <sup>6</sup> cells)	INH (ng/10x10 <sup>6</sup> cells)	PZA (ng/10x10 <sup>6</sup> cells)	RFP (ng/10x10 <sup>6</sup> cells)
STD 1	5.86	0.781	0.781	1.95
STD 9	1500	200	200	500
QC H	800	150	150	400
QC M	80	15	15	40
QC L	16	3	3	8

**Table S2.** Standard and quality control levels used for quantification in plasma and PBMCs. STD, standard; QC, quality control; H, high; M, medium; L, low; EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RFP, rifampicin.





**Figure S1.** Representative chromatograms relative to the stock solutions of the four antituberculars and internal standards dissolved in water and acetonitrile (95:5, v/v) at the concentration of 10000 ng/mL. EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RFP, rifampicin, THY, thymidine; QX, dimethyl-2,3-di(2-pyridyl)quinoxaline.

	Mean recovery (%)	R.S.D.%	Mean matrix effect (%)	R.S.D.%
Plasma				
EMB	28.59	12.12	88.97	10.44
INH	11.45	14.04	94.51	8.02
PZA	12.64	14.73	104.93	3.53
RFP	9.90	13.55	92.96	4.20
THY	91.22	11.86	114.62	10.00
QX	61.74	14.96	111.68	4.98
PBMCs				
EMB	65.90	12.28	34.36	13.28
INH	57.54	11.31	50.39	12.54
PZA	94.30	4.77	82.40	6.42
RFP	60.92	7.81	99.94	13.89
THY	84.31	10.19	95.05	12.87
QX	88.47	9.17	103.53	3.87

**Table S3.** Mean recovery and mean matrix effect obtained from the method of extraction from plasma and PBMCs. R.S.D, relative standard deviation; EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RFP, rifampicin, THY, thymidine; QX, dimethyl-2,3-di(2-pyridyl)quinoxaline.

