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Simultaneous quantitation of azole antifungals, antibiotics, imatinib, and raltegravir in human plasma by two-dimensional high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

High-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a standard analytical technique for therapeutic drug monitoring (TDM). A rapid LC–MS/MS method was developed for simultaneous quantitation of 3 antifungals and one active metabolite (posaconazole, voriconazole, itraconazole, and hydroxy-itraconazole), 5 antibiotics (daptomycin, ciprofloxacin, oxacillin, levofloxacin, and rifampicin), an antineoplastic agent (imatinib), and an antiretroviral (raltegravir) in human plasma. Protein precipitation of 10 μL of plasma with acetonitrile was used as a single-extraction procedure. After 2-dimensional LC, all drugs were quantified by electrospray ionization-triple quadrupole mass spectrometry by selected reaction monitoring detection in the positive mode. The method was validated per FDA recommendations including the study of extraction recovery (from 79.3% to 105.9%) and matrix effect via ion suppression/enhancement phenomenon. This method is precise (intra- and inter-assay coefficients of variation of 1.95–12.77%, 2.56–8.16% and 2.12–11.38% for low, medium and high levels of internal quality controls respectively) and accurate (intra- and inter-assay biases of 0.19–12.67%, 0.04 to −12.17% and 0.22–12.98% respectively). This method is an efficient tool for routine TDM and optimization of laboratory resource utilization.

1. Introduction

High-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been a standard analytical technique for therapeutic drug monitoring (TDM) for more than 10 years in clinical pharmacology–toxicology laboratories. In recent years, many laboratories have invested in this system, which has become cheaper and easier to use routinely.

The benefits of LC–MS/MS have been well described. This technique provides greater sensibility (lower quantities of detected compounds), better specificity (limited cross-reaction with potential metabolites), wider dynamic ranges (no necessity to dilute samples with high concentration levels), less expensive analysis costs, faster run times, and versatility [1–4].

These features have encouraged the switch of many immunochemical techniques and LC methods with ultraviolet detection to LC–MS/MS methods, which, however, has led to the rapid saturation of analytical systems, consequently reducing available time for new development and for technical transfer to daily routine. Further, after several years of LC–MS/MS use, the analytical devices become overflowed by all the new transferred methods. This pattern leads to difficulties in managing time schedules, necessitating multiple analyses being chained and operated using disparate methods that it is not always possible to check and reprocess on the same day.

The multiplicity of analyses also creates problems with regard to the preparation of biological samples, the preparation of different mobile phases, and the use of multiple analytical methods that are laborious, creating sources of error by the technical staff.

Our aim was to develop an analytical method that enables one to gather as many new molecules as possible to transfer to LC–MS/MS quantitation, independent of their pharmacological class. We initially examined antifungals, for which we developed a method that was versatile enough to analyze them and their metabolites, for which chromatographic separation was required due to the electrospray in-source transformation phenomenon [5–8]. The 4 antifungals were voriconazole (VORI), itraconazole (ITRA) and its active metabolite hydroxy-itraconazole (OH-ITRA), and posaconazole (POSA). In this preliminary method, we focused

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on separating interfering metabolites, such as glucuronides. Based on this method, we aimed to integrate new compounds from disparate therapeutic families: an antineoplastic agent, imatinib (IMA); 5 antibiotics (daptomycin (DAPTO), rifampicin (RIFAM), oxacillin (OXA), ciprofloxacin (CIPRO); and levofloxacin (LEVO)); and the antiretroviral integrase inhibitor raltegravir (RALTE).

Several LC–MS/MS analytical methods have been described for the quantitation of antifungals [8–22], IMA [23–29], DAPTO [30–32], LEVO [33–36], RIFAM [35–38], and CIPRO [39,40]. The quantitation of RALTE has recently been reported, including or not its glucuronide interference [5,41–45]. Thus, we concluded that at least 5 methods would be necessary to quantitate these 11 compounds.

To combine the analyses of these compounds, including an optimized chromatographic separation of interfering metabolites, we developed a single, rapid method with simplified sample preparation. To this end, a quick sample preparation step was performed, and the core–shell chromatographic column was selected. This new type of column technology enables one to perform ultrahigh-performance liquid chromatography (UHPLC) while keeping the backpressure compatible with conventional LC systems. Furthermore, the 2-dimensional chromatography including an online sample clean-up step, allows a simple and fast sample pretreatment (protein crash) while avoiding the generation of matrix effects such as ion suppression and/or enhancement phenomenon. Online sample clean-up is commonly described using different type of materials (turbulent flow chromatography, Restricted Access Materials (RAM)[46], Large Particle Supports technologies as Turbulent Flow Chromatography[47], Perfusion Chromatography[17]) to enhance the purity of the sample which has been quickly prepared offline.

We described the development and technical validation of a versatile, simple, sensitive, and robust 2-dimensional LC–MS/MS method for the simultaneous quantitation of 11 molecules in human plasma from routine therapeutic drug monitoring (TDM).

2. Experimental

2.1. Chemical and reagents

OXA sodium salt, CIPRO, and LEVO were purchased from Sigma Aldrich Chemicals (St. Quentin Fallavier, France). RIFAM and the internal standards (IS) voriconazole-d_3 (VORI-d_3), itraconazole-d_5 (ITRA-d_5), hydroxy-itraconazole-d_5 (OH-ITRA-d_5), and posaconazole-d_3 (POSA-d_3) were purchased from LGC standards (Molsheim, France). Levofloxacine-^{13}C-d_3 (LEVO-^{13}C-d_3) was purchased from AlsaChim (Strasbourg, France). VORI was kindly provided by the Central Research Division of Pfizer (Gronton, CT, USA); ITRA and OH-ITRA by the Quality and Compliance Division of Janssen Pharmaceutica N.V. (Beerse, Belgium); DAPTO, IMA, and imatinib-d8 (IMA-d_8) by Novartis Pharma (Basel, Switzerland); POSA by the Research Division of Schering-Plough (Schaumburg, IL, USA); and RALTE potassium salt and raltegravir-$^{13}C_6$ (RALTE-$^{13}C_6$) by the Chemical Department of Merck (Darmstadt, Germany).

HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from Carlo Erba Reagents (Val de Reuil, France), HPLC-grade ammonium acetate and formic acid were provided by Prolabo (Paris, France). Ultrapure water (resistivity ≥18.0 MΩ/cm) was obtained using a Milli-Q Plus (Millipore, Molsheim, France). Polypropylene 2-milliliter (mL) centrifuge tubes, 2-mL tubes with 200-microliter (μL) restrictor screw cap vials, and pipette tips were purchased from Eppendorf (Le Pecq, France), Interchim (Montluçon, France) and Gilson (Middletown, WI, USA), respectively. Drug-free plasma from volunteers was provided by Etablissement Français du Sang (Grenoble, France), and patient's plasmas were obtained from patients treated by at least one on the drugs included in our analytical method and for whom TDM was performed. Blood samples were collected in sodium heparinate, centrifuged at 1700 × *g* for 10 min at 4 °C, and stored at −20 °C until analysis.

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

Considering the solubility of the powders, various concentrations of stock solutions were prepared in MeOH or water: in MeOH: DAPTO at 5 mg/mL; IMA at 2 mg/mL; CIPRO, RALTE, and RIFAM at 1 mg/mL; and VORI, POSA, ITRA, and OH-ITRA at 0.2 mg/mL; in water: LEVO at 0.2 mg/mL and OXA at 1 mg/mL.

All stock solutions were ultrasonicated for 10 min to improve dissolution and stored at −80 °C. Two batches of stock solutions were made: one for calibration curves and the other for internal quality control (IQC) samples.

Appropriate volumes of each stock solution were added to a centrifugation tube and evaporated under nitrogen. An aliquot of 1.00 mL of drug-free plasma was added to obtain the highest concentration of the calibration curve (see Table 1 for details). Beginning with this spiked plasma, a series of dilutions was made to obtain the 6 levels of calibration (see Table 1 for details of the dilution and concentrations for each compound). This process was repeated for the 3 levels of IQC beginning with a separate 1 mL spiked plasma (see Table 1).

Stock solutions of ISs were prepared in MeOH at 1 mg/mL for RALTE-$^{13}C_6$, VORI-d_3, ITRA-d_5, OH-ITRA-d_5, and POSA-d_4 and at 0.25 mg/mL for LEVO-$^{13}C_3$ d_3 and IMA-d_8.

2.3. Sample preparation

In polypropylene tubes, 10 μL of plasma was treated with 60 μL of precipitation reagent (acetonitrile/methanol (50/50, v/v) + 0.1% perchloric acid), containing ISs at their respective concentrations (1000 ng/mL for all ISs excepted 800 ng/mL for RALTE-$^{13}C_6$).

Samples were vortexed immediately for 10 s. The mixture was centrifuged 10 min at 25,000 × *g* (Eppendorf Centrifuge 5417c), and 40 μL of supernatant was transferred to integrated microinsert polypropylene HPLC vials.

2.4. Instrumentation

2.4.1. Chromatographic conditions: 2-dimensional chromatography configuration

The LC system comprised an Ultimate 3000 RS quaternary pump (Pump A) and an Ultimate 3000 quaternary pump (Pump B), equipped with an autosampler and a column compartment (Ultimate 3000 RS, Dionex, Thermo Scientific, Germering, Germany). Online sample clean-up was performed on a Perfusion column (POROS R1/20, 20 μm, 2.1 mm × 30 mm, Applied Biosystems, Darmstadt, Germany). Chromatographic separation was performed on a pentafluorophenyl (PFP) analytical column (Phenomenex Kinetex, 2.6 μm, 2 mm × 50 mm, Aschaffenburg, Germany).

The LC-integrated online sample clean-up consisted of 2 steps: first, 5 μL of deproteinized sample (thermostated at 4 °C in the autosampler) was injected into the system and transferred onto the POROS column, onto which the analytes adsorbed, whereas potentially interfering matrix compounds (mainly salts, protein residues) were washed directly into the waste by mobile phase A (water + 0.1% formic acid) delivered at a flow rate of 4.0 mL/min over 0.70 min (Fig. E1A in Online Supplemental Material). Next, the 6-port valve was switched at 0.75 min.

To obtain good separation between the compounds and their isobaric metabolites, the extract was eluted in a backflush

Table 1
Preparation of points of calibration (C1 to C7) and internal quality controls (IQC) for each analyte with their respective concentration levels, regression models, weighting factors, and internal standards. From C1 to C7, concentrations are obtained by successive dilution from the highest to the lowest level (first lot of 1 mL spiked plasma) From IQC1 to IQC3, concentrations are obtained by diluting the second lot of 1 mL spiked plasma.

Levels of concentrations for each point of the calibration curve and the three internal quality controls													
Calibration points and IQC		C1		C2		C3	C4	C5	C6	C7	IQC1	IQC2	IQC3
Volume of spiked plasma (μL)		250		400		400	500	500	500	1000	15	200	750
Volume of drug-free plasma (μL)		750		600		600	500	500	500	0	985	800	250
Corresponding concentrations (mg/L) for each analyte											Regression model	Weighting factor	Internal standard
ITRA	0.1	0.4	1.0	2.5	5.0	10	20	0.3	4.0	15	Quadratic	1/*x*	ITRA-d_5
OH-ITRA	0.1	0.4	1.0	2.5	5.0	10	20	0.3	4.0	15	Quadratic	1/*x*	OH ITRA-d_5
POSA	0.1	0.4	1.0	2.5	5.0	10	20	0.3	4.0	15	Quadratic	1/*x*	POSA-d_4
VORI	0.1	0.4	1.0	2.5	5.0	10	20	0.3	4.0	15	Quadratic	1/*x*	VORI-d_3
IMA	0.025	0.1	0.25	0.625	1.25	2.5	5.0	0.075	1.0	3.75	Quadratic	1/*x*	IMA-d_8
RALTE	0.01	0.04	0.10	0.25	0.50	1.0	2.0	0.03	0.4	1.5	Linear	1/*x*	RALTE-$^{13}C_6$
DAPTO	0.6	2.4	6.0	15	30	60	120	1.8	24	90	Quadratic	1/*x*	IMA-d_8
CIPRO	0.04	0.16	0.4	1.0	2.0	4.0	8.0	0.12	1.6	6	Linear	1/*x*	LEVO-$^{13}C_3$ d_2
LEVO	0.04	0.16	0.4	1.0	2.0	4.0	8.0	0.12	1.6	6	Quadratic	1/*x*	LEVO-$^{13}C_3$ d_2
OXA	0.2	0.8	2.0	5.0	10	20	40	0.6	8.0	8	Quadratic	1/*x*	VORI-d_3
RIFAM	0.2	0.8	2.0	5.0	10	20	40	0.6	8.0	8	Quadratic	1/*x*	VORI-d_3

mode and transferred to the analytical column (maintained at 60 °C). The elution was performed at 0.9 mL/min under the following conditions: water + 10 mM ammonium formate, pH 3.0 with formic acid (mobile phase C) and ACN + 0.1% formic acid (mobile phase D): 0–0.75 min: 10% D; 0.75–3.20 min: 10–98% D; 3.20–3.80 min: 98% D; 3.80–3.81 min: 10% D; and 3.81–4.00 min: 10% D (Fig. E1B in Online Supplemental Material). During this step, the valve was switched back at 2.9 min to its original configuration, allowing a mobile phase B (MeOH + 0.1% formic acid) to wash the POROS purification column for 0.90 min before being equilibrated using mobile phase A for the next run.

A supplemental diverter valve was set to waste from 0 to 1.9 min, to mass spectrometer from 1.9 to 3.5 when the analytes were eluted, and then to waste till the end of the run

2.4.2. Mass spectrometric conditions

Measurements were performed on an API 4000 Tandem Mass Spectrometer (ABSciex, Toronto, Canada) equipped with a Turbo Ion Spray® source. Quantification was achieved in the multiple reaction monitoring (MRM) mode, monitoring 2 ion transitions per analyte (one for quantitation and one for confirmation) and one ion transition for each IS. For each analyte, the monocharged molecular ion $[M+H]^+$ was selected as the parent ion except for DAPTO for which the doubly-charged molecular ion $[M+2H]^{2+}$ was selected.

The source was operated in positive ion mode with an ESI potential of +5500 V and the following parameters: turbo heater gas at 60 psi, ion source temperature at 600 °C, nebulizer gas setting at 50 psi, and curtain gas setting at 40 psi.

Due to the high analytical flow rate (0.9 mL/min) and narrowness of the chromatographic peaks, the dwell times for each ion transition were set relatively low (≤10 ms) to obtain at least 15 points per chromatographic peak to obtain good reproducibility in peak areas. The MRM settings are listed in Table 2.

2.5. Method validation

Validation of the method was based on guidance of the Food and Drug Administration Guidance for Industry Bioanalytical Method Validation [48].

The selectivity was tested by the analysis of 10 sources of blank plasma samples, treated with the precipitation reagent without IS, and 10 blank plasma samples treated with the precipitation reagent containing the ISs to measure putative interference by isotope-labeled IS impurities. For each molecule, the MRM signal was monitored to confirm the absence of interfering matrix compounds.

The calibration model (linearity) was examined for each compound by choosing a suitable regression model and the appropriate weighting factor to compensate for heteroscedasticity.

Within- and between-day accuracy (bias) and precision were examined by replicate analyses ($n = 6$) of the 3 levels of the IQC. Per the guidance of the FDA, the first level of the IQC was 3 times higher than the first level of the calibration curve. Inter-day accuracy and precision were assessed with the same IQCs by injections ($n = 6$) of the same IQCs within 6 various days (including 3 consecutive days among these six) and by several analysts, and were calculated as the percentage deviation of the average calculated concentration from the nominal concentration. Precision was expressed as the percentage of coefficient of variation (CV%). The acceptance limits were <15% for precision and within ±15% of the nominal value for accuracy, except for the lower limit of quantitation (LLOQ), for which <20% for precision and within ±20% of the nominal value for accuracy were accepted.

The upper limit of quantitation (ULOQ) has been evaluated and validated with the same levels of precision and accuracy criteria than those required for the three levels of IQC.

Two dilution factors (2- and 4-fold) were tested by spiking blank plasma ($n = 6$) with all analytes at concentrations above the ULOQ.

Extraction recovery was performed to evaluate the sample preparation steps: 1 series of spiked plasma samples was processed as follows: 1. Blank plasmas from 6 sources were precipitated, vortexed, and centrifuged. The supernatants were then evaporated and reconstituted with the appropriate diluted stock solutions. These samples were considered to reflect the loss of compounds during sample preparation. 2. Six blank plasma samples spiked with known concentrations of analytes were prepared in the same way as the IQC.

The extraction recoveries were calculated as the ratio of absolute peak area responses of the samples, spiked before and after the protein precipitation step.

Qualitative matrix effects were studied by analyzing ion suppression and enhancement phenomenon. Extracted blank plasma samples were injected into the LC system while a methanolic solution that contained all compounds and ISs (each at 200 ng/mL) was continuously post-column infused in the ionization source through a tee [49]. The choice of the most appropriate IS was clear for 7 compounds for which stable isotope-labeled analogs were available. For the remaining 4 compounds (CIPRO, DAPTO, OXA and RIFAM), the

Table 2
MRM transitions, voltage settings declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) for each analyte. The collision gas (CAD) was set to 10 psi. Dwell times were set automatically by the Scheduled MRM mode of the software to obtain at least 15 points per peak. Underlined ions were used as quantifiers.

Analyte	Q1 *m/z*	Q3 *m/z*	Dwell time (ms)	DP (V)	EP (V)	CE (V)°	CXP (V)
ITRA	705.0	392.2	10	116	10	49	12
	705.0	159.1	2	116	10	105	10
OH-ITRA	721.0	408.2	10	106	10	51	12
	721.0	174.2	2	106	10	65	10
POSA	701.1	127.1	10	131	10	91	12
	701.1	203.1	2	131	10	71	8
VORI	350.1	281.0	10	61	10	23	18
	350.1	127.0	2	61	10	45	8
IMA	494.3	394.1	10	106	10	35	10
	494.3	247.2	2	106	10	65	6
RALTE	445.2	109.1	10	71	8	51	4
	445.2	361.0	2	71	8	23	4
DAPTO	811.2	159.2	10	76	9.5	67	10
	811.2	304.3	2	76	9.5	23	8
CIPRO	332.1	231.0	10	61	12	49	8
	332.1	288.1	2	61	12	21	8
LEVO	362.1	261.1	10	71	7.5	39	8
	362.1	318.2	2	71	7.5	23	8
OXA	402.1	160.1	10	40	8	19	4
	402.1	114.1	2	40	8	51	4
RIFAM	823.5	791.5	10	71	8	23	12
	823.5	399.5	2	71	8	34	8
LEVO-$^{13}C\ d_3$	366.2	320.2	10	86	10	31	8
RALTE-$^{13}C_6$	451.2	115.1	10	71	8	51	4
IMA-d_8	502.3	225.2	10	111	11.5	33	4
VORI-d_3	353.1	284.0	10	61	10	23	18
ITRA-d_5	710.0	397.2	10	116	10	49	12
OH ITRA-d_5	726.0	413.2	10	106	10	51	12
POSA-d_4	705.3	127.2	10	131	10	91	12

ion suppression or enhancement analysis guided the selection of appropriate ISs among the ones that were available for the first 7 compounds.

Stability experiments should establish the conditions that are likely to be encountered during sample transfer, handling, and analysis. The stability of the extracted analytes in the injection vials at ambient temperature or at −20 °C for 24 h was evaluated for 1 concentration (same level of spiked plasma used for the extraction recovery test, $n = 6$). To ensure that the analytes were stable in the 4 °C thermostated autosampler throughout the entire analytical procedure, samples were analyzed again 6 h (overestimation of the time needed to analyze all the samples from patients) after their initial injection.

3. Results and discussion

3.1. Choice of analytical column

In addition to the new approaches of sub-2 μm particles and monolith columns, core–shell particle technology improves chromatographic efficiency (UHPLC). In our study, core–shell technology was selected through the Kinetex analytical column. The 2.6 μm particle size consists of a 1.9 μm nonporous core and a 0.35 μm porous silica layer. This highly optimized process, combined with uniform particle size distribution, yields a column that has a low height equivalent to a theoretical plate (HETP) by reducing the Van Deemter equation factors i.e. eddy dispersion (band broadening), longitudinal diffusion, and resistance to mass transfer [50].

Further, the small particle size allows high linear velocity without generating any significant backpressure, which is incompatible with conventional HPLC instruments that are limited to 400 bars. These 2 factors significantly reduce the HETP and allow a greater interval for optimal flows [50].

During the second step of the chromatographic run, when the purification column and the analytical column were placed in series, the maximum backpressure did not exceed 280 bars at the optimized flow rate of 0.9 mL/min. These backpressure conditions were compatible with the conventional HPLC system.

A run was completed within 5 min.

3.2. Choice of internal standard

Matrix effects are significant problems particularly with ESI ionization [51]. When possible, the use of isotope-labeled ISs is required [51,52]. Except for a slight lag in retention time when deuterated are used (which can impact the precision [53]), these analogs have similar chromatographic behaviors and almost identical physiochemical properties for ionization. Unfortunately, we did not have an isotope-labeled analog for all 11 drugs. The choices for CIPRO, DAPTO, OXA and RIFAM were based on the ion suppression/enhancement test. The aim was to select analyte-SI couples with similar MRM signals at their respective retention times. For instance, the IMA-d8 MRM signal was more suitable for DAPTO than ITRA-d5 due to the large slope of the signal at the ITRA-d5 retention time (Fig. 1).

Moreover, the choice of ISs for these 4 drugs was, based on a reproducibility study of spiked plasma, which confirmed the selections.

Table 1 lists the ISs that were chosen for each analyte.

3.3. Chromatograms

A representative chromatographic profile of calibration point number 6 is shown in Fig. 2.

It was necessary to separate POSA and RALTE from their isobaric glucuronide metabolites, and the presence of isotope-labeled ISs of the parent compounds confirmed their retention times (Fig. 3).

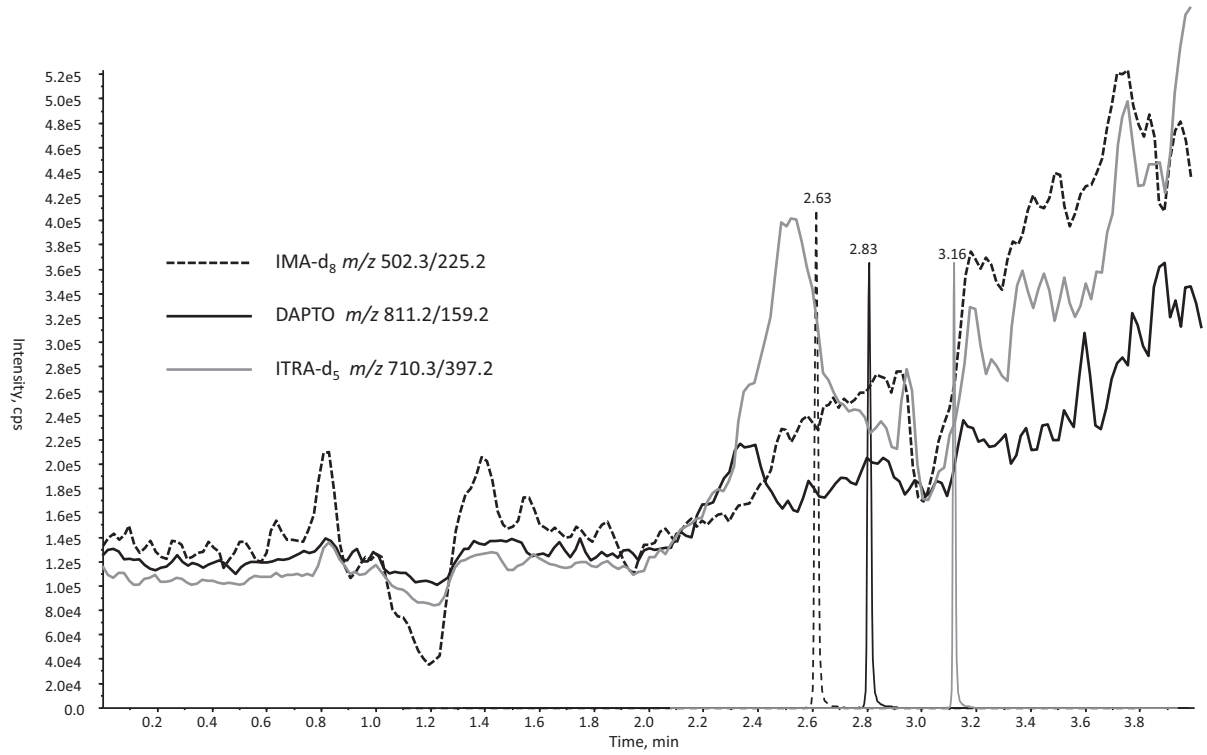


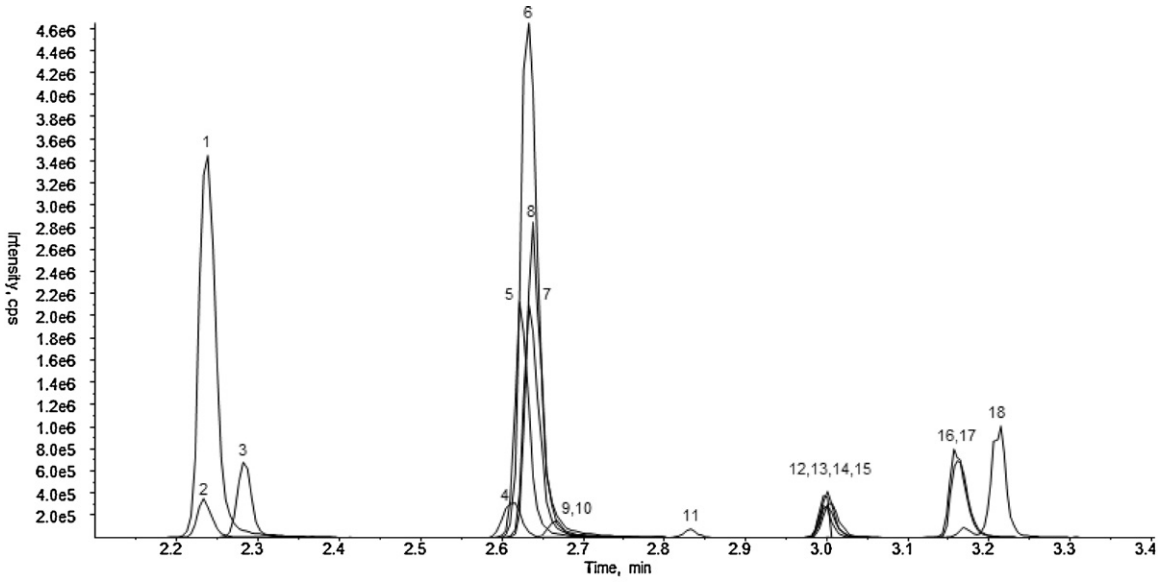
Fig. 1. Choice of internal standard for DAPTO. Post-column infusion of DAPTO, IMA-d_8, and ITRA-d_5 with injection of an extract of drug-free plasma, overlaid with typical chromatographic peaks of the same compounds.

3.4. Selectivity

The analysis of 10 blank plasmas in the absence of ISs showed that there was no interference between the 11 compounds and endogenous compounds in the matrix.

3.5. Within- and between-day accuracy and precision, LLOQ, ULOQ

Within-day and between-day precision and accuracy for the 3 levels of IQC samples are shown in Table 3.



1. **Levofloxacin** *m/z* 362.1/261.1
2. **Levofloxacin-^{13}C d_3** *m/z* 366.2/320.2
3. **Ciprofloxacin** *m/z* 332.1/231.1
4. **Oxacillin** *m/z* 402.1/160.1
5. **Imatinib-d_8** *m/z* 502.3/225.2
6. **Imatinib** *m/z* 494.4/394.2
7. **Voriconazole-d_3** m/z 353.2/284.1
8. **Voriconazole** m/z 350.2/281.0
9. **Raltegravir-$^{13}C_6$** *m/z* 445.2/109.1
10. **Raltegravir** *m/z* 450.2/114.1
11. **Daptomycin** *m/z* 811.2/159.2
12. **Posaconazole-d_4** *m/z* 705.2/127.1
13. **Posaconazole** *m/z* 701.3/127.1
14. **OH-Itraconazole-d_5** *m/z* 726.3/413.3
15. **OH-Itraconazole** *m/z* 721.2/408.1
16. **Itraconaole-d_5** *m/z* 710.3/397.2
17. **Itraconazole** *m/z* 705,0/392,2
18. **Rifampicin** *m/z* 823.5/399.5

Fig. 2. Typical chromatogram of the sixth calibration point (level of concentrations reported in Table 1) containing all of the analytes and the internal standards.

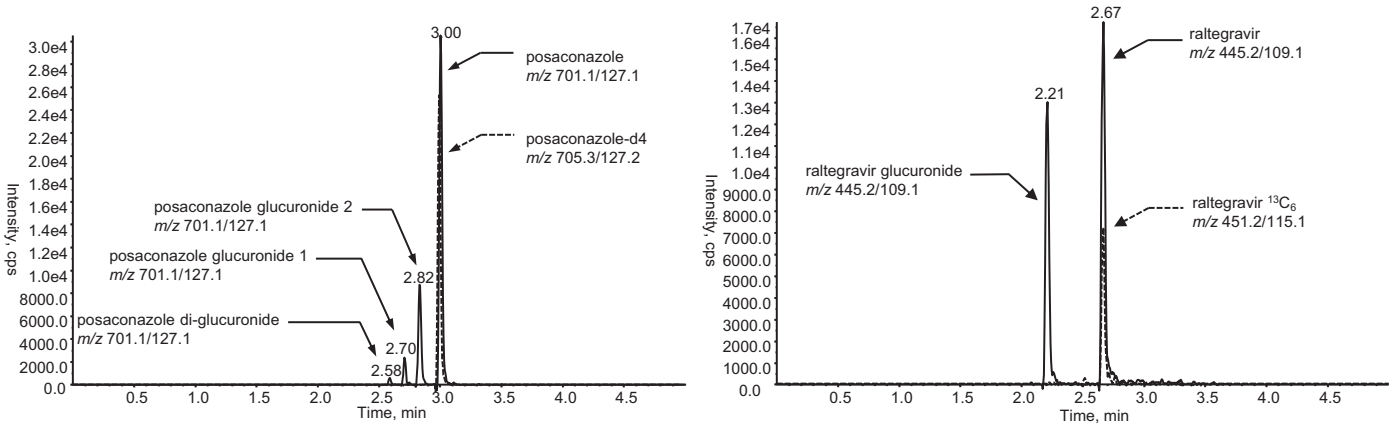


Fig. 3. Chromatograms presenting the analyses of samples from patients receiving (a) posaconazole and (b) raltegravir. In both cases, the isobaric glucuronide metabolites from in-source transformation are represented. Solid-line chromatograms for the molecules and their metabolites, dotted-line chromatograms for isotopic labeled internal standards.

All calculated concentrations were within the ranges of the FDA guidance: ±15% for the low, middle and high quality control levels.

The LLOQ and ULOQ were respectively determined as the lowest and highest concentrations of the calibration curve (Table 1). All concentrations met the FDA recommendations—ie, ±20% for precision and accuracy for LLOQ, ±15% for precision and accuracy for ULOQ (data not shown).

3.6. Linearity

Ratios of analyte peak area and IS peak area (y-axis) were plotted against ratios of analyte and IS concentrations (x-axis), and calibration curves were calculated by a least square regression with $1/x$ weighting with linear or quadratic regression, depending on the compounds (Table 1).

Table 3
Within- and between-day precisions (expressed as coefficients of variation) and accuracies (expressed as bias) of the LC–MS/MS method.

Analyte	Nominal concentration (ng/mL)	Within-day precision and accuracy ($n=6$)			Between-day precision and accuracy ($n=6$)		
		Mean concentration (ng/mL)	CV (%)	Bias (%)	Mean concentration (ng/mL)	CV (%)	Bias (%)
ITRA	0.30	0.29	5.5	−3.7	0.32	7.4	7.8
	4.00	3.51	6.1	−12.2	4.02	2.6	0.0
	15.0	14.3	3.2	−4.7	14.7	6.0	−2.0
OH-ITRA	0.30	0.31	6.0	2.9	0.33	8.7	1.4
	4.00	3.67	3.8	−8.3	8.68	4.7	0.4
	15.0	13.0	2.2	−11.3	1.44	5.5	2.8
POSA	0.30	0.30	11.9	−1.4	0.32	11.9	7.6
	4.00	3.59	5.2	−10.2	4.05	7.0	1.2
	15.0	14.7	2.2	−2.0	15.3	4.0	2.1
VORI	0.30	0.32	9.2	7.9	0.33	6.4	8.7
	4.00	3.91	4.0	−2.2	4.32	6.4	8.0
	15.0	13.8	4.2	−7.9	15.5	6.6	3.0
IMA	0.075	0.073	2.0	−3.3	0.08	7.7	6.0
	1.00	0.93	3.2	−6.8	1.02	5.1	1.7
	3.75	3.26	4.5	−13.0	3.79	5.6	1.0
RALTE	0.030	0.029	10.0	−3.1	0.031	5.1	2.2
	0.40	0.39	7.6	−0.2	0.42	6.4	6.2
	1.50	1.50	5.7	0.0	1.62	9.2	8.2
DAPTO	1.80	1.89	9.0	4.8	1.80	12.4	0.2
	24.0	22.3	3.7	−7.2	23.82	6.9	−0.8
	90.0	84.6	6.1	−6.1	91.25	11.4	1.3
CIPRO	0.12	0.13	7.0	4.6	0.12	13.2	1.4
	1.60	1.59	3.0	−1.0	1.63	8.2	1.9
	6.00	5.37	6.1	−10.6	6.29	7.5	4.8
LEVO	0.12	0.12	2.0	2.4	0.12	3.4	2.1
	1.60	1.53	4.1	−4.4	1.66	3.9	3.7
	6.00	5.34	2.1	−10.9	6.09	3.2	1.5
OXA	0.60	0.68	12.8	12.7	0.66	10.9	9.4
	8.00	7.87	5.8	−1.7	8.28	4.9	3.5
	30.0	26.6	8.1	−11.2	30.32	5.7	1.1
RIFAM	0.60	0.60	8.0	−0.5	0.65	9.2	8.3
	8.00	7.24	7.2	−9.6	8.21	4.3	2.6
	30.0	27.2	5.1	−9.8	29.74	6.3	-0.9

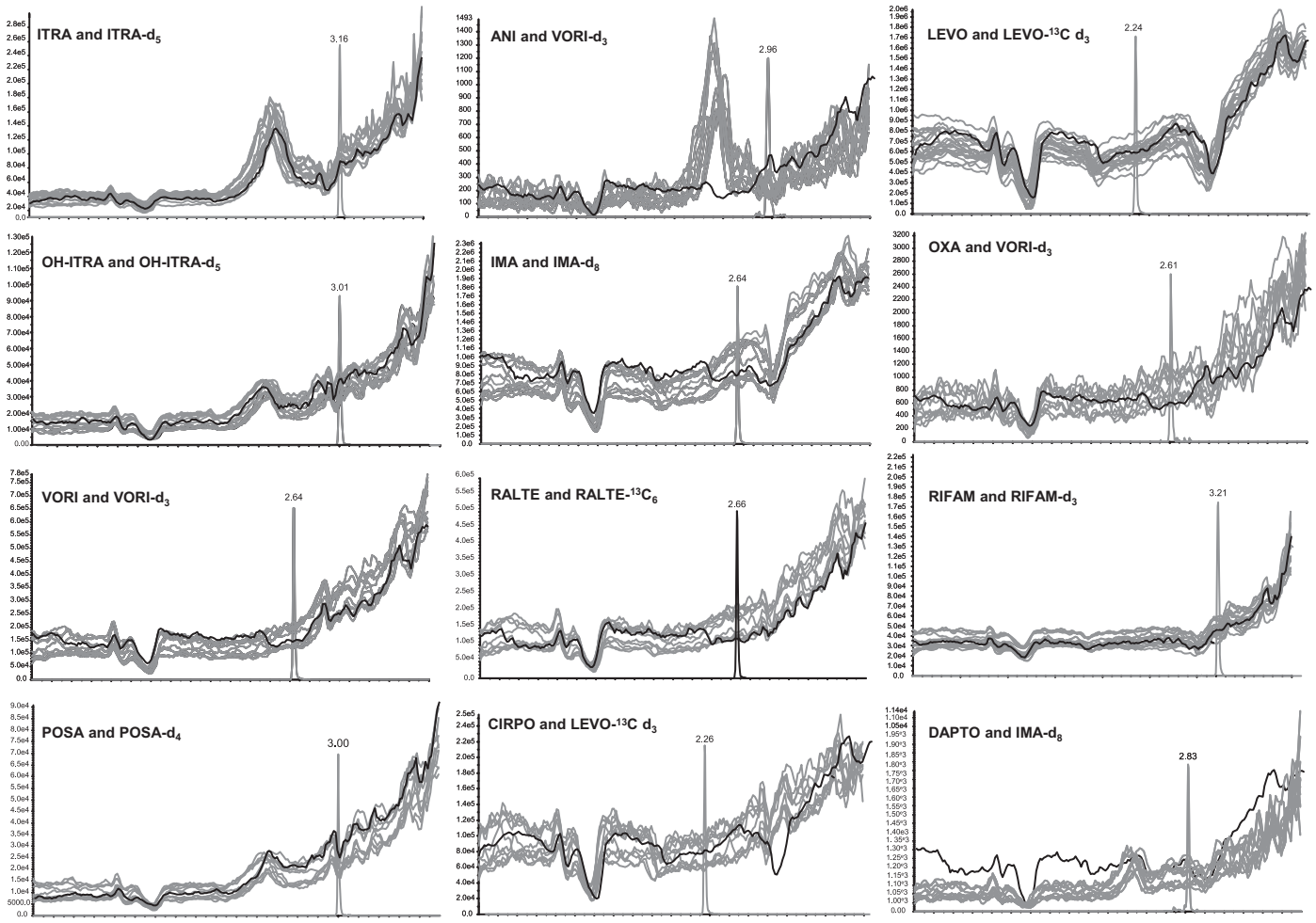


Fig. 4. Post-column infusion chromatograms depicting the matrix effects (ion suppression/enhancement) from 10 different blank plasma samples extracted by protein precipitation and online sample clean-up (overlay of 10 signals of each post-column infused compound – gray signals), following direct infusion of the analytes with their respective internal standards (single bold black signal representing a typical ion current among the ten initially obtained). When the SI was not the isotopically labeled analog (not co-eluted), two peaks were represented on the chromatogram in order to compare the similarities between the signals of the analyte and the signal of the IS.

3.7. Sample test dilution

During the validation, we diluted plasma samples ($n = 6$) spiked with concentrations of analytes 2- and 4-fold higher than the ULOQ. The resulting concentration values calculated with an adapted dilution factor were always ±15% of the target value (data not shown).

3.8. Matrix effects

3.8.1. Extraction recovery

Extraction recovery efficiencies are shown in Table 4. For all drugs, the extraction efficiency was between 79.3% and 105.9% (all CV <15%), indicating an acceptable loss of compounds during the protein precipitation step of the sample preparation.

Since all CV were <15% (Table 4.), we limited the extraction recovery study to a single level, whereas the FDA guidance requires to study this parameter on 3 levels of concentration. We acknowledge that this could represent a potential limitation of the validation of the method.

3.8.2. Ion suppression phenomenon

For each compound, there was an overlay of 10 signals of each postcolumn-infused compound (Fig. 4) and a signal that corresponded to the selected IS (in bold). Thus, at the retention times of the compounds, the signal variations were equivalent to those of the selected ISs (similar patterns).

3.9. Sample stability

Table 5 showed that the concentrations of samples stored for 24 h at room temperature, +4 °C and −20 °C were within 85–115% of the original concentrations.

These experiments were completed with a bibliographic study (Table E1 in Online Supplemental Material) demonstrating that some compounds were sensitive to storage conditions.

Table 4
Extraction recovery ($n = 6$).

	Nominal concentration (mg/L)	Extraction yield (%)	CV (%)
ITRA	0.5	93.9	7.7
OH-ITRA	0.5	100.2	7.3
POSA	0.5	91.0	5.1
VORI	0.5	92.5	4.0
IMA	0.1	105.9	6.8
RALTE	0.03	79.3	3.0
DAPTO	3.0	105.8	5.7
CIPRO	0.2	104.1	5.0
LEVO	0.2	94.7	4.8
OXA	1.0	97.2	8.6
RIFAM	1.0	103.2	8.4

Table 5
Stability of drug concentration after 24 hours in the injection vials at room temperature (RT), +4 °C, and −20 °C (n = 6).

Nominal concentration (mg/L)		Deviation from the nominal concentration (%)		
Temperature and delay		24 h RT	24 h +4 °C	24 h −20 °C
ITRA	0.5	+0.0	−9.0	+11.0
OH ITRA	0.5	+5.2	−10.4	+5.8
POSA	0.5	−6.9	−14.6	−2.1
VORI	0.5	+11.4	+5.7	+8.6
IMA	0.1	+1.2	+3.6	+3.9
RALTE	0.03	−7.7	−0.26	−5.1
DAPTO	3.0	−1.1	+5.5	+2.2
CIPRO	0.2	+10.0	+11.7	+13.7
LEVO	0.2	+6.1	+5.0	+6.6
OXA	1.0	−14.9	−0.5	+9

Most stock solutions remained stable at RT for several days, except for LEVO and IMA, the stabilities of which have not been examined after 24 h and 6 days, respectively. The most common method was to store highly concentrated stock solutions at −20 °C. The shortest duration was described for CIPRO (within one month) [39]. The compounds were also stable in plasma at −20 °C and −80 °C for at least 1 month for OXA [54], 2 months for all antifungals [10] and IMA [26], 3 months for LEVO [34], and 4.5 months for CIPRO [39].

The data on RIFAM stability were contradictory. Whereas Le Guellec et al. [55] showed that RIFAM concentrations decreased rapidly after 14 days in plasma (even with the addition of ascorbic acid), Allanson et al. [56] and Fang et al. [36] failed to note any degradation after 2 years at −20 °C and 1 month at −70 °C for low and high concentrations, respectively. Three freeze-thaw cycles had no effect on the stability of the compounds, except for POSA and OH-ITRA, which decreased by 16% [10] for 1 of 4 concentrations tested, and for RIFAM, for which a loss of 40.7% at low concentration [56] was reported.

Regarding the protocol constraints of our study, the significant parameters were storage of stock solutions and compound stability in plasma for several hours at room temperature. Thus, stock solutions of IMA, OXA, and CIPRO must be renewed monthly and every 2 months for POSA, VORI, ITRA, and OH-ITRA. For RALTE, LEVO, DAPTO, and RIFAM, fresh stock solutions were prepared when the quality control concentrations fell under the limits of acceptance (±15% of the nominal concentration, as supplied by the manufacturer).

Table 6
Average, minimal, and maximal drug concentrations in plasma from patients treated with one of the following drugs: POSA, VORI, ITRA, OH-ITRA, DAPTO, IMA, RALTE. [a]: trough concentration, [b]: maximal concentration, n = number of patients.

	Average concentration (min–max) (mg/L)	n
ITRA[a]	0.64 (0.10–1.3)	31
ITRA[b]	1.08 (0.40–1.60)	6
OH-ITRA[a]	1.31 (0.20–2.10)	31
OH-ITRA[b]	1.36 (0.20–1.90)	7
POSA[a]	1.20 (0.10–4.80)	339
VORI[a]	2.32 (0.10–70.00)	243
VORI[b]	4.57 (1.10–11.50)	18
IMA[a]	0.95 (0.14–3.57)	35
DAPTO[a]	21.29 (4.40–53.90)	23
DAPTO[b]	81.58 (53.50–169.0)	19
RALTE	0.23 (0.02–1.80)	133

[a] Trough concentrations.
[b] Maximal concentrations.

3.10. Clinical application

The maximum and trough plasma concentrations of drugs that have been measured in the context of routine TDM are shown in Table 6. All concentrations were within the ranges of calibration (Table 1); data for CIPRO, LEVO, OXA, and RIFAM are not shown.

4. Conclusion

Our 2D-LC–MS/MS method is the first rapid and robust procedure for determining the concentrations of 11 drugs belonging to disparate pharmacological classes—ie, antifungals, antibiotics, antineoplastic, and antiretroviral agents.

Our protocol is compatible with routine activities in TDM: simple and single-patient sample preparation, single-calibration standard points for all 11 analytes, fast run time (5 min), and LC–MS/MS resource optimization of clinical pharmacology laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.12.028.

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