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Short communication

Lack of specificity for the analysis of raltegravir using online sample clean-up liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

Background: Raltegravir is the first antiretroviral agent to target the human immunodeficiency virus-1 (HIV-1) integrase. It is indicated, in association with other antiretrovirals, in the treatment of acquired immunodeficiency syndrome (AIDS) in antiretroviral treatment-experienced adult patients with viral resistance. To evaluate the feasibility of raltegravir therapeutic drug monitoring, we developed a rapid and specific analytical method for the quantification of raltegravir in human plasma by online sample clean-up liquid chromatography–tandem mass spectrometry (LC–MS/MS).
Methods: After protein precipitation (with 100 μL of acetonitrile/methanol (50/50)) of 25 μL of plasma, fast online matrix-clean-up was performed using a column switching program. The chromatographic step was optimized to separate raltegravir and its glucuronide metabolite (G-raltegravir). Multiple reaction monitoring (MRM) was used for detection of raltegravir and G-raltegravir. In the absence of G-raltegravir standard, G-raltegravir identification was confirmed by β-glucuronidase pre-treatment.
Results: A total analysis of 3.8 min was needed to separate raltegravir to G-raltegravir. The method was linear between 10 and 3000 ng/mL for raltegravir. Analytical recovery was 94 ± 1%. Variation coefficients ranged between 5% and 8.4%. Pre-treatment of plasma from a patient under raltegravir treatment with β-glucuronidase suppressed G-raltegravir peak.
Conclusion: We describe a fast online LC–MS/MS assay that is valid and reliable for the quantification of raltegravir, despite the lack of specificity that could occur in MRM scanning mode experiments.
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1. Introduction

Liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) represents a highly attractive and versatile technology for therapeutic drug monitoring (TDM).

Most of the time, the high specificity offered by scanning mode multiple reaction monitoring (MRM) allows the simultaneous analyses of several molecules without chromatographic separation.

However, when these analyses are realized on complex biological matrices, the specificity of such a technique is questioned because of the presence of interfering physiological isobaric compounds. Indeed, in-source transformation phenomenon of conjugated drug metabolites into the target analytes can sometimes be observed [1] and must be studied when a new analytical method development is initiated.

Raltegravir (MK-0518, Isentress) is the first commercially available antiretroviral agent to target the human immunodeficiency virus-1 (HIV-1) integrase, one of the three enzymes that play a crucial role in viral replication. It is indicated, in association with other antiretrovirals, in the treatment of human acquired immunodeficiency syndrome (AIDS) in antiretroviral treatment-experienced adult patients with viral resistance [2].

Raltegravir is mainly metabolized by glucuronidation via the uridine 5′-diphospho-glucuronosyltransferase (UDP-GT) A1 [3]. Its pharmacokinetics may display important inter- or intra-individual variability [4]. There are no therapeutic ranges of concentrations actually defined, consequently, raltegravir TDM is not still recommended. However, within the framework of clinical trials, its dosage will allow better understand of concentration–efficiency or concentration–toxicity relationships.

Several liquid chromatography–tandem mass spectrometry (LC–MS/MS) analytical methods have already been described [3,5–8]. Two of them mentioned an in-source transformation phenomenon responsible for an interference between the glucuronide-raltegravir (G-raltegravir) and raltegravir [7,8] suggesting that chromatographic separation of both compounds is required to avoid overestimation of raltegravir concentration.

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The present study describes a rapid and specific analytical method for the quantification of raltegravir in human plasma by online LC–MS/MS, and provides a complementary enzymatic approach confirming that G-raltegravir interferes with raltegravir both in electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

2. Experimental

2.1. Materials

MK-0518 and [4-fluorobenzyl]-$^{13}C_6$ raltegravir used as internal standard (IS) were kindly provided by the chemical department of Merck (Darmstadt, Germany). Methanol (LiChrosolv®) and acetonitrile (Pestipure®) were purchased from Merck (Darmstadt, Germany). Ammonium acetate (Normapure), acetic acid and formic acid (analytical grade) were provided by Prolabo (Paris, France). β-Glucuronidase was from Sigma chemical Co. (St. Quentin Fallavier, France). Ultra pure water (resistivity ≥18.0 MΩ/cm) was generated with a Milli-Q Plus (Millipore, Molsheim, France). Drug-free plasmas (blood was collected on sodium heparinate as anticoagulant) from volunteers were provided by Etablissement Français du Sang (Grenoble, France) and patient's plasmas were obtained from patients treated with raltegravir. Blood samples were centrifuged at 1700 × *g* for 10 min at 4 °C, and stored at −80 °C until later analysis.

2.2. Preparation of solutions

Stock solutions of raltegravir and IS at a concentration of 10 mg/mL were prepared in methanol and stored at −20 °C.

A solution of β-glucuronidase at a concentration of 15 mg/L was prepared by diluting 15 mg of β-glucuronidase powder in 1 mL of ammonium acetate 2 M/acetic acid 2 M (32.2/37.8).

2.3. Sample preparation and enzymatic hydrolysis

25 μL of plasma were treated with 100 μL of precipitation reagent (acetonitrile/methanol (50/50, v/v) containing IS at 200 ng/mL) in polypropylene centrifuge tubes. Samples were immediately vortexed for 10 s and then centrifuged for 10 min at 25,000 × *g*.

80 μL of supernatant were transferred into integrated microinsert polypropylene vials ready to be injected in the chromatographic system.

To confirm the presence of G-raltegravir as an interfering compound in ESI, 200 μL of one sample from a patient under raltegravir treatment was treated before precipitation procedure by either β-glucuronidase (25 μL) for 16 h at 56 °C, or vehicle (25 μL).

Lastly, to determine the influence of the ionization mode, a plasma from a patient under raltegravir treatment was analysed on APCI mode.

2.4. Instrumentation

2.4.1. Chromatographic conditions

The LC–MS/MS conditions were based, in part, on a previously published method [9]. The LC system consisted of two Shimadzu series Prominence LC 20AD quaternary pumps, equipped with a Prominence SIL 20AC 70-vials autosampler (operated at 4 °C) and a Shimadzu column oven Prominence CTO-20AC.

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 phenyl-hexyl analytical column (Phenomenex Luna, 5 μm, phenyl-hexyl, 2 mm × 50 mm, Aschaffenburg, Germany).

The operating procedure for the HPLC-integrated online sample clean-up consisted on two steps: first 5 μL of deproteinized sample was injected into the system and transferred onto the POROS column. Here the analytes were adsorbed, whereas potentially interfering matrix compounds (mainly salts, protein residues) were washed directly into the waste by a mobile phase consisting of ammonium acetate 15 mM delivered at a flow rate of 2.700 mL/min (see Fig. 1A). Following this first step, the six-port valve was switched at 1.2 min. In order to obtain a good separation between raltegravir and G-raltegravir, the extract was then eluted in back-flush mode and transferred to the analytical column (maintained at 84 °C) with a methanol/ammonium acetate 15 mM 68/32 (v/v), 0.1% acetic acid mobile phase, at a flow rate of 0.700 mL/min (see Fig. 1B).

After this chromatographic step, the valve was switched back on its original configuration from 2.4 to 4 min.

2.4.2. Mass spectrometric conditions

MS/MS analyses were performed on an API 3200 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) equipped with ESI and APCI probes, on a Turbo V® ion source.

The mass spectrometer was operated in positive mode under the following conditions: ESI electrospray voltage: 5500 V; nebulisation gas flow rate: 50 psi; turbo heater gas flow rate: 60 psi (no turbo heater gas for APCI); turbo heater temperature: 500 °C. The analyses were performed in multiple reaction monitoring (MRM). Three ion transitions were monitored: $[M+H]^+$ *m/z* 445.2/109.1 (quantification) and $[M+H]^+$ *m/z* 445.2/361.1 (confirmation) for raltegravir and $[M+H]^+$ *m/z* 451.2/115.2 for IS. The chemical structures of raltegravir, G-raltegravir and IS and their respective ways of MS/MS fragmentation are shown in Fig. 2. Each monitored transition dwell time was set to 500 ms in order to obtain at least 15 points per peak. Analyst 1.4.2 software was used for data acquisition and processing.

2.5. Analytical method validation

The validation of the method was based on the guidelines provided by the Food and Drug Administration guidance for Industry Bioanalytical Method Validation [10]. Data are presented as mean ± standard deviation.

3. Results

3.1. Assay validation

3.1.1. Selectivity

To differentiate and quantify raltegravir in the presence of other components in the sample, the selectivity of the method has been tested with six samples of six different lots of blank plasmas. In each case, no interference with the MRM ion transitions of raltegravir have been noted (data not shown).

Selectivity has also been ensured at the lower limit of quantification (LLOQ) from six different lots of plasma. The mean concentration at the lower limit of quantification was 11.2 ± 0.4 ng/mL.

3.1.2. Linearity

The linearity of the assay was verified by spiking aliquots of free-drug plasma samples at concentrations of 10, 15, 25, 50, 100, 200, 500, 1000, 2000, 3000 ng/mL. After adding IS at 200 ng/mL contained in the protein precipitation solution, linearity was evaluated using least-square linear regression fitted by $1/x$ of raltegravir-to-internal standard (IS) peak area ratios *versus* theoretical concentrations and described by an equation of slope = 10.04 ± 0.52 (correlation coefficient 0.9987 ± 0.0009; $n = 6$)

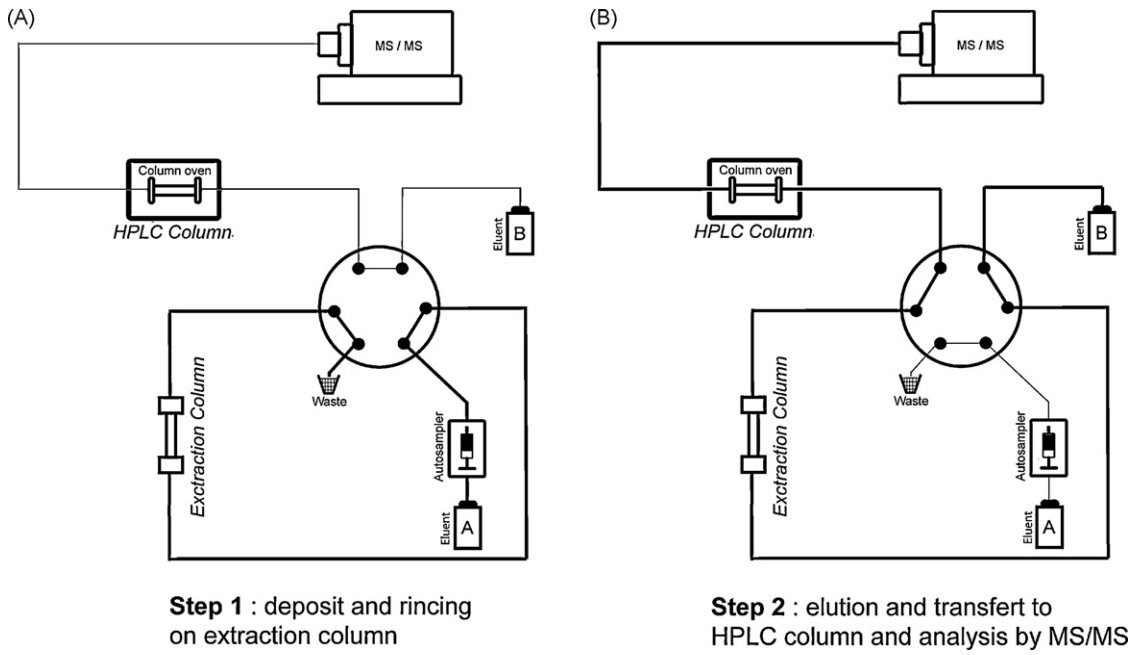

Fig. 1. Representation of the 2-D LC system: (A) deposit of the sample on a sample clean-up column and then rinsing; (B) elution of the analytes and transfer towards a chromatographic column followed by the analyse in the mass spectrometer.

3.1.3. Within- and between-day accuracy and precision

Within-day precision and accuracy were calculated from six repeated analysis of spiked plasmas (three levels of quality controls (QC)) during one working day, by the same operator. Between-day precision and accuracy were calculated from six analysis of spiked plasmas (three same levels of QC), one analysis being performed a day. The precision was expressed as the coefficient of variation (CV%) and the accuracy as the percentage of deviation between nominal and measured concentrations.

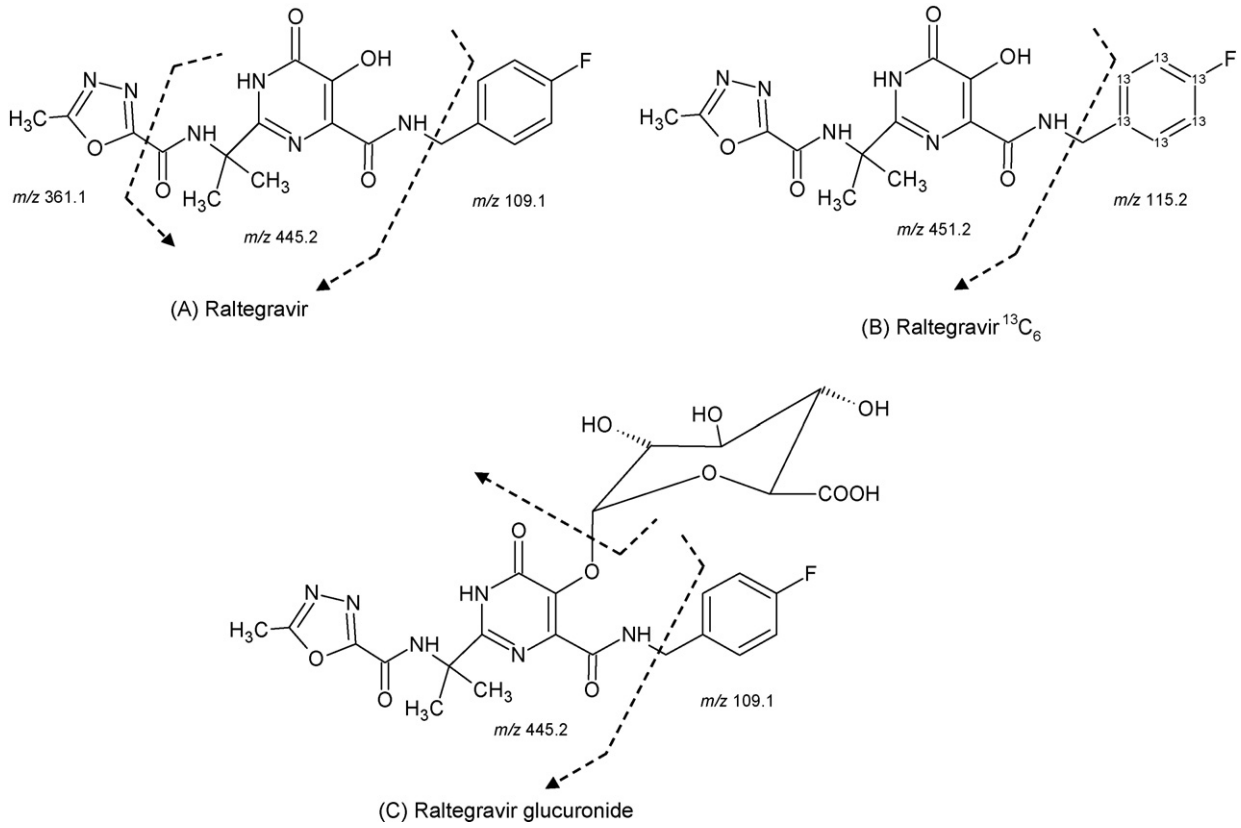

Fig. 2. (A) Structure of raltegravir and typical way of MS/MS fragmentation $[M+H]^+$ *m/z* 445.2/109.1 and $[M+H]^+$ *m/z* 445.2/361.1; (B) structure of $^{13}C_6$ raltegravir (IS) and typical way of MS/MS fragmentation $[M+H]^+$ *m/z* 451.2/115.2; (C) structure of G- raltegravir and typical ways of first in-source transformation (loss of the glucuronide) and consecutive MS/MS fragmentation $[M+H]^+$ *m/z* 445.2/109.1.

Table 1
Precision and accuracy of the assay for raltegravir in human plasma with QC samples at low, medium and high concentrations.

Nominal concentration (ng/mL)	Inter-day essay ($n=6$)[a]			Intra-day essay ($n=6$)		
	Mean concentration found (ng/mL)	Precision CV (%)	Accuracy bias (%)[b]	Mean concentration found (ng/mL)	Precision CV (%)	Accuracy bias (%)
30	32	6.3	6.7	32	6.2	6.7
75	76	7.3	1.3	74	8.4	−1.3
750	759	5.0	1.2	742	6.1	−1.1

[a] n represents the number of experiments.
[b] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Each calibration level and quality control samples were less than ±15%, and less than ±20% at the limit of quantification (defined as the lowest calibrator).

3.1.4. Extraction recovery

Extraction recovery experiments were performed by comparing the peak areas for extracted samples after 2D-LC–MS/MS analysis with unextracted standards after direct LC–MS/MS analysis, which represented 100% recovery. The average percentages of recovery based on six assays for the three levels of QC (30, 75 and 750 ng/mL) were, respectively 97.04 ± 1.47%, 96.24 ± 2.14%, 97.13 ± 1.91% (Table 1).

3.1.5. Sample test dilution

Sample dilution test was assessed to determine whether a sample with a concentration above the upper limit of quantification could be diluted with drug-free plasma for accurate quantitation within the range of the calibration curve.

During the validation procedure, we made tests by diluting 3 times 6 plasma samples spiked with 6000 ng/mL and the results were always ±15% of the target value (5928 ± 174 ng/mL).

3.1.6. Ion suppression phenomenon

Ion suppression was investigated by injecting 10 different drug-free-human plasma samples in the LC system while a methanolic solution containing raltegravir and IS (each at 200 ng/mL) was post-column continuously infused in parallel in the ionization source through a tee.

No ion suppression phenomenon was observed at the raltegravir and IS retention times. On the other hand, even if a signal perturbation would occur, the presence of a stable isotope labelled internal standard should cancel potential inaccuracies in the quantification results.

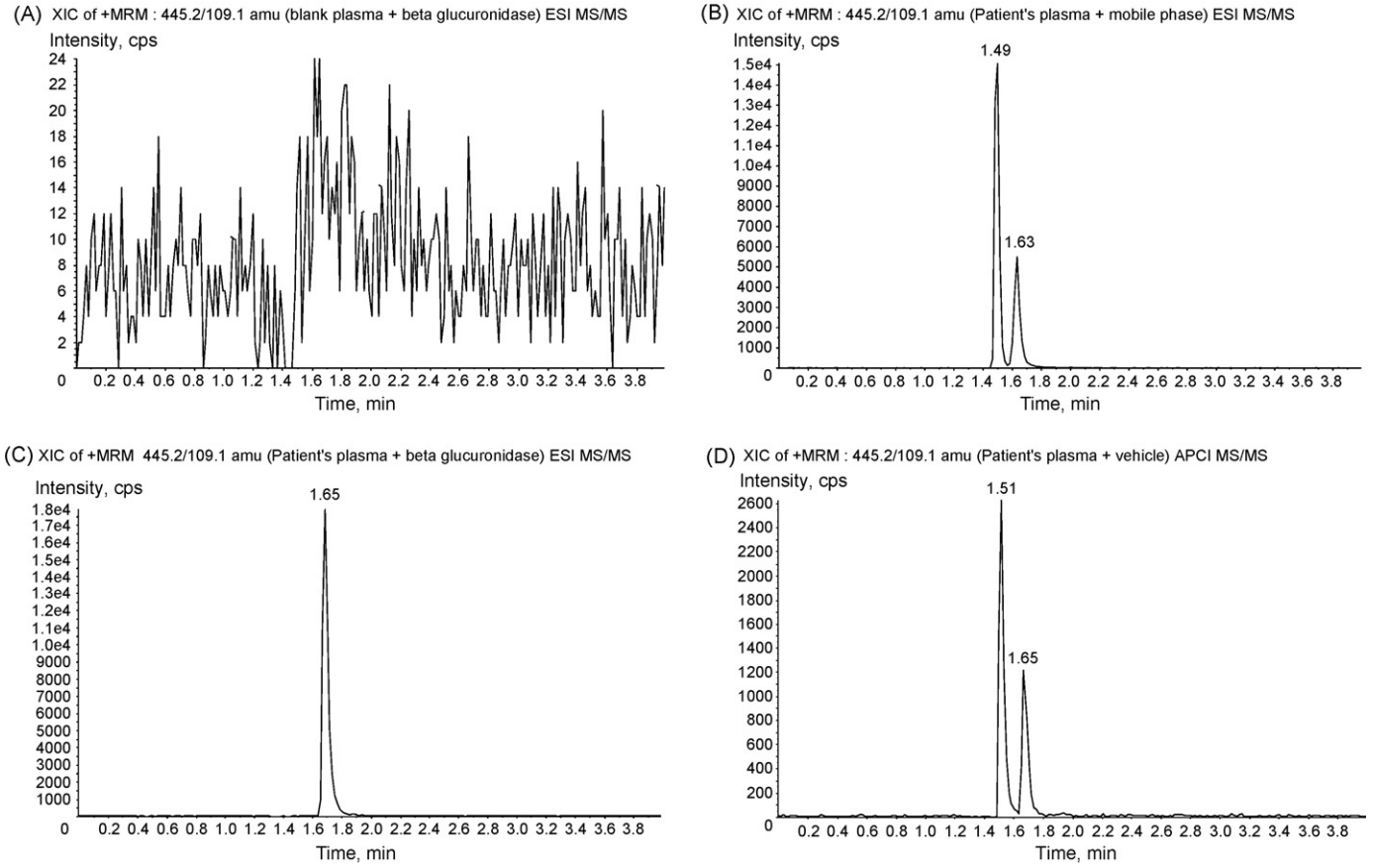

Fig. 3. Representative MRM chromatograms of raltegravir ion transition $[M+H]^+$ m/z 445.2/109.1: (A) a blank plasma obtained by LC–ESI-MS/MS; (B) a plasma from a patient treated by raltegravir obtained by LC–ESI-MS/MS; (C) the same plasma (from the same patient under raltegravir treatment) and treated with β-glucuronidase obtained by LC–ESI-MS/MS); (D) another plasma (from another patient under raltegravir treatment) obtained by LC–APCI-MS/MS.

3.2. Chromatograms

Four representative chromatograms of the assays for the raltegravir ion transition $[M+H]^+$ m/z 445.2/109.1 and one for the IS ion transition $[M+H]^+$ m/z 451.2/115.2 are represented in Fig. 3.

Fig. 3A presented the chromatogram of a blank sample and showed that there was no interference with endogenous compounds.

Fig. 3B presented the chromatogram of a patient's plasma and showed two peaks with retention times of 1.49 and 1.63 min, respectively.

Fig. 3C presented the chromatogram of the same patient's plasma but treated with β-glucuronidase, and showed only one peak at the raltegravir retention time. In these conditions, the peak corresponding to raltegravir was wider and higher than that measured in the absence of treatment with β-glucuronidase and revealed a strong generation of the raltegravir molecular ion from G-raltegravir during ESI-LC–MS/MS analysis of patient's plasma. The surface of the single peak obtained on plasma treated by β-glucuronidase (Fig. 3C, surface = $8.5e^5$ arbitrary units) was equivalent that the sum of the surfaces of the two peaks obtained on the plasma not treated with β-glucuronidase (Fig. 3B, surface = $8.3e^5$ arbitrary units).

Fig. 3D presented the chromatogram from another patient's sample but ionised with an APCI ion source. As with ESI-LC–MS/MS, the chromatogram obtained with APCI showed two peaks.

4. Clinical sample analysis

Human plasma samples were analysed following oral dose of raltegravir (400 mg BID). At steady state, the mean trough raltegravir concentration (at T 12 ± 2 h) was 207 ± 239 ng/mL (n = 62).

5. Discussion and conclusion

The present study is the first to describe an automated online sample clean-up method by a LC–MS/MS method for the analysis and quantification of raltegravir in human plasma. In addition, our work confirmed the need of the chromatographic separation of raltegravir from its glucuronide metabolite since ion in-source transformation occurred.

Several analytical methods for the quantification of raltegravir by LC–MS/MS have recently been published [3,5–8]. Three of these studies mentioned the presence of G-raltegravir on samples from patients under raltegravir treatment [3,7,8] but only two of them suggest the potential generation of the raltegravir molecular ion from G-raltegravir by LC–MS/MS [8]. In addition, in the absence of pure G-raltegravir-standard, the putative interference of G-raltegravir was provided by indirect evidence. Lowering declustering potential in the ion source induced the presence of a $[M+H]^+$ m/z 621 ion, corresponding to the G-raltegravir, associated on the same mass spectra with the $[M+H]^+$ m/z 445 ion corresponding to the raltegravir [7]. Fayet et al. [8] confirmed this identification of G-raltegravir by injecting a patient's sample and by monitoring in negative ionization mode the transition $[M-H]^-$ m/z 619 corresponding to G-raltegravir. Our work provided further evidence of the identification of G-raltegravir as the second peak since pre-treatment of patient's sample with β-glucuronidase abolished the second peak. Indeed, β-glucuronidase is an enzyme that specifically catalyzes hydrolysis of β-D-glucuronic acid chemical functions of molecules. Therefore, pre-treatment of patient's sample with β-glucuronidase broke the bond between raltegravir and glucuronic acid and released free raltegravir.

Collectively, these studies confirm the identification of the interfering compound as the glucuronide metabolite and point out the importance to chromatographically separate it from raltegravir to avoid overestimation of raltegravir concentration.

Such in-source transformation of drug conjugate metabolites to the respective target analytes has previously been described for the LC–MS/MS quantification of mycophenolic acid [1]. Our data provided another illustration of the potential pitfall of LC–MS/MS when no chromatographic separations are operated.

Surprisingly, Merschman et al. [5] did not mention this interference. In this later study [5] raltegravir quantification was performed using LC–APCI-MS/MS methods in which, the Turbo V® ion source of the spectrometer was exactly the same as the one we used in the present study. However, we also observed these two chromatographic peaks for the raltegravir ion transition with the APCI source (present study), suggesting that the mode of ionization is not involved. In addition, Merschman et al. [5] provided the representative LC–APCI-MS/MS chromatogram of a healthy subject's plasma that was collected 1 h after a first single oral dose of 400 mg of raltegravir. The presence of a single chromatographic peak corresponding to raltegravir could be explained by the fact that it was a single dose in a healthy volunteer. Since the T_{max} value of raltegravir is about 4 h [2], it could be hypothesized that the glucuronide metabolite was not present or was undetectable because it was the first dose and the steady state of raltegravir plasma concentration could have not been reached. Lastly, the sample preparation (present study) is different from that described by Merschman et al. [5]. This could also partly explain why the glucuronide peak is not observed in this latter method. Liquid–liquid extraction optimized for the analyte of interest may not extract the glucuronide whereas protein precipitation could extract all analytes.

In the present study, a high temperature (84 °C) was required to obtain a good and rapid chromatographic separation. Assays with lower column oven temperature were experimented but chromatographic performances were worse (data not shown). Indeed temperature has a direct effect on column efficiency, selectivity and peak shape [11].

In conclusion, using the analytical conditions described in this study, correct calibration and quantification of raltegravir can be achieved with a simple sample preparation, despite the lack of specificity provided by the MRM scanning mode.

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