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LCMS using a hybrid quadrupole time of flight mass spectrometer for impurity identification during process chemical development of a novel integrase inhibitor

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ABSTRACT

LCMS incorporating a quadrupole time of flight mass spectrometer was used to identify impurities found in a chemical process development sample of a novel integrase inhibitor, raltegravir. The combination of accurate mass measurement in full scan mode followed by construction of targeted masses for further MSMS interrogation allowed for the determination of atomic composition and connectivity. The fragmentation pattern of raltegravir was used as a model compound, and the product ion spectra of an impurity was compared to both the model fragmentation pattern and the atomic composition generated in the full scan experiment to deduce a structure.

1. Introduction

Process research plays an extremely important role in the drug development process within the pharmaceutical industry. Developmental efforts can encompass early lead discovery through product launch [1]. The primary objectives of process chemical research are the development of cost effective, efficient, scaleable and safe reproducible synthetic routes to drug candidates within the developmental space including the support of pre-clinical and clinical programs and acting as a framework for commercial production. Some recent reviews have described elegant synthetic chemical routes to a wide array of pharmaceutically active agents, illustrating the challenges and creativity of the process chemist [1,2]. Tangential benefits are the development of enabling technologies supporting synthetic efforts and leveraging of intellectual property by patent protection of chemical routes [3].

Development and optimization of viable processing routes to investigational materials operates under the constraint of generating compounds not having a wide degree of variability. This ensures that drug metabolism, safety and clinical studies are not jeopardized by inconsistent purity or impurities having potential harmful toxological properties. Regulatory guidelines promulgated by the International Conference on Harmonization (ICH) dictates rigorous identification of impurities at levels of 0.1% and greater depending upon dosage, followed by appropriate toxicological studies, if needed, for qualification [4].

Raltegravir, a 6-oxo-1,6-dihydropyrimidine-4-carboxamide, 1, belongs to a new class of compounds chosen for development as inhibitors of HIV integrase, an enzyme catalyzing the integration of viral DNA into the host genomic DNA, thus preventing further virus replication [5–7]. It has been approved by the US Food and Drug Administration for sale and is marketed as ISSENTRESS™ as a complimentary agent to existing anti retroviral therapies. During chemical process development of raltegravir, we were required to identify several impurities appearing in a sample of drug substance which was produced by a potential commercial route. LCMS has been a popular approach for impurity identification in pharmaceutical drug substances, and recent reports using high resolution MS with accurate mass measurements for determination of atomic composition have illustrated the advantages of this approach over low resolution/nominal mass measurement using quadrupoles [4,8,9]. We describe the use of LCMS and LCMSMS using a quadrupole time of flight mass spectrometer (QTof) for the identification of the impurities of interest.

2. Experimental

2.1. Materials

Acetonitrile and water were Optima grade and were purchased from Fisher Scientific (Bridgewater, NJ, USA). Trifluoroacetic

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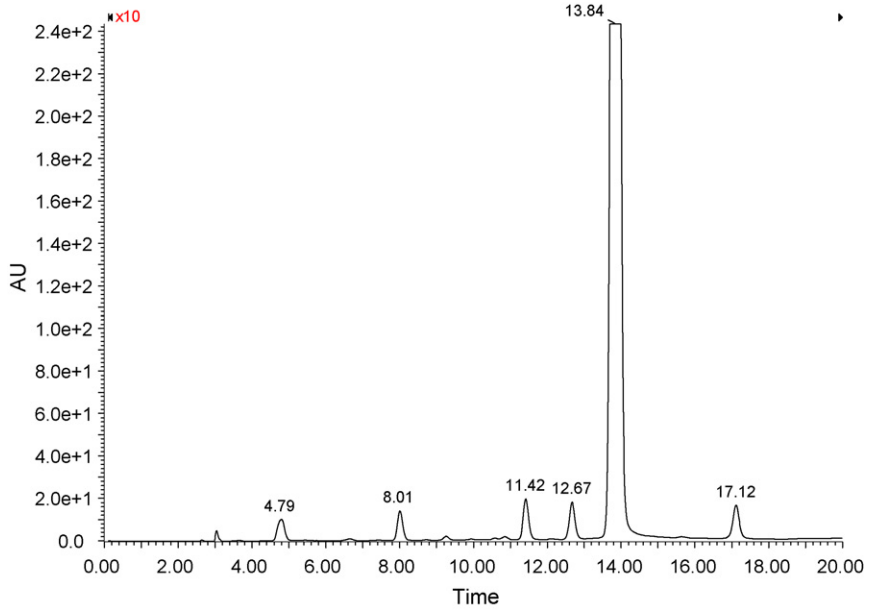


Fig. 1. HPLC–UV chromatogram of impurities observed in a process development sample of raltegravir drug substance.

acid, 99.9+%, spectrophotometric grade, trifluoroacetic acid-*d* (99 at.%), deuterium oxide (99 at.%), leucine enkephalin, alanine and polyalanine were purchased from Aldrich (St. Louis, Mo, USA). A sample of raltegravir was prepared by the Process Research Department of Merck Research Laboratories (Rahway, NJ, USA).

2.2. LC/MS

Chromatographic separations were performed on a Waters Alliance model 2695 HPLC equipped with a photodiode array detector monitored in wide band from 220 to 400 nm. The column used was a Waters Atlantis C_{18}, 250 mm × 4.6 mm, 5 μm particle size operated at a flow rate of 1 ml/min at a temperature of 30 °C. The mobile phase was water and acetonitrile with 0.02% (v/v) trifluoroacetic acid added to each. A linear gradient from 20% acetonitrile to 85% acetonitrile in 20 min was used. The injection volume was 1 μl. Deuterium exchange experiments were performed substituting trifluoroacetic acid-*d*, and deuterium oxide for the protic components. The mass spectrometer was a Waters/Micromass QTof API Ultima US equipped with an electrospray interface connected in series to the diode array detector and operated in positive ion mode. The electrospray voltage was 1.5 kV and the sample cone voltage was 25 V. The Tof voltage was 9.1 kV and the MCP plates were operated at 2200 V. The source temperature was 100 °C and the desolvation temperature was 350 °C. Nitrogen was used for the desolvation and cone gas and was set at 550 and 30 l/h, respectively. The analyzer was operated in V optics mode at a resolution (FWHM) of 10,000. The collision gas was argon operated at a cell pressure of 12 psi. For MSMS measurements, the collision energy was set at 25 eV. Full scan data acquired in continuum mode was collected from 100 to 1200 amu and MSMS data collected from 70 to 600 amu. The data acquisition rate was 1 s with an interscan delay rate of 0.1 s. In order to alleviate dead time effects, intensities less than 200 counts/s were used for data analysis. The lockmass was leucine enkephalin (*m*/*z* 556.2771) and was infused at 10 μl/min at a concentration of 1 ng/μl. A solution of polyalanine containing 20% (w/w) alanine was used for mass calibration. Instrument control and data analysis was performed using Mass Lynx 3.5.

2.3. Sample preparation

The sample concentration was 100 μg/ml using 50:50 water acetonitrile as the diluent. The sample was maintained at 10 °C in the autosampler.

3. Results and discussion

3.1. Chromatography

Previous bioanalytical methods used for the measurement of raltegravir in human plasma have utilized reversed phase HPLC. One LC–MS approach was a combination of water:methanol with formic acid for pH control [10]. EDTA has been used as an additive in combination with water:methanol and formic acid to alleviate complexation with alkali cations, thus simplifying the MS measurement [11]. An alternative approach used water:acetonitrile with triethylammonium phosphate as an additive for pH control and fluorescence detection [12]. All of these approaches resulted in good chromatographic efficiency for raltegravir.

In this study, we were required to reproduce the HPLC gradient impurity control assay which used water:acetonitrile and phosphate salt for pH control. Since phosphate is not compatible with atmospheric pressure ionization, we incorporated dilute trifluoroacetic acid as a substitute for phosphate. This resulted in a reasonable match to the phosphate-based control assay with good efficiency and selectivity. A representative separation employing UV detection is shown in Fig. 1. Five impurities were separated and detected and all were subjected to further MS analysis for identification. The UV spectrum of raltegravir, retention time 13.84 min, acquired using the diode array detector showed a shoulder at 240 nm and a maxima at 304 nm. All the impurities of interest exhibited similar UV characteristics with the exception of the impurity eluting at retention time 4.79 min. This impurity showed maxima at both 245 and 300 nm, suggesting a subtle structural dissimilarity compared to the other impurities and raltegravir.

3.2. Mass spectrometry of raltegravir

The product ion spectrum of raltegravir was used as a template for assignment of the atomic composition of fragment ions and

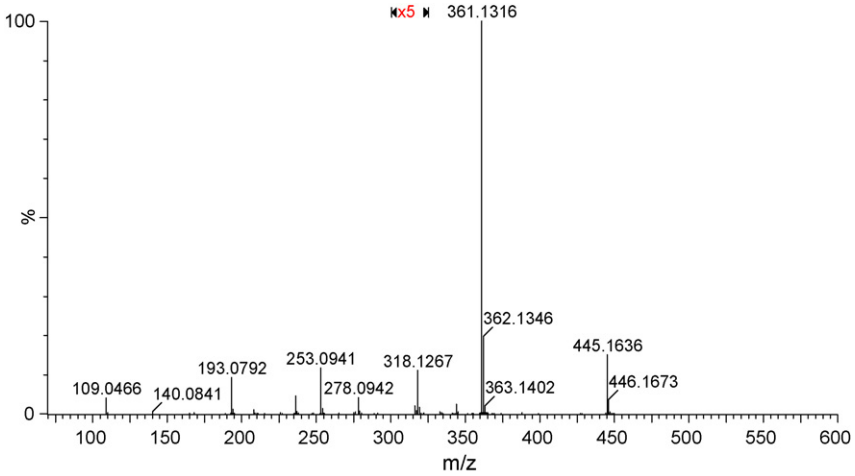


Fig. 2. Product ion spectrum of raltegravir.

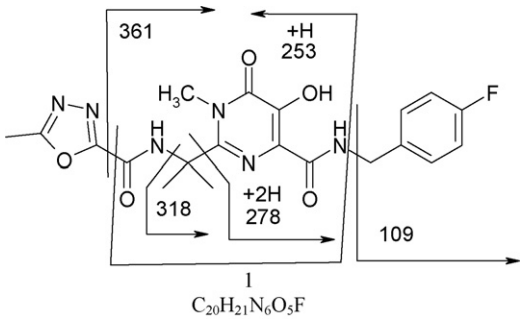


Fig. 3. Structure of raltegravir and proposed fragmentation pattern observed in this study.

to establish connectivity. This was accomplished by flow injection introduction of raltegravir into the mass spectrometer and selected introduction of the protonated molecule, *m*/*z* 445 into the collision cell. The collision energy was varied until approximately 10–20% of the intensity of the protonated molecule relative to the base product ion, *m*/*z* 361, was observed. The collision energy associated with this criteria was 25 eV, and was used for further structural elucidation. The collision cell pressure was kept constant at 12 psi.

The product ion spectrum of raltegravir observed under these conditions is shown in Fig. 2. The theoretical protonated molecule of raltegravir, *m*/*z* 445.1636, was used as an internal lock mass. Although several product ions were observed, five were chosen for comparison to the product ion spectra observed for the impurities of interest. These were *m*/*z* 361, 318, 278, 253 and 109. These assignments are shown in Fig. 3 and accurate masses and atomic compositions are shown in Table 1. In general, the assignments cover most of the molecule with some redundancies with transparency noted for the methyloxadiazoyl moiety. Accurate mass measurements of the fragments ranged from 0.4 to 12 ppm deviation from theoretical atomic composition. The *m*/*z* 361 and 109 ion assignments have been reported previously and were used as the basis of LCMSMS assays using standard multiple reaction monitoring procedures [10,11].

Table 1
Accurate mass data for the product ions of raltegravir observed in this study.

Ion composition	*m*/*z* (theory)	*m*/*z* (measured)	ppm error
$C_{17}H_{18}N_4O_4F$	361.1312	361.1316	1.1
$C_{16}H_{17}N_3O_3F$	318.1254	318.1267	4.0
$C_{13}H_{13}N_3O_3F$	278.0941	278.0942	0.4
$C_{10}H_{13}N_4O_4$	253.0937	253.0941	1.6
C_7H_6F	109.0453	109.0466	12

3.3. LCMS and LCMSMS of unknowns

Full scan LCMS was performed initially for exact mass measurement of the impurities of interest to establish the atomic composition. Subsequent LCMSMS studies were performed by construction of a mass list of the impurities of interest and performing the product ion experiments by interrogation of the mass list only. The mass list was constructed using a narrow mass window of ±20 mDa around each target mass. In this way, the product ion experiments for all of the impurities could be completed in a single chromatographic run. The full scan and product ion spectra are shown in Figs. 4 and 5. Table 2 shows the accurate mass data for the protonated molecule as well as structural assignments.

The first impurity at retention time 4.79 min showed a protonated molecule of 335.1530 with a significant amount of a sodium adduct, *m*/*z* 357. By limiting the potential atomic composition to carbon, nitrogen, oxygen and fluorine, which seemed reasonable given the process chemistry, and applying an error constraint of no more than 5 ppm error relative to the theoretical composition, the assignment was consistent with $C_{16}H_{19}N_4O_4F$. The experimental error was 3.3 ppm. The atomic difference relative to raltegravir, $C_{20}H_{21}N_6O_5F$, was $C_4H_2N_2O_2$ which suggested the loss of the methyloxadiazoyl group. The product ion spectrum obtained using leucine enkephalin as the lock mass showed only two fragments at *m*/*z* 109.0460 and 278.0939, which were also observed for raltegravir and assigned as C_7H_6F and $C_{13}H_{13}N_3O_3F$. The assignment of the impurity was the free amine, 2 (Table 2), the penultimate intermediate used in the synthesis, and was confirmed by a spike experiment.

The second impurity at retention time 8.01 min showed a protonated molecule of 390.1940. Using the same criteria as that applied to the free amine impurity, 2, the atomic composition was consistent with $C_{19}H_{24}N_5O_3F$, with an experimental error of −0.3 ppm. The product ion spectra using the theoretical value of the protonated molecule as the internal lock mass did show *m*/*z* 318.1255 and a low intensity *m*/*z* 109.0459, which suggested the methylethyl dihydropyrimidine carboxamide portion, $C_{16}H_{17}N_3O_3F$ was intact. The mass difference, $C_3H_7N_2$ suggested three possibilities for connectivity in the remaining portion of the molecule based upon the process chemistry; $(CH_3)_2NCH{=}N–$, $NH{=}CHCH_2CH_2NH–$ or $NH_2CH{=}CHCH_2NH–$. A second line of evidence was the prominent *m*/*z* on at 113.1076, consistent with

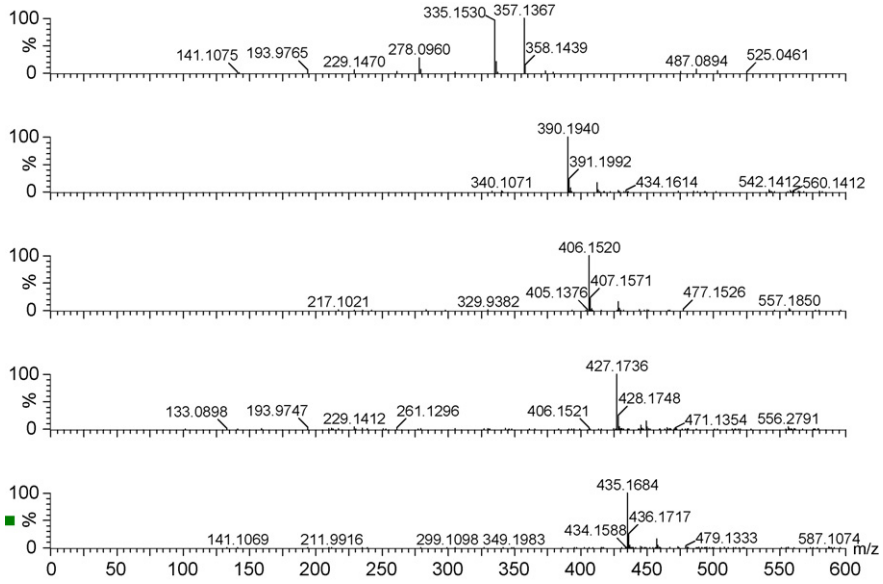


Fig. 4. Full scan mass spectra observed for impurities 2 (top) to 6 (bottom). Leucine enkephalin was used as the external lock mass.

$C_6H_{13}N_2$ and suggesting the methylethyl group intact at the 2 position of the dihydropyrimidine again leaving $C_3H_7N_2$ accounting for the difference. Hydrogen/deuterium LCMS exchange experiments using deuterated mobile phase additives in place of protic ones showed an *m*/*z* of 393 for the impurity indicating 2 exchangeable protons. Thus the correct assignment was the N,N dimethyl imine, 3. This was subsequently confirmed by ^{1}H NMR.

The third impurity at retention time 11.42 min showed a protonated molecule of 406.1520, consistent with an atomic composition of $C_{18}H_{20}N_5O_5F$ with an experimental error of −1.7 ppm. The product ion spectra using the theoretical mass for the protonated impurity as the internal lock mass showed *m*/*z* 109.0457, 278.0935, 318.1237 and 361.1320 suggested the methylethyl dihydropyrimidine carboxamide was intact up to the 2 position side chain carboxamide. The *m*/*z* 363.1467 ion, arising from the transfer of 2 protons to the *m*/*z* 361 ion, confirmed this sub assignment. The atomic difference of the *m*/*z* 363 and 361 product ions relative to the unknown moity adjacent to the carboxamide at the 2 position was CH_2NO. This indicated the structure was the carboxamide, 4.

The fourth impurity at retention time 12.67 min showed a protonated molecule of 427.1736, consistent with an atomic composition of $C_{20}H_{22}N_6O_5$ with an experimental error of 1.4 ppm. The lack of product ion masses at *m*/*z* 361 and 109 with product ion masses *m*/*z* 343.1411 and 91 0551 using $C_{20}H_{23}N_6O_5$ as the internal lock mass was consistent with the assignment as the desfluoro analog of raltegravir, 5.

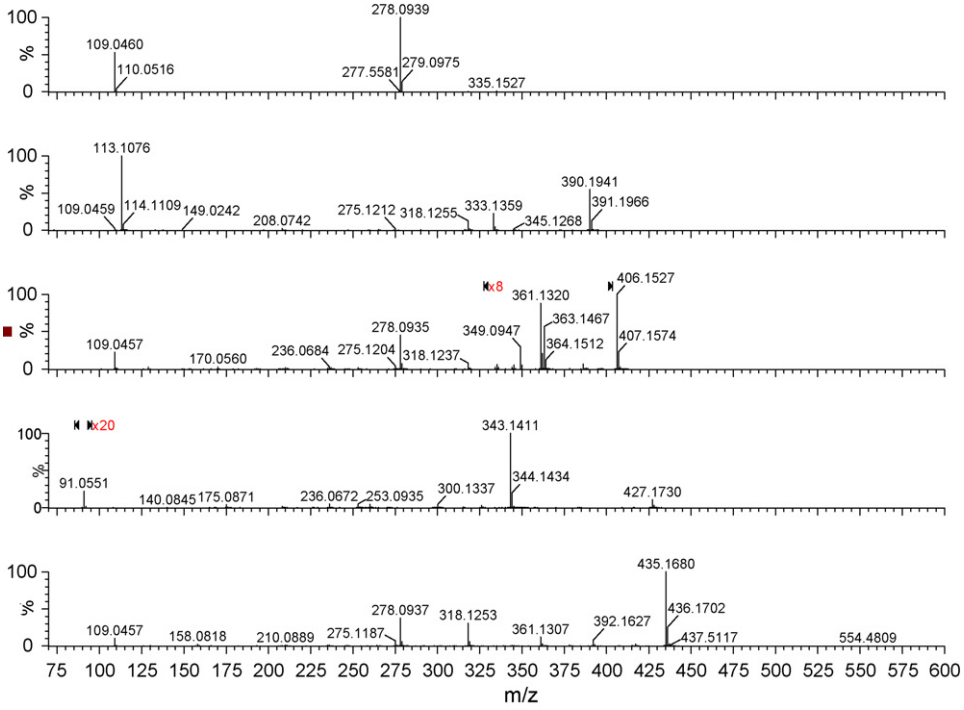
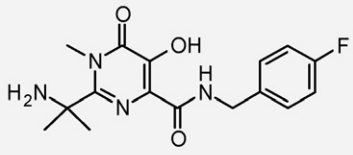
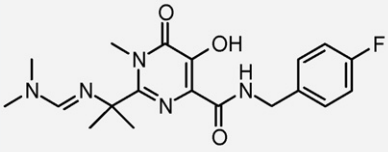
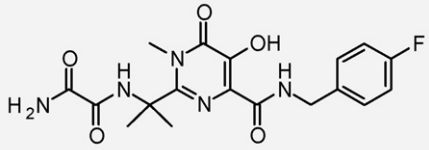
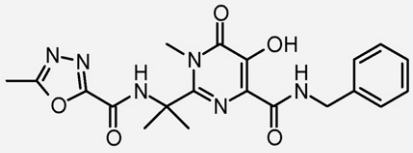
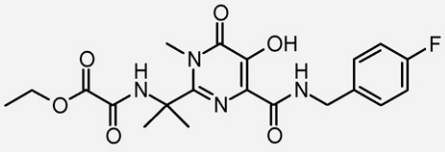


Fig. 5. Product ion spectra observed for impurities 2 (top) to 6 (bottom) at a collision energy of 25 eV. Leucine enkephalin was used as an external lock mass for impurity 2.

Table 2
Accurate mass data and structural assignments of the impurities observed in a process development sample of raltegravir drug substance.

Structure	Formula	M+H (measured)	ppm error
 2	$C_{16}H_{19}N_4O_3F$	335.1530	3.3
 3	$C_{19}H_{24}N_5O_3F$	390.1940	−0.3
 4	$C_{18}H_{20}N_5O_5F$	406.1520	−1.7
 5	$C_{20}H_{22}N_6O_5$	427.1736	1.4
 6	$C_{20}H_{23}N_4O_6F$	435.1684	0.9

The fifth impurity at retention time 17.12 min showed a protonated molecule of 435.1684, consistent with an atomic composition of $C_{20}H_{23}N_4O_6F$ and experimental mass error of 0.9 ppm. The product ion spectra using the impurity theoretical mass as the internal lock mass, showed fragment ions at *m*/*z* 109.0457, 278.0937, 318.1253 and 361.1307, suggesting a difference in the methyloxadiazoyl group. The atomic difference of the impurity relative to the *m*/*z* 361 fragment was $C_3H_5O_2$. The assignment of the impurity was the ethyl oxamide ester, 6.

4. Conclusions

Impurities found in a potential commercial route to raltegravir drug substance were identified by a determination of atomic composition and interpretation of product ion spectra made possible by accurate mass measurements using a QTof mass spectrometer.

Full scan experiment accuracy ranged for −0.3 to 3.3 ppm while product ion accuracy ranged from 0.4 to 12 ppm. Most of the impurities originated during construction of the methyloxadiazoyl portion of raltegravir. The desfluoro analog of raltegravir can be attributed to an impurity introduced in a raw material. This information allowed the process chemistry team to further optimize the process to generate a cleaner drug substance profile.

References

[1] H.-J. Federsel, Searching for scaleable processes: addressing the challenges in times of increasing complexity, Curr. Opin. Drug Disc. Dev. 6 (2003) 838–847.
[2] M.C. Hillier, P.J. Reider, Stereoselective synthesis from a process research perspective, Drug Disc. Today 7 (2002) 303–314.
[3] A.E. Rubin, S. Tummala, D.A. Both, C. Wang, E.J. Delaney, Emerging technologies supporting chemical process R&D and their increasing impact on productivity in the pharmaceutical industry, Chem. Rev. 106 (2006) 2794–2810.
[4] M. Vuletic, M. Cindric, J.D. Koruznjak, Identification of unknown impurities in simvastatin drug substance and tablets by liquid chromatography/tandem mass spectrometry, J. Pharm. Biomed. Anal. 37 (2005) 715–721.
[5] P. Pace, S.A.H. Spieser, V. Summa, 4-Hydroxy-5-pyrrolinone-3-carboxamide HIV-1 integrase inhibitors, Bioorg. Med. Chem. Lett. 18 (2008) 3865–3869.
[6] J.D. Croxtall, K.A. Lyseng-Williamson, C.M. Perry, Raltegravir, Drugs 68 (2008) 131–138.
[7] V. Summa, A. Petrocchi, F. Bonelli, B. Crescenzi, M. Donghi, M. Ferrara, F. Fiore, C. Gardelli, O.G. Paz, D.J. Hazuda, P. Jones, O. Kinzel, R. Laufer, E. Monteagudo, E. Muraglia, E. Nizi, F. Orvieto, P. Pace, G. Pescatore, R. Scarpelli, K. Stillmock, M.V. Witmer, M. Rowley, Discovery of raltegravir, a potent selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection, J. Med. Chem. 51 (2008) 5843–5855.
[8] R.S. Plumb, M.D. Jones, P. Rainville, J.M. Castro-Perez, The rapid detection and identification of the impurities of simvastatin using high resolution sub 2 μm

particle LC coupled to a hybrid time of flight MS operating with alternating high-low collision energy, J. Sep. Sci. 30 (2007) 2666–2675.
[9] C. Eckers, N. Haskins, J. Langridge, The use of liquid chromatography combined with a quadrupole time of flight analyzer for the identification of trace impurities in drug substance, Rapid Commun. Mass Spectrom. 11 (1997) 1916–1922.
[10] M.C. Long, C. Bennetto-Hood, E.P. Acosta, A sensitive HPLC–MS/MS method for the determination of raltegravir in human plasma, J. Chromatogr. B 867 (2008) 165–171.
[11] S.A. Merschman, P.T. Vallano, L.A. Wenning, B.K. Matuszewski, E.J. Woolf, Determination of the HIV integrase inhibitor, MK-0518 (raltegravir) in human plasma using 96-well liquid–liquid extraction and HPLC–MS/MS, J. Chromatogr. B 857 (2007) 15–25.
[12] J.-M. Poirier, P. Robidou, P. Jaillon, Quantification of the HIV-integrase inhibitor raltegravir (MK-0518) in human plasma by high performance liquid chromatography with fluorescence detection, J. Chromatogr. B 867 (2008) 277–281.