Talanta 82 (2010) 125–128

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Characterization of emtricitabine related substances by liquid chromatography coupled to an ion trap mass spectrometer

Murali Pendela, Dunge Ashenafi Mamade, Jos Hoogmartens, Ann Van Schepdael, Erwin Adams*

Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Analyse, O&N2, PB 923, Herestraat 49, B-3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form 6 April 2010
Accepted 7 April 2010
Available online 13 April 2010

Keywords:
Emtricitabine
LC/MS^n
Impurity profiling

ABSTRACT

Emtricitabine (FTC) is an antiretroviral compound that inhibits the HIV-1 reverse transcriptase. For the quantification of FTC related substances, a liquid chromatography (LC) method coupled with ultraviolet (UV) detection was developed earlier in our laboratory. Several unknown compounds were detected during the analysis of a commercial sample. However, most of these impurities were not characterized. In this study, impurity profiling in a selected FTC sample was done using LC–mass spectrometry (MS). Due to the presence of a non-volatile buffer, a desalting procedure was carried out before sending the impurity into the MS. Totally, nine peaks were studied and most of them could be characterized.

1. Introduction

Emtricitabine (FTC) or 5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine (see Fig. 1a) is an HIV-1 reverse transcriptase inhibitor. Due to lack of a 3-hydroxyl group, it does not support continued synthesis of the newly made DNA strand and thus terminates or aborts the polymerization process catalyzed by the HIV reverse transcriptase [1].

Enantiomers of FTC could be separated by LC using an amylose-based chiral stationary phase under polar organic elution mode [2]. Unnam et al. developed a LC–UV method for the determination of FTC related substances using an Inertsil ODS-3V column. They performed forced degradation experiments and the degradation products were well separated [3]. Rebiere et al. developed two LC–UV methods for the determination of 19 antiretroviral agents in pharmaceuticals. The method using YMC-Pack ODS-AM allows the determination of FTC along with eight other antiretrovirals [4]. In addition, several LC methods using UV [5,6] and MS [7–9] were published to quantify FTC in biological samples. As per our knowledge, no literature is available on the MS characterization of FTC related substances. Recently, Mamade et al. proposed a gradient LC–UV method to the International Pharmacopoeia for the quantification of FTC and its related substances [10]. It was developed on a Hypersil BDS column using 0.2 M phosphate buffer, acetonitrile (ACN) and water as mobile phase constituents. However, several peaks in the chromatogram could not be attributed to a structure. Direct transfer of this method to MS is not possible due to the non-volatile buffer. For erythromycin and its related substances, Pendela et al. have described a desalting procedure to couple a LC method with a non-volatile mobile phase to MS [11]. As per our knowledge, no literature is available on the characterization of FTC related substances by LC–MS. This is important since impurities present in bulk drugs may often be toxic. The aim of this study was to apply the desalting procedure described by Pendela et al. to characterize the FTC related substances by LC–MS.

2. Experimental

2.1. Reagents and samples

Acetonitrile MS grade and formic acid were purchased from Biosolve LTD (Valkenswaard, The Netherlands). Potassium dihydrogen phosphate was purchased from Acros Organics (Geel, Belgium). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify demineralised water. A commercial FTC sample was obtained from the World Health Organization (Geneva, Switzerland). For tuning of the MS, a solution of FTC was prepared in methanol–water (4:6, v/v) at 0.01 mg/mL. 0.5 mg/mL sample solution in water was injected into the non-volatile system.

2.2. LC instrumentation and chromatographic conditions

Non-volatile system for LC–UV: the LC system from Dionex (Germering, Germany) consisted of a P680 HPLC pump, an ASI-100 automated sample injector and a UVD 170U detector. Chromeleon

* Corresponding author. Tel.: +32 16323444; fax: +32 16323448.
E-mail addresses: Erwin.Adams@pharm.kuleuven.be, Erwin.Adams@pharm.kuleuven.ac.be (E. Adams).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.04.004

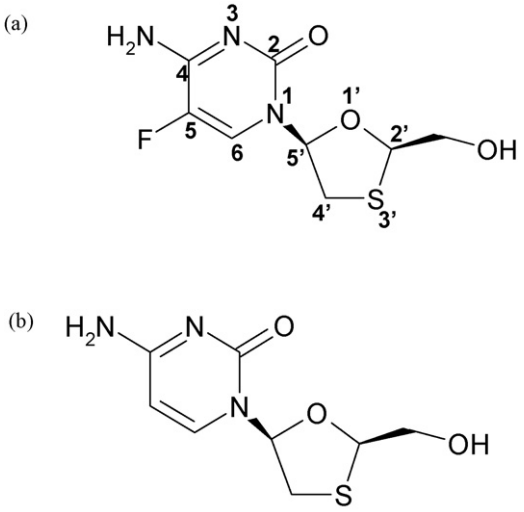

Fig. 1. Chemical structures of (a) FTC and (b) lamivudine.

Table 1
Gradient program for the analysis of FTC and its related substances.

Time (min)	Mobile phase A (%, V/V)	Mobile phase B (%, V/V)	
0–9	93	7	Isocratic
9–15	93–0	7–100	Linear gradient
15–19	0	100	Isocratic
19–19.1	0–93	100–7	Linear gradient
19.1–30	93	7	Isocratic re-equilibration

software (Dionex) was connected to the detector to record the signals.

A gradient LC method containing mobile phase A: 0.2 M potassium dihydrogen phosphate and water (5/95, v/v) and B: 0.2 M potassium dihydrogen phosphate, ACN and water (5/70/25, v/v/v) was used at a flow rate of 1.0 mL/min. Hypersil BDS C_{18} (250 mm × 4.6 mm) 5 μm, 120 Å (Thermo Electron Corporation, Kleinostheim, Germany) was immersed in a water bath, maintaining the temperature at 35 °C using a Julabo heating circulator (Seelbach, Germany). The injection volume was 20 μL. The gradient program used is shown in Table 1.

Desalting system for LC–MS: the desalting LC system consisted of a P680 HPLC pump from Dionex Corporation (Sunnyvale, CA, USA), a switching valve (VICI AG International, Schenkon, Switzerland) equipped with a 500 μL loop and a variable wavelength TSP Spectra 100 UV–Vis detector (San Jose, CA, USA) set at 280 nm. ChromPerfect 4.4.23 software (Justice Laboratory Software, Fife, UK) was connected to the detector to record the signals. The LCQ (Thermo Finnigan, San Jose, CA, USA) ion trap mass spectrometer was equipped with an electro spray ionisation (ESI) source operated in the positive ion mode. As stationary phase, an XTerra RP C18, 5 μm, 250 mm × 4.6 mm column was kept at room temperature.

Each peak eluted in the LC–UV non-volatile system was collected and injected into the desalting system for the LC–MS investigation. In this system, mobile phases A and B consist of 0.1% formic acid and ACN respectively. Until 5 min, a mixture of 95% mobile phase A and 5% mobile phase B was pumped at a flow rate of 1.0 mL/min. This was done to remove the salts and it was sent to waste. After that, the flow rate was decreased from 1.0 mL/min to 0.2 mL/min while mobile phase B was increased to 12% in order to decrease the elution time and obtain a better ESI spray. For early eluted peaks (before 5 min) in the non-volatile system, mobile phase A only was used up to 5 min, to avoid elution of the impurity along with the salts.

3. Results and discussion

3.1. Study of the fragmentation behavior of emtricitabine

Tuning and MS investigation of the reference substance was carried out as described by Pendela et al. [11]. The $[M+H]^+$ 248 precursor ion was isolated with an isolation width of 3 u and collisionally activated at 35% collision energy level (CEL). The $[M+H]^+$ CID spectrum of FTC is shown in Fig. 2. Most fragmentation involves a cleavage of the bond between the six (5-fluoro cytosine) and five (2′-hydroxymethyl-1′3′-oxathiolan) membered rings. After a loss of 118 u, a characteristic ion at *m*/*z* 130 corresponding to 5-fluoro cytosine was observed.

3.2. Investigation of impurities present in the emtricitabine sample

A typical chromatogram of a commercial FTC sample is shown in Fig. 3. The peaks are numbered according to their elution order in the chromatogram. For the characterization of peaks, the procedure described in Section 2.2 was carried out. The identity of the FTC was established from its *m*/*z* ratio and MS^2 data in comparison with its known structure. The unknown (UNK) impurities were characterized by comparing their MS^2 spectra with that of FTC.

3.2.1. Peak 1 (UNK1 $[M+H]^+$ *m*/*z* 262), Peak 2 (UNK2 $[M+H]^+$ *m*/*z* 264) and Peak 8 (UNK8 $[M+H]^+$ *m*/*z* 265)

Peak 1 (UNK1), Peak 2 (UNK2) and Peak 8 (UNK8) are related to each other due to the presence of an extra oxygen attached to sulphur at C-3′ position. Peak 8 is composed of two impurities UNK8 (*m*/*z* 265) and UNK9 (*m*/*z* 248). UNK9 is described in Section 3.2.6. UNK1, UNK2 and UNK8 are 14, 16 and 17 u higher respectively than FTC. MS^2 investigation of the protonated molecules

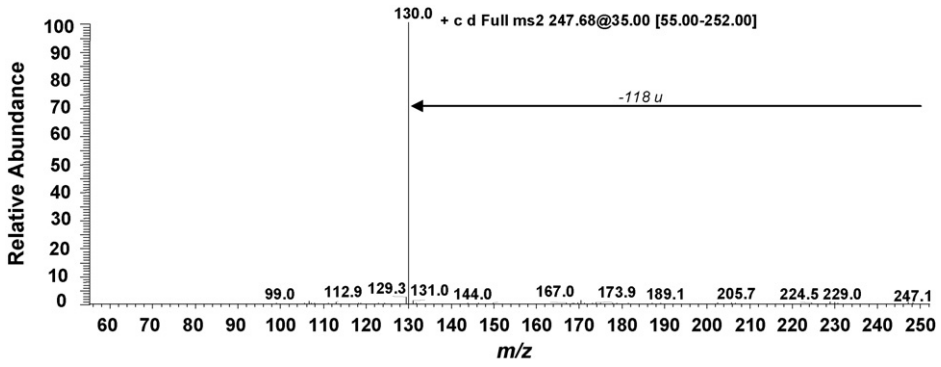

Fig. 2. CID MS^2 $[M+H]^+$ CID spectrum acquired for FTC ($[M+H]^+$ *m*/*z* 248); the result of isolation and collisional activation of the precursor ions in the ion trap at 35% CE.

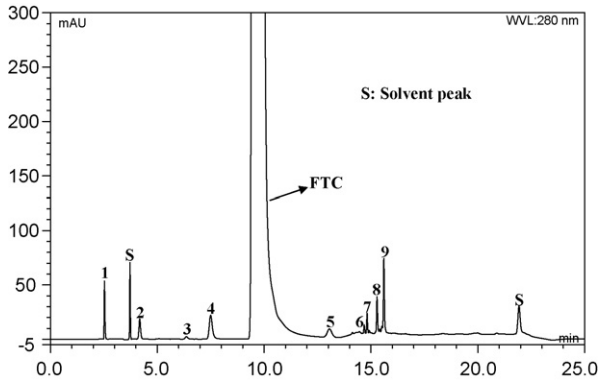

Fig. 3. UV chromatogram of a FTC sample using the non-volatile eluent LC system.

of UNK1, UNK2 and UNK8 showed losses of 132, 134 and 134 u respectively instead of 118 u to yield product ions *m*/*z* 130, 130 and 131 respectively (data not shown). The yielded spectra showed that UNK1 and UNK2 have a non-modified 5-fluorocytosine moiety while UNK8 has a modified one (*m*/*z* 131 instead of *m*/*z* 130). However, initial losses suggested that these impurities have a peculiar 2′-hydroxymethyl-1′,3′oxathiolan ring. For UNK2 and UNK8 the difference compared to FTC is 16 u, suggesting that an extra oxygen atom is attached to the 5-membered ring. Unnam et al. described a FTC-sulphoxide impurity, which was formed due to acid degradation [3]. The same degradation procedure was carried out in our lab. The degradation product found showed the same *m*/*z* and when the FTC sample was spiked with this degradation product, it was eluted along with UNK2. So, UNK2 was identified as 3′-sulphoxo FTC. Similar to this, UNK1 and UNK8 also have an extra oxygen. However, the 5-membered ring of UNK1 is 2 u less than this of UNK2. For the characterization of this impurity three possibilities could be considered: (1) presence of a hydroxyethyl instead of hydroxymethyl group in the C-2′ position of FTC, (2) attachment of a carboxylic acid group in the C-2′ position of FTC and (3) a formyl substituent instead of a hydroxymethyl group in the 3′-sulphoxo FTC. Hence, three compounds, 2′-dehydroxymethyl-2′-hydroxyethyl FTC, 2′-dehydroxymethyl-2′-carboxylic acid FTC (acid form) and 2′-dehydroxymethyl-2′-formyl-3′-sulphoxo FTC (aldehyde form) can be considered. NMR experiments are necessary to confirm the structure. However, on the basis of retention time (polarity), the latter two compounds are somewhat more favorable. Further, the collected fraction of the UNK1 was injected using the non-volatile mobile phase with pH 1.5. The elution time of the impurity was increased and it evidences that UNK1 could have an acidic functional group rather than an aldehyde. So, UNK1 is proposed to be 2′-dehydroxymethyl-2′-carboxylic acid FTC.

UNK8, with an extra oxygen at the S-3′ position, contains a 6-membered ring which is 1 u higher than 5-fluorocytosine, probably due to the presence of a hydroxyl group instead of an amino substituent at the C-4 position. This thus suggested UNK8 as 4-desamino-4-hydroxy-3′-sulphoxo FTC.

3.2.2. Peak 3 (UNK3 $[M+H]^+$ *m*/*z* 246)

The full mass spectrum showed that $[M+H]^+$ of UNK3 is 2 u lower than FTC. The MS^2 CID experiment on the protonated molecule showed a typical loss of 118 u (5-membered ring) to yield a characteristic ion at *m*/*z* 128 (data not shown). It indicates the presence of an aberrant 5-fluorocytosine group (128 u instead of 130 u). A difference of 2 u could be attributed to the replacement of the fluorine atom at the C-5 position by a hydroxyl group. Thus, UNK3 was assigned as 5-desfluoro-5-hydroxy FTC.

3.2.3. Peak 4 (UNK4 $[M+H]^+$ *m*/*z* 230)

Compound UNK4 has an $[M+H]^+$ which is 18 u lower than FTC. MS^2 investigation of the protonated molecule showed a loss of 118 u (data not shown), corresponding to the 2′-hydroxymethyl-1′,3′-oxathiolan moiety. This yielded a product ion at *m*/*z* 112, indicating a modified 5-fluorocytosine. A difference of 18 u is due to the absence of the fluorine atom at the C-5 position as is the case in lamivudine (see Fig. 1b). So, UNK4 was identified as lamivudine.

3.2.4. Peak 5 (UNK5 $[M+H]^+$ *m*/*z* 249)

The full mass spectrum showed that UNK5 is 1 u higher than FTC. Further CID investigation on this protonated molecule, yielded a product ion at *m*/*z* 131 with a loss of 118 u. It indicates that it has a modified 5-fluorocytosine group with 1 u extra. Similar to UNK8, UNK5 could have a hydroxyl group at the C-4 position instead of an amino group. This suggested that UNK5 is 4-desamino-4-hydroxy FTC. The retention time of UNK5 is similar to the degradation product, which was formed due to alkaline treatment [10]. When the degradation product was introduced in the MS, it showed the same fragmentation pattern as UNK5. Hence, it was confirmed that UNK5 and the degradation product are the same. However, desamino FTC impurity formed due to alkaline treatment [3,10] was not found in the sample and also in the degradation product.

3.2.5. Peak 6 (UNK6 $[M+H]^+$ *m*/*z* 364) and Peak 7 (UNK7 $[M+H]^+$ *m*/*z* 364)

Compounds corresponding to Peak 6 ($[M+H]^+$ *m*/*z* 364) and Peak 7 ($M+H]^+$ *m*/*z* 364) were identified as isomers of 4-N-(2″-dehydroxymethyl-2″-formyl-1″,3″-oxathiolan) FTC. The MS^2 CID spectra of these compounds show the initial losses of 116 u to yield a characteristic ion at *m*/*z* 248 which was further confirmed as FTC (see Fig. 4). It evidences that a 2″-dehydroxymethyl-2″-formyl-

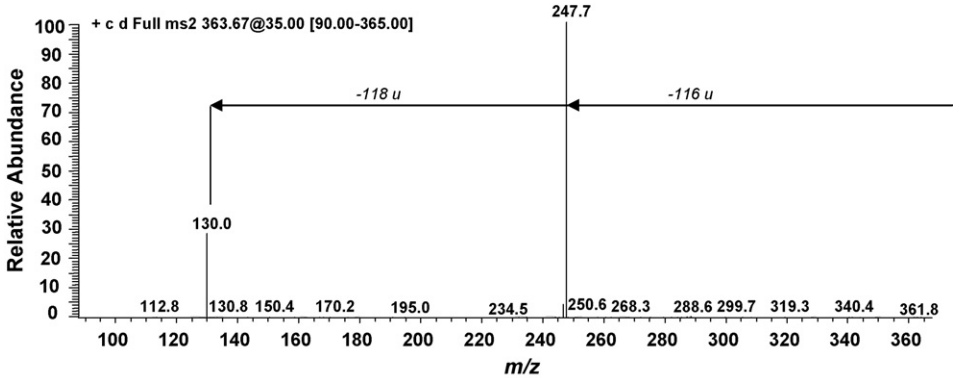

Fig. 4. CID MS^2 spectra of $[M+H]^+$ acquired for UNK6 and UNK7 ($[M+H]^+$ *m*/*z* 364); the result of isolation and collisional activation of the precursor ions in the ion trap at 35% CE.

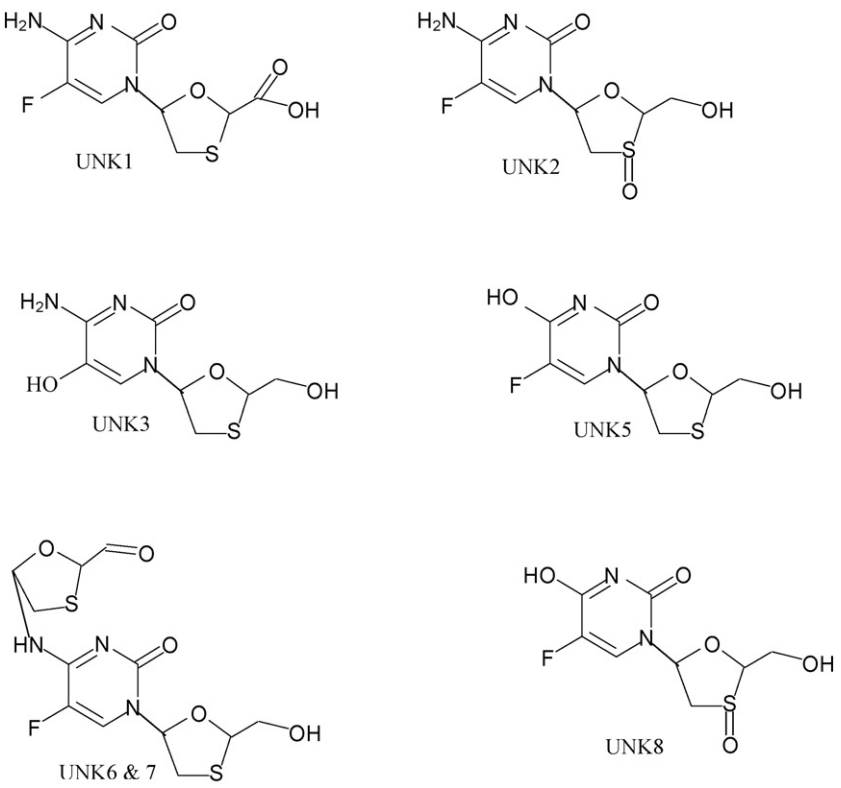

Fig. 5. Proposed structures for the unknown impurities present in the FTC sample.

Table 2
Overview of the compounds present in the FTC sample according to their elution order (see Fig. 3) and some proposed names for the unknown compounds.

Peak no.	Proposed name for unknowns (UNK)
1	UNK1 (*m*/*z* 262): 2′-dehydroxymethyl-2′-carboxylic FTC
2	UNK2 (*m*/*z* 264): 3′-sulphoxo FTC
3	UNK3 (*m*/*z* 246): 5-desfluoro-5-hydroxy FTC
4	UNK4 (*m*/*z* 230): Lamivudine
5	UNK5 (*m*/*z* 249): 4-desamino-4-hydroxy FTC
6 and 7	UNK6 and 7 (*m*/*z* 364): 4-N-(2″-dehydroxymethyl-2″-formyl-1″,3″-oxathiolan) FTC
8	UNK8 (*m*/*z* 265): 4-desamino-4-hydroxy-3′-sulphoxo FTC
	UNK9 (*m*/*z* 248): isomer of FTC
9	UNK10 (*m*/*z* 248): isomer of FTC

1″,3″-oxathiolan group is attached to FTC. The most probable position is the amino group on C-4. UNK6 and UNK7 are identified as isomers of 4-N-(2″-dehydroxymethyl-2″-formyl-1″,3″-oxathiolan) FTC.

3.2.6. Peak 8 (UNK9 [M+H]$^+$ m/z 248) and Peak 9 (UNK10 [M+H]$^+$ m/z 248)

Based on their full mass spectrum, Peaks 8 and 9 were found to have a *m*/*z* value of 248. Careful investigation of the spectrum of Peak 8 shows a high abundant ion at *m*/*z* 248 (UNK9) and a low abundant at *m*/*z* 265 (UNK8). UNK8 (*m*/*z* 265) has already been described in 3.2.1. In the MS^2 spectra of UNK9 and UNK10 (data not shown), the product ion *m*/*z* 130 was observed after a loss of the 2′-hydroxymethyl-1′,3′-oxathiolan moiety. This shows that UNK9 and UNK10 are isomers of FTC. NMR experiments are necessary for further confirmation.

An overview of the compounds present in the FTC sample and proposed names for the UNKs are given in Fig. 5 and Table 2 respectively.

4. Conclusion

Structure characterization of FTC related substances was carried out. Most of the impurities are due to modifications at the C-4 and S-3′ positions. Among nine peaks studied, six new compounds were identified. A peak corresponding to lamivudine which is an other antiretroviral drug was also identified. Due to the limitation of mass spectrometry, two peaks corresponding to isomers of FTC could not be fully characterized.

Acknowledgment

The MS detector was made available through project G.0355.98 of the Flemish Fund for Scientific Research (Belgium).

References

[1] M.J. Otto, Curr. Opin. Pharmacol. 4 (2004) 431–436.
[2] Q.B. Cass, C.S.F. Watanabe, J.A. Rabi, P.Q. Bottari, M.R. Costa, R.M. Nascimento, J.E.D. Cruz, R.C. Ronald, J. Pharm. Biomed. Anal. 33 (2003) 581–587.
[3] S. Unnam, H. Bodepudi, B.C. Kottapalli, J. Sep. Sci. 30 (2007) 999–1004.
[4] H. Rebiere, B. Mazel, C. Civade, P. Bonnet, J. Chromatogr. B 850 (2007) 376–383.
[5] A. Darque, G. Valette, F. Rousseau, L.H. Wang, J.P. Sommadossi, X.J. Zhoui, Antimicrob. Agents Chemother. 43 (9) (1999) 2245–2250.
[6] N.L. Rezk, R.D. Crutchley, A.D.M. Kashuba, J. Chromatogr. B 822 (2005) 201–208.
[7] S. Notari, C. Mancone, M. Tripodi, P. Narciso, M. Fasano, P. Ascenzi, J. Chromatogr. B 833 (2006) 109–116.
[8] R.W. Sparidans, J.M. Prins, J.H.M. Schellens, J.H. Beijnen, Biomed. Chromatogr. 21 (2007) 621–627.
[9] T. Le Saux, S. Chhun, E. Rey, O. Launay, L. Weiss, J.P. Viard, G. Pons, V. Jullien, J. Chromatogr. B 865 (2008) 81–90.
[10] D.A. Mamade, A. Verbeek, J. Hoogmartens, A. Van Schepdael, E. Adams, J. Sep. Sci. 32 (2009) 1823–1830.
[11] M. Pendela, L. Van den Bossche, J. Hoogmartens, A. Van Schepdael, E. Adams, J. Chromatogr. A 1180 (2008) 108–121.