Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 731–737

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

"In-source" fragmentation of an isobaric impurity of lamotrigine for its measurement by liquid chromatography tandem mass spectrometry after pre-concentration using solid phase extraction

Dan J. Carrier*, Christine Eckers, Jean-Claude Wolff

GlaxoSmithKline, Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, UK

ARTICLE INFO

Article history:
Received 18 October 2007
Received in revised form 27 February 2008
Accepted 2 March 2008
Available online 14 March 2008

Keywords:
LC–MS/MS
In-source fragmentation
SPE
Isobaric impurity

ABSTRACT

An analytical method has been developed for trace analysis (i.e. sub-ppm levels) of a key synthetic impurity, 14W80 ((*Z*)-2-(2,3-dichlorophenyl)-2-(guanidinylimino)acetonitrile) in lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine). 14W80 is isobaric with lamotrigine, which gives an extra layer of complexity to its determination and the various problems associated with development of an appropriate methodology are discussed in this work. Ultimately, a liquid chromatography tandem mass spectrometry (LC–MS/MS) method using in-source fragmentation with Atmospheric Pressure Chemical Ionisation (APCI) followed by multiple reaction monitoring (MRM) has been found to provide adequate sensitivity and specificity. A detection limit of 25 ppb mass fraction relative to lamotrigine was achieved for 14W80. The use of solid phase extraction (SPE) enhanced the detection limit to 2 ppb mass fraction relative to lamotrigine.

1. Introduction

Lamotrigine ((3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) is the active pharmaceutical ingredient in Lamictal®, which is a novel antiepileptic that has a membrane-stabilising mechanism via blockade of voltage-dependent sodium channels and inhibition of glutamate release [1,2]. 14W80 ((*Z*)-2-(2,3-dichlorophenyl)-2-guanidinylimino)acetonitrile is a synthetic intermediate in some routes of synthesis for lamotrigine (refer to Fig. 1 for structures). Since 14W80 is an intermediate in some synthetic routes, it is a route indicative impurity. To determine the route of synthesis, it is necessary to monitor for its presence/absence in final drug substance and drug products. In this paper we describe an LC–MS/MS method using in-source fragmentation with Atmospheric Pressure Chemical Ionisation (APCI) followed by multiple reaction monitoring (MRM).

The major difficulty with analysing 14W80 is that it is isobaric with lamotrigine (Fig. 1). As it is more hydrophobic than lamotrigine, it elutes with a longer retention time using reverse phase chromatography. A high concentration of lamotrigine (drug substance or drug product) is required to be introduced into the chromatographic system in order to detect 14W80 at trace level (sub-ppm mass fraction levels relative to lamotrigine). Even with good chromatographic separation, a method based on single mass alone such as Single Ion Monitoring would be unsuitable for detection of 14W80, as tailing from the lamotrigine peak would mask detection of low levels of 14W80. In addition, the similarity of structures and thus polarity for lamotrigine and 14W80 makes development of a sample preparation technique complex. In previously published work [3], solvent extraction with normal phase chromatography followed by reverse phase chromatography was used to achieve a detection limit for 14W80 in lamotrigine of 50–100 ppb mass fraction. In this work, we have looked at both mass spectrometric and sample preparation techniques, to develop a simpler method, with improved detection limits over the previously reported work [3].

In atmospheric pressure ion sources, e.g. APCI or electrospray ionisation (ESI), fragmentation of ions can occur in the ion source (in-source fragmentation) before ions reach the analyser. Although this method of fragmentation is less selective than tandem mass spectrometry, as all ions in the source will be fragmented simultaneously, it has been used by several research groups [4–8]. The most common method employed to achieve best specificity, selectivity and sensitivity in LC–MS/MS is MRM [9–12]. In this work, we combine the usage of in-source fragmentation with MRM to overcome the challenges posed by the fact that 14W80 is isobaric with lamotrigine.

* Corresponding author.
E-mail addresses: dan.j.carrier@gsk.com, Jean-Claude.Wolff@gsk.com (D.J. Carrier).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.03.002

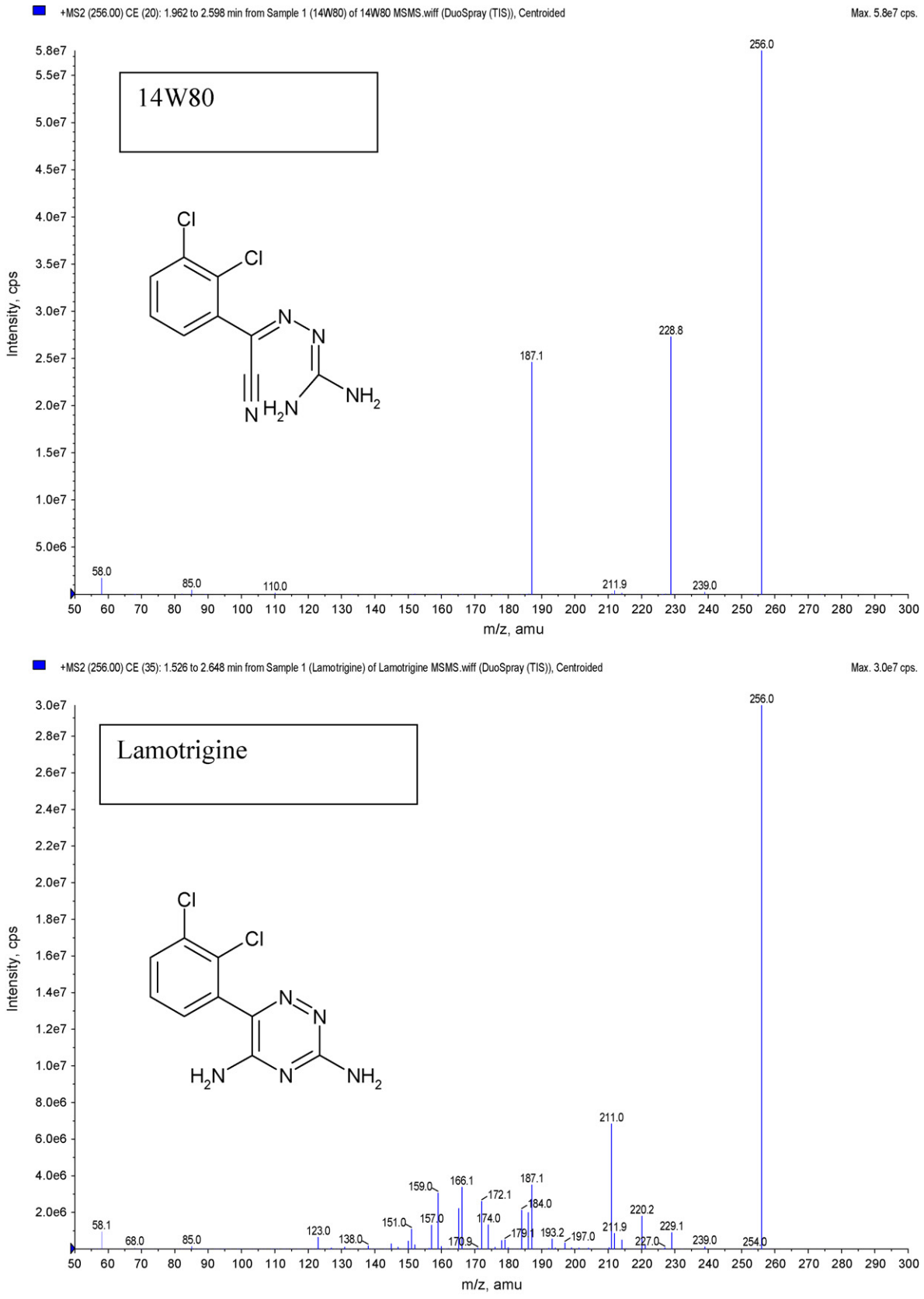

Fig. 1. MS/MS and structures for lamotrigine ($C_9H_7Cl_2N_5$) and 14W80 ($C_9H_7Cl_2N_5$).

To achieve better detection limits, solid phase extraction (SPE) [13–19] can be used to enrich trace analytes of interest. Analytes which show hydrophobic characteristics can be retained and enriched on non-polar stationary phases such as a C18 phase, whilst analytes, which show hydrophilic characteristics, can be washed off. To achieve the sensitivity required (sub-ppm mass fraction levels) for the measurement of 14W80 in lamotrigine, this sample preparation technique has been combined with LC–MS/MS employing in-source fragmentation followed by MRM. The assay developed was designed as a presence/absence test so that only semi-quantitative results were reported. Only limited validation was carried out because the method was developed as a limit test.

2. Experimental

2.1. Reagents, solvents and standard solutions

14W80 and lamotrigine were supplied by GlaxoSmithKline, Barnard Castle, UK. HPLC grade Acetonitrile (MeCN), Ammonium acetate, Phosphoric acid was purchased from Fisher Scientific UK (Loughborough, Leicestershire, UK). Distilled deionised water (18.2 ΩM) was obtained using an Elga Maxima Purification System.

2.2. LC–MS/MS

An Agilent 1100 HPLC (Agilent, Stockport, UK) coupled to an API4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) was used for this analysis using MRM detection. A gradient profile using Acetonitrile (MeCN) (eluent A), and 0.05 M ammonium acetate pH unadjusted (eluent B) was used to give sufficient separation between lamotrigine and 14W80. The HPLC gradient was set to 15/85 A/B volume fraction for 10 min with linear gradient up to 95/5 A/B volume fraction over 10 min. A Luna C18 (2) 50 mm × 2 mm, 3.5 μm (Phenomenex, Macclesfield, UK) with a flow rate of 0.4 mL/min was used for this analysis.

The API4000 was operated in positive APCI mode. The source temperature was set to 600 °C. The declustering potential was set to 80 V for fragmentation of 14W80 in the ion source. Ion Spray voltage was 4.5 kV. The collision energy used for MRM analysis was 17 eV.

14W80 ($[M+H]^+ = 256$) was fragmented in-source after optimisation of the declustering potential to give a fragment ion at m/z 229. For MRM, the transition monitored were m/z 229–187 and m/z 229–152. The transition m/z 229–187 is the most abundant, but m/z 229–152 is used for confirmatory purposes.

2.3. Sample preparation

Samples were either supplied as bulk lamotrigine (drug substance) or tablets containing lamotrigine. Lamotrigine drug substance, labelled A–E for proprietary reasons, was obtained from different sources. The drug products, in form of tablets, and labelled F–H, were from different sources, too.

Lamotrigine samples were prepared in [15/85 MeCN/water] + 1% phosphoric acid volume fraction at a mass concentration of 20 mg/mL. For lamotrigine containing tablets, a pestle and mortar was used to crush tablets. The powder was then weighed and diluted in 1 mL to give an equivalent mass concentration (i.e. amount of crushed tablet weighed adjusted accordingly to tablet weight and dosage) of lamotrigine (20 mg/mL). The solution was then centrifuged at 15,000 rpm for 5 min, and the supernatant was pipetted into a HPLC vial for analysis. Due to low percentage of organic solvent in the diluent, a high injection volume (100 μL) could be used without chromatographic peak deterioration.

For SPE analysis, a Mega Bond Elute (1 g) C18 Cartridge was used with a negative vacuum using a flow rate of approximately 1 mL/min through the cartridge. A 10 mL solution of lamotrigine in [15/85 MeCN/water] + 1% phosphoric acid volume fraction at 20 mg/mL was prepared. This solution was passed through a pre-conditioned SPE cartridge. Lamotrigine was washed off the cartridge with 15/85 MeCN/water + 1% phosphoric acid volume fraction. Retained 14W80, was then collected from the cartridge by eluting with MeCN. The solution was evaporated to dryness and the residue was reconstituted in 1 mL of diluent.

2.4. Method validation

Limited validation was carried out. Full validation was not required since the method was only developed as a limit test for 14W80.

Linearity of the method for measurement of 14W80 in the presence of lamotrigine was performed. 14W80 was spiked into lamotrigine at six mass fractions (0.2, 0.8, 2, 4, 10 and 20 ppm). External standards for 14W80 were prepared at 2 ppm mass fraction without lamotrigine present to calculate recovery of 14W80 in lamotrigine. Six replicate injections of 14W80 in the presence of lamotrigine were performed to calculate repeatability [20].

3. Results and discussion

3.1. Mass spectrometry method development

Fig. 1 shows the tandem mass spectrometry spectra obtained from the protonated molecule (m/z 256) for both lamotrigine and 14W80. Although, on initial inspection the two MS/MS appear quite different, there is no product ion in the MS/MS spectrum for 14W80 which is not observed in the spectrum obtained for lamotrigine. Thus, there is no transition, which is not interfered by lamotrigine, that can be used to develop a selected or multiple reaction monitoring (SRM or MRM) assay for 14W80 in lamotrigine. This is detrimental to the detection limits obtained for 14W80 as it elutes after lamotrigine under all the reverse phase chromatographic conditions investigated as part of this work. The only literature reference found where 14W80 elutes prior to lamotrigine is the work by Ashton et al. [3], which uses preparative normal phase chromatography. This suggests that normal phase chromatography is required to change the elution order. However, normal phase chromatography is not well suited for atmospheric pressure ionisation mass spectrometry. A possible approach would be to use a Hilic phase for LC [21]. However, this was not investigated during development of this method.

In order to improve selectivity, in-source fragmentation was investigated, since lamotrigine is a more stable molecule than 14W80, which is reflected in its MS/MS spectrum (Fig. 1). Indeed, higher collision energy is needed to fragment lamotrigine compared to 14W80, i.e. 35 eV versus 20 eV (Fig. 1). Fig. 2 shows the in-source fragmentation spectra obtained for both compounds. The major product ion in the MS/MS of 14W80, i.e. m/z 229, can easily be obtained in the ion source by adjusting the declustering potential. This ion is not observed in the corresponding in-source fragmentation spectrum for lamotrigine (Fig. 2). The declustering potential was optimised so as to yield the maximum ion abundance for m/z 229 in the MS spectrum of 14W80 without yielding the ion in the lamotrigine spectrum. A value of 80 V was found to give the best result. MS/MS was then carried out on m/z 229 for 14W80 and two significant product ions at m/z 187 and m/z 152 were observed.

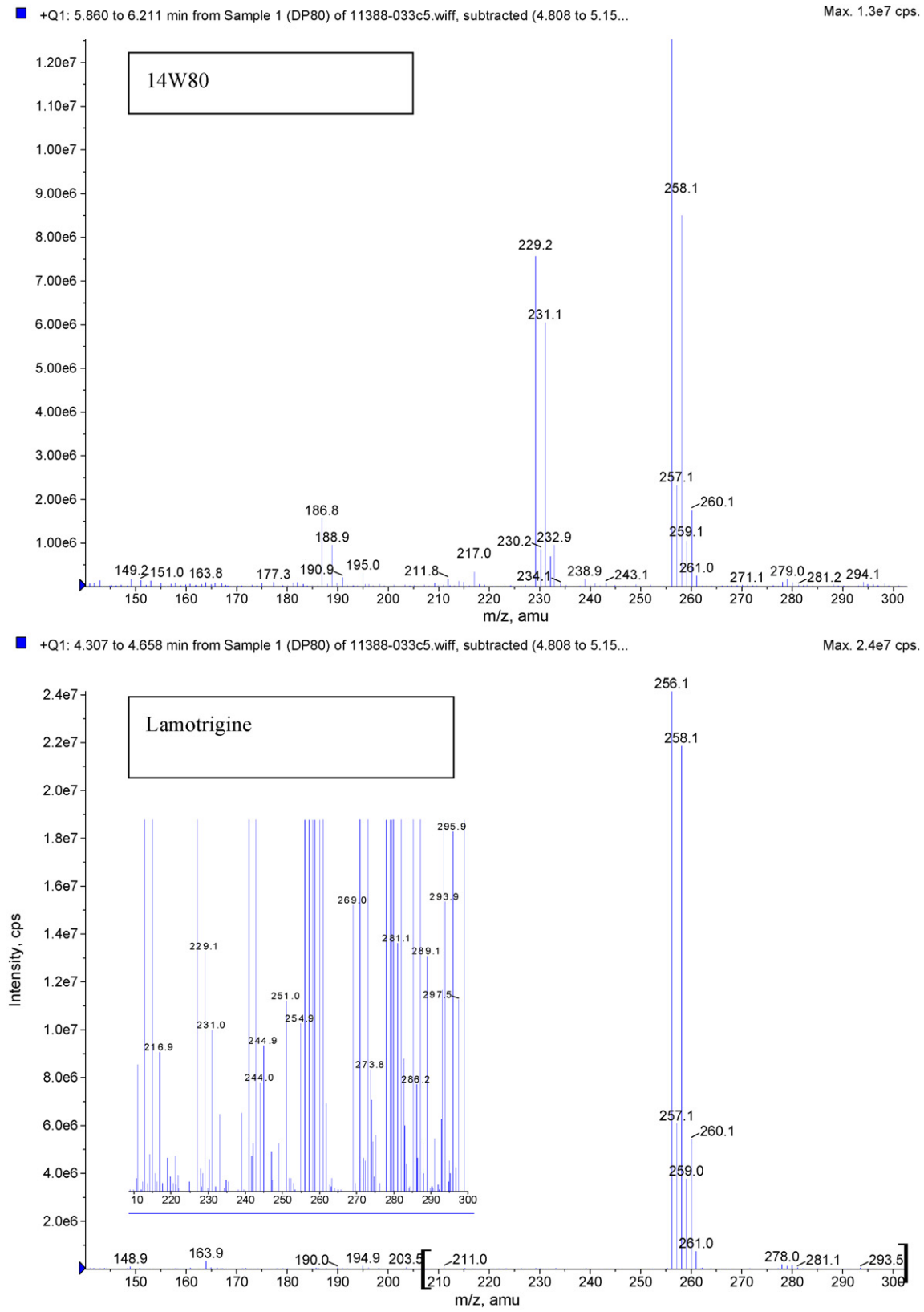

Fig. 2. In-source fragmentation MS spectra for lamotrigine and 14W80 (inset zoomed view of noise in region of 229 m/z).

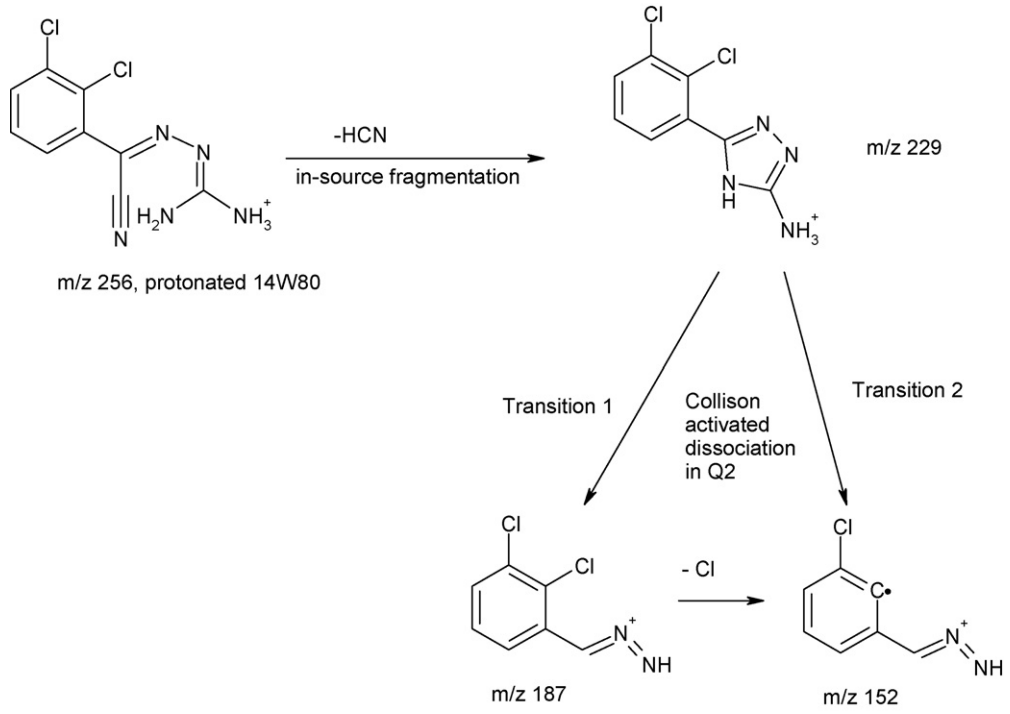

Fig. 3. Fragmentation pathway for 14W80.

Fig. 3 shows a potential fragmentation pathway for the generation of both the in-source and MS/MS product ions for 14W80.

The two MS/MS product ions (m/z 187 and m/z 152) can be used to develop an MRM assay for 14W80. As m/z 229 was not generated in-source for lamotrigine no peak for these two transitions was observed during the assay development for lamotrigine itself. Fig. 4 shows the transition m/z 229–187 for a sample of lamotrigine at a mass concentration of 20 mg/mL. Lamotrigine has a retention time of 8 min, and clearly no peak can be seen at that retention time. Two transitions were used to increase confidence in the detection of

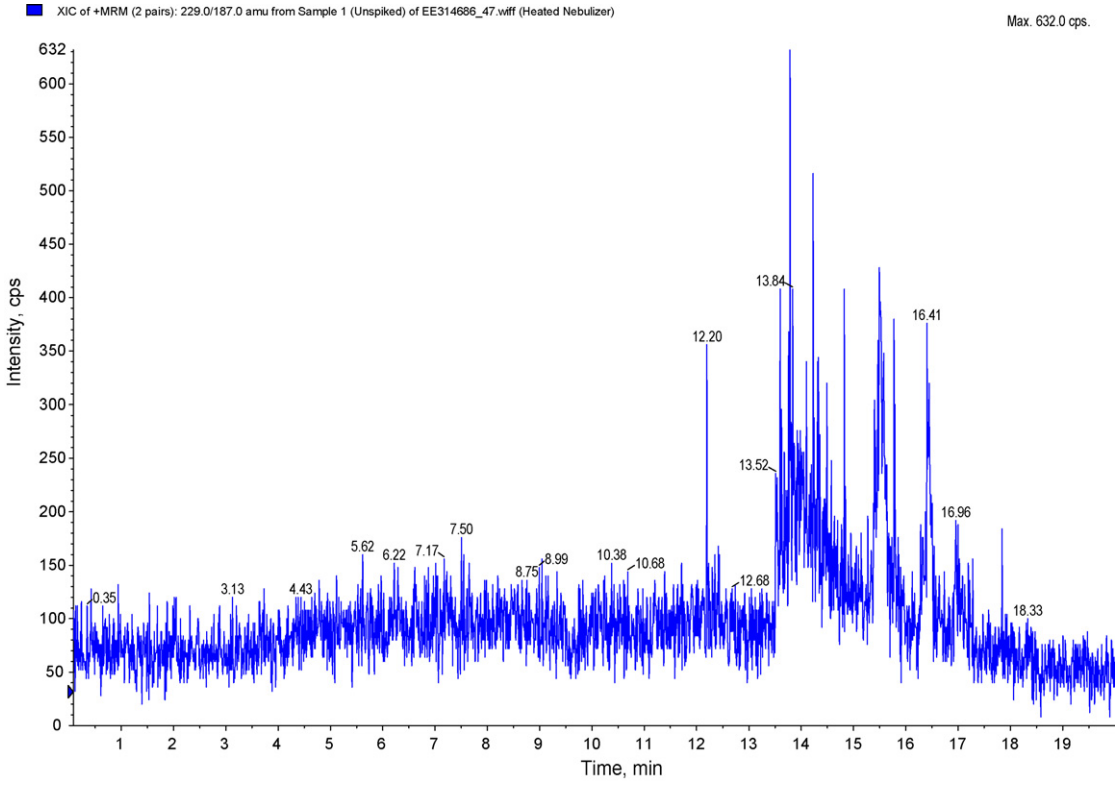

Fig. 4. MRM ion chromatogram (transition m/z 229–187) for lamotrigine sample.

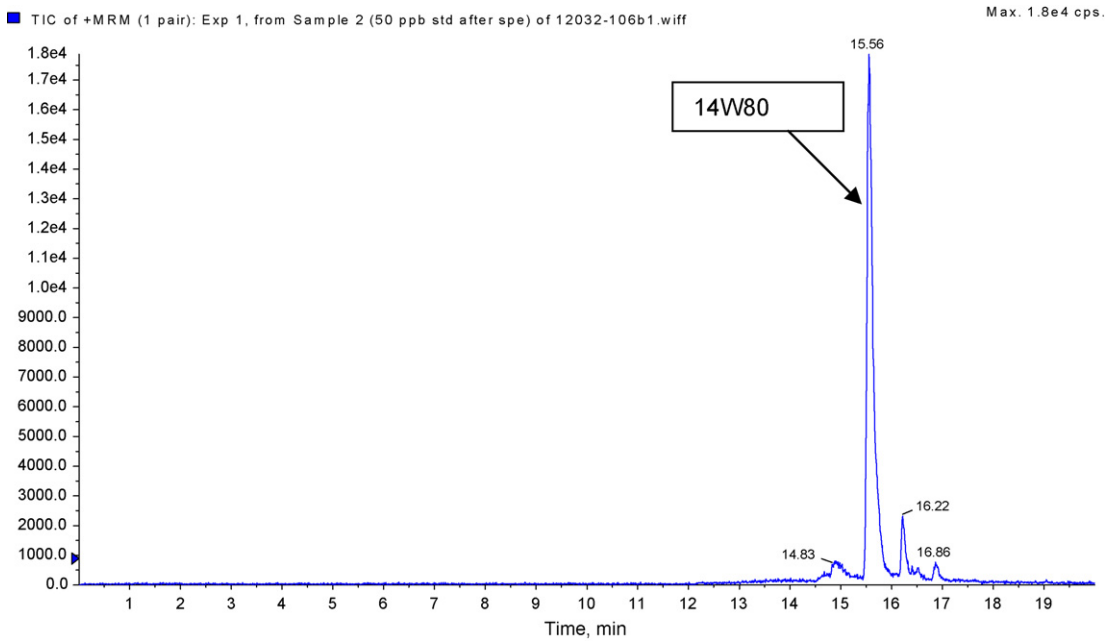

Fig. 5. MRM ion chromatogram (transition *m*/*z* 229–187) for 50 ppb mass fraction 14W80 in lamotrigine using SPE as sample pre-concentration.

14W80, using *m*/*z* 229–187 for semi-quantitation and *m*/*z* 229–152 being the qualifier transition.

Using this methodology the detection limit for 14W80 was 25 ppb mass fraction relative to lamotrigine. Hence, without any sample pre-treatment such as preparative normal phase chromatography, an improvement of the detection limit with respect to the work by Ashton et al. [3] was achieved. This is greatly due to the fact that the usage of in-source fragmentation of 14W80 is specific and has significantly reduced/removed the interference from lamotrigine. In-source fragmentation of 14W80 permits its analysis by reverse phase chromatography without the removal of lamotrigine.

3.2. Limited validation

Good linearity was shown over two orders of magnitude for 14W80 (0.2–20 ppm mass fraction) in lamotrigine. The correlation coefficient was calculated to be 0.998. Coefficient of variation for six injections of 14W80 in lamotrigine was calculated to be 6%. Recovery of 14W80 in lamotrigine was found to be above 70% for all mass fractions (0.2–20 ppm). All calculations were carried out in accordance with ICH guidelines on validation [20].

3.3. Pre-concentration of 14W80 using solid phase extraction

For some samples better detection limits were required, and the use of SPE was investigated. 14W80 is more hydrophobic than lamotrigine, therefore it is possible to develop a method where 14W80 can be retained on the SPE cartridge and lamotrigine, which is more hydrophilic, washed off. A series of cartridges and eluent conditions were investigated. The conditions described in the experimental section were found to give the best results. Recovery for 14W80 from the SPE cartridge was calculated to be between 80 and 100% and a 10-fold increase in sensitivity for 14W80 was observed from this approach. Thus, the detection limit was calculated to be approximately 2 ppb mass fraction. Fig. 5 shows the extracted MRM ion chromatogram for the transition *m*/*z* 229–187, for 50 ppb mass fraction of 14W80 (retention time 15.5 min) in lamotrigine (retention time 8 min) using SPE. No response is observed for lamotrigine at 8 min. The other peaks in the chromatogram are due to impurities with $[M+H]^+$ 229 and which give a product ion at *m*/*z* 187, too. The qualifier transition *m*/*z* 229–152 is monitored in parallel. For samples, where 14W80 is present at a mass fraction close to the detection limit, the reduced sensitivity for the transition *m*/*z* 229–152 means that no peak be observed for this transition. In this case, a 14W80 standard and a spiking experiment of samples suspected to contain 14W80 are used to confirm the presence/absence of 14W80.

Although SPE gives a significant improvement in detection limits (at least one order of magnitude better than previously published methodology [3]), it was not possible to eliminate all the lamotrigine in the samples to be analysed, because such high levels of material were used in the preparation of the samples. Thus, the in-source fragmentation step in the methodology was required to reduce/avoid interference.

3.4. Analysis of lamotrigine drug substance and drug product samples

The methodology described above (combining in-source fragmentation with SPE when and if required) was used to screen both

Table 1
Semi-quantitative results for the measurement of 14W80 in drug substance and drug product

Sample description	Approximate mass fraction of 14W80 relative to lamotrigine	With/without SPE
Drug substance A–E	Not detected	With
Drug product F	30 ppb, both transitions observed	With
Drug product G	600 ppb, both transitions observed	Without
Drug product H	100 ppb, both transitions observed	Without

drug substance and drug product for presence/absence of 14W80, to understand the route of synthesis of the samples. A series of samples, both drug substance and drug products (tablets), were analysed first only using in-source fragmentation. If 14W80 was not detected in the samples or close to the detection limit, i.e. 25 ppb mass fraction relative to lamotrigine, SPE, which is more labour intensive was used. Using this approach 14W80 was detected in some tablets at varying levels between 30 and 600 ppb mass fraction relative to lamotrigine. In the drug substances analysed, 14W80 was below detection limit, i.e. 2 ppb mass fraction relative to lamotrigine. Results are summarised in Table 1. The results shown are semi-quantitative since the aim of the assay was to test samples for absence/presence of 14W80. However, the limited validation carried out on the method shows it is fit for purpose.

4. Conclusion

It can be clearly seen that the methodology developed can be used to measure low levels (i.e. sub-ppm or even sub-ppb mass fraction relative to lamotrigine) of 14W80 in both drug product and drug substance. Depending on the levels present different levels of complexity of the methodology can be used. The final method developed met the original aim of this work of providing a simpler method with an enhanced detection limit, i.e. at least one order of magnitude better, over the previously published method [3].

This work demonstrated the importance of investigating possible approaches for improving detection limits when trace level analysis is required. In this example, in-source fragmentation gives improved specificity and the selection of appropriate sample preparation techniques—SPE was able to provide a detection limit of 2 ppb mass fraction for 14W80 relative to lamotrigine.

References

[1] M.J. Leach, C.M. Marden, A.A. Miller, Epilepsia 27 (1986) 490–497.
[2] A.A. Miller, P. Wheatley, D.A. Sawyer, M.G. Baxter, B. Roth, Epilepsia 27 (1986) 483–489.
[3] D. Ashton, A. Ray, K. Valko, Int. J. Pharm. 189 (1999) 241–248.
[4] A. Putschew, M. Jekel, Rapid Commun. Mass Spectrom. 17 (2003) 2279–2282.
[5] A. Huetteroth, A. Putschew, M. Jekel, Int. J. Environ. Anal. Chem. 87 (2007) 415–424.
[6] J.H. Gil, J. Hong, J.C. Choe, Y.H. Kim, Bull. Korean Chem. Soc. 24 (2003) 1163–1168.
[7] Q. Tian, C.J.G. Duncan, S.J. Schwartz, J. Mass Spectrom. 38 (2003) 990–995.
[8] Z. Yan, G.W. Caldwell, W.J. Jones, J.A. Masucci, Rapid Commun. Mass Spectrom. 17 (2003) 1433–1442.
[9] C.R. Mallet, J.R. Mazzeo, U. Neue, Rapid Commun. Mass Spectrom. 15 (2001) 1075–1083.
[10] K. Srinivasan, P. Wang, A.T. Eley, C.A. White, M.G. Bartlett, J. Chromatogr. B 745 (2000) 287–303.
[11] G.K. Poon, G. Kwei, R. Wang, K. Lyons, Q. Chen, V. Didolkar, C.E.C.A. Hop, Rapid Commun. Mass Spectrom. 13 (1999) 1943–1950.
[12] L. Di Donna, F. Mazzotti, G. Sindona, A. Tagarelli, Rapid Commun. Mass Spectrom. 19 (2005) 1575–1577.
[13] K.M. Li, L.P. Rivory, S.J. Clarke, Curr. Pharm. Anal. (Aust.) 2 (2006) 95–102.
[14] M.W.J. Van Hout, C.M. Hofland, H.A.G. Niederlander, G.J. De Jong, Rapid Commun. Mass Spectrom. 14 (2000) 2103–2111.
[15] C.H.P. Bruins, C.M. Jeronimus-Stratingh, K. Ensing, W.D. Van Dongen, G.J. De Jong, J. Chromatogr. A 863 (1999) 115–122.
[16] J. Ding, U.D. Neue, Rapid Commun. Mass Spectrom. 13 (1999) 2151–2159.
[17] J. Pis, J. Hykl, T. Vasar, J. Harmatha, J. Chromatogr. A 658 (1994) 77–82.
[18] B.L. Inman, L.E. Chovan, P.J. Dandliker, Y.Y. Lau, J. Pharm. Sci. 96 (2007) 1619–1624.
[19] A. Schellen, B. Ooms, M. van Gils, O. Halmingh, E. Van der Vlis, D. Van de Langemaat, E. Verheij, Rapid Commun. Mass Spectrom. 14 (2000) 230–233.
[20] ICH, Validation of analytical procedures: methodology (Q2R1), International Conference on Harmonization, Food and Drug Administration, USA, November 1996 and November 2005.
[21] M. Michel, T. Baczek, S. Studzinska, K. Bodzioch, T. Jonsson, R. Kaliszan, B. Buszewski, J. Chromatogr. A 1175 (2007) 49–54.