Implementation of a single quad MS detector in routine QC analysis of peptide drugs

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Original Article

Abstract

A newly developed single quad mass spectrometry (MS) detector was coupled to a ultra-high performance liquid chromatography (UPLC) system and implemented in the routine quality control (QC) and impurity analysis of four therapeutic peptides, namely bleomycin sulfate, tyrothricin, vancomycin HCl and bacitracin, which were selected given their multi-component drug nature and their closely structurally related impurity profiles. The QC and impurity profiling results obtained using the ultra-high performance liquid chromatography ultraviolet/mass spectrometry (UPLC-UV/MS) detection system were analyzed against the results obtained using traditional high performance liquid chromatography-ultraviolet detection (HPLC-UV) methods derived from pharmacopoeial methods. In general, the used stationary phases of sub-2 μm particle (UPLC) technology resulted in lower limits of detection and higher resolution separations, which resulted in more detected impurities and shorter overall run times contrasting the traditional HPLC columns. Moreover, online coupling with a single quad MS detector allowed direct peak identification of the main compounds as well as small impurities, hereby increasing the information content without the need of reference standards.

Keywords:
Quality control
Peptide
Impurity profiling
Single quad MS detector

1. Introduction

Over the past few decades, mass spectrometry (MS) has become an essential tool in fundamental disease research [1–4]. Mass spectrometry-based proteomics, metabolomics, peptidomics, glycomics, phosphoproteomics and lipidomics are used to quantitatively and qualitatively differentiate protein, peptide, sugar and lipid structures of patients and healthy volunteers, in order to identify new biomarkers or seek treatment opportunities [5]. Due to the complexity of these human samples, as well as the quest to obtain greater sensitivity, a wide variety of high-end mass spectrometry instruments have been developed in recent years. An excellent review written by Domon and Aebersold provides a performance overview of different types of MS used in proteomic field. For example, triple quadrupole instruments were used for quantification of proteins and ultra-high resolution Fourier transform ion cyclotron resonance (FT-ICR) for their identification [3]. MS has also been applied in drug development such as drug metabolism studies [6,7]. The factors that limit the widespread use of high-end MS instruments are high purchasing costs and expensive maintenance. Furthermore, highly skilled operators for method optimization and subsequent data interpretation are needed.

Factors like those mentioned above have limited the use of MS in routine pharmaceutical quality control (QC) environments. The performance demands on MS instrumentation in routine pharmaceutical setting are less challenging than those in the “omics”-field, as matrix effects are less evident and drug concentrations are much higher. However, simplified operation and maintenance within a good manufacturing practice (GMP) environment are paramount, which leads to the need for downsized and lower-end MS systems. Recently, a number of small single quad MS detectors that are directly compatible with the existing high performance liquid chromatography (HPLC)/ultra-high performance liquid chromatography (UPLC) equipment and software platforms used in QC, have been developed and introduced into the pharmaceutical setting [8–10]. The availability of these new MS detectors has created the potential to bring routine use of MS to the QC laboratory and pharmaceutical QC to a higher level. The present work considered a new ultra-high performance liquid chromatography ultraviolet/mass spectrometry detection (UPLC-UV/MS) equipment set-up in relation to the traditional high performance liquid chromatography-ultraviolet detection (HPLC-UV) set-up for the QC and impurity profiling of complex therapeutic peptides.

2. Materials and methods

2.1. Materials

Tyrothricin, bacitracin, propionic acid, sodium pentane sulfate,
sodium sulfate and triethylamine were bought from Sigma-Aldrich (Diegem, Belgium), whereas vancomycin HCl was purchased from Bufa (Ijsselstein, the Netherlands). These peptides were ordered without explicit pharmacopeial quality requirements, in order to increase the probability of containing related impurities. Bleomycin sulfate was obtained from Sanoﬁ Aventis (Brussel, Belgium). Acetonitrile, methanol and tetrahydrofuran of HPLC grade were acquired from Fisher Scientiﬁc (Aalst, Belgium), whereas acetoni- trile, methanol and triﬂuoroacetic acid (TFA) of UPLC grade were bought from Biosolve (Valkenswaard, the Netherlands). Water was puriﬁed using an Arium 611 puriﬁcation system (Sartorius, Gottingen, Germany) yielding Z18.2 MΩ/cm quality water. Potassium dihydrogen phosphate and ammonium sulfate were purchased from Merck (Overijse, Belgium), whereas dipotassium hydrogen phosphate was obtained from Panreac Chimica (Barcelona, Spain).

### 2.2. Peptide selection

Three different antimicrobial peptides, i.e., tyrothricin (cyclic polypeptide), vancomycin HCl (glycopeptide), bacitracin (cyclic polypeptide), and the chemotherapeutic peptide bleomycin sulfate (glycopeptide) were selected as test compounds, given their complex and multi-component drug nature and their closely structurally related impurity proﬁles. An overview of the selected peptides, as well as their major components and structural properties, is given in Table 1.

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**Table 1**

| Name                  | Major compounds | Formula                                      | Molecular weight | Sample solvent | Ph. Eur. concentration |
|-----------------------|-----------------|----------------------------------------------|------------------|----------------|-----------------------|
| Bleomycin sulfate     | Bleomycin A₂    | C₅₅H₈₄N₁₇O₂₁S₃                            | 1414.52          | H₂O            | Test solution: 0.5 mg/mL |
|                       | Bleomycin B₂    | C₅₅H₈₄N₂₀O₂₁S₂                            | 1424.56          |                |                       |
| Tyrothricin            | Gramicidin A₁   | C₈₃H₁₄₂N₂₇O₂₇                            | 1881.07          | 15 volumes H₂O | Test solution: 1.0 mg/mL |
|                       | Gramicidin A₂   | C₁₀₀H₁₈₅N₂₉O₂₇                           | 1895.09          | 85 volumes MeOH | Ref. solution B: 0.02 mg/mL |
|                       | Gramicidin C₁   | C₂₉H₅₃N₉O₂₆                             | 1858.05          |                |                       |
|                       | Gramicidin C₂   | C₂₉H₅₃N₉O₂₆                             | 1872.07          |                |                       |
|                       | Tyrocidin A     | C₅₀H₆₅N₂₁O₂₁                           | 1270.66          |                |                       |
|                       | Tyrocidin B     | C₅₀H₆₅N₂₁O₂₁                           | 1309.67          |                |                       |
|                       | Tyrocidin C     | C₅₀H₆₅N₂₁O₂₁                           | 1348.68          |                |                       |
|                       | Tyrocidin D     | C₅₂H₆₅N₂₁O₂₁                           | 1371.70          |                |                       |
|                       | Tyrocidin E     | C₅₀H₆₅N₂₁O₂₁                           | 1254.67          |                |                       |
|                       | Vancomycin HCl  | Vancomycin B                               | 1447.43          | 10 volumes THF | Test solution A: 2.0 mg/mL |
|                       |                 |                                              |                  | 70 volumes ACN | Test solution B: 0.08 mg/mL |
|                       |                 |                                              |                  | 920 volumes H₂O| Test solution C: 0.002 mg/mL |
| Bacitracin             | Bacitracin A    | C₆₅H₁₀₃N₁₇O₁₆S                        | 1421.75          | 40 volumes ACN | Test solution: 2.0 mg/mL |
|                       | Bacitracin B₁   | C₆₅H₁₀₁N₁₇O₁₆S                        | 1407.73          | 400 volumes H₂O| Ref. solution C: 0.01 mg/mL |
|                       | Bacitracin B₂   | C₆₅H₁₀₁N₁₇O₁₆S                        | 1407.73          | 520 volumes MeOH|                       |
|                       | Bacitracin B₃   | C₆₅H₁₀₁N₁₇O₁₆S                        | 1407.73          |                |                       |

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**Table 2**

| Compound        | Mobile phase A | Mobile phase B | Run time (min) | Flow rate (mL/min) | Column temperature (°C) | Injection volume (µL) | Quant. wavelength (nm) |
|-----------------|----------------|----------------|----------------|---------------------|-------------------------|-----------------------|------------------------|
| Bleomycin sulfate | MeOH          | Pentanesulfonate buffer | 100            | 1.2                 | Room                   | 20                    | 254                    |
| Tyrothricin      | 75 MeOH       | 25 sulfate buffer | 60             | 1.2                 | Room                   | 25                    | 280                    |
| Vancomycin HCl   | 70 ACN        | 10 THF 920 triethylamine | 35             | 1                   | Room                   | 20                    | 280                    |
| Bacitracin       | 40 ACN 300 H₂O 520 MeOH 100 phosphate buffer | 75 | 1 | Room | 100 | 254 |

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**Table 3**

| Sample            | Time (min) | Mobile phase A (%) v/v | Mobile phase B (%) v/v |
|-------------------|------------|------------------------|------------------------|
| Bleomycin sulfate | 0          | 100                    | 0                      |
|                   | 10         | 70                     | 30                     |
|                   | 12.50      | 70                     | 30                     |
|                   | 14         | 100                    | 0                      |
|                   | 20         | 100                    | 0                      |
| Tyrothricin       | 0          | 60                     | 40                     |
|                   | 10.50      | 10                     | 90                     |
|                   | 11.86      | 10                     | 90                     |
|                   | 12.50      | 60                     | 40                     |
|                   | 16.80      | 60                     | 40                     |
| Vancomycin HCl    | 0          | 100                    | 0                      |
|                   | 10         | 90                     | 10                     |
|                   | 20         | 70                     | 30                     |
|                   | 22         | 100                    | 0                      |
|                   | 30         | 100                    | 0                      |
| Bacitracin        | 0          | 95                     | 5                      |
|                   | 10.50      | 50                     | 50                     |
|                   | 11.86      | 50                     | 50                     |
|                   | 12.60      | 95                     | 95                     |
|                   | 16.80      | 95                     | 95                     |
2.3. Sample preparation

Peptide samples were analyzed using HPLC-UV method derived from European Pharmacopoeia (Ph. Eur.), as well as by newly developed UPLC-UV/MS method [11–14]. As the latter also comprised MS detection, peptide sample solutions were prepared by replacing the buffer components with H₂O, whilst maintaining the same target concentrations as stipulated in the Ph. Eur. Details regarding peptide concentrations and solvents used for the samples are given in Table 1.

2.4. Chromatography

The HPLC apparatus consisted of a Waters Alliance 2695 separations module and a Waters 2996 Photodiode Array (PDA) detector with Empower 2 software for data acquisition (all Waters, Milford, MA, USA). Chromatographic analysis of the four selected peptides was based upon the methods as described in the Ph. Eur. [11–14]. Tyrothricin, vancomycin HCl and bleomycin sulfate were analyzed with a Lichropher 100 RP-18 (250 mm × 4 mm, 5 μm) (Merck, Overijse, Belgium), whereas bacitracin was analyzed with a Hypersil ODS (250 mm × 4 mm, 5 μm) (Thermo Scientific, Erembodegem-Aalst, Belgium). A brief overview of the chromatographic conditions is given in Table 2.

The UPLC equipment consisted of a Waters UPLC Quaternary Solvent Manager, a Waters ACQUITY Sample Manager, a Waters Ultra Performance LC (UPLC) PDA, a Waters ACQUITY Isocratic Solvent Manager and a Waters ACQUITY QDa detector (QDa) which is a compact single quad mass detector equipped with an electrospray ionization (ESI) interface. For data acquisition and instrument control, the Empower 3 FR 2 software was used. The ACQUITY UPLC CSH C18 column (100 mm × 2.1 mm, 1.7 μm) (Waters, Zellik, Belgium) was maintained at 40 °C [15]. Mobile phase A consisted of a 95/5 H₂O/ACN+0.1% TFA, whilst mobile phase B consisted of a 5/95 composition of the same solvents. Different gradient methods were used for peptide analysis (Table 3). The flow rate was set at 0.5 mL/min and the injection volume was 2 μL. A post-column 10/1 PDA/QDa split ratio was employed, together with a post-column addition of 40/10/50 H₂O/propionic acid/2-propanol at a flow rate of 0.35 mL/min to the portion going to the QDa. This post-column addition neutralized the TFA ion suppression effect [16] and sustained a sufficient flow rate to the QDa detector. The QDa was operated in an electrospray positive ion mode by applying a voltage of 0.8 kV to the ESI capillary and the
cone voltage was set at 15 V. The desolvation temperature was set at 600 °C. A full mass spectrum between m/z 100 and 1250 was acquired at a sampling rate of 2.0 points/sec.

The HPLC and UPLC methods were compared based upon (i) the system suitability tests (SST), i.e., resolution, peak to valley ratio (p/v), signal-to-noise ratio (S/N) and symmetry factor (Aₜₓ), listed in the Ph. Eur.; (ii) the quality limits of the active pharmaceutical ingredient (API); (iii) the limit of detection (LOD) based on S/N ratio; (iv) the number of peaks detected above reporting threshold (reported peaks); (v) the number of peaks (tentatively) identified; and (vi) the total run time.

3. Results

Chromatograms obtained from HPLC-UV and UPLC-UV/MS analysis of bleomycin sulfate are given in Fig. 1. Using the HPLC-UV method, bleomycin A₂ and B₂ were identified by their high normalized peak areas (62.79% and 34.05%) [11]. Impurity D (demethylbleomycin A₂) was identified by its relative retention time (RRT) to bleomycin A₂, i.e., 1.5 to 2.5. Although this HPLC-UV method adheres to the individual monograph SST (resolution between bleomycin A₂ and B₂ is higher than 5; peak width at half height), significant tailing was observed, i.e., above the maximal value of 1.5 as given in the Ph. Eur. 2.2.46, and the amount of bleomycin B₂ surpassed the limit stipulated in the Ph. Eur. This is an example of how an averagely performing HPLC-UV QC method, combined with inadequate integration, can result in a false low quality conclusion. Using the UPLC-UV/MS method, which resulted in a better chromatographic separation of all compounds as evidenced by the reduction in tailing of the major bleomycin compounds and increased resolution with the present impurities, the amount of bleomycin B₂ was determined to be 29.0%, thus complying with the Ph. Eur. limit. Moreover, using MS detection, the identity of bleomycin A₂ (m/z 708.08 and 472.44; z = 2, 3) and B₂ (m/z 713.52 and 476.06; z = 2, 3) was confirmed. Furthermore, two additional Ph. Eur.-listed impurities were identified by their mass spectra, i.e., bleomycinic acid (m/z 657.34; z = 2) and bleomycin A₅ (m/z 720.42 and 480.73; z = 2, 3). The identity of demethylbleomycin A₂ (impurity D) was also confirmed by MS (m/z 701.04; z = 2). Finally, it was noted that the total run time of the UPLC-UV/MS method was reduced to 20 min, whereas the run time of HPLC-UV was 100 min.

Fig. 2. Tyrothricin (1 mg/mL) HPLC-UV (A) and UPLC-UV/MS (B) analysis with identification of major compounds and method comparison. Exemplary MS¹ spectra of main compounds tyrocidin C and gramicidin C₁ are given.
Chromatograms obtained from HPLC-UV and UPLC-UV/MS analysis of tyrothricin are given in Fig. 2. Due to the complex nature of the tyrothricin sample that contains different tyrocidins and gramicidins, formal identification was not possible with the HPLC-UV method without an additional analysis for retention time matching to reference standards. Moreover, in the absence of certified reference materials, calculation of the SST (peak to valley ratio between gramicidin A1 and A2) could not be performed to verify whether the method was Ph. Eur. compliant. Alternatively, using the UPLC-UV/MS method, tyrocidins A-D (m/z: 636.01, 655.67, 675.11 and 686.47, respectively; z = 2) and gramicidin A1, A2 and C1 (m/z: 941.65, 948.76 and 930.22, respectively; z = 2) were identified based upon their MS spectra and the presence of K⁺ adducts.

Chromatograms obtained from HPLC-UV and UPLC-UV/MS analysis of vancomycin HCl are given in Fig. 3. Identification of vancomycin B using the traditional HPLC-UV method was done by its high relative peak area, whereas aglucovancomycin B (Ph. Eur. impurity C) was identified based upon its relative retention time to vancomycin B [17]. Analysis of the same sample using the UPLC-UV/MS method improved the resolution, resulting in the separation of more impurity peaks. Moreover, using the MS detection, the majority of these impurity peaks could be tentatively identified based upon their MS² spectra and the work was performed by Diana et al. (Table 4) [17]. The mass spectra of two vancomycin-related impurities, which were tentatively identified as a demethylated impurity (peak 3) and aglucovancomycin B (peak 13), are given in Fig. 3. Even though their individual impurity amount, relative to the main compound vancomycin B, was well below 1%, i.e., 0.1% for peak 3 and 0.3% for peak 13, these mass spectra could be used for immediate identification purposes.

A similar observation was made for analysis of bacitracin by UPLC-UV/MS. Confirmation of the identity of the main compound, as well as tentative identification of major impurity peaks was done with reference to the previous MS research performed by Govaerts et al. [18]. The mass spectra of the main compound bacitracin A, as well as of two small impurities, i.e., bacitracin E (1.5% relative to bacitracin A) and bacitracin H (1.7% relative to bacitracin A), are given in Fig. 4 and were positively used for identification purposes. The total run time of the UPLC-UV/MS method was also drastically reduced compared with the HPLC method, i.e., 16.8 min vs. 75 min.

A comparison between the performances of the traditional HPLC-UV and new UPLC-UV/MS method for the QC analysis of bleomycin sulfate, vancomycin HCl and bacitracin is given in Fig. 5.
As tyrothricin components could not be identified using the HPLC-UV method, performance comparison could not be made.

4. Discussion

The advantages of sub-2 μm chromatography (UPLC) over traditional HPLC chromatography for analysis of pharmaceutical compounds have already been extensively documented [19–23]. The use of smaller particle size with an optimized LC system gives improved efficiency, resulting in faster and higher resolution separations than those of traditional LC systems with column particle sizes in the 3–10 μm range. For the transfer of a method from traditional HPLC to UPLC (and vice versa), guidance about the adjustment of column size, injection volume, flow rate and time program for gradient elution are available [24]. However, this was not the main objective of this study. The goal of this study was to inspire pharmaceutical analysts to implement new technologies in method development. Currently, most LC-based quality control methods for APIs listed in the Ph. Eur. generally still utilize stationary phases with a particle size of 3–10 μm. Small variations in particle size are permitted for isocratic methods, i.e., reduction of 50% in particle size, whereas no alteration in particle size is allowed with gradient elutions [25]. As a result, the use of UPLC

Table 4
Tentatively identified vancomycin HCl related impurities.

| # | RT (min) | m/z        | Identification                                           |
|---|----------|------------|---------------------------------------------------------|
| 1 | 1.134    | 661.24     | Oxidized desvancosaminylvancomycin, Demethyleucylvancomycin B |
| 2 | 5.028    | 734.78     | Crystalline degradation product (demethylated), Deamidated vancomycin B (demethylated), N-demethylvancomycin B |
| 3 | 5.095    | 718.40     | Crystalline degradation product (demethylated), Deamidated vancomycin B (demethylated) |
| 4 | 5.209    | 725.51     | Oxidized vancomycin B (demethylated), Crystalline degradation product major, Crystalline degradation product minor |
| 5 | 5.318    | 717.77     | Crystalline degradation product (demethylated), Deamidated vancomycin B (demethylated), N-demethylvancomycin B |
| 6 | 5.950    | 718.47     | Crystalline degradation product (demethylated), Deamidated vancomycin B (demethylated) |
| 7 | 6.009    | 724.95     | Crystalline degradation product major, Crystalline degradation product minor |
| 8 | 6.098    | 725.51     | Oxidized vancomycin B (demethylated) |
| 9 | 6.302    | 724.95     | Crystalline degradation product major, Crystalline degradation product minor |
| 10| 6.369    | 725.44     | Vancomycin B |
| 11| 6.518    | 725.92     | Oxidized vancomycin B (demethylated) |
| 12| 6.707    | 725.58     | Oxidized vancomycin B (demethylated) |
| 13| 12.694   | 1143.48    | Aglucovancomycin B |

Fig. 4. HPLC-UV (A) and UPLC-UV/MS (B) analysis of bacitracin (2 mg/mL) with identification of major compounds and method comparison. The MS² spectra of a main compound, bacitracin A, and two exemplary impurity peaks, are given.
converting existing API quality control methods in Ph. Eur. into faster and more efficient sub-2 μm methods is currently not allowed without validation. Hence, simple transferring HPLC–UPLC methods in pharmaceutical QC method validation is currently not allowed. Moreover, it is emphasized that when a method is changed, the corresponding acceptance limits are also to be reconsidered [26]. However, newly developed quality control methods have already used this sub-2 μm stationary phase as evidenced by the related substance methods for quetiapine fumarate and nevirapine hemihydrate [27,28]. Moreover, the revised Ph. Eur. 8.3 general chapter 2.2.29 liquid chromatography covers sub-2 μm particle technology [29].

Although MS is listed as an analytical technique in the Ph. Eur. [30], its use in the quality control of APIs is currently limited to a few cases, e.g. detection of impurity B of oseltamivir phosphate, detection of impurity F of imatinib mesilate, and determination of the interferon β-1a isoform distribution [31–33]. However, as the newly developed single quad MS detector is smaller, cheaper and easily used, it is expected that its application will increase exponentially. Coupled to UPLC separation module, it adequately answers the current shift in QC emphasis from API assay towards impurity profiling [34,35].

This study has demonstrated the applicability of identifying related impurities and/or multiple active components in complex API samples using a single quad MS detector. The current equipment set-up, using a post-column split, coupled with post-column addition of a propionic acid containing solvent, even permits the use of TFA to improve chromatographic peak shape [36], without compromising the MS detection too much. As liquid chromatographic methods with volatile buffers often show inferior chromatographic separation compared with analogous methods with non-volatile buffers [37], the use of the anionic ion-pairing reagent TFA for peptide separation is important to counterbalance the loss in chromatographic performance. As expected, resolution and LOD were improved by the use of sub-2 μm column technology and the overall run time was significantly reduced, when compared with traditional 3–10 μm HPLC methods listed.

5. Conclusion

A newly developed, single quad MS detector was coupled to a UPLC separation module and used in routine quality control analysis of bleomycin sulfate, tyrothricin, vancomycin HCl and bacitracin peptide APIs. The results were compared with the results obtained by traditional HPLC-UV methods which were based upon the Ph. Eur. As expected, the UPLC separation resulted in a higher resolution and a lower limit of detection, as well as a significant reduction in run time. Furthermore, MS detector may enable to directly identify impurities or components even at low levels without the need of reference standards. Currently, MS is only sparsely applied in the Ph. Eur. However, recent technical advances deliver fit for using single quad MS detectors to the pharmaceutical field, and its applications in QC analysis are expected to rise in the near future.

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Fig. 5. Relative performance comparison between UPLC-UV/MS and HPLC-UV method for bleomycin sulfate, vancomycin HCl and bacitracin QC analysis. HPLC-UV performance was set at 100%.
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