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Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 707–714

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

The use of hyphenated LC–MS technique for characterisation of impurity profiles during drug development

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Received 15 May 1998; received in revised form 28 August 1998; accepted 13 September 1998

Abstract

As part of an integrated quality concept for impurities during drug development, the multidimensional evaluation of impurity profiles by LC–MS coupling is presented using peptide drugs as an example. This quality concept can be regarded as an adaptation of the ICH-requirements to the special situation during the drug development process. The primary goal is to obtain qualitative molecular weight information for impurity peaks detected at the same UV wavelength as for the impurity test procedure. The approach is focused on the investigation, if the impurities in a clinical batch were also present in the toxicologically qualified batch(es). Depending on the relevance of individual impurities in further batches or as degradation products, the molecular weight can be used as a starting point for further characterization and identification. Often, eluents with volatile buffers required for MS result in different selectivities and/or inferior chromatographic separation and sensitivity compared with nonvolatile buffers (e.g. phosphates). In these cases, peak 'tracking' especially for small peaks can become critical. A procedure is presented for on-line coupling of LC methods with non-volatile eluents to mass spectrometry.

Keywords: Impurity profile; Impurity qualification; LC–MS; Multidimensional; On-line coupling; Quality control

1. Introduction

Over the last decade, the 'International Conference on the Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use' (ICH) has achieved a great deal towards a worldwide standardisation of the registration process. The ICH guidelines, which are being gradually implemented, can also serve as a framework and basis of orientation for areas which are not covered such as the drug development process.

The presence of impurities in pharmaceuticals can have a significant effect on their quality and safety. Therefore, the testing for and evaluation of

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0731-7085/98/$ - see front matter
PII: S0731-7085(98)00267-2

impurities have been important topics of the ICH process [1–4]. In the guidelines, thresholds are given for the reporting, qualification and identification of impurities. At the time of submission of the registration dossier, unknown impurities must be limited to 0.1% and known impurities to the amount present in the bio-batches, i.e. batches which are used in toxicological and clinical studies [1].

The thresholds of the ICH guidelines cannot be applied in the early stages of drug development due to a normally higher impurity level, the limited experience and information as well as a larger variability in the synthetic process, especially during the scale-up. Here, as part of an integrated quality concept [5] the crucial aspect for impurities is qualification [6]. Once an impurity profile is qualified by the first toxicological studies, attention is focused on the comparison of qualified impurities with those in new batches. This requires a comprehensive analytical monitoring of the impurity profiles. The addition of further dimensions to chromatographic separations by hyphenated techniques offers new possibilities.

MS and LC–MS are widely used for impurity identification [7] and for quantification of drugs and metabolites in biological fluids [8]. However, only few examples [9,10], are published with regard to a systematic, multidimensional evaluation of impurity profiles during drug development or drug synthesis. The growing importance of demonstrating the consistency of impurity profiles of the batches during the clinical development in order to maintain their toxicologically qualified status implies a systematic approach [5,6].

The use of LC–MS coupling for such an 'impurity inventory' during the quality control in the development will be discussed using the example of peptide drugs.

2. Experimental

2.1. Materials

The LHRH antagonist ramorelix (INN) is a synthetic glycosylated decapeptide (acetyl-[D-3(2-naphthyl) - alanyl - D - *p* - chlorophenylalanyl - D - tryptophyl-L-seryl-L-tyrosyl-*O*-à-L-rhamnosyl-D-seryl-L-leucyl-L-arginyl-L-proline semicarbazide, monoisotopic M_r 1530.7).

HMR xx is a synthetic tripeptide composed of non-proteinogenic amino acids (monoisotopic M_r 550.3). Both substances were synthesized at Hoechst Marion Roussel, Germany.

HPLC-grade acetonitrile (Lichrosolv™) and spectroscopy-grade trifluoroacetic acid (Uvasol™) were obtained from Merck, Darmstadt, Germany. Water was purified with a Milli-Q system from Millipore (Milford). All other reagents were at least of analytical grade.

2.2. Apparatus

A HP 1090 liquid chromatograph (Hewlett Packard, Palo Alto) was equipped with a SP-6 variable wavelength UV detector (Gynkotek, Germering, Germany).

For mass spectrometric investigations, an LCQ™ ion trap instrument with an electrospray ion source (Finnigan MAT, San Jose) was used.

2.3. LC–MS-method for ramorelix

The chromatographic separation was performed on a 250 × 4 mm LiChrospher™ PAH column, 5 μm particle size (Merck, Darmstadt, Germany, Cat. no. 1.50149) using an initial mixture of the components A and B of 68 and 32%, respectively, at a flow rate of 0.5 ml min^{-1}. A and B are composed of acetonitrile–water–trifluoroacetic acid (100:900:2, v/v/v) and acetonitrile-water-trifluoroacetic acid (900:100:1.5, v/v/v), respectively. After 35 min of isocratic elution, a gradient was applied with 2% B min^{-1} for 20 min. The UV detection wavelength is set to 230 nm because of an optimized signal-to-noise ratio (instead of the relative maximum wavelength of 223 nm). The column was adjusted to 30°C. 25 μl of a sample solution of about 1.5 mg peptide ml^{-1} water were injected. The following mass spectrometric conditions were used: capillary temperature 200°C, source voltage 5.2 kV, full scan 50–2000 u, positive ion mode.

2.4. On-line coupling of the phosphate LC-method for HMR xx to MS

The chromatographic separation was performed on a 250 × 4 mm Superspher™ 60 RP-select B column (Merck, Darmstadt, Germany, Cat. no. 1.50973) using the components A and B, composed of acetonitrile–water–orthophosphoric acid 85% (50:950:14, v/v/v) and acetonitrile–water–orthophosphoric acid 85% (400:600:14, v/v/v), respectively. The two eluents were adjusted to an apparent pH of 4.0 with 6N aqueous NaOH. After 15 min of isocratic elution at a flow rate of 1.0 ml min^{-1} with a mixture of 93% A and 7% B, a two-stage linear gradient was applied resulting in 29% B at 35 min and 100% B at 60 min. The UV detection wavelength is set to 240 nm. The column was adjusted to 30°C. A volume of 50 μl of a sample solution of about 0.5 mg peptide ml^{-1} water was injected.

A switching valve (Model 7010, Rheodyne, USA) is placed behind the UV-detector (Fig. 1).

Normally, the flow of the phosphate system is directed to the waste. When peaks of interest are eluted, fractions of 0.05–0.1 ml are switched into the second LC-system which is operated with volatile eluents. Mobile phase mixtures of acetonitrile–water–trifluoroacetic acid (130:870:1, v/v/v) and (230:770:1, v/v/v) were used for rechromatography of fractions of impurity peaks eluted before and after 40 min in the phosphate method, respectively. A Model 510 pump (Waters, Eschborn, Germany) was used to provide a flow rate of 1.0 ml min^{-1}. The separation of the buffer salts from the analyte was perfomed on a CC Lichrospher 60-5 select B, 125 × 2.0 mm (Macherey-Nagel, Düren, Germany). After 0.8 min, which was proved to be sufficient for the elution of the phosphate, the flow is directed by the second switching valve (which is incorporated in the LCQ) into the mass spectrometer. The overall run time was 4 min. The following mass spectrometric conditions were used: capillary temperature 200°C, source voltage 5.0 kV, full scan 50–1000 u positive ion mode.

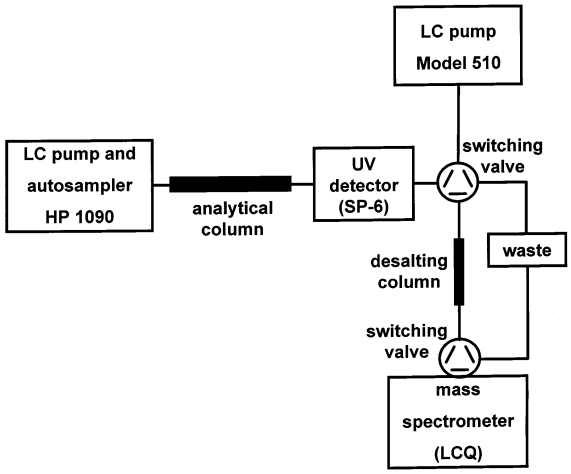

Fig. 1. Schematic coupling of a LC method using non-volatile eluents with mass spectrometry. Horizontally, non-volatile eluent system; vertically, volatile eluent system.

3. Results and discussion

LC-methods with UV-detection have gained a widespread acceptance for quality control of impurities in pharmaceuticals due to their robustness and high sensitivity. Therefore, the impurity profiling must also be based on the UV chromatogram. The primary goal for the LC–MS coupling is to obtain qualitative molecular weight information for impurity peaks detected at the same wavelength as for the impurity test procedure.

For the multidimensional evaluation of the impurity profiles of the peptide drug ramorelix, mass spectra are taken at the position of impurity peaks in the UV chromatogram. The assignment is confirmed by extracting the ion chromatogram of the relevant mass-to-charge ratio (m/z) and comparing it with the UV trace (Fig. 2). This is performed for all relevant UV impurity peaks, adding the molecular weights (or m/z) as a further dimension to the UV-chromatogram. The batch used in toxicological trials serves as a standard with which all other batches must be compared (Fig. 3). The procedure focuses primarily on a qualitative assignment of molecular weights to UV area% peaks to check, if a certain impurity in a batch for clinical trials was also present in the toxicologically qualified batch. Of course, also

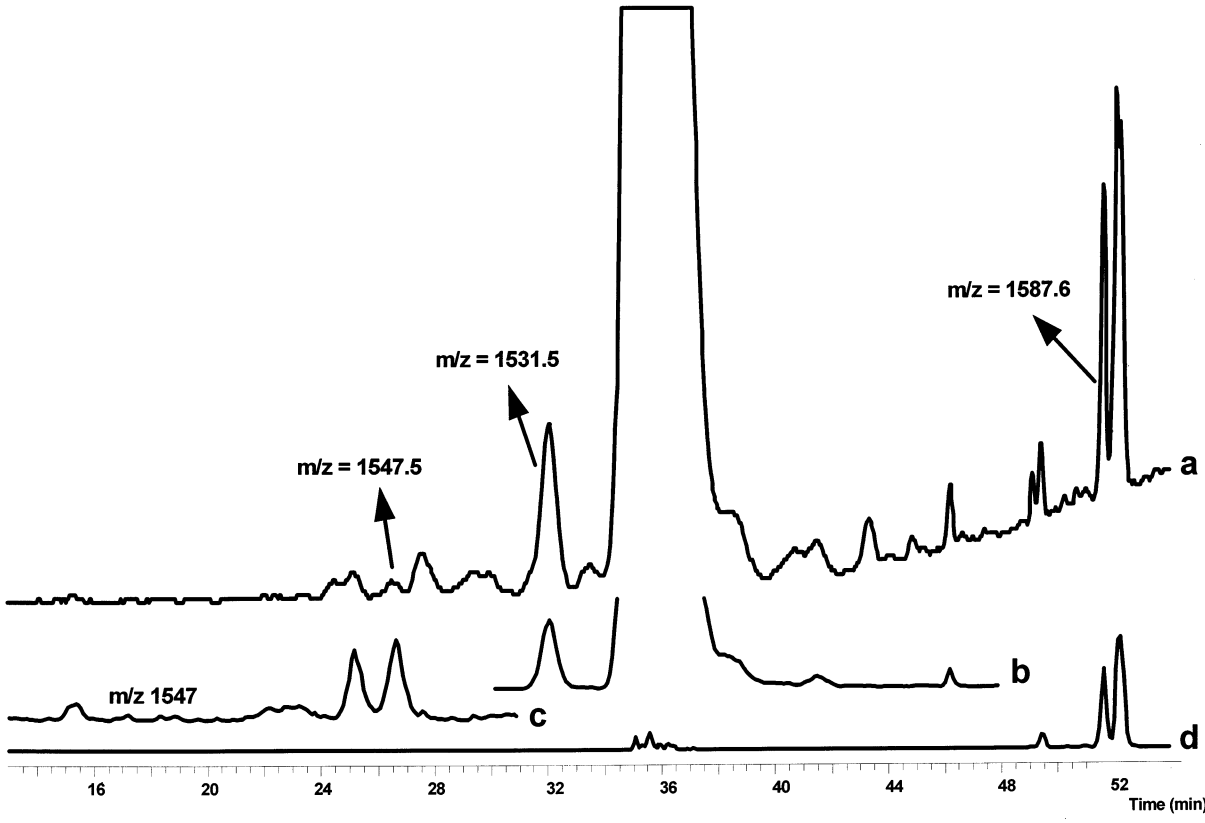

Fig. 2. Assignment of molecular weights to ramorelix impurity peaks detected in UV. (a) RP-chromatogram at a detection wavelength of 230 nm. Mass spectra are taken at the position of impurity peaks in the UV chromatogram, shown representatively for m/z 1531, 1547, and 1587. (b) (c) and (d) extracted ion chromatograms shown for representative impurities with m/z 1531, 1547, and 1587, respectively.

first conclusions can be drawn from the molecular weights of the impurities. For example, with respect to synthetic peptides, impurities with the same molecular weight as the drug substance itself can be assumed to represent diastereomers, i.e. a peptide sequence with one of the amino acids in the opposite enantiomeric form. For ramorelix, four of such isomers can be detected. The impurities with m/z 1547 and 1563 may be formed by oxidation (M + 16) and (M + 32), respectively.

If the (UV) amount is larger than the qualified level, it must be discussed, if there are any concerns with regard to safety taking into consideration safety factors, dosage, administration, etc. This is especially important for batches to be used in clinical trials and batches from the scale-up in order to maintain a state of constant impurity qualification. In batch B (Fig. 3), for example, a new isomeric impurity appeared indicating a diastereomeric peptide. Compared to the toxicologically qualified batch A, the total amount of impurities is lower. Furthermore, the administered dose in the clinical study (Phase I) was very low. Therefore, the new impurity in batch B should not present a safety risk.

The impurity inventory could be further supported by obtaining not only the molecular weight but also the fragmentation pattern. Based on the molecular weight information and the relevance of the impurities (with respect to amount and consistency), approaches can be made towards a structural identification.

If impurities are only detected with mass spectrometry, comparison should strictly be per-

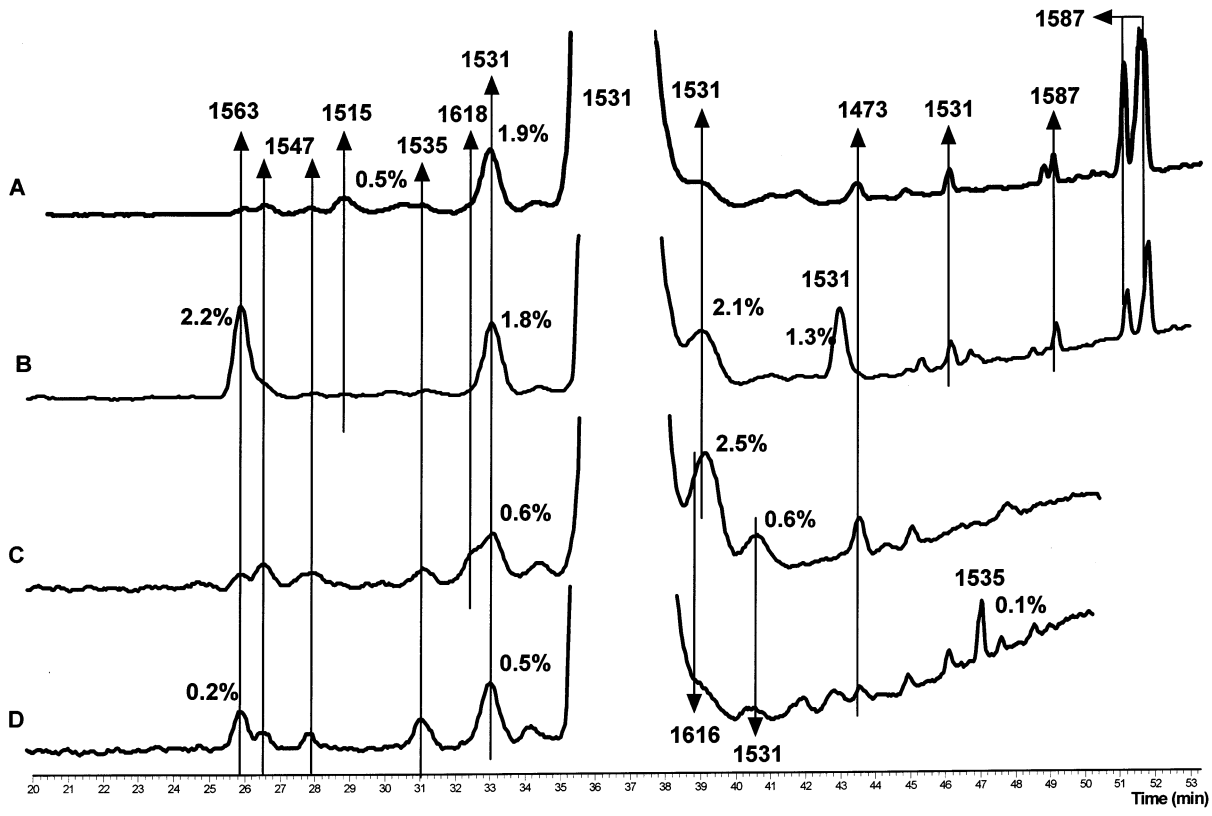

Fig. 3. Comparison of impurity profiles for various batches of ramorelix used in toxicological studies (A), clinical studies (B and C), and from scale-up (D). The chromatograms at a detection wavelength of 230 nm are shown. Mass-to-charge ratio for relevant peaks and area percent (UV, 230 nm) for some peaks are indicated. Crossings of the vertical lines with chromatograms indicate the presence of impurities with the m/z values shown above (or below) the arrows.

formed on a qualitative basis. As long as the structure is unknown, the magnitude of the response is difficult to estimate (and therefore the significance of this impurity) and careful interpretation is required.

For LC–MS coupling, eluents with volatile buffers such as trifluoroacetic acid, ammonium acetate and formic acid are required. In many cases, eluents with nonvolatile buffers (e.g. phosphate) result in different selectivities and/or superior chromatographic separation and sensitivity compared with volatile eluents. Therefore, if the two systems are used in parallel, peak'tracking' is required. This can be performed by sampling the peak fractions in the non-volatile LC-method and rechromatography using MS compatible elution conditions, if required after sample concentration. However, this procedure may be susceptible to sample artifacts such as adsorption, degradation, etc.

An on-line coupling of the impurity control test procedure for HMR xx (using phosphate buffer) to the mass spectrometer was performed (Fig. 4). The UV-chromatogram of a sample batch is shown in Fig. 4a. The procedure is demonstrated for an impurity with a retention time of 20.2 min. The total ion current for a rechromatographed peak fraction of about 0.05 ml (switching time about 3 s) is shown in Fig. 4b (trace a). After 0.8 min, the flow is directed into the mass spectrometer (indicated by an arrow). The void volume of the column was about 0.4 ml. After 0.8 ml, the amount of phosphate ions further eluted was determined in previous investigations to be de-

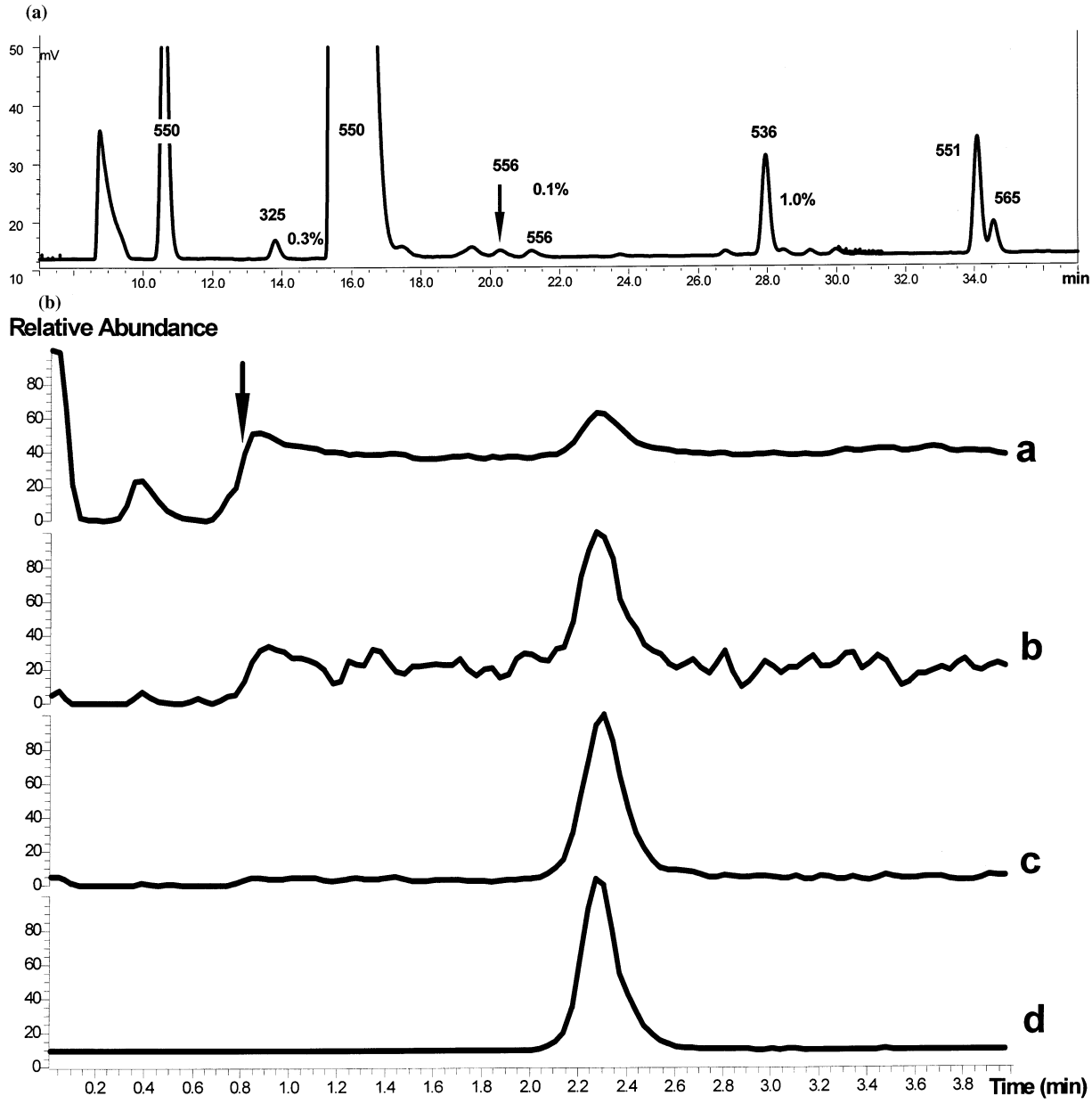

Fig. 4. On-line investigation of peak fractions from an LC method using 0.1 M phosphate buffer by direct coupling to mass spectrometry. (a) RP-chromatogram of a HMR xx batch at a detection wavelength of 240 nm. The molecular weights (m/z of the singly charged molecular ions) of all impurities obtained are indicated above the peaks. Some peaks are also labelled with their area percent (at 240 nm). The peak fraction, the further results of which are given in 4b and 4c, is marked by an arrow. (b) Ion chromatograms for a rechromatographed peak fraction from about 20.3 to 20.35 min of the run represented in 4a. (a) total ion current; (b), (c) and (d) extracted ion chromatograms at m/z 556.4, 278.9, and 298.5. (c) Mass spectrum taken from the total ion current chromatogram shown in 4b. The spectrum was obtained by averaging six scans (2.21–2.37 min) using a 2-fold baseline substraction from 1.84 to 2.14 min and from 2.54 to 2.84. Because of the highlighting of the main ion signals, their isotope peaks can not be distinguished.

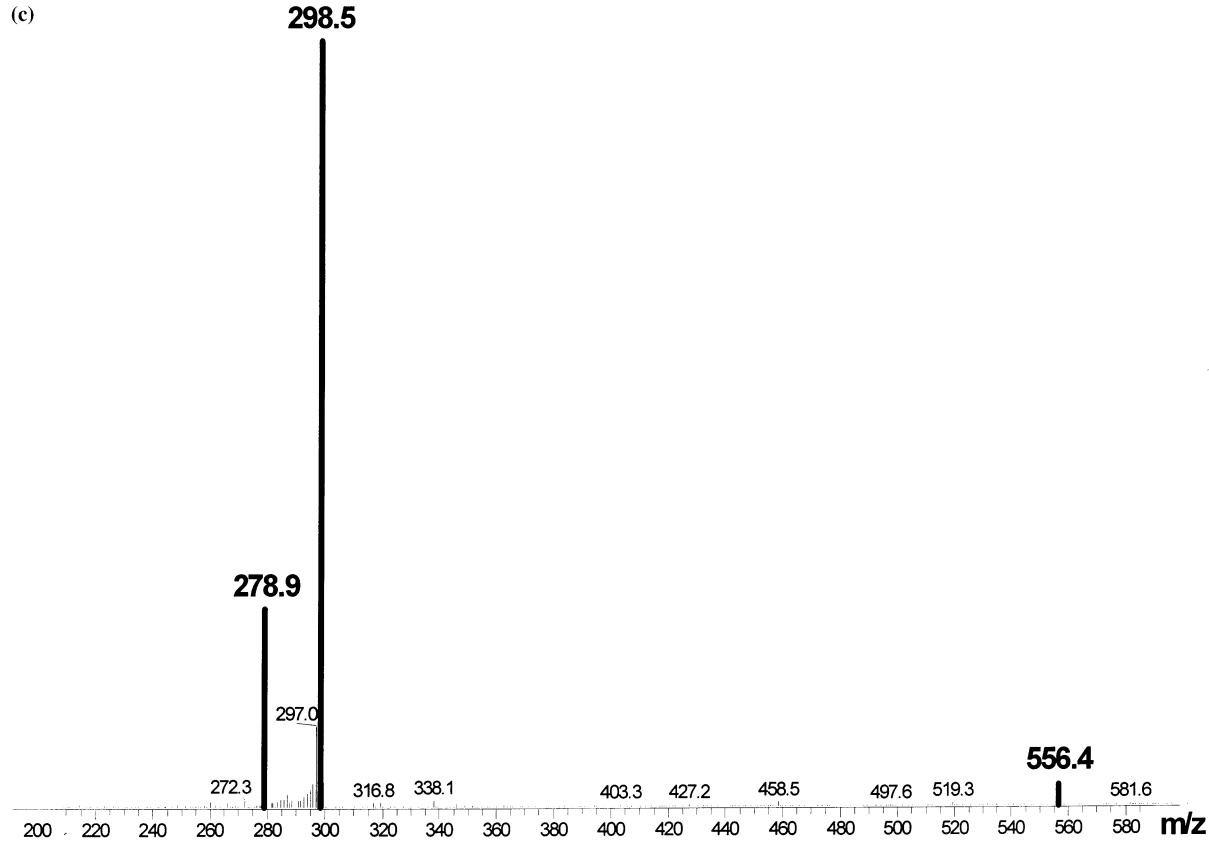

Fig. 4. (*Continued*)

creased below a tolerable level. The mass spectrum is obtained from the total ion current chromatogram (Fig. 4c) and the corresponding ion chromatograms are extracted (Fig. 4b, traces b–d). The identical retention times of the three m/z 278.9, 298.5, and 556.4 indicate that all ions belong to the same molecule. This was later confirmed by a direct LC–MS method with improved resolution. Using the zoom scan mode, the m/z 278.9, 298.5, and 556.4 could be assigned to the doubly charged molecular ion, a cluster of the doubly charged ion with acetonitrile, and the singly charged molecular ion, respectively (data not shown). After 4 min, the desalting system is ready for the next impurity fraction. Four chromatographic runs were needed for the presented example of HMR xx to obtain molecular weights (m/z) for all impurity peaks of interest. Even impurities in the range of 0.1 UV-area% are accessible (Fig. 4a).

Following the assignment of molecular weights (m/z), the LC-method with non-volatile eluents can then be compared with a direct LC–MS-method as described for ramorelix. Alternatively, the on-line coupling can also be applied routinely if no adequate separation is possible with volatile eluents. Such a system can also be extended to a true orthogonal coupling of two chromatographic separations, e.g. a combination of size exclusion chromatography or ion chromatography with reversed-phase (RP) chromatogrography and subsequent MS detection [11].

The on-line coupling has the advantage of a direct transfer of a known peak fraction to MS investigation. On the other hand, the on-line coupling requires a suitable adjustment of the elution conditions in the volatile system, especially with respect to the content of organic modifier. The

latter must not be significantly lower than its content in the sample from the non-volatile separation. Therefore, the selection of the desalting RP-column (stationary phase, column dimensions) becomes an important optimisation parameter.

4. Conclusions

The development of analytical equipment and techniques, especially in the last two decades, offers tremendous possibilities. Techniques which a few years ago were only suitable for specific research applications (and experts) have been developed to routine use such as the described coupling of chromatography with mass spectrometry. These are exciting developments but such a tremendous increase in the degrees of freedom requires a careful focus on information which is relevant to ensure the quality and safety of pharmaceuticals.

A formalised, structured approach may facilitate an efficient drug development process. Such a quality concept can be focused on (toxicologically) qualified impurity profiles by means of initial toxicological studies and subsequent comparison of impurity profiles. Hyphenated LC–MS techniques can be used as part of this comprehensive characterization of impurity profiles.

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