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Use of collision induced dissociation mass spectrometry as a rapid technique for the identification of pharmacologically active peptides in pharmacopoeial testing

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ABSTRACT

The applicability of collision induced dissociation mass spectrometry (CID-MS) for the pharmacopoeial identification of pharmacologically active peptides was examined. Two different classes of related peptides were selected, i.e. four synthetic gonadotropin releasing hormone analogues (gonadorelin, goserelin, buserelin and leuprorelin) being either nona- or decapeptides, and human insulin and 2 insulin analogues (insulin lispro and insulin aspart). For all these substances the pharmacopoeial identification currently requires a combination of several partly rather laborious tests using sophisticated equipment. In contrast, CID-MS as a stand alone test can provide increased reassurance about the identity and is rapid and efficient. Moreover, the substance consumption for testing is significantly lower, which is a non-negligible factor for very expensive substances.

1. Introduction

Compliance with the requirements of the monographs of a Pharmacopoeia is a key criterion for the use of active pharmaceutical ingredients all over the world. In Europe, the European Pharmacopoeia [1] (Ph.Eur.) defines the minimum quality standards for human and veterinary medicines. It is obligatory in all 36 member states of the European Pharmacopoeia Convention and was also made legally mandatory by EU-legislation [2,3].

Amongst other elements, a European Pharmacopoeia monograph usually consists of an identification and a test section as well as an assay determination, each describing the analytical methods to be used as well as the corresponding specifications. Whilst the test section is intended to control the purity of a pharmaceutical substance or material by state-of-the-art methods, the assay determination allows the verification of the content or potency. With regard to the identification section of a monograph, it is important to note that the scope of the tests described is not the structure elucidation of an unknown substance or the determination of the composition of an unknown mixture. The aim of identifying a material should also not be mixed up with the assessment of its purity or the determination of its strength, which is the subject of the further parts of a monograph. Rather, the series of physical and/or chemical identification tests ensures, as far as possible, the specificity to an extent that substances exhibiting similar structures are distinguished [4,5]. In the majority of cases the identification of a drug substance is relatively straightforward and can easily be achieved by simple techniques like infrared spectroscopy, either on its own or in combination with further tests, e.g. identification reaction for the counter-ion of the active ingredient or an optical rotation test.

Unfortunately this is not always the case and it may happen for some substances that laborious tests or sophisticated equipment need to be described for the identification test. Furthermore it is important to note that some substances, e.g. synthetic peptides, are very expensive and should preferably be used for the production of medicines than for quality control testing. Therefore, the reduction of the amount of substance to be invested in a test should also be reduced as much as reasonably possible.

1.1. Gonadotropin releasing hormone analogues

The European Pharmacopoeia contains monographs for the synthetic nonapeptides goserelin [6], buserelin [7] and leuprorelin [8] and for the decapeptide gonadorelin acetate [9]. In the United States Pharmacopeoia [10] (USP) monographs for gonadorelin acetate and leuprorelin acetate are described. These peptides belong to the group of gonadotropin releasing hormone (GnRH) agonists and are used in various applications in gynaecology, reproductive medicine, and oncology [11]. As presented in Fig. 1, the 4 substances are structurally closely related. Differences are found in the amino

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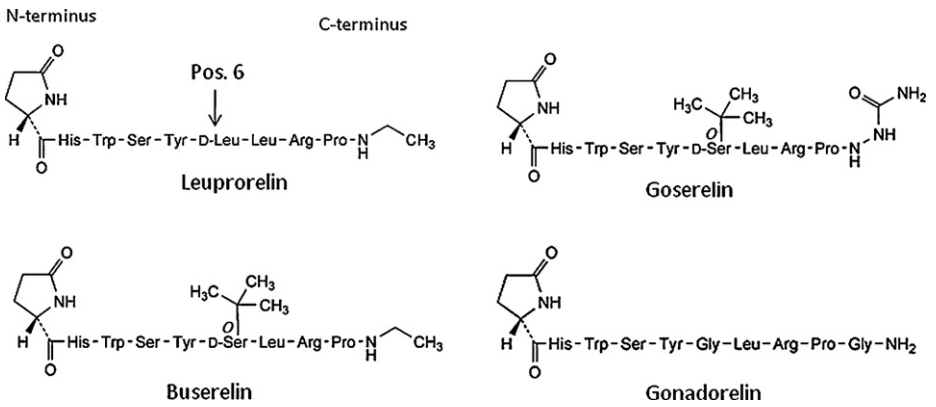

Fig. 1. Structures of gonadorelin, goserelin, buserelin and leuprorelin.

acid (AA) in position 6 as well as in the AA on the C-terminal side of the peptides.

As already pointed out by Vergote et al. [12], the situation regarding the identification of these peptides in the pharmacopoeial monographs is not consistent. For buserelin and goserelin, the Ph.Eur. prescribes a combination of three different tests, i.e. a liquid chromatography (LC), a nuclear magnetic resonance spectroscopy (NMR) and an amino acid analysis (AAA) for identification purposes. Interestingly in the monograph of buserelin ^{1}H NMR is given whilst the technique to be applied for goserelin is ^{13}C NMR. For leuprorelin a combination of infrared spectroscopy (IR), LC and AAA are prescribed in the Ph.Eur. whereas the USP requires identification by IR and LC only. According to Ph.Eur. gonadorelin acetate is identified by a combination of thin layer chromatography (TLC) and LC whilst USP employs a combination of mass spectrometric determination of the molecular mass and LC. Considering the limitations of the above techniques—at least in the way they are currently described, none of them can be considered sufficiently specific to provide reliable information about the AA sequence and therefore the identity. Moreover, the requirement to perform several tests in combination is very demanding in terms of time, equipment and amount of analyte to be used.

1.2. Insulin

Human insulin is a 2-chain antidiabetic peptide hormone produced by the human pancreas. It has a molecular mass of 5808 u and consists of 51 amino acids [13] organized in 2 chains, an A-chain with 21 amino acids (AAs) and a B-chain with 30 AAs both linked via 2 cysteine (Cys) disulfide bridges. Human insulin is nowadays mainly manufactured biosynthetically using recombinant DNA technology. Moreover, several slightly modified versions of human insulin, such as insulin lispro have been developed exhibiting different absorption or duration of action characteristics [14–16]. The structurally related insulin lispro differs from human insulin in a sequence switch of two beta-chain AAs, i.e. reversal of B-28 proline (Pro) and B-29 lysine (Lys). A further rapid acting insulin analogue is insulin aspart. Compared with human insulin, the AA Pro in position 28 of the B-chain is substituted in insulin aspart by aspartic acid (Asp) [17].

In the Ph.Eur. monographs the identification of the three insulins [13,18,19] consists of a combination of two tests. In the first test the LC-retention times of the substance to be examined and of the corresponding insulin reference standard are compared. As a second test peptide mapping employing a Glu-C-protease for digestion and subsequent separation of the fragments by LC is described. In the USP monographs of human insulin and insulin lispro [10] the same approach is used.

Unfortunately, identification by comparison of the LC retention times is not very specific. According to the "Technical Guide for the Elaboration of Monographs for the European Pharmacopoeia" [4] LC is only used for identification purposes if no suitable alternative is available. These reservations against the use of LC for substance identification are confirmed by the fact that peptide mapping LC results in identical chromatographic profiles for insulin lispro and insulin aspart (Fig. 2a and b).

Taken together, the current situation of the pharmacopoeial identification of peptides is not satisfactory. Thus, it was the aim of this study to demonstrate how direct infusion electrospray ionization (ESI) collision induced dissociation mass spectrometry (CID-MS) could be used as a tool to make identity testing more simple, rapid and resource saving whilst at the same time keeping, if not improving, the scientific quality of the corresponding monograph.

In a first series of experiments a relatively straightforward approach for the identification of a group of four synthetic peptides (gonadotropin releasing hormone analogues) was verified. In a second series of experiments, the possibility of identifying and distinguishing closely related polypeptides was tested by using human insulins and two insulin analogues.

2. Materials and methods

2.1. Samples, reagents and chemicals

For gonadorelin, goserelin, buserelin, leuprorelin human insulin, insulin lispro and insulin aspart the corresponding reference standards of the Ph.Eur. (EDQM, Strasbourg, France) were used. Acetonitrile of LC grade was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany), water for chromatography was delivered by an ELGA PureLab Ultra system (Elga Antony, France), HCl solutions were prepared from dilutions of 0.1 M HCl TitriPUR from Merck (Darmstadt, Germany), ammonium hydrogen carbonate p.a., formic acid 98–100% p.a. and ammonium acetate puriss. for HPLC were either delivered by Sigma–Aldrich (St.Quentin Fallavier, France), Fluka (St.Quentin Fallavier, France) or Acros (Noisy le Grand, France). Endoproteinase Glu-C sequencing grade (V8 protease) was supplied by Roche (Mannheim, Germany). Nitrogen +99% used as nebulizer and drying gas was delivered by a Peak Systems NM30LA nitrogen generator (Lab Gaz Systems, Massy, France). Nitrogen N 50 used as MS/MS collision-gas came from Air Liquide (Illkirch, France).

2.2. Apparatus

CID-MS experiments were performed using an Agilent Triple Quad LC/MS 6410 (m/z range: 20–2000) equipped with an

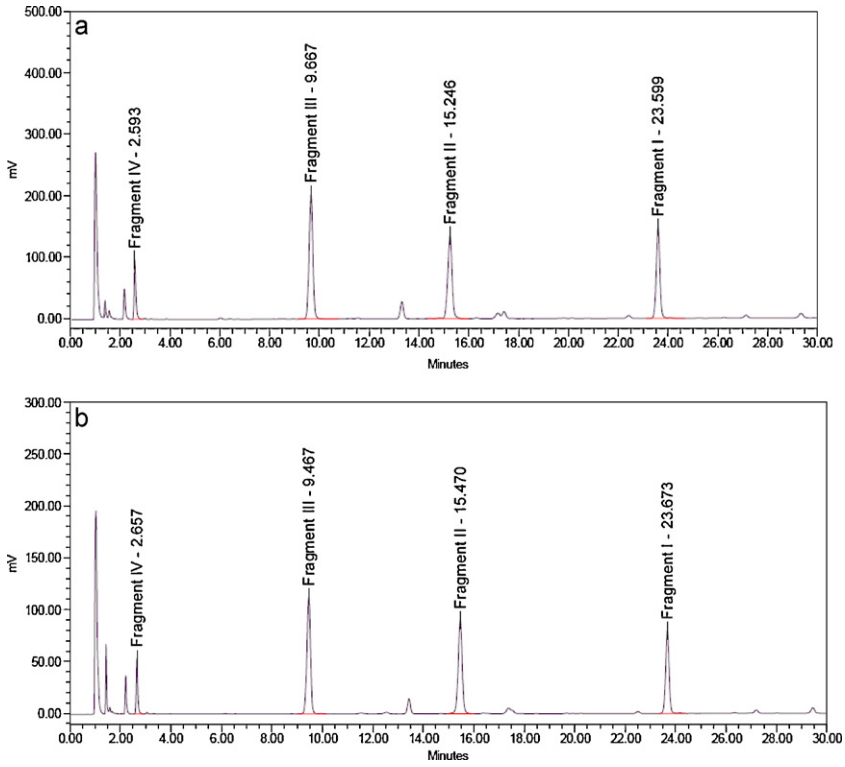

Fig. 2. Chromatographic profile of (a) insulin lispro and (b) insulin aspart applying peptide mapping according to the conditions described in the corresponding Ph.Eur. monograph.

ESI-source and coupled to an Agilent 1200 Binary SL liquid chromatography system (Waldbronn, Germany).

2.3. Methods

1 mg/ml solutions of gonadorelin acetate, goserelin, buserelin and leuprorelin, respectively, in acetonitrile/water (80/20, v/v) were prepared. For human insulin, insulin lispro and insulin aspart, respectively, 1 mg/ml solutions in 1 mM hydrochloric acid were used. Digested human insulin, insulin lispro and insulin aspart was prepared by addition of 20 μl of a 12 mg/ml solution of insulin dissolved in 10 mM hydrochloric acid to 0.5 ml of 0.1 M ammonium hydrogen carbonate. After addition of about 50 μg of V8 protease the solutions were heated in a water bath at 37 °C for 4 h, and subsequently quenched with 20 μl of formic acid.

Sample infusion was performed by flow injection analysis (FIA) using an injection volume of 10 μl (MS2-scan) and 20 μl, respectively, at a flow rate of 0.2 ml/min of a mixture of acetonitrile/water (4/1, v/v) for the synthetic peptides and a mixture of acetonitrile/0.07 g/l ammonium acetate solution (1/1, v/v) for the insulins. An ESI source (positive mode) was used for all experiments. The triple-quadrupole MS was operated in MS2-scan mode from a mass-to-charge-ratio (m/z-ratio) of 200–2000, scan time 500 ms, fragmentor voltage at 135 V. For the CID experiments collision energy of 75 eV for the singly charged ions and of 20 eV for the doubly charged ions was applied. The ESI product ion scan was performed for an m/z-ratio of 50–2000. MS-source parameters: capillary −4000 V, nebulizer pressure 25 psi, dry gas-flow 10 l/min, gas temperature 330 °C.

3. Results and discussion

"De Novo Peptide Sequencing" using tandem mass spectrometry is a widely used technique for the determination of the AA sequence of peptides via the ions produced by fragmentation at low energy CID as it is the case in triple quadrupole or ion trap MS [20–25]. The ESI-source of these instruments generates predominantly multiply charged peptide precursors, which is advantageous since these ions are easier to fragment in low energy CID than singly charged precursors [26–28].

If the charge of a CID-fragment is retained on the N-terminal side, the ion is classed as *b*. If the charge is retained on the C-terminus, the ion type is *y*. A subscript indicates the number of residues in the fragment. Consequently, a b_2-ion along with a neutral fragment would be produced from a singly protonated precursor ion (cf. Fig. 3) if the charge is retained within the first two amino acids of the N-terminal end. In turn, if the charge is retained on the C-terminus, a y_2-fragment and a neutral species containing the peptide N-terminus would be generated [27,29,30]. Following this scheme, the masses of the expected fragments can easily be predicted. This task can be significantly facilitated by the use of cor-

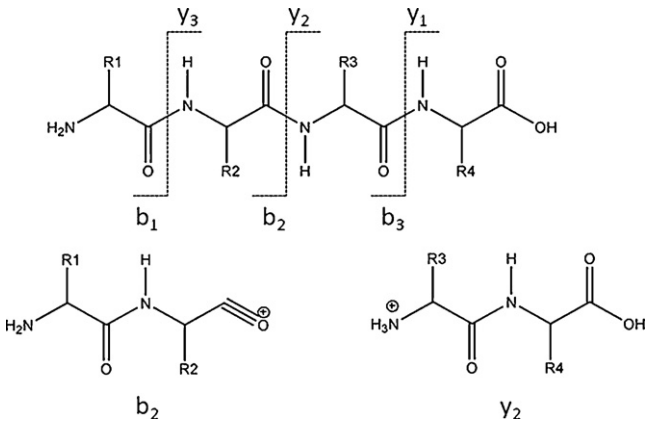

Fig. 3. Peptide cleavage by CID-MS.

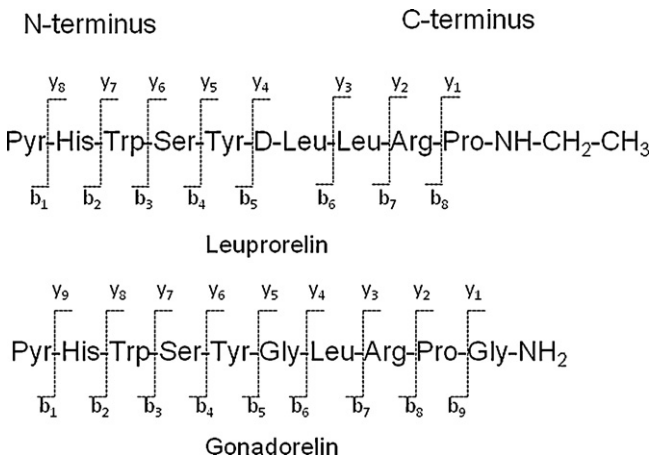

Fig. 4. Theoretical b and y low energy CID-MS fragments of leuprorelin and gonadorelin.

responding software packages provided by most MS manufacturers [20,31–33].

However, it should be noted that other fragmentation pathways can occur during CID, some of which can also provide composition-specific information. An example for such type of ions is the "immonium ions" which have the general structure $RCH{=}NH_2^+$ (where R is the amino acid side chain) and a mass of 27 u less than the mass of the fragment AA they are formed from [34].

To delineate the complete sequence, fragmentation must occur at each peptide bond. Unfortunately, this is a situation that rarely occurs [30,35,36]. Nevertheless, by combining the results obtained from C- and N-terminal fragmentation of a peptide, confirmation of the AA sequence is mostly possible. However, it should be noted that low energy CID-MS has some limitations: first, the distinction of D- and L-forms of AAs is possible, but not straightforward [37] and second, the distinction of the constitutional isomers Leu and Ile is not possible.

3.1. Identification of gonadotropin releasing hormones

The expected molecular masses of gonadorelin, leuprorelin, buserelin and goserelin were confirmed by direct mass measurement using the $[M+H]^+$ and $[M+2H]^{2+}$ ions. For CID-MS, precursors with a single positive charge ($[M+H]^+$) and with a double positive charge, $m/z = [M+2H]^{2+}$ of the 4 peptides were selected. Whilst for the singly charged precursors mainly intense b-fragments were found after CID, b and y fragments were formed from the double charged precursor ions. A summary of the results is given in Table 1. Moreover a fragmentation scheme showing all theoretical b and y fragments of leuprorelin (as an example for the nonapeptides) and of gonadorelin is depicted in Fig. 4.

For the nonapeptides leuprorelin, goserelin and buserelin, the complete AA sequence could be confirmed via the corresponding sequence of y-fragment ions (C-terminal fragments) and by the expected series of b-fragment ions (N-terminal fragmentation) with the exception that no y_8 fragment, resulting from the splitting of the N-terminal pyroglutamic acid (Pyr) from the remaining 8 AAs of the nonapeptide, was found. Considering that intense b_2 fragments with an m/z-ratio of 249 (cf. Fig. 5) were found, it was concluded that the formation of a Pyr-His b_2-fragment is privileged and that therefore y_8-fragment with N-terminal His could not be detected [36].

The fragments marked with an asterisk in Table 1 correspond to the so-called "satellite-ions" (ions produced by split-off of the side chain modification; in this case the *tert.*-butyl residue) [26] of the

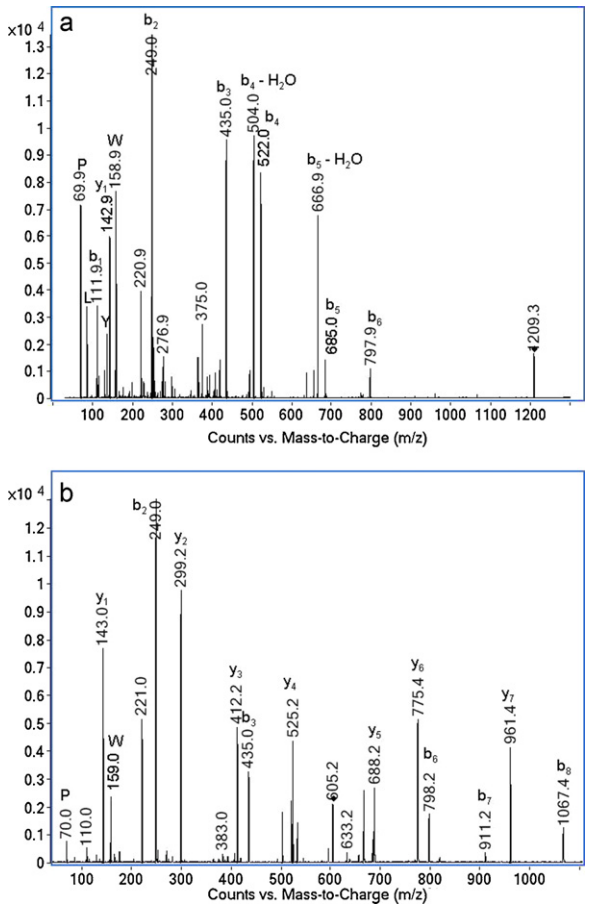

Fig. 5. Mass spectra of leuprorelin after CID of the precursor with (a) $z = 1$ and (b) $z = 2$; b and y fragments detected are indicated in the spectrum. A single letter code is used to indicate the AAs (P, proline; L, leucine; Y, tyrosin; W, tryptophan).

b- and y-fragments containing modified D-Ser which is present in position 6 of goserelin and buserelin (cf. Fig. 1).

In the case of gonadorelin, the y_1 (C-terminal Gly) and y_9 (resulting from the splitting of the N-terminal Pyr from the remaining 9 AAs of the decapeptide) fragments are missing. The reason for the absence of the y_9 fragment, corresponding to the y_8 fragment of the nonapeptides, was already discussed above. The absence of the y_1 fragment (C-terminal Gly) can be explained by the fact that the AA in position 9 is Pro (cf. Fig. 4). For Pro is known that the N-terminal peptide bond is particular labile whereas the bond on the C-terminal side is not. Consequently N-terminal cleavage of Pro is privileged and C-terminal fragments (in this case the y_1-ion) show only a very low abundance or are even absent [26,27,36]. Based on this explanation, the missing y_1-fragment (C-terminal Gly) can even be interpreted as an indirect confirmation of the sequence since in case of an inversion of Gly and Pro at the C terminal end, the Gly ion should have appeared as y_1-fragment.

The CID-MS spectra of leuprorelin precursors with a single positive charge ($z = 1$) and with a double positive charge ($z = 2$) are given in Fig. 5 for information.

3.2. Identification of human insulin, insulin lispro and insulin aspart

Since the difference between human insulin and insulin lispro is an inversion of the amino acids proline and lysine in position B-28/29, their molecular masses (M_r) are identical ($M_r = 5808$ u). However, due to substitution of the AA Pro in position 28 of the B-chain by Asp, insulin aspart exhibits a different molecular mass ($M_r = 5826$ u).

Table 1
Synthetic peptides—summary of the MS results.

Precursors found in MS2 scan						
Peptide:		Gonadorelin	Leuprorelin		Goserelin	Buserelin
Sum formula $[M+H]^+$		$C_{55}H_{75}N_{17}O_{13}$	$C_{59}H_{84}N_{16}O_{12}$		$C_{59}H_{84}N_{18}O_{14}$	$C_{60}H_{86}N_{16}O_{13}$
Theoretical *m/z*	*z* = 1	1182.6	1209.7	*z* = 1	1269.6	1239.6
Theoretical *m/z*	*z* = 2	591.8	605.3	*z* = 2	635.3	620.3
m/z found	*z* = 1	1182.6	1209.7	*z* = 1	1269.6	1239.6
m/z found	*z* = 2	591.9	605.4	*z* = 2	635.5	620.4

Detected fragments in CID-MS

AA sequence				*b*-Ion	N-terminal *m/z*			*y*-Ion[a]	C-terminal *m/z*			
Gonadorelin	Leuprorelin	Goserelin	Buserelin		Gonadorelin	Leuprorelin	Goserelin Buserelin		Gonadorelin	Leuprorelin	Goserelin	Buserelin
Pyr	Pyr	Pyr	Pyr	1		112.1					Precursor	
His	His	His	His	2		249.1		9/8	–	–	–	–
		Trp		3		435.2		8/7	934.5	961.5	965.5*	935.4*
		Ser		4		522.2		7/6	748.4	775.4	835.4	805.4
		Tyr		5		685.3		6/5	661.4	688.4	692.4*	662.4*
Gly	D-Leu		D-Ser-C_4H_9	6	742.3	798.4	772.3*	5/4	498.3	525.3	585.3	555.3
		Leu		7	855.4	911.5	885.4*	4/3	441.3	412.3	442.3	412.3
		Arg		8	1011.5	1067.6	1097.5	3/2	328.2	299.2	329.2	299.2
Pro-Gly-NH_2	Pro-NH-C_2H_5	Pro-N_3H_4CO	Pro-NHC_2H_5			Precursor		2/1	172.1	143.1	173.1	143.1
Additionally detected immonium ions (*m/z*):				Pro (70), His (110), Trp (159), Leu/Ile (86), Tyr (136)								

[a] The first figure stands for the position in the decapeptide gonadorelin. The second figure indicates the position of the AA residue in the three nonapeptides.
* Further fragmentation occurred and only the [M+H]+-ion minus the *tert.*-butyl residue of D-Ser could be detected.

Table 2
Summary of the MS results for the digested fragments III of human insulin, insulin lispro and insulin aspart, respectively.

		Human insulin	Insulin lispro	Insulin aspart
Precursors of digestion fragment III in MS2-scan				
Precursor *m/z*	*z* = 1	*1116.6*	*1116.6*	*1134.6*
Precursor *m/z*	*z* = 2	**558.9**	**558.9**	**567.8**
Tandem-MS results after CID of digestion fragment III				
Internal cleavage ions (*m/z*):		[Pro-Lys]$^+$(**226.1**)[a]	[Thr-Lys]$^+$(*230.1*)[a]	[Asp-Lys]$^+$(244.1)

AA sequence			*b*-Ion	N-terminal *m/z*			y-ion	C-terminal *m/z*		
Human	Lispro	Aspart		Human	Lispro	Aspart		Human	Lispro	Aspart
	Arg-Gly		2	–	–	–			Precursor	
	Phe		3	*361.2*	–	*361.2*	7	–	–	–
	Phe		4	508.3	508.3	508.3	6	–	–	–
	Tyr		5	*671.3*	*671.3*	671.3	5	–	–	–
	Thr		6	**772.4**	772.4	772.4	4	–	–	–
Pro	Lys	Asp	7	–	**900.5**	**887.4**	3	345.2	345.2	–
Lys	Pro	Lys	8	997.5	*997.5*	*1015.5*	2	*248.2*	**217.1**	*248.2*
	Thr				Precursor		1	*120.1*	*120.1*	*120.1*

[a] Bold face indicates strong signals, italic indicates weak signals.

Since the upper mass range limit of the MS instrument used here was at a *m/z*-ratio of 2000, a direct confirmation of the expected molecular masses of the three polypeptides could only be performed focusing on multiply charged molecular ions. However, analyzing the $[M+4H]^{4+}$ and the $[M+5H]^{5+}$ ions, the expected molecular masses of the peptides could be confirmed and insulin aspart could be clearly distinguished from human insulin and insulin lispro. Since additional structural information about the AA sequence was required to discriminate between human insulin and insulin lispro and also to confirm the position 28 modification of insulin aspart, CID-MS experiments were performed after digestion of the insulins with *Staphylococcus aureus* V8 protease. This enzyme splits the sequence of amino acids at the C-terminal side of each Glu.

As expected [38], four peptide fragments were obtained. Fragment III is a linear nonapeptide with a *m/z*-ratio of 1116.6 ($[M+H]^+$): It consists of the 9 final AAs of the C-terminal side of the B-chain [36]. This peptide was selected for CID-MS. The theoretical CID-MS fragmentation scheme of the fragment III nonapeptide of human insulin is depicted in Fig. 6. Ions b_1 to b_6 and y_1 are identical with insulin lispro and insulin aspart, whilst the b_7, b_8 and y_2, y_3-fragments are different.

The AA sequencing was not as straightforward as with the GnRHs, because not all expected b-fragments and only very few y-fragments were detected (Fig. 7). However, with two exceptions

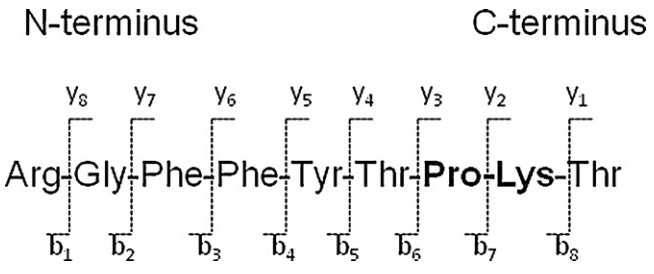

Fig. 6. Theoretical b and y low energy CID-MS fragments of digested fragment III of human insulin.

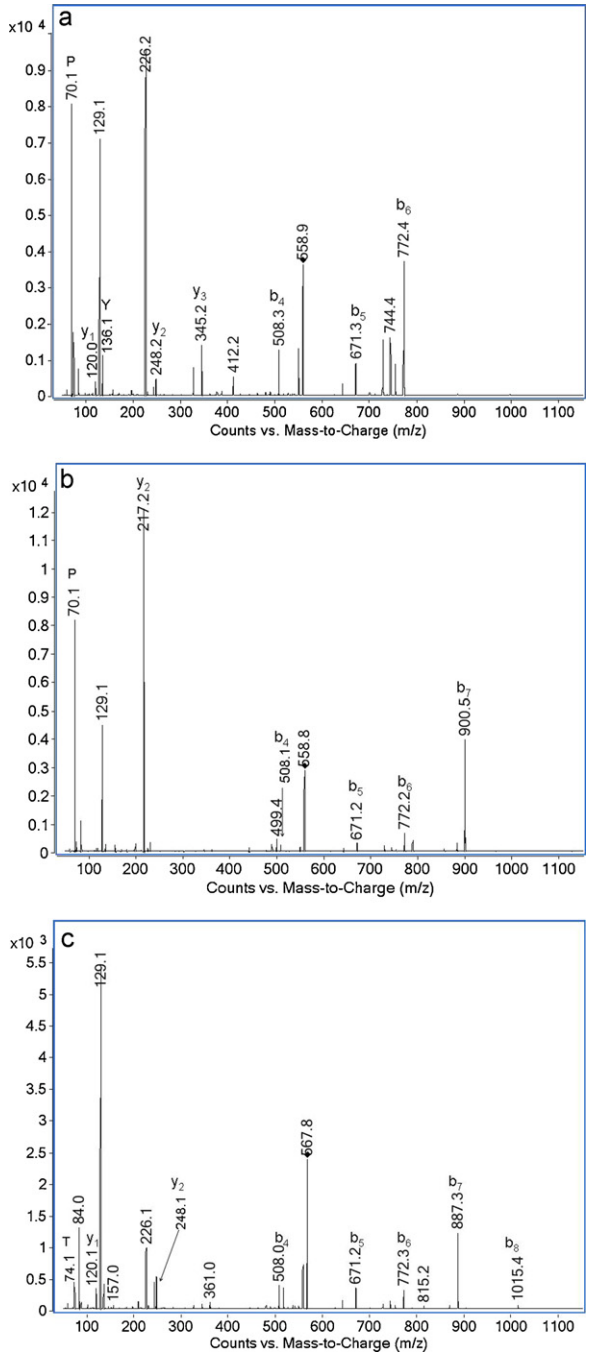

Fig. 7. CID-MS spectra obtained from the $[M+2H]^+$ precursor ions of the digested fragment III of (a) human insulin, (b) insulin lispro, and (c) insulin aspart. A single letter code is used to indicate the AAs (P, proline; T, threonin; W, tryptophan).

(y_3-fragment of insulin aspart and b_7-fragment of human insulin) the fragments essential for a differentiation of the three insulins could be detected, which is summarized in Table 2.

The fact that no b_7-ion was found for human insulin indirectly confirms the sequence. As already outlined above a cleavage of the N-terminal peptide bond of Pro is particularly labile and cleavage on the C-terminal side is not favorable. Therefore a b_7 fragment of human insulin was not expected to occur. The reason for the absence of a y_3-fragment for insulin aspart is seen in the fact that for Asp C-terminal cleavage is privileged [26]. Nonetheless, for both, human insulin and insulin aspart, three out of four discriminating fragments could be detected. In the case of insulin lispro all four could be found. Moreover, some fragments resulting from internal cleavage could be assigned to $[Pro\text{-}Lys]^+$ ($m/z = 226.1$) for human insulin, to $[Thr\text{-}Lys]^+$ ($m/z = 230.1$) for insulin lispro and to $[Asp\text{-}Lys]^+$ ($m/z = 244.1$) for insulin aspart. These internal sequence combinations can only occur in the corresponding insulin analogues and are therefore also considered to be indicative of the corresponding structures.

In summary, the verification of the molecular masses of the insulins by means of m/z-ratio of the $[M+4H]^{4+}$ and $[M+5H]^{5+}$ ions combined with the specific differences found in the AA sequence by CID-MS allowed a quick and efficient identification and distinction of human insulin, insulin lispro and aspart with a degree of certainty that is considered superior to what is currently described in the pharmacopoeial monographs.

4. Conclusion

In the past, the CID-MS technique in which a precursor ion is mass-selected and fragmentation is induced to obtain structurally relevant product ions, was limited to specialized mass spectometrists. Today, instrument development has progressed in a way that CID-MS spectra can easily be recorded under the control of automated software. Due to dropped instrument prizes more laboratories have access to this technology.

Here, we have demonstrated by means of two different clusters of pharmacologically active peptides (GnRHs and insulins) how CID-MS can successfully be utilized for identification testing in pharmacopoeial monographs. In this respect, the combination of a direct confirmation of the molecular mass via the m/z-ratio of the molecule ions with structural sequence information obtained by CID-MS experiments was found to deliver a higher degree of certainty of the identity of a given substance than the set of tests currently described in the monographs. With an appropriate set of pre-defined acceptance criteria a pass/fail decision could probably be made by a standard laboratory operator even without in-depth knowledge of mass spectrometry.

As an example, the acceptance criteria for leuprorelin could consist of the confirmation of the m/z-ratio of the precursor ion and the detection of either the b or the corresponding y-fragment for each step of the AA sequence. For insulin lispro, the confirmation of the m/z ratio of the intact molecule (e.g. via the $[M+4H]^{4+}$ or the $[M+5H]^{5+}$ ion) combined with the detection of the b_7 fragment at an m/z ratio of 900 and the y_2 fragment at an m/z ratio of 217 could be defined as acceptance criteria. In order to avoid any misinterpretation, the CID-MS spectra of the test sample could additionally be evaluated against those of a corresponding reference standard.

Compared with the current situation where several partly rather laborious tests have to be performed to come to a pass/fail decision an identification by MS offers the following advantages: a significant gain in efficiency and throughput due to easy sample preparation, fast measurement and result interpretation; an important reduction of the amount of costly peptides consumed during testing; a reduction of the amount of reference standards required per test; and a higher degree of certainty of the identity of the substance tested.

In the case of the GnRHs, a further improvement of the monographs would probably be possible by the development of a LC-CID-MS method which could combine the identification of the peptide with the determination of the purity and also with the assay determination.

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