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Quantitative analysis of polypeptide pharmaceuticals by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract

An accurate method based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been developed for quantitative analysis of calcitonin and insulin in different commercially available pharmaceutical products. Tryptic peptides derived from these polypeptides were chemically modified at their C-terminal lysine-residues with 2-methoxy-4,5-dihydro-imidazole (light tagging) as standard and deuterated 2-methoxy-4,5-dihydro-imidazole (heavy tagging) as internal standard (IS). The heavy modified tryptic peptides (4D-Lys tag), differed by four atomic mass units from the corresponding light labelled counterparts (4H-Lys tag). The normalized peak areas (the ratio between the light and heavy tagged peptides) were used to construct a standard curve to determine the concentration of the analytes. The concentrations of calcitonin and insulin content of the analyzed pharmaceutical products were accurately determined, and less than 5% error was obtained between the present method and the manufacturer specified values. It was also found that the cysteine residues in CSNLSTCVLGK from tryptic calcitonin were converted to lanthionine by the loss of one sulfhydryl group during the labelling procedure.

Keywords: Calcitonin; Human insulin; MALDI-TOF-MS; Quantification; Injection solution and suspension

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a powerful technique for identification and qualitative analysis of proteins and peptides [1–3]. MALDI, despite the ease of operation, high speed and high-throughput, frequently suffers from a strong variation in the signal intensities at different positions of the sample that is related to an inhomogeneous distribution of the analyte molecules among the matrix crystals [4–6]. This spot-to-spot variation hampers quantitative analysis by MALDI, since peak area does not correlate to the true quantity of the analyte in the sample. The peak area is also affected by other factors, such as the analyte concentration, competitive ionization effects, the type of matrix, rate of crystallization, and the chemical properties of the amino acid side chains [4,7–9].

However, it has been demonstrated that MALDI can be employed for quantitative measurements [6,10–13]. The fluctuation in the signal intensity caused by inhomogeneous analyte distributions can significantly be reduced by the use of isotope-labelled species, chemically identical to the analyte, as internal standard (IS) [4,14]. The analyte and the IS will be distributed similarly among the matrix crystals. As a result, the ratio between the signal intensities of standard and IS remains constant all over the analyte–matrix surface. There are other approaches for the improvement of sample homogeneity of MALDI samples which have been discussed in a recently published review article [6].

The aim of the present study has been to develop a strategy based on stable isotope coding for the quantification of calcitonin and insulin in different commercially available parenteral pharmaceutical products by MALDI-TOF-MS. The lysine residues of the tryptic peptides were covalently labelled with light and heavy 2-methoxy-4,5-dihydro-imidazole. The modification

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reaction converts lysine to its imidazol-2-yl derivative [15,16]. The lysine derivatization was originally introduced to enhance ionization and fragmentation pattern of lysine containing peptides [15]. The light modified tryptic peptides (4H-Lys tag) were used as the standards and the corresponding heavy modified tryptic fragments (4D-Lys tag) as the IS for the assays. Non-deuterated Lys-tag and deuterated Lys-tag add 68 and 72 Da per lysine residue, respectively, to the molecular mass of the peptides. This mass difference enables resolution and thus integration of the mono isotopic peaks from the light and the heavy labelled peptides.

2. Experimental

2.1. Chemicals and reagents

Calcitonin and human recombinant insulin used as reference standards were supplied by the European Directorate for the Quality of Medicines (EDQM, Strasbourg, France). Pharmaceutical products containing calcitonin and insulin were purchased from a local pharmacy (Uppsala, Sweden). 2-Methoxy-4,5-dihydro-H-imidazole (4H-Lys tag) and deuterium labelled 2-methoxy-4,5-dihydro-imidazole (4D-Lys tag) was obtained from QMX Laboratories Ltd. (Thaxed Essex, United Kingdom). Porcine trypsin was purchased from Promega (Madison, WI, USA), ammonium bicarbonate (AbC) from Fluka BioChemica (Buchs, Switzerland) and α-cyano-4-hydroxy-*trans*-cinnamic acid solution (ACHCA) from Agilent Technologies (Palo Alto, CA, USA). Sequencing kit (Ettan CAF MALDI Sequence, Kit) used for the derivatization of the peptides was purchased from GE Healthcare (Uppsala, Sweden).

2.2. Equipment and methods

MALDI-TOF was performed in an Autoflex (Bruker Daltonics, Bremen, Germany) of reflector type time-of-flight mass spectrometer, equipped with a pulsed nitrogen laser working at 337 nm. The instrument was operated in the positive ion mode with delayed extraction at an accelerating voltage of 20 kV and a variable voltage reflectron. The parameter settings were optimized to analyze peptides in reflector mode. Before analysis the instrument was externally calibrated with Bruker Daltonics standard peptide mixture, consisting of seven peptides ranging from *m/z* 1046 to 3147, i.e., Angiotensin-II, Angiotensin-I, Substance-P, Bombesin, Reinin-substrate, ACTH-clip (1–7), ATCH-clip (18–39) and Somastatin. 1 μl sample was carefully mixed with 1 μl matrix (ACHCA ready to use from Agilent). 1 μl of the mixture was then applied on the MALDI sample plate and allowed to air-dry (dried-droplet method) before being placed in the mass spectrometer. The laser intensity was set in the interval 15–25% and mass spectra were obtained by averaging 250 laser shots (5× 50 shots) at different positions on the sample surface. In order to evaluate the repeatability and precision of the measurements each sample spot was analyzed at least five times.

All samples used for post source decay (PSD) analysis were analyzed in the reflector mode. The ion selector was set at chosen *m/z* value and the presence of neighboring ions was considered by setting a detection window around the target mass (±20 Da). The instrument was set for PSD and ion selector was set to *m/z* of precursor ions one at a time. The laser intensity was adjusted to obtain unit mass resolution. For each voltage segment 250 laser shots (5× 50 shots) were collected. Spectra were finally pasted together to yield a single PSD data set, which was interpreted manually.

Peak integration was executed with the X-mass software (Bruker Daltonics).

The pipettes used during the experiments were calibrated.

2.3. Proteolysis

Calcitonin reference was dissolved in water to a final concentration of 1 mg/ml and from this solution 8.3 μl (i.e., 8.3 μg) was diluted in 242 μl of 50 mM AbC, pH 7.9. From a commercially available pharmaceutical product containing 100 IU (≈16.7 μg in 1 ml) 0.5 ml (8.3 μg) calcitonin was lyophilized and dissolved in 250 μl of AbC to concentrate the sample. Both reference and samples were digested with 1.2 μg trypsin, over night at RT. The reaction was stopped after around 20 h by freezing in −20 °C. Insulin standard and formulations were treated in the same way. The reaction was stopped by freezing to prevent decomposition of the peptides.

After digestion the samples and the standards of both calcitonin and insulin were frozen by liquid nitrogen (−80 °C) and lyophilized.

2.4. Lysine labelling

The lyophilized samples were dissolved in 79 μl H_2O containing 0.8 mg 4H-Lys tag or 4D-Lys tag. The pH of the reaction solution was measured to be approximately 11. The reaction was carried out at 55 °C for 3 h. The reaction was then stopped by addition of 1 μl 100% TFA followed by 20 μl 0.1% TFA in water.

2.5. CAF derivatization of tryptic peptides for PSD analysis

Fragmentation efficiency of tryptic peptides is improved by CAF modification, i.e., chemical assisted fragmentation, which introduces a negatively charged sulfone group at the N-terminus of the tryptic peptide [17]. As a consequence CAF-peptides yield PSD spectra containing exclusively y-ions, i.e., C-terminal fragment ions. CAF label (136 Da) is lost from the precursor ion and then the rest of the y-ions are observed. The tryptic peptides were modified according to the protocol provided by GE Healthcare and others [18,19].

2.6. Standard curves for quantification of salmon calcitonin and human insulin

The peak area ratios between the 4H-Lys tag labelled and the 4D-Lys tag labelled peptides were plotted as functions of the reference standard concentration. The concentrations of calcitonin and insulin standards were in the range 7–22 and 3–30 μg/ml,

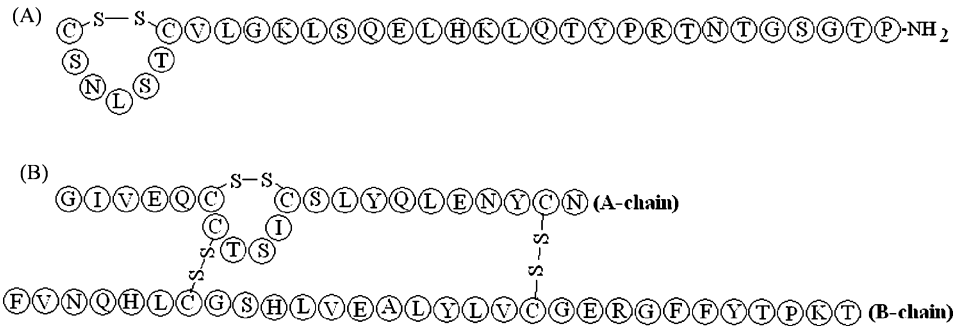

Fig. 1. The primary structure of calcitonin (A) and human insulin (B).

respectively. IS was added to each standard sample as well as to each analyte sample at a concentration of 12 and 5 μg/ml in the calcitonin and the insulin samples, respectively. The digestion of the polypeptides and modification of the corresponding tryptic peptides were performed in duplicate at different occasions. Each standard point and sample concentration was analyzed at least five times by MALDI-TOF-MS.

3. Results and discussion

3.1. Quantification of calcitonin in an injection solution

Salmon calcitonin, as shown in Fig. 1A, consists of 32 amino acids with a 1–7 intra-chain disulfide bridge and proline-amide at the C-terminal [20]. Trypsin digest of calcitonin yields four tryptic fragments, among them two lysine containing peptides (Table 1). After modification of lysine residues, the derivatization efficacy was tested by analyzing the modified sample with MALDI. A comparison between the spectra obtained from the tryptic calcitonin before (Fig. 2A) and after lysine modification (Fig. 2B) revealed the presence of trace amount of the native peptides in the lysine-modified sample. However, this did not significantly affect the accuracy of the measurements, since the standard samples and the analyte samples were treated under the same conditions.

In order to examine the resolution between the standard and the IS, a mixture of heavy and light modified calcitonin was analyzed with MALDI-TOF, respectively. The mass spectrum is shown in Fig. 3. Peptides corresponding to the same peptide are identified as pairs in mass spectrum at *m/z* 922.5, 926.5; 1158.7, 1162.7 and 1190.7, 1194.7 due to the mass difference between light and heavy isotopes (Δmass = 4 Da). The peak at *m/z* 1158.7 appeared after the C-terminal modification and was suggested to stem from decomposition of the cysteine residues in the peptide chain T_1 during the lysine labelling, i.e., at high temperature (55 °C) and basic condition (pH ≈ 11). Intra-chain disulfide bridges may be ruptured by base-catalyzed β-elimination, producing an intermediate amino acid, i.e., dehydroalanine, and a cysteine residue [21–23]. Dehydroalanine in turn interacts with cysteine to form a thioether amino acid, that is lanthionine [4,21,23–26] (will be discussed below). Lanthionine is actually composed of two alanine residues connected to each other by a thioether bond (Table 1) [27].

The normalized peak area of the abundant ion of LSQELHK-4H-tag (T_2) at *m/z* 922.5 and its corresponding heavy tagged ion at *m/z* 926.5 were used for the assays (Fig. 3).

In order to verify the influence of the IS on the precision of the method, the performance of the signal intensity and the peak area of T_2 at different calcitonin concentrations were evaluated. The results summarized in Table 2 demonstrate the contribution of the IS to the precision of the measurements. The R.S.D. values in the presence of the IS were between 2 and 6%, being at least four times smaller than those obtained in the absence of the IS.

The concentration of the calcitonin content of one injection solution was determined to be 17.0 (±2.1%) μg/ml, which was 1.8% larger than the labelled concentration, i.e., 16.7 μg/ml or 100 IE. In order to verify the results, another standard curve

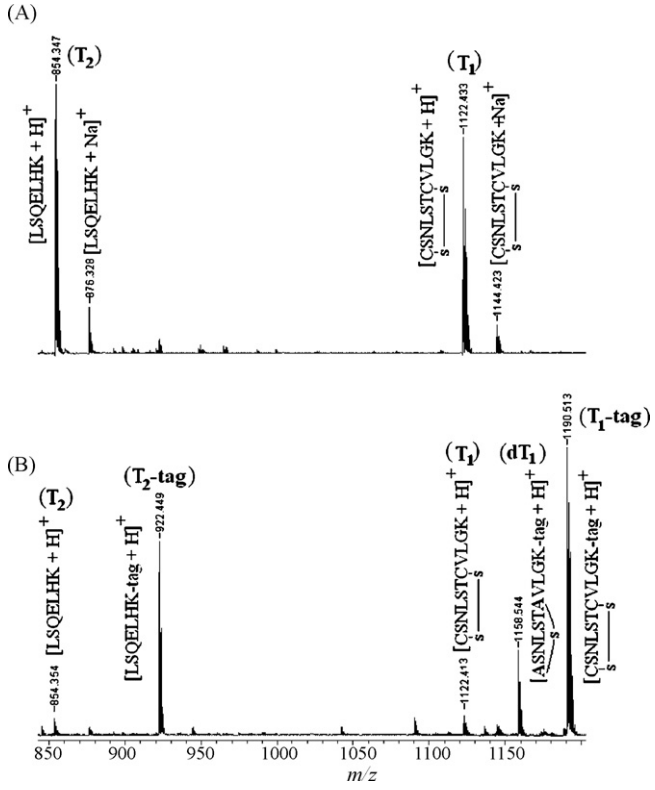

Fig. 2. Positive ion mode MALDI mass spectra of tryptic digest calcitonin before (A) and after (B) reaction with 2-methoxy-4,5-dihydro-H-imidazole. The lysine containing peptides exhibit an increase in mass by 68 Da after the derivatization (peptides T_1 and T_2). Operation conditions as those given in Section 2.

Table 1
Summary of tryptic digest masses of calcitonin and human insulin before and after reaction with 2-methoxy-4,5-dihydro-H-imidazole (masses are monoisotopic)

Tryptic peptide		Molecular mass (Da)	
		Native peptide	Modified peptide (light tagged)
Calcitonin			
CSNLSTCVLGK	T_1	1121.53	1189.53
ASNLSTAVLGK	dT_1[a]	–	1157.53
LSQELHK[b]	T_2	853.47	921.47
LQTYPR	T_3	776.42	–
TNTGSGTP-NH_2	T_4	732.34	–
Human insulin			
GIVEQCCTSICSLYQLENYCN	I_1 (A-chain)	2381.99	–
FVNQHLCGSHLVEALYLVCGER	I_2	2486.22	–
GFFYTPK[b]	I_3	858.43	926.42
T	I_4	119.06	–

[a] Cysteine residues at positions 1 and 7 are converted to lanthionine during the lysine modification.
[b] These peptides were used for the determinations.

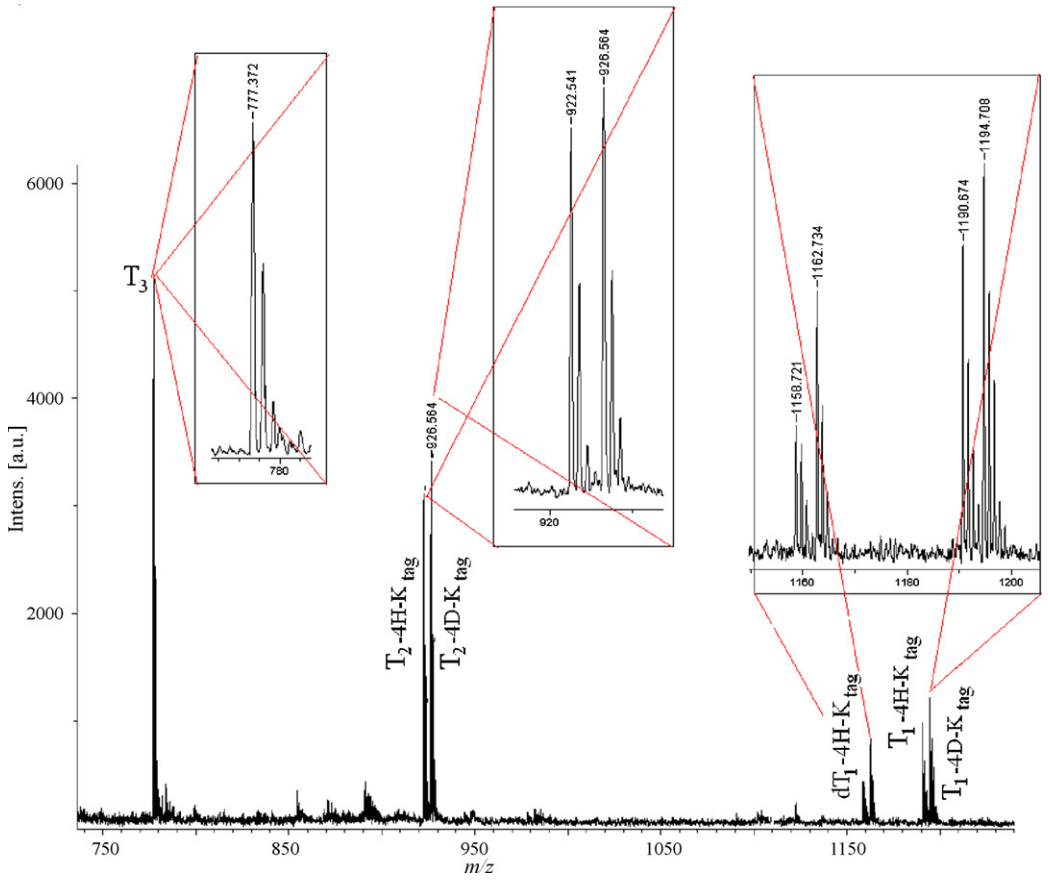

Fig. 3. Full scan spectrum from a mixture of light and heavy derivatized tryptic calcitonin. The heavy and light samples were generated under identical conditions. Operation conditions as those given in Section 2.

Table 2
Reproducibility of MALDI signal intensities and areas for the tryptic peptide T_2, derived from calcitonin, after lysine labelling[a]

Concentration (μg/ml)	Signal intensity (R.S.D., %)		Peak area (R.S.D., %)	
	S[b]	S/IS[c]	S[b]	S/IS[c]
33	30.7	5.8	31.5	5.5
28	28.2	4.2	37.7	4.7
26	30.9	6.2	32.1	5.3
23	33.6	5.4	36.6	5.4
22	38.2	4.6	40.7	4.5
20	21.5	4.9	23.0	4.1
15	33.0	1.5	37.4	1.5
13	31.8	2.0	34.5	1.8
10	42.5	4.6	48.1	4.3
8	40.5	3.0	41.4	3.3
7	48.6	6.6	50.0	6.4
6.5	23.4	3.0	21.6	2.4

[a] Based on at least five replicate measurements.
[b] R.S.D. (%) of signal intensity or peak area of T_2-4H-Lys tag as standard (S).
[c] R.S.D. (%) of signal intensity or peak area ratios of S and T_2-4D-Lys tag as IS.

Table 3
Precision and accuracy study for the determination of calcitonin in a pharmaceutical product in different days and by different persons ($n = 2$)

[Labelled] (μg/ml)	[a][Found] $_{(1)}$ (μg/ml)	[a][Found] $_{(2)}$ (μg/ml)
16.7	17.0 (±2.1%)	16.9 (±3.2%)

[a] The concentration of calcitonin was determined by different standard curves constructed in different days as indicated by the prefix number, and by two different persons using new standards. The measurements 1–3 were performed using the following equations:

$$y = 0.0816\,x + 0.099 \quad (r^2 = 0.999) \tag{1}$$

$$y = 0.0817\,x + 0.091 \quad (r^2 = 0.998) \tag{2}$$

was constructed by using new standard and IS solutions. The concentration of calcitonin sample by this standard curve was determined to be 16.9 (±3.2%) μg/ml, i.e., 1.2%, higher than the declared concentration (Table 3). The good agreement between the determined concentrations demonstrated the ruggedness of the method.

LOD and LOQ were determined to be approximately 2 and 7 μg/ml, respectively.

3.2. MALDI-PSD analysis of the dT_1 peptide

The mass signal at *m/z* 1158 (Figs. 2 and 3), being 32 atomic mass units lighter than the abundant ion at *m/z* 1190, was suggested to arise from the conversion of cysteine residues to lanthionine during the lysine modification procedure (Fig. 4) [21,22,24–26]. In order to confirm the identity of the peptide and this hypothesis, a MALDI-PSD experiment was performed. Prior to the PSD analysis the peptide was sulfonated by using CAF reagents to facilitate the fragmentation of the peptide and confirm the fragment assignment. The PSD fragmentation pattern was shown to be consistent with the suggested amino acid sequence of the peptide dT_1 as shown in Table 1 and Fig. 5. The presence of two pairs of fragment ions at *m/z* 484.2 and 553.6, and 1089.5 and 1158.6 spaced by 69 Da, assigned to the loss of a sulfhydryl group (34 Da) from the cysteine residues, i.e., conversion of cysteine to dehydroalanine (Fig. 4). The presence of thioether bond between the Ala 1 and Ala 7 displayed an enhanced stability against fragmentation. As seen the fragmentation pattern of the peptide at positions 1–7 in contrast to the remaining part of the peptide is not good.

The cysteine conversion can be inhibited by alkylating the cysteine residues with 4-vinylpyridine prior to tryptic digestion. Alkylation of cysteine residues with iodoacetic acid and iodoacetamide brings another problem, arising from the cyclization of the N-terminus carboxyamide methyl cysteyl and carboxy methyl cysteyl residues [28]. Therefore, these alkylating agents cannot be used for alkylation of N-terminal cystein residues. Lanthionine formation could also be prevented by addition of β-mercaptoethanol into lysine modification reactions. Cystein residues will be methylated under such conditions [29].

3.3. The quantification of insulin in two pharmaceutical products

Tryptic digestion of human insulin provides one lysine containing peptide (Fig. 1B and Table 1). The A-chain escapes tryptic digestion because of the lack of arginine or lysine in its sequence. The lysine containing peptide (I_3) from the B-chain was modified at the C-terminal to be used for the determinations.

The concentration of insulin in a test sample and an injection solution were measured to be 3.0 and 3.5 mg/ml, respectively,

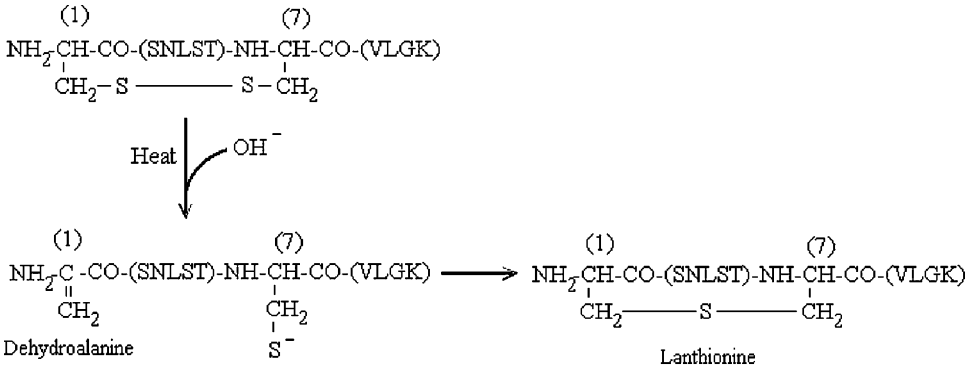

Fig. 4. The cysteine residues at positions 1 and 7 are converted to lanthionine under derivatization conditions, i.e., 55 °C and pH 11.

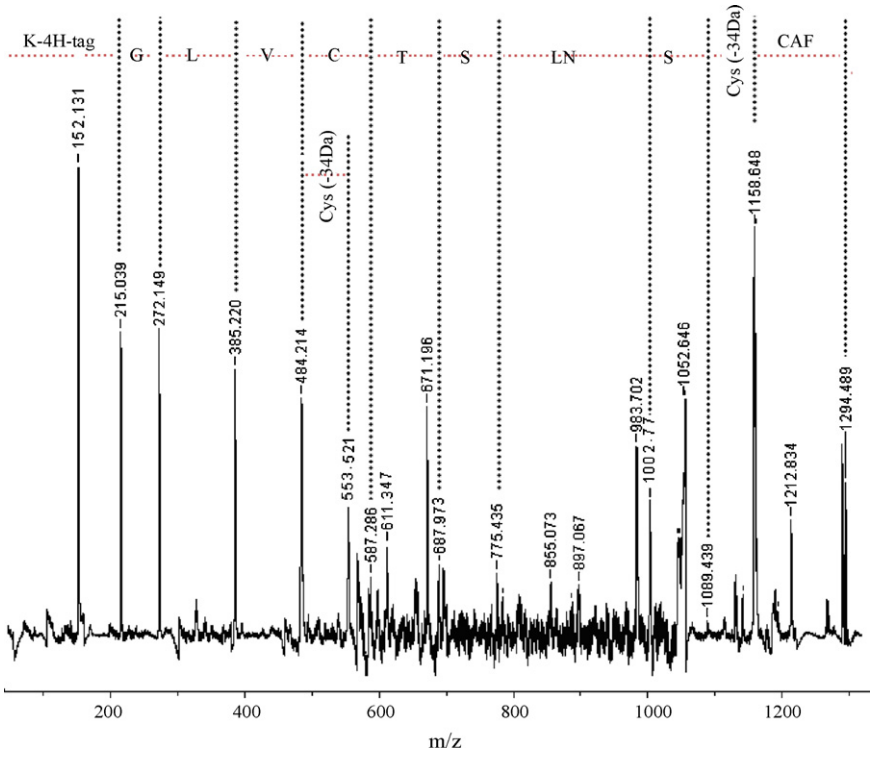

Fig. 5. MALDI-PSD analysis of lysine modified peptides at *m/z* 1158.6. The peptide was modified with CAF reagent prior to PSD analysis. Operation conditions as those given in Section 2.

which were in good agreement with the original concentrations, i.e., 3.0 and 3.47 mg/ml. An injection suspension, being stored at room temperature for 1 year, was also subjected to the quantification. The concentration of free insulin was found to be 1.2 mg/ml, which constituted 34% of the total insulin concentration. 70% of the insulin content should be present as crystals. The slight deviation from the declared concentration can partly be explained by the extreme storage conditions.

Insulin in formulations has been found to be chemically transformed by formation of dimerization and deamidation products [30–32]. The method, however, is not able to distinguish between the native and denatured insulin since the peptide used for the assays does not reflect the quality of the whole polypeptide. Therefore, the obtained results cannot be used for evaluating the quality of the preparations.

4. Conclusion

It was shown that stable isotope labelling of lysine containing peptides derived from calcitonin and human insulin was a good strategy for the MALDI-TOF-MS quantification. An important advantage with quantitative MALDI-TOF-MS is its high sensitivity which only requires samples of a few microliters with low concentrations.

The addition of the isotopic tags described in this study can be readily applied to other protein and polypeptide pharmaceuticals. The only prerequisite is the presence of at least one lysine residue in the polypeptide chain.

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