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Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 487–494

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Kinetic degradation study of insulin complexed with methyl-beta cyclodextrin. Confirmation of complexation with electrospray mass spectrometry and ^{1}H NMR

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Received 19 December 2001; received in revised form 17 January 2002; accepted 1 March 2002

Abstract

Hydrolysis of insulin has been studied during storage of various preparations at different temperatures. Deamidation is the predominant degradation process in acid solution resulting in a desamido product. The current study examines whether the interaction of insulin with methyl-beta cyclodextrin (metβCD) improves its stability. Hydrolysis of insulin was monitored by an HPLC assay with ultraviolet detection. The stability constant of insulin–metβCD complex was calculated by Lineweaver–Burke linear equation. Furthermore, the complexation of insulin with metβCD was characterized by ^{1}H NMR and Electrospray Mass Spectrometry (ESI-MS). MetβCD had a stabilizing effect on insulin degradation according to the kinetic parameters, leading to a decreased chemical deterioration. Furthermore, the stability constant K_{st} and the activation energy E_a were calculated by fitting the kinetic results to Lineweaver–Burke and to Arrhenius linear equations, respectively. Finally, the complexation of insulin with metβCD was characterized in aqueous media by ^{1}H NMR chemical shift displacements of assignable aromatic protons of specific amino acids upon the addition of the cyclodextrin, as well as by ESI-MS, since additional m/z peaks, which were attributed to insulin-metβCD complex, were detected. It is concluded that addition of metβCD resulted in a significant increase in the stability of complexed insulin compared with free insulin.

Keywords: Insulin degradation; Methyl-beta cyclodextrin; Electrospray ionization; Stability constant; ^{1}H NMR; Arrhenius plot

1. Introduction

Insulin has been in therapeutic use for almost 80 years but its stability has been concerned primarily with the changes in biological potency on storage of insulin formulations. In the last few years much information has appeared on the chemical transformation of insulin and most studies have dealt with its hydrolysis into desamido products.

All previous work has shown that insulin degrades by two main mechanisms: deamidation and polymerization [1]. Deamidation is a progres-

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00076-6

sive loss of $-NH_2$ groups from side-chain amide groups in glutaminyl (Gln) or asparaginyl (Asn) residues forming free carboxylic acids. Insulin contains six such residues, Gln^{A5}, Gln^{A15}, Asn^{A18}, Asn^{A21}, Asn^{B3} and Gln^{B4}, of which the three asparagine residues are likely to be the most labile sites [2]. In acid medium, deamidation takes place at position 21 in the A-chain of insulin (Asn^{A21}) [3,4].

Hydrolysis of amides is H^+ and OH^- catalysed. The formation of the higher molecular weight transformation products (polymerization), which are mainly covalent insulin dimers (CID), is pH-dependent and takes place at $pH > 3$. At pH 7–8, formation of higher molecular weight products, mainly due to disulphide interchange reactions, dominates [5]. To avoid complications by polymerization, pH 2 was chosen to investigate the effect of metβCD on the hydrolysis rate.

Cyclodextrins [6,7] are cyclic oligosaccharides made up of glucopyranose units bonded together via α(1, 4)-linkages. The most common CDs are α, β and γCD, containing six, seven and eight units, respectively. In view of the enormous research interest in CDs, a great variety of βCD derivatives [8] have been studied for a number of applications. CDs are 'cone-like' toroid molecules with a central cavity whose size varies according to the CD type. Due to the arrangement of hydroxyl groups within the molecule, the internal surface of the cavity is hydrophobic whereas the outside of the torus is hydrophilic. This arrangement permits the CD to accommodate a guest molecule within its cavity, forming an inclusion complex with enhanced solubility and improved chemical and physical stability [9].

The interaction of insulin with CDs has been studied mostly in terms of improving its absorption through skin, nasal and pulmonary mucus membranes of animals. These studies have shown that methylated CDs were more potent absorption enhancers than the parent and hydroxypropylated CDs [10]. This increase of the absorption is probably due to the interaction of CDs with lipids and/or divalent cations on the membrane surface [11]. In the present study, the effect of metβCD (a methylated CD) on the stability of insulin was monitored by carrying out an accelerated stability assay of the protein with and without the cyclodextrin. An increase of insulin stability of about 20% was observed due to the complexation with metβCD. Complexation was also studied by ^1H NMR spectroscopy at 400 MHz and ESI-MS.

2. Experimental

2.1. Materials

Bovine insulin was purchased from Sigma Chemical Co. (St. Louis, MO, USA), with a nominal activity of 28 IU/mg and was used without further purification. MetβCD was obtained from Wacker-Chemie GmbH (München, Germany). This product is characterised by an average degree of substitution of 1.8 which indicates the number of CH_3-groups per anhydroglucose unit. All other materials were HPLC grade, and de-ionised doubly distilled water was obtained from a Millipore Milli-Q Plus System (Resistivity > 18 MΩ cm). All solvents were filtered with 0.45 μm (pore size) filters.

2.2. HPLC method

All analyses of insulin and its degradation product were carried out by reverse phase HPLC with the isocratic system described previously [12]. GBC LC 1120 pump system with a GBC LC 1210 UV–vis detector (Darmstadt, Germany) were used. The analysis was performed on a Nucleosil 100-5 C18 (4.6×250 mm) column (Macherey-Nagel, Düren, Germany). The mobile phase used to separate and determine insulin and its desamido product was: acetonitrile–sodium dihydrogen phosphate (pH 2.2; 0.1 M) (30:70, v/v). The flow rate was 1.6 ml/min; the system was at room temperature and the detection wavelength was 214 nm.

2.3. Stability studies

Crystalline bovine insulin was diluted with a sodium dihydrogen phosphate buffer (pH 2) to obtain a final concentration of 40 IU/ml ($2.5 \times$

10^{-4} mM). These solutions were heated, protected from light, at temperatures of 50, 55 and 60 °C with variations less than ±1 °C, without shaking. At the same time, solutions of insulin were prepared containing metβCD; [insulin]:[metβCD] 1:20 (molar ratio) and were heated at the same temperatures. Certain proportions of the samples were removed at designated times for the measurement of insulin and its desamido product. At 55 °C, a kinetic study was performed by monitoring insulin degradation in the presence of increasing concentrations of metβCD using the HPLC assay described above. Pseudo-first-order rate constants for the disappearance of insulin (k_{obs}) were determined from the slopes given by the linear plots of the logarithm of the percentage of the remaining insulin against time. Hydrolysis of cyclodextrins at low pH [13] is very slow ($t_{1/2} = 170$ h at pH 1.5 and 60 °C) and did not affect the present stability studies.

2.4. Proton-nuclear magnetic resonance (^{1}H NMR) spectroscopy

The ^{1}H NMR spectra of insulin (2 mM) in the absence and presence of metβCD (40 mM) were obtained with a Brucker Analytik GmbH spectrometer (Avance DRX 400, Houston, USA), operating at 400.13 MHz at 25 °C, using 30% (v/v) CD_3COOD in D_2O as solvent. ^{1}H NMR chemical shifts were given in parts per million (ppm) relative to that of the solvent signal (HOD: 4.84 ppm) with an accuracy of ±0.001. The ^{1}H NMR signals of the aromatic region of insulin were assigned according to previous reports [14,15].

2.5. ESI-mass spectrometry

The LC/MS spectra of insulin solutions were measured by an AQA LC/MS system (Finnigan, Thermoquest, Argenteuil Cedex, France) equipped with an electrospray ionisation (ESI) source and a quadrupole mass analyser. Insulin solutions (0.1 mM) in the absence and presence of metβCD (2 mM) in water/methanol/acetic acid (47/47/6) were transferred from AQA reservoir to ESI probe via a fused silica capillary by pressurising the reservoir. A pressure of approximately 6 psi was needed to produce the desired flow rate of 10 μl/min. The probe heater temperature was set at 130–140 °C and probe and cone voltage were held at 3500 and 40 V, respectively.

3. Results and discussion

The quantification of insulin degradation was carried out from data obtained by HPLC. This degradation in the presence and absence of metβCD, follows a first order process [16] as can be seen from Fig. 1. Table 1 shows data obtained from the accelerated stability study, as well as degradation rate constant and half-life time values $t_{1/2}$.

At 50 °C, addition of metβCD in a 20:1 metβCD:insulin molar ratio was resulted in a 20% increase in the stability of complexed insulin compared with free. From Table 1 it is becoming also evident that increasing further the amount of metβCD can further increase insulin stability (see data at 55 °C).

3.1. Determination of stability constant for insulin–metβCD

In an attempt to get an idea of how 'stable' an inclusion complex is, the stability constant K_{st}

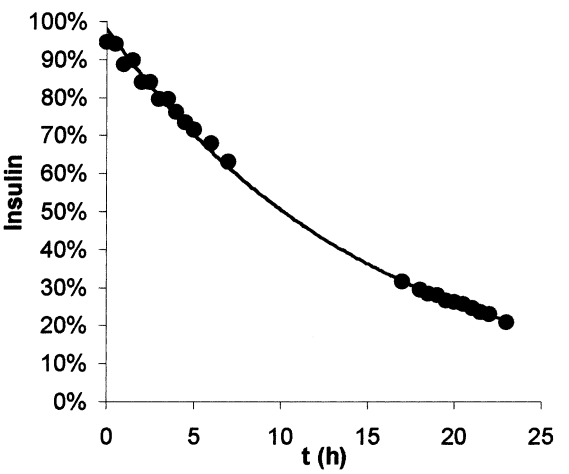

Fig. 1. Plot of the percentage of the remaining insulin against time at 60 °C.

Table 1
Degradation rate constants and half-life time values obtained at three different temperatures

T (°C)	Insulin: metβCD	$k \times 10^{-3}$ (per h)[a]	$t_{1/2}$ (h)	r[b]
50	1:0	23.8 (±1.9)	29.12	0.998
	1:20	19.2 (±1.7)	36.10	0.999
55	1:0	41.0 (±2.4)	16.91	0.996
	1:20	35.3 (±2.3)	19.64	0.998
	1:50	30.6 (±1.9)	22.65	0.999
	1:100	28.8 (±1.7)	24.06	0.998
	1:150	27.5 (±1.5)	25.21	0.997
	1:200	26.0 (±1.6)	26.66	0.998
60	1:0	66.4 (±3.7)	10.43	0.999
	1:20	56.6 (±2.9)	12.24	0.999

[a] Experimental rate constant. The numbers represent mean values of three experiments ± S.D.
[b] Coefficient of correlation

must be calculated. Linear and non-linear models [17] have been used to illustrate the kinetic behaviour of the 'guest' molecule in the presence of CD. In this study, the stability constant was determined kinetically, where the degradation of insulin could be characterised by the following equation [18]:

$$\frac{-\mathrm{d(ins.)}}{\mathrm{d}t} = k_o(\mathrm{ins.}) + k_c(\mathrm{ins.-CD})$$

where k_o and k_c are the rate degradation constant for decomposition of free and totally complexed insulin, respectively. The observed reaction rate for the degradation of insulin in the presence of metβCD is an average of the degradation rate of free and complexed insulin. In addition, K_{st}, the apparent stability constant of the complex can be determined by relating the observed rate constant with the concentration of the added metβCD. Actually, the measurable rate constant is

$$k_{obs} = k_o + \frac{(k_c - k_o)[\mathrm{CD}]}{1 + K_{st} + [\mathrm{CD}]}$$

which on rearrangement gives the Lineweaver–Burke transformation:

$$\frac{1}{k_o - k_{obs}} = \frac{1}{K_{st}(k_o - k_c)}\frac{1}{[\mathrm{CD}]} + \frac{1}{k_o - k_c}$$

The plot of $1/k_o - k_{obs}$ versus 1/[CD] gives $1/K_{st}(k_o - k_c)$ as the slope and $1/k_o - k_c$ as the intercept. Fig. 2 gives the plot for data obtained at 55 °C. The stability constant was calculated from that linear plot and its value was 472.5 (±54.8) l/mol ($n = 3$) for insulin–metβCD complex at 55 °C. Furthermore, the activation energy for the deamidation of insulin, was obtained graphically from Arrhenius plots of the degradation rate constants in the presence and absence of metβCD against three temperatures (50, 55 and 60 °C). The Arrhenius plot (Fig. 3) in the presence of metβCD was parallel to that in its absence, indicating that the mechanism of the degradation remained the same.

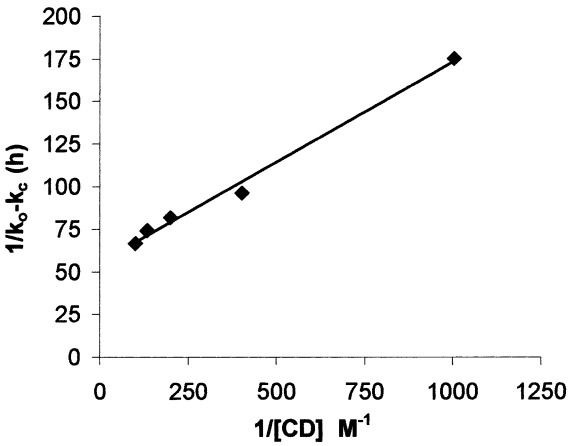

Fig. 2. Graph of the rate data according to Lineweaver–Burke transformed equation. Determination of K_{st} at 55 °C.

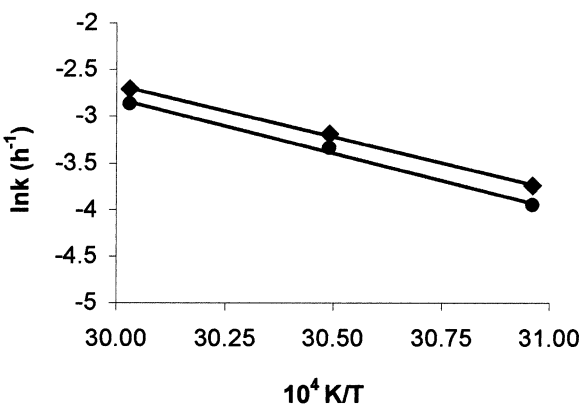

Fig. 3. Arrhenius diagrams for the degradation of insulin alone (◆) and insulin–metβCD complex (●).

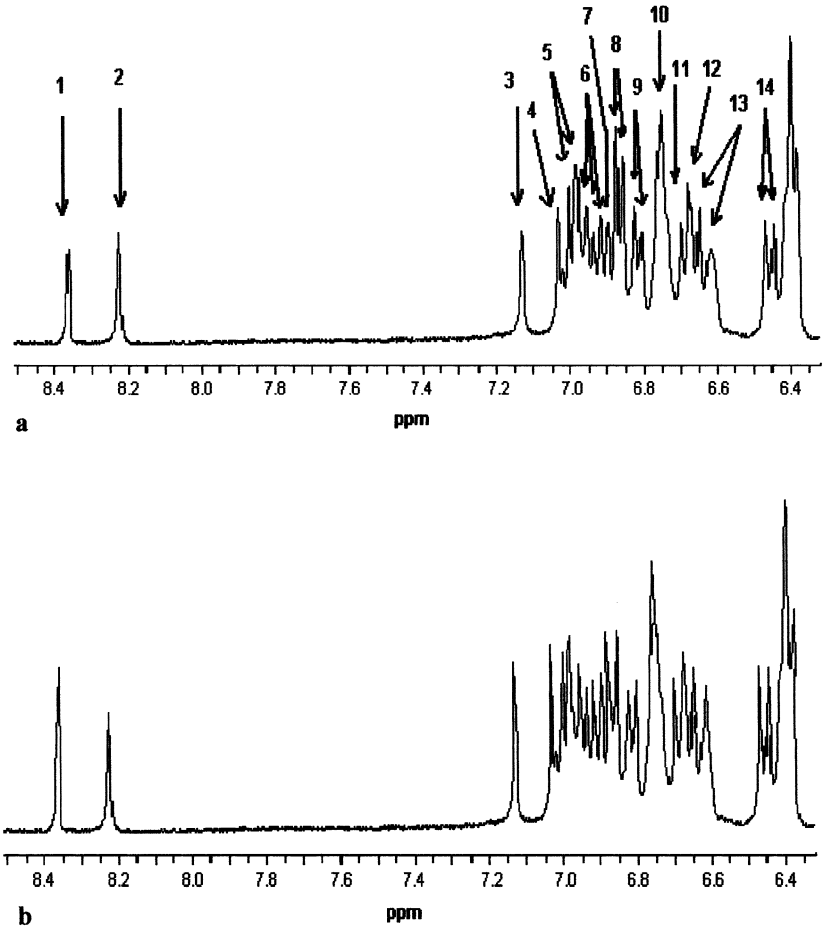

Fig. 4. ^{1}H NMR spectrum of aromatic region of insulin (2 mM) in the presence of metβCD (40 mM) (a) and insulin (2 mM) alone (b) in D_2O containing 30% (v/v) CD_3COOD at 25 °C.

The activation energy (E_a) for insulin degradation was calculated to be 22.0 ($\pm$0.4) kcal/mol. In the presence of metβCD, when insulin is totally complexed, E_a is increased to 23.1 ($\pm$0.4) kcal/mol. This increase could be attributed to steric hindrance that prevents the formation of the cyclic intermediate and protects from the nucleophilic attack by the aqueous medium.

3.2. Effects of metβCD on the ^{1}H NMR spectrum of insulin

In an attempt to investigate the interaction of insulin with metβCD and evidence their complexation, ^{1}H NMR spectroscopy was employed in 30% CD_3COOD. This solvent weakened the self-association of insulin, enabling the monomer to be the predominant species [19]. Fig. 4 shows the effects of metβCD (40 mM) on the ^{1}H NMR spectrum of the aromatic region of insulin. The presence of metβCD did not allow monitoring any chemical shift displacements of non-aromatic protons of insulin due to CD addition. Table 2 summarises all chemical shifts which are attributed to the addition of the CD.

The first two peaks (Fig. 4), well dislocated from the main aromatic region (6.2–7.0 ppm) are attributed to C2 protons of the B5-and B10-Histidines. The inclusion of the aromatic side chains within the cavity of metβCD induced chemical

shift displacements, characteristic for each amino acid. βCD derivatives are reported to complex with free aromatic amino acids at nearly neutral pH, with the efficacy decreasing in the order of tyrosine > phenylalanine ≫ histidine depending on their hydrophobicity [20] and the complementary forces between host and guest molecules. Under the acidic condition used in this study (pH ≈ 1.9), the complexation of the protonated imidazole moieties of the B5 and B10 histidines was probably destabilized. The fact that the changes in chemical shifts of the B5 and B10 Histidines are among the largest, could be explained by the stabilising effect of sugar-like compounds on protein structure [21], resulting in reduced mobility of protein backbone or just changes in the conformation of insulin.

Based on the results in Table 2, the presence of CD may affect amino acid residues such as B5, B10, A14 and B26 (significant chemical shift changes), whereas other amino acids such as B1, B25 and B16 are not significantly perturbed in the presence of the CD. Furthermore, the proton signals for almost all proton signals of the residues where shifted upfield (negative $\Delta\delta$ values) with the exception of C2,6 protons of A14. In general, the topological constraints of the peptide backbone may increase or reduce the formation of inclusion complexes. For example, the B24-phenylalanine is known to be directed towards the hydrophobic interior of the insulin molecule and its ring rotation is considerably restricted [22], as indicated by a line broadening of the resonances. Therefore, a relatively small change in the ^{1}H NMR signal of the B24-phenylalanine in the presence of metβCD indicated a degree of difficulty of the CD in approaching the side chain.

3.3. ESI-mass spectrometry of insulin complexed with metβCD

Electrospray ionisation mass spectrometry provides further confirmation of complexation of relatively polar compounds with cyclodextrins. With this 'soft' ionisation technique, ions existing in solution can be transferred into the gas phase without breaking non-covalent interactions which are the predominant forces in this 'host–guest' interaction.

Basic compounds (e.g. amines) can form a protonated molecule, $[M+H]^+$, which can be analysed in positive ion mode (ESI+) and give a peak at m/z $M+1$, where M represents the molecular weight of the compound. Electrospray can produce multiply charged ions for analytes [23,24] that contain multiple basic or acidic sites, e.g. proteins and peptides. Preliminary studies [25,26] for peptides and their complexes with CDs, have shown the potential of the technique to

Table 2
Effects of metβCD (40 mM) on ^{1}H NMR chemical shifts of insulin (2 mM) in 30% (v/v) deuterated acetic acid at 25 °C

Number	Side chain	Position	Insulin (2 mM), chemical shift (δ)	With metβCD (40 mM) $\Delta\delta$[a] ppm
1	Histidine (B 10)	C2	8.3297	−0.0032
2	Histidine (B 5)	C2	8.1935	−0.0086
3	Histidine (B 10)	C4	7.0979	−0.0060
4	Histidine (B 5)	C4	7.0000	−0.0070
5	Tyrosine (A 19)	C2 and 6	6.9488	−0.0042
6	Phenylalanine (B 1)	C4	6.8802	−0.0016
7	Phenylalanine (B 25)	C3 and 5	6.8620	−0.0026
8	Phenylalanine (B 1)	C2 and 6	6.8235	−0.0044
9	Phenylalanine (B 25)	C2 and 6	6.7714	−0.0016
10	Phenylalanine (B 24)	C3 and 5	6.7202	−0.0030
11	Tyrosine (B 16)	C2 and 6	6.6653	−0.0030
12	Tyrosine (A 14)	C2 and 6	6.6342	0.0012
13	Tyrosine (B 26)	C2 and 6	6.5903	−0.0064
14	Tyrosine (A 14)	C3 and 5	6.4139	−0.0058

[a] $\Delta\delta = \delta_{\text{with CD}} - \delta_{\text{insulin alone}}$. Negative signs indicate upfield displacement.

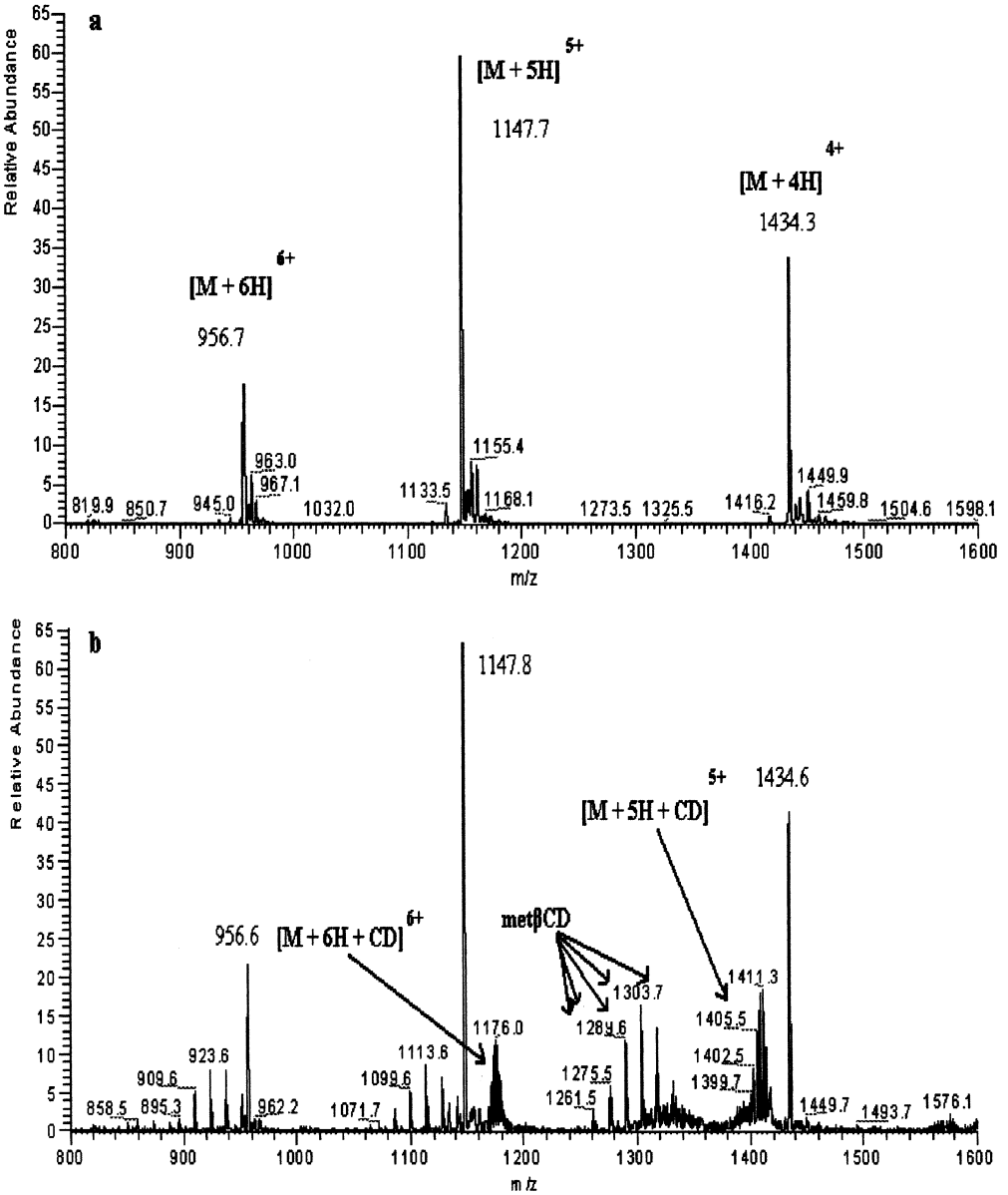

Fig. 5. ESI mass spectra of insulin (0.1 mM) in positive ion mode in the absence (a) and presence (b) of metβCD (2 mM).

characterise their formation. The extended mass-range acquisition (m/z: 800–1.600) allowed us to detect the three last peaks related to the respective multiply-charged insulin molecular ions as indicated in Fig. 5a. In the presence of metβCD (Fig. 5b) two additional 'bell-shaped' multiple charge peaks appeared at m/z 1176.0 and 1399.7–1411.3, corresponding to the complex of the charged insulin with metβCD. Five more peaks at m/z 1261.5, 1275.5, 1289.6, 1303.7 and 1317.7 are all attributed to metβCD, indicating a varying degree of substitution of the physical βCD with the methyl group and explaining the appearance of the 'bell-shaped' peaks. The difference between two adjacent peaks of the previous five is 14 m/z in all cases, due to an additional $-CH_2$-group.

4. Conclusion

In conclusion, the present study examines the stabilization of insulin complexed with metβCD from deamidation. These results suggest that metβCD had a pronounced stabilizing effect on insulin decomposition. Furthermore the present study highlights the usefulness of ESI-MS and ^{1}H NMR for the characterisation of complexation between cyclodextrins and complicated guests such as insulin, a polypeptide drug that has attracted the interest for improved and more efficient pharmaceutical formulations. To these efforts, cyclodextrins could offer, at least, some positive and useful results in designing rapid or long-acting insulin preparations (e.g. nasal), since they are potent absorption enhancers.

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