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A protocol for quality assessment of illegally distributed human growth hormone with respect to identity, purity, endotoxin level and microorganism content

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Abstract

Different methods based on MALDI-TOF-MS and double injection capillary zone electrophoresis (DICZE) were used for the identification and purity determination of somatropin in illegally distributed products. During the past few years more than 150 products suspected to contain somatropin have been analysed. Some of the samples were also subjected to control microorganisms and endotoxins. The identification of somatropin was carried out by peptide mapping using trypsin as proteolytic enzyme. A double chain peptide cross-linked via a disulfide bond was used as the signature peptide. Capillary electrophoresis in double injection mode was applied to both identification and purity determination of the samples. The identification was based on the comparison between the observed migration time of the reference standard and the calculated migration time of the analyte, being present in the second injection plug. The DICZE provides electrophoretic finger prints of intact somatropin and the related proteins which facilitates the identification. In addition, some of these samples revealed the presence of microorganisms as well as high level of endotoxins. Taken together, the doubtful quality of the analysed samples and the microbiological findings represent a serious threat for the consumers and public health.

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1. Introduction

Growth hormone or somatropin (Fig. 1) promotes proteinogenesis and fat mobilization and oxidation.[1-6] In the belief that such effects can be extrapolated to the healthy individual somatropin is abused by athletes and bodybuilders.[7] Increasing demand for somatropin on the black market paves the way to counterfeit somatropin, more so than any other hormonal compound. It has been attributed to cheap imports from China being often sold via the Internet.[8] Most off-label users are unaware of the correct doses and on how to mix the solution and give an injection. It has been demonstrated that supra physiological dosages can have fatal consequences.[9] However, apart from the undesired consequences following the use of somatropin, the illegally marketed products often contain high levels of impurities, e.g. endotoxins, which can have a significant public health risk.[1-3] During the last five years more than 150 lyophilized samples have been analysed in our laboratory, each representing 50 to 200 ampoules confiscated by the police and custom officials, Fig. 2A.

Somatropin consists of a single polypeptide chain containing 191 amino acid residues, with two disulfide bridges connecting Cys_{53} to Cys_{165} and Cys_{182} to Cys_{189}, respectively,[10,11] Fig. 1. The protein undergoes decomposition through oxidation and deamidation both in the solid state, e.g. the lyophilized form, and in aqueous solutions. The Met_{14}, Met_{125} and Asn_{149}, Asn_{152}, and Gln_{137} residues are the predominant residues for oxidation and deamidation reactions, respectively.[12-14] Deamidated forms, oligomeric aggregates, cleaved, and oxidized forms are the major somatropin-related proteins.[15] One of the somatropin isoforms, i.e. somatropin Gln_{18}, is suggested to be generated through replacement of His by Gln during the translation in *Escherichia Coli*.[16]

In order to analyse these products several techniques based on physico-chemical and biological methodologies have been applied. CZE in double injection mode has been used for both purity determination and identification of somatropin.[17,18] MALDI-TOF-MS has been

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applied to the identification of somatropin through protein mass finger printing (PMF).[19] Combination of DICZE and MALDI-TOF-MS techniques provides finger-prints over the native and digested protein, Fig. 2B and 3. Somatropin is administered parenterally, therefore sterility testing is required to ensure safety of the product. The low water activity (Aw) of the lyophilized products will not promote the proliferation of microorganisms. Most bacteria do not grow at Aw < 0.91 and most molds cease grow at Aw < 0.80.[20] However, lyophilisation and/or drying of a preparation does not imply that microbes or spores are killed; they may remain viable under dry state and stored under suitable conditions for many years.

Endotoxin test is another control test for quality assessment. Endotoxins are associated with gram-negative bacteria which can cause severe immune response and diseases in humans.[21-23] They act through activation of monocytes and macrophages, with the release of a range of pro-inflammatory cytokines, e.g. interleukin (IL-6 and IL-1) and tumour necrosis factor (TNF). The maximum level of endotoxin for intravenous applications of pharmaceutical and biological product is defined to 5 endotoxin units (EU) per kg of body weight per hour by all pharmacopoeias.[24,25] Endotoxins are large biomolecules consisting of a polysaccharide moiety and a glycolipid moiety. In comparison to proteins, endotoxins are very stable molecules, resisting extreme pH values and temperatures.[26,27] Endotoxins are also able to form aggregates with proteins, especially basic proteins, mainly through electrostatic interactions.[26] Endotoxines are continuously liberated into the environment with cell dead and during growth and division. Somatropin is produced in *Escherichia coli* (E-Coli) as a gram-negative bacterium, for this reason endotoxin testing is necessary to ensure it is endotoxin-free.

Both capillary zone electrophoresis and MALDI-TOF-MS are considered efficient and fast techniques for the analysis of polypeptides, and were chosen on the basis of their ability to provide adequate and reliable results within a reasonable time period and at a reasonably low cost. The aim of the present paper is to provide a short summary of the methods used for the

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identification and control of illegally marketed somatropin products. We want to highlight the associated potential health risks with the administration of such products, with reference to their doubtful quality.

2. Experimental

2.1. Chemicals and reagents

Somatropin CRS batch 2 (1.69 mg somatropin) and batch 3 (3.86 mg somatropin), were obtained from the European Directorate for the Quality of Medicines & Health Care (EDQM, Strasburg, France). The somatropin reference standard was kept at -20°C until use. Soya bean casein-digest agar, sabourad agar, Phosphoric acid and NaOH were obtained from Merck (Darmstadt, Germany). Chondroitin sulfate A sodium salt (CS) from bovine trachea, ammonium phosphate dibasic, and hexadimethrine bromide (PB) used for capillary coating were purchased from Sigma-Aldrich (St Louis, MO, USA). Porcine trypsin was purchased from Promega (Madison, WI, USA), ammonium bicarbonate from Fluka BioChemica (Buchs, Switzerland) and α- cyano- 4- hydroxy-trans-cinnamic acid solution (ACHCA) from Agilent Technologies (Palo Alto, CA, USA). Sinapinic acid was purchased from Bruker Daltonics (Bremen, Germany). The LAL assay, endotoxin standard, Pyrosol buffer, endotoxin-free water and pyrotube-K were purchased from Associates of Cape Cod (MA, USA). Soda glasses were purchased from VWR (PA, USA). Water was purified by Elga Maxima LC water cleaning system. All solutions were filtered through 0.22 µm PVDF filters (Pall, Ann Arbor, MI, USA). Confiscated samples were provided by police authority and customs service, where lyophilized materials were packed into either labelled or unlabelled sample holders, see Fig 2A.

2.2. Equipments and methods

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2.2.1. CZE. Capillary electrophoresis experiments were performed on a ProteomeLab PA 800 system (Beckman Coulter, Fullerton, CA, USA). Detection was performed at 200 nm. The background electrolyte (BGE) was prepared by adjusting the pH of 13.2 g (100 mM) of ammonium phosphate dibasic to pH 6.0 with Ortho-Phosphoric acid 85 %, water was added to 1000.0 ml[17]. A 70 cm fused silica capillary (60 cm effective length) x 50 µm I.D. (O.D. 375 µm) from Polymicro Technologies, Phoenix, AZ, USA was used. The capillary was cut to the desired length using a SGT Shortix capillary column cutter (Middelburg, The Netherlands). A new fused silica capillary was coated as follows: The capillary was preconditioned with water (5 min), 0.1 M HCl (5 min) and 0.1 M NaOH (10 min) followed by 5.0 mg/ml Polybrene (PB) in water (5 min), 5 mg/ml Chondroitin sulfate A (CS) in water (5 min) and finally BGE for 10 min at 60 psi (413 kPa). Between the injections the capillary was washed with the BGE for 5 minutes. The coating was, however, regenerated every five runs by washing the capillary with 0.1 M NaOH followed by PB and CS solutions as described above. After the coating or recoating the system is ready to be used. Injections were performed hydrodynamically at 0.8 psi (5.5 kPa) for 10 seconds, followed by a second injection of the BGE at 0.3 psi for 5 seconds. Separations were performed in normal polarity mode by applying a potential of 10 to 17.4 kV across the capillary for 30 to 70 minutes. The observed electric current was 40 and 90 µA. As seen a higher voltage brings a faster separation at the expense of a higher current. The ramp time (t_{ramp}) was set to 0.17 min. The capillary cartridge and the sample storage were thermostated at 30°C and 10°C, respectively.

The analyses were carried out by performing two injections, where the first injected sample contains the reference standard while the analyte to be identified is presented in the second sample injection. However, the injection sequence does not affect the result. The first injected sample plug was electrophoresed for 10 or 20 min in order to get the sample plugs well

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separated. Following the second injection, a constant voltage of 10 to 17.4 kV (143-249 V/Cm capillary) was applied to complete the separation and finally acquisition of data was initiated.

2.2.2. MALDI-TOF MS. Measurements were performed on an Autoflex (Bruker Daltonics, Bremen, Germany) 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 and linear modes. Before analysis the instrument was externally calibrated with Bruker Daltonics standard protein or peptide mixtures. The peptide calibration standard II from Bruker consisted of seven peptides ranging from *m/z* 1046 to 3147, i.e., Angiotensin-II, Angiotensin-I, Substance-P, Bombesin, ACTH-clip (1-7), ATCH-clip (18-39) and Somatostatin (28). The protein calibration kit consisted of Trypsinogen (23982 Da), Protein A (44613 Da) and Albumin-Bovin (66431 Da). One µl sample was carefully mixed with 1 µl matrix consisting of either α-cyano-4-hydroxy-trans-cinnamic acid solution (for tryptic peptides) or sinapinic acid in 50 % acetonitrile and 0.1 % TFA. One µl of the mixture was 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 25-35 % and mass spectra were obtained by averaging 200 laser shots (4 x 50 shots) at different positions on the sample surface. The peptides were analysed in refelectron mode while undigested somatropin was analysed in linear mode. All samples used for post source decay (PSD) analysis were analysed in the reflector mode. The ion selector was set at chosen *m/z* value and the presence of neighbouring ions was considered by setting a selection window around the target mass (± 10 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 x 50

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shots) were collected. Spectra were finally pasted together to yield a single PSD data set, which was interpreted manually. Annotation of the mass signals was executed with the X-mass software (Bruker Daltonics).

2.2.3. Proteolysis. Somatropin reference standard was dissolved in water to a final concentration of approximately 1 mg/ml. 10 µl of the solution was diluted in 190 µl ammonium bicarbonate buffer (50 mM at pH 7.9) to make a 50 µg/ml somatropin solution. Unknown samples, where neither the presence nor the concentration of somatropin are known, were dissolved in ammonium bicarbonate buffer. To 10 µl of the protein solution in the buffer, 2.5 µl trypsin (200 µg/ml in 10 mM HCl) was added. The reaction was carried out for at least 30 minutes at either room temperature or at 4°C. The protein was digested in unreduced condition.

2.2.4. Endotoxins. The European Pharmacopeia monograph 2.6.14 method C (Turbidometric kinetic LAL method) was used for determination of bacterial endotoxins. The LAL test (limulous amoebocyte lysate test) is based on the biology of the horseshoe crab (Limulous). These animals produce LAL enzymes in blood cells (amoebocytes), as a primitive immune response, to bind and inactivate endotoxin from invading bacteria. The LAL test exploits the action of this enzyme, by adding LAL reagent to the tested product. Upon the analysis the test solution becomes turbid prior to gel-formation. The time required to produce a specified level of turbidity is inversely proportional to the amount of endotoxin in a sample.

The control standard endotoxin (0.5 mg/vial) with a potency of 4000 EU per vial was diluted with endotoxin-free water in endotoxin-free glassware. A series of standard dilutions with concentrations of 5 EU/ml, 0.5 EU/ml, 0.05 EU/ml and 0.005 EU/ml, were achieved. The samples were dissolved in 400 ml LAL water and diluted (1:10,000 and 1:100,000) with LAL water in soda glass tubes which were autoclaved at 200°C for 240 min before being used. The

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lysate was diluted with Pyrosol buffer. The experiments were performed with a Cape Cod spectrophotometer (Pyros Kinetix)

2.2.5. Sterility test. The European Pharmacopeia monograph 2.6.1 “STERILITY” (part of chapter 2.6 Biological tests) was used for the testing sterility. Two different samples were suspended aseptically with 1 ml of sterile water. The sterility test was performed under aseptic conditions in a cleanroom facility. The method used was membrane filtration (Steritest Pump Equinox, Millipore). Samples were filtrated and incubated in fluid thioglycollate medium and Trypticase soy broth (TSB) at +30-+35°C and +20-+25°C, respectively, for 14 days. The media containers were viewed daily for microbial growth. The first growth was observed after 72 h incubation in both media. A 100 µl aliquot was further plated on Tryptic soy agar (TSA) and Sabourad agar plates. The TSA plates were incubated at +30-35°C for 3 days and Sabourad plates at +20-25°C for 5 days. Growth on TSA plates was observed after 18 h and identification was started with gram-staining. A gram-positive rod from one ampoule and two different gram-negative rods from the other ampule were identified.

3. Results and discussion

The illegally distributed samples were analysed with two techniques based on physico-chemical methodologies. CZE in double injection mode was used for the purity determination and the identification of somatropin[17,18] and MALDI-TOF-MS was applied to the identification of somatropin through protein mass finger printing (PMF).[19]
Furthermore, in order to make a reasonable judgment on the safety of illegally distributed products some of the samples were subjected to endotoxin and microbial sterility testing, see Fig. 3.

3.1. Double injection capillary zone electrophoresis

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The p*I* of somatropin and its charge variants varies between 4.9 and 5.2.[28] Therefore, the proteins will migrate, as depicted in Fig 2B, in the following order at the used pH, i.e., pH 6.0, $I_1 > I_2 >$ somatropin $> I_3 > I_4$. The I_2, I_3, and doublet I_4 peaks correspond to a cleaved form of somatropin, Gln_{18} somatropin, and deamidated forms, respectively.[11]

Capillary zone electrophoresis in double injection mode (DICZE) was applied to both identification and purity determination of somatropin in unknown samples. DICZE provides the possibility of simultaneous analysis of the analyte to be identified and the reference standard. The pharmacopeia method for the analysis of somatropin was transferred to double injection mode, where the first or second injected sample plug consisted of the reference standard at a concentration of 1.0 mg/ml.[11] The method was validated for the quality control of somatropin reference standard.[11,17] In DICZE the peaks of the first injection will appear within a time window corresponding to the $t_{mig(0)}$ (migration time of water, Fig 2B). The time period for the partial electrophoresis (t_{PE}) of the first injection plug should therefore be shorter than $t_{mig(0)}$ and longer than Δt_{mig} in order to avoid inter-plug interference (i.e., $t_{mig(0)} > t_{PE} > \Delta t_{mig}$). The electropherogram in Fig. 2B illustrates the DICZE separation of a suspected somatropin sample (Plug II) along with the reference standard (Plug I). The difference between the migration times of water (the negative peak), and the last-migrating analyte ($t_{mig(n)}$), i.e., I_4, was approximately 16 min. Therefore, the first injected plug could be electrophoresed for at least 16 min prior to the second injection, which was electrophoresed for 20 min. It should be noted that for the analysis of other samples the t_{PE} was adjusted between 10 to 20 min depending on the applied voltage, capillary conditions, i.e., coated or uncoated capillaries. During the t_{PE} the analytes present in the first injected sample are separated in single-injection mode. The injected plugs are finally analysed simultaneously under a time period corresponding to the partial migration time of somatropin ($t_{mig(P)}$).[18]

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The identification was carried out by comparing the ratio (RMT) between the calculated migration time ($t_{\mathrm{mig(C)}}$) and the migration time of the reference standard, Eq. (1).

$$\mathrm{RMT} = t_{\mathrm{mig(c)analyte}} / t_{\mathrm{mig\ (Reference)}} \quad \text{Eq. (1)}$$

The migration time of the analyte being exposed to the partial electrophoresis was calculated as follows.[18]

$$t_{\mathrm{mig(c)}} = t_{\mathrm{mig(p)}} + t \quad \text{Eq. (2)}$$

and

$$t = t_{\mathrm{mig(IM)}}\ (\text{or } t_{\mathrm{migEM}}) - t_{\mathrm{mig(P)IM}}\ (\text{or } t_{\mathrm{mig(p)EM}}) \quad \text{Eq. (3)}$$

where IM, EM and $t_{\mathrm{mig(P)}}$ stand for internal marker (used to be presented in both analyte and the standard samples), external marker and partial migration time, respectively. The reference standard is used as external marker (EM_i), i.e., that reference standard is analyzed in double-injection mode after or prior to the sample analysis. The t_{EM} values will be used for the migration time calculations in the bracketing runs.[18] An accurate identification requires a RMT value of 1.0000.

The migration times were calculated by using either internal or external markers. The salt or water (the negative peak in the electropherogram, Fig. 2B) peaks were used as internal markers (IM). The RMT values from more than 200 analyses of different somatropin samples, i.e., both the CRS and illegal samples, were determined to be in the range of 0.995 and 1.006, which was considered to be acceptable for a successful identification, based on the experimental data.[18] The method provides an electrophoretic finger print of somatropin and its related proteins, which facilitates the final identification. The electrophoretic pattern of unknown sample and the reference standard should be comparable (see Fig. 2B). The

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hydrodynamic transport of the protein zones during the second injection, results in band broadening. This is depicted in Fig. 2B where the peaks from somatropin analogs in the first injected plug are broader than the peaks originating from the same analytes in the second plug. [18]

The method was also applied for the quality control of somatropin in suspected products. However, in order to get reliable results the concentration of unknown samples was adjusted to approximately 1 mg/ml, i.e., the same concentration as the reference standard, by comparing the peak area and the peak height of somatropin in the analyte sample with those of the reference standard. In order to supress matrix-induced migration time shifts, and thereby improve accuracy and precision of the identification, the analyte sample were desalted prior to the DICZE analysis.[29,30]

The impurity levels of the successfully identified samples are reported in Table 1. The results summarized in the Table show that none of the analysed samples met the specification requirements regarding impurity levels determined by the European Pharmacopeia.[11] In addition to the identified peaks, many of the analysed samples contained an extra unknown peak.

3.2. MALDI-TOF-MS analyses

In order to determine the identity of suspected somatropin samples, they were exposed to the MALDI-TOF-MS analysis together with somatropin reference standard. The analysis of more than 200 intact somatropin samples revealed that the measured molecular mass of somatropin deviated by less than ±15 Da from the theoretical average molecular mass, i.e., 22125.1 Da, Fig. 4.

However, in order to explicitly determine the identity of an illegally distributed product it is subjected to peptide mass finger printing (PMF) after trypsination of the sample content,[19] Fig

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4. *In silico* trypsination of the protein yields 20 peptide fragments and a single amino acid, i.e., K_{168}. The most frequently observed peptides are listed in Table 2.

The results from analysis of many somatropin samples showed that MALDI fingerprinting, using trypsin as a proteolytic enzyme, covered approximately 70 % of the primary structure. There is a unique mass signal at *m/z* 1400.638, which has been utilized as the signature peptide to confirm the identity of somatropin[19]. This peptide arose from a double peptide consisting of two tryptic peptide fragments (i.e., $IVQC_{182}R$ and $SVEGSC_{189}GF$), being bound to each other through the C_{182}-C_{189} disulfide-bridge,[19] Fig. 5. The mass signal disappeared from the spectrum upon reduction and/or alkylation of the peptide.[19] Mass signals originating from incomplete digestion fragments further confirm the identity of somatropin. A tryptic peptide containing oxidized methionine, i.e., $DM_{(ox)}DKVETFLR$ (at *m/z* 995.5), was also identified (Table 2).

A successful identification requires a molecular mass in the range of 22125.1 ± 15 Da and a tryptic map being consistent with the theoretical map as well as the presence of the signature dipeptide. A few of the tryptic peptides were also analyzed by MALDI in the PSD mode, to confirm the amino acid sequence.[19]

The information obtained from the tryptic peptide map provides confidence in the identification of somatropin, which was further confirmed by DICZE as described above, see Fig 3.

The analyses showed that only 48% of the 200 tested illegal samples contained somatropin. The remaining 52% contained either no active ingredients or other peptides and proteins, which will be reported in a future paper.

3.3. Endotoxin and microbial testing

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Four randomly selected samples were analysed to determine their endotoxins levels. It was found that the endotoxin levels were in the range of 342-533 IU/vial. The upper limit for endotoxin levels is 5.0 IU/kg of body mass per injection.[11] The high amount of endotoxins can cause serious side effects.[21-23] It should be mentioned that common procedures such as ultrafiltration[31] or affinity chromatography[32], are used to remove endotoxins.

Sterility test performed on two of the samples demonstrated that one of the samples was contaminated with an aerobic gram-positive bacillus while the other one with aerobic gram-negative rods. The gram positive and catalase-positive colony was identified as *Bacillus cereus* endopores. Endospores produce harmful toxins which can cause food poisoning. *B. cereus* has also great potential of being an opportunistic pathogen and sometimes even a primary pathogen. One of the gram-negative, oxidase-positive colonies was found to be *Pseudomonas fluorescens biovar* . The results indicated that the other gram-negative colony could be a member of group CDC *biovar*. Gram-negative oxidase-positive aerobic rods are often associated with spoilage of food and are usually isolated from the environment, e.g. soil, dust and water. The high count of gram-negative rods is responsible for the high endotoxin content of the samples. In addition, a heavy bacterial growth on the plate indicated that there may have been other gram-negative species. The identification of these minor colony types was not performed.

The endotoxin levels and microbiological findings strongly indicate that these products were not manufactured under GMP controlled conditions. Therefore, it can be concluded that administration of these products are associated with potential health risks.

4. Conclusions

The present paper presents an approach based on physicochemical and biological methodologies for the identification and quality control of somatropin in illegally distributed

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somatropin products. The protein mass finger printing using MALDI-TOF-MS demonstrated to be a reliable method for the identification of somatropin. The presence of the 'signature peptide' at *m/z* 1400.638 in the mass spectrum was considered to be crucial for the identification. The DICZE identification approach was based on a comparison between the calculated migration time of the analyte and the observed migration time of the reference standard, being analysed in a double injection run. The migration time ratios (RMT) of positively identified samples varied between 0.995 and 1.006. The DICZE is also used for the purity determination of the analysed samples. The method is able to provide a finger print of the intact somatropin and its related proteins, which facilitates the final identification. The quality and safety of the analysed samples, regarding purity and endotoxin level as well as the microbiological quality indicate that the products present potential serious threat for the users and public health. It can be mentioned that approximately half of the analysed samples did not contain somatropin but other ingredients, which may further question and complicate the safety of these products.

Abbreviations

MALDI-TOF-MS Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

BGE Background electrolyte

CRS Chemical reference substance

RMT Relative migration time

DICZE Double injection capillary zone electrophoresis

PMF Peptide mass finger printing

PSD Post source decay (MALDI-TOF-MS)

Analytical Methods Accepted Manuscript

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Acknowledgements

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Figure legends

Fig. 1- Primary structure of somatropin.

Fig. 2- Examples of three different confiscated glass vials that suspected of containing somatropin (A) and DICZE-analysis of somatropin reference standard (plug I) along with an illegally distributed somatropin sample, plug II (B). Separation conditions: the separation was performed in a PB/CS double coated capillary over which a voltage of 10.0 kV was applied. The t_{PE} was adjusted to 20 min. Other separation conditions are given in experimental section.

Fig. 3- The flowchart of somatropin identification by MALDI-TOF-MS and DICZE. Both the molecular mass of the native protein and its tryptic peptides (PMF) were used for the identification. The identification by DICZE was carried out by determining the relative migration time between the reference standard and unknown analyte. The separation pattern between the reference standard and the analyte were also compared. The purity of the sample was determined by calculating the peak area ratio between each somatropin related protein to the sum of the peak areas.

Fig. 4- MALDI-TOF-MS analysis of native somatropin CRS. The theoretical average molecular mass of somatropin is 22125.1 Da. Experimental conditions as those in the experimental section.

Fig. 5- MALDI mass spectrometry analysis of digested somatropin. Experimental conditions as those in the experimental section.

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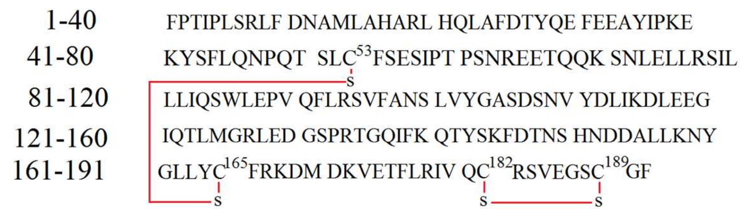

Figure 1

Fig. 1- Primary structure of somatropin.
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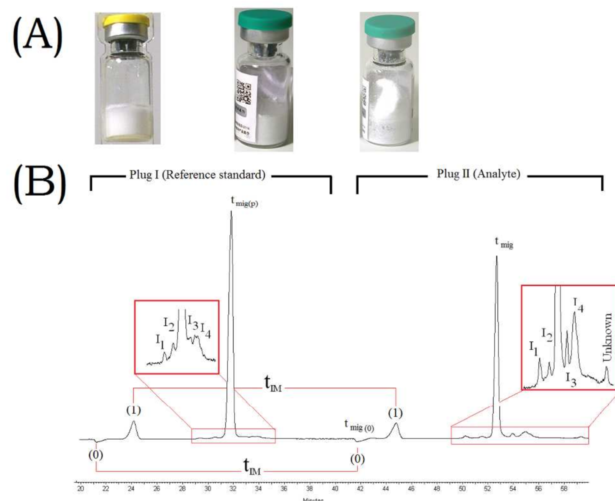

Figure 2

Fig. 2- Examples of three different confiscated glass vials that suspected of containing somatropin (A) and DICZE-analysis of somatropin reference standard (plug I) along with an illegally distributed somatropin sample, plug II (B). Separation conditions: the separation was performed in a PB/CS double coated capillary over which a voltage of 10.0 kV was applied. The tPE was adjusted to 20 min. Other separation conditions are given in experimental section.
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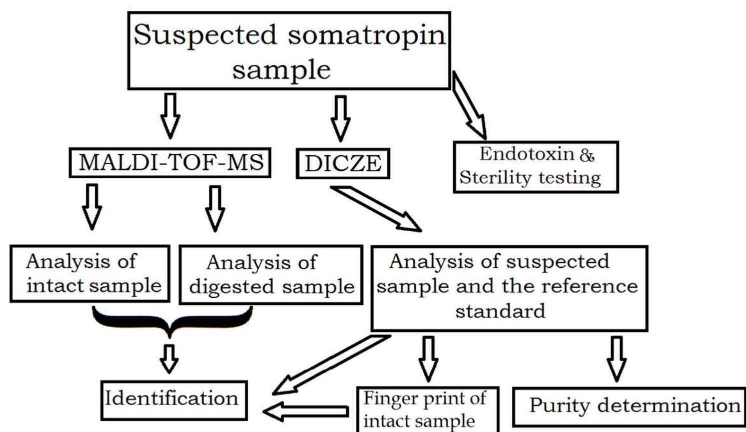

Figure 3

Fig. 3- The flowchart of somatropin identification by MALDI-TOF-MS and DICZE. Both the molecular mass of the native protein and its tryptic peptides (PMF) were used for the identification. The identification by DICZE was carried out by determining the relative migration time between the reference standard and unknown analyte. The separation pattern between the reference standard and the analyte were also compared. The purity of the sample was determined by calculating the peak area ratio between each somatropin related protein to the sum of the peak areas.
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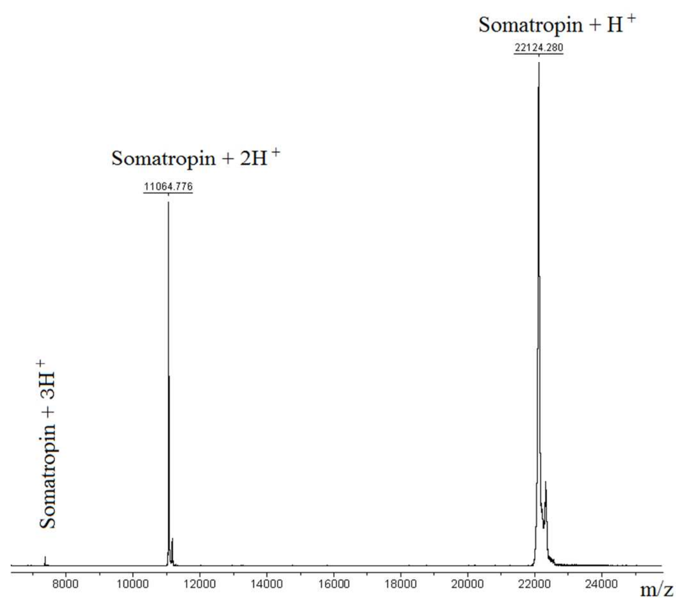

Figure 4

Fig. 4- MALDI-TOF-MS analysis of native somatropin CRS. The theoretical average molecular mass of somatropin is 22125.1 Da. Experimental conditions as those in the experimental section.

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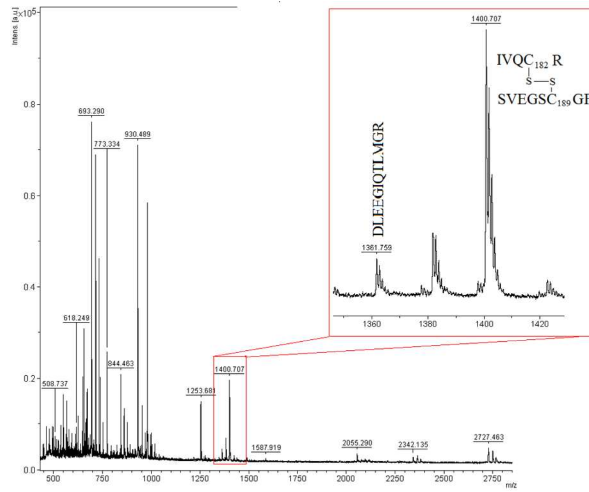

Figure 5

Fig. 5- MALDI mass spectrometry analysis of digested somatropin. Experimental conditions as those in the experimental section.

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Table 1 A comparison between the purity of 32 different illegally distributed somatropin products and somatropin CRS (two different batches)

Peak (see Fig. 3)	Average (Illegal samples) (±%CV)	Average (CRS) (±%CV)	Specification[a]
Somatropin (Main peak)	76.5% (±14.7%) Range: 56.3% - 88.9%	> 98.0%	>90%
I_1	2.3% (±35.4%) Range: 0.2% - 6.5%	0.20% (±0.13%)	< 2%
I_2	2.7% (±72.6%) Range: 0.3% - 4.1%	0.34% (±0.16%)	< 2%
I_3	5.2% (±103.6%) Range: 0.03% - 26.2%	0.36% (±0.38%)	< 2%
I_4	10.88% (±45.4%) Range: 0.01% - 22.3%	0.50% (±0.42%)	< 5%
Total	21.1% (±44.3%) Range: 9.3% - 36.6%	1.4% (±0.30%)	< 10%
Other impurities	2.6% (±86.3%) Range: 0.1% - 7.7%	-	-

[a]According to the European Pharmacopoeia.[11]

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Table 2 The most frequently observed tryptic peptides in the MALD-TOF-MS MFP map of somatropin (n=200).[19]

Mass (Da)	Peptide sequence	Position
929.533	FPTIPLSR	1-8
978.496	LFDNAMLR	9-16
994.495	LFDNAM$_{(oxid)}$LR (Oxidized Met)	9-16
2341.127	LHQLAFDTYQEFEEAYIPK	20-38
2726.323	LHQLAFDTYQEFEEAYIPKEQK[a]	20-41
761.355	EETQQK	65-70
1586.180	EETQQKSNLELLR[a]	65-77
843.481	SNLELLR	71-77
2054.193	ISLLLIQSWLEPVQFLR	78-94
2077.193	ISLLLIQSWLEPVQFLR (+Na^+)	78-94
2261.122	SVFANSLVYGASDSNVYDLLK	95-115
2284.122	SVFANSLVYGASDSNVYDLLK (+Na^+)	95-115
1360.666	DLEEGIQTLMGR	116-127
772.371	LEDGSPR	128-134
692.386	TGQIFK	135-140
625.307	QTYSK	141-145
1488.684	FDTNSHNDDALLK	146-158
501.199	DMDK	169-172
1252.612	DMDKVETFLR	169-178
617.332	IVQ**C**R	179-183
1399.638	IVQ**C**R/SVEGS**C**GF (**-S-S-** bound)[b]	179-183 & 184-191

[a] Miscleaved peptide. [b] The signature peptide (see Fig. 4)

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Table 3 Endotoxin test assay for 4 different illegally distributed somatropin samples were analyzed. The analyses were performed according to the European Pharmacopeia monograph 2.6.14 method C (kinetic turbidometric LAL method).

Sample	Result[a]	Acceptance criterium[b]
#1	425 EU / ml	<5.0 EU / ml
#2	480 EU / ml	<5.0 EU / ml
#3	533 EU / ml	<5.0 EU / ml
#4	521 EU / ml	<5.0 EU / ml
#5	342 EU / ml	<5.0 EU / ml

[a] EU per ml or per vial since the content of each vial was dissolved in 1.0 ml LAL water.

[b] According to the European Pharmacopoeia

Analytical Methods Accepted Manuscript

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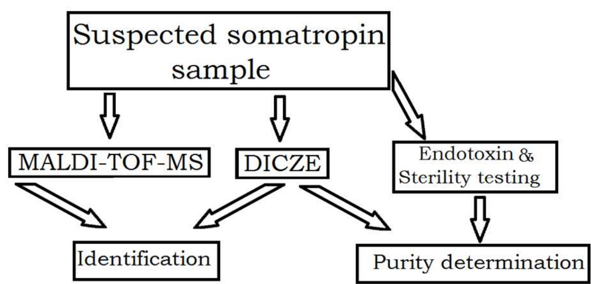

Grafical Figure

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