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Determination of insulin in innovative formulations by means of LC coupled to fluorescence detection

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ABSTRACT

A fast and simple method based on LC with fluorescence detection has been developed for the determination of insulin in innovative formulations consisting of microparticles and inserts for oral and nasal drug administration, respectively.

A reverse-phase C8 column and a mobile phase composed of pH 3.7, 40 mM sodium sulphate solution and acetonitrile (24%, v/v) were employed. Using isocratic elution at 1.0 mL/min flow, analysis is completed within 7 min.

Three different kinds of spray-dried microparticles were analysed, consisting of an insulin loaded core composed of chitosan salts (chitosan succinate, chitosan adipate or chitosan suberate) coated with stearic acid. Nasal inserts consisted of chitosan/hyaluronate polyelectrolyte complexes which were loaded with insulin and freeze-dried.

Insulin was extracted from both the oral and nasal formulations using pH 7.4 phosphate buffer. The employment of fluorescence detection (λ_{exc} = 276 nm, λ_{em} = 306 nm) granted high selectivity, with no interference from the matrix.

Full method validation was performed with good results in terms of linearity (insulin concentration range 0.10–30.0 μg/mL), LOD (0.03 μg/mL) and LOQ (0.10 μg/mL), precision (R.S.D.% < 3.6) and accuracy (recovery percentage > 90.0%).

Insulin content in innovative formulations, expressed as percentage w/w, resulted to be between 0.90 and 0.97 for oral innovative formulations, while an average value of 342 μg of insulin was found in a single nasal insert, in good agreement with preparative protocols.

1. Introduction

Insulin, a peptide hormone, consists of two chains (51 aminoacids in total), named A (21 amino acids) and B (30 amino acids), connected by two disulfide bridges (Fig. 1a).

Nowadays insulin represents the main therapy for insulin-dependent diabetes mellitus (IDDM or Type I diabetes), while Type 2 diabetes patients require insulin administration when other medications become inadequate [1,2].

It is usually administered via subcutaneous injection, since proteins and peptides are digested into their constitutive aminoacids by several enzymes along the gastrointestinal tract [2].

Insulin therapy requires at least a two daily dose regimen and repeated subcutaneous injection may cause local adverse effects, such as lipoatrophy and lipohypertrophy, which may affect the absorption kinetics; erythema, pruritus, and induration are the most common local allergic reactions to insulin [3]. This represents a serious problem for diabetic patients who need a chronic insulin therapy for life.

For these reasons, great efforts were recently carried out in order to find easier and more compliant administration routes, such as the intranasal and oral ones. The strategies adopted for oral insulin delivery include the use of permeation enhancers, protease inhibitors, enteric coatings and microsphere formulations [2]. The latter, in particular, are attractive, since they can be up-taken in small intestine [4] and their delivery can easily be controlled by their physicochemical properties.

The development of innovative formulations for the administration of insulin is a key-issue and we have already carried out studies in this field, developing and analysing chitosan microparticles for controlled insulin release [5]. Now we have implemented a novel method to reliably determine insulin in innovative formulations for oral and nasal delivery.

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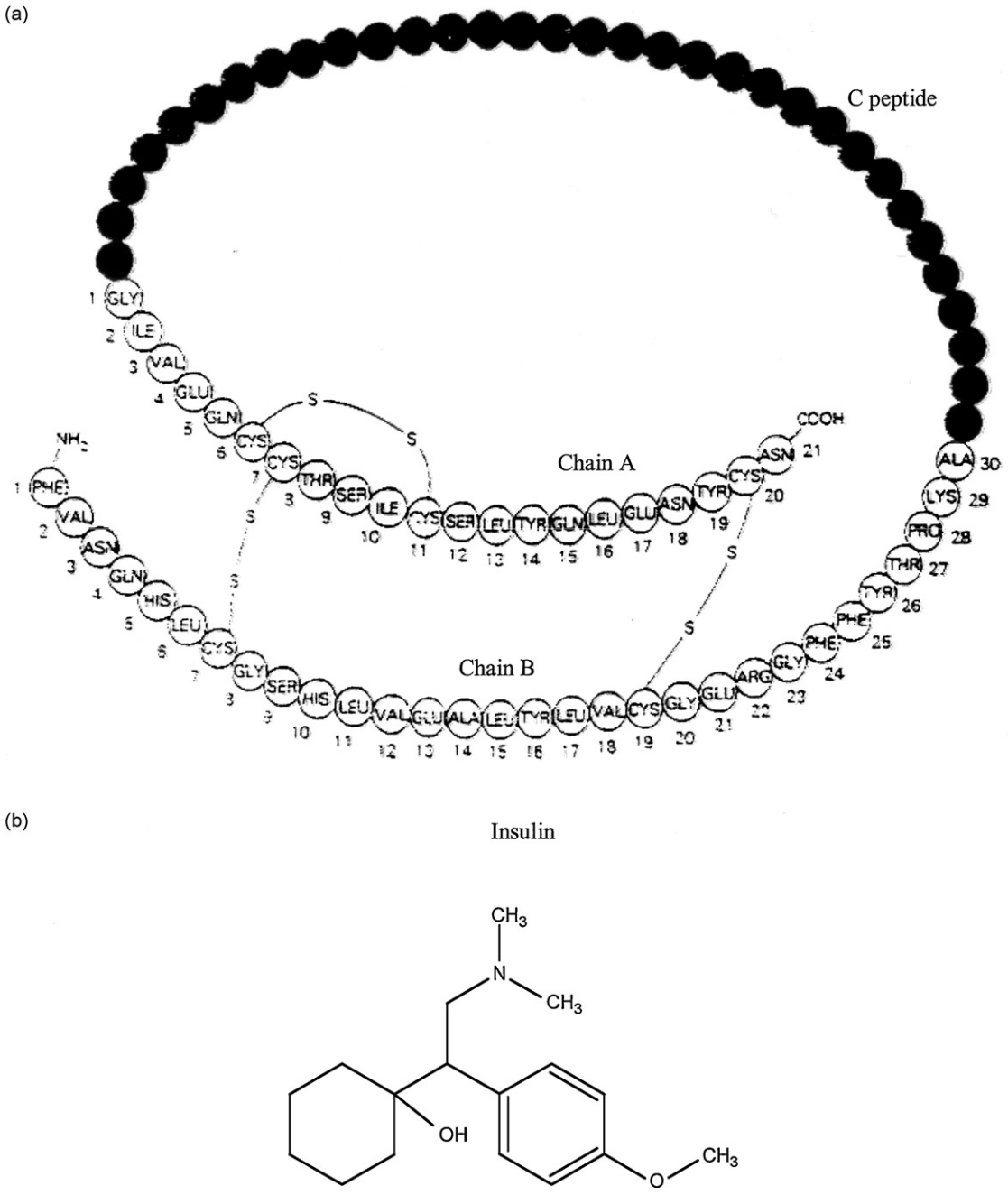

Fig. 1. Chemical structure of (a) insulin (and proinsulin) and (b) venlafaxine (IS).

Oral formulations consist of microparticles with an inner core loaded with insulin and an external coating represented by stearic acid and SPAN 60. The formulation should prevent gastric degradation of insulin, allowing a controlled release in the colon site. In order to evaluate the best composition of these oral formulations, different kinds of microparticles with different inner cores (consisting of chitosan and different acids, namely succinic, adipic and suberic) have been prepared.

Nasal inserts consist of freeze-dried chitosan/hyaluronate polyelectrolyte complexes, loaded with insulin (350 μg) and finally lyophilized.

It is clear that the first step when developing such a complex formulation is the accurate and reliable determination of the insulin content. This is particularly valid during the development and optimisation of the formulation production protocol, but a quality control is always required also during routine production of the final pharmaceutical product.

LC has been currently employed for insulin analysis in pharmaceutical formulations, often using UV or diode-array detection [6–14].

As regards the analysis of innovative formulations, insulin content in microspheres for subcutaneous delivery were analysed by reverse-phase and size-exclusion LC using UV detection (at 276 nm) [7]. LC with UV detection at 220 nm (together with electron microscopy, laser light scattering and SDS-PAGE) was used for the analysis of insulin in nanoparticles for injection [9]. A water-in-oil emulsion for oral delivery of insulin was analysed by LC with UV detection at 215 nm [10].

Insulin is a fluorescent molecule and can be determined using LC with fluorescence detection, with great advantage in terms of selectivity and sensitivity of the method, compared to UV detection.

Anyway, derivatisation reactions have been employed for the analysis of insulin [15–18]: using LC with fluorescence detection, insulin was determined in spiked serum after derivatisation with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole [15]; insulin was labelled with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate for competitive chromatographic immunoassays [17]; 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate was used to derivatise and analyse insulin isomers in the islets of Langerhans [18]. Only one paper reports a method based on LC with fluorescence detection applied to the quality control of insulin vials for injection [19]. The method was not validated in terms of precision and accuracy. Analysis time is quite long (about 15 min) and a gradient mode elution is employed. Moreover, the method was only applied to injectable formulations, whose analysis does not require any extraction procedure or sample pre-treatment.

The method presented herein is based on LC with fluorescence detection, it does not require a derivatisation step nor gradient mode elution. Moreover, it allows a fast, accurate and selective determination of insulin in microparticles for oral delivery and nasal inserts.

2. Experimental

2.1. Chemicals

All chemicals were analytical grade. Bovine insulin was purchased from Sigma–Aldrich (St. Louis, MO, USA); Venlafaxine (1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol; Fig. 1b) used as the internal standard (IS) was kindly provided by Wyeth (Madison, NJ, USA).

Methanol and acetonitrile (both LC grade) were purchased from Sigma–Aldrich, 37% (w/w) hydrochloric acid (HCl), 85% (w/w) phosphoric acid and monobasic potassium phosphate pure for analysis (>99%) were purchased from Carlo Erba (Milan, Italy).

Low-viscosity chitosan (96% deacetylated), butanedioic acid (succinic acid), hexanedioic acid (adipic acid), octanedioic acid (suberic acid) and SPAN 60 were purchased from Fluka (Buchs, Switzerland).

Sodium hyaluronate (M_r 1,650,000) and chitosan low-viscous (viscosity $\leq$ 200 mPa s at C = 1% in 1% acetic acid, T = 20 °C; deacetylation degree 97%) were obtained from Fluka. Mannitol was obtained from Carlo Erba.

Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus from Millipore (Milford, USA).

2.2. Solutions

Stock solutions of insulin (1 mg/mL) were prepared in 0.1 M HCl and were stored at −80 °C; stock solutions of the IS (1 mg/mL) were prepared in methanol and were stored at −20 °C. Stock solutions were stable for at least 1 month, as assessed by LC assays.

Working standard solutions were prepared fresh every day by diluting stock solutions with the mobile phase.

2.3. Instrumentation and chromatographic conditions

Analysis were carried out using a Jasco (Tokyo, Japan) PU-2089 PLUS chromatographic pump. Separation was performed using an Agilent Zorbax Eclipse XDB C8 reversed-phase column (150 mm × 4.6 mm i.d.; 5 μm; pore size 80 Å) and a Phenomenex (Torrance, CA, USA) SecurityGuard C8 pre-column (4.0 mm × 3.0 mm i.d.; 5 μm); elution was isocratic at 1 mL/min.

The mobile phase was composed of a 40 mM sodium sulphate solution, whose pH was adjusted to 3.7 using 85% (w/w) phosphoric acid, and acetonitrile (24%, v/v). Before use, it was filtered through a Phenomenex membrane filter (47 mm membrane, pore size 0.2 μm, nylon) and degassed by an ultrasonic bath.

Samples were injected through a 20-μL loop and chromatograms were recorded by means of a Jasco FP-2020 spectrofluorimetric detector set at λ_{exc} = 276 nm, λ_{em} = 306 nm.

Data processing was handled by means of a Jasco Borwin 3.0 software.

A Crison (Barcelona, Spain) Basic 20 pHmeter was used for pH measurement.

2.4. Insulin-containing oral microparticles preparation

Three different microparticles were prepared, by combining chitosan with adipic, succinic and suberic acid, respectively, according to the procedure described below.

In particular, 20 mM solutions of adipic and succinic acid were prepared by weighing 236 and 292 mg of adipic and succinic acid, respectively, which were dissolved in 100 mL of water; the 20 mM solution of suberic acid was prepared by weighing 174 mg of suberic acid and dissolving in 100 mL of a water/acetone (4/1, v/v) mixture.

The pH of the obtained solutions was adjusted to 4 using 1 M HCl, then an amount corresponding to 325 mg of chitosan was added to each solution; the chitosan solutions were kept under agitation at room temperature for 24 h.

An amount corresponding to 10.4 mg of insulin (300 UI) was added into each chitosan solution, which was finally dried using a spray-drying apparatus (Büchi Mini Spray Dryer, B-191, Flawil, Switzerland).

Microparticles were then coated by suspending 300 mg of microparticles in 50 mL of an ethanolic solution containing 250 mg of stearic acid and 50 mg of SPAN 60; the suspension obtained was kept under agitation, nebulised and dried using a spray-drying apparatus.

2.5. Insulin-containing nasal inserts preparation

Chitosan/hyaluronate complexes were prepared by separately dissolving 0.75 mmol of sodium hyaluronate and 0.75 mmol of low-viscous chitosan in 150 μL of a pH 2.0 hydrochloric acid solution. The chitosan solution was added to the sodium hyaluronate solution in a 3/7 molar ratio and stirred for 24 h at room temperature. After centrifugation (10 min, 10,000 rpm, ALC 4239R centrifuge, Milan, Italy) the precipitate was separated, washed with deionised water and dried under vacuum to constant weight.

Inserts were prepared by adding 100 μL of a 3.5 mg/mL insulin solution in pH 7.4 phosphate buffer to 10 mg of chitosan/hyaluronate complexes and mannitol (in a 9/1, w/w ratio). The resulting suspension was filled into polypropylene microcentrifuge tubes, allowed to settle to swell and remove air, then lyophilised. Inserts were stored in a dessiccator.

2.6. Extraction and determination of insulin content in microparticles for oral administration

Amounts corresponding to 1.0 mg of microparticles were weighed and transferred into a vial containing 1.0 mL of pH 7.4 50 mM phosphate buffer and 10 μL of IS standard solution (final concentration: 50 μg/mL). The resulting mixture was shaken for 10 min and then centrifuged (4000 × *g*, 25 °C). The supernatant was separated by means of a pasteur pipette, while the microparticle fraction was extracted again with 1.0 mL of pH 7.4, 50 mM phosphate buffer. Finally, both collected supernatant fractions were put

together into a vial, aliquots were diluted with the mobile phase to the suitable concentration and injected into the LC apparatus.

Insulin content was calculated as [($mg_{insulin}/mg_{microparticles}$) × 100] and expressed as w/w%.

2.7. Determination of insulin content and insulin release from nasal inserts

For the evalutation of insulin release from nasal inserts, a self-made diffusion cell was employed. Inserts (V = 0.1 mL) were placed on the sintered-glass filter plate (pore size 90–150 μm) of a Borosil® glass filter crucible (inner diameter = 2.0 cm, capacity 15 mL) and the system was closed using Parafilm®. The crucible was placed vertically into a release medium container filled with 10 mL of a pH 7.4 phosphate buffer and adjusted to the height of the release medium surface, in order to maintain the porous glass membrane wetted but not submersed. Magnetic stirring and a 37 °C temperature were maintained. 300 μL aliquots were taken after 30, 60, 120, 180, 240, 300 and 360 min, diluted with the mobile phase to the appropriate concentration and then injected into the LC system. The withdrawn volumes were replaced by fresh medium.

For the evaluation of the total insulin content in nasal inserts, inserts were treated with 10 mL of pH 7.4 phosphate buffer and then stirred for 7 h at 37 °C. Aliquots of the supernatant were then diluted with the mobile phase and analysed with the LC system.

Insulin content was expressed as $\mu g_{insulin}$/insert.

2.8. Method validation

The method was validated in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy, according to the USP XXVIII [20] and "Crystal City" [21] guidelines.

2.8.1. Linearity

Linearity was assessed by analysing insulin standard at six different concentrations (between 0.10 and 30.0 μg/mL), while maintaining the concentration of the IS constant at the value of 50.0 ng/mL.

Each analysis was repeated in triplicate and the calibration curve was determined by measuring the area insulin/IS peak area ratios as a function of the corresponding insulin concentrations. The calibration equation was calculated by means of the least square method.

2.8.2. LOD and LOQ

The values corresponding to LOQ and LOD were estimated as the insulin concentrations that give rise to peaks whose heights correspond to 10 and 3 times the background noise (both automatically determined by the integration software).

2.8.3. Precision

In order to evaluate the precision of the method, insulin standard solutions were prepared at three different concentrations, corresponding to the lowest, the middle and the highest point of the calibration curve, containing the IS at the concentration of 50.0 ng/mL. Each analysis was repeated six times within the same day and in six different days to calculate the repeatability and the intermediate precision, respectively. Values were expressed as the relative standard deviation percentage (R.S.D.%).

2.8.4. Accuracy

Accuracy was evaluated by analysing microcapsules and nasal inserts as such and after being spiked with standard insulin at three different concentrations (always maintaining the IS constant at the concentration value corresponding to 50.0 ng/mL). Accuracy was finally calculated as the recovery percentage.

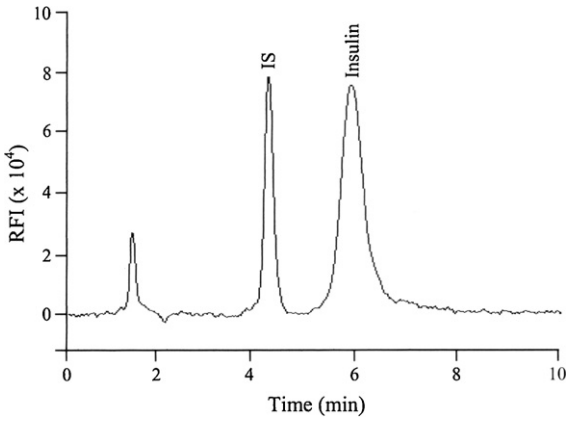

Fig. 2. Chromatogram obtained from the analysis of insulin standard solution (2 μg/mL), containing the IS (50 ng/mL). LC conditions: see Section 2.

3. Results and discussion

3.1. Chromatographic conditions

Starting from our previous paper [22], where a spectrofluorimetric method was used for the determination of insulin in pharmaceutical formulations, we decided to verify whether the chosen wavelengths were suitable also for the present work, being the fluorescence emission influenced by the solvent. In the present case, fluorescence is recorded in a solution (mobile phase) consisting of a pH 3.7, 40 mM sodium sulphate solution and acetonitrile (24%, v/v), while in our previous paper [22] fluorescence was recorded in 0.01N HCl solutions. After preliminary spectrofluorimetric assays, an excitation wavelength of 276 nm and an emission wavelength of 306 nm were chosen as the best conditions for insulin detection during LC analysis.

As regards the choice of the optimal chromatographic conditions, different columns with different stationary phases (C18 and C8) were tried. It was found that the less hydrophobic stationary phase (C8) granted appropriate insulin retention with shorter analysis times (6.0 min).

A mobile phase composed of a mixture of phosphate buffer (pH 3.7) and acetonitrile (76/24, v/v) was initially tested out. Using these conditions insulin peak showed remarkable broadening and asymmetric shape. When the phosphate buffer was substituted by a 40 mM, pH 3.7 sodium sulphate solution, the problem could be solved and neat and symmetric peaks were obtained. The explanation for this fact is the particular affinity of sulphate anionic groups toward cationic insulin (whose pI is about 5). Thus, insulin can form an ion pair with sulphate, in turn allowing the formation of stronger interactions with the lipophilic mobile phase. By comparing to the previously published paper [19], it can be observed that the present method is faster, more feasible and grants a better peak resolution.

Several substances were tested, in order to find the suitable internal standard: quinine, fluorescein, dichlorofluorescein, mirtazapine, citalopram, fluoxetine, paroxetine and venlafaxine. The latter resulted to be the best one, because it showed an intense fluorescence emission and shorter retention time compared to insulin, thus not influencing the total analysis time.

A chromatogram obtained from the analysis of insulin (2.0 μg/mL), containing the IS (50.0 ng/mL) is represented in Fig. 2. As one can note, both insulin and IS standard peaks are symmetric

Table 1
Precision data for insulin standard solutions

Insulin concentration ($\mu g\,mL^{-1}$)	Repeatability[a] (R.S.D.%)	Intermediate precision[a] (R.S.D.%)
0.10	2.5	3.0
10.0	1.8	2.4
30.0	1.2	1.6

[a] $n = 6$.

and well resolved. The retention times correspond to 4.6 min for IS and 6.0 min for insulin.

3.2. Method validation

Linearity was assessed as described in Section 2, by analysing insulin standard solutions at six different concentrations (between 0.10 and 30.0 μg/mL), while maintaining constant the concentration of the IS to the value of 50.0 ng/mL.

Good linearity ($r^2 = 0.9996$) was found in the insulin concentration range 0.10–30.0 μg/mL, which corresponds to the 1.72×10^{-8}–5.17×10^{-6} molar range. The corresponding equation, obtained by means of the least square method, is $y = 1.721x + 0.012$.

The limit of quantitation (LOQ) and limit of detection (LOD) were evaluated as the insulin concentrations that give rise to peaks whose heights correspond to 10 and 3 times the background noise, respectively; the values obtained correspond to 0.10 and 0.03 μg/mL, respectively, confirming that sensitivity obtained is appropriate for the present purpose.

Precision was evaluated by analysing insulin standard solutions at three different concentrations and the results, expressed as R.S.D.% values, are reported in Table 1.

Good results were obtained, with R.S.D.% values always lower than 3.1.

3.3. Insulin extraction from microparticles for oral delivery

In the view of evaluating the formulations and choosing the best ones for future investigations (e.g. in vivo studies), the accurate and reliable determination of the insulin content is crucial.

At the beginning, the optimal pH for insulin extraction was investigated. It should be kept in mind that these microparticle formulations have been developed to protect insulin from degradation in the stomach and allow a drug release in the colons site; this means that the prepared formulations will release the drug in a basic (or neutral/slightly basic) but not in an acidic environment. Anyway, insulin is known to be unstable in basic media [23]. For this reason, using 50 mM phosphate buffer, pH values between 7.0 and 7.8 were investigated. Best results (extraction yields) were obtained at both pH 7.8 and pH 7.4 and the latter was chosen because of the above mentioned insulin stability.

Different volumes and extraction steps were then investigated. It was found that two extraction steps (using 1.0 mL of phosphate buffer each time) were required to ensure complete insulin extraction. In fact, a single extraction step procedure using a double volume of phosphate buffer (2.0 mL) resulted to be not satisfactory, giving insulin extraction corresponding to about 80% of the total insulin content.

To verify that complete extraction was obtained, a third extraction step was carried out and the supernatant obtained was analysed: no insulin peak was detected (i.e. insulin peak lower than the LOD value).

As a tentative to speed up the procedure, a filtration (using 0.4 μm cellulose filters) was also tested out as an alternative to centrifugation and separation of the supernatant fraction. Anyway, this procedure resulted to be not suitable, as no insulin was recovered, most probably due to absorption of insulin on the filter membrane.

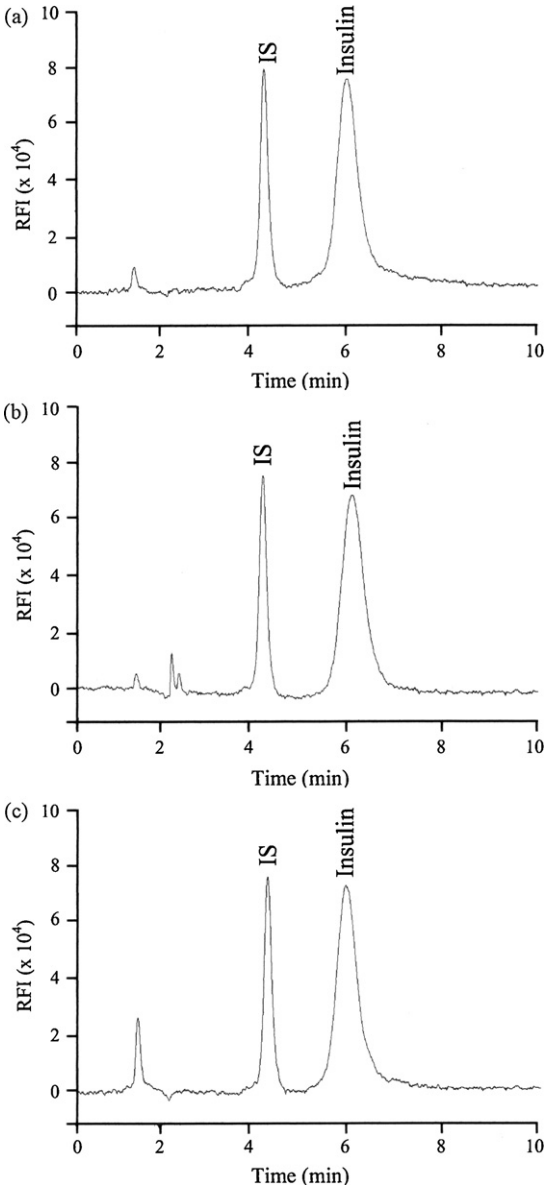

Fig. 3. Chromatogram obtained from the analysis of insulin extracted from microparticles prepared with chitosan and (a) adipic acid, (b) succinic acid, (c) suberic acid. IS concentration: 50 ng/mL. Conditions: see Section 2.

3.4. Analysis of insulin content in microparticles for oral delivery

All different microparticle formulations for oral delivery were treated as described and the supernatant solutions obtained from the extraction procedure were diluted with the mobile phase and analysed. Representative chromatograms are reported in Fig. 3. They correspond to the analysis of insulin extracted from microparticles whose core was prepared with chitosan and adipic acid (Fig. 3a), chitosan and succinic acid (Fig. 3b), chitosan and suberic acid (Fig. 3c). As one can note, insulin is detected without any influence from the matrix, e.g. no difference is observed if compared to the chromatogram obtained when analysing insulin standard solutions. The fluorescence detection resulted to be a good choice

Table 2
Determination of insulin content in innovative formulations

Formulation		Insulin content	Repeatability[a] (R.S.D.%)	Intermediate precision[a] (R.S.D.%)
Oral formulations	Type 1[b]	0.97% (w/w)	3.1	3.5
	Type 2[b]	0.91% (w/w)	2.4	2.8
	Type 3[b]	0.90% (w/w)	2.0	2.2
Nasal insert		342 ($\mu g_{insulin}$/insert)	3.0	3.6

[a] $n = 6$.
[b] Microparticle inner core composition: Type 1: chitosan/adipic acid; Type 2: chitosan/succinic acid; Type 3: chitosan/suberic acid.

because, albeit sensitivity does not represent a crucial issue for the present topic, selectivity does, considering that several potential interfering compounds are present in the formulations developed.

Insulin peak was identified by comparison of the retention time in the chromatograms obtained from the analysis of standard solutions. In order to rule out a possible interference from the matrix, "blank" microparticles (without addition of insulin) were prepared and analysed as described: no peak was observed in the chromatogram, thus demonstrating the selectivity of the method.

Insulin content in the different microparticles was evaluated by interpolating the chromatographic peak areas obtained in the calibration curve. Data obtained, calculated as $[(mg_{insulin}/mg_{microparticles}) \times 100]$, are reported in Table 2. As one can note, the highest loading value was found for the chitosan/adipic acid microparticles, even if all the three microparticle types can be considered acceptable. Repeatability and intermediate precision data are satisfactory (R.S.D.% < 3.6), as can be observed in Table 2.

3.5. Analysis of insulin content in nasal insert

The pre-treatment procedure for nasal inserts is faster if compared to the procedure adopted for microparticles for oral delivery. This is due to the fact that these nasal inserts are biodegradable: it was found that after 7 h complete swelling and therefore a complete insulin release were obtained.

For this reason, nasal inserts were treated with 10 mL of pH 7.4 phosphate buffer, stirring the solution for 7 h at 37 °C. Aliquots of the solution were then diluted with the mobile phase and injected into the LC system.

Insulin content in nasal inserts was calculated and expressed as $\mu g_{insulin}$/insert. The obtained results (average insulin content for each insert corresponding to 342 μg) are in good agreement with the total insulin content expected according to the preparation protocol (350 μg/insert). Complete results are reported in Table 2.

3.6. Analysis of insulin release from nasal inserts

In order to evaluate the release kinetic from nasal formulations, inserts were placed in the diffusion cell together with 10 mL of pH 7.4 phosphate buffer, as described in Section 2. Insulin solutions withdrawn from the diffusion cell at the different times were appropriately diluted with the mobile phase and analysed.

Representative chromatograms, obtained at different withdrawal times (30, 120 and 360 min) are reported in Fig. 4a–c, respectively. As expected, the chromatograms obtained do not show any difference compared to the one obtained from the analysis of insulin standard solutions. No interference from the matrix was observed.

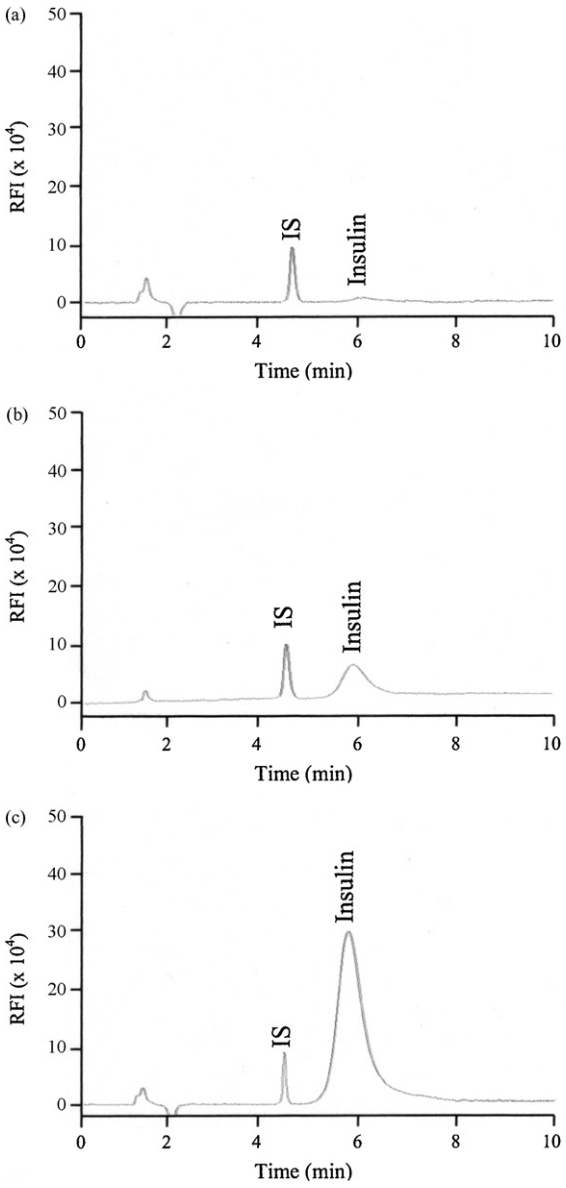

Fig. 4. Chromatograms obtained from the analysis of insulin released from nasal inserts after (a) 30 min, (b) 120 min, (c) 360 min. Conditions: see Section 2.

As expected from the insert composition a combination of different factors are involved in insulin release such as swelling, drug solubility and drug diffusion. The release profile obtained, suggested that insulin diffusion in the hydrated network of polyelectrolyte complex was the rate limiting step ("Novel mucoadhesive nasal inserts based on chitosan–hyaluronic acid polyelectrolyte complexes for peptide and protein delivery"; Luppi, submitted for publication.).

3.7. Accuracy of the method

Accuracy of the method was evaluated by analysing the different formulations (both oral microparticles and nasal inserts) as such and after being spiked with insulin standard at three different concentrations (corresponding to 0.10, 5.0 and 15.0 μg/mL). Very good accuracy values were obtained, expressed as recovery percentage values, always higher than 90.0%. Results obtained are reported in Table 3.

Table 3
Accuracy of the method

Formulation		Insulin added (μg/mL)	Accuracy (recovery%, average value)[b]
Oral formulations	Type 1[a]	0.1	96.3
		5.0	97.2
		15.0	97.8
	Type 2[a]	0.1	96.0
		5.0	98.2
		15.0	99.1
	Type 3[a]	0.1	95.1
		5.0	96.2
		15.0	97.8
Nasal inserts		0.1	91.1
		5.0	92.0
		15.0	92.3

[a] Microparticle inner core composition: Type 1: chitosan/adipic acid; Type 2: chitosan/succinic acid; Type 3: chitosan/suberic acid.
[b] $n = 6$.

4. Conclusions

An original method based on LC with fluorescence detection has been developed for the analysis of insulin in formulations consisting in innovative nasal inserts and coated microparticles for drug delivery after oral administration.

Three different microparticles for oral insulin administration consisting of an inner core composed of chitosan and adipic, succinic or suberic acid, respectively (and an outer coating composed of stearic acid and SPAN 60) were analysed.

Moreover, the method was applied to the analysis of nasal inserts consisting of chitosan/hyaluronate complexes, loaded with insulin and lyophilised.

Complete insulin extraction from all the formulations was obtained without any interference from the matrix and the use of fluorescence detection granted high selectivity.

The method has been fully validated in terms of linearity, LOD and LOQ, precision and accuracy.

Good and reliable results were obtained and the method resulted to be suitable for the determination of insulin in innovative formulations.

Compared to the only published method based on LC coupled to fluorescence detection for the determination of insulin in pharmaceutical injectable formulations [19], the method presented herein is more feasible (making use of isocratic elution instead of gradient elution), allows a faster analysis (about 7 min instead of 15 min) and has the advantage of a good precision and accuracy.

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