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Original Article

Development and validation of a new robust RP-HPLC method for simultaneous quantitation of insulin and pramlintide in non-invasive and smart glucose-responsive microparticles

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Abstract

Background and purpose: Since insulin and pramlintide cooperate in glucose hemostasis, co-administration and quantitation of them in pharmaceutical preparations are imperative. A simple, rapid, sensitive, and isocratic RP-HPLC method was developed and validated for simultaneous quantitation of insulin and pramlintide in loading and *in-vitro* release studies of a glucose-responsive system to improve the control of hyperglycemic episodes in diabetic patients.
Experimental approach: The isocratic RP-HPLC separation was achieved on a C18 µ-Bondopak column (250 mm × 4.6 mm) using a mobile phase of water:acetonitrile:trifluoroacetic acid (65:35:0.1%) at a flow rate of 1 mL/min in an ambient temperature. Both proteins were detected using a UV detector at 214 nm. The method was validated for specificity, linearity, precision, accuracy, the limit of detection, the limit of quantification, and robustness.
Findings/Results: Linearity was obtained in the concentration range of 30 to 360 µg/mL for insulin and 1.5 to 12 µg/mL for pramlintide. The results were validated statistically and recovery studies confirmed the great accuracy and precision of the proposed method. The robustness of the method was also confirmed through small changes in pH, mobile phase composition, and flow rate.
Conclusion and implications: The method was found to be simple, specific, precise, and reproducible. It was applied for the determination of loading capacity, entrapment efficiency, and *in-vitro* release studies of insulin and pramlintide in a smart glucose-responsive microparticle. Co-delivery of insulin and pramlintide could be a new intervention in diabetes management and concurrent quantitation of these two proteins is, therefore, essential.

Keywords: Diabetes; Insulin; Pramlintide; RP-HPLC; Smart-glucose responsive microparticles.

INTRODUCTION

Insulin is the first-line therapy in diabetes type 1 and is one of the important therapies in type 2 diabetes which was known as the only responsible hormone for the control, transportation, utilization, and storage of glucose in the body for many years (1,2). In 1987 another hormone secreted from the β-cells of the pancreas "amylin" was discovered, which complements insulin action by a reduction in the rate of glucose entrance into the blood (1,2). In summary, amylin works to regulate the rate of blood glucose appearance from both endogenous (liver-derived) and exogenous (meal-derived) sources, and insulin regulates the rate of blood glucose disappearance (3).

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Because of co-localization and co-secretion of both hormones within β-cells, patients with diabetes have an absolute or relative deficiency of both insulin and amylin (3,4). According to this fact and the cooperation of these two hormones in the regulation of blood glucose levels, co-delivery of them in a single smart delivery system is beneficial and mimics the β-cell secretion profile of a healthy body in response to elevated blood glucose levels (4-6). Besides the co-delivery results in less frequent administration and better patient compliance. Being unstable in dosage forms, amylin was replaced with its therapeutically equivalent analog "pramlintide" in pharmaceutical preparations (3,6). The chemical structures of insulin and pramlintide are shown in Fig. 1.

Glucose-responsive drug delivery systems are a promising strategy enabling the drug carriers to release their content only in response to elevated blood glucose levels. A pancreas-like, closed-loop, glucose-responsive insulin delivery system that "secrete" insulin only in response to elevated blood glucose levels, would provide a desirable way of hyperglycemia management, mimicking the physiological condition of glucose control with minimal patient intervention, side effects elimination, and potential improvement in glycemic control and quality of life (2). Therefore, we have recently designed and developed a non-invasive smart glucose-responsive insulin and pramlintide co-delivery system to improve the control of hyperglycemic episodes in diabetic patients, taking the advantage of both glucose hemostatic proteins co-delivery and responsivity of the system to hyperglycemic condition and also eliminating the complications of subcutaneous administration of insulin and pramlintide (unpublished study).

Secreting by the pancreas, the insulin-to-amylin molar ratio in portal circulation is approximately 50:1. Because of disproportional hepatic extraction of insulin, this ratio falls to ~ 20:1 in the peripheral circulation (5). Considering this ratio in the design of the dual delivery system, the loading content weight ratio of insulin to amylin in pharmaceutical preparation should be 30:1. Therefore, following the release of insulin and pramlintide from smart glucose-responsive microparticle in response to elevated glucose levels, a very low yet effective concentration of pramlintide in comparison to insulin (which is about 30 times greater than pramlintide) is achieved. Thus, characterization and quantitation methods should be sensitive enough to quantify the low levels of pramlintide alongside relatively higher levels of insulin in the delivery systems.

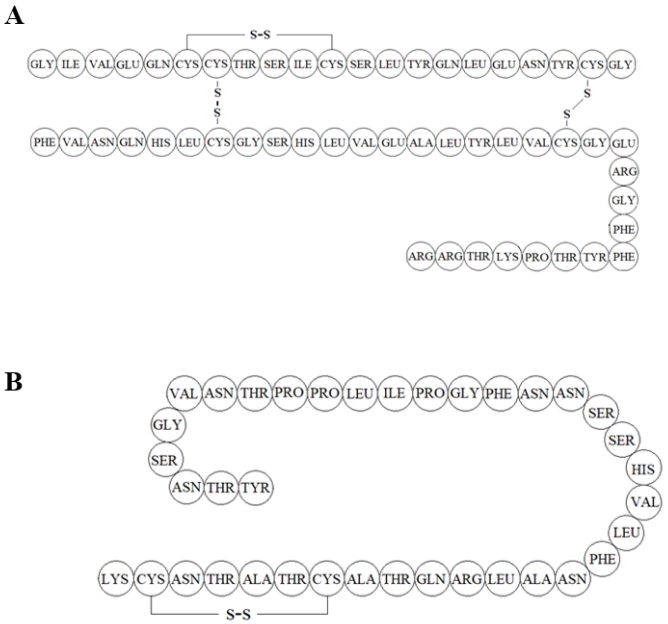

Fig. 1. Chemical structures of (A) insulin and (B) pramlintide

Several immune and non-immune methods have been reported for the quantitation of proteins. The drawbacks of the immune methods such as radioimmunoassay and enzyme immunoassay (*e.g*. enzyme-linked immunosorbent assay (ELISA)) are high costs, the short shelf life of kits, the inability to differentiate between protein and its analogs or degradation products. Moreover, these methods are unable to quantify more than one protein simultaneously with the same setup (7-9). Among non-immune methods, colorimetric assays such as Lowry, bicinchoninic acid, and Bradford are not suitable due to the wide spectrum of interferences and also the inability to distinguish between two or more proteins at the same time despite their simplicity and sensitivity (7,9). UV spectrophotometry as a non-colorimetric method is simple and samples are recoverable but the same as calorimetric methods it is unable to differentiate between two proteins simultaneously in the same wavelength (7,9).

Chromatographic methods, high-performance liquid chromatography (HPLC) in particular, have been widely used for the determination of proteins as a part of non-colorimetric methods (7,10-15). It is versatile, sensitive, reproducible, replicable, and able to work together with techniques such as mass spectrometry and different detectors such as UV, fluorescence, and diode array (7,8,15-18). The separation selectivity of this technique can be manipulated through changes in mobile phase characteristics, operating temperature, and ionic modifier and also can separate complex mixtures of peptides and proteins with low picomolar to femtomolar amounts (7,9,18-25). Insulin has been analyzed by HPLC using fluorescence and UV detection and also mass spectrometry. Although fluorescence detection and mass spectrometry techniques are very sensitive, they are not commonly available in most laboratories (20,21) but UV detectors are more accessible and also acceptable in results (8,14,15). There are also few studies utilizing HPLC for the purification of pramlintide from its degradation products (23-25). However, HPLC methods for simultaneous measurements of insulin and pramlintide per se and their application for the determination of these two proteins in one delivery system have not yet been reported. To the best of our knowledge, this is the first report of the development and validation of a simple, rapid, sensitive, and isocratic reverse-phase (RP)-HPLC method for separation and concurrent quantitation of insulin and pramlintide using a UV detector with a short run time. The developed method was validated for its selectivity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ) as per International Conference on Harmonization (ICH) guidelines. The validated method was applied in the *in vitro* studies of the smart glucose-responsive microparticle delivery system containing insulin and pramlintide.

MATERIALS AND METHODS

Materials and instruments

Pure regular human recombinant insulin was supplied by Ronak Daru Company (Iran, Tehran), pramlintide acetate was purchased from Dayangchem Company (China), HPLC grade acetonitrile, methanol, water, and trifluoroacetic acid (TFA) were procured from Merck (Germany). Waters 515 HPLC pump with a Rheodyne 7725I autoinjector, Waters 2487 Dual λ absorbance detector, and Waters 746 Chromatopac integrator (Waters, USA) were used in this project.

Selection of suitable column and optimization of mobile phase and ion-pairing reagent concentration

Novapak (150 mm × 4.6 mm, particle size 4 µm, pore size 60 Å, 7% carbon-loaded, low purity silica), Novapak (250 mm × 4.6 mm, particle size 4 µm, pore size 60 Å, 7% carbon-loaded, low purity silica), and C18 µ-Bondapak (250 mm × 4.6 mm, particle size 10 µm, pore size 125 Å, 10% carbon-loaded, low purity silica) columns were tested to evaluate the effect of stationary phase types, length and pore size on separation of the two proteins.

The effect of various concentrations of TFA as ion-pairing reagent and pH modifier (0%, 0.05%, and 0.1%) in the mobile phase and different proportions of organic to the aqueous

phase (acetonitrile to water ratios of 30:70, 35:65, 40:60 v/v) were evaluated on the separation of two proteins.

Resolution factor (Rs), asymmetry factor (As), and tailing factor (Tf) as separation factors were calculated using the following equations:

$$Rs = \frac{RT2 - RT1}{0.5 \times (W1 + W2)} \quad (1)$$

where, RT is the peak retention time and W is the width of the peaks.

$$Tf = \frac{W5\%}{2F} \quad (2)$$

where, W5% is the peak width at 5% of the peak height and F is the front peak half-width at 5% of the peak height.

$$As = \frac{B}{A} \quad (3)$$

where, A is the distance from the leading edge of the peak to the peak midpoint at 10% of the peak height and B is the distance from the peak midpoint to the trailing at 10% of peak height.
The mobile phase consisted of HPLC-grade premixed aqueous and organic components, along with an ion-pairing agent, freshly prepared daily, filtered through a 0.22 µm membrane filter, and degassed *via* an online degasser. All practices were performed at ambient temperature, and 30 µL of samples were injected into the HPLC system each time.

After optimization of the above-mentioned variables, insulin and pramlintide samples were also prepared in loading and release media and analyzed under the optimum conditions of the column, mobile phase, and TFA concentration to evaluate the Rs, As, and Tf of both proteins.

Preparation of solutions

Stock solutions of insulin and pramlintide

Quantities of insulin and pramlintide acetate powder were precisely weighed out to make stock solutions of 1 mg/mL and 25 µg/mL, respectively. Insulin powder was first dissolved in HCl 0.01 M and then neutralized with NaOH 0.01 M to attain pH 7 and then diluted with water to the final volume. Pramlintide acetate was dissolved in and diluted with water to the final volume.

Standard solutions of insulin and pramlintide

As mentioned, stock solutions of insulin and pramlintide were prepared and according to the ratio of these two proteins in the fabricated delivery system, standard solutions containing 30, 60, 105, 150, 195, 240, 300, and 360 µg/mL of insulin and 1.5, 2, 3.5, 5, 6.5, 8, 10, 12 µg/mL of pramlintide acetate were prepared in the mobile phase, phosphate buffer (0.1 M, pH 7.4) containing 0.46% polyvinyl alcohol used as the external phase of the double w1/o/w2 emulsion to formulate the smart glucose responsive microparticles (referred to as the loading medium), and phosphate buffer (0.005 M, pH 7.4) containing 400 mg/dL glucose (designated as the release medium).

Calibration curve

Calibration curves were constructed for insulin and pramlintide in standard solutions of the proteins by plotting the concentration of compounds versus peak area response. Standard solutions containing 30, 60, 105, 150, 195, 240, 300, 360 µg/mL of insulin and 1.5, 2, 3.5, 5, 6.5, 8, 10, 12 µg/mL of pramlintide acetate in the mobile phase, loading and release media. They were prepared in triplicate and after filtration injected into the HPLC column.

Specificity and selectivity

The specificity and selectivity of the analytical method were confirmed by the analysis of a solution containing insulin and pramlintide in the mobile phase as the control with a clean baseline, the supernatant of the plain, as well as the drug-loaded smart glucose responsive microparticles prepared in the loading medium and samples withdrawn from release medium, were injected into the HPLC system. The ability to resolve insulin and pramlintide from all the probable excipients which may have been driven from the formulation during the emulsification process was demonstrated by the presence or absence of the excipients and also by assessing the resolution between the resolved peaks. Identification was performed by comparing the retention time of major peaks in the chromatogram of the assay and release solution with those in the chromatogram of the standard or control solutions (17,26,27). Also, the Rs and Tf for the peaks of both proteins were calculated in the chromatograms.

Linearity, the LOD and LOQ

The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The calibration curves were evaluated by the correlation coefficient, slope, and intercept. The linear regression coefficient of determination will be accepted if it is greater than or equal to 0.995 (26). The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as LOD. The LOQ was identified as the lowest concentration of the standard curve that could be quantified with acceptable accuracy, precision, and variability (27-29).

Precision and accuracy

The precision of the HPLC method was determined by intra-day and inter-day variations. Each level of precision was investigated by repeated analysis of standard solutions at concentrations ranging from 30 to 360 µg/mL for insulin and 1.5 to 12 µg/mL for pramlintide acetate in triplicate and three different media of mobile phase, loading and release media (26-29). The percent coefficient of variance (% CV) or relative standard deviation indicating the precision of the assay, was determined using the following equation:

$$\%\ Precision\ =\ (SD/Mean) \times 100 \quad (4)$$

where, SD is the standard deviation of the samples and mean is the average concentration. The percent differences of the back-calculated concentrations from the nominal concentrations were also determined to validate the accuracy or error percent of the assay. The calibration model will be accepted if the residuals are within ± 20% for the lower limit of quantification and within 15% for all other calibration levels. In addition, at least 2/3 of the standards should meet this criterion (29). Accordingly, the % accuracy was determined using the equation below.

$$\%\ Accuracy = \left(Cm - \frac{Cn}{Cn}\right) \times 100 \quad (5)$$

where, C_m is the measured concentration and C_n is the nominal concentration.

Robustness

The robustness of the HPLC method was determined by analysis of samples under a variety of conditions such as small changes in the composition, pH, and the flow rate of the mobile phase, and the effects on peak parameters were studied (26-29).

Method application

Loading capacity and entrapment efficiency analysis

Insulin and pramlintide were loaded in a smart glucose-responsive microparticle system by multiple emulsion methods (2). Such a smart delivery system is composed of a glucose monitoring module (glucose sensor) and a glucose-triggered drug-releasing module (responsive material). The glucose sensor, can sense the increase in blood glucose levels and trigger the responsive material to release a certain amount of loaded drug (mainly insulin). Ten mg insulin and 0.33 mg pramlintide and defined amounts of glucose sensor were dissolved in the aqueous phase (2 mL) and emulsified in the chloroform (4 mL), as an organic phase containing responsive polymer (75 mg), by sonication for 30 s on ice. This primary w/o emulsion was added to the second aqueous phase (polyvinyl alcohol, PVA) 1% in phosphate buffer (pH 7, 8 mL) and sonicated on ice for another 30 s. This emulsion was immediately poured into a final solution of PVA 0.1% in phosphate buffer (pH 7, 24 mL), and stirred until the complete evaporation of the organic phase. After removal of the organic phase, the resultant dispersion was centrifuged for 30 min at 12,000 rpm and 30 µL of the supernatant was injected into the HPLC column for quantitation of unloaded insulin and pramlintide. Insulin and pramlintide concentrations were determined from the calibration equation derived in the linearity study of the loading medium. Loading capacity and entrapment efficiency were calculated by the following equations (9):

$$\%\ Entrapment\ efficiency = \frac{(A-B)}{A} \times 100 \quad (6)$$

$$\%\ Loading\ capacity = \frac{(A-B)}{C} \times 100 \quad (7)$$

where A is the amount of drug initially fed in the formulation and B is the free amount of the drug in the supernatant, and C is the total weight of smart glucose-responsive microparticles.

In vitro release analysis

In vitro release analysis of insulin and pramlintide from smart glucose-responsive microparticles was conducted in a phosphate buffer solution containing 400 mg/dL glucose, mimicking hyperglycemic blood conditions in diabetes (2). At different time intervals of 1, 2, 4, 6, 8, and 12 h, samples were withdrawn and centrifuged, then 30 µL of the supernatant was filtered and injected into the HPLC column. Insulin and pramlintide concentrations were determined from the calibration equation derived in the linearity study of the release medium.

RESULTS

Determination of a suitable wavelength

To determine an appropriate wavelength for the simultaneous determination of insulin and pramlintide, solutions of these two proteins in the mobile phase were scanned by UV spectroscopy over the range of 200-400 nm. The maximum absorption for both proteins was found to be 214 nm. Typically, peptides and proteins exhibit maximum absorption at a wavelength between 210-220 nm, which is specific for the peptide bond, or at 280 nm which belongs to the aromatic amino acids, tryptophan, and tyrosine (16-18,20). Samples containing a mixture of the compounds were injected into the HPLC column at both 214 and 280 nm. Both tested compounds jointly have considerable absorbance at 214 nm. As there is no tryptophan in insulin and pramlintide structure and just a limited number of tyrosine, low absorption intensities at 280 nm seem reasonable.

Selection of a suitable column and optimization of mobile phase and ion-pairing reagent concentration

Different types of C18 columns including Novapak150 mm × 4.6 mm and Novapak 250 mm× 4.6 mm both with particle size 4 µm, pore size 60 Å, surface area 120 m^2/g, 7% carbon loaded low purity silica; as well as a µ-Bondapak, 250 mm × 4.6 mm, particle size 10 µm, pore size 125 Å, surface area 330 m^2/g, 10% carbon loaded low purity silica were tested to optimize the resolution of insulin and pramlintide (30-32). Using two similar Novapak columns with different lengths (150 *vs* 250 mm), respectively insulin was eluted at 2 and 6 min after sample injection as a tailed peak and pramlintide did not appear even after 20 min. The µ-Bondapak column was able to resolve insulin and pramlintide with reasonable retention time, resolution, asymmetry, and tailing factors. The column wider pore size, larger surface areas, and a sufficient number of theoretical plates resulted in better interaction of proteins with the stationary phase and more efficient resolution.

Different parameters such as Tf, As, and Rs indicating column efficiency are displayed in Table 1. Resolution values given for µ-Bondapack 250 mm column are within the accepted limits outlined in ICH guidelines.

Three different proportions of TFA (0, 0.05, and 0.1%) in the mobile phase were tested. Based on the Rs, As, and Tf of two protein peaks, 0.1% TFA was selected as the optimum concentration in the mobile phase (Fig. 2).

Table 1. Insulin and pramlintide peak parameters eluted from different columns.

Column type	Rs Insulin-pramlintide	Tf insulin	Tf pramlintide	As insulin	As Pramlintide
Novapak 150 mm	Pramlintide ND	3.5	ND	4	ND
Novapak 250 mm	Pramlintide ND	1.57	ND	2	ND
µ-Bondapack 250 mm					
In mobile phase	2.32	1.2	1.11	1.2	1.18
In loading medium	2.81	1	1.07	1.03	1.2
In release medium	2.81	1.1	1	1.23	1.07
ICH acceptable range (17)	> 1.5	1-1.5		1-1.25	

Tf, Tailing factor; Rs, resolution factor; As, asymmetry factor; ND, not detected; ICH, International Conference on Harmonization.

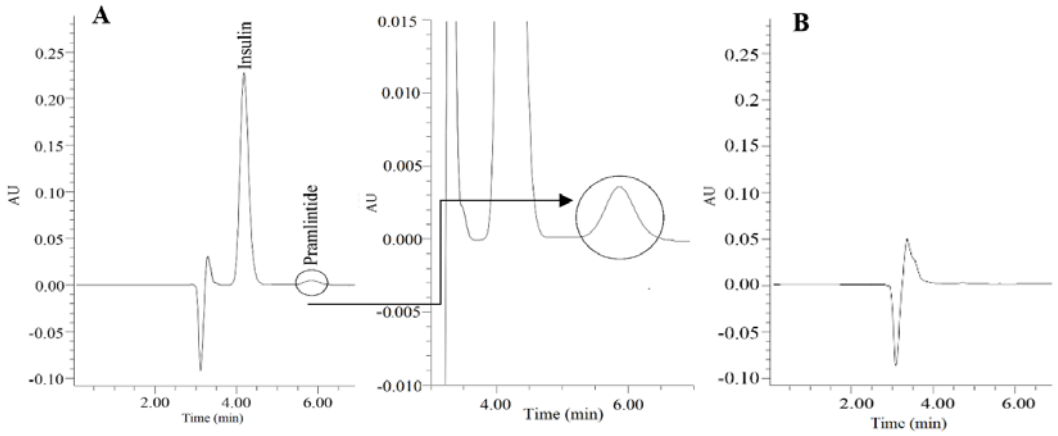

Fig. 2. Chromatogram of (A) insulin and pramlintide prepared in the mobile phase, and (B) mobile phase injected as the control. Insulin concentration: 195 µg/mL, pramlintide concentration: 6.5 µg/mL.

The impact of different ratios of organic to the aqueous phase (acetonitrile to water: 30:70, 35:65, and 40:60 v/v) on the separation and retention time of two proteins were evaluated. Based on the Rs and run-time assessment the mobile phase was composed of 65, 35, and 0.1% of water, acetonitrile, and TFA, which provided complete resolution of proteins at 4.12 min for insulin and 5.88 for pramlintide with an Rs of 2.32 (Fig. 2) was chosen as the optimized ratio for mobile phase composition

Finally, it was established to perform RP-HPLC analysis using the µ-Bondapak column, with the flow rate of 1 mL/min and the mobile phase consisting of water: acetonitrile: TFA at 65:35:0.1% adjusted at pH 2. Insulin and pramlintide samples were also prepared in loading and release medium and analyzed under the optimum condition of column type, mobile phase, and TFA concentration. Both peaks were completely resolved in the same above-mentioned retention times (as depicted in Figs. 3 and 4) with Rs of more than 2.

Calibration curve

Calibration curves of insulin and pramlintide in the mobile phase, loading medium, and release medium, were constructed and related equations were derived (Fig. 5). Also, the chromatogram of the low medium and high concentrations of insulin and pramlintide in the range of the standard curve were reported in Fig. 6.

Specificity and selectivity

The HPLC chromatograms recorded in the mobile phase, for the plain and insulin/ pramlintide containing smart glucose responsive microparticles (Fig. 2), and in the loading and release media shown in Figs. 3 and 4, revealed almost no interfering peaks during the run time, and two substances eluted with good resolution as two separate resolved peaks within 8 min.

Linearity, the limit of detection and limit of quantitation

Calibration curves for insulin and pramlintide were constructed in the mobile phase, loading, and release media. As it is shown in Fig. 5, the developed method demonstrated excellent linearity for insulin and pramlintide in three tested media ($R^2 > 0.995$).

The mean correlation coefficient (R) of the linear regression analysis was 0.9999 ± 0.0002 for insulin and 0.998 ± 0.0006 for pramlintide in the mobile phase, 0.999 ± 0.0005 for insulin and 0.9976 ± 0.0006 for pramlintide in loading medium, and 0.999 ± 0.0003 for insulin and 0.9973 ± 0.0006 for pramlintide in release medium and linear regression coefficients of determination were greater than 0.995 in all the cases. Table 2 lists the linearity parameters of insulin and pramlintide calibration curves in mentioned matrices as well as the LOD and LOQ. According to the acceptance criteria of the ICH and the Food and Drug Administration (FDA) guidelines, these calibration curves of insulin and pramlintide in different matrices with relatively similar slopes, and square regression over the studied range of concentrations are linear (17,26).

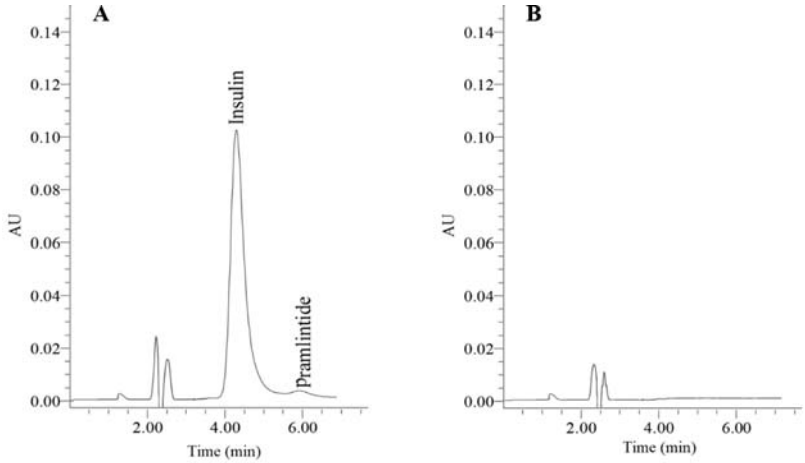

Fig. 3. Chromatogram of unloaded proteins in the supernatant of (A) smart glucose-responsive microparticles containing insulin at 101 µg/mL and pramlintide at 2.9 µg/mL, and (B) plain smart-glucose responsive microparticles in loading medium.

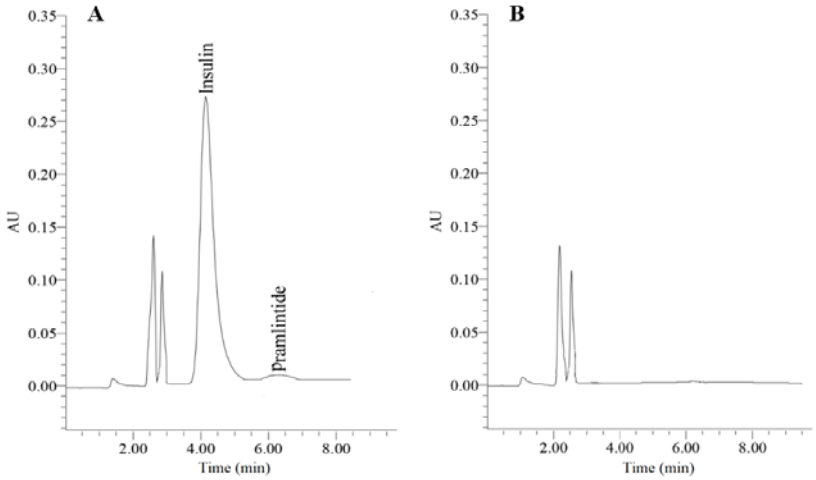

Fig. 4. Chromatogram of released proteins from (A) smart glucose-responsive microparticles containing insulin and pramlintide and (B) plain smart glucose-responsive microparticles in release medium (released insulin concentration: 241 µg/mL; pramlintide concentration: 9.3 µg/mL).

Table 2. Linearity of calibration curves for insulin and pramlintide in the mobile phase, loading and release medium solutions.

Compounds	Calibration range (µg/mL)	R^2	Slope	Intercept	LOD (µg/mL)	LOQ (µg/mL)
In the mobile phase						
Insulin	30-360	0.999	24591	82100	0.2	30
Pramlintide	1.5-12	0.995	14402	12607	0.5	1.5
In the loading medium						
Insulin	30-360	0.999	24313	49427	0.2	30
Pramlintide	1.5-12	0.995	14644	12268	0.5	1.5
In the release medium						
Insulin	30-360	0.998	24464	15566	0.2	30
Pramlintide	1.5-12	0.995	14882	14485	0.5	1.5

LOD, Limit of detection; LOQ, limit of quantitation.

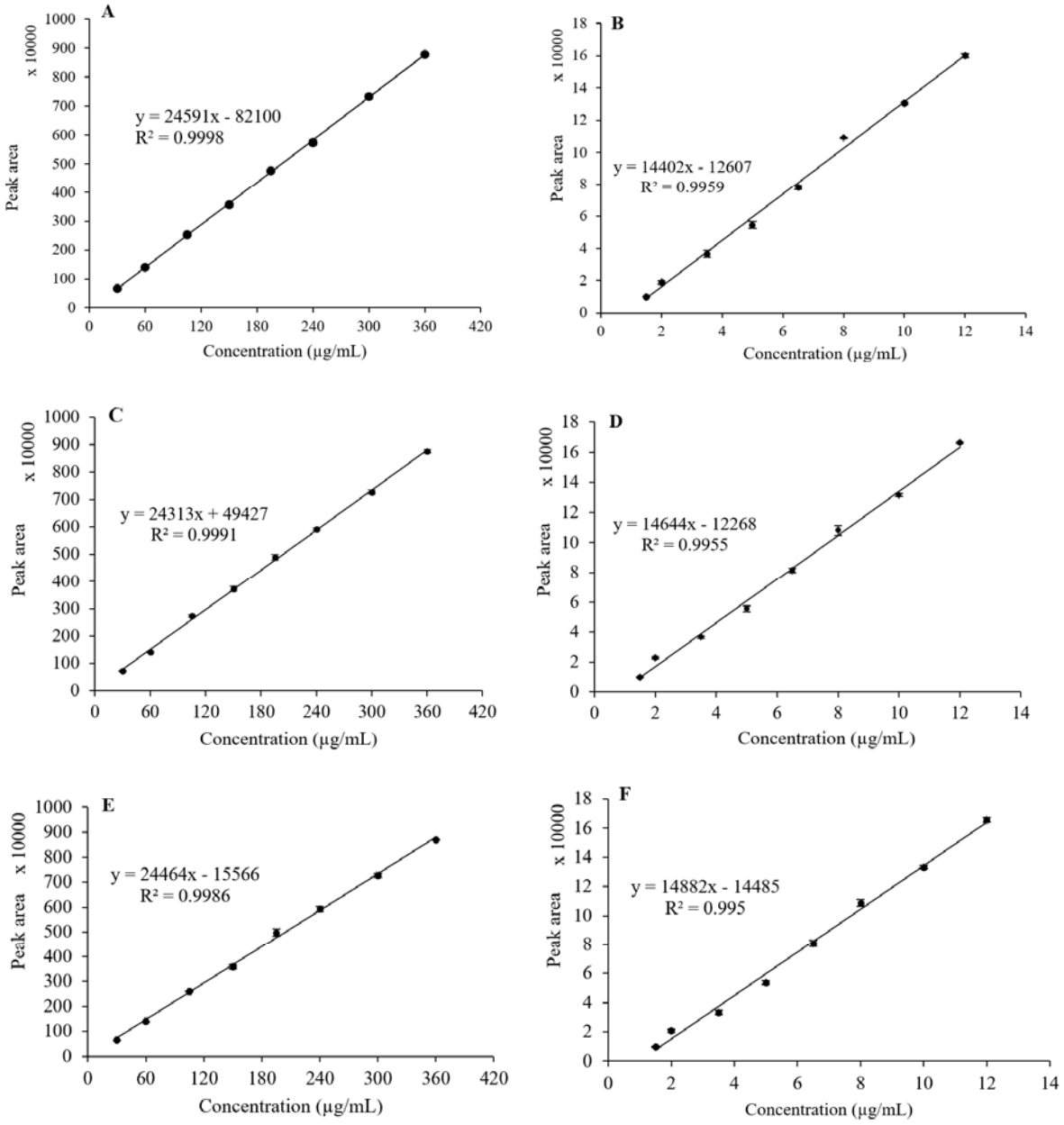

Fig. 5. Calibration curves of (A, C, E) insulin and (B, D, F) pramlintide in the mobile phase, loading, and release media. Each point represents means ± SD, n = 3. Due to small variations amongst experiments, in many data points, SD bars are not visible.

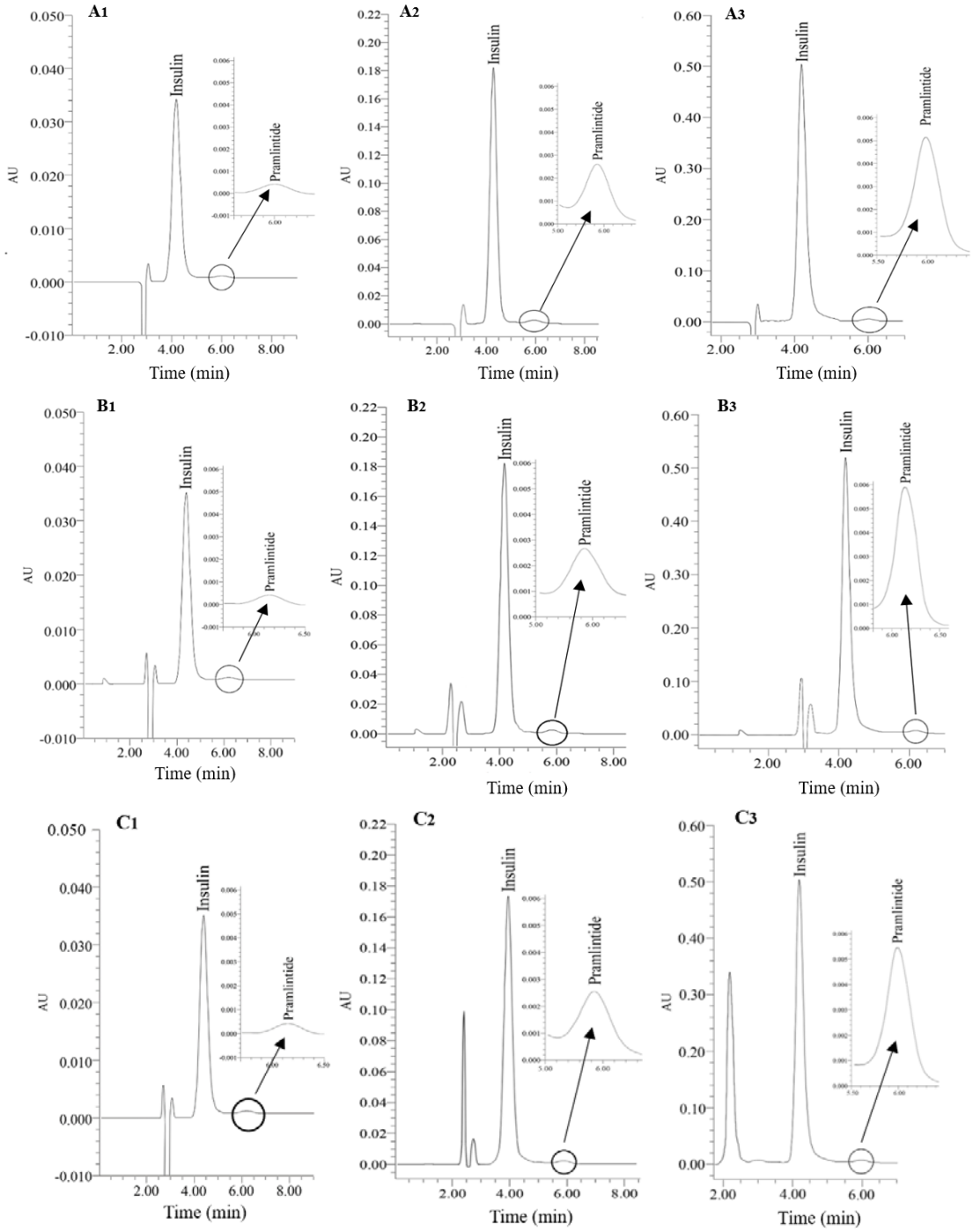

Fig. 6. Chromatograms of different concentrations of insulin and pramlintide in (A) mobile phase, (B) loading medium, and (C) release medium. In each set of chromatograms A_1, B_1, and C_1 represent low concentration, insulin at 30 µg/mL and pramlintide at 1.2 µg/mL; A_2, B_2, and C_2 represent medium concentration, insulin at 150 µg/mL and pramlintide at 5 µg/mL; and A_3, B_3, and C_3 show the highest concentration, insulin at 360 µg/mL and pramlintide at 12 µg/mL.

Precision and accuracy

Results attained for precision and accuracy studies of the method in the mobile phase, Table 3, loading medium, Table 4, and release medium, Table 5 are presented, respectively. All results comply with the acceptance criteria defined in the ICH and the FDA guidelines showing well precision and accuracy of the method (17,26).

Table 3. Intra and inter-day variations of the HPLC method for determination of insulin and pramlintide in the mobile phase

Insulin									Pramlintide							
Concentration (µg/mL)	**Intra-day precision and accuracy**				**Inter-day precision and accuracy**					**Intra-day precision and accuracy**				**Inter-day precision and accuracy**			
	Mean	**SD**	**CV (%)**	**Error (%)**	**Mean**	**SD**	**CV (%)**	**Error (%)**	**Concentration (µg/mL)**	**Mean**	**SD**	**CV (%)**	**Error (%)**	**Mean**	**SD**	**CV (%)**	**Error (%)**
30	30.17	0.57	1.09	0.56	29.92	0.46	1.56	0.26	**1.5**	1.54	0.005	0.366	2.66	1.53	0.01	1.20	2.00
60	59.32	1.15	1.94	1.13	60.48	1.05	1.74	0.80	**2**	2.17	0.06	0.030	8.50	2.17	0.08	1.41	8.50
105	107.02	2.03	1.90	1.92	105.41	1.57	1.49	0.39	**3.5**	3.39	0.15	0.046	3.14	3.36	0.01	1.87	4.00
150	147.42	1.70	1.15	1.72	148.15	2.64	1.78	1.23	**5**	4.64	0.15	0.034	7.20	4.57	0.13	1.96	8.60
195	198.88	3.04	1.53	1.98	197.29	2.50	1.26	1.17	**6.5**	6.28	0.09	0.015	3.38	6.27	0.01	2.01	3.53
240	235.73	3.11	1.32	1.77	237.65	3.43	1.44	0.98	**8**	8.44	0.02	0.002	5.50	8.47	0.12	1.41	5.87
300	301.58	3.43	1.13	0.52	301.02	3.68	1.22	0.34	**10**	9.93	0.04	0.004	0.70	9.89	0.01	1.44	1.10
360	360.65	1.27	0.35	0.18	361.15	0.57	0.15	0.31	**12**	12.00	0.09	0.77	0.00	12.13	0.12	0.98	1.08

SD, standard deviation; CV, coefficient of variation

Table 4. Intra and inter-day variations of the HPLC method for determination of insulin and pramlintide in loading medium

Insulin									Pramlintide							
Concentration (µg/mL)	**Intra-day precision and accuracy**				**Inter-day precision and accuracy**					**Intra-day precision and accuracy**				**Inter-day precision and accuracy**			
	Mean	**SD**	**CV (%)**	**Error (%)**	**Mean**	**SD**	**CV (%)**	**Error %)**	**Concentration (µg/mL)**	**Mean**	**SD**	**CV (%)**	**Error (%)**	**Mean**	**SD**	**CV (%)**	**Error (%)**
30	27.37	0.86	3.17	8.76	27.39	0.31	1.15	8.70	**1.5**	1.56	0.008	0.36	4.00	1.55	0.01	1.11	3.33
60	55.10	0.70	1.27	8.16	56.22	0.41	0.74	6.30	**2**	2.19	0.03	0.029	9.50	2.22	0.04	1.90	11.00
105	110.01	2.0	1.82	4.77	108.95	0.89	0.81	3.76	**3.5**	3.41	0.05	0.04	2.57	3.37	0.01	0.34	3.71
150	151.19	3.36	2.22	0.79	150.59	4.28	2.84	0.39	**5**	4.66	0.13	0.03	6.80	4.59	0.13	2.95	8.20
195	199.20	4.36	2.19	2.15	198.60	1.29	0.65	1.84	**6.5**	6.29	0.08	0.014	3.23	6.28	0.01	0.17	3.38
240	241.15	1.82	0.75	0.47	242.13	4.15	1.71	0.88	**8**	8.44	0.22	0.002	5.50	8.47	0.12	1.46	5.87
300	297.03	2.91	0.98	0.99	301.17	2.87	0.95	0.39	**10**	9.93	0.06	0.004	0.70	9.88	0.009	0.09	1.20
360	358.41	2.64	0.73	0.44	359.23	0.90	0.25	0.21	**12**	11.99	0.03	0.77	0.08	12.12	0.12	1.01	1.00

SD, standard deviation; CV, coefficient of variation

Table 5. Intra and inter-day variations of the HPLC method for determination of insulin and pramlintide in the release medium

	Insulin								Pramlintide							
Concentration (µg/mL)	**Intra-day precision and accuracy**				**Inter-day precision and accuracy**					**Intra-day precision and accuracy**				**Inter-day precision and accuracy**			
	Mean	**SD**	**CV (%)**	**Error (%)**	**Mean**	**SD**	**CV (%)**	**Error (%)**	**Concentration (µg/mL)**	**Mean**	**SD**	**CV (%)**	**Error (%)**	**Mean**	**SD**	**CV (%)**	**Error (%)**
30	26.89	0.43	1.6	10.35	27.66	0.27	1.00	7.80	**1.5**	1.64	0.01	0.92	9.33	1.62	0.008	0.51	8.00
60	57.82	0.01	0.02	3.63	57.62	0.05	0.10	3.96	**2**	2.43	0.07	3.15	21.5	2.36	0.07	2.24	18.00
105	106.80	0.07	0.07	1.71	105.10	0.54	0.52	0.09	**3.5**	3.27	0.09	3.02	6.57	3.20	0.099	3.08	8.57
150	147.06	3. 6	2.5	1.96	153.57	3.36	2.19	2.38	**5**	4.65	0.08	1.74	7.00	4.59	0.08	1.78	8.20
195	204.01	6.21	3.04	4.60	204.99	5.46	2.56	5.12	**6.5**	6.42	0.11	1.71	1.23	6.39	0.15	2.39	1.69
240	242.80	4.9	2.03	1.16	243.56	6.09	2.32	1.48	**8**	8.23	0.11	1.43	2.87	8.27	0.15	1.88	3.37
300	297.76	3.03	1.01	0.74	299.18	3.30	1.10	0.27	**10**	9.85	0.07	0.73	1.50	9.92	0.07	0.72	0.80
360	356.02	0.24	0.06	1.11	356.15	5.69	1.60	1.07	**12**	12.18	0.004	0.04	1.50	12.11	0.10	0.90	0.91

SD, standard deviation; CV, coefficient of variation.

Table 6. Influence of changes in experimental parameters on the performance of the chromatographic system indicating the robustness of the assay.

Parameters	**Modifications**	**Insulin**		**Pramlintide**	
		Accuracy (%)	**RT (min)**	**Accuracy (%)**	**RT (min)**
Mobile phase composition (v/v/%) Acetonitrile: water: TFA	63:37:0.1	98.88	4.01	89.84	5.71
	64:36:0.1	97.75	4.11	93.71	5.82
	68:32:0.1	93.02	4.23	98.93	6.02
pH	2	92.95	4.11	98.67	5.87
	2.5	95.72	4.12	103.28	5.89
	3	97.51	4.13	95.72	5.88
Flow rate (mL/min)	0.75	99.00	4.01	100.18	5.63
	1	102.0	4.12	98.68	5.88
	1.25	97.74	4.23	99.76	6.13

RT, retention time, TFA, trifluoroacetic acid

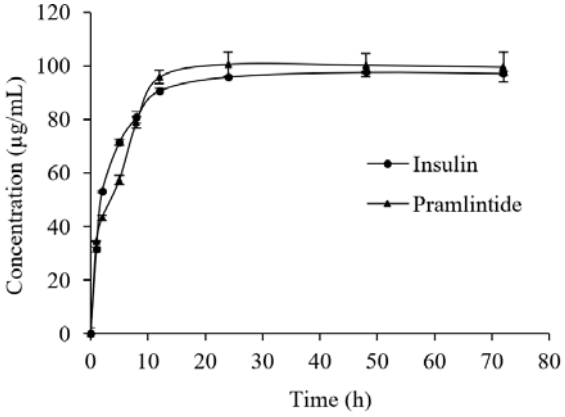

Fig. 7. Release profiles of insulin and pramlintide liberated from smart glucose-responsive microparticles. Each point represents means ± SD, n = 3.

Robustness

The results of the robustness test demonstrated in Table 6 show that after conscious alterations of mobile phase composition, flow, and pH as operational parameters, the performance of the chromatographic system does not change essentially. The Tf for insulin and pramlintide always ranged from 1.1 to 1.5 and the two proteins were well separated under all the changes carried out. The percent accuracy of insulin and pramlintide was acceptable in most cases and did not change significantly. Considering the result of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

Application to the assay of smart glucose-responsive microparticles

The developed and validated method was used to determine loading capacity, entrapment efficiency, and *in-vitro* release profile of smart glucose-responsive microparticles containing insulin and pramlintide. In all cases assumed peaks of insulin and pramlintide were compared with the position of standard solution peaks in the medium. Entrapment efficiency and loading capacity of insulin were 68% and 11%, in the case of pramlintide entrapment efficiency was 75% and loading capacity was 4%. The smart glucose-responsive microparticles released their contents in about 12 h. The release profile of insulin and pramlintide is depicted in Fig. 7.

DISCUSSION

The purpose of the current study was to develop a simple, sensitive, and reliable HPLC method for the simultaneous determination of insulin and pramlintide for the characterization of loading capacity, entrapment efficiency, and *in vitro* assessment of smart glucose-responsive microparticles loaded with these two drugs. Assessing the dissolution of both drugs as a function of time measures the rate of drug release *in vitro*, can guide the formulation, and is the only test that predicts drug release *in vivo.* To achieve the highest resolution, sensitivity, and elution under an isocratic condition, the column type, ion-pairing reagent proportions, and organic to aqueous solvent fractions as the mobile phase, were assessed. Amongst three different types of HPLC columns employed, in the case of Novapak columns with different lengths (150 *vs* 250 mm), respectively insulin was eluted at 2 and 6 min after sample injection as a tailed peak and pramlintide did not appear even after 20 min. Using the Novapak 150 mm column, the shorter column length and fewer theoretical plates of the column contributed to a shorter retention time. Ionic interaction between insulin-protonated amine groups and some free ionized silanol may have also caused peak broadening and tailing. The presence of some metals in the silica with low purity that is able to convert free silanols to extremely acidic groups that can interact electrostatically with some analytes (even in the presence of ion-pairing reagent) might be another reason for peak tailing (16-18,20,29). The µ-Bondapak presented the most acceptable features for the stationary phase, due to the wider pore size, larger surface areas, higher percentage of carbon loading, and also a sufficient number of theoretical plates, which resulted in a more efficient resolution. Larger silica particle size, especially in the porous types creates more surface areas for more hydrophobic adsorptions and better separation of the analytes. Given that proteins cannot enter small pores of silica and stay on the exterior surface of the stationary phase until the separation occurs, the smaller pore size is not favorable for proteins, unlike for other chemical entities. Contrariwise, wider pore size silica allows proteins to enter and fully

interact with the surface, thus resulting in a better peak shape and separation (16-20).

For optimizing the mobile phase composition and TFA content, different proportions of acetonitrile: water in the mobile phase were assessed and the binary mixture of water/acetonitrile at 65:35 (v/v) combined with 0.1% TFA proved to be the most effective combination as evidenced by more efficient resolution, lack of tailing, noiseless baseline, and satisfactory retention and run times. TFA changes the mobile phase pH up to about 3 and prevents undesirable ionic interactions between protein protonated amine groups and any free, non-capped silanol groups which are less ionized at low pHs and negatively charged at pH values above 3. Also, TFA has ion-pairing properties such that at low pHs (below the isoelectric pH of proteins), proteins are predominantly positively charged and are less hydrophobic to interact with the stationary phase, thus adding TFA to the mobile phase as an anionic counter ion, makes the protein more hydrophobic and interactive with the stationary phase, resulting in acceptable elution and retention time (16,18). Moreover, the presence of TFA enhances the peak resolution and shapes as a result of minimizing the effect of metal impurities on peak shape in low-purity silica columns (16,18,20,28,29). The volatility of ion-pairing reagents is an important issue because the risk of salt precipitation in the column is minimized (in comparison with phosphoric acid or buffers with acidic pH) (16-20). There are many reports on using non-volatile ion-pairing reagents in protein quantification, which are difficult to remove from the protein and column (10-13). The separation of small molecules in the reverse-phase columns occurs by continuous partitioning of the molecules between the mobile phase and the hydrophobic stationary phase, while the adsorption of the proteins to the stationary phase is something like “sitting”, with most of the molecules exposed to the mobile phase, and only a part of the molecule, “hydrophobic foot” is in contact with the column surface. After desorption, they slightly interact with the surface until they elute down the column; thus practically, the adsorption/desorption step takes place only once for the proteins in the column. Consequently, the separation of proteins is very sensitive to the organic modifier concentration and small changes in the concentration in the mobile phase result in a significant change in the retention time (16-18,20). This is why only a 5% change in the acetonitrile component has led to a larger effect on insulin and pramlintide retention time, as mentioned before.

Having established the above-mentioned conditions, respectively insulin and pramlintide eluted as sharp symmetrical peaks at about 4.12 and 5.88 min in the mobile phase, loading and release media, and overall separation run time lasted 8 min in all cases. Previous studies that have investigated insulin determination or its degradation products have reported longer run times of 10-20 min, which are relatively long, especially when multiple samples are being run (10,12,13). Both insulin and pramlintide eluted completely without peak tailing, indicating that the assay method involves high specificity and selectivity from other associated agents. The linearity was confirmed due to the acceptance criteria of the squared regression coefficient (recommended by the FDA) of greater than 0.995 in all the tested media (26). The precision (repeatability) and accuracy of the calibration standard concentrations for the three tested media were within acceptable limits, as defined in the ICH guidelines (17,26). As evident in Tables 3, 4, and 5, the highest error% values for the lowest and the highest insulin concentrations in all media tested were between 10.35-1.11% and for those of pramlintide concentrations were between 9.33-1.50% which are less than 20%. In the meantime, respectively 100% and 96% of other insulin and pramlintide concentrations will meet the criteria of error% < 15. These values indicate that the developed method is precise, accurate, and reproducible.

The robustness of the method checked after deliberate alterations of the mobile phase composition, pH, and flow rate showed that the changes in the operational parameters did not lead to any essential changes in the performance of the chromatographic system. The Tf for insulin and pramlintide always ranged from 1.0-1.2, and drugs were well separated under all of the changes carried out.

The accuracy of insulin and pramlintide was acceptable under most conditions and did not show significant changes when the critical parameters were altered. Considering the result of alterations in the system suitability parameters and the specificity of the method, it could be concluded that the method conditions are robust. Using an internal standard, which is not common for substance determination in pharmaceutical preparations, was reported by Najjar *et al.* to overcome any chromatographic condition changes during the quantification of insulin and its degradation products using a photodiode array detector, which is not accessible in most laboratories. Similar to our study, the CVs reported were acceptable and in range, showing that utilization of an internal standard in the assay may not be crucial in protein measurements *in vitro*. Also, the lack of an ion-pairing reagent in the mobile phase resulted in tailed peaks and poor resolution (12).

The gradient mode has been repeatedly used to resolve insulin or pramlintide peaks from their degradation products (12,23-25) but is complex and expensive (11) with difficulty to maintain a constant flow rate while there are constant changes in the mobile phase composition, establish a complete equilibration after each change where the re-equilibration time adds on analysis time resulting in elongation of the run time (8,9). The gradient mode, however, is a better method than the isocratic method, when separating a wide range of components with different polarities (20-22). Keeping in mind the above mentioned limitations of the gradient mode and considering the polarities of insulin and pramlintide, effective separation of the two proteins will be achievable with isocratic mode through manipulation of the mobile phase mixture.

Compared with chromatography-based methods for protein separation in biological samples, ELISA is considered a sensitive and specific method with no special pretreatment procedure and is supplied as validated commercial kits. The only drawback is its inability to separate insulin from its analog or proinsulin. While HPLC methods based on mass spectrometry are very sensitive and accurate, they are not affordable or readily available in most laboratories. On the other hand, the HPLC-UV method is sensitive and specific with the ability to effectively separate all analogs and interfering compounds, but pretreatment methods are critical in reaching clear samples. Dilution is not a suitable pretreatment method for mutual quantification of insulin and pramlintide as dilution makes the sample less concentrated, and the concentration of the pramlintide may fall below the quantification limit of the assay. Protein precipitation is also not suitable, because both drugs are proteins and may precipitate with the endogenous proteins of the sample, resulting in erroneous results (33-35). In the case of liquid-liquid extraction, the presence of an organic solvent can lead to protein instability and misfolding (7,36). Solid-phase extraction can be used for insulin and pramlintide, but the high cost and the time-consuming process are the main disadvantages. Besides the pretreatment requirement of the HPLC methods, validation of the developed method is necessary. As the necessity of mutual quantification of insulin and pramlintide, gradient elution will be required to separate these two proteins from unwanted endogenous peptides.

Keeping in mind all of the above-mentioned points, insulin, and amylin ELISA kits are readily available in the market with high sensitivity (LOD of 0.17 pg/mL (5 µIU/mL) (7) or even ultra-sensitivity, LOD in the range of nIU/mL (37)) in comparison to the sensitivity of the HPLC-UV method (LOD of 0.1 µg/mL); therefore, the use of ELISA method is in priority for biological samples, and the development of HPLC-UV methods are not very common, except for use in research settings. On the other side, for *in vitro* studies of pharmaceutical preparations, higher concentrations facilitate drug quantification, and using techniques like ELISA is not reasonable or cost-effective (38).

CONCLUSION

Mimicking the physiological condition of glucose hemostasis, a novel smart glucose-responsive microparticle-containing insulin and pramlintide were fabricated. A rapid and

reliable isocratic RP-HPLC method for the determination of insulin and pramlintide was developed and validated. Specificity, linearity, precision, repeatability, accuracy, and robustness were proved. It was a highly specific and precise analytical procedure for the separation of insulin and pramlintide in a short run time of 8 min which allows the analysis of a large number of samples in a short period of time. It needed no temperature intervention instruments and precipitation of the mobile phase doesn't occur because of its volatility. The developed method showed no interference with the associated excipients or matrices and also presented good resolution between two proteins; the method was also linear and precise. Finally, the method was rapid and suitable for loading content and release profile analysis of insulin and pramlintide in previously fabricated smart glucose-responsive microparticles. The main novelty, therefore, exists in the simultaneous quantification of the two essential proteins in the management of diabetes, with a simple, rapid, sensitive, accurate, and robust method. Validation of the method in different matrices appropriate to its practical application was different from similar studies. Because there is no pharmacopeial method and even reliable publication for co-delivery and co-detection of insulin and pramlintide, this method is recommended for quality control of insulin and pramlintide content in novel pharmaceutical preparations.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

J. Emami supervised the project, conceived the methodology and validation, edited the final version, and provided grammatical revisions to the manuscript. M. Haghighi carried out the experiments and wrote the original draft of the manuscript. M. Rostami assisted in synthesizing the polymer needed for the formulation. M. Minaeian conceptualized and verified the analytical method. The final version of the manuscript was approved by all authors.

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