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A simple HPLC method for the simultaneous analysis of insulin and ovomucoid

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An analytical HPLC method is reported for the simultaneous determination of insulin and its enzyme inhibitor, chicken ovomucoid. Verapamil was used as an internal standard. The elution was achieved using a gradient technique (10–15% B for 4 min, 15–35% B from 5th to 11th min and 35–10% B from 12th to 22nd min). The mobile phase used was 0.05% v/v trifluoroacetic acid (TFA) in water and 0.05% v/v TFA in acetonitrile with a flow rate of 1.2 ml/min. The analytes were detected at 210 nm after resolution using a reversed phase C-18 column. Insulin, ovomucoid and verapamil (IS) were eluted at 11.9, 14.2, and 18 min, respectively, free from any interfering endogenous peaks during a run time of 22 min. Linear relationships were observed between the detector response and the concentrations of the analytes (0.05–1 I.U/ml for insulin ($r^2 = 0.9975$) and 5–100 μg/ml for the chicken ovomucoid ($r^2 = 0.9993$)). The assay was found to be highly selective and sensitive due to the absence of any interfering peaks. The lower C.V and % error values of the assay indicates that the assay could accurately and precisely quantitate both insulin and ovomucoid in the examined concentration range. This method can be used for the simultaneous quantitation of insulin and chicken ovomucoid.

1. Introduction

Insulin, a 51 amino acid polypeptide, is extensively used in the treatment of type I diabetes mellitus. Enzymatic degradation and epithelial permeability across the gastrointestinal tract are two important factors that affect its oral bioavailability. The feasibility of oral administration of insulin has been widely studied. It has been demonstrated that insulin can be absorbed in the gastrointestinal lumen when protected by appropriate concentrations of enzyme inhibitors [1].

Enzymatic degradation of insulin in the gastrointestinal tract is mediated by α-chymotrypsin and trypsin in the lumen [2]. Consequently, inhibitors of these enzymes have the potential to enhance the oral delivery of insulin. Some examples of the inhibitors evaluated include aprotinin, bowman birk inhibitor, trypsin inhibitor, and bacitracin. Among the trypsin inhibitors come chicken ovomucoid and duck ovomucoid. Recently chicken and duck ovomucoids have shown promising results in inhibiting the degradation of insulin [3].

Ovomucoids represent a relatively new class of enzyme inhibitors derived from the egg white of the avian species. Extensive information entailing their source, active domains and mechanism of inhibitory action can be found elsewhere [4]. Their inhibitory activity depends on the species from which they are isolated. They inhibit pancreatic enzymes by binding to the corresponding enzymes through their reactive site. Since they inhibit digestive enzymes such as bovine trypsin and bovine α-chymotrypsin, they might be useful as absorption enhancers for oral proteins in general, and insulin in particular.

A number of assays have been reported regarding the quantification of insulin [5–7] and fractionation of glycopeptides [8]. However, the available literature did not indicate any assay on simultaneous analysis of insulin and chicken ovomucoid. The purpose of this study was to develop a novel, simple and selective HPLC assay for the quantitative evaluation of both insulin and chicken ovomucoid. Verapamil was used as an internal standard in the chromatographic procedure.

2. Investigations, results and discussion

The gradient reversed-phase chromatographic conditions described allowed the separation of insulin, chicken ovomucoid and verapamil (internal standard) within a run

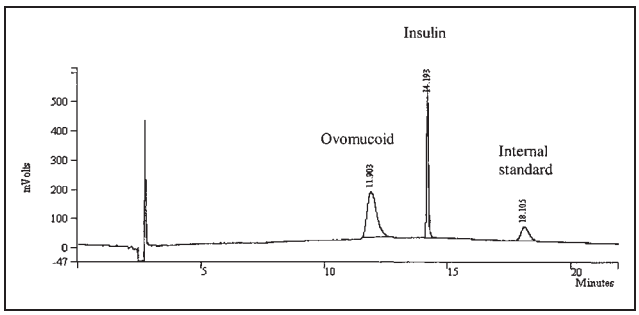

Fig.: Chromatograms of insulin and ovomucoid with the internal standard (verapamil)

Table 1: Intra run accuracy and precision of the assay (insulin)

Added conc. (I.U/ml)	Calculated conc. (I.U/ml)	Error %	% C.V.
0.05	0.053	7.6	9.3
0.5	0.489	−2.2	3.15
1	0.98	−1.9	5.4

Table 2: Inter run accuracy and precision of the assay (insulin)

Added conc. (I.U/ml)	Calculated conc. (I.U/ml)	Error %	% C.V
0.05	0.057	14	4.8
0.5	0.516	3.3	1.75
1	1.04	4.4	1.1

Table 3: Intra run accuracy and precision of the assay (ovomucoid)

Added conc. (μg/ml)	Calculated conc. (μg/ml)	Error %	% C.V
5	5.28	5.7	10.5
50	48.4	−3.07	3.6
100	99.7	−0.26	5.5

Table 4: Inter run accuracy and precision of the assay (ovomucoid)

Added conc. (μg/ml)	Calculated conc. (μg/ml)	Error %	% C.V
5	5.8	16.9	9.7
50	49.9	−0.05	3.16
100	104	4.8	1.8

time of 22 min. Insulin, ovomucoid and verapamil (IS) were eluted at 11.9, 14.2, and 18 min, respectively. No interfering peaks were observed in the chromatograms. For the given standards of the insulin and chicken ovomucoid, the response of the detector to the analytes were linear ($r^2 = 0.998$ and 0.999 respectively) over the range of 0.05–1 I.U/ml for insulin and 5–100 μg/ml for chicken ovomucoid. The standard chromatograms of insulin and chicken ovomucoid are shown in the Fig. The limits of detection were 4 μg/ml and 0.04 I.U/ml for chicken ovomucoid and insulin. Typical calibration curves for insulin and chicken ovomucoid were $y = 2.6774x - 0.053$ and $y = 0.0468x - 0.0514$ respectively, where y was the peak area ratio of insulin and chicken ovomucoid to that of the internal standard and x was concentrations of insulin and chicken ovomucoid, respectively. Calibration curves for insulin and ovomucoid were linear. The C.V's of insulin and ovomucoid were found to be ≤ 9.3 and ≤ 10.5 respectively. The intra and inter run validation data for insulin and chicken ovomucoid are reported in Tables 1, 2, 3 and 4 respectively. The precision and accuracy of the elaborated methods are also reported in Tables 1–4. The results indicate a good linear proportionality between the detector response and the concentrations of insulin and chicken ovomucoid.

The stability of insulin in the presence of chicken and duck ovomucoids against α-chymotrypsin and trypsin mediated degradation has been studied by Agarwal et al. [9]. Thus the inhibitors of the enzymes [ovomucoids] have the potential to enhance the oral delivery of insulin. In this context, it appears beneficial to have an assay for the simultaneous and quantitative evaluation of both the insulin and chicken ovomucoid.

A review of the literature did not indicate any assay for the simultaneous quantification of insulin and chicken ovomucoid while a number of assays have been reported for the individual quantification of insulin [5–7] and for the fractionation of glycoproteins [8]. Additionally, there have been no available chromatographic assays for the quantification of chicken ovomucoid. Therefore, by the current assay method, chicken ovomucoid can be quantified per se or it can be used for the simultaneous analysis of both insulin and chicken ovomucoid.

A number of compounds were tried as the internal standards (e.g. benzoic acid). Most of them were co-eluted with either the peaks of insulin or chicken ovomucoid. Verapamil was clearly separated in the current assay from insulin and chicken ovomucoid, and the total analysis time was 22 min.

The mobile phase consisting of 0.05% v/v TFA – water (A) and 0.05% v/v TFA – acetonitrile (B) and the gradient conditions described above at a flow rate of 1.2 ml/min was found to be appropriate allowing an adequate separation of the insulin, chicken ovomucoid and the internal standard (retention times 14.2, 11.9 and 18 min respectively). As shown in the Fig. the substances were eluted forming symmetrical single peaks well separated from the solvent front with out any interfering peaks.

The gradient conditions maintained are also very important for the elution. During the development of the assay it was observed that an increase in the percentage of acetonitrile (B) above 37% was affecting the sensitivity and the retention times of the peaks. Precipitation of the chicken ovomucoid was observed at higher concentrations of the acetonitrile (B), which could be one of the probable reasons for the differences in the peak sensitivity and the retention times. Therefore, the concentration of acetonitrile in the mobile phase might be one of the critical points governing the elution time and reproducibility of the chicken ovomucoid peaks.

In conclusion, a gradient, reversed phase- HPLC method is reported for the simultaneous quantification of insulin and chicken ovomucoid using verapamil as the internal standard. The satisfying recoveries and low coefficient of variation confirms the suitability of the proposed method for the simultaneous quantification of insulin and chicken ovomucoid.

3. Experimental

3.1. Chemicals and reagents

Recombinant human insulin was obtained from the Intergen Company (Purchase, NY, USA). Chicken ovomucoid, Verapamil and trifluoroacetic acid were obtained from Sigma (St.Louis, MO, USA). For chromatography, HPLC-grade acetonitrile was obtained from EM Sciences (Gibbstown, NJ, USA). Other chemicals were of analytical grade and were used as received. Distilled and deionized water was used for all the experiments.

3.2. Standard solutions

Stock solutions of insulin (100 I.U/ml) were prepared by dissolving 34.84 mg of insulin (1 I.U = 34.84 μg/ml) in 10 ml of 0.01 N HCl. This solution was stored at 4 °C. Further dilution of the stock solution for preparation of the calibration standards was carried out daily using the same 0.01 N HCl. Stock solutions of chicken ovomucoid were prepared by dissolving chicken ovomucoid in distilled and deinonised water to obtain a final concentration of 500 μg/ml. Dilutions of the stock solutions for the preparation of the calibration standards were carried out using distilled and deionised water. Further stock solution of verapamil (15 μg/ml) was also prepared in distilled and deionized water.

Calibration standards were prepared with stock solutions of both insulin and chicken ovomucoid, to produce concentrations of 0 (blank), 0.15, 0.3, 0.6, 0.9, 1.5, 2.7 and 3 I.U/ml and 0 (blank), 15, 30, 90, 150, 270, and 300 μg/ml of insulin and chicken ovomucoids respectively.

3.3. Sample preparation

Equal volumes of calibration standards of insulin, chicken ovomucoid and verapamil were added to the vials to obtain concentrations of 0 (blank), 0.05, 0.1, 0.2, 0.3, 0.5, 0.9 and 1 I.U/ml of insulin and 0 (blank), 5, 10, 20, 30, 50, 90 and 100 μg/ml for chicken ovomucoid and 5 μg/ml for verapamil. One hundred microlitres of the injection solution was injected into the system.

3.4. Chromatography

A computer controlled Varian Chromatography workstation consisting of the following components was used; Two Dynamax SD-200 pumps, an AI-200A autosampler fitted with a 100 μl injection loop, a Dynamax UV-1 detector and Star 5.3 chromatography software. Room temperature was maintained for the column and chromatographic separations were carried out on a C-18 Vydac 218MS54 column (5 μm, 4.6×250 mm) with a pore size of 300 A°. Samples were analyzed by the reversed phase HPLC method. The mobile phase consisted of 0.05% v/v TFA-Water (A) and 0.05% v/v TFA-Acetonitrile (B). The gradient conditions were 10–15% B for 4 min, 15–35% B until the 11^{th} minute and 35–10% B until the 22^{nd} minute at a flow rate of 1.2 ml/min. The detection was achieved at a wavelength of 210 nm.

3.5. Assay validation

The intra and inter-run precision and accuracy of the assay ($n = 5$) were determined by percent coefficient of variation (C.V) and percent error values, respectively, based on reported guidelines [10]. Control samples of insulin and chicken ovomucoid containing lowest, midpoint and highest concentration in the calibration curve were run along with the calibration curve. The data were weighed by 1/concentration. The concentrations of the quality control samples were then determined against the calibration curve and used for the calculation of the percent C.V and percent error values. The percent error values were calculated by the following equation.

$$\text{Percent error} = \frac{(\text{Observed concentration} - \text{Expected concentration})}{\text{Expected concentration}} \times 100$$

The quality control samples were run at the insulin concentrations of 0.05, 0.5, and 1 I.U/ml and at the chicken ovomucoid concentrations of 5, 50 and 100 μg/ml respectively.

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