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A validated HPLC assay for simultaneous analysis of salmon calcitonin and duck ovomucoid

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A highly sensitive and selective analytical HPLC method is reported for the simultaneous measurement of salmon calcitonin (sCT) and its enzyme inhibitor, duck ovomucoid (dOVM). The method used a reversed phase C-18 column (4.6 × 250 mm, 5 μm) at room temperature. The elution was achieved using a gradient technique (20–35% B for 10 min, 35–37% B from 10th to 20th min and 37–20% B from 20th to 25th 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 ml/min. Detection was carried out by UV spectrophotometry at 210 nm. sCT and dOVM were eluted at 7.8 and 15.4 min respectively, free from any interfering endogenous peaks during a run time of 25 min. Linear relationships were observed between the detector response and the concentrations of the analytes (10–100 μg/ml for CT ($r^2 = 0.996$) and 10–100 μg/ml for the dOVM ($r^2 = 0.999$)). 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 sCT and dOVM in the examined concentration range. This method can be used for the simultaneous quantitative analysis of sCT and dOVM.

1. Introduction

Calcitonin (CT) is a polypeptide of 32 amino acid residues arranged in a single linear chain. It is an endogenous polypeptide hormone, and plays a crucial role in both calcium homeostasis and bone remodeling [1–4]. It causes hypocalcaemia by inhibiting the release of calcium from bone and by stimulating urinary calcium excretion. Preparations of CT available for clinical use include salmon, human, procine, and a derivative of eel CT. sCT is one of the most potent hypocalcemic agents of the CT preparations [5–7]. In an effort to formulate oral dosage form of sCT, proteolytic degradation in GI tract is of prime concern. Trypsin and chymotrypsin have been shown to degrade sCT. It has been demonstrated that sCT can be absorbed in the gastrointestinal lumen when protected by appropriate concentrations of enzyme inhibitors [8]. Some examples of the inhibitors evaluated include aprotinin, bowman birk inhibitor, trypsin inhibitor, and bacitracin. Among the trypsin inhibitors come dOVM, chicken OVM and turkey OVM. Recently chicken and duck OVM have shown promising results in inhibiting the degradation of insulin in our laboratory [9].

Ovomucoids represent a recent group of enzyme inhibitors derived from the egg white of the avian species. 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 protease inhibitors for oral proteins in general, and salmon calcitonin in particular.

A number of assays have been reported for the quantification of sCT. Reported analytical methods of sCT include LC-Mass Spectroscopy [10–12]. Some of the analytical methods of sCT typically utilize biological or radioimmunoassay techniques which have high variability and low accuracy [13, 14]. These limitations can be overcome by use of chromatographic techniques. HPLC is one of the most widely used and rapidly expanding techniques for the separation of polypeptide and peptide hormones [15–18]. Many of these assays include precolumn derivatization with a fluorescence labeling agent [19, 20]. However, this includes one additional step before injection and variability is possible. There are assays reported for fractionation of glycopeptides also [21]. However, the available literature did not indicate any assay on simultaneous analysis of sCT and dOVM. Therefore, the purpose of this study was to develop a simple and selective HPLC assay for the quantitative evaluation of both sCT and dOVM.

2. Investigations and results

The gradient reversed-phase chromatographic conditions described allowed the separation of sCT and dOVM within a run time of 25 min. sCT and dOVM were eluted at 7.8 and 15.4 min respectively. No interfering peaks were

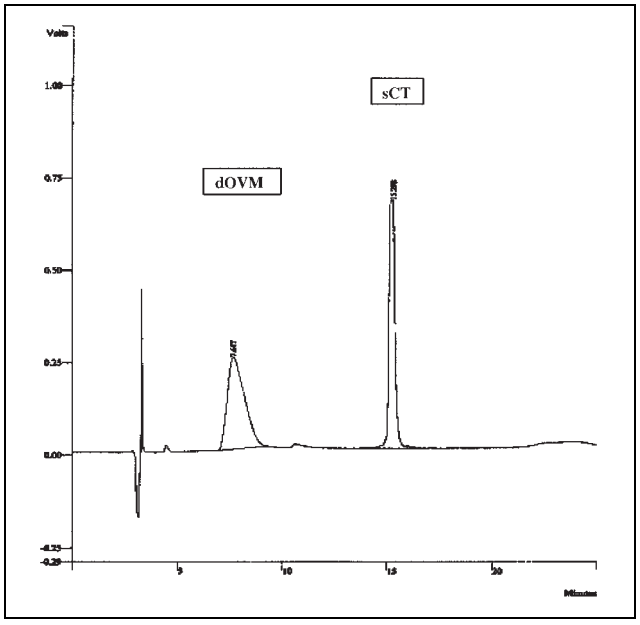

Fig.: Chromatograms of sCT and dOVM

observed in the chromatograms. For the given standards of the sCT and dOVM, the response of the detector to the analytes were linear (r^2 = 0.996 and 0.999 respectively) over the range of 10–100 μg/ml for both sCT and dOVM. The standard chromatograms of sCT and dOVM are shown in the Fig. The limits of detection were 5 μg/ml and 1 μg/ml for dOVM and sCT. Typical calibration curves for sCT and dOVM were $y = 25335x - 128249$ and $y = 16885x - 31063$ respectively, where y was the peak area of sCT and dOVM and x was concentrations of sCT and dOVM respectively. The C.V's of sCT and dOVM were found to be $\leq$12.6 and $\leq$17.1 respectively. The intra and inter run validation data for sCT and dOVM are reported in Tables 1, 2, 3 and 4 respectively. The precision and accuracy of the elaborated methods are also reported in Tables 1, 2, 3 and 4 respectively. The results indicate a good linear proportionality between the detector response and the concentrations of sCT and dOVM.

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

Added conc. (μg/ml)	Calculated conc. (μg/ml)	Error (%)	% C.V.
10	10.988	9.88	3.79
50	48.51	−2.97	7.94
100	106.29	6.29	3.78

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

Added conc. (μg /ml)	Calculated conc. (μg/ml)	Error (%)	% C.V
10	8.11	−18.89	3.793
50	47.71	−4.565	5.268
100	106.6	6.605	5.879

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

Added conc. (μg/ml)	Calculated conc. (μg/ml)	Error (%)	% C.V
10	12.237	22.37	6.352
50	59.09	18.18	19.82
100	97.01	−2.985	6.117

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

Added conc. (μg/ml)	Calculated conc. (μg/ml)	Error (%)	% C.V
10	9.645	−3.545	16.521
50	50.332	0.664	15.82
100	105.836	5.836	13.752

3. Discussion

Ovomucoids represent a recent class of enzyme inhibitors derived from the egg white of the avian species. Extensive reviews entailing their source, active domains and mechanism of inhibitory action can be found elsewhere [22]. The stability of insulin in the presence of duck and chicken ovomucoids against α-chymotrypsin and trypsin mediated degradation has been studied by Agarwal et al. [23]. Thus the inhibitors of the enzymes [ovomucoids] have the potential to enhance the oral delivery of proteins, in this case sCT. In this context, it appears beneficial to have an assay for the simultaneous and quantitative evaluation of both the sCT and dOVM.

A review of the literature did not indicate any assay for simultaneous quantification of sCT and dOVM although a number of assays have been reported for the individual quantification of sCT [10–14, 19, 20] and for the fractionation of glycoproteins [21]. Additionally, there have been no available chromatographic assays for the quantification of dOVM. Therefore, by the current assay method, dOVM *per se* can be quantified or it can be used for the simultaneous analysis of both sCT and dOVM.

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 ml/min was found to be an appropriate mobile phase allowing adequate separation of the sCT and dovm (retention times 7.8 and 15.4 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 as no peak was observed at isocratic conditions. The influence of organic modifier (acetonitrile) on retention time of sCT has already been studied [24]. The retention of calcitonin was found to be very sensitive to the concentration of the organic modifier. During the development of the assay it was observed that an increase in the percentage of acetonitrile above 40% was affecting the sensitivity and the retention times of the peaks. Precipitation of the dOVM was observed at higher concentrations of acetonitrile, 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 components governing the elution time and reproducibility of the dOVM peaks also.

TFA was used in the mobile phase as an ion-pair reagent as it affected the peak shape. CT is hydrophilic, therefore, mobile phase is often adjusted below pH 3 to reduce the polarity of the protein [25] and to increase interaction with the nonpolar stationary phase. At this pH value, most

of the carboxylic groups of the amino acid residues are in an undissociated form. However, at these low pH values amine residues of basic amino acids are charged. Therefore, an appropriate reagent that can pair with these ions is essential to produce a molecular complex with sufficient hydrophobicity to interact with the stationary phase. TFA has high protein solubilizing and adsorption characteristics, and does not disrupt the native protein conformation [26–29]. Also, TFA is a powerful partition reagent and has high surface-active properties [30]. Therefore, it can be used to mask the remaining silanol groups on the support surface. The influence of TFA concentration on the retention time of sCT is studied in another study [24]. Increasing TFA concentration directly affects the retention of sCT and it was recommended that the TFA concentration of more than 0.1% should be avoided because it might result in cleavage of the bound phase. Therefore, 0.05% TFA was used in the mobile phase.

In conclusion, a gradient, reversed phase – HPLC method is reported for the simultaneous quantification of sCT and dOVM. The consistent recoveries and low coefficient of variation confirms the suitability of the proposed method for the simultaneous quantification of sCT and dOVM.

4. Experimental

4.1. Chemicals and reagents

Recombinant human sCT was obtained from the Calbiochem Novabiochem (La Jolla, CA, USA). dOVM was obtained as a gift from the Massachusetts College of Pharmacy (MA, USA). Trifluoroacetic acid was 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.

4.2. Standard solutions

Stock solutions of sCT (1000 μg/ml) were prepared by dissolving in distilled deionized water. 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 water. Stock solutions of dOVM were prepared by dissolving it in distilled and deinonised water to obtain a final concentration of 1000 μg/ml. Dilutions of the stock solutions for the preparation of the calibration standards were carried out using distilled and deionised water.

Calibration standards were prepared with stock solutions of both sCT and dOVM, to produce concentrations of 0 (blank), 10, 30, 50, 70, and 100 μg/ml and 0 (blank), 10, 30, 50, 70, and 100 μg/ml of sCT and dOVM respectively.

4.3. Sample preparation

Equal volumes of calibration standards of sCT and dOVM were added to the vials to obtain concentrations of 0, (blank), 10, 30, 50, 70, and 100 μg/ml of sCT and 0 (blank), 10, 30, 50, 70 and 100 μg/ml for dOVM. The dilutions were made with deionized and distilled water. Fifty microlitres of the injection solution was injected into the system.

4.4. 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 100μl injection loop, a Dynamax UV-1detector 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 (5μm, 4.6 × 250 mm) with a pore size of 300 Å. 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 20–35% B for 10 min, 35–37% B from 10^{th} to 20^{th} min and 37–20% B from 20^{th} to 25^{th} min at a flow rate of 1 ml/min. The detection was achieved at a wavelength of 210 nm.

4.5. 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 [31]. Control samples of sCT and dOVM containing lowest, midpoint and highest concentration in the calibration curve were run along with the calibration curve. The data were weighed by concentration^{-1}. 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 sCT concentrations of 10, 50, and 100 μg/ml and at the dOVM concentrations of 10, 50 and 100 μg/ml respectively.

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