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Hydrophilic Interaction Liquid Chromatography Coupled with Fluorescence Detection (HILIC-FL) for the Quantitation of Octreotide in Injection Forms

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Abstract: Octreotide is a synthetic cyclic octapeptide analogue of somatostatin-14. It is mainly administered for the treatment of acromegaly, severe diarrhea, and neuroendocrine neoplasias. In this work, a hydrophilic interaction liquid chromatography (HILIC) method with fluorescence (FL) detection was developed and validated for the quantitation of octreotide in solutions for injection. Chromatographic separation was performed on an XBridge®-HILIC analytical column under isocratic elution with a short chromatographic run time of less than 10 min. The mobile phase consisted of ammonium bicarbonate 8.6 mM (pH 8.1)/acetonitrile 35/65 (*v/v*). The high sensitivity and selectivity of the fluorescence detection, with the excitation wavelength ($\lambda_{excitation}$) set at 280 nm and the emission wavelength set at ($\lambda_{emission}$) 330 nm, enabled a simple sample preparation procedure that included only dilution steps. The calibration curve showed good linearity with a correlation coefficient greater than 0.998. The method was successfully applied to the analysis of commercially available octreotide injection forms.

Keywords: hydrophilic interaction liquid chromatography; fluorescence detection; octreotide; octreotide acetate; SMS 201-995; somatostatin

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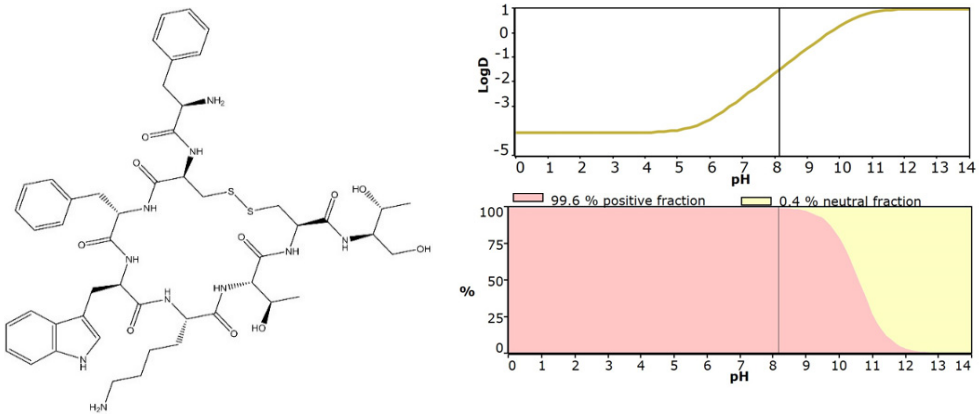
1. Introduction

The neuropeptide somatostatin is a cyclic tetradecapeptide originally isolated from ovine hypothalamus and has been shown to be distributed in the peripheral and central nervous system [1,2]. It inhibits the secretion of growth hormone, glucagon, insulin, secretin, and gastrin, and takes part in neural transmission [3–6]. Somatostatin-14 is derived from the prohormone somatostatin-28, which is also biologically active [7]. The clinical use of somatostatin-14 is limited due to its short half-life (less than 3 min) and the post-infusion rebound hypersecretion of hormones [8]. To overcome these issues, many somatostatin analogues were synthesized [9].

Octreotide (SMS 201-995) is an eight amino acid peptide and the first synthesized analogue of somatostatin-14 [10,11]. It is a cyclic octapeptide and the retention of the disulfide bond is essential for its biological activity (Figure 1a) [12]. Octreotide binds to somatostatin receptor 2 (SSTR2), which inhibits the secretion pathways of growth hormone, as well as glucagon, angiogenesis, T-cell proliferation, and pro-inflammatory cytokines [13–15]. When compared to somatostatin-14, octreotide has been shown to have similar pharmacological properties but a longer half-life (approximately 2 h), in addition to greater potency and antihormonal specificity, with no rebound effect after administration [16,17]. It is

indicated for the treatment of acromegaly, severe diarrhea, neuroendocrine tumors of the pancreas and gastrointestinal tract, and for the treatment of acute bleeding due to esophageal varices [18,19]. It is currently used to control the hypersecretion of hormones in pituitary, endocrine pancreatic, and carcinoid tumors [20–23]. Octreotide is also an option as a therapeutic in neonates with congenital and acquired chylothorax, but its therapeutic role has not yet been substantiated [24].



(**a**) (**b**)

Figure 1. (**a**) Octreotide chemical structure; (**b**) plots of the LogD values (top) and the ionization fraction (bottom) as a function of pH.

A literature survey revealed various analytical methods for the detection of octreotide. These methods include the determination of octreotide levels in human plasma using ultrahigh-performance liquid chromatography with tandem mass spectrometric detection (UHPLC-MS/MS) [25], the quantification of octreotide acetate in formulation and stability studies in sodium bisulfate using reversed phase liquid chromatography with ultraviolet (UV) detection at 210 nm [26,27], the assessment of octreotide stability under stress conditions via a capillary electrophoresis-mass spectrometric method (CE-MS) [28] and the determination of long-acting release octreotide in human plasma by means of liquid chromatography coupled to tandem mass spectrometry [29]. Reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection was used to separate poly(ethylene glycol)octreotide derivatives with various molecular weights [30] and also to find optimal conditions to produce mono-poly(ethylene glycol) octreotide [31]. Recently, an on-line electrochemistry–tandem mass spectrometry was developed for the structural characterization of the octreotide-related impurities generated during the production of octreotide-loaded poly(D, L-lactic-co-glycolic acid) microspheres [32].

In the last decade, high performance liquid chromatography (HPLC) has been widely used in the analysis of peptides in various fields of research and development, using different modes of separation. Nowadays, hydrophilic interaction liquid chromatography (HILIC) is ideal for the analysis of peptides [33–36]. In HILIC, the separation mechanism is complex, including partition, normal phase/absorption, hydrogen bonding, electrostatic and reversed phase interactions [37–40]. The analytes' retention in HILIC is increased by increasing the proportion of the organic modifier (typically acetonitrile) in the mobile phase. In this work, a hydrophilic interaction liquid chromatographic method with fluorescence (HILIC-FL) detection was developed and validated for the quantification of octreotide in formulations for injection. To the best of the authors' knowledge, this is the first reported application of HILIC and fluorescence detection for the quantification of octreotide in pharmaceutical preparations leading to a rapid, selective, and accurate method with simple sample preparation procedure.

2. Materials and Methods

2.1. Chemical and Reagents

Acetonitrile of HPLC grade was supplied by Merck (Darmstadt, Germany). Analytical purity grade ammonium formate, ammonium acetate and ammonium bicarbonate were obtained from Acros Organics (Morris Plains, NJ, USA). Octreotide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethylamine was purchased form Sigma-Aldrich–Fluka and the water was distilled and purified using the Synergy® water purification system (Merck Millipore, Burlington, MA, USA).

Commercially available injection forms of octreotide acetate were purchased from community pharmacies. Each injection form was labelled to contain 0.1 mg octreotide per 1 mL, in the form of octreotide acetate, and inactive ingredients including lactic acid, mannitol (E421), sodium hydrogen carbonate, and water for injections.

2.2. Instrumentation and Chromatographic Conditions

Chromatography was performed using a Spectra physics SP8810 HPLC system with an isocratic pump, a Rheodyne 7725i injection valve with a 20 μL loop and a PerkinElmer LS300 fluorescence detector. The analytical column used for the analysis of octreotide was the BEH XBridge®-HILIC (150.0 × 2.1 mm i.d., particle size 3.5 μm, 135 Å) (Water's, Milford, MA, USA) with an XBridge®-HILIC (20 × 2.1 mm, 3.5 μm) guard column. A mobile phase of ammonium bicarbonate 8.6 mM (pH 8.1)/acetonitrile 35/65 (*v/v*) was used at a flow rate of 0.25 mL min^{-1}. The mobile phase was always degassed under vacuum while filtering through a 0.45 μm nylon membrane filter prior to use. The excitation wavelength was set at 280 nm and the emission wavelength was set at 330 nm. Chromatographic experiments were performed at ambient temperature with a chromatographic run time of less than ten minutes. Water's Empower software was used for data acquisition and processing.

2.3. Preparation of Stock and Working Standard Solutions

A stock solution of octreotide (100 μg mL^{-1}) was prepared by dissolving exactly 2 mg of the reference substance in a 20 mL volumetric flask with acetonitrile. This solution was further diluted in acetonitrile to prepare working standard solutions. These solutions were stored in the dark and under refrigeration at 4°C and remained stable for several weeks.

Calibration standard solutions and quality control (QC) samples were freshly prepared every working day. Calibration standard solutions of octreotide were prepared in acetonitrile at the concentration levels of 0.15, 0.25, 0.5, 1.0, 1.25 and 1.5 μg mL^{-1}. Quality control samples were prepared in the same way at three concentration levels 0.15, 1.0 and 1.5 μg mL^{-1}.

2.4. Preparation of Sample Solution

For the analysis of the pharmaceutical formulation, 1 mL of the injectable solution (corresponding to 0.1 mg octreotide) was transferred to a 10 mL volumetric flask and then diluted to volume with acetonitrile/water mixture 80/20 (*v/v*). Then, 500 μL of the solution was transferred to a 5 mL volumetric flask and brought to volume with acetonitrile. The resulting test solution contained octreotide at 1 μg mL^{-1} and it was analyzed immediately without any filtration step.

2.5. Method Validation

The method was validated in terms of linearity, precision, accuracy, robustness, and stability according to the ICH guidelines [41].

2.6. Stability Studies

The stability of octreotide injection forms was evaluated under forced degradation studies. The conditions for accelerated degradation studies for octreotide were designed to assure that some degradation occurred, but not enough to generate secondary products. The concentration of octreotide in the stability samples was 1 μg mL^{-1}. The stress con-

ditions involved acidic hydrolysis in 0.01 M hydrochloric acid (HCl) at 25 °C for 75 min, alkaline hydrolysis in 0.01 M sodium hydroxide (NaOH) at 25 °C for 70 min and oxidation in 0.001 % hydrogen peroxide (H_2O_2) at 25 °C for 125 min.

3. Results and Discussion

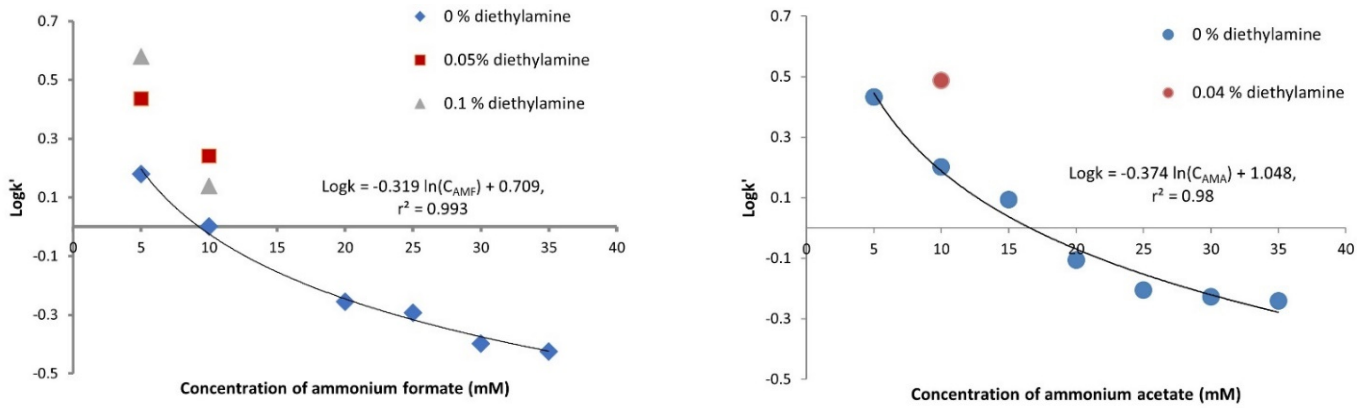
3.1. Method Development

Octreotide consists of an eight-amino-acid chain, NH-D-Phe-Cys(1)-Phe-D-Trp-Lys-Thr-Cys(1)-Thr-ol, as shown in Figure 1a. The LogD values and the ionization fraction of octreotide, as a function of pH, were calculated by the ADME boxes software, ver. 3.0.3, build 45, Pharma Algorithms Ltd., and the results are presented in Figure 1b.

As shown by the plots of the ionization fraction presented in Figure 1b, octreotide is a hydrophilic peptide. HILIC chromatography appears to be ideal for the analysis of polar compounds such as octreotide. The BEH XBridge®-HILIC analytical column used in this work consisted of bis-triethoxysilylethane groups with ethylene bridges (BEH particles). To achieve better efficiency of the chromatographic system, the mobile phase and the wavelength of the detection were optimized. One factor at a time was changed while the others remained at a constant value.

During the preliminary experiments, the salts that were used in the mobile phase eluent were ammonium formate (AMF) and ammonium acetate (AMA), and their concentration levels ranged from 5 to 35 mM in mobile phases that consisted of acetonitrile/aqueous buffer 70/30 *v/v*. In the case of ammonium formate, the pH of the aqueous content of the mobile phase was 6.4; in the case of ammonium acetate, the pH was 6.9.

As shown in Figure 2, an increase in the concentration of the ammonium formate (Figure 2a) and ammonium acetate (Figure 2b) decreased the retention of octreotide. At the pH values tested, the residual silanols of the stationary phase were negatively charged. Therefore, an increase in the concentration of the buffering salt in the mobile phase decreased the electrostatic attractions between the negatively charged residual silanols of the stationary phase and the positively charged molecules of the analyte. To improve the peak symmetry, a proportion of diethylamine was added at 5 mM and 10 mM of ammonium formate (Figure 2a) and at 10 mM of ammonium acetate (Figure 2b). It was then observed that, through the addition of diethylamine, the retention of octreotide was increased with concomitant improvements in the peak symmetry.



(a) (b)

Figure 2. Plots of the logarithm of capacity factor (Logk) of octreotide versus the concentration of (**a**) ammonium formate (C_{AMF}) and (**b**) ammonium acetate (C_{AMA}), in the aqueous component of the mobile phase that consisted of acetonitrile/aqueous buffer 70/30 *v/v*.

Continuously, ammonium bicarbonate (AMC) was also tested as a salt in the mobile phase aqueous eluent (30%, *v/v*) and at concentration levels ranging from 5 mM to 25 mM at a pH of 8.1. It was found that the use of AMC as buffering salt improved the peak symmetry without adding diethylamine in the mobile phase. Typical chromatograms representing the effect of the concentration of AMC on the peak shape and the retention time of octreotide are shown in Figure 3.

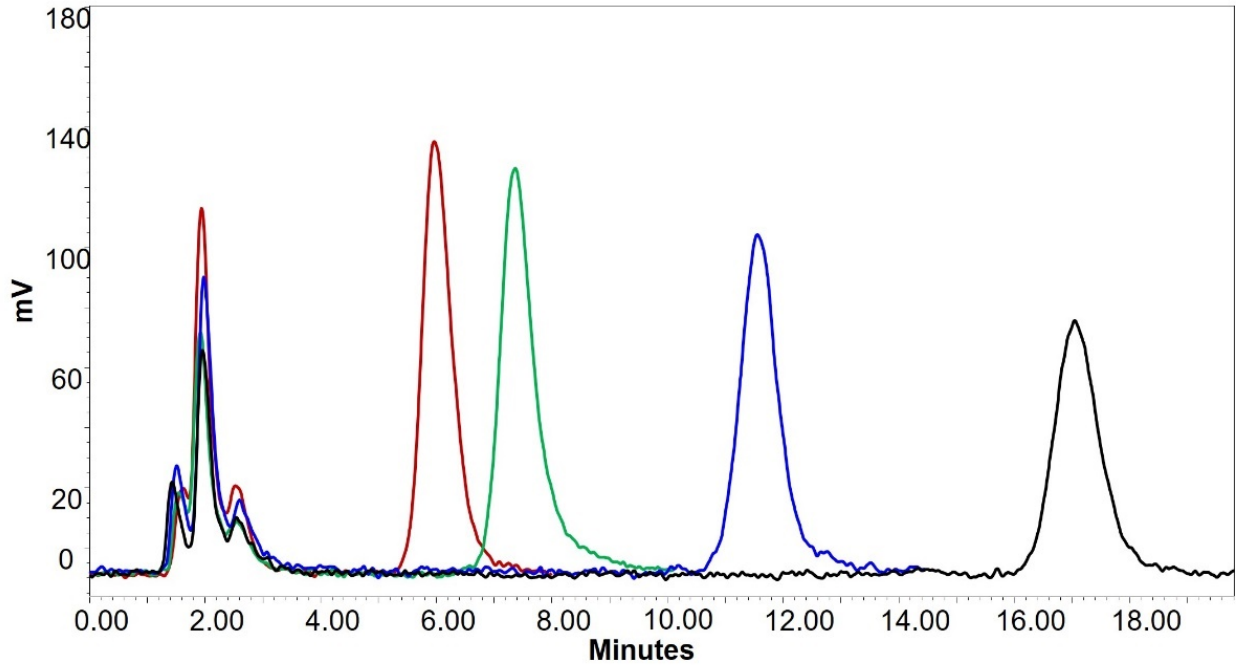


Figure 3. Superimposed HILIC-FL chromatograms of octreotide test solution at 1 μg mL^{-1} using various concentrations of ammonium bicarbonate (AMC) as a buffering salt in the mobile phase. Black line: 5 mM AMC; blue line: 10 mM AMC; green line: 15 mM; red line: 25 mM AMC. Chromatographic conditions: BEH XBridge®-HILIC analytical column; mobile phase acetonitrile/aqueous solution of ammonium bicarbonate (70:30, *v/v*); flow rate—0.25 ml min^{-1}.

It was observed that when 10 mM of ammonium bicarbonate was used in the mobile phase, adequate retention of octreotide at 11 minutes was achieved, with a good symmetry factor of 1.1. The best detector response for the analyte was observed at the excitation wavelength (λ_{ex}) of 280 nm and at the emission wavelength (λ_{em}) of 330 nm.

The effect of the percentage of water, φ_{water}, was also evaluated in experiments where the mmoles of ammonium bicarbonate in whole mobile phase were kept constant at the optimum value of 0.3 mmoles (pH 8.1), while φ_{water} varied from 15% to 35%. An exponensial relationship between the logk' value of the octreotide in relation to the percentage of water in the mobile phase (φ_{water}) was observed (data not shown).

According to the above studies, a mobile phase consisting of ammonium bicarbonate 8.6 mM (pH 8.1)/acetonitrile 35/65 (*v/v*) was chosen as the optimum. The selectivity of the proposed HILIC-FL method is illustrated in Figure 4, where it is depicted that a chromatogram obtained from the analysis of the octreotide quality control sample at 1 μg mL^{-1} (black line) was superimposed to a chromatogram obtained from the analysis of the octreotide injection form (red line). Under the optimum chromatographic conditions, octreotide was eluted at 7.7 min. The high sensitivity and selectivity of the fluorescence detection, with the excitation wavelength ($\lambda_{excitation}$) set at 280 nm and the emission wavelength set at ($\lambda_{emission}$) 330 nm, enabled a simple sample preparation procedure that included only dilution steps.

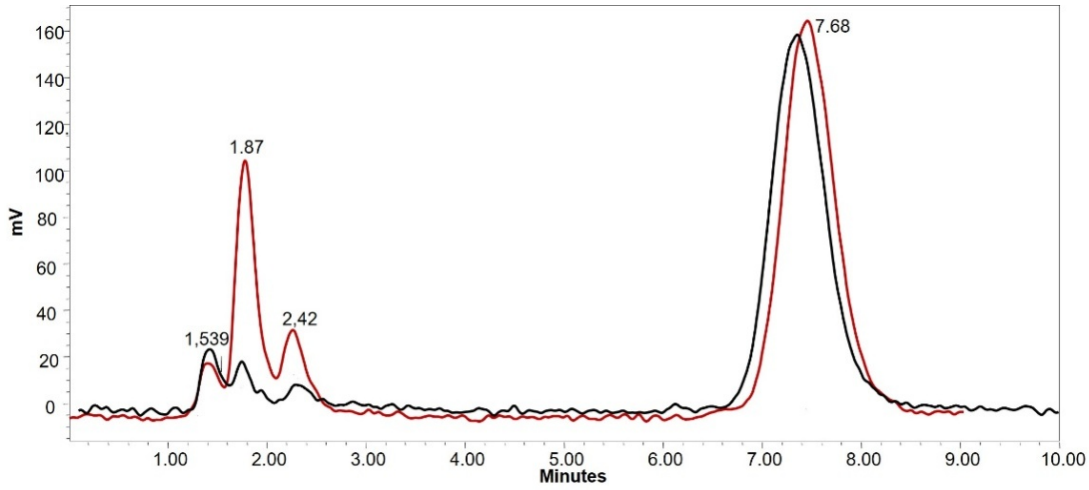


Figure 4. Superimposed HILIC-FL chromatograms obtained from the analysis of a quality control sample at 1 μg mL^{-1}(black line) and of an octreotide injection form sample at 1 μg mL^{-1}(red line). Chromatographic conditions: BEH XBridge®-HILIC analytical column; mobile phase acetonitrile/8.6 mM (pH 8.1) aqueous solution of ammonium bicarbonate (65:35, *v/v*); flow rate—0.25 ml min^{-1}.

3.2. Statistical Analysis of Data

3.2.1. Linearity

To evaluate the linearity, three calibration curves were constructed in three different analytical runs during a two-week period, each one with six different analyte concentrations in the range of 0.15 to 1.5 μg mL^{-1}. The peak area signal of the peptide multiplied by 10^{-6}, S, versus the corresponding concentration, C, exhibited linear relationships. The results showed good linearity; in all cases, the correlation coefficient was greater than 0.998. The back-calculated concentrations were within 4.0% to 4.6% of the nominal values; this is in agreement with international guidelines. The results of a typical calibration curve are shown in Table 1.

Table 1. Linearity data for the quantitation of octreotide as assessed by the HILIC-FL method.

Concentration Range (μg mL^{-1}).	
Regression equation	0.15–1.5
Correlation coefficient, r	S = 4.91 (±0.11) × C + 0.258 (±0.034)
Standard error of estimation, Sr	0.998
Limit of Detection, LOD (μg mL^{-1})	0.18
Limit of Quantitation, LOQ (μg mL^{-1})	0.02

The limit of detection (LOD) and limit of quantitation (LOQ) values for octreotide were calculated as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively. This was achieved by the analysis of dilute solutions of octreotide at known concentrations [42], and the results are reported in Table 1.

3.2.2. Accuracy and Precision

To evaluate the accuracy and precision of the proposed method, quality control samples were analyzed in five replicates and at three concentration levels (0.5, 1.0 and 1.5 μg mL^{-1}) within three different analytical runs. The accuracy was assessed by the percentage of relative recovery (% Relative recovery) and the precision was assessed by the percentage coefficient of variation(% CV). The results are presented in Table 2. Intra-day % CV values ranged between 0.95 % and 1.28 %, the inter-day % CV values ranged from 0.95 % to 1.15 %, and the overall accuracy ranged from 99.6 % to 104.5 %.

Table 2. Accuracy and precision data of the HILIC-FL method for the quantitation of octreotide (n = 3 runs in 5 replicates).

Parameters	Concentration Levels (μg mL^{-1})		
Added Concentration	**0.15**	**1.0**	**1.5**
Overall mean	0.1494 (±0.0004)	1.045 (±0.003)	1.471 (±0.005)
Intra-day, CV (%) [1]	0.95	1.11	1.28
Total precision, CV (%) [1]	0.99	0.95	1.15
Total accuracy, Relative recovery (%) [2]	99.6	104.5	98.1

[1] Coefficient of variation. [2] Relative recovery percentage.

3.2.3. Stability Studies

After the acidic hydrolysis, 24.5% of the octreotide was degraded using 0.01 M HCl for 75 min at 25°C. After 70 min of basic hydrolysis in 0.01 M NaOH at 25°C, 16.7% of the octreotide was degraded. Under oxidative degradation in 0.001% H_2O_2 for 125 min at 25°C, 16.3% of the sample was degraded. The results of the accelerated stability studies indicated that any degradation products could not be detected under the current conditions and, therefore, that they did not interfere in the quantitation of the parent drug.

3.3. Application to the Analysis of Real Samples

The method was applied to the analysis of three different batches of commercially available injection forms labelled to contain 0.1 mg of octreotide per 1 mL. The preparation of the sample solutions is described in Section 2.4. The concentration of octreotide was estimated according to the calibration curve. The results in Table 3 show a good recovery, ranging from 101.37 to 100.9 %. These results are in agreement with the results obtained by Kyaterekera et al [26] for the analysis of octreotide in Sandostatin® injection forms.

Table 3. Results of the HILIC-FL method for the quantitation of octreotide in injection forms.

Octreotide Injection Form	% Label Claim (±SD) [1] $_{(n=5)}$	% CV [2]	%Recovery (±SD) [1] $_{(n=5)}$
Batch No 1	0.00163 (±0.00076)	0.75	101.63 (±0.76)
Batch No 2	0.10137 (±0.00087)	0.86	101.37 (±0.87)
Batch No 3	0.1009 (±0.0011)	1.1	100.9 (±1.1)

[1] Standard deviation. [2] Coefficient of variation.

4. Conclusions

Hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FL) was applied for the first time to the analysis of octreotide in pharmaceutical dosage forms. Fluorescence detection offers selectivity, as well as higher sensitivity, when compared to UV detection methods. The proposed method was fully validated in terms of linearity, accuracy, and precision for the quantitation of octreotide in injection forms. Furthermore, stability studies performed under acidic, basic, and oxidative conditions indicated that the degradation products could not be detected under fluorescence detection and, therefore, that they did not interfere in the analysis of octreotide. The simplicity of the sample preparation procedure and the short chromatographic run time of less than 10 min allowed for high sample throughput. Based on the above, the proposed method has practical value for pharmaceutical manufacturers and analytical laboratories, and it can be used to support the routine quality control of octreotide in injection forms.

Author Contributions: Conceptualization, N.A.C., K.T. and I.P.; methodology, I.P. and E.D.; validation, E.D., A.P. and I.P.; formal analysis, E.D. and I.P.; investigation, M.K., E.D. and A.P.; resources, I.P., N.A.C. and K.T.; writing—original draft preparation, A.P. and I.P.; writing—review and editing, I.P., K.T. and N.A.C.; visualization, E.D.; supervision, I.P. and N.A.C.; All authors have read and agreed to the published version of the manuscript.

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