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Analysis and Preparation of Linaclotide by High Performance Liquid Chromatography

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Abstract

Linaclotide, a synthetic peptide, is a guanylate cyclase-C (GC-C) agonist for treating irritable bowel syndrome with constipation and chronic idiopathic constipation. A simple, precise and selective RP-HPLC method for linaclotide analysis was developed and validated. Analysis was achieved with a SinoChrom ODS-BP column, mobile phase A and B comprised 30 mol·L^{-1} phosphate (pH 2.8) solution and acetonitrile respectively, gradient elution B% set to 7%→25% from 0 to 32 min and to 25% from 32 to 50 min, flow rate of 1.0 mL·min^{-1}, oven temperature at 40℃, injection volume of 20 μL, and UV detection wavelength at 214 nm. Method validation was carried out in linearity, LOD, LOQ, precision and stability. Modified method for linaclotide preparation on a preparative liquid chromatography was developed from the analytical method and applied successfully.

Keywords: Linaclotide; HPLC; Analysis; Preparation

Introduction

Linaclotide is a guanylate cyclase-C (GC-C) agonists, which is aimed for treating constipation type of irritable bowel syndrome (IBS-C) and chronic idiopathic constipation (CIC) [1]. One third of IBS patients are IBS-C around the world. These patients have had influenced greatly in the quality of their lives, and had given a large burden to the society. Linaclotide have brought expectation for these patients since it was approved by FDA in 2012. Linaclotide and its active metabolite bind to GC-C, which is found on the intestinal epithelium, to stimulate the release of intracellular and extracellular cyclic guanosine monophosphate (cGMP) [2]. The rise of intracellular cGMP concentration promotes the intestinal fluid secretion, strengthen the gastrointestinal peristalsis, and lead to an increased frequency of defecation. The rise of extracellular cGMP concentration can reduce the sensitivity of the nere, which reduces patient's pain, so as to relieve the symptoms of abdominal pain and so on. Other medicines, which treat constipation, generally do not reduce adverse reactions in patients with abdominal distension, abdominal pain, etc.

Linaclotide [3] is a peptide with 14 amino acids (CCEYCCNPACTGCY) and three disulfide bonds (1-6, 2-10, 5-13). The molecular weight is 1526.8 [3]. The structure was shown in Figure 1. Solid phase peptide synthesis was applied to synthesise linaclotide. Impurities in crude product mainly include incompletely synthesised peptides, incorrectly-formed disulfides of peptides, etc. Therefore, it is crucial to set up HPLC methods for the analysis and the preparation of linaclotide.

Materials and Methods

Materials

Linaclotide were synthesised by Fmoc solid phase peptide synthesis in our laboratory. Acetonitrile was obtained from Anaqua Chemicals Supply (Houston, TX, USA). Formic acid was obtained from Aladdin (Shanghai, China). Phosphoric acid and Sodium dihydrogen phosphate were purchased from XiLong Chemical Company (Shantou, Fujian, China). Distilled water was purified with Millipore's Milli-Q analytical deionization system (Bedford, MA, USA).

Instruments

Dionex P680 HPLC (Sunnyvale, CA, USA) equipped with 170UV detector was applied for analysis. The chromatographic separation was performed on an Elite 250 × 4.6 mm column (Sinochrom ODS-BP 5 μm) with injecting volume of 20 μL. Gilson Preparative HPLC (322 pump) equipped with 156 detectors and GX-281 host (Middleton, WI, USA) was applied for preparation. The preparation was performed on an Elite 250 × 30 mm column (Sinochrom ODS-BP 10 μm).

Sample preparation

Accurately weighed crude linaclotide product was dissolved in mixed solvents, which was water: acetonitrile=2:1 (v/v), with 0.1% formic acid. The sample solution and mobile phases were filtered through a 0.22 μm PTFE filter (Jinan, Shandong, China) prior to analysis and preparation.

HPLC determination

Mobile phases of the HPLC were phosphate solution (A) and acetonitrile (B) [4]. The maximum absorption wavelength was determined at 214 nm by full range scan of linaclotide on UV spectrometry. The eluting gradient was shown in Table 1. The volume of injection was 20 μL. The flow rate was 1.0 mL/min. Optimised analytical conditions were as follows: the analytical column was SinoChrom ODS-BP (5 μm, 250 mm × 4.6 mm); mobile phase A was 30 mmol·L^{-1} phosphate (pH-2.8) and mobile phase B was acetonitrile; column temperature was set to 40°C.

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Optimisation of the HPLC column

To separate linaclotide by HPLC, three kinds of HPLC columns, i.e., SinoChrom ODS-BP (250 mm×4.6 mm, 5 mm, pH 2-8), Ultimate XB-C18 (250 mm × 4.6 mm, 5 mm, pH 1-12) and Kinetex Evo C18 (250 mm × 4.6 mm, 5 mm, pH 1.5-10), were examined.

Optimisation of solvents

Five different proportions of acetonitrile as solvent to dissolve crude linaclotide were compared in the solubility and the peak shape [5].

Optimisation of pH

In order to investigate the effect of pH on linaclotide analysis, phosphate was applied to adjust the pH of mobile phase A from 2.0 to 3.2.

Optimisation of phosphate concentration

Phosphate concentration of mobile phase A was optimised in the range of 10~50 mmol·L^{-1} to investigate the effect of buffer concentration on linaclotide analysis [6].

Optimisation of oven temperature

Oven temperature was compared as an effect on resolution in the range of 17~40°C controlled by column oven.

Linearity and Limit of Detection (LOD)/Limit of Quantification (LOQ)

The calibration curve was plotted in the range of 0.1-1.6 mg/mL of linaclotide and fitted with least squares linear regression. The LOD and LOQ were estimated considering the ratio of back-ground noise and analytical signal. Linaclotide LOD and LOQ were determined based on the calibration curve and calculated according to Eq. (1) and Eq. (2).

$$LOD=3\sigma/S \quad \text{Eq. (1)}$$

$$LOQ=10\sigma/S \quad \text{Eq. (2)}$$

Where σ represents the standard deviation of the response, S represents the slope of the calibration curve.

Precision

Precision was evaluated in terms of repeatability. Repeatability (intra-assay) was verified for one concentration of linaclotide with 6 replicates.

Stability

Linaclotide was placed at room temperature (24°C) and kept at this temperature for 24 hours. Samples were analysed at 0, 2, 4, 8, 12, 24 h.

Preparation of linaclotide

In chromatographic preparation of linaclotide, analytical method was modified due to the modification of flow rate, the volume of sample, and the diameter of column [7]. The preparative chromatographic conditions were as follows: the column was SinoChrom ODS-BP (250 × 30 mm, 10 μm); mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid; the elution gradient was 7%→25% B% from 0 to 32 min and was 25% from 32 to 50 min; room temperature was applied; the flow rate was 30 mL·min^{-1}; wavelength of detection was at 214 nm.

Results and Discussion

Optimisation of the HPLC column

Three types of chromatographic columns were compared to analyse crude linaclotide. The analytical results were shown in Table 2. The SinoChrom ODS-BP was selected for the further analysis due to its best resolution results with impurities before and after linaclotide.

Optimisation of solvents

Results (Table 3) showed that with increasing portion of acetonitrile in mobile phase, the chromatographic peaks of linaclotide were deformed and open. Optimised portion was set to water: acetonitrile=2:1 (v/v).

Optimisation of pH

Results were shown in Figure 2. The resolution of the impurity before linaclotide and linaclotide (R1) was gradually increased with the increase of pH. The resolution of linaclotide and the impurity after linaclotide (R2) was increased till pH 2.8, and then decreased. The optimised pH was set to 2.8 adjusted by phosphate buffer.

Optimisation of phosphate concentration

Results were shown in Figure 3. The R1 gradually decreased with the increase of phosphate concentration. The R2 increased with the increase of phosphate concentration. 30 mmol·L-1 was chosen as the optimised concentration of phosphate solution as mobile phase A.

Optimisation of oven temperature

Results was shown in Figure 4. With the increase of oven temperature, R1 and R2 increased. The oven temperature was optimised to 40°C. The optimised chromatography diagram was shown in Figure 5.

Linearity and LOD/LOQ

The linear equation of the calibration curve is: y=231.9, x=4.310, r=0.9995, where y represents the peak area and x represents the concentration of linaclotide. Limit of detection (LOD) and limit of quantification (LOQ) were 0.5 μg/mL and 1.0 μg/mL with the signal-

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	93	7
32	75	25
50	75	25

Table 1: Eluting gradient for the linaclotide analysis by HPLC.

Column	pH range	t_R (min)	Asymmetry	R_1	R_2
SinoChrom ODS-BP	2-8	30.8	1.16	5.91	6.13
Ultimate XB-C_{18}	1-12	27.1	1.02	4.16	5.52
Kinetex Evo C_{18}	1.5-10	24.6	1.27	2.38	3.06

R_1: the resolution of impurity before linaclotide with linaclotide, R_2: the resolution of impurity after linaclotide with linaclotide.

Table 2: Comparison of different columns for linaclotide analysis on HPLC.

Solvent (water: acetonitrile, v/v)	Solubility	Peak shape
2:3	soluble	Split
1:1	soluble	Split
2:1	soluble	symmetry
3:1	Slightly soluble	symmetry
4:1	Insoluble	/

Table 3: Influence of different ratio of solvents on the chromatographic peak of linaclotide in HPLC.

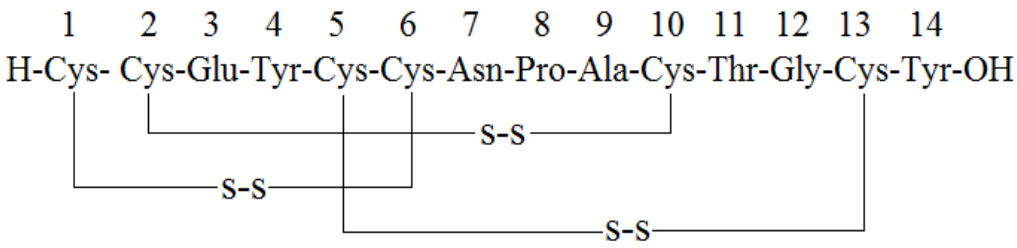

Figure 1: The structure of Linaclotide.

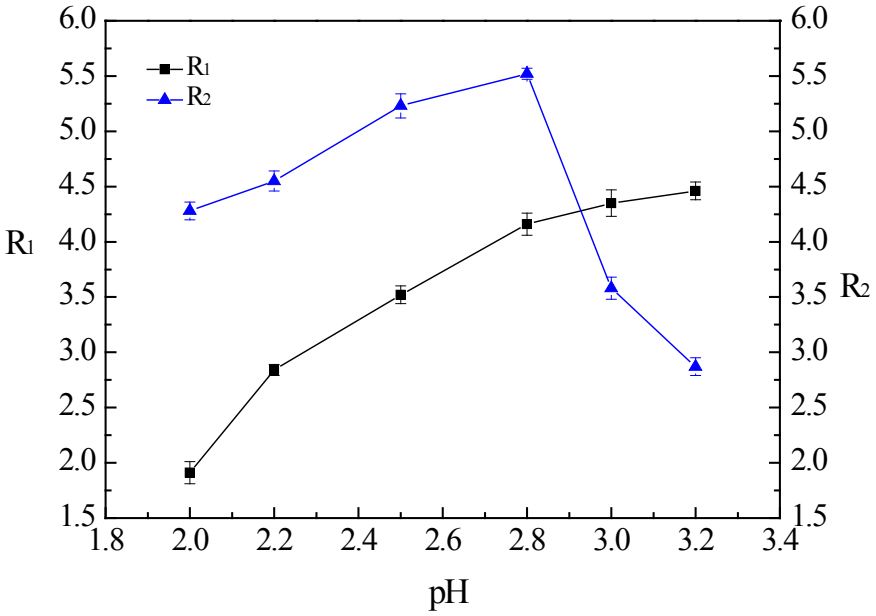

Figure 2: pH influence of phosphate in the mobile phase A on resolution, R_1: the resolution of impurity before linaclotide with linaclotide, R_2: the resolution of impurity after linaclotide with linaclotide.

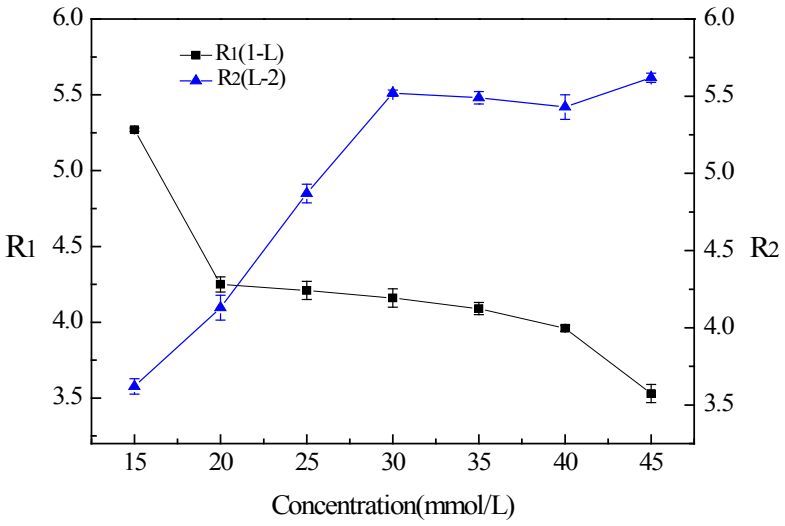

Figure 3: Influence of phosphate concentration in the mobile phase A on resolution, R_1: the resolution of impurity before linaclotide with linaclotide, R_2: the resolution of impurity after linaclotide with linaclotide.

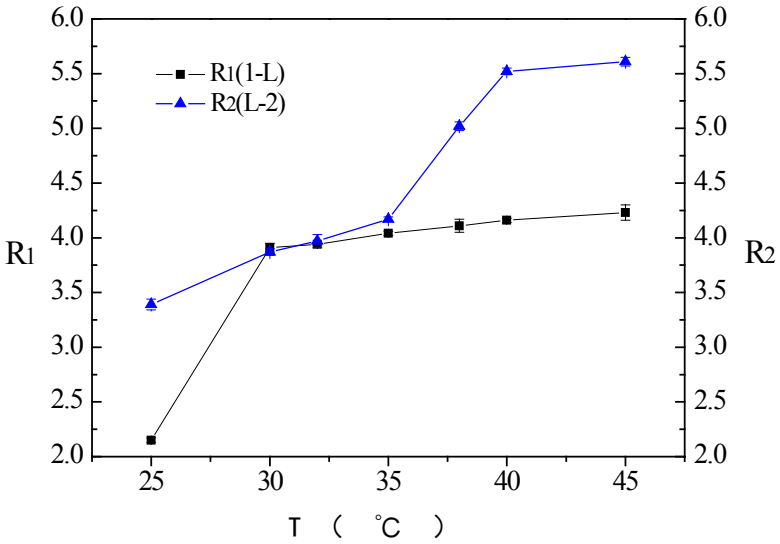

Figure 4: Influence of oven temperature on resolution, R_1: the resolution of impurity before linaclotide with linaclotide, R_2: the resolution of impurity after linaclotide with linaclotide.

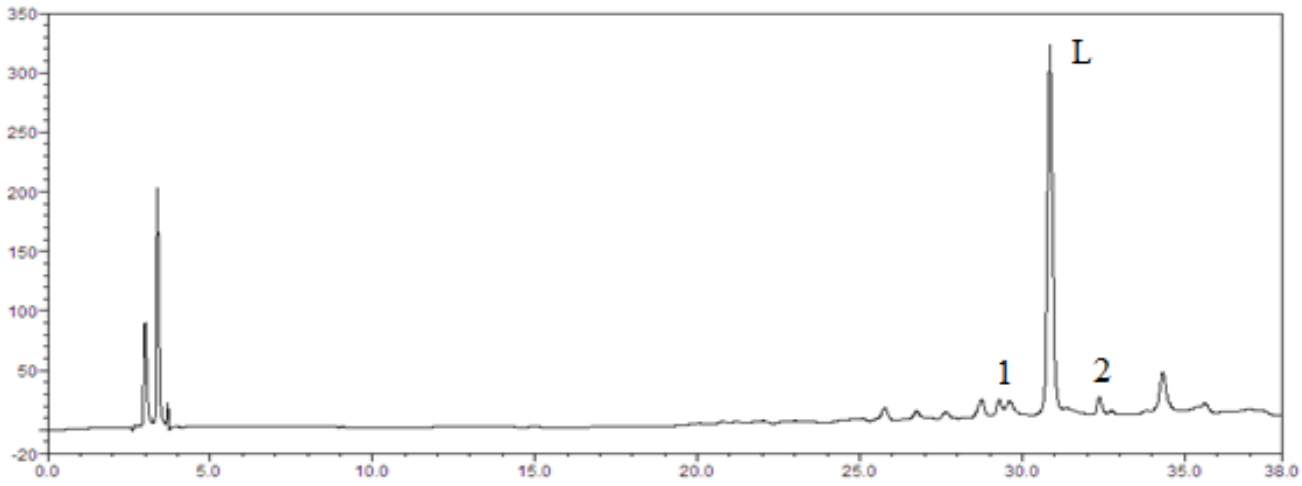

Figure 5: HPLC chromatogram of Linaclotide, L represents the peak of linaclotide, 1 represents the impurity peak before linaclotide and 2 represents the impurity peak after linaclotide.

noise ratio 3:1 and 10:1, respectively.

Precision

The RSD of intraday results were less than 1%. Results were listed in Table 4.

Stability

Peak area and retention time were compared. Results suggested that linaclotide solution was stable in 24 hours, shown in Table 5. RSD was less than 1%.

Application in preparative chromatography

Preparative chromatography diagram was shown in Figure 6, in accordance with linaclotide was collected between the vertical lines. Analysis of freeze-dried linaclotidewas shown in Figure 7. Batch preparation results were shown in Table 6. The purity of linaclotide after preparation was over 98%.

Conclusion

Analytical RP-HPLC method was developed for analysis of linaclotide. Optimisation of solvent and chromatographic conditions including pH of mobile phase, phosphate concentration and oven temperature was studied. Method validation was carried out in linearity, LOD, LOQ, precision and stability. Linaclotide was successfully prepared on preparative HPLC with this method. Modified method for linaclotide preparation on a preparative liquid chromatography was

	1	2	3	4	5	6	Avg.	RSD (%)
Peak area	148.78	148.70	148.37	148.01	148.66	148.61	148.52	0.19
Rt(min)	30.30	30.18	30.25	30.08	30.35	30.71	30.31	0.72

Table 4: Intraday precision results of linaclotide with HPLC analysis (n=6).

T (h)	0	2	4	6	12	24	Avg.	RSD (%)
Peak area	48.786	48.372	48.534	48.661	48.104	47.987	48.407	0.65
t_R (min)	30.253	30.353	30.012	30.813	30.543	30.307	30.380	0.89

Table 5: The stability of linaclotide solution with HPLC analysis.

Batch	Injected sample (mg)	Content (%)	Recovery (mg)	Yield (%)	Purity (%)
150527-1	120	54.3	44.2	67.83	98.47
150527-2	78.5	53.1	26.2	62.86	98.62
150527-3	57.8	50.8	19.5	66.67	98.97

Table 6: HPLC preparation results of three batches of crude linaclotide.

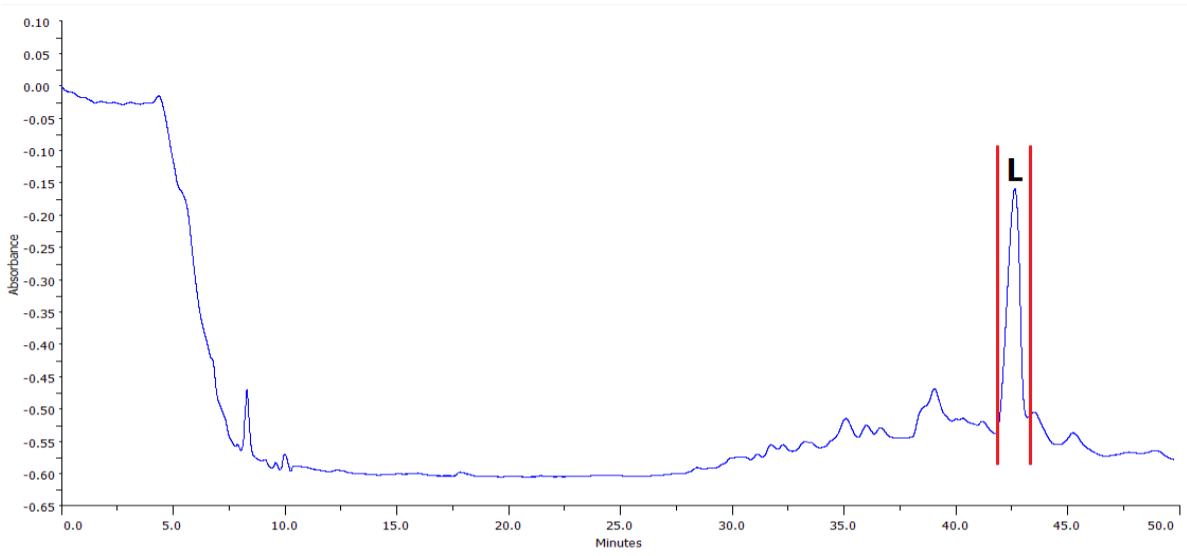

Figure 6: The preparative chromatogram of crude linaclotide.

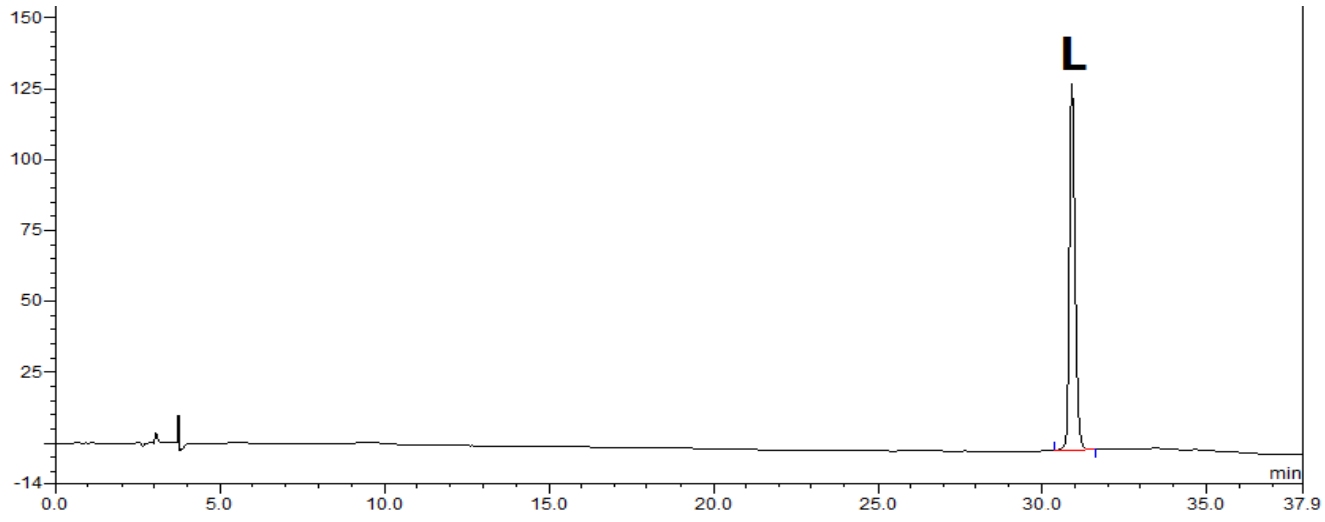

Figure 7: The HPLC chromatogram of purified linaclotide.

developed from the analytical method. The purity of linaclotide was achieved over 98% after preparation.

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