Hacettepe University Journal of the Faculty of Pharmacy
Volume 31 / Number 2 / July 2011 / pp. 97-108

Analytical Method Development and Validation for the Anticancer Peptide, Lyp-1

Received : 03.09.2011
Revised : 11.11.2011
Accepted : 30.11.2011

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Introduction

Lyp-1 is a nine amino acid cyclic peptide, which has specificity of tumor cells especially on breast cancer cells[1-4]. LyP-1 is not only a special marker for the tumor endothelial cells but also specific to the tumor lymphatic endothelia to which vascular peptides (F3, CREKA peptide, RGD peptides) are not specific[5]. It has been shown that following iv administration of LyP-1, the peptide accumulates heavily at the tumor and metastatic regions[2,3]. In addition to localization and internalization in the tumor and endothelial cells, LyP-1 also induces apoptosis (programmed cell death) in the cells that it has been internalized. This renders LyP-1 invaluable among the other tumor-specific peptides. It has been demonstrated that following administration of LyP-1 to tumor-grown mice, this peptide inhibits tumor growth[6]. The fact that LyP-1 plays a crucial role in lymphatic targeting in cancer therapy brings out the possibility of the use of this peptide as itself or by conjugation to another anticancer drug for targeted cancer therapy. Zhang et al. have described peptides spesific to tumor lymphatics[7]. They have shown that there were site spesific differences on blood and lymph vessels during tumor growth. Lyp-1 is one of

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these peptides, which is spesific to lymphatic endothelial cells in breast carcinomas. In another study, increased fluorescence intensity in tumor cells has shown that the Lyp-1 peptide could internalize into the cell nucleus[6]. Also, quantum dots (q-dots) coated with site spesific peptides, F3 and Lyp-1, have been shown to be potential systems for drug delivery and imaging as it was possible to target tumors with these peptides[2]. Interestingly, q-dots coated with F3 peptide have been localized in blood vessels whereas Lyp-1-coated q-dots have been shown to accumulate in lymph vessels of the tumor. Albumin-bound paclitaxel nanoparticles (Abraxane®) have been used in another study with tumor homing peptides[8]. For tumor targeting, CREKA and Lyp-1 peptides have been conjugated to Abraxane®, and Lyp-1 conjugated to Abraxane has been shown to localize in tumor lymph vessels. Luo et al. have prepared Lyp-1 conjugated to nanoparticles for treatment of metastatic cancer, and they have shown that targeted therapy could be possible using Lyp-1[9]. Recently, lipid based formulations, namely self micro-emulsifying drug delivery systems (SMEDDS), of Lyp-1 have been developed. In this study, suitable SMEDDS formulations have been designed and characterized for entrapment of Lyp-1 to target lymphatic vessels of solid tumors[10]. Finally, Lyp-1 conjugated to doxorubicin-loaded PEGlyated liposomes have been targeted to metastatic tumors, and it has been shown that Lyp-1 conjugation has increased liposome uptake in tumor lymphatics compared to normal lymphatics[11].

Although Lyp-1 has been shown to be a very potent peptide, there is not a quantitative analysis method of this peptide at present. Therefore, in this study, a RP-HPLC method has been developed and validated for the quantitative analysis of Lyp-1, which could be utilized for a quantitation of this peptide in pharmaceutical formulations.

Materials and Methods

Materials

The HPLC system of Agilent 1200 series with degasser G1322A, quaternary pump G1311A, autosampler G1329A, thermostatted column compartment G1316A, variable wavelength dedector G1314B was used. Chromatographic analyses were carried out at 25 °C using a reversed

phase Waters XTerra RP18, 5 μm, 4.6x250 mm column and Waters XTerra, RP18, 5 μm, 3.9x20 mm guard column. Lyp-1 peptide was purchased from Anaspec (USA). Spectrophotometric grade trifloroacetic acid (TFA) and HPLC grade acetonitrile were purchased from Sigma-Aldrich.

Methods

An isocratic elution technique was used where the mobile phase was 95 % water: 5 % acetonitrile with 0,1 % TFA. The flow rate was set at 1 ml/min and wavelength was 204 nm. The injection volume was 35 μl. A stock solution of Lyp-1 (200 μg/ml) was prepared using ultrapure water (Milipore Milli-DI). Other samples were prepared by diluting the stock solution with ultrapure water.

The analytical validation parameters were selected according to the "Validation of Analytical Procedures (Q2, R1) Guideline" of ICH[12]. Analytical validation parameters were accuracy, precision (repeatability and reproducibility), specificity, sensitivity (limit of detection, LOD; limit of quantification, LOQ) and linearity.

Results

Accuracy

Three different samples including high, medium and low concentrations were used for determination of accuracy. Accuracy was expressed as the percentage of coefficient of variation (% CV). All values found for the three different concentrations of Lyp-1 were lower than 2 % (n=3) (Table I).

Precision

The precision parameter was evaluated in terms of repeatability and reproducibility. For injection repeatability, six analyses of one concentration were performed in the same sample (Table II). For method repeatability and reproducibility, analyses of six different samples of one concentration were performed in the same day and 2 different days (Tables III and IV). All CV values were found to be lower than 2 %.

TABLE I

Accuracy values for three different concentrations of Lyp-1 (n=3)

Theoretical Concentration (µg/ml)	**Area(mAu)**	**Practical Concentration (µg/ml)**	**% Recovery**
25	594,1	24,794	99,177
25	595,2	24,842	99,366
25	538,6	22,416	89,665
Ave*		24,017	96,070
Bias %		3,931	
100	2355,4	100,26	100,26
100	2347,6	99,926	99,261
100	2348,6	99,969	99,969
Ave		100,052	100,052
Bias %		0,031	
200	4669,9	199,429	99,715
200	4663,9	199,172	99,586
200	4676,6	199,716	99,858
Ave		199,439	99,72
Bias %		0,142	

*Average

Specificity

SMEDDS formulations were prepared as previously described[10]. Briefly, surfactants, oils and cosolvents were heated at 40°C and an isotropic formulation was obtained following 1:10 dilution with pH 7,4 buffer. Specifity of the analytical method was determined by analyzing the formulation excipients and blank formulation using the newly developed HPLC method. The HPLC chromatograms of the excipients, blank SMEDDS formulation (diluted to 1:10 with pH 7,4 buffer) and Lyp-1 containing SMEDDS are given in Figure 1. It can be easily seen from the chromatograms that the formulation excipients and the blank formulation did not have a peak at 204 nm using the newly developed

TABLE II

Injection Repeatability values (n=6)

Theoretical Concentration (µg/ml)	**Area (mAu)**	**Practical Concentration (µg/ml)**
200	5027,68	214,759
200	4997,38	213,461
200	4981,31	212,772
200	4961,84	211,938
200	4944,09	211,177
200	4925,01	210,360
Ave		212,411
SD		1,594
CV		0,750

TABLE III

Repeatability values (n=6)

Theoretical Concentration (µg/ml)	**Area (mAu)**	**Practical Concentration (µg/ml)**
200	4658,7	198,949
200	4631,3	197,775
200	4669,9	199,429
200	4663,9	199,172
200	4676,6	199,716
200	4595,6	196,246
Ave		198,548
SD		1,311
CV		0,660

TABLE IV

Reproducability values (n=6)

Injection numbers	Day 1	Day 2			
1	4129,787	5027,68			
2	4868,065	4997,38			
3	4876,091	4981,31			
4	4922,404	4961,84			
5	4943,106	4944,09			
6	4982,01	4925,01			
Ave Area (mAu)	4786,911	4972,885	**Average**	**SD**	**CV**
Theoretical Conc. (µg/ml)	205,755	213,723	209,739	3,984	1,899

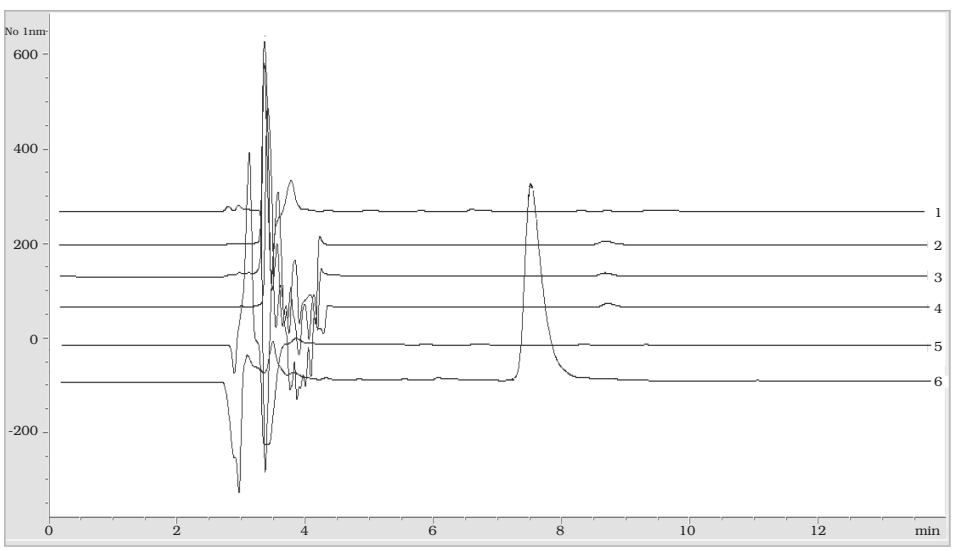

Figure 1

RP-HPLC chromatograms of formulation excipients and Lyp-1 in the SMEDDS formulation; 1) PEG 300, 2) Peceol, 3) Gelucire 44/14, 4) Labrasol, 5)Blank SMEDDS, 6) Lyp-1 in SMEDDS

analytical method; and thus, the method could be considered as specific to the Lyp-1 peptide.

Sensitivity

Sensitivity was calculated based on the residual standard deviation (RSD) of the response and the slope. Average values of six calibration curves were used for this purpose. By this method LOD was found as 7,75 μg/ml and LOQ was found as 23,50 μg/ml using the following equations, respectively.

$$LOD = 3{,}3\ (SD/Slope) \qquad \text{Equation 1}$$

$$LOQ = 10\ (SD/Slope) \qquad \text{Equation 2}$$

Linearity

The HPLC chromatogram of the Lyp-1 peptide could be seen in Figure 2. The retention time (t_R) was 10.6 min at 204 nm with 95 % water: 5 % acetonitrile (0,1 % TFA) solvent system. The calibration curve was prepared from five sample concentrations within the range of 25 – 200 μg/ml and found linear in this range. There were six series of samples prepared, and average values were used to prepare the calibration curve. The r^2 value was found as 0,9999 (Table V).

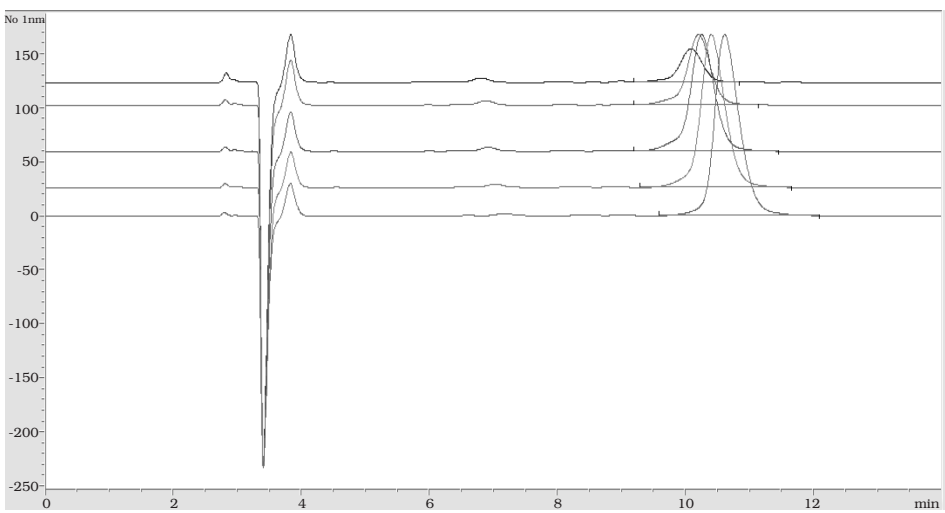

Figure 2
RP-HPLC chromatogram of Lyp-1 in different concentrations (25 – 200 μg/ml)

Table V
Linearity Parameters of the analytical method

Parameters	**Values**
Linearity Range	25 – 200 μg/ml
Slope	23,34
Intercept	-15,425
RSD % of r^2	0,030
RSD % of m	1,475
LOD	7,75 μg/ml
LOQ	23,50 μg/ml

System Suitability Test

The system suitability test results have been given in Table VI. Tailing factor and column efficiency were calculated as described in the United States Pharmacopoeia (USP)[13].

Discussion

Lyp-1 is a potent anticancer peptide, which binds specifically to tumor lymphatic vessels especially in breast cancer. This property can be useful for the prevention of lymphatic metastasis of cancer or achievement of lymphatic targeting. For example, lipid based formulations have been prepared and characterized for lymphatic targeting of this peptide[10]. Besides that nanoparticle formulations[9] and q-dots[14] have also been prepared, *in vivo* and *in vitro* studies have been carried out. These studies have shown that this peptide can be used for targeted drug delivery by itself or conjugation to another drug/ drug carrier system. However, there has not been an analytical method reported in the literature for this peptide. Thus, a RP-HPLC method has been developed and validated for the quantitative analysis of Lyp-1 in the present study.

Validation parameters have been selected as accuracy, precision (repeatability and reproducibility), specificity, sensitivity (LOD, LOQ) and linearity. Accuracy and precision of the method have been established by

Table VI
System Suitability Parameters

Parameters	**Results**
% RSD of t_R (min)	0,702*
Tailing Factor (T_f)	1,33
Capacity Factor (k')	2,02
Column Efficiency (N)	2722,96

* (n=6)

the CV values. All values have been found less than 2 %. Using this newly developed method, Lyp-1 peptide has been successfully determined from SMEDDS formulations. The LOD and LOQ of the method have been found to be 7,75 μg/ml and 23,50 μg/ml, respectively. The method has been found linear in the 25 – 200 μg/ml range with a 0,9999 determination coefficient. Finally the system suitability test values have been found between required ranges. Tailing factor is a measure of peak asymmetry and has been found between 0,9 – 1,4, capacity factor is a term that measures the degree of retention and has been found between 1 – 20. Column efficiency has been calculated by the number of theoretical plates and has been found 2722,96. To the best of our knowledge, this is the first study showing the development and validation of an analytical method for Lyp-1.

Conclusion

Lyp-1 is a very potent anticancer peptide and shows high specificity to the lymphatic vessels of solid tumors. Thus, it is a promising candidate for the targeted therapy of metastatic cancers, such as breast cancer. Because of the potential and increased interest in Lyp-1, a validated quantitative analytical method has become of paramount necessity. Therefore, in this study, a suitable RP-HPLC method was successfully developed and validated for the quantitative analysis of Lyp-1.

Summary

Lyp-1 is a nine amino acid peptide, which is spesific to tumor lymphatics. It has been shown that the Lyp-1 peptide accumulates in tumor lymphatics, and it could be internalized in the tumor cell. It has also been shown to induce apoptosis in the cell that it binds to. On the other hand, there is not a quantitative analysis method of this peptide in the literature at present. Therefore, a RP-HPLC method has been developed and validated for the quantitative analysis of the Lyp-1 peptide in this study. Analytical validation parameters were accuracy, precision (repeatability and reproducibility), specificity, sensitivity (limit of detection and limit of quantification) and linearity. The accuracy and precision of the method have been tested and the coefficients of variation have been found to be less than 2 % according to ICH Validation Guideline. The method has been found to be linear in the 25 – 200 μg/ml concentration range. The specificity of the method has been determined using the newly developed SMEDDS formulations. In conclusion, a new RP-HPLC method has been developed and validated successfully for the quantitative analysis of the Lyp-1 peptide in this study.

Keywords: Cancer, Lyp-1 peptide, RP-HPLC, quantitative analysis, analytical method, validation

Özet

Antikanser Lyp-1 Peptidi için Analitik Yöntem Geliştirilmesi ve Validasyonu

Lyp-1, dokuz amino asitli bir peptid olup, tümör lenf damarlarına özgünlük göstermektedir. Yapılan çalışmalarda, peptidin tümör lenf damarlarında toplandığı ve hücreye internalizasyon özelliği gösterdiği belirlenmiştir. Ayrıca bağlandığı hücrede apoptozu indüklemesi de peptidin diğer önemli bir özelliğidir. Ancak literatürde bu peptide ait miktar tayini yöntemi henüz bulunmamaktadır. Dolayısıyla bu çalışmada, Lyp-1 peptidine ait bir RP-HPLC miktar tayini yöntemi geliştirilmiş ve valide edilmiştir. Validasyon parametreleri doğruluk, kesinlik (tekrarlanabilirlik ve tekrar elde edilebilirlik), özgünlük, duyarlılık (saptanabilme sınırı ve tayin edilebilme sınırı) ve doğrusallık olarak belirlenmiştir. Yöntemin doğruluğu ve kesinliği test edilmiş ve varyasyon katsayıları ICH

Validasyon Kılavuzu'na göre % 2'den küçük bulunmuştur. Geliştirilen metot 25 – 200 μg/ml konsantrasyon aralığında doğrusal bulunmuştur. metodun özgünlüğü tasarlanan SMEDDS formülasyonları kullanılarak ile gösterilmiştir. Sonuçta, bu çalışma ile Lyp-1 peptidinin miktar tayini için geçerli bir RP-HPLC metodu geliştirilmiş ve validasyonu başarıyla gerçekleştirilmiştir.

Anahtar kelimeler: Kanser, Lyp-1 peptidi, RP-HPLC, miktar tayini, analitik yöntem, validasyon

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