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RP-HPLC Method Development for Determination of Vasopressin From Nanoparticles

Dipti Desai[1,*], Dushyant Shah[2]

1.Department of Pharmaceutics, Pioneer Pharmacy Degree College, Gujarat Technological University, Vadodara, Gujarat, India,

2.Department of Pharmaceutics, APMC College of Pharmaceutical Education & Research, Himatnagar, Gujarat, India

ABSTRACT

Vasopressin, nonapeptide, used as an antidiuretic hormone. Very few method has been reported for analysis of Vasopressin from pharmaceutical dosage form. A simple and rapid high performance liquid chromatography (HPLC) method was developed for the quantitative analysis of arginine vasopressin released from polymeric nanoparticles. Chromatographic analysis was performed on an RP C18 column with a mobile phase consisting of acetonitrile and phosphate buffer (13:87 v/v) at a flow rate of 1.6 ml/min at a wavelength of 220 nm, with a retention time 4.1 min. The method was shown to be specific and linear in the range of 1-50 IU/ml (r^2 = 0.9997). Developed method was validated for various evaluation parameters as per ICH guidelines. The method showed no peak interference in presence of formulation excipients. The limit of detection and quantitation were 0.32 and 1.06 IU/ml, respectively. The method was applied to the quantitative analysis of drug to study in vitro drug release from polymeric nanoparticles.

Keywords: Vasopressin, Nanoparticles, RP-HPLC, method validation.

*Corresponding Author Email: diptidesai2001@yahoo.com

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INTRODUCTION

Vasopressin is a nine amino acid peptide secreted from the posterior pituitary. Antidiuretic hormone binds to receptors in the distal or collecting tubules of the kidney and promotes re-absorption of water back into the circulation[1].

Vasopressin is an antidiuretic hormone indicated for the prevention and treatment of postoperative abdominal distention, in abdominal roentgenography to dispel interfering gas shadows, and in diabetes insipidus. Vasopressin can cause contraction of smooth muscle of the gastrointestinal tract and of all parts of the vascular bed, especially the capillaries, small arterioles and venules. It has less effect on the smooth musculature of the large veins. Vasopressin may also be used to control bleeding in some forms of von Willebrand disease and to treat extreme cases of bed wetting in children[2,3].

Vasopressin is a hormone naturally present in the body. Vasopressin was developed as gel for transdermal iontophoretic drug delivery[4]. Recently, Vasopressin is developed as parenteral solution[5]. But existing dosage form suffers from drawbacks like low bioavailability, frequent administration and patient incompliance. In preceding study, nanoparticles incorporating Vasopressin were prepared to improve and sustain the availability of the drug after parenteral administration. The current study aims to develop and validate analytical method to quantify the drug from polymeric nanoparticles.

Some bioanalytical techniques have been developed like Ion exchange chromatography[6], liquid chromatography/mass spectrometry[7] and Time of flight mass spectrometry[8] for assaying Vasopressin in plasma. Method has been cited in the United States pharmacopeia (USP)[9] for analysis of drug in bulk and injection.

USP method describes use of Buffer solution and acetonitrile in ratio of 87:13 as mobile phase, operated at flow rate of 1ml/min. The method suggests use of buffers with high concentration of salts. With low flow rate, extended retention time was observed, and vice versa.

Aim of present study is to develop a simple, fast, accurate and precise reversed-phase HPLC (RP-HPLC) method to quantify vasopressin in PLGA nanoparticles in the release media (i.e., phosphate buffer with pH=7.4 and ionic strength=0.03). Further, developed method was applied to assay drug released from nanoparticles. Developed method was validated for various parameters.

Furthermore, this method was very friendly with the type of release media and was tested in an in vitro environment.

MATERIALS AND METHOD

Vasopressin was purchased from Hysel, India. Poly (lactide -co-glycolide) PLGA (50:50) obtained as gift sample from Boehringer Ingelheim, Germany. Poly vinyl alcohol (PVA) and other chemicals were purchased from S.D. Fine Chem., Mumbai, India. Analytical grade potassium di hydrogen phosphate and di sodium hydrogen o-phosphate, dibasic ammonium phosphate, phosphoric acid were purchased from S. D. Fine Chemicals Ltd., Mumbai, India. Solvents for mobile phase like water and acetonitrile used were of HPLC grade. Nylon 0.45 µm, 47 mm membrane filter purchased from Hi-media, India.

HPLC (Shimadzu- LC 20AD, Japan) equipped with a UV- Visible detector, manual injector with 20 µl loop, Shim pack XR ODS (Shim pack XR ODS II 150 mm x 3 mm x 5 µm id) and LC solution software used. Optimized mobile phase was prepared by mixing ammonium buffer/ acetonitrile (85:15, v/v). The flow rate was set to 1.6 ml/min. Injection volume of 20 µl was made and the column eluents were monitored at 220 nm over a run time of 10 min. All the separations were carried out at ambient conditions (25°C) after baseline stabilization for at least 30 min.

Preparation of Nanoparticles

The nanoparticles were prepared by double emulsion- solvent evaporation technique based on the formation of a W/O/W-multiple emulsion. An aqueous solution of Vasopressin (1.0 ml) (water phase) was emulsified into a solution of PLGA (50:50) and PCL (85:15) in dichloride methane (3.0 ml) (Oil phase). The water and oil phases are vigorously mixed on vortex at 2800 rpm. Primary emulsion is subjected to sonication at 55 amplitude, 4 kHz frequency for 100 sec. Prepared emulsion was re-emulsified into 50 ml of aqueous solution of 1%w/v PVA with mechanical stirrer at 1500 rpm to form the double emulsion [(W1/O)/W2]. After 4-5 hr., the nanoparticles were collected by centrifugation, rinsed thrice with water and lyophilized (LABCONCO, Trait).

The nanoparticles were digested with 2 ml acetonitrile by ultra-sonication (10 min, 25°C). The volume was made with acetonitrile and the samples were centrifuged at 10000 rpm for 15 min. Finally, 0.5 ml of supernatant was transferred to 10 ml volumetric flask and the volume was made with 0.25% glacial acetic acid.

Preparation of mobile phase

6.6 gm of dibasic ammonium phosphate was dissolved in about 950 ml of water, pH was adjusted with concentrated phosphoric acid to 3 and the final volume was set up to 1000 ml with water.

Preparation of Calibration curve

Primary stock solution of Vasopressin containing 1000 IU/ml was prepared by dissolving 18.8 mg of drug in 10 ml of 0.25% glacial acetic acid solution. Second stock solution of 100 IU/ml was prepared by diluting 5 ml of primary stock solution to 50 ml. Into a series of 10 mL volumetric flasks, different concentrations of Vasopressin 1, 5, 10, 20, 30, 40 and 50 IU/mL were prepared.

Method validation

Validation was carried out assessing the following parameters: linearity, range, specificity, precision, accuracy, and detection and quantification limits, according to the International Conference on Harmonization (ICH) guidelines[10].

Specificity

Specificity was evaluated by analyzing solutions containing all the components of the Vasopressin loaded nanoparticles, except the drug (blank Nanoparticles). The system response was examined for the presence of interference.

Linearity, limits of detection, and quantification

Linearity was evaluated by the analyzing various(three) concentrations of standard solutions of Vasopressin on three days. Three independent calibration curves were constructed, and linearity was evaluated by the least-square regression analysis. Calibration curve was used to calculate the predicted concentrations.

Accuracy

Accuracy was evaluated assaying, in triplicate, samples of known concentrations (Nanoparticles) spiked with standard solution at three different levels (lower, medium, and higher) i.e. spiking at 80%, 100 % and 120%.

Precision

Repeatability (intra-day precision) was evaluated by measuring, in triplicate, three different samples under the same experimental conditions and on the same day. Intermediate precision was calculated from results obtained by the analysis of samples with three concentration, in triplicate, on three different days (inter-day precision). Precision (repeatability and intermediate precision) was expressed as relative standard deviation [RSD (%)].

LOD and LOQ

Limits of detection (LOD) and quantification (LOQ) were calculated directly from the calibration plot. LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of intercept and S is the slope of the calibration[11].

Robustness

Robustness was evaluated by the deliberate variation of the mobile phase, flow rate, and wavelength. Sample solutions were evaluated for each variation of the method conditions.

System suitability

System suitability tests were carried out on freshly prepared standard stock solutions of Vasopressin and it was calculated by determining the standard deviation of Vasopressin by injecting standards in five replicates and the values were recorded[12].

Formulation analysis

As an application, the proposed method was used for determination of drug content from in-house prepared nanoparticles. For nanoparticles, amount equivalent to 5 mg of Vasopressin was accurately weighed and processed as described in sample preparation section. Finally, 20 µl of resulting solution was injected in triplicates and analyzed.

***In vitro* release study of nanoparticles**

In vitro release studies were performed using dialysis sac method[16]. The freeze dried nanoparticles drug equivalent to (2mg) were suspended in phosphate buffer saline pH 7.4[13] and sealed in a dialysis membrane (molecular weight cut off 12 KDa) clips. The sealed dialysis membrane was then placed in a beaker containing 50 ml of dissolution media, and maintained at 37 ºC with continuous magnetic stirring. Samples of 5 ml were withdrawn at specified time points over a period of 168 h and same amount of blank dissolution media was added. The obtained samples were centrifuged and analyzed by proposed HPLC method. The obtained in vitro release data was fitted into various mathematical models like zero order, first order, Higuchi model and Hixon Crowell model[14].

RESULTS AND DISCUSSION

HPLC has been widely studied in pharmaceutical analysis, including drug assay in products based on nanotechnology[15-17]. Developed method was optimized using variety of different parameters. The wavelength of 220 nm was chosen based on the amount of absorbance of drug in buffer solution. Likewise, different mobile phase compositions were analyzed prior to validation.

In order to establish optimized HPLC conditions for Vasopressin assay in the samples, systematic variations of the effective parameters comprising mobile phase composition, such as aqueous pH, ratio of aqueous to organic mobile phase constituents and flow rate were investigated. Based on the results, the best chromatographic conditions considering total run time, retention time, solvent elution time and peak shape (i.e., symmetry and analytical power) were fixed at a flow rate of 1.6 ml/min and mobile phase containing of acetonitrile/buffer (13:87 v/v), at retention time of 4.1 min.

Calibration curve

Calibration data of Vasopressin is shown in Table 1. Correlation coefficient is a statistical tool used to measure the degree or strength of this type of relationship, and here, a high correlation coefficient value (a value very close to 1.0) indicates a high level of linear relationship between the concentration of vasopressin and peak area. The calibration curve obtained by least square analysis showed linear relationship with regression coefficient (R^2) of 0.9997. The best fit equation obtained was mean peak area= 1117×concentration (IU/ml) + 578.04. At all concentration levels, the standard deviation was low and %RSD did not exceed 2%. The predicted concentrations were in close agreement with the theoretical concentrations. The linearity range was found to be 1-50 IU/ml (Figure 1).

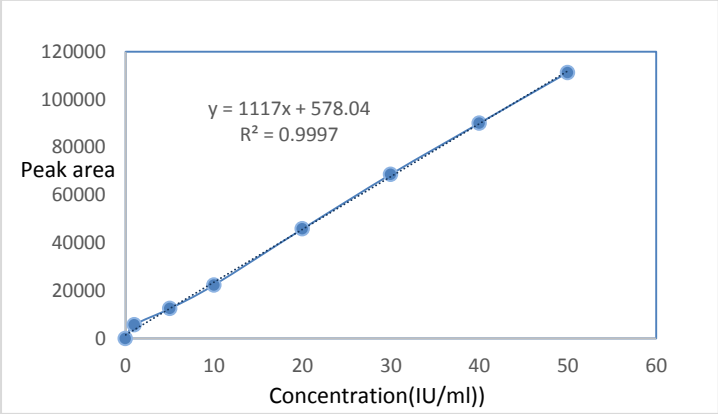
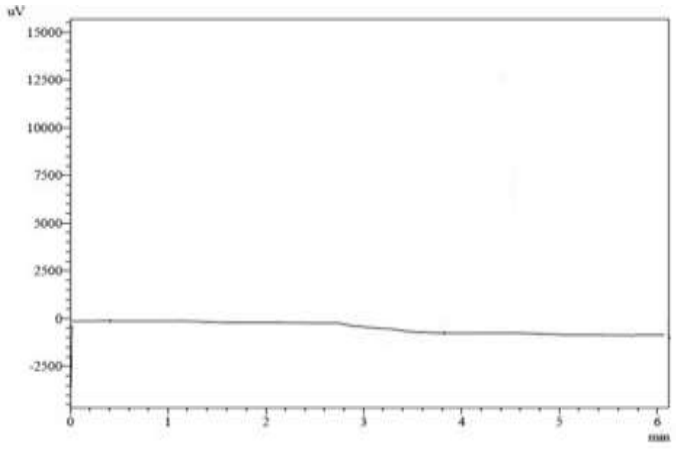


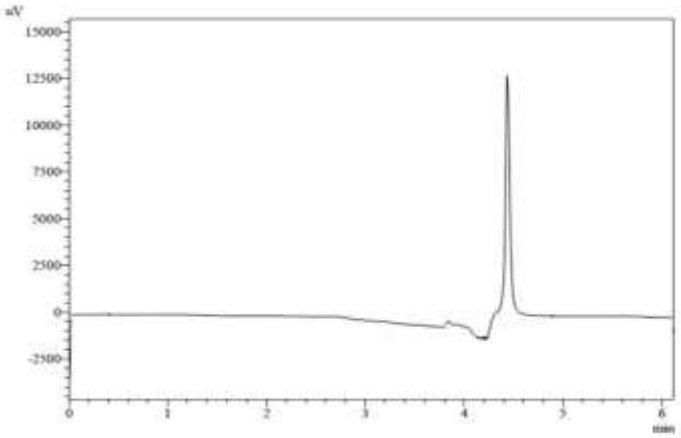
Figure1: Calibration curve of Vasopressin in Phosphate buffer (pH 7.4)

Specificity

Placebo standards showed no interference in the vicinity of vasopressin peak, when compared with freshly prepared calibration standards indicating the selectivity of developed method for Vasopressin in presence of formulation excipients(Figure 2).



(a)



(b)

Figure 2: Chromatogram of (a) placebo, (b) drug from nanoparticles

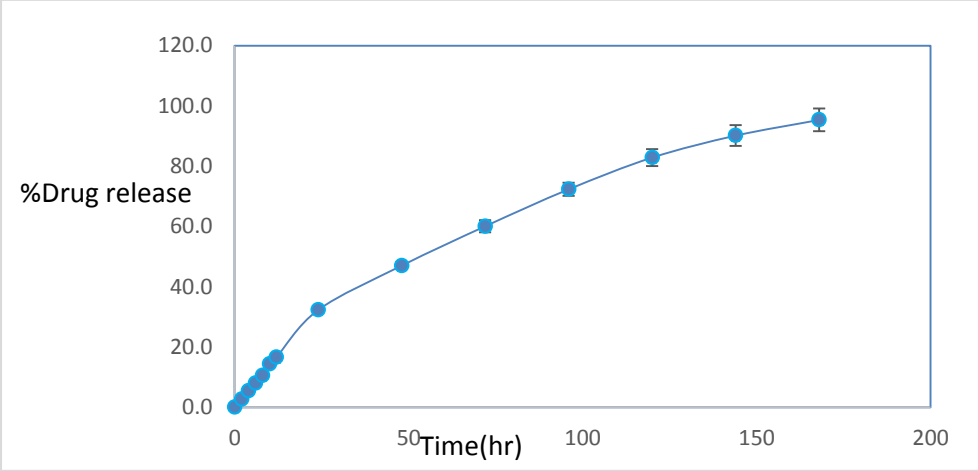


Figure 3: *In vitro* drug release of Vasopressin from polymeric nanoparticles

Accuracy (Recovery)

The recovery studies were carried out to check the sensitivity of the method to estimate drug. The standard addition technique was carried out by adding 80%, 100%, and 120% of vasopressin concentration in the sample. The percentage recoveries of the three concentrations were found to be 99.50% to 100.3%, which is indicative of high accuracy. The values of percentage recovery and %RSD are displayed in Table 1. The mean percentage recovery values, close to 100%, and their low %RSD values indicated high accuracy of the analytical method.

Precision

The repeatability of developed HPLC method, by intraday assay, is expressed in the terms of %RSD, and the results (Table 2) demonstrated the repeatability of the method. The interday variation of vasopressin at three different concentration levels of 10, 20, and 30 IU/ml establishes

the intermediate precision of the method. The low values of %RSD for repeatability and intermediate precision suggested an excellent precision of the developed HPLC method.

Table 1 Results of recovery study

Level	%Recovery	%RSD
80	99.5	1.2
100	99.67	0.77
120	100.3	0.92

Table 2 Results of Intraday and Interday Precision

Intraday Precision			Interday Precision		
Level	Conc. (IU/ml)	%RSD	Level	Conc. (IU/ml)	%RSD
LQC	10	0.77	LQC	10	1.50
MQC	20	0.59	MQC	20	0.90
HQC	30	0.32	HQC	30	0.77

LOD and LOQ

The LOD and LOQ of the method were found to be 0.32 and 1.06 IU/ml respectively. The method has demonstrated high value of slope with minimal standard error. Insignificant change in chromatographic peak properties (retention time and peak area) were observed upon re-injection at quantification limit. Hence, the method was found to be highly sensitive for determination of Vasopressin.

Slightly variation in buffer-solvent ratio and flow rate has not shown any significant changes in validation parameter. However, major deliberate variations have shown significant effect on retention time, peak area and tailing factor.

The literature describes chromatographic methods for Vasopressin quantitation in bulk drug, dosage forms[9]. Each experimental work utilizes different composition of mobile phase with varied pH. Most of them have applied isocratic system which was not effective as that of gradient system. The mobile phase was comprised of buffer: Acetonitrile (85:15), and the results showed a retention time of 1.6 min. The method was sensitive but retention time was inadequate and unsuitable for chromatographic conditions. Thus, the RP-HPLC method developed and validated in present work represents an alternative to developed methods for the analysis of Vasopressin in nanoparticles and fulfills the requirement for detailed data.

***In vitro* drug release study**

Nanoparticles showed 95.7% release after 168 hours. The results showed the sustained release of drug from Nanoparticles. The release profile followed biphasic with an initial burst attributed to the drug associated near particles surface, followed by a linear release phase. The drug release profile of formulation confirmed to the Higuchi model (R^2= 0.9918), suggesting the drug release to

be a diffusion controlled process based on the Fick's law in which the diffusion coefficient depends upon both the concentration and the time.

CONCLUSION

The simple, sensitive, precise and accurate RP-HPLC method was developed and validated according to ICH guidelines. The developed analytical procedure has several advantages like short chromatographic run time, low proportion of acetonitrile which allows no. of samples can be analyzed in less time and reduce damage to environment respectively. Method showed no interference in presence of formulation excipients. Further, developed method was found to be suitable for estimation of Vasopressin released from polymeric nanoparticles.

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