DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DESMOPRESSIN FROM POLYMERIC NANOPARTICLES

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<table>
<thead>
<tr>
<th>ARTICLE INFO</th>
<th>ABSTRACT</th>
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<tbody>
<tr>
<td><strong>Article history</strong></td>
<td>The objective of this work was to develop and validate high performance liquid chromatography (HPLC) method for determination of desmopressin (DDAVP) in polymeric nanoparticles. Chromatographic analysis was performed on an RP C18 column with a mobile phase consisting of acetonitrile and phosphate buffer (15:85 v/v) at a flow rate of 1.8 mL/min at a wavelength of 220 nm. The method was shown to be specific and linear in the range of 500-10000 ng/mL ($r^2 = 0.9987$). The good precision (intra- and inter-day) was demonstrated because the maximum relative standard deviation was 0.731%. The method is robust relative to changes in flow rate, column and temperature. The limits of detection and quantitation were 41 and 136.8 ng/mL, respectively. The method fulfilled the requirements for reliability and feasibility for application to the quantitative analysis of DDAVP in polymeric nanoparticles. Developed method proposed short run time and less use of organic solvent. Developed method was successively employed for analysis of samples of in vitro drug release from nanoparticles.</td>
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</table>

| **Keywords** | Desmopressin, Nanoparticles, RP-HPLC, Method Validation. |

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INTRODUCTION

1-Desamino-8-D-arginine vasopressin (DDAVP) is analogue of Vasopressin, natural hormone found in body. It prevents
reabsorption of water and thereby decreases frequency of urination. Hence, it can be used to treat polyuria in diabetic patients and bed
wetting in children [1,2].

Presently, DDAVP is available as tablet, Nasal spray and parenteral solution [3-6]. But existing dosage form suffers from
drawbacks like low bioavailability, frequent administration and patient compliance respectively. Efforts have been made by
researchers to develop bio adhesive microsphere [7] and liposome [8] for nasal delivery; coated micro needle by transdermal delivery
[9] for sustaining the drug release. Nanoparticles were also developed to improve oral bioavailability. In present study, an attempt has
been made to develop analytical method to quantify desmopressin from prepared nanoparticles aimed to be deliver by depot injection
for sustained drug delivery.

Many analytical techiques have been reported for assaying DDAVP in dosage forms like high performance liquid
chromatography (HPLC) [10-11], liquid chromatography in combination with mass spectrometry[12-13] and capillary

Chromatographic procedure has been also cited in the United States pharmacopeia (USP) and European Pharmacopeia as an
officially described method using DDAVP. USP method describes use of Buffer solution and acetonitrile in ratio of 78:22 as mobile
phase, operated at flow rate of 1ml/min[14]. EP method discusses Phosphate buffer and acetonitrile as a mobile phase in ratio of
60:40, at a flow rate of 2.0 ml/min [15]. Both the method suggests use of buffers with high concentration of salts. With low flow rate,
extended retention time was observed, and vice versa. Drug is freely water soluble. But still it was found to be stable in Phosphate
buffer pH 7.4 rather than water. Hence, buffer could not replaced with water but use of organic solvent have been minimized to obtain
optimum retention time and greater peak resolution. In this work, an attempt has been made to develop and validate HPLC method to
determine drug released from polymeric nanoparticles.

MATERIAL AND METHODS

Desmopressin acetate (EP 7.0, Purity- 101.5%) was purchased from Hemmo Pharmaceuticals, Mumbai, India. PLGA (50:50)
[Resomer® RG 502 H (MW 13600)] and Poly caprolactone (PCL) polymers, were obtained as gift sample from Boehringer Ingelheim
(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 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 (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 were used. The mobile phase was optimized at
ratio of 85:15 Phosphate buffer saline, pH 7.4/ acetonitrile in a gradient mode over a wavelength of 220 nm. Other chromatographic
parameters adjusted were injection volume 20 μl, flow rate at 1ml/min[14]. Baseline was stabilized for 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 [16]. An aqueous solution of DDAVP (1.0 ml) (water phase) was emulsified into a solution of PLGA and PCL
(85:15) in dichloro methane (3.0 ml) (Oil phase). The water and oil phases are vigorously mixed on vortex at 2800 rpm. Primary
emulsion was subjected to sonification 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, TriadTM).

Preparation of stocks and standards

Stock solution of drug was prepared in Phosphate buffer pH 7.4. Three separate series of seven calibration standards 500,
1000, 2000, 4000, 6000, 8000 and 10000 ng/ml were prepared by serial dilution in Phosphate buffer pH 7.4. All the samples were
prepared in triplicate for plotting calibration graphs.

Sample preparation

Calculated amount of nanoparticles containing drug equivalent to 1 mg was digested with 2 ml of Acetonitrile and further
diluted to 10 ml. Samples were centrifuged at 10000 rpm for 15 min. After collecting supernatent, further dilution was carried out
using Phosphate buffer pH 7.4.

Analytical method validation

Method was developed to quantify drug released from polymeric nanoparticles and validated for various parameters as per
ICH guidelines [17].
Specificity
The specificity of the method was determined by observing interference of any encountered ingredients present in the formulations. It was determined by comparing nanoparticle carrier samples with and without desmopressin (placebo)[18].

Selectivity
The selectivity of the method in presence of formulation excipients was assessed by injecting the processed placebo and sample standard (with drug) in three triplicates on three different days. The obtained chromatograms were compared with freshly prepared calibration standards.

Linearity and range
The linearity of the method was assessed by analyzing the calibration standards in three replicates on three different days. Calibration curve was obtained by plotting average peak area vs concentration of drug and coefficient of variation was found to confirm the linearity.

Accuracy and precision
Accuracy study was performed by recovery study of DDAVP. Known amount of standard was added to the placebo sample and subjected to the proposed HPLC analysis. The study was performed at triplicate levels [19].

Sensitivity
The sensitivity of developed method was determined by using standard deviation of intercept (σ) and slope (s) of calibration equation. Limit of detection (LOD) and limit of quantification (LOQ) were calculated using 3.3 σ/s and 10 σ/s respectively [20].

Robustness
Influence of small changes in chromatographic conditions such as change in flow rate and wavelength of detection was studied to determine the robustness of the method and its %RSD was determined.

System suitability and sample solution stability
The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. HPLC system was allowed to stabilize for 60 min. The stock solution containing 1000 ng/ml was injected and repeated five times and the chromatograms were recorded to evaluate the system suitability parameters like retention time, tailing factor (NMT 1.5) and theoretical plate count (NLT 3000). Further, the stability of DDAVP in mobile phase was determined by injecting calibration standard 1000 ng/ml at 0, 6, 12, 24, 36, and 48 h in three replicates.

Formulation analysis
The proposed method was aimed to assay drug released from prepared polymeric nanoparticles. Amount of nanoparticles equivalent to 1 mg of drug was 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 study was performed using dialysis bag (Mol. Wt. cut off 12 kDa) by dispersing weighed amount of nanoparticles in 2 ml of phosphate buffer saline (pH 7.4) as release media. Release studies were carried out using modified USP Type II apparatus (Electrolab, India) with 50 ml of dissolution media, set at 50 rpm, 37±2°C. 5 ml sample was withdrawn at specific time interval over a period of 168 hr and sink condition was maintained throughout experiment. Collected samples were subjected to centrifugation and processed for analysis by HPLC. Release kinetic study was performed for obtained data.

RESULTS AND DISCUSSION
HPLC method was developed to determine drug content from polymeric nanoparticles. Detection wavelength was found to be 220 nm under given set of conditions which is same as that of pure drug i.e. formulation excipient didn’t interfere spectrum of drug. To optimize HPLC conditions for quantification of drug in sample, some of the parameters have been varied like aqueous pH, ionic strength, ratio of aqueous to organic mobile phase constituents and flow rate. 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 1.8 ml/min and mobile phase containing of acetonitrile/water (15:85, v/v).

The aim of the present study was to develop a simple, accurate and precise HPLC method for determination of DDAVP in nanoparticles. Based on the UV-profile of DDAVP, the wavelength was optimized at 220 nm for better sensitivity and selectivity in presence of formulation excipients.
Due to the high aqueous solubility of DDAVP, hydrophilic solvents were used to reduce the affinity of the drug for the column and thus obtain short retention times. Initially, the methodology described in the European Pharmacopoeia (EP) 7.0 was tested, describing Phosphate buffer pH 7.0 and acetonitrile (60:40) as mobile phase, with a rate of 2.0 mL/min. However, for samples, irregular peaks were observed using low resolution chromatography, possibly because of instrumental or column differences. Analysis were performed using acetonitrile and water in many proportions. Noticeable tailing and an irregular shape of the DDAVP peak were observed when the proportion of acetonitrile was higher than the phosphate buffer. Increasing the phosphate buffer proportion in the mobile phase, the DDAVP peak became more regular, and, when the proportion of acetonitrile:water of 15:85 (v/v) was used, a regular and symmetric peak was observed. Under these conditions, the DDAVP peak was detected in approximately 3.8 min and run time was 8 min, which is short enough to analyze no. of samples in less time period. Chromatogram of blank nanoparticles showed no interference in the vicinity of DDAVP peak, when compared with freshly prepared calibration standards indicating the selectivity of developed method for DDAVP in presence of formulation excipients (Figure 1 and Figure 2).

![Figure 1: Chromatogram of placebo.](image1)

![Figure 2: Chromatogram of drug from nanoparticles.](image2)

The calibration curve obtained by least square analysis showed linear relationship with regression coefficient ($R^2$) of near to 1 (Figure 3). 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.

![Figure 3: Calibration curve of DDAVP in Phosphate buffer (pH 7.4).](image3)
Validation

Specificity

The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method. Furthermore, the well-shaped peaks also indicate the specificity of the method (Table 1).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>No peaks</td>
</tr>
<tr>
<td>Placebo</td>
<td>No peaks</td>
</tr>
<tr>
<td>Desmopressin (4000 ng/ml)</td>
<td>3.7 min.</td>
</tr>
</tbody>
</table>

Selectivity

Placebo standards showed no interference in the vicinity of Desmopressin peak, when compared with freshly prepared calibration standards indicating the selectivity of developed method for drug in presence of formulation excipients.

Linearity and Range

The linearity of an analytical procedure obtained was found to be directly proportional to the concentration (amount) of analyte in the sample. The linearity range was found to be 500-10000 ng/ml.

Precision

Low inter-day and intraday precision %RSD was observed for intermediate precision. The %RSD values were very well within the acceptable range indicating the repeatability and intermediate precision of the developed method (Table 2).

<table>
<thead>
<tr>
<th>Intraday Precision</th>
<th>Interday Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>Conc.(ng/ml)</td>
</tr>
<tr>
<td>LQC</td>
<td>2000</td>
</tr>
<tr>
<td>MQC</td>
<td>4000</td>
</tr>
<tr>
<td>HQC</td>
<td>6000</td>
</tr>
</tbody>
</table>

Accuracy (Recovery)

The developed method showed high and consistent absolute recoveries at all studied levels for nanoparticles (Table 3). At all studied concentration levels, the standard deviation was low (< 5%) representing the accuracy of the proposed method. Additionally, the obtained recoveries were found to be normally distributed with low and uniform %RSD at all concentration levels. Hence, the recovery study demonstrated the suitability of proposed method for determination of Desmopressin from nanoparticles.

<table>
<thead>
<tr>
<th>Level</th>
<th>%Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>99.9</td>
<td>0.3</td>
</tr>
<tr>
<td>100</td>
<td>100.08</td>
<td>0.26</td>
</tr>
<tr>
<td>120</td>
<td>100.02</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Sensitivity

Method validation indicated that the developed method had high sensitivity with LOD of 41 ng/ml and LOQ of 136.8 ng/ml respectively (Table 4). The method was found to be highly sensitive for determination of Desmopressin as no significant changes were observed in chromatogram upon repeated injection of formulation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>500-10000 ng/ml</td>
</tr>
<tr>
<td>Slope</td>
<td>9.896</td>
</tr>
<tr>
<td>Intercept</td>
<td>1843.5</td>
</tr>
<tr>
<td>Correlation coefficient (R²)</td>
<td>0.9987</td>
</tr>
<tr>
<td>LOD</td>
<td>41 ng/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>136.8 ng/ml</td>
</tr>
<tr>
<td>Accuracy (%RSD)</td>
<td>0.731</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>0.345</td>
</tr>
</tbody>
</table>
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.

Robustness

The method for the development of RP-HPLC method for the estimation of Desmopressin was found to be robust as the % RSD was found to be less than 2 (Table 5).

Table 5: Result of Robustness.

<table>
<thead>
<tr>
<th>Flow rate (±0.2 ml/min.)</th>
<th>% RSD</th>
<th>Wavelength (±2nm)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>0.62</td>
<td>218</td>
<td>0.42</td>
</tr>
<tr>
<td>2.0</td>
<td>0.78</td>
<td>222</td>
<td>0.57</td>
</tr>
</tbody>
</table>

System suitability and solution stability

The retention time, number of theoretical plates, and tailoring factors were calculated and obtained result was complied with the recommended limit (Table 6). Low variability in peak area and retention time were observed upon re-injection indicating that the developed method was specific, precise and stable for estimation of Desmopressin.

Table 6: System suitability parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chromatographic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>3.7 min.</td>
</tr>
<tr>
<td>Tailoring factor</td>
<td>1.037</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>10693</td>
</tr>
</tbody>
</table>

The literature describes chromatographic methods for Desmopressin quantitation in bulk drug [21], dosage forms and nanoformulations [22]. 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. S. M. TAGHIZADEH [23] developed HPLC method for determination of desmopressin from chitosan nanoparticles.

The mobile phase was comprised of Water: Acetonitrile (75:25), 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 desmopressin in nanoparticles and fulfills the requirement for detailed data.
Figure 4: *In vitro* drug release of DDAVP from polymeric nanoparticles.

Nanoparticles showed 95.7% release after 168 hours (Figure 4). The results showed the sustained release of drug from Nanoparticles. The drug release profile of optimized formulation confirmed to the Higuchi model ($R^2 = 0.9907$), 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 number of samples can be analyzed in less time and reduced damage to environment respectively. Further, this method found to be suitable for estimation of DDAVP from polymeric nanoparticles. Recommended future research- HPLC method can be developed for Peptide based drugs to quantify it from various pharmaceutical dosage forms.

**Conflict of interest**
Authors declare that there is no conflict of interest.

**List of Abbreviation**

DDAVP- 1-Desamino-8-D-arginine vasopressin  
PLGA- Poly lactic- co- glycolic acid  
PCL- Poly e-caprolactone

**ACKNOWLEDGEMENT**

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