

## ANALYSIS

DEVELOPMENT AND VALIDATION OF RP HPLC METHOD TO DETERMINE  
NANDROLONE PHENYLPROPIONATE IN DIFFERENT  
PHARMACEUTICAL FORMULATIONSJAYANTI MUKHERJEE, AYAN DAS, UDAY SANKAR CHAKRABARTY, BIJAY KUMAR SAHOO,  
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**Abstract:** This study describes development and subsequent validation of a reversed phase high performance liquid chromatographic (RP-HPLC) method for the estimation of nandrolone phenylpropionate, an anabolic steroid, in bulk drug, in conventional parenteral dosage formulation and in prepared nanoparticle dosage form. The chromatographic system consisted of a Luna Phenomenex, CN (250 mm × 4.6 mm, 5 μm) column, an isocratic mobile phase comprising 10 mM phosphate buffer and acetonitrile (50:50, v/v) and UV detection at 240 nm. Nandrolone phenylpropionate was eluted about 6.3 min with no interfering peaks of excipients used for the preparation of dosage forms. The method was linear over the range from 0.050 to 25 μg/mL in raw drug ( $r^2 = 0.9994$ ). The intra-day and inter-day precision values were in the range of 0.219–0.609% and 0.441–0.875%, respectively. Limits of detection and quantitation were 0.010 μg/mL and 0.050 μg/mL, respectively. The results were validated according to International Conference on Harmonization (ICH) guidelines in parenteral and prepared nanoparticle formulation. The validated HPLC method is simple, sensitive, precise, accurate and reproducible.

**Keywords:** nandrolone phenylpropionate, RP-HPLC, anabolic-androgenic steroid, nanoparticles

Nandrolone phenylpropionate (NPP) (Fig. 1), 17β-hydroxyestr-4-en-3-one 3-phenylpropionate (1), is an anabolic-androgenic steroid. The low androgenicity and enhanced anabolic activity of NPP has shown to positively influence calcium metabolism and to increase bone mass in osteoporosis.

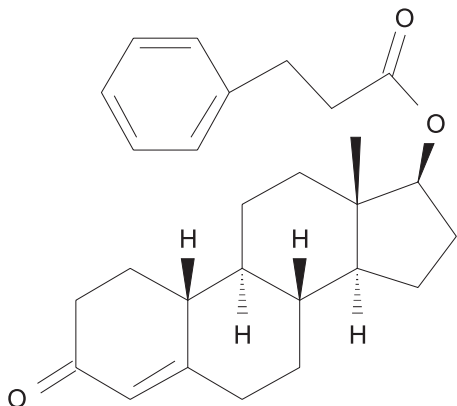


Figure 1. Nandrolone phenylpropionate (CAS number 62-90-8)

In women with disseminated mammary carcinoma, this has been reported to produce objective regressions for many months. Furthermore, it has a nitrogen-saving action. This effect on protein metabolism has been established by metabolic studies and is utilized therapeutically in conditions where a protein deficiency exists such as during chronic debilitating diseases and after major surgery and severe trauma (2, 3). Nandrolone has been reported to improve respiratory muscle function in COPD (Chronic Obstructive Pulmonary Disease) (4). The administration of anabolic androgenic steroids increases skeletal muscle mass (hypertrophy) and protein synthesis (5).

NPP is commercially available as injections, a conventional pharmaceutical formulation. The UV spectrophotometric method of assay has been reported in British Pharmacopoeia and United States Pharmacopoeia (6, 7). Analysis of nandrolone esters in oily injections to discriminate them from nandrolone alcohol, a potential impurity, by reversed-phase high performance liquid chromatography has

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also been reported (8). The work on the development and validation of a multi-residue method for the determination of a wide range of hormonal anabolic compounds in hair using gas chromatography-tandem mass spectrometry had been carried out (9). Findings on the effect of side chain, the injection site and the volume of the injection on the pharmacokinetics and pharmacodynamics of nandrolone esters in an oil vehicle in men is also available (10). However, HPLC assay for determination of NPP in any novel drug delivery system has not yet been reported and therefore, this study claims to be a novel one.

The objective of the present study was to develop a simple, precise, accurate and validated RP-HPLC method for the estimation and routine analysis of NPP in nanoparticulate drug delivery system and also in commercially available injectables. The results of the analysis were validated according to ICH guidelines (11).

## EXPERIMENTAL

### Chemicals and reagents

NPP was obtained from Govind Laboratories Pvt. Ltd., Ahmedabad, Gujarat. Injection of brand Durabolin (25 mg/mL) (Batch No. 013895), manufactured by Organon, Kolkata, West Bengal, India were procured from a local pharmacy. Dextran sulfate (MW 500,000), mannitol, and zinc sulfate were all purchased from Sigma Chemical (St. Louis, MO, USA). Chitosan (MW 30,000 with 95% deacetylation) was purchased from Aquapremier, Bangkok, Thailand. Acetonitrile and water of HPLC grade were purchased from Merck, India. All other chemicals were of analytical grade and purchased from Merck, India. Milli-Q water was used for the mobile phase preparation. Cellulose ester dialysis membrane tubing with a molecular weight cutoff (MWCO) of 1,000,000 (Spectra/Por CE) was purchased from Fischer Scientific (Chicago, IL, USA).

### Preparation and characterization of nanoparticles

#### Nanoparticles preparation

The preparation of nanoparticles was done by polyelectrolyte complexation at room temperature (12). The required amount of aqueous solutions of polymers and drug in ethyl alcohol was stirred at 600 rpm. Then, the aqueous chitosan solution was added dropwise and the resulting nanoparticles were stirred for 5 min. A few milliliters of zinc sulfate solution (1 M) were added as a stabilizer. The resulting stabilized NPP nanoparticles were dialyzed for

24 h in the dark (as the drug is light sensitive) against DI water using Spectra/Por CE MWCO 1,000,000 dialysis membrane. Mannitol was added as lyoprotectant to the purified, loaded nanoparticles during lyophilization (13, 14).

#### Particle size determination

The particle size was determined by laser diffractometry using Mastersizer 2000 (Malvern laser diffraction particle size analyzer, Malvern Instruments, U.K.). The Mastersizer comprises of helium-neon laser as a light source. This is then focused by a Fourier lens to a detector, which consists of a large number of photosensitive elements radiating outward from the center. The intensity of the scattered light is measured. The volumetric particle size distribution and specific surface area is calculated using an optical model and mathematical deconvolution procedure. For particle size analysis, the samples were dispersed in Milli-Q water. Measurements were carried out at 30°C using a helium-neon laser at an angle of 90°.

### Instrumentation and chromatography

The HPLC system was of Knauer (Berlin, Germany), consisting of a solvent delivery pump (LC-1000 Knauer), a Rheodyne injector and 2500 variable wavelength UV/VIS detector with EZChrom (version 3.1.6) software for integration.

Separation was achieved using a Luna Phenomenex CN column (250 mm × 4.6 mm, 5 µm). The isocratic mobile phase pumped at a flow rate of 1 mL/min consisted of 10 mM phosphate buffer and acetonitrile (50:50, v/v) freshly prepared, filtered through 0.45 µm filter (Millipore, Milford, MA, USA) and degassed by sonication for 15 min. The injection volume was 50 µL and the wavelength used was at 240 nm. All separations were performed at room temperature.

### Standard and sample preparation

#### Standard preparation

Stock solution (1 mg/mL) of NPP was prepared in ethyl alcohol. From this stock, a solution of concentration 10 µg/mL was prepared with the mobile phase. Standard solutions of NPP (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10, 15, 20 and 25 µg/mL) were prepared by subsequent dilution using the mobile phase.

#### Sample preparation for assay and recovery study

For the analysis of the dosage form, Durabolin injection manufactured by Organon Ltd. (India) was selected. The content of the ampoule was poured in a 250 mL volumetric flask and ethyl alcohol (ana-

lytical grade) was added and shaken thoroughly to extract the drug from the excipients. Dried powdered sample equivalent to 10 mg NPP was dissolved in about 40 mL of ethanol in a separate 100 mL volumetric flask and shaken for 2 h. Both the solutions were filtered through Whatman filter paper (No. 41). A volume of 1 mL of each sample was pipetted out and the final volume was made up to 10 mL with mobile phase to prepare a concentration of 10 µg/mL. After suitable dilution, the drug concentration with six replicates for the parenteral solutions, as well as solutions of prepared dosage form was determined by HPLC using the calibration curve. The recoveries were determined by adding known amounts of NPP reference substance (2.5, 5.0, 7.5 and 10.0 µg) to the formulated samples. These samples were compared with the standard solutions (i.e., 2.5, 5.0, 7.5 and 10.0 µg).

#### Method validation

The method was validated according to the ICH guidelines for the validation of analytical procedures. The parameters which were used to validate the method of analysis were linearity range, limit of detection (LOD), limit of quantification (LOQ), specificity, accuracy, precision and recovery.

## RESULTS AND DISCUSSION

Nanoparticles were successfully prepared by polyelectrolyte complexation technique. The speed

was kept constant at 600 rpm. to obtain the desired results concerning average particle size and narrow particle size distribution profile. The model formulation used in the proposed RP-HPLC analysis showed an average particle diameter of 530.97 nm. The chromatographic conditions were optimized and separation was performed on a Luna Phenomenex CN column using a mobile phase consisting of phosphate buffer and acetonitrile. The proposed mobile phase composition allowed suitable retention time of NPP and good selectivity towards interference from the excipients of the formulation. Calibration curve was constructed using standard NPP solutions in the range of 0.05–25 µg/mL. The linearity of the calibration curve (Fig. 2) was validated by high value of correlation coefficient ( $r^2 = 0.9994$ ).

Under the chromatographic conditions described, NPP was eluted after about 6.3 min. Good baseline resolutions and peak shape can be observed (Fig. 3).

The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.010 and 0.05 µg/mL, respectively. Low values of LOD and LOQ indicate that the method is sensitive.

The specificity of the proposed method demonstrated that the excipients present in market formulation and also in the prepared formulation do not interfere with the drug peak. Furthermore, well-resolved peaks indicate the specificity of the method (Fig. 3). Thus the proposed method is useful to

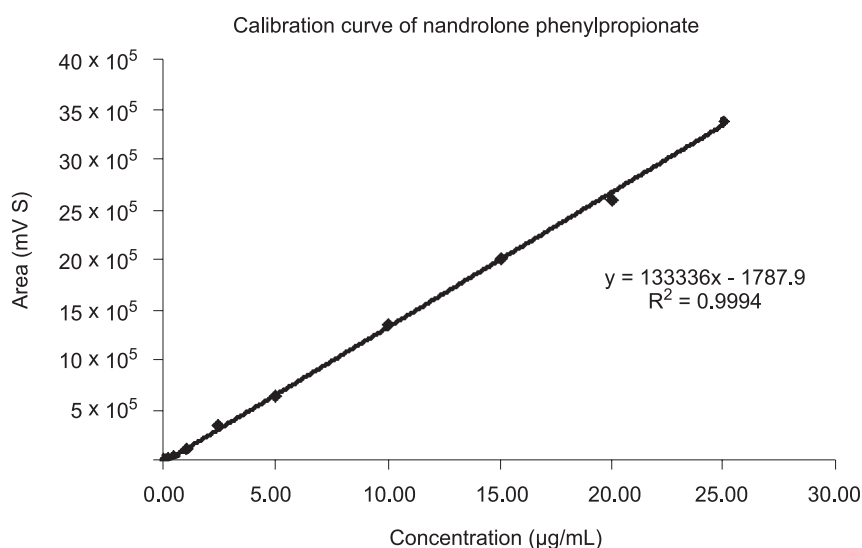


Figure 2. Linearity curve of nandrolone phenylpropionate

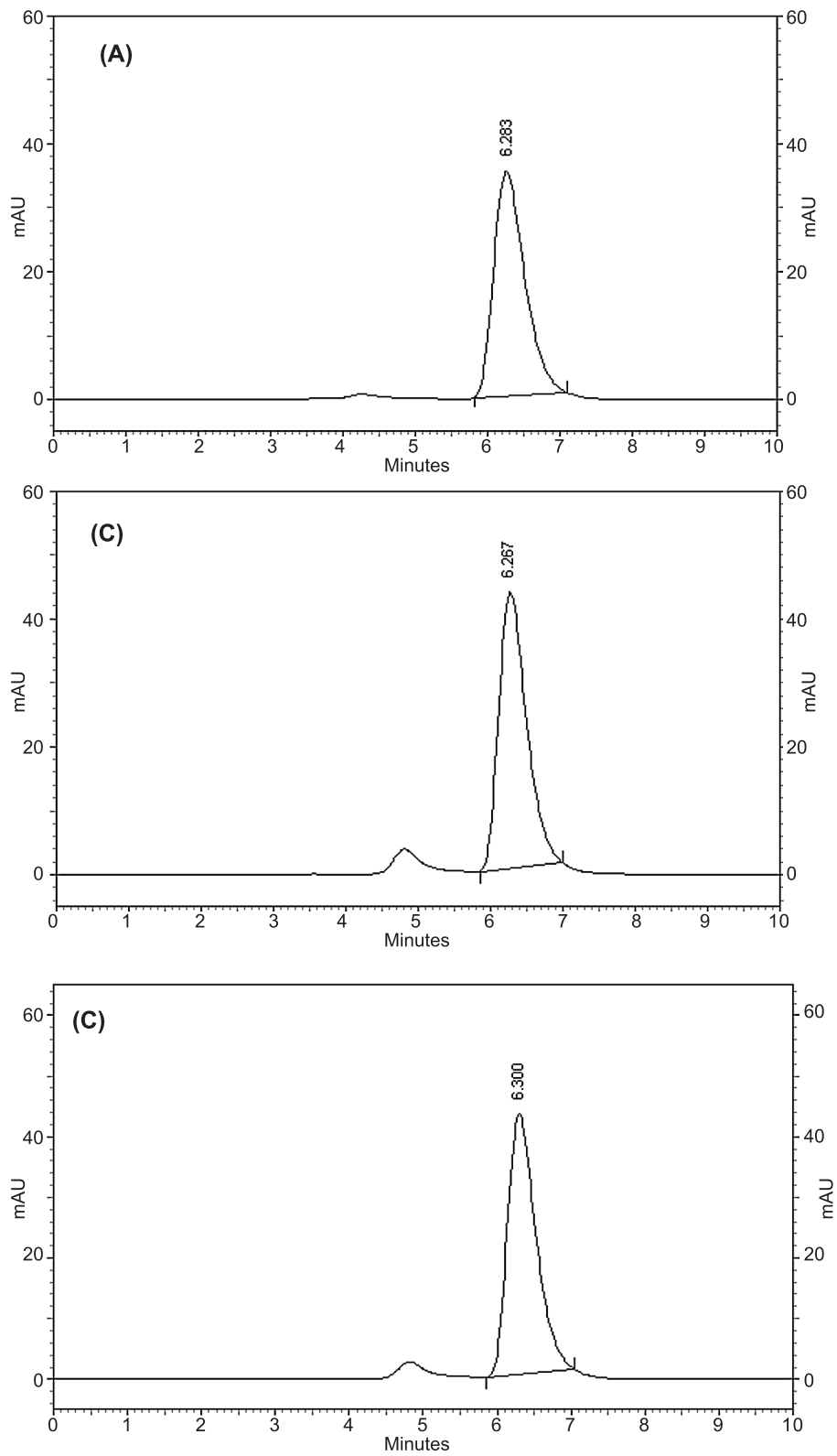


Figure 3. (A) Chromatogram of nandrolone phenylpropionate standard solution, (B) chromatogram of NPP in parenteral dosage form, (C) chromatogram of NPP in nanoparticle solution

Table 1. Intraday precision and accuracy.

Concentration ( $\mu\text{g/mL}$ )	Mean (n = 6)	$\pm$ SD*	Accuracy (%)	Precision (% RSD)**	Relative error
8.0	8.03	0.03	101.40	0.31	1.40
8.5	8.52	0.05	100.28	0.61	0.28
9.0	9.14	0.04	101.56	0.47	1.56
9.5	9.60	0.04	101.04	0.43	1.04
10.0	10.03	0.02	100.34	0.22	0.34
10.5	10.51	0.02	100.12	0.26	0.12
11.0	11.10	0.03	100.99	0.40	0.99
11.5	11.58	0.05	100.69	0.44	0.70
12.0	12.13	0.05	101.04	0.44	1.05

\* Standard deviation ; \*\* relative standard deviation

Table 2. Interday precision and accuracy.

Concentration ( $\mu\text{g/mL}$ )	Mean (n = 6)	$\pm$ SD*	Accuracy (%)	Precision (% RSD)**	Relative error
8.0	8.05	0.04	100.64	0.54	0.64
8.5	8.56	0.06	100.69	0.73	0.69
9.0	9.09	0.08	101.01	0.88	1.01
9.5	9.56	0.05	100.62	0.54	0.62
10.0	10.09	0.05	100.93	0.53	0.93
10.5	10.56	0.08	100.59	0.78	0.59
11.0	11.12	0.05	101.05	0.44	1.05
11.5	11.58	0.06	100.68	0.53	0.67
12.0	12.08	0.06	100.66	0.50	0.66

\* Standard deviation ; \*\* relative standard deviation

Table 3. Assay of pharmaceutical formulations.

Sample (n = 6)	Assay (%)	$\pm$ SD*	RSD**
Durabolin (Injection) (10 $\mu\text{g/mL}$ )	02.48	0.65	0.63
Formulation (10 $\mu\text{g/mL}$ )	100.87	0.71	0.71

\* Standard deviation ; \*\* relative standard deviation

Table 4. Recovery studies in different formulations.

Dosage form	Mean recovery (n = 6)	$\pm$ SD*	RSD**
Durabolin Injection (10 $\mu\text{g/mL}$ )	98.91 %	0.80	0.81
Formulation (10 $\mu\text{g/mL}$ )	97.84 %	0.93	0.95

\* Standard deviation; \*\* relative standard deviation

quantify NPP in different pharmaceutical formulations.

The precision was determined by analyzing nine different concentrations of the bulk drug on three different days. The intra-day and inter-day precisions were calculated as 0.218–0.875% and 0.441–0.875%, respectively, whereas the accuracies

were calculated as 100.284–101.564% and 100.587–101.053% as shown in Table 1 and 2, respectively. The recovery results are very close to 100%, which prove the suitability and accuracy of the proposed method for both the formulations.

The accuracy of both the formulations was tested by assaying the marketed formulation and the

prepared nanoparticles on the same day and three different days. The results of formulation assay are shown in Table 3.

## CONCLUSION

The described HPLC method provides simple, sensitive, precise, accurate and reproducible quantitative method for routine analysis of NPP in conventional dosage form such as injections as well as in novel drug delivery system like nanoparticles. Simple sample preparation procedure and a retention time less than 10 min make this method unique and suitable for analyzing a number of samples in a short period of time. The formulations assays were within the acceptable limit. High percentage of recovery (Table 4) ensures the method to be free from interference of excipients and additives, used in the formulations.

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