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Original article

Validated HPLC Method for the Determination of Nocodazole in Solid Lipid Nanoparticle Formulations

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Nocodazole (NCD) is an anti-neoplastic agent which exerts its effect in cells by interfering with the polymerization of microtubules. As NCD affects the cytoskeleton, it is often used incell biology experiments as a control. In this study, NCD was loaded into Solid Lipid Nanoparticle (SLN) systems. A reverse phase high performance liquid chromatography (HPLC) method was validated and applied forthe determination of NCD in SLN. Determination and validation studies were carried out on a 4.6 × 150 mm, 5µm C_{18} Thermo column using an optimized mobile phase (MP) of methanol:water:phosphate buffer (45:42.5:12.5, v/v/v , pH 5.03) at a flow rate of 0.8 mL/min. Diode array detection was performed at 256 nm and the column temperature was adjusted to 40^{o}C. Naproxen was used as an internal standard (IS). The retention times for naproxen and NCD were 2.2 and 9.8 min, respectively. The specified working range was derived from linearity studies and kept in the concentration range 0.5-100 ppm. Limit of dedection (LOD) and limit of quantitation (LOQ) values were determined to be 0.065 ppm and 0.196 ppm, respectively. NCD recovery % results of the SLN formulations which are stored at 25^{o}C, 4^{o}C and 40^{o}C were investigated and compared to the freshly prepared samples.

Key words: Nocodazole, HPLC, Validation, Nanoparticle

Katı Lipid Nanopartikül Formülasyonları İçindeki Nokodazol'ün Belirlenmesi için Onaylanmış HPLC Metodu

Nokadazol (NCD), mikrotübüllerin polimerizasyonuyla etkileşerek hücre içinde etkisini gösteren anti neoplastik bir ajandır. NCD hücre iskeletini etkilediği için hücre biyoloji deneylerinde kontrol grubu olarak kullanılmaktadır. Bu çalışmada, katı lipid nanopartikül içine NCD yüklendi. Ters faz yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak NCD'nın ve SLN içindeki NCD için metot ve validasyon çalışması yapıldı. Tayin ve validasyon çalışmalarında, 4.6 × 150 mm, 5µm C_{18} Thermo kolon ve metanol:su:fosfat çözeltisi(45:42.5:12.5, h/h/h , pH 5.03) olan hareketli faz kullanılarak 0.8 mL/dk akış hızında yapıldı. Çalışmalar 256 nm dalgaboyunda ve kolon sıcaklığı 40^{o}C'ye ayarlanarak yapıldı.İç standart madde (IS) olarak naproksen kullanıldı. Naproksen'in ve NCD'nin alıkonma zamanları sırasıyla 2.2 ve 9.8 dk. olarak belirlendi. Özel çalışma aralığı doğrusallık çalışmalarından belirlendi ve 0.5-100 ppm derişim aralığında tutuldu. Belirme sınırı (LOD) ve tayin sınırı (LOQ) değerleri sırasıyla 0.065 ppm ve 0.196 ppm olarak belirlendi.25^{o}C, 4^{o}C ve 40^{o}C'de saklanan SLN formülasyonlarının NCD % geri kazanım değerleri boş nanopartiküller ile karşılaştırıldı.

Anahtar kelimeler: Nokodazol, HPLC, Validasyon, Nanopartikül

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INTRODUCTION

NCD is a classical aneugen, which binds to beta-tubulin with high affinity and affects polymerization kinetics even at very low concentrations. In addition, chemicals like colchicine or NCD may alter the morphology of centromeres and kinetochores, the sites of attachment for spindle microtubules on the chromosome, and induce malorientation and lagging of chromosomes in mitotic and meiotic cells(1-4).

DNA content of the reconstructed embryos can be controlled by altering cytoskeletal structures and function by using cytoskeletal modifiers such as cytochalasins, demecolcine, or NCD. It has been successfully used for chemically assisted enucleation because it has induced condensation of metaphase II chromosomes and membrane protrusion with the condensed chromosomes(5-7).

Recently, a growing body of studies indicated that the use high pressure liquid chromatography (HPLC) and coupled techniques, especially DAD and MS, is a powerful approach for the rapid identification of the constituents in medicinal plants and their preparations. High-performance liquid chromatography is very important for analyzing a wide range of chemical species.The performance of this technique is strongly related to the properties of the stationary phaseused. Thus,chromatographers attach special importance to the design of efficient stationary phases for HPLC. The development of new silica-based stationary phases for HPLC remains a challenge for chromatographers. Silica modified with C8 and C18 alkyl chains are the most widely used stationary phases for reversed-phase HPLC (8-10).

There are a lot of studies about validation and optimization studies of various compounds. Some of these studies are with respect to compounds in drugs, while others are with regard to compound in blood or some foods (11-14).

This study describes sensitive, accurate and precise method forthe determination of NCD using HPLC. The method has been validated with respect to precision of peak response, linearity range, specificity and accuracy, LOD and LOQ. The proposed method validated was used for determination of NCD in the novel SLN formulation (15).

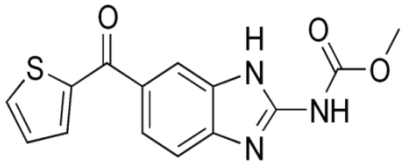

Figure 1. Structure of Nocodazole (NCD)

EXPERIMENTAL

Materials

Compritol, NCD, and polyoxyethylene sorbitan monooleat (Tween 80) were purchased from Merck Schuchardt (Germany) and Across Organics (USA), respectively. The mobile phase, analytical reagent grade methanol for the high performance liquid chromatography was purchased from Merck KgaA (Germany). Used as IS materials naproxen was provided by Abdi Ibrahim (Turkey).

A Shimadzu HPLC device in AUBIBAM was used for the HPLC process validation and active ingredient determinations.Inalab WTW Series 720 pH meter in AUBIBAM was used for adjust pH values of mobile phase.

Preparation of solid lipid nanoparticles (SLN)

Hot homogenization technique was used to prepare NCD loaded SLN. According to this technique, 3% lipids, 5% NCD and 1.2% surface active agent (Tween 80) were used. Lipids were melted about 80^0C. After melting of the lipids, NCD added at the same temperature. Then, tween 80 was added slowly through ultraturaks 20500 rpm, approximately about 1 min NCD loaded SLNs have been obtained(16) (Table 1).

HPLC method validation

The analytical process validation method Q2(R1) of the International Harmonization Committee was used in this study and the parameters such as linearity, accuracy, precision, specificity were evaluated (17,18).

The operating conditions which were applied in validation process are given in Table 2.

Table 1. Nocodazole loaded SLN formulations

Code	Empty Formulation	Nocodazole Formulation
Compritol	3 %	3 %
Tween 80	1.2 %	1.2 %
Nocodazole	-	5 %
Water	95.8 %	90.8 %
Stirred	ultraturrax	ultraturrax
Temperature	80°C	80°C
Stirring rpm	20 500	20 500
Stirring Time	5 min	5 min
Particle size	100 - 250 nm	100 - 250 nm

Table 2. Operating conditions of HPLC

Mobile Phase	Methanol:Water:Phosphate buffer solution (45:42.5:12.5, v/v/v, pH 5.03)
Injection Volume	20µL
Flow Rate	0.8 mL/min
Column	4.6 × 150 mm, 5µm C_{18} Thermo
Detection	256 nm
Oven Temperature	40°C

Naproxen was selected as internal standard (IS). 5 mg accurately weighted naproxen was mixed with the MP and 20mL solution was obtained. Later, this solution was diluted to 1:10 ratio.

5mg NCD was accurately weighted and dissolved in DMSO and volume adjusted to 5 mL. Nine samples of 5µL, 25 µL, 50 µL, 250 µL and 500 µL were taken from this stock solution and diluted to 5 mL with dimethly sulfoxide (DMSO).

In order to establish linearity, a minimum of 5 different concentrations are recommended. The stock solutions of IS and NCD in MP were used to prepare 5 sample solutions of varying NCD concentration.

In order to determine linearity, three different series of the same concentrations were prepared. The NCD series are 0.5 (low), 10 (medium) and 100 (high) ppm.

The obtained series were injected into the HPLC. For the individual peak normalizations (PN) of IS and NCD were calculated by using the area and the retention times (Rt) of chromatograms.

MP, NCD, IS and IS- NCD chromatograms were shown in Figure 2.

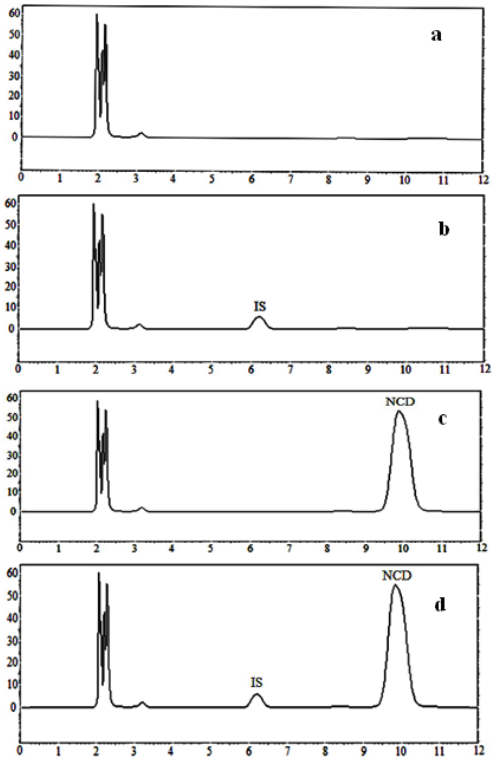

Figure 2. a:chromatograms of the MP, b: chromatograms of IS, c: chromatograms of NCD, d: chromatograms of IS and NCD

The prepared series were injected into the HPLC and the individual PN for IS and NCD were calculated using the area of recorded chromatograms and the Rt.

$PN_{IS}=Area_{IS} / Rt_{IS}$
$PN_{NOCODAZOLE}=Area_{NOCODAZOLE} / Rt_{NOCODAZOLE}$
$PN_{RATIO}=PN_{NOCODAZOLE} / PN_{IS}$

The mean PN ratios of the three series versus the NCD concentration values were used to calculate the linearity equation.

Preparation of samples

Experiments were repeated 6 times for each sample. Amount of NCD in the SLN formulations were expressed as % recovery. In addition, for the NCD amount in the formulations standard error (SE), relative standard deviation (RSD) and 95% confidence interval (CI) values were calculated (19,20).

Determination of nocodazole in SLN

Approximately 20 mg of SLN suspension was mixed with DMSO to obtain a 10 mL suspension. This suspension was centrifuged at 4000 rpm for 15 min following 5 min in an ultrasonic bath. 1mL of the transparent portion of the suspension was added to 0.6 mL mobile phase with 0.4 mL IS to obtain 2mL of solution and filtered through a 0.2 µm polypropylene filter. 1 mL of this transparent filtrate was applied to the column to calculate the total NCD in the formulation.

RESULTS AND DISCUSSION

Method validation was carried out and accuracy and reliability were proven. The specificity of the method was analyzed for SLN systems. It was determined that IS peak and active ingredient did not effected by component in the SLN formulation.

In this study, the correlation equation was found to be y = 0.2105x+0.0305 and according to this equationwhose values are depicted in the Table1 were calculated.

In order to determinate the intraday repeatability of the method, 3 different concentrations of NCD containing samples were prepared. The samples were performed 6 times for each concentration. For inter day repeatability of the method, the same series were prepared on different days. Results obtained from these studies were provided as standard error and relative standard deviation. Intra and inter day repeatability results are given in Table 3.

Table 3. Intraday and inter day (pool-days) linearity results

Linearity Parameters	Intra day (n=5)			Inter day (n=15) Mean
	Day 1	Day 2	Day 3	
Slope	0.1909	0.1886	0.1873	0.2080
Slope SD	0.0009	0.0007	0.0007	0.0030
y-intercept	0.06438	0.1243	0.09634	-0.02633
y-intercept SD	0.03747	0.02824	0.03067	0.0069
CI 95%	0.0023	0.0017	0.0026	0.0063
r^2	0.9996	0.9999	0.9996	0.9997

Measurements performed on three different concentrations (low, medium, high) for evaluation the repeatability and reproducibility of the analytical method have been used to verify the precision of the method. Because the coefficient of variation % is below 2%. The method precision was found to be within the targeted intervals for reparability and reproducibility test.Placebo SLN formulations were prepared forthis study. Any interruption on nocodazole specificity with other components were investigated using the chromatograms obtained (Table 4,5).

The recorded chromatograms have showed that peaks of NCD and IS were separated (Fig1). Thus, it was therefore concluded that the analysis method for the determination of NCD in this study is a specific method.

Chromatograms of the calibration set which were prepared within the NCD were used forthe calculation of LOD and LOQ values (9).

Table 4. Series prepared for determination of accuracy and % recovery results (n=6)

Nocodazole (Concentration is prepared) (ppm)	Recovery (Concentration is measured) (ppm)	Recovery %	SE	% RSD
0.5	0.4983	99.66	0.0032	1.56
1	1.0202	102.02	0.0069	1.69
5	5.0742	101.48	0.032	1.56
10	10.3180	103.18	0.068	1.66
50	49.0870	98.17	0.39	1.91
100	100.4207	100.42	0.8	1.95

(SE: Standard Error)

Table 5. Intraday and interday precision and accuracy results (n=6)

Nocodazole (ppm) (n=3)	Day 1			Day 2			Day 3		
	Measured conc.	SE	% RSD	Measured conc.	SE	% RSD	Measured conc.	SE	% RSD
0.5	0.45	0.002	0.69	0.41	0.004	1.38	0.7	0.003	1.03
10	9.2	0.06	1.03	9.3	0.09	1.55	8.9	0.07	1.2
100	94	0.7	1.2	95	0.82	1.42	99.6	0.8	1.39

(SE: Standard Error)

LOD = 3.3 σ/ S = (3.3 × 0.003747) / 0.1909 = 0.065 ppm
LOQ = 10 σ / S = (10 × 0.003747) / 0.1909 = 0.196 ppm
(σ= standard deviation of response; S= slope of the calibration curve)

The lowest concentration level used in this method is 0.5 ppm. Because LOD and LOQ values are less than this concentration, it can be concluded that our method is sensitive.

Methanol: water and Methanol (MeOH):water:phosphate buffer mixtures at different ratios were tested as the mobile phase. Methanol:water: phosphate buffer (45:42.5:12.5, pH 5.03, 25mM) were selected as the MP. Because the best retention time, separation and distance betweenNCD and IS peaks (Figure 3).

After ratios of the solvents were determined in the MP, first molarity scanning was performed for phosphate buffer solution which used in the MP. These scans were performed at 10, 25, 75 and 200 mM. Subsequently, pH scanning was carried out at pH 3.07, 4.02, 5.03, 6.00, 6.80 and 7.40forthese solutions. According to width of peak was minimum value, values of theoretical number of layer were maximum values and value of peak asymmetry was minimum, 25 mM, pH 5.03 phosphate buffer solution was selected (Figure 4 and 5).

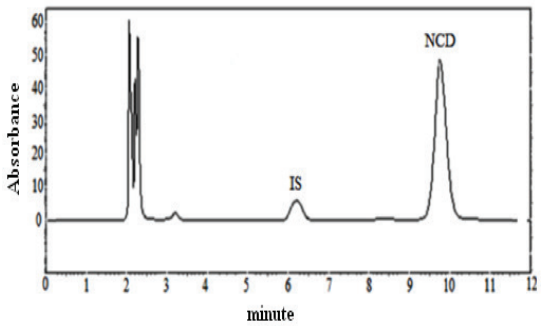

Figure 3. Peaks of IS and NCD(mobile phase MeOH:H_2O: Phosphate Buffer, 45:42.5:12.5 (pH 5.03), 254 nm UV-Vis detector, flow rate 1 mL/min)

Flow rate of the MP was tested in the range of 0.8-1.5 mL/min. Due to the most appropriate peak shape and retention time, a

flow rate was selected 0.8mL.min^{-1}. Appropriate back pressure was determined to be 102 bar in this flow rate. Injection volume was kept constant at 20µL for all validation studies. Solvent front time was observed almost at 2.2 minutes.

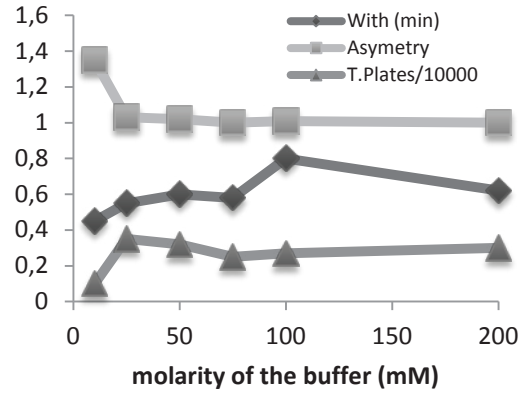

Figure 4. Molarity selected of phosphate buffer solution

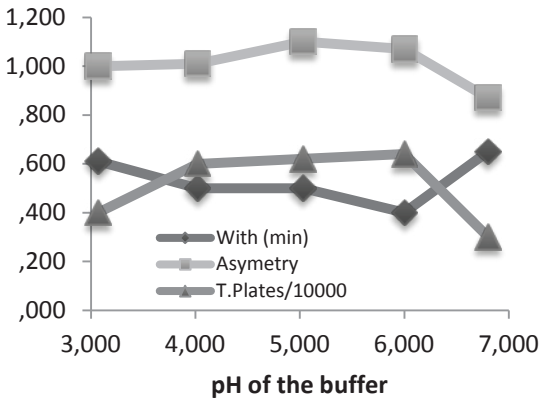

Figure 5. pH selected of phosphate buffer solution

Column volume was calculated by multiplying the solvent front time with the flow rate and was found 1.8 mL.

Due to nearly 1% of column volume was used as the injection volume, the volume was used at 20 µL for all validation studies.

Oven temperature was tested at 25^{o}C and 40^{o}C.It was observed that NCDhas indicated sharper peak symmetry by the increase in temperature. Although, retention time of NCD was raised in thelow temperature,noeffect on Rt of IS. Oven temperature was found at nearly 40^{o}C. All validation studies were carried out at 40^{o}C.

Firstly, due to the apolar structure of the substance 4.6 × 250 mm, 5µm C_{18} Thermo column was tested. Subsequently in an afford to decrease the retention times and to ensure the best separation of the IS material peak and NCD peak, 4.6 × 150 mm, 5µm Thermo column was tested and as a results 4.6 × 150 mm, 5µm Thermo column was used in the study.

In this study, naproxen, diclofenac and paracetamol were tested as IS materials.Naproxen was chosen as an IS because of good resolution between the compounds and suitable retention time of the compounds.

System suitability testing is an integral part of many analyticalprocedures. The tests are based on the concept that the equipment, electronics, analyticalseparations and samples to be analyzed.

Naproxen and NCD peak morphologies were calculated by the software of the HPLC device using the chromatograms derived from the investigations in Table 6.

Table 6. Peak morphology results of nocodazole formulations

Parameters	Nocodazole
Capacity factor	3.98
Asymmetry factor	1.18
Theoretical plate number	2374.08
Width	1.17

CONCLUSION

In literature, no HPLC validation and optimization method forthe NCD have been reported. In this study, application by HPLC method for the determination of NCD was proposed forthe first time. Suggested method can be used as an alternative determination method in solid lipid nanoparticle formulations containing NCD and NCD. Moreover, a simple and stability indicating gradient HPLC assay was developed forthe

analysis of NCD and NCD in solid lipid nanoparticles.

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