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A Sensitive UPLC Method Development and Validation with LC-MS Compatible for the Determination of 1-Deoxynojirimycin In Mulberry Leaves using Fluorescence Detection

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ABSTRACT

A simple, rapid, precise LC-MS compatible method for the degradation impurities if any UHPLC method was developed for the determination of 1-deoxynojirimycin (DNJ) in Mulberry leaves. The DNJ in 200 mg dried leaves was extracted twice with 50 mL aqueous 0.05 mol/L HCl, then derivatized by 9 fluorenylmethyl chloroformate (9 FMOC-Cl) in Potassium Borate buffer, and analyzed using a high performance liquid chromatograph equipped with a fluorescence detector. Chromatographic separation of the same was performed by using a Shimpack XR ODS-III (150*3.0mm) 2.1µm as stationary phase with a mobile phase of 0.1% Acetic acid in water: Acetonitrile (60: 40, v/v) at the rate of 0.5 mL/min. Wavelengths used were 254 nm for excitation and 322 nm for emission, which were suitable for detecting the native fluorescence of all the pigments assayed. The derivatized DNJ was well dissolved from the hydrolysis products of 9 FMOC-Cl. The linearity ranged from 0.45 to 66 mg/L, and the detection limit was 0.2 mg/L (S/N = 3). The content of DNJ in Mulberry leaves was 0.09%, the recovery was 90.0%-110.0%.

Keywords: UPLC, Method development, Method Validation, 1-deoxynojirimycin and Mulberry leave

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INTRODUCTION

Mulberry (*Morus alba* L.; Moraceae) has been cultivated in many Asian countries such as China, Korea, Japan and Thailand. The infusion of its leaves is consumed as antihyperglycemic nutraceutical foods for patients with diabetes mellitus[1-2]. The leaves are rich in alkaloid components including 1-deoxynojirimycin (DNJ) [3] which is known as one of the most potent glycosidase inhibitors[4–11]. It inhibits the enzyme from decomposing starch and sugar and preventing the glucose absorption, resulting in a decrease of blood sugar level. DNJ (Figure. 1) is a polyhydroxylated piperidine alkaloid, which lack of chromophore in its molecule and therefore difficult to be detected by HPLC-UV analysis. The sample pretreatment such as derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) was necessary for HPLC-fluorescence detection[12-17]. Evaporative light scattering detection (ELSD)[18] as well as mass spectrometry[19-20] was an alternative detection method for compounds without a chromophore like DNJ. Another difficulty of DNJ analysis is the separation method as DNJ is highly polar. The interaction between the stationary phase of reversed phase columns and DNJ is so weak that DNJ does not retain in the column. Method development[21-22] and Method Validation[23] are performed to analyse this compound at very lower level and to establish the limit of detection and limit of quantization well the specification limits.

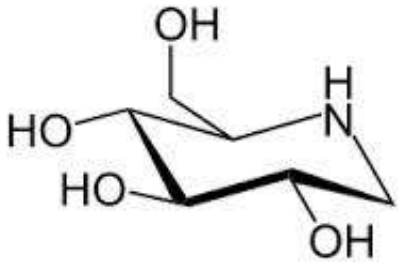

Figure 1: Chemical structure of 1-deoxynojirimycin

MATERIALS AND METHODS

Chemicals and Standards

The reference substance, 1-deoxynojirimycin hydrochloride, was purchased from Sigma–Aldrich (Mumbai, india) purity 99%. , HPLC grade acetonitrile and Hydrochloric acid were purchased from Merck (Darmstadt, Germany). Acetic acid HPLC grade was purchased from Qualigens (Mumbai, India). Deionised water (HPLC Grade) was purchased from Rankem.

Analytical mode Ultra High Performance liquid chromatography

A Shimadzu LC Prominence HPLC module equipped quaternary gradient pump, column

oven, auto sampler and DAD system were used for the analysis and validation of the proposed method of analysis. The data was recorded using LC solution software for Shimadzu Prominence. The chromatographic column used was Shimpack XR ODS-III length 150 mm, internal diameter 3.0 mm with 2.1µm particle size.

Standard and sample solutions

Stock solution of DNJ standard was made in mobile phase. A working solution was prepared in 0.05M HCl solution and diluted to provide a series of analytical standards ranging from 3mg/L to 100mg/L for constructing calibration curves. The stock solution was stable up to 16 days when kept at room temperature before used. Leaves from different positions of the tree and classified into three groups, i.e., the shoots, the young leaves, and the mature leaves. The leaves were harvested, cleaned, air dried and ground into powder and sieved. The material that passed through an 80 mesh sieve was collected and stored in a glass bottle at 4°C until used. 100mg of the powder was added to 10 ml of mobile phase, and then sonicated in an ultrasonic bath for 30 min. The extracts were filtered through filter paper. The filtrate was adjusted to a volume of 50 ml with mobile phase. The filtrate was filtered through a 0.2_m nylon syringe filter (Chromtech, USA) before injected into the UPLC system and the chromatogram was shown in the Figure 2.

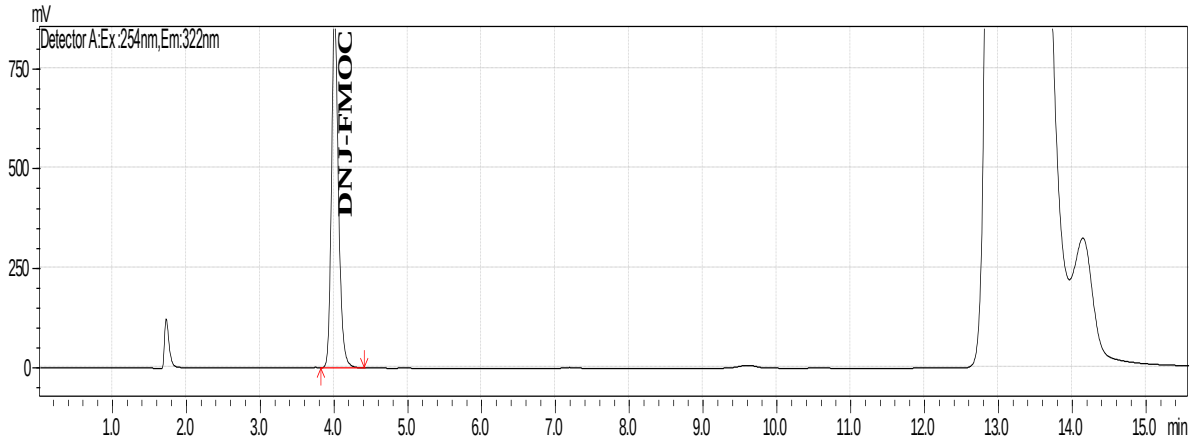

Figure 2: Standard UPLC chromatogram of DNJ

UPLC instrument and conditions

A series Shimatzu Prominence UPLC system was equipped with a quaternary pump, a degasser, an autosampler, a thermostated column compartment and a control module The chromatographic separation was on a Shimpack XR ODS-III (150*3.0mm) 2.1µm as stationary phase with a mobile phase of 0.1% Acetic acid in water: Acetonitrile (60: 40, v/v) at the flow rate of 0.5 mL/min. Wavelengths used were 254 nm for excitation and 322 nm for emission. The column temperature was used in this method is 25°C with 15 min run time.

Method development and optimization

Aim of this study was to develop the chromatographic system which was capable to separate DNJ-FMOC from its all peaks with reduced run time. There was a limited analytical literature was available on this compound. Since there may be any degradant peaks, it was proposed to keep the mobile phase was LC-MS compatible method to identify if any degradant impurities in the same method. Trails were initiated using 10 mM (millimolar) Ammonium Aceate buffer as mobile phase-A and Acetonitrile as mobile phase-B. The peak was retained at dead volume, but it was observed with distorted shape or it may be due to pH or mobile phase incompatibility. Even after adjusting pH to 4, same peak shape was observed so mobile phase was changed to TFA, Initially different gradient programs at a flow rate of 0.4 mL per minutes were proposed for optimum separation with the other peaks. Different column chemistries were tried during initial trail purpose. However Shimpack XR ODS-III length 150 mm, internal diameter 3.0 mm with 2.1µm particle size column has shown better specificity. The same column was used for entire method development work. The method development was summarized in the Table 1.

Table 1: Summary of the Solvents used to optimise the method

Solvent-A (Sol-A)	Solvent-B (Sol-B)	Observation/Remarks
10mM Ammonium Acetate solution	Acetonitrile	Distorted peak shape
10mM Ammonium Acetate solution, pH adjusted to 3.5	Acetonitrile	Distorted peak shape
0.1% Trifloro acetic acid	Acetonitrile	The API peak was not eluted
0.1% Acetic acid	Acetonitrile	Good peak shapes with better resolution

Solution preparation

A stock solution of DNJ (40.0 mg/L) was prepared by dissolving appropriate amount of drug substance in 0.05M HCl. Working solutions of 4.0 and 66 µg/mL were prepared from stock solution for the quantification. This stock solution was stored at around 5°C and found to be stable for a month.

Method Validation for the proposed method

Linearity and Range

Calibration curve obtained by the least square regression analysis between peak area and concentration showed linear relationship with a correlation coefficient of greater than 0.995 over the calibration ranges tested. Linear calibration plot for the quantification method is obtained over the calibration range LOQ to 150%. A graph of peak area versus concentration (on X-axis concentration and on Y- axis Peak area) was plotted and the correlation coefficient was

calculated. The results were shown in the table-2.

Precision

The precision of the related substance method was checked by injecting six individual preparations of 16µg/mL and the % RSD of area for DNJ was calculated. The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory. The results were reported in % RSD. The results for precision are given in table-3.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for DNJ were determined at a signal-to-noise ratio of 3:1 and 10:1, by injecting a series of dilute solutions with known concentration. The lower levels of LOD and LOQ were discussed in the results.

Accuracy

Accuracy was assessed from three replicate determinations of three different levels including 50%, 100% and 150% of the specification levels and the method is accurate within the desired range. The mean percentage recovery values were calculated and reported in the table-4.

Robustness

Close observation of analysis results for deliberately changed chromatographic conditions Flow rate, wave length (both excitation and emission) is no significant change observed in the relative retention times of the DNJ illustrating the robustness of the method.

Solution stability and mobile phase stability

The solution stability of DNJ in the quantification method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed for 6 h interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h interval up to 48 h. Mobile Phase prepared was kept constant during the study period. The percentage assay values were calculated and reported in the table-5.

RESULTS AND DISCUSSION

Method Validation Parameters

Linearity and Range

The results show an excellent correlation obtained between peak area and concentration of DNJ as shown in Figure 3.

Table 2: Linearity table for DNJ

S.No.	Conc (µg/mL)	Response
1	3.97	394745
2	7.94	670698
3	15.88	1311872
4	39.70	3549859
5	66.05	5904720

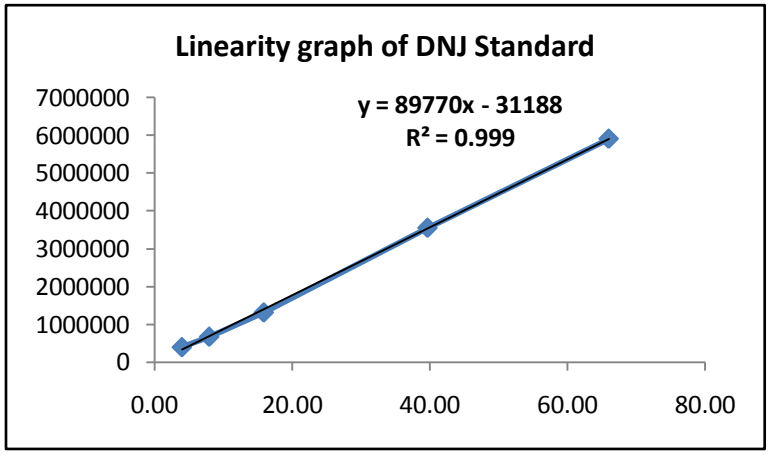

Figure 3: Linearity curve of the DNJ

Precision

The standard and sample solutions were injected for six times and the areas were measured. The %RSD for the area of six replicate injections was found to be within the specified limits and the result was 0.18.

Table 3: Precision for DNJ

Injections	Area
1	1312125
2	1310352
3	1320127
4	1311258
5	1311897
6	1312354
Avg	1313018.83
SD	2369.39
%RSD	**0.18**

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of Detection and Limit of Quantification were calculated and observed like 0.05µg/mL and 0.2µg/mL respectively with respect to analyte concentration and the chromatogram as shown in the Figure 4.

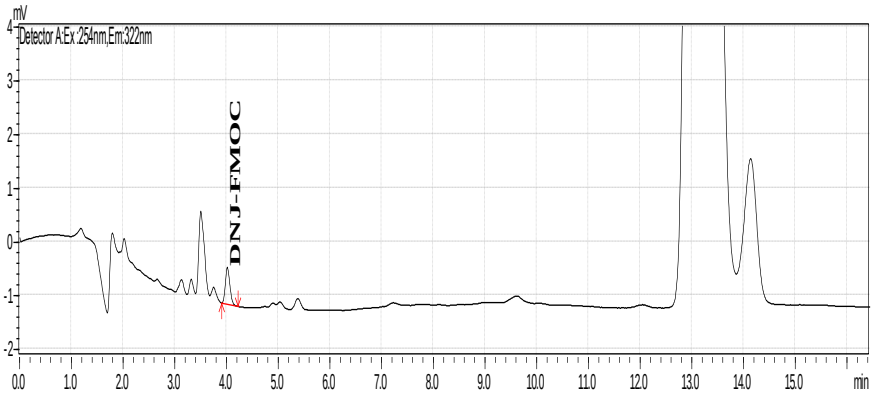

Figure 4: LOQ Chromatogram of DNJ

Accuracy

The observed recovery results were found in the range between 90 to 110% .

Table 4: Recovery for the determination of DNJ

S.No.	Sample spiked at level	Amount added (µg/mL)	Amount recovered (µg/mL)	% Recovery
1	50% sample -1	0.082	0.081	98.8
2	50% sample -2	0.081	0.082	101.2
3	50% sample -3	0.08	0.081	101.3
4	100% sample -1	0.159	0.154	96.9
5	100% sample -2	0.156	0.151	96.8
6	100% sample -3	0.155	0.158	101.9
7	150% sample -1	0.258	0.251	97.3
8	150% sample -2	0.251	0.25	99.6
9	150% sample -3	0.252	0.257	102.0

Robustness

Close observation of analysis results for deliberately changed chromatographic conditions Flow rate, wave length (both excitation and emission) is no significant change observed in the relative retention times of the DNJ illustrating the robustness of the method.

Solution stability and mobile phase stability

The percentage of RSD of asssy of DNJ was calculated for the study period during mobile phase and solution stability experiments.

Table 5 Solution stability and mobile phase stability

Assay	Initial	After 12hrs	After 24hrs	After 48hrs
	99.5	99.8	99.9	100.6

CONCLUSION

The UPLC method developed is accurate, precise, reproducible and specific. The method is

linear over a wide range, economical and utilizes a mobile phase which can be easily prepared and LCMS compatible for the unknown impurities identification. All these factors make this method suitable for quantification of DNJ in bulk drugs and in intermediates. It can therefore be concluded that use of the method can save much time and money and it can be used even in small laboratories with very high accuracy and precision. The method for the quantitative determination of DNJ in mulberry leaves was developed and validated. The method gave high sensitivity and selectivity. It can be applied as routine quality control procedure for the determination of DNJ both in raw materials used for manufacturing and the herbal products in the market.

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