JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF NITISINONE IN BULK AND TABLET DOSAGE FORM.

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ABSTRACT

An accurate, simple, sensitive, precise, fast isocratic reverse phase HPLC (RP-HPLC) method has been developed and validated for quantification of Nitisinone in bulk and pharmaceutical tablet dosage forms. With acetonitrile as the organic solvent, the best separation was achieved on a 250 mmx 4.6 mm i.d, 5µ-particle size Inertsil®-Octadecyl-silyl-3V-Reverse-Phase-C_{18}-column with 0.03M ammonium acetate in water: acetonitrile (50:50 v/v) in the isocratic mode of elution as mobile phase solvent at a speed of 0.8 ml.min^{-1}. UV detection was at 212 nm. Retention time of Nitisinone was 3.0 minutes. With a correlation coefficient of about 1.000, peak-response was obtained as function of concentration over the range of 40 to 120 µg/ ml for Nitisinone. Nitisinone was shown to have a percentage assay of 110.31 %. Nitisinone had a limit of detection of 0.1 mcg/ ml and a limit of quantification (LOQ) of 0.3 mcg/ ml. The presence of excipients in the formulation Orfadin had no effect on the assay method. The procedure is appropriate for use in QC- laboratories since it is economical and precise.

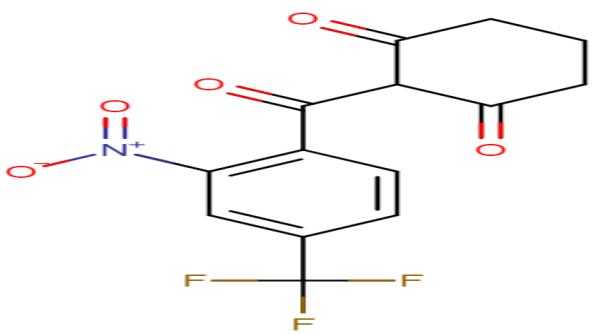
Keywords: Nitisinone, Orfadin, RP-HPLC, Isocratic, Acetonitrile.

INTRODUCTION

Nitisinone is a hydroxy phenyl pyruvate dioxygenase inhibitor used as an adjunct to dietary restrictions for the treatment of hereditary tyrosinemia type 1 (HT-1), which causes intolerance to tyrosine containing foods. Nitisinone has the chemical name 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (Fig 1). Nitisinone acts by preventing the body from breaking down an amino acid called tyrosine and by keeping other toxic substances from building up and causing harm to your liver or kidneys [1].

Nitisinone is a competitive inhibitor of 4-hydroxyphenyl-pyruvate dioxygenase, an enzyme upstream of fumaryl acetoacetatehydrolyase (FAH) in the tyrosine catabolic pathway. By inhibiting the normal catabolism of tyrosine in patients with hereditary tyrosinemia type 1 (HT-1), Nitisinone prevents the accumulation of the catabolic intermediates maleyl acetoacetate and fumaryl acetoacetate [2].

Figure 1: Structure of Nitisinone



Molecular formula of Nitisinone is $C_{14}H_{10}NO_5F_3$ and it has a relative mass of 329.23 g/ mol. It is marketed under the brand names Orfadin, Nityr under the class of drug medication for metabolic and endocrine disorders for the treatment of HT1[3]. Very few techniques for the determination of Nitisinone in oral dosage form have been published [4,5] and mostly bioanalytical techniques are reported for the estimation if Nitisinone [6-11]. Furthermore, no official or preliminary monograph on this analyte has been published in any of the compendial pharmacopoeias. The goal of this study was to develop an accurate and efficient RP-HPLC method to estimate Nitisinone in unit dosage forms for oral administration. The validation of the devised approach is also addressed in this study, as per ICH guidelines [12,13].

EXPERIMENTAL

Chemicals and Reagents:

Nitisinone 99% pure was acquired from Laurus labs Ltd, Hyderabad, India. HPLC Grade- Acetonitrile and water was procured from Rankem-Fine-Chemicals. Ammonium acetate was supplied by Qualigen-Fine chemicals.

Chromatographic-Instrument

Quantitative RP- HPLC was carried out on a Waters 2996 high-performance liquid chromatograph with a PDA detector module, which included an automated injector with a 20 microliters injection volume and a quadra-pump. The column utilized was a Reverse Phase Inertsil Octa Decyl-S-3V-C_{18} column (250mmx 4.6 mm internal diameter with particle size 5µm). EMPOWER Software was installed on the HPLC equipment. The column temperature was adjusted to 25^0C and eluted over 15.0 minutes at a mobile solvent speed of 0.8 ml.min^{-1} under isocratic conditions. The mobile phase used was 0.03M ammonium acetate in water: acetonitrile (50:50 v/v). It was degassed and filtered via 0.45µm Nylon membrane filters before use. For the analyte, UV detection at 212 nm was used as wavelength of detection with a PDA detector. Acetonitrile was used as the diluent to make the standard dilutions. Nitisinone was eluted at 3.0 minutes.

Preparation of the Primary Standard Drug Solution: To make the primary standard stock solution, 100 mg of Nitisinone was dissolved in a volumetric flask (100ml) and diluted with some amount of diluent, sonicated for 15 minutes and finally diluted up to 100ml mark with the diluent to get the primary standard stock solution containing 1000µg-ml^{-1}of Nitisinone.

Preparation of Working Standard Drug Solution: After adding 5 ml of the primary working standard solution to the 50-ml volumetric flask, the flask was filled with 50 ml of the diluent. This solution, which includes 100 ug/ml of Nitisinone, was suitable for use as a working standard solution. The stock solutions were kept in a cool, dark place that was controlled to be four degrees Celsius.

Sample Preparation: After measuring the weight of each individual tablet, the average weight of twenty Orfadin® pills was calculated. The tablets were crushed into a powder form and a sample containing 100-mg of Nitisinone was calculated, which was then weighed, shifted to a 100ml pre-calibrated-measuring flask, and dissolved in acetonitrile. The sample was sonicated in the diluent and strained via Whatman 41 filter paper; the resultant primary working sample solution contained 1000 mcgs-ml-^{1} of Nitisinone. After quantitatively transferring 5ml of the filtrate to a 50-ml pre-calibrated-measuring flask, the diluents were added to bring the volume of the solution to 50 ml. This served as a working testing solution having 200 µg/ ml of Nitisinone. The stock solution was kept in a dark place at 4 degrees centigrade.

RESULTS AND DISCUSSION

The purpose of this research was to create a chromatographic technique for the quantifiable determination of unit-dose of Nitisinone.

Optimized Chromatographic Conditions:

Elution solvents: 0.03M ammonium acetate in water: acetonitrile (50:50v/v)
Elution mode: Isocratic
Column: Inertsil ODS C-18-3V (250 x 4.6mm, 5µm particle size)
Flow rate: 0.8 ml/ min
Injection volume: 20 µl
Detector: Photo diode array (PDA)
Wavelength (λ_{max}): 212 nm
Column temperature: Ambient
Diluent: CH_3CN
Run time: 10 minutes
Retention time: 3.0mins

Linearity: Aliquots of Nitisinone working stock solutions was placed in various 10ml volumetric flasks and the volume was made up to the 10ml with the mobile phase, yielding in final strengths of 40-120 µg-ml^{-1}(Table 2). The peak areas and retention times of each

of these drug solutions (loaded at 20µl) were measured thrice in the column. Using a PDA-detector set at 212 nm, a linearity-graph was generated by plotting peak areas-vs- Nitisinone concentrations in µg-ml^{-1}.

Accuracy: The accuracy of the method was found by evaluating the drug recovery using the standard-spiking method. To assess if the analyte contained in the formulation caused positive or negative interventions, known amounts of the drug equivalent to 12 percent standard drug solution was added to 80 percent, 100 percent and 120 percent of the target test concentrations of the formulation. Each set-of-addition was replicated thrice at each dilution level. The results were compared to a competent reference standard after extraction of sample preparation. The percentage of the analyte recovered by the assay was used to assess the accuracy. Table-3 shows the results of accuracy investigations on standard solution and process-related impurity; recovery measurements suggest that the procedure was accurate.

Precision: Quality-control samples in 100 % (w/v) dilution were used to assess intraday and inter-day precision. On the same day, six replicates of the target concentrations were examined for intra-day variation, and six replicates were examined for inter-day variation on three different days. The method's repeatability was indicated by the low RSD value (1%). (Table-4)

Limits of Detection and Quantification: The method's LOD was set at the lowest concentrations of active pharmaceutical component with a signal-to-noise (S/N) ratio of around 3(LOD). The lowest active therapeutic medication concentration that can be assessed with acceptable precision and accuracy while maintaining a signal-to-noise (S/N) ratio of roughly 10 (LOQ) was also determined.

Method Applicability: The newly created method was evaluated by applying it to pharmaceutical tablets for the estimation of Nitisinone.

Optimization of Chromatographic Conditions:

An isocratic RP- HPLC procedure for assaying the active ingredient was developed due to lack of an easy, economical, reproducible, and quick-to-use method for the determination of Nitisinone concentrations in formulary matrices. The effect of various HPLC technique variables was examined on the result of the study to optimize the chromatographic parameters, various proportions of CH_3CN-KH_2PO_4, CH_3CN-H_2O, and CH_3CN: *O*-H_3PO_3 buffer were tested. After several early investigatory tests, 0.03M ammonium acetate in water: acetonitrile (50:50 v/v) was chosen over other mobile phases because it resulted in improved resolution of active component. This procedure gave the good detection of analyte after multiple exploratory & investigatory trail runs. The active pharmaceutical analyte had excellent UV sensitivity and was interference-free at 212 nm. The analyte peak was highly defined and showed no incidence of tailing under these conditions. The set of conditions previously noted in this article were chosen for additional validation after considering the entire body of data acquired from this extensive study.

Method Validation Tests:

Method precision (RSD percent), method accuracy (recovery percent & % RSD,), linearity range (r^2) and LOD & LOQ were explored as recommended method validation characteristics.

Linearity: With a correlation coefficient of 1.000, the graph of chromatographic-peak areas of the analyte versus respective concentration was shown to be linear in the band of 40-120 µg. ml^{-1} for Nitisinone (Table 2). The least square fit data of linear regression analysis derived from the measurements is given in Table 1. Nitisinone is y = 64486x. Table 1 presents the regression parameters for this technique that include slope, intercept, and % RSD. These findings suggest that there was a significant correlation (Fig 3).

Accuracy: Individual recovery of analyte at 80 %-dilution level on w/v basis, 100 %-dilution level on w/v basis and 120 %-dilution level on w/v basis of prescribed concentrations was 107 percent to 108.75 percent for Nitisinone demonstrating the method's accuracy. The % RSD was usually less than 1% in these data, demonstrating that the technique seems to be very accurate and generates consistent results (Table 3)

Precision: Table 4 summarizes the intraday and interday fluctuation in precision analysis. The method's repeatability is indicated by the low RSD value (less than-1%). These results show that the approach has a high level of precision and repeatability, both within a single analytical run and across multiple runs.

Limit-of-Detection & Limit-of-Quantifications: Nitisinone has a limit of detection of 1 mcg/ml and a limit of quantification (LOQ) of 3 mcg/ ml. These numbers illustrate the method's high sensitivity, which is essential in most investigations, as well as the fact that it can be used to detect and quantify the analyte over a wide concentration range.

Specificity: The Retention time for Nitisinone was determined to be 3.0 minutes, according to the representative chromatogram given in Figure 2. When the pharmaceutical tablet matrices were evaluated, no indication of excipient interference signal was observed in the respective retention time of the chromatogram. It indicates that the analyte was not disturbed of probable merging peaks. As a result, this technique can be employed with certainity.

Table 1: Regression analysis & Operating-System Suitability Results:

Study-Parameter	Nitisinone
Retention Time (min)	3.046
Peak areas	6675373
Percentage of peak areas	99.22
USP-Tailing	1.19
Theoretical Plates	2087.14
Resolution	2.43
Linear range in (µg/ml)	40-120
Limit-of-Detection in µg. ml^{-1}	1
Limit-of-Quantification in µg. ml^{-1}	3
Correlation-Coefficient (r^2)	1.000
Assay-in-Percentage (%)	110.31

Table 2: Summary of the standard calibration Curve for Linearity experiment

Calibration Standard Dilution Level	Concentration of Nitisinone (µg/ml)	Peak Area
40 %	40	2484180
60 %	60	3872895
80%	80	5149339
100 %	100	6515007
120 %	120	7719250

Table 3: Accuracy evaluation by Spike-analysis method

Accuracy study at 80% target level	Injection Number	Nitisinone	
		Standard Soln.	Spiked Soln.
Orfadin-® tablet dosage form solution at 80% level was spiked with 12% of standard solution of API	1	5235489	5941822
	2	5175654	5906601
	3	5215994	5989243
	Mean area	5206319	5928050

	Std. Dev	31732	16914.00
	% RSD	0.62	0.31
	% Recovery		107.00
80% of the target concentration is equivalent to 80 µg/ mlin acetonitrile as diluent.			
Accuracy study at 100% target level	Injection Number	Nitisinone	
		Standard Soln.	Spiked Soln.
Orfadin-® tablet dosage form solution at 100% level was spiked with 12% of mixed standard solution of API's	1	6570782	7291615
	2	6619342	7244384
	3	6589411	7224596
	Mean area	6590062	7250337
	Std. Dev	16577	44288.00
	% RSD	0.33	0.62
	% Recovery		101.16
100% of the target concentration is equivalent to 100 µg/ ml in acetonitrile as diluent.			
Accuracy study at 120% target level	Injection Number	Nitisinone	
		Standard Soln.	Spiked Soln.
Orfadin-® tablet dosage form solution at 120% level was spiked with 12% of mixed standard solution of API's	1	7863745	8558776
	2	7876805	8561203
	3	7872671	8559912
	Mean area	7870943	8559936
	Std. Dev	23917	3060.00
	% RSD	0.33	0.42
	% Recovery		108.75
120% of the target concentration is equivalent to 120µg/ml in acetonitrile as diluent.			

Table 4: Evaluation of precision with-in-day and day-to-day analysis

Intra-Day Precision study of 100% standard dilution containing 200 µg/ ml of Nitisinone			Inter-Day Precision study of 100% standard dilution containing 200 µg/ ml of Nitisinone	
S. No	Nitisinone		Nitisinone	
	Rt. time	Peak area	Rt. time	Peak area
1	3.054	6512314	3.050	6685049

2	3.055	6518225	3.054	6660696
3	3.048	6541915	3.046	6687918
4	3.056	6555805	3.046	6696846
5	3.057	6560015	3.047	6662322
6	3.059	6552111	3.055	6679936
Average	3.055	6538925	3.049	6678140
Std. Dev	0.004	49777	0.004	12130
% RSD	0.12	0.9	0.13	0.19

Figure 2: Chromatogram of Nitisinone100µg/ml analyzed by optimized Isocratic RP-HPLC method.

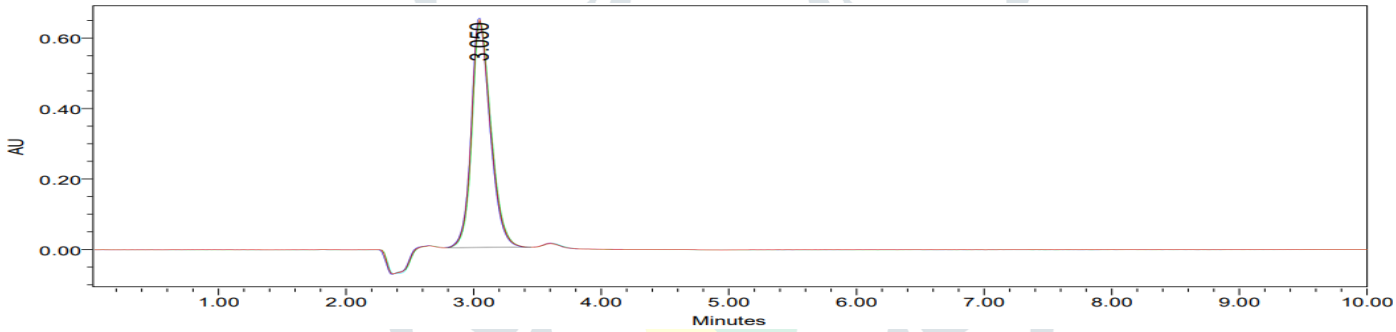
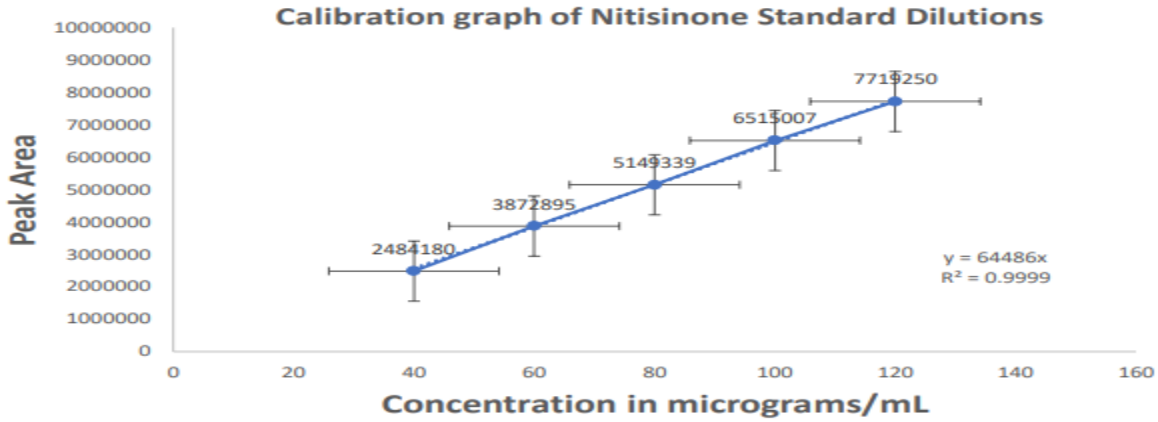


Figure 3: Linearity graph of Nitisinone standard solution



CONCLUSION

In this study, an economical, efficient and commonly available HPLC method for the analysis of Nitisinone in pharmaceutical matrices was devised. This method's key advantages are its significantly reduced cost, ease of use, reduced run time and ease of operation. All these features are critical in operation, especially when analyzing a large number of samples. The validation experiments demonstrated that the procedural approach has a large calibration concentration range, adequate precision & accuracy, and practically reliable sensitivity. The method can be used for regular analysis in formulation QC-studies and allows for a straightforward, selective, sensitive, and specific assessment of Nitisinone.

REFERENCES

1. Anibh Martin Das, Clinical utility of Nitisinone for the treatment of hereditary tyrosinemia type-1 (HT-1), ApplClin Genet. 2017; 10: 43–48.
2. Jessie Neuckermans, Alan Mertens, Dinja De Win, Ulrich Schwaneberg and Joery De Kock; A robust bacterial assay for high-throughput screening of human 4-hydroxyphenylpyruvate dioxygenase inhibitors; Sci Rep. 2019; 9: 14145.
3. Drugs.com on Aug 30, 2022; Cerner Multum.
4. Effat Souri, Farnaz R. Lahiji, Tannaz Nourhashemi, and H. Jalalizadeh. A Stability Indicating HPLC Method for the Determination of Nitisinone in Capsules; Indian J Pharm Sci. 2015 May-Jun; 77(3): 348–351.
5. Zana Mustafa1, Zorica Arsova-Sarafinovska1, Maya Shishovska, Aleksandra Grozdanova. A simple RP-HPLC method for determination of Nitisinone in pharmaceuticals; Macedonian Pharmaceutical Bulletin; Vol. 65(2) 2019.
6. Ridvan Murat Oktem, AsliInci,GurselBiberoglu, IlyasOkur, FatihSuheylEzgu, LeylaTumer. Simultaneous succinylacetone-Nitisinone measurement in tyrosinemia type I patients and evaluation of Nitisinone therapeutic range; Research Square; December 20th, 2022.
7. Cansever MS, Aktuglu-Zeybek AC, Erim FB. Determination of NTBC in serum samples from patients with hereditary tyrosinemia type I by capillary electrophoresis. Talanta. 2010; 80: 1846–8.
8. Prieto JA, Andrade F, Lage S, Aldamiz-Echevarria L. Comparison of plasma and dry blood spots as samples for the determination of Nitisinone (NTBC) by high-performance liquid chromatography-tandem mass spectrometry. Study of the stability of the samples at diferent temperatures. J Chromatogr B AnalytTechnol Biomed Life Sci. 2011; 879: 671–6.
9. La Marca G, Malvagia S, Materazzi S, Della Bona ML, Boenzi S, Martinelli D, Dionisi-Vici C. LC-MS/MS method for simultaneous determination on a dried blood spot of multiple analytes relevant for treatment monitoring in patients with tyrosinemia type I. Anal Chem. 2012; 84:1184–8.
10. DiranHerebian, Ute Spiekerkötter, Marc Lamshöft, Eva Thimm, Maurice Laryea, ErtanMayatepek. Liquid chromatography tandem mass spectrometry method for the quantitation of NTBC (2-(nitro-4-trifluoromethylbenzoyl)1,3-cyclohexanedione) in plasma of tyrosinemia type 1 patients. J Chromatogr B AnalytTechnol Biomed Life Sci. 2009 May 15; 877(14-15):1453-9.
11. Anne Davit-Spraul , HoudaRomdhane, Josephine Poggi-Bach. Simple and fast quantification of Nitisinone (NTBC) using liquid chromatography-tandem mass spectrometry method in plasma of tyrosinemia type 1 patients. J Chromatogr Sci. 2012 May; 50 (5): 446-9.
12. Shabir GA. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, The US Pharmacopeia and the International Conference on Harmonization. *J Chromatogr A.* 2003; 987: 57–66.
13. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q2B: Guideline on Validation of Analytical Procedure/ Methodology, 1996.