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OPEN PEER REVIEW

# Validated Spectrofluorimetric and Derivative Spectrophotometric Method for the Determination of Nitisinone in Pharmaceutical Dosage Form

Maliheh Barazandeh Tehrani, Marzieh Sedighy Tajan, Effat Souri, Hassan Jalalizadeh, Roshanak Hariri

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## About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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## 1 Original submission

5 comments · 6 May 2025 – 18 May 2025

### Referee 1 Reviewer

MINOR REVISION 6 May 2025

Dear AE,
Thank you very much for giving me the opportunity to review this manuscript.
This study presents the development and validation of spectrofluorimetric and derivative spectrophotometric methods for quantifying nitisinone in capsules, offering simpler and more accessible alternatives to HPLC. The manuscript is well-structured, with robust methodology and comprehensive validation data. The use of fluorescein quenching for spectrofluorimetry and the use of derivative spectrophotometry for interference resolution are notable innovations.

@ Strengths:
* Novel application of fluorescein quenching for nitisinone detection.
* Thorough validation (linearity, LOD/LOQ, precision, accuracy) per ICH guidelines.
* Comparison with HPLC demonstrates method reliability.

@ Weaknesses:
* Limited discussion on potential interferences from capsule excipients.
* No environmental impact assessment of methanol usage despite its classification as a "greener" solvent.

@ Detailed Review
# Abstract:
1. Clearly outlines objectives but could emphasize the clinical relevance of nitisinone monitoring in HT1 patients. Add a sentence on the translational impact (e.g., "These methods enable cost-effective quality control in resource-limited settings").

# Introduction:
2. Background on HT1 and nitisinone is well-referenced. However, the rationale for avoiding HPLC (cost, complexity?) is implied but not explicitly stated. Why was fluorescein selected over other fluorophores? Discuss selectivity advantages.

# Materials & Methods
* Methodology:
* Spectrofluorimetry (Method I):
3. Correct "fluorescence intensity (λem) at 517 nm" duplication (lines 126–127). Justify the choice of pH 6 with a reference to fluorescein's pKa (6.4) earlier in the text.

* Derivative Spectrophotometry (Methods II/III):
4. Why were second- and third-order derivatives selected? Provide a brief rationale (e.g., noise reduction, peak resolution).

* Validation:
5. Precision/Accuracy: Table 2 shows excellent CV values (<3%) but lacks discussion on outliers (e.g., 3.86% CV for λex = 326 nm at 0.2 µg/ml). Address potential causes (e.g., pipetting errors, fluorescein stability).

# Results & Discussion
* Spectrofluorimetry:
4. Figure 2: Needs labels for excitation/emission peaks. Editing Note: "Fluorescein ion pair complex" should be "fluorescein-nitisinone complex."

5. Stability Data: Claims 5% variation after 24 hours but does not specify storage conditions (e.g., light protection).

* Derivative Spectrophotometry:
6. Table 3: LOD/LOQ values are impressive (0.01–0.03 µg/ml) but lack comparison to prior methods (e.g., HPLC LODs). Contrast with literature values to highlight advancements.

* Application to Capsules:
7. Table 5: Recovery rates (96.5–102.8%) are acceptable, but excipient interference (e.g., cellulose derivatives) could be discussed.

* Figures 1–5:
8. Figure 5: Add axis labels and units (e.g., "Wavelength (nm)").

# Conclusion
9. Overstates derivative methods as "alternatives to HPLC" without addressing HPLC's gold-standard status. Acknowledge limitations (e.g., specificity in complex matrices).

Final Decision: Minor Revisions

Kind Regards | Publisher Supervisor

**Referee 2** Reviewer

MAJOR REVISION 7 May 2025

The manuscript entitled "Validated Spectrofluorimetric and Spectrophotometric Methods for the Determination of Nitisinone in Pharmaceutical Dosage Forms" requires re-review after the following major revisions have been implemented:
1- The writing should be checked in general.
2- The use of the derivatization method should be included in the title.
3—In some tables, the results are compared with the HPLC method, which was not used in this study.
4- The use of an unusual method to measure a pharmaceutical dosage form, when the method has not been used or accepted in most reputable pharmacopoeias, such as USP, cannot be well justified, especially for measuring a molecule that has good UV absorption, and this has called into question the generality of the study.
5- Examples, with valid references, of the use of the derivatizing agent used in this study (fluorescein) to measure other drugs should be given in the Introduction and Discussion sections.
6- Many sections of the Discussion should be moved to Results.

**Hamid Yaghooti** Reviewer

MAJOR REVISION 13 May 2025

Dear Author/Editor,

I have reviewed the manuscript titled "Validated Spectrofluorimetric and Spectrophotometric Methods for the Determination of Nitisinone in Pharmaceutical Dosage Form". The study proposes simpler and more accessible alternatives to HPLC for the analytical determination of nitisinone. The work is valuable and the use of spectrofluorimetry based on fluorescence quenching of fluorescein is novel for nitisinone analysis, but some aspects need more discussion to ensure the applicability of the proposed methods.
The study should comply with the ICH validation guidelines.
Results from the new method should be compared with a reference method (HPLC) using regression analysis, and the correlation between the new method and the reference method should be reported ($R^2$, slope).

The study mentions the absence of interference from excipients, but no detailed specificity tests or assessments of possible interferences (for example, analysis of placebo formulations and others) are provided. These data are important to confirm the method's selectivity for real samples.
The extraction efficiency of nitisinone from capsules is not quantified. Recovery studies for sample preparation can show the completeness of drug extraction, especially given the poor solubility of nitisinone in water.
The article is not well-structured. The Methods section is incomplete and the methodological descriptions should be moved from the results and discussion sections to the methods section. Additionally, there are some results described in the discussion section that need to be moved to the results section. The results in subsection 3.1.2.4 are not results of this study, as no methods are provided for them. It would be better to delete this subsection from the results section and discuss its content in the discussion section with references.
The Discussion needs to be improved with more explanation of the new methods' performance parameters and limitations.
Best regards,
Reviewer

**Associate Editor** MAJOR REVISION 18 May 2025

Dear Authors
The manuscript needs major revision.
Best regards,
EIC

**Editor-in-Chief** MAJOR REVISION 18 May 2025

Dear Authors
The manuscript needs major revision.
Best regards,
EIC

2

# Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor

Thank you very much for your kind consideration and reviewing the article entitled **“Validated spectrofluorimetric and derivative spectrophotometric method for the determination of nitisinone in pharmaceutical dosage form”**. The suggestions were quite helpful for us and we incorporate them in the revised paper. The article has been fully revised as the respected reviewers suggested and you will kindly find the point-to-point response to the comments as followed. The changes in the article are marked in yellow color.

@ Weaknesses:

* Limited discussion on potential interferences from capsule excipients.

Thanks for your valuable comment. The relative recovery was checked using standard addition method. By using this method, the effect of all of the excipients is considered.

* No environmental impact assessment of methanol usage despite its classification as a "greener" solvent.

As referenced in the text to “Alternative green solvents in sample preparation”, methanol is considered a green solvent according to the information in this reference:

Current green solvents such as subcritical water, supercritical fluids, bio-based solvents, such as methanol, ethanol, d-limonene, and cyrene, surfactant-based solutions including supramolecular solvents and hydrotopes, deep eutectic solvents with special emphasis in natural deep eutectic solvents, and ionic liquids have been evaluated as alternatives to conventional solvents.

And

Solvents such as ethanol, ethyl acetate, ethyl lactate, 2- methyltetrahydrofuran, methanol, glycerol, or d-limonene could be used as green extraction solvent.

@ Detailed Review

# Abstract:

1. Clearly outlines objectives but could emphasize the clinical relevance of nitisinone monitoring in HT1 patients. Add a sentence on the translational impact (e.g., "These methods enable cost-effective quality control in resource-limited settings").

Thank you very much for your valuable comment. Inn this study, a simple method was developed for the determination of nitisinone in pharmaceutical dosage forms. For this reason, it seems that detailed information about monitoring in patients is not necessary. The needed relevant sentences were added to the text as per the reviewer's comment.

#Introduction:

2. Background on HT1 and nitisinone is well-referenced. However, the rationale for avoiding HPLC (cost, complexity?) is implied but not explicitly stated. Why was fluorescein selected over other fluorophores? Discuss selectivity advantages.

Thank you very much for your kind consideration. Actually, we have worked with fluorescein and eosin in our laboratory for other research projects. So, we decided to check the usage of this reagent to see whether it works or not? The results showed that fluorescein could be used for spectrofluorimetric determination of nitisinone. The purpose of this study was to develop a simple and not expensive method using available instruments for the determination of nitisinone in pharmaceutical dosage forms which could be used in quality control laboratories instead of HPLC methods which are more expensive and time consuming.

#Materials&Methods

Methodology:

Spectrofluorimetry (MethodI):

3. Correct "fluorescence intensity (λem) at 517 nm" duplication (lines 126–127).

The intended repetition in the text was removed.

Justify pH 6 choice with a reference to fluorescein's pKa (6.4) earlier in the text.

Thanks to the kind reviewer's attention. The justification of pH has been explained in the results and discussion under the effect of pH on the complex formation.

*Derivative Spectrophotometry (Methods II/III):

4. Why were second- and third-order derivatives selected? Provide a brief rationale (e.g., noise reduction, peak resolution).

The first to fourth order derivative spectra were compared and finally, considering the resolution of the peaks and changes in the absorption intensity, the best results were obtained from the second and third derivatives.

* Validation:

5. Precision/Accuracy: Table 2 shows excellent CV (<3%) but lacks discussion on outliers (e.g., 3.86% CV for λex =326 nm at 0.2 µg/ml). Address potential causes (e.g., pipetting errors, fluorescein stability).

Thank you very much for your prompt comment. Unfortunately, there was some problems with the statistical calculations by transferring the data from excel file. All the calculations were checked again and the corrected data was added in Table 2.

#Results & Discussion

* Spectrofluorimetry:

4. Figure 2: Needs labels for excitation/emission peaks.

Editing Note: "Fluorescein ion pair complex" should be "fluorescein-nitisinone complex."

Thanks for your comment. The items in question were revised according to the referee's opinion.

5. Stability Data: Claims 5% variation after 24 hours but does not specify storage conditions (e.g., light protection).

Thank a lot for your prompt comment. The storage conditions were added to the text.

* Derivative Spectrophotometry:

6. Table 3: LOD/LOQ values are impressive (0.01–0.03 µg/ml) but lack comparison to prior methods (e.g., HPLC LODs). Contrast with literature values to highlight advancements.

The results of other studies on this drug were added to the manuscript (Table 7) at the discretion of the reviewer.

* Application to Capsules:

7. Table 5: Recovery rates (96.5–102.8%) are acceptable but could discuss excipient interference (e.g., cellulose derivatives).

Relative recovery was checked using standard addition method. By using this method, the effect of all of the excipients is considered.

* Figures 1–5:

8. Figure 5: Add axis labels and units (e.g., "Wavelength (nm)").

Thank a lot for your comment. In Figure 5, axis labels and units were added.

# Conclusion

9. Overstates derivative methods as "alternatives to HPLC" without addressing HPLC’s gold-standard status. Acknowledge limitations (e.g., specificity in complex matrices).

Thank you for highlighting this point. Necessary revisions were made to the text as recommended by the referee.

Reviewer 2:

1- The writing should be checked in general

Thank you very much for your kind consideration. The necessary checks were carried out again, and the relevant corrections were made.

2- The use of the derivatization method should be included in the title.

According to the reviewer, the title of the article was changed to:" Validated spectrofluorimetric and derivative spectrophotometric method for the determination of nitisinone in pharmaceutical dosage form".

3—In some tables, the results are compared with the HPLC method, which was not used in this study.

Thank you very much for your prompt consideration. The HPLC method was just used for determination of nitisinone in pharmaceutical dosage form to be compared with developed methods. Unfortunately, the used HPLC method was not addressed in the text. This reference was added in the text.

4- The use of an unusual method in measuring a pharmaceutical dosage form that has not been used and welcomed in most reputable pharmacopoeias, such as USP, especially for measuring a molecule that has good UV absorption, cannot be very justified, and this has called into question the generality of the study.

Thanks to the respected reviewer for paying attention to this point. Spectrophotometric and spectrofluorimetric methods are simpler and cheaper than HPLC. Therefore, these methods enable cost-effective quality control in resource-limited settings. As you know in most in-house methods used in quality control methods, these simple methods are used which are cheaper and time-consuming. For this reason, in this study, nitisinone was investigated with such new methods to allow the investigation of this drug under different conditions, if needed. Also, spectrofluorimetric methods are most of the times more sensitive and specific.

5- Examples with valid references of the use of the derivatizing agent used in this study (fluorescein) to measure other drugs should be given in the Introduction and Discussion sections.

Fluorescence derivatization in HPLC is a technique used to enhance the detection and analysis of various drugs, especially those that don't naturally fluoresce. This method involves chemically modifying the drug molecule with a fluorescent "tag" or reagent, making it detectable by fluorescence detectors in HPLC. This allows for increased sensitivity and selectivity in drug analysis, particularly for compounds that may be present in low concentrations or in complex matrices like biological samples.

Examples of drugs analyzed using fluorescence derivatization in HPLC: Steroids (1), Peptides (2), Nitrofuran drugs (3), Methylphenidate (4),….

6-Many sections of the Discussion should be moved to Results.

Thank you very much for your valuable comment. By reviewing examples of the latest articles published in this journal and to remove ambiguity from the relevant sections, the Results and Discussion sections were presented together and relevant corrections were made in the text of the article.

Reviewer 3:

The study should accord to the ICH validation guidelines.

The authors thank the respected reviewer for this comment. The developed method was validated based on guidelines and the data for the linearity, accuracy, precision, and relative recovery were checked.

Results from the new method should be compared with a Reference Method (HPLC) with Regression Analysis and the correlation between the new method and the reference method should be reported ($R^2$ , slope).

The authors thank the respected reviewer for this comment. In most of the reported new validated methods, after finding the validity of the method based on the guidelines, a comparison made for

the analysis of the final product and in this study the developed methods were compared with a previously reported HPLC method which shows no significant differences between the results

The study mentions the absence of interference from excipients, but no detailed specificity tests or possible interferences (for example analysis of placebo formulations and others) are provided. These data are important to confirm the method's selectivity for real samples.

Thank a lot for your valuable comment. The relative recovery was checked using standard addition method. By using this method, the effect of all of the excipients is considered.

The extraction efficiency of nitisinone from capsules is not quantified. Recovery studies for sample preparation can show the completeness of drug extraction, especially in case of poor solubility of nitisinone in water.

Thanks for your comment. Actually, due to the poor water solubility of nitisinone, the experiments were performed in methanol. There is no need for extraction. After sonicating of the capsule powder with methanol, nitisinone dissolved in methanol and this solution could be used for sample preparation. The results of recovery are given in Table 5.

The article is not well-structured. The Methods section is incomplete and the methodological descriptions should be removed from the results and discussion sections to the methods section. Additionally, there are some results described in the discussion section that need to be removed to the results section.

Thank you very much for your valuable comment. As you have mentioned there was some problems with the article structure. By reviewing examples of the latest articles published in this journal and to remove ambiguity from the relevant sections, the Results and Discussion sections were presented together and relevant corrections were made in the text of the article.

The subsection 3.1.2.4 are not the results of this study as there are no methods for them. Its better to be deleted from the results and be discussed in the discussion section with references.

Thank you very much for your valuable comment. This subsection was deleted.

The Discussion needs to be improved with more explanation of the new methods performance parameters and limitations.

The results of other studies on this drug were added to the manuscript (Table 7) at the discretion of the reviewer.

1. Hameedat F, Hawamdeh S, Alnabulsi S, Zayed A. High performance liquid chromatography (HPLC) with fluorescence detection for quantification of steroids in clinical, pharmaceutical, and environmental samples: a review. Molecules. 2022;27(6):1807.
2. Rhodes GR, Boppana VK, Rubenfield MJ. Fluorescence derivatization approaches for quantitative high-performance liquid chromatography of peptides. Journal of Controlled Release. 1990;13(2-3):121-8.
3. Sheng L-Q, Chen M-M, Chen S-S, Du N-N, Liu Z-D, Song C-F, et al. High-performance liquid chromatography with fluorescence detection for the determination of nitrofuran metabolites in pork muscle. Food Additives & Contaminants: Part A. 2013;30(12):2114-22.
4. Zhu H-J, Wang J-S, Patrick KS, Donovan JL, DeVane CL, Markowitz JS. A novel HPLC fluorescence method for the quantification of methylphenidate in human plasma. Journal of Chromatography B. 2007;858(1-2):91-5.

## 2 Revision 1

3 comments · 19 Aug 2025 – 20 Sep 2025

### Referee 2 Reviewer

ACCEPT 19 Aug 2025

The manuscript 160785 is recommended for publication.

### Referee 1 Reviewer

ACCEPT 20 Sep 2025

Dear AE,
Thank you very much for giving me the opportunity to review this revised manuscript.
I have carefully reviewed the revised manuscript titled "Validated spectrofluorimetric and derivative spectrophotometric method for the determination of nitisinone in pharmaceutical dosage form" and am pleased to recommend its acceptance for publication in the Journal of Reports in Pharmaceutical Sciences.
My evaluation confirms that the authors have addressed all the concerns raised by the reviewers in the previous revision, and the authors have revised their manuscript substantially.
This study addresses an important gap in
pharmaceutical dosage form, derivative spectrophotometry, and spectrofluorimetry and offers valuable insights in this regard. The experimental/analytical approach is well-designed, with appropriate controls/validation. The results are robust and support the conclusions. Furthermore, the manuscript is clearly written, with logical flow and sufficient detail. Although minor language editing can further strengthen the text, this is not critical for acceptance.
Thank you for considering my assessment. Please feel free to contact me if further clarification is needed.

Final Decision: Accept

Kind Regards | Publishing Assistant

### Associate Editor

ACCEPT 20 Sep 2025

Dear
The manuscript was accepted.
BW