Simple chromatographic detection modes for antitumor agent and its degradants

Waleed A. Hassanain, Maha A. Hegazy, Laila E. Abdel Fattah & Hamed M. El-Fatatry


To link to this article: https://doi.org/10.1080/10826076.2017.1378674

Accepted author version posted online: 18 Sep 2017.
Published online: 15 Nov 2017.

Submit your article to this journal

Article views: 48

View related articles

View Crossmark data
Simple chromatographic detection modes for antitumor agent and its degradants

Waleed A. Hassanain\textsuperscript{a}, Maha A. Hegazy\textsuperscript{b}, Laila E. Abdel Fattah\textsuperscript{a}, and Hamed M. El-Fatatry\textsuperscript{c}

\textsuperscript{a}Analytical Chemistry Department, Faculty of Pharmacy, Misr University for Science and Technology, Al-Motamayez District, 6th of October City, Egypt; \textsuperscript{b}Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt; \textsuperscript{c}Analytical Chemistry Department, Faculty of Pharmacy, Tanta University, Tanta, Egypt

\textbf{ABSTRACT}
Degradation of active ingredients is common during the production, transportation, and storage of the pharmaceutical preparations. The resulted degradation products can affect the pharmaceuticals' therapeutic effect. Also, they may have some toxic properties that make them have deleterious effects on patients. Nifuroxazide, antitumor, antimetastasis, and antidiarrheal agent, has been detected in pharmaceutical formulation by two sensitive, selective, and cheap methods. The first method was densitometric thin-layer chromatography technique. The compound was detected in the presence of its degradation products without any interference. Limits of quantification and detection values were 200 and 94.3 ng/band, respectively. The method was linear in the range 0.2–12 µg/band with mean recovery\% ± relative standard deviation (RSD)\% = 99.97\% ± 1.414. The second method was developed using simple high-performance liquid chromatography technique. The method was successfully able to separate the proposed compound from its degradation forms in the presence of pentoxifylline as an internal standard without any interference in less than 5 min. The method also detected nifuroxazide degradation products down to 500 ng/mL. Both methods were statistically compared to a reported method with no significant difference in performance.

\textbf{GRAPHICAL ABSTRACT}

\textbf{KEYWORDS}
Antitumor agent; degradation products; high-performance liquid chromatography; nifuroxazide; stability studies; thin-layer chromatography

\section*{Introduction}
Degradation products are resulting from chemical reactions that occur during the drug manufacturing, storage, and/or transportation due to changes in light, temperature, pH, and humidity. The presence of these products can affect the pharmaceuticals safety and quality. Therefore, it is necessary to know and follow certain protocols to assess the presence of such products in the pharmaceutical formulations. Even small amounts of degradation products can affect pharmaceuticals' safety because of their possible potential to...
cause adverse effects on patients. If the degradation products are toxic, it would be very important to perform more thorough assessments to evaluate their levels in the final product. Controlling critical variables during drug manufacture and conducting a follow-up study of these impurities can prevent degradation impurities present at high concentrations above the permissible limits.[1]

Nifuroxazide (NX) is an oral nitrofuran antibiotic.[2] It is chemically designated as 4-hydroxybenzoic acid [(5-nitro-2-(furanyl)methylene) hydrazide].[3] It has an antitumor activity as it can inhibit the growth and metastasis of melanoma[4,5] through the inhibition of Stat3 phosphorylation. In addition, NX has been well known for its effect in the treatment of acute and chronic diarrhea, gastroenteritis, and colitis[6,7].

Many studies have been reported before for the detection of NX in a single form,[6,8] in the presence of its degradation products,[7,10] and in its binary mixture with other compounds.[7,10] The developed methods include chromatographic,[11,12] electrochemical,[13,14] spectrophotometric,[15,16] and spectrofluorometric techniques.[17]

To the best of our knowledge, no methods have been reported for the detection of NX and its degradation products in bulk powder and/or pharmaceutical dosage form. In this work, we present two new, rapid, selective, and cost-effective chromatographic methods for the detection of antitumor drug, NX, and its alkaline-induced degradation products, NXD. First, mixtures containing NX and NXD were spotted onto thin-layer chromatography (TLC) plates and placed in a chromatographic jar. The mobile phase was allowed to run; three spots were separated representing NX and its two NXD compounds. TLC plates were scanned densitometrically and the compound was quantitatively detected in the presence of its two degradation products without any interferences. Second, rapid high-performance liquid chromatography (HPLC) method was developed for the detection of NX and NXD in the presence of pentoxifylline as an internal standard (IS). Mixtures containing NX, NXD, and IS were injected into HPLC under certain specified chromatographic conditions, the compounds were completely separated in a good resolution and quantitatively determined in the presence of each others. Following this success, these two methods were applied to detect NX in the pharmaceutical dosage form. The alkaline-induced degradation products were qualitatively cross-validated using LC–MS and the two methods were statistically compared to the manufacturer reported method with no significant difference in performance.

Experimental

Chemicals and reagents

Ethanol, chloroform, acetone, sodium hydroxide, and hydrochloric acid were purchased from El-Nasr Pharmaceuticals Chemicals Company (Egypt). Methanol was purchased from Sigma-Aldrich (Germany). Acetonitrile was purchased from Fisher scientific (UK). Nifuroxazide powder was kindly supplied from Hikma Pharmaceuticals (Egypt). Pentoxifyllin powder was kindly supplied from Sanofi Aventis Company (Egypt). Antinal capsules 200 mg, batch No. 2488, manufactured by Amoun Pharmaceuticals (Egypt) were obtained from local pharmacy in Egypt. TLC plates (20 × 10 cm²) coated with silica gel 60 F254 with 0.25 mm thickness were purchased from E-Merck (Germany). Milli-Q water (18.2 MΩ cm) was used for all aqueous preparations. All chemicals were of analytical grade and used without further purification.

Preparation of standard solutions

200 µg/mL solution of NX was prepared by dissolving 20 mg of NX in 100 mL of ethanol. 200 µg/mL solution of NXD was prepared by dissolving 20 mg of NX in 30 mL 0.1 N NaOH and heating in oven for 2 hr at 70°C then cooling. The solution was brought to neutralization using 0.1 N HCl; finally, the volume was completed to 100 mL using ethanol. Complete degradation was tested every 30 min interval and confirmed by TLC using chloroform:acetone (7:3) as a mobile phase. The identity of degradation products was checked by LC–MS. 200 µg/mL solution of IS was prepared by dissolving 20 mg of the pentoxifylline in 100 mL of ethanol. Further dilutions were performed to obtain 100 µg/mL solutions of NX, NXD, and IS.

Preparation of pharmaceutical dosage form

To prepare 200 µg/mL solution of the pharmaceutical dosage form, 10 antinal capsules (200 mg NX/capsule) were accurately evacuated and weighed. An accurate weight of the powder equivalent to 20 mg NX was transferred into 100-mL measuring flask. A total of 60 mL of ethanol was added to the flask and left in ultrasonic bath for 45 min, then left in the fridge overnight. The sample was filtered and the volume was completed to 100 mL with ethanol. Suitable dilutions were performed to prepare the required concentrations.

Construction of calibration curves

For TLC method, six aliquots of NX solution (200 µg/mL) equivalent to 20–120 µg/mL of NX were prepared. A total of 10 µl of each aliquot was applied onto TLC plate by the automatic microsyringe to prepare different concentrations for the calibration (0.2, 0.4, 0.6, 0.8, and 1.2 µg/band). The plate was positioned into a chromatographic jar containing chloroform:acetone (7:3). The plate was left for 20 min till the mobile phase reached 9.5 cm of the plate height and then removed to dry. The plate was visualized under UV lamp and scanned by TLC scanner at 254 nm. The slit dimension was kept at 6.0 × 0.30 µm² and 20 mm/s scanning speed was used. A linear relationship was obtained between the area under the peak and the corresponding concentration.

For HPLC method to construct NX calibration curve, eight aliquots of NX solution (100 µg/mL) ranged from 0.05 to 1.4 µL were mixed with 1.2 mL of IS solution (100 µg/mL) and the volumes were completed to 10 mL using HPLC mobile phase. The final concentrations used for the calibration were 0.5, 2, 4, 6, 8, 10, 12, and 14 µg/mL. The solutions were injected into HPLC at room temperature on RP-C18 column. The mobile phase composed of acetonitrile:water (25:75) and...
was delivered at a rate of 1.5 mL/min. The injection volume was 20 μL and the effluent was detected at 285 nm. A linear relationship was plotted between the relative peak area [peak area of NX/peak area of IS (12 μg/mL)] and the corresponding concentrations.

To construct the calibration curves for the two alkaline-induced degradation products of NX, NXD1, and NXD2, the same steps of NX HPLC calibration curve were repeated again. The concentrations used for NXD1 were 0.5, 2, 4, 6, 8, 10, and 12 μg/mL with IS of concentration 5 μg/mL. For NXD2, the used concentrations were 6, 8, 10, 12, 14, and 16 μg/mL with 1 μg/mL IS.

**Preparation of laboratory mixtures**

For TLC method, to prepare mixtures containing from 5 to 70% of NXD, aliquots ranged from 4.75 to 1.5 mL of NX solution (200 μg/mL) were mixed with aliquots ranged from 0.05 to 3.5 mL of NXD solution (200 μg/mL) and the volumes were completed to 10 mL with ethanol. A total of 10 μL of each aliquot was applied onto TLC plate by the automatic microsyringe to prepare serial concentration of NX from 0.3 to 0.95 μg/band.

For HPLC method, to prepare mixtures containing from 8 to 90% of NXD, aliquots ranged from 1.38 to 0.15 mL of NX solution (100 μg/mL) were mixed with aliquots ranged from 0.12 to 1.35 mL of NXD solution (100 μg/mL). Each aliquot was mixed with 1.2 mL of IS solution (100 μg/mL) and the volume was completed to 10 mL with HPLC mobile phase. The final concentrations of NX were ranged from 1.5 to 13.8 μg/mL.

**Application of standard addition technique**

Three different concentrations of NX solutions were mixed with pharmaceutical formulation before proceeding in the previously mentioned methods. These concentrations were arranged to be more, less, and equal to the added concentration of the pharmaceutical formulation.

**Instruments**

Thin-layer chromatography scans were performed using CAMAG TLC scanner 3 controlled by winCATS software version 3.15 (SUI). The samples were spotted with TLC linomat 5 sample applicator with 100-μL syringe (SUI). UV 254/365 nm lamp was used for scanning (FR).

High-performance liquid chromatography measurements were performed using Agilent 1260 infinity liquid chromatograph (Germany). HPLC separations were performed on Zorbax-SB/RP C-18 column (4.6 × 150 mm²), particle size 5 μm (USA). The data were processed using Agilent Chem Station for LC 3D systems software.

Liquid chromatography–mass spectometry measurements were performed on Shimadzu LC–MS-8050 (USA) that is equipped with “LabSolutions 5.65” software to control the mass spectrometer working parameters.

**Results and discussion**

**Identification and separation of NX degradation products**

The degradation process of the pharmaceuticals can result in producing new unwanted degradation products and/or toxic impurities. Also, it can modify the activity of the principal active component. Therefore, it would be very necessary to monitor and detect these compounds, especially if the proposed drug has a critical effect on health as antivirals and antitumors. Trukhacheva et al. reported that alkaline hydrolysis of NX is accompanied with the removal of nitro group from its structure, therefore losing or reducing its pharmacological antimicrobial activity.

To be able to assess NX and its degradation products in a fair way, we aimed to do this under very mild hydrolysis conditions. We tested the hydrolysis of NX under different parameters. We tried mild acidic hydrolysis for different times with different strengths but the compound withstands hydrolysis. The compound was only hydrolyzed under drastic acidic conditions. Also, the use of 30% H₂O₂ solution did not affect NX stability. The mild alkaline hydrolysis condition was able to decompose NX and produce its suggested degradation products. The suggested degradation pathway was shown in Scheme 1. It was suggested that the interaction with standard alkali lead to detachment of nitro group in the form of nitrite ion and forming two new degradation products.

The degradation process was monitored by TLC, HPLC, and LC–MS. Figure 1a shows TLC separation of NX solution from NXD solution with different retardation factor values (Rf) after applying a mixture of these solutions onto a TLC plate and using chloroform:acetone (7:3) as a mobile phase. Figure 1b and c shows the appearance of new two peaks corresponding to the new two degradation products using the previously mentioned HPLC chromatographic parameters. To check for the identity of the alkaline-induced degradation products, solution contains NXD was scanned by LC–MS. The appearance of new two peaks at 126.9 m/z and 137.8 m/z confirmed the presence of the suggested degradation products (Figure 1d).

**Scheme 1.** Suggested degradation pathway of NX.
Method optimization

The TLC method was based on the difference in the migration rates of the three compounds using the developing system. It was necessary to test the effect of different variables to optimize the method. Different developing systems of different compositions were tested to obtain optimum resolution. Satisfactory separation was achieved upon using chloroform:acetone (7:3). This system was found to give compact sharp symmetrical bands for NX and its two degradation product with suitable $R_f$ values at 0.41, 0.26, and 0.19 using UV lamp at 254 nm. Figure 2 shows a typical chromatogram of the three components.

High-performance liquid chromatography methods have been investigated and validated for quantitative analysis of NX and NXD using internal standard. The use of internal standards is to correct for analytical variability and thus improve the precision and accuracy of the results. The procedures were optimized with a view to develop a quantitative and stability indicating method in a convenient time analysis and with high-quality separation of NX, NXD, and IS. The chromatographic operational conditions were selected by considering the peak resolution and retention times of the first and the last eluted components. Parameters affecting the efficiency of the chromatographic separation had been tested and optimized in a trial to obtain the maximum separation of the cited components. Several trials were performed to obtain good and optimum separation of NX from its degradation products and internal standard. Different mobile phases’ compositions with different ratios have been tested such as acetonitrile:water (2.5:7.5), (3:7), (4:6) and acetonitrile:methanol:water (1.5:1.5:7) at different flow rates such as 0.5, 1, and 1.5 mL/min. The best resolution was obtained upon using acetonitrile:water (2.5:7.5) at 1.5 mL/min flow rate. Under these chromatographic conditions, the whole separation for the four compounds was performed in less than 5 min. NXD1, NXD2, IS, and NX were eluted at retention time ($R_t$) 0.8, 1.1, 2.1, and 4.4, respectively (Figure 3).

Quantification of NX and NXD

Thin-layer chromatography method was used for the quantitative detection of NX. The area under the peak was found responsive to the change in the concentration of NX and

Figure 1. (a) Schematic representation for the separation of NX and NXD on TLC plate, (b) HPLC chromatogram of NX, (c) HPLC chromatogram of NXD, (d) LC–MS chromatogram of NXD. Note: TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

Figure 2. TLC densitometric chromatogram of NX ($R_f = 0.41$) and NXD ($R_f = 0.19$ and 0.26).
increased with an increase in NX concentration in the range 0.2–1.2 µg/band. Therefore, linear relationship between the concentration of NX and area under the peak was obtained. The regression equation was found to be $y = 10.976x + 0.5531$ and the $r$ value = 0.9997. The RSD% of the regression line was found to be 1.414. The RSD% value was calculated by: (average standard deviation value of the method/mean R% of the concentration values) $\times$ 100.

High-performance liquid chromatography method was used also for the quantitative detection of NX, NXD1, and NXD2. Linear relationships were obtained by plotting the concentrations of NX (0.5–14 µg/mL), NXD1 (0.5–12 µg/mL), and NXD2 (6–16 µg/mL) against their relative peak areas. The regression equations were found to be $y = 0.1801x - 0.001$, $y = 0.1485x + 0.0358$, and $y = 0.0849x + 0.1343$ with $r$ values = 0.9998, 0.9997, and 0.9991 for NX, NXD1, and NXD2, respectively.

High-performance liquid chromatography method was also successful in the detection of NXD1 as low as 1% in NX pure powder form with recovery% = 98.95%.

**Method validation**

The international council for harmonization of technical requirements for pharmaceuticals for human use (ICH) guidelines for method validation[21] were followed for the validation of the developed methods.

**Linearity and ranges**

The limits of detection (LOD) was calculated mathematically by the relationship between the standard deviation (SD) of the calibration curve and its slope (S) using this equation $LOD = (3.3 \times SD/S)$. The limits of quantification (LOQ) was determined as the lowest concentration that could be quantified by the corresponding method.

The corresponding concentration ranges, LOD, LOQ, and other statistical parameters for the two chromatographic methods are listed in Table 1.

**Precision**

Precision was evaluated by calculating intra- and inter-day precision by repeating the assay of three different concentrations of NX (0.4, 0.8, and 1.2 µg/band for TLC and 2, 4, and 6 µg/mL for HPLC) thrice in the same day and assaying the same samples in triplicate on three successive days. The developed chromatographic methods were used for the calculation of recovery% and RSD%. Results in Table 1 indicate satisfactory precision of the proposed methods.

![HPLC chromatogram of mixture of contains NXD1, NXD2, IS, and NX using acetonitrile:water (2.5:7.5) as a mobile phase.](image)

**Table 1.** Validation parameters of the proposed methods for the detection of NX, NXD1, and NXD2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NX (TLC)</th>
<th>NX (HPLC)</th>
<th>NXD1 (HPLC)</th>
<th>NXD2 (HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.2–1.2 µg/band</td>
<td>0.5–14 µg/mL</td>
<td>0.5–12 µg/mL</td>
<td>6–16 µg/mL</td>
</tr>
<tr>
<td>Slope</td>
<td>10.976</td>
<td>0.1801</td>
<td>0.1485</td>
<td>0.0849</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.5531</td>
<td>-0.001</td>
<td>0.0358</td>
<td>0.1343</td>
</tr>
<tr>
<td>Standard error of the slope</td>
<td>0.1383</td>
<td>0.0013</td>
<td>0.0017</td>
<td>0.0018</td>
</tr>
<tr>
<td>Standard error of the intercept</td>
<td>0.1077</td>
<td>0.0109</td>
<td>0.0121</td>
<td>0.0209</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9997</td>
<td>0.9997</td>
</tr>
<tr>
<td>LOD</td>
<td>0.0311 µg/band</td>
<td>0.2807 µg/mL</td>
<td>0.3520 µg/mL</td>
<td>0.5277 µg/mL</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.2 µg/band</td>
<td>0.5 µg/mL</td>
<td>0.5 µg/mL</td>
<td>6 µg/mL</td>
</tr>
<tr>
<td>Mean R% ± RSD%</td>
<td>99.97 ± 1.414</td>
<td>100.46 ± 1.871</td>
<td>100.18 ± 1.735</td>
<td>100.09 ± 1.370</td>
</tr>
</tbody>
</table>

TLc, thin-layer chromatography; HPLC, high-performance liquid chromatography; LOD, limits of detection; LOQ, limits of quantification.
**Table 2.** Accuracy of the proposed methods for the detection of NX, NXD1, and NXD2.

<table>
<thead>
<tr>
<th>Method</th>
<th>TLC (µg/band)</th>
<th>HPLC (µg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Added</td>
<td>Found</td>
<td>Rec. %</td>
<td>Added</td>
</tr>
<tr>
<td>0.300</td>
<td>0.293</td>
<td>97.76</td>
<td>1.00</td>
</tr>
<tr>
<td>0.500</td>
<td>0.490</td>
<td>98.00</td>
<td>3.00</td>
</tr>
<tr>
<td>0.700</td>
<td>0.689</td>
<td>98.57</td>
<td>5.00</td>
</tr>
<tr>
<td>0.900</td>
<td>0.879</td>
<td>97.67</td>
<td>7.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>98.00</td>
<td>1.265</td>
<td>100.22</td>
</tr>
<tr>
<td>RSD%</td>
<td>0.413</td>
<td>0.398</td>
<td>1.589</td>
</tr>
</tbody>
</table>

TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

*Recovery% calculated by: (Found/added concentration) × 100.

**Accuracy**

The accuracy of the developed methods was validated by analyzing samples of NX, NXD1, and NXD2 along their linearity range. The found concentrations of the compounds were calculated from the related regression equation. The results are shown in Table 2.

**Specificity**

Specificity was ascertained by analyzing different mixtures containing NX and NXD in different ratios. The resulted concentrations of NX in the prepared mixtures were calculated using the corresponding regression equation as shown in Table 3.

**HPLC system suitability**

To validate the suggested HPLC method, an overall system suitability testing was performed for NX, NXD1, NXD2, and IS separation to determine if the operating system is performing properly. Good results were obtained and are shown in Table 4.

**Application to commercial capsules**

The suggested methods were successfully applied for the determination of NX in antinal capsules. The dosage form solution was prepared as previous and the same procedures were repeated as described for NX quantification. The analysis of NX by both the methods was satisfactory and with a good agreement with the labeled amount. The mean recovery% ± RSD% values were equal to 100.29 ± 1.186 and 99.97 ± 1.196 for TLC and HPLC methods, respectively. When standard addition technique was applied for the detection of NX in both the methods, no interferences from the excipients were observed. This reflected the selectivity of the methods for the detection of NX only. The concentrations of NX in each method were calculated from the corresponding regression equation (Table 5).

**Cross-validation**

The results obtained by TLC and HPLC methods for the detection of NX were statistically compared with those obtained by applying the manufacturer method. The calculated t and F values for both the methods were less than the tabulated ones (Table 6). Therefore, there is no significant difference between the developed and manufacturer methods with respect to accuracy and precision.
Conclusion

Degradation products can be toxic and affect the dosage form safety. This work presented simple, sensitive, accurate, and reproducible methods for the detection of antitumor drug and monitor its degradation products. The two developed chromatographic methods were successful in the detection of NX and NXD without any cross-interference. TLC method was simple, sensitive, and cost-effective and can be used for the detection of NX in biological fluids. It has the advantages of large sample capacity and use of minimal volume of solvents. HPLC method was rapid as it gave a good resolution between NX and its degradation products in the presence of internal standard in less than 5 min. HPLC method was used for the first time in the detection of the degradation products of nifuroxazide with a very good selectivity and sensitivity. HPLC methods detected successfully the drug degradation product down to 1% in the pure powdered form of the drug. The developed methods can be useful for stability investigation of other active entities and checking the extent of degradation in the pharmaceutical formulations in quality control laboratories.

Conflict of interest and novelty statement

The authors declare that there are no financial/commercial conflicts of interest. The authors also declare that this manuscript has not been published elsewhere and that it has not been submitted simultaneously for publication elsewhere.

References