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1. AU: The text has been lightly edited throughout for fluidity and syntax. Not all edits have been queried. Please review the text carefully for accuracy to ensure your original intent has been retained.
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16. AU: Please define the em dash in footnote “b”. Also, footnote “d” does not appear to be necessary in Table 2 because of the straddle headings (eg, 10–100 µg/band) that appear beneath the component list. In addition, because the footnote is not cited anywhere in the table, can footnote “d” be deleted?
17. AU: Please define the em dash in footnote “e”.
18. AU: Please confirm that footnote “a” indeed does not apply to Imp-A found by the HPLC method.
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# Quantitative Determination of Synthesized Genotoxic Impurities in Nifuroxazide Capsules by Validated Chromatographic Methods

NADA S. ABDELWAHAB, NOURUDDIN W. ALI, and MARCO M. ZAKI<sup>1</sup>

Beni-Suef University, Faculty of Pharmacy, Pharmaceutical Analytical Chemistry Department, Alshaheed Shehata Ahmed Hegazy St, 62514 Beni-Suef, Egypt

M. ABDELKAWY

Cairo University, Faculty of Pharmacy, Pharmaceutical Analytical Chemistry Department, Kasr El-Aini St, 11562 Cairo, Egypt

MOHAMMED T. EL-SAAD

Beni-Suef University, Faculty of Pharmacy, Pharmaceutical Medicinal Chemistry Department, Alshaheed Shehata Ahmed Hegazy St, 62514 Beni-Suef, Egypt

AU1

**Two accurate, selective, and precise chromatographic methods, namely TLC-densitometric and reversed-phase (RP)-HPLC, were developed and validated for the simultaneous determination of nifuroxazide (NIF) and its four synthesized impurities, which are also reported to be its related substances in the range of 10–100 µg/band and 10–100 µg/mL for NIF in the TLC and RP-HPLC methods, respectively. The developed TLC-densitometric method depended on the separation and quantitation of the studied components on silica gel 60 F<sub>254</sub> TLC plates. Ethyl acetate–acetone–methanol–ammonia (85 + 25 + 5 + 0.5, v/v/v/v) was used as the developing system, and the separated bands were UV-scanned at 230 nm. On the other hand, the developed RP-HPLC method depended on chromatographic separation using a C8 column at 25°C and an aqueous solution of 0.1% sodium lauryl sulfate–acetonitrile as the mobile phase delivered according to the gradient elution program. Factors affecting the developed methods were studied and optimized. Also, method validation was carried out according to International Conference on Harmonization guidelines. The proposed methods were successfully applied for the determination of the studied drug in its bulk powder and in its pharmaceutical formulation. The developed methods showed no significant difference when compared with the reported RP-HPLC one. Their advantage is being the first stability-indicating methods for NIF and its genotoxic impurities.**

AU2

**N**ifuroxazide (NIF) is a synthetic drug of a nitro furans derivative (1, 2), which is chemically named (*E*)-4-hydroxy-*N*-[(5-nitrofuran-2-yl)methylidene]benzohydrazide (1; Figure 1). NIF acts as an intestinal anti-infectious drug. It has the advantage of rapid action and is active against a broad spectrum of organisms known to cause diarrhea (3).

An intensive literature survey revealed that there are some analytical methods reported for the estimation of NIF either individually or in combination with other drugs. These methods include spectrophotometry (4–6), HPLC (7–12), voltammetry (13–16), differential pulse polarography (17), and colorimetry (9). All these published methods aimed for the determination of NIF without caring about the possibility of the presence of drug impurities in the NIF samples.

The impurity profile of active pharmaceutical impurities (APIs) is one of the greatest challenges of pharmaceutical analytical chemists in the pharmaceutical industry (18). The presence of unwanted matter, even in small amounts, may influence the safety of the pharmaceutical preparations rather than the therapeutic efficacy (19). Therefore, the pharmacopeias have established maximum allowed limits of related compounds for both bulk and formulated APIs. Impurity profile studies of a pure drug powder (20) and drug formulation (21) need to be carried out using a suitable analytical method, a requirement of various regulatory authorities. This was the motivation for establishing our research, which aimed to synthesize the biohazardous and toxic impurities of NIF and develop analytical methods for the simultaneous determination of NIF with its impurities. The synthesized impurities were named Imp-A [4-hydroxybenzohydrazide(*p*-hydroxybenzohydrazide)], Imp-B [methyl 4-hydroxybenzoate], Imp-C [(5-nitrofuran-2-yl)methylidene diacetate], and Imp-D {[*E,E*]-*N,N*-bis[(5-nitrofuran-2-yl)methylidene]hydrazine(5-nitrofurfural azine)}; Figure 1}. This research paper presents, for the first time, different stability-indicating chromatographic, TLC-densitometric, and reversed-phase (RP)-HPLC methods for the separation and quantification of NIF and its process-related toxic impurities. Both developed methods were successfully applied for resolving the five components in a single run using a single detection wavelength. Also, the methods were successfully able to determine the studied impurities with high sensitivity (up to 0.1% of NIF), which agreed with the impurity profile criteria reported by the International Conference on Harmonization (ICH; 22) and *British Pharmacopoeia* (BP; 1).

AU3

AU4

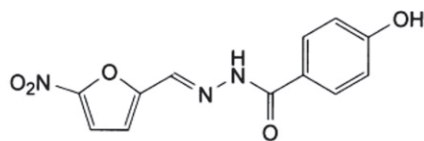
## Toxicology

The seriousness of these impurities ranges from just a skin rash to human carcinogenic and genotoxic effect.

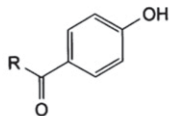
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<sup>1</sup>Corresponding author's e-mail: marcomounir11@yahoo.com

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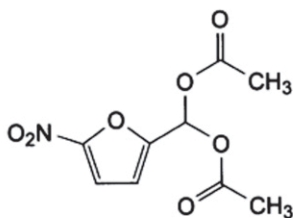


Chemical structure of Nifuroxazide (NIF).

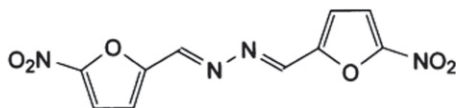


A. R = NH-NH<sub>2</sub>: 4-hydroxybenzohydrazide (*p*-hydroxybenzohydrazide),  
 B. R = OCH<sub>3</sub>: methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

Chemical structure of Imp A & Imp B



Chemical structure of Imp C. (5-nitrofuran-2-yl) methylidene diacetate



Chemical structure of Imp D (*E,E*)-*N,N'*-bis[(5-nitrofuran-2-yl)methylidene]hydrazine (5-nitrofurfural azine),

Figure 1. Chemical structures of NIF and Imp-A, -B, -C, and D.

Imp-A and -D have been reported to be related to the substance, NIF, and are fairly common synthetic intermediates (1) that are related to hydrazide and azine families. Moreover, Imp-A has been reported as a NIF principal hydrolytic degradation product (4) and as a metabolite (8). It is known that azines and hydrazides show conventional structural alerts for genotoxic potential (23), and they are known as human carcinogens (24). These harmful effects are due to DNA damage, gene mutations, and chromosome (25).

Concerning Imp-C, reduction of the nitro group seems to be the most important metabolic pathway for nitrofurans, potentially leading to reactive intermediates that are capable of binding to proteins and to DNA. Nitroreduction and subsequent redox-cycling results in the generation of reactive species (including oxygen species) might be responsible for some adverse effects (26).

Finally, Imp-B, methylparaben, is generally recognized as a safe antibacterial preservative for food and cosmetics (27). Methylparaben is readily absorbed by the gastrointestinal tract or through the skin (28). In a population with normal skin, methylparaben is practically nonirritating and nonsensitizing; however, its adverse effects start from allergic reactions (from ingested parabens in some people; 29) to carcinogenic stimulatory action in vitro effects on breast cancer cell proliferation (30). Studies indicate that methylparaben applied

to the skin may react with UV-B rays, leading to increased skin aging and DNA damage (31, 32).

Recently, the European Medicines Agency published a draft guideline for setting limits in pharmaceutical studies for genotoxic impurities (33). Kean et al. (34) defined the limits for these genotoxic impurities in drugs, which are only used in short-term administration, such as NIF, with a posology of 4 × 200 mg dose daily for 10 days; the acceptance limit is equivalent to 120 µg hydrazine in 800 mg pharmaceutical substance, i.e., 150 parts per million. From here we decided to establish different sensitive methods that are able to cover the acceptance limit, assisting in furthering toxicological studies.

## Experimental

### Pure Samples

(a) *NIF*.—Kindly supplied by Amriya Pharmaceutical Industries (Alexandria, Egypt). Its purity was reported to be 99.5%, according to the company's certificate of analysis.

(b) *Imp-B*.—Kindly supplied by Sigma-Aldrich (Cairo, Egypt). Their purity was reported to be 99.50%, according to the company's certificate of analysis.

(c) *Imp-A*, *-C*, and *-D*.—Laboratory-prepared.

### Pharmaceutical Formulation

Nifunal (Batch No. 731302), labeled to contain 200 mg NIF, was manufactured by Amriya Pharmaceutical Industries.

### Chemicals and Solvents

All chemicals and solvents used throughout this work were of analytical grade and used without further purification.

(a) *Ethyl acetate, ammonia solution, methanol, acetone, sodium lauryl sulfate, sulfuric acid, and nitric acid.*—From El Nasr Pharmaceutical Chemicals Co. (Abu-Zabaal, Cairo, Egypt).

(b) *Acetonitrile, furfural, acetic anhydride, absolute ethanol, and dimethyl sulfoxide (DMSO).*—HPLC grade (SDS, France).

(c) *Deionized water.*—Sedico Pharmaceuticals Co. (Cairo, Egypt).

(d) *Hydrazine hydrates and semicarbazide.*—Sigma-Aldrich.

### Instruments

(a) *Synthesis and structural elucidation.*—Melting points (mp) were uncorrected and carried out with an open capillary tube method using an IA 9100MK Digital Melting Point Apparatus.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were obtained on a Bruker APX400 at 400 and 100 MHz, respectively. Chemical shifts were reported at the  $\delta$ -scale and were related to those of the solvent;  $J$ -values were in Hz. The mass spectra were recorded on a Waters Acquity Ultra Performance LC system with a ZQ detector in electrospray ionization mode. IR spectra were obtained with a PerkinElmer Spectrum 100FT-IR spectrometer. TLC plates (20 × 20 cm) coated with silica gel 60 F<sub>254</sub> (Merck, Germany) were used. Elemental analysis [carbon (C), hydrogen (H), and nitrogen (N)] were recorded on a PerkinElmer CHN/SO series II elemental analyzer.

(b) *TLC-densitometric method.*—This method was carried out using a sample applicator for TLC Linomat 5 with a 100  $\mu\text{L}$  syringe (CAMAG, Muttenz, Switzerland). A TLC Scanner 3 densitometer (CAMAG) was used for scanning controlled with WIN CATS software (Version 3.15, CAMAG). Slit dimensions were 3.00 × 0.45 mm, with a scanning speed of 20 mm · s<sup>-1</sup> and data resolution of 100  $\mu\text{m}/\text{step}$ . All of these requirements were taken into consideration. Finally, a UV lamp with a short wavelength (254 nm, Model No. VL-6.LC; Marne la Vallée, France) was used for scanning until the proposed method was optimized. TLC plates (20 × 20 cm) coated with silica gel 60 F<sub>254</sub> with 0.2 mm thickness were used as the stationary phase.

(c) *HPLC method.*—The instrument used was an HPLC Agilent 1260 Infinity (Germany), equipped with an Agilent 1260 infinity preparative pump (Model No. G1361A), Agilent 1260 infinity diode array detector VL (Model No. G131SD), Agilent 1260 infinity thermostated column compartment (Model No. G1316A), and Agilent 1260 infinity preparative autosampler (Model No. G2260A). The stationary phase was a Zorbax Eclipse plus C8 column (250 × 4.6 mm id, 5  $\mu\text{m}$  particle size; United States). A Sonix TV ss-series ultrasonicator (United States) was also used. For filtration of the formulation solution, a Nylon 66 membrane syringe filter (Npore, Ghaziabad, India) was used.

### Prepared Solutions

(a) *Surfactant solution.*—The 0.1% sodium lauryl sulfate solution was prepared by dissolving 1 g sodium lauryl sulfate

in deionized water and diluting it to a volume of 1 L. The prepared solution was filtered through the previously mentioned membrane filter.

(b) *Stock solutions (5 mg/mL and 0.5  $\mu\text{g}/\text{mL}$ , respectively) for NIF and impurities.*—Stock solutions of NIF and its impurities, which were prepared by accurately weighing 500 mg NIF and 50 mg of each impurity, were separately transferred into five separate 100 mL amber glass volumetric flasks (due to the photosensitivity of NIF). For increasing solubility of the impurities, 2 mL DMSO were used and the solutions shaken well and diluted volume with absolute ethanol to obtain stock solutions of 5 mg/mL for NIF and 0.5 mg/mL for Imp-A, B, C, and D.

(c) *First working solutions (0.5 and 0.05 mg/mL, respectively) for NIF and impurities.*—Working solutions of NIF and Imp-A, B, C, and D were prepared by accurately transferring 10 mL of each from their respective stock solutions into five separate 100 mL amber glass volumetric flasks and diluting to volume with absolute ethanol (for the TLC-densitometric method) or a 0.1% aqueous solution of sodium lauryl sulfate-acetonitrile (60 + 40, v/v; for the HPLC method) to get 500  $\mu\text{g}/\text{mL}$  NIF and 50  $\mu\text{g}/\text{mL}$  Imp-A, B, C, and D working solutions.

(d) *Second working solutions (1  $\mu\text{g}/\text{mL}$ ) of Imp-A, B, C, and D.*—Second working solutions of Imp-A, B, C, and D were prepared by accurately transferring 2 mL of each from their respective first working standard solutions (50  $\mu\text{g}/\text{mL}$ ) into four separate 100 mL amber glass volumetric flasks and diluting to volume with a 0.1% aqueous solution of sodium lauryl sulfate-acetonitrile (60 + 40, v/v; for the HPLC method) to get 1  $\mu\text{g}/\text{mL}$  working solution of each.

(e) *Laboratory-prepared mixtures.*—Different laboratory-prepared mixtures containing different ratios of NIF and Imp-A, B, C, and D were prepared by accurately transferring different volumes of each from their respective stock solutions of NIF and working solutions of each impurity (for the TLC method) or working standard solutions (for the HPLC method) into 10 mL amber glass volumetric flasks and diluting to volume using a suitable solvent.

### Procedures

(a) *Preparation of NIF impurities.*—(1) *Scheme I for Imp-A.*—A mixture of methyl 4-hydroxy benzoate (1.54 g, 10 mmol) and hydrazine hydrate (99%, 1.3 g, 25 mmol) in absolute ethanol (50 mL) was heated under reflux for 2 h. The formed precipitate was filtered off, washed with ethanol, and dried. Recrystallization from ethanol gave white crystals of a 4-hydroxybenzohydrazide derivative (Figure 2).

(2) *Scheme II for Imp-C and D.*—(a) *Imp-C.*—A mixture of concentrated nitric acid (5 mL) and sulfuric acid (36 N; 0.5 mL) was added dropwise to acetic anhydride (50 mL) at low temperature (0–5°C). To this solution a double-distilled

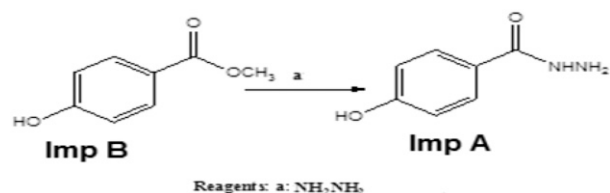
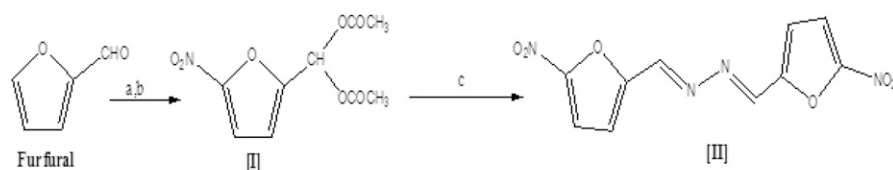


Figure 2. Scheme I for Imp-A.



Reagents: a : Conc $n$  HNO $_3$ /Conc $n$  H $_2$ SO $_4$ , b: (CH $_3$ CO) $_2$ O, c: NH $_2$ NHCONH $_2$

**Figure 3.** Scheme II for Imp-C and -D.

furfural (6 mL) was added, under the same conditions. The mixture was stirred in an ice bath for 2 h. Water (60 mL) was added to the mixture at room temperature. After 30 min, the pH was adjusted to 2.5–2.7, and the mixture warmed on a hot plate (60°C, 1 h) and left overnight at room temperature. The precipitate that formed was collected and recrystallized from ethanol (Figure 3).

(b) *Imp-D*—5-Nitrofurfural diacetate (20 mmol) and semicarbazide HCl (25 mmol) were dissolved in ethanol–water–sulfuric acid (6 + 25 + 5 mL) and refluxed for 3 h. The precipitate was collected and recrystallized from ethanol (Figure 3).

#### Method Validation

(a) *Linearity and range.*—(1) *TLC-densitometric method.*—Aliquots equivalent to 250–2500  $\mu$ g/mL NIF were prepared from its stock standard solution (5 mg/mL) and transferred into a series of 10 mL amber glass volumetric flasks equivalent to 25–250  $\mu$ g/mL for each. Impurities from their working standard solutions (0.05 mg/mL) were prepared and transferred into four separate series of 10 mL amber glass volumetric flasks and diluted to volume with methanol. An accurate 40  $\mu$ L was applied in triplicate to NIF plates to obtain NIF in concentrations in the range of 10–100  $\mu$ g/band and 0.1–1  $\mu$ g/band of each impurity and applied in triplicate on the TLC aluminum plates (20  $\times$  20 cm), which had been prewashed with methanol and preactivated at 100°C for 15 min. Samples were applied as bands using the CAMAG TLC autosampler. The band length was 4 mm, and the bands were applied 15 mm from the bottom edge of the plate. Ascending development was performed in a chromatographic tank previously saturated for 0.5 h with an ethyl acetate–acetone–methanol–ammonia solution (85 + 25 + 5 + 0.5, v/v/v/v). The migration distance was 80 mm from the lower edge, and the developed plates were air-dried. NIF and Imp-A, B, C, and D bands were scanned at 230 nm, and the calibration curves were constructed by plotting the mean integrated peak area versus the corresponding concentrations from which the regression equations were computed.

(2) *HPLC method.*—Accurately measured aliquots equivalent to 10–100  $\mu$ g/mL NIF were separately transferred from NIF's stock standard solution (1000  $\mu$ g/mL) and aliquots equivalent to 0.01–0.15  $\mu$ g/mL each of Imp-A, B, C, and D were separately transferred from their respective second working standard solutions (1  $\mu$ g/mL) into five series of 10 mL amber glass volumetric flasks and diluted to volume with mobile phase. Triplicate 20  $\mu$ L injections were made for each concentration. Chromatographic separation was performed on the C8 column at 25°C, and the effluent was UV-scanned at 220 nm. A gradient solvent mixture was used in which solvent A consisted of a 0.1%

aqueous solution of sodium lauryl sulfate, whereas solvent B was acetonitrile. The optimized program is given in Table 1. The peak areas were recorded, and calibration curves relating the obtained integrated peak areas to corresponding concentrations were constructed.

(b) *Accuracy.*—The accuracy of the proposed methods was assessed by analyzing the samples with different concentrations of pure NIF and Imp-A, B, C, and D within their linearity ranges by the developed methods. The concentrations of NIF and Imp-A, B, C, and D were calculated from their corresponding regression equations, and the mean recoveries were calculated.

(c) *Precision.*—Repeatability was evaluated by assaying three concentrations of NIF (30, 50, and 80  $\mu$ g/band) for both the TLC and HPLC methods and Imp-A, B, C, and D (0.3, 0.5 and 0.7  $\mu$ g/band) for the TLC and (0.02, 0.04 and 0.06  $\mu$ g/mL) for the HPLC method three times intraday. Intermediate precision was evaluated by assaying the three chosen concentrations of NIF and Imp-A, B, C, and D in triplicate on 3 successive days using the procedure stated in the *Linearity and range* section. The mean recoveries and SD values were calculated.

(d) *Specificity.*—The specificity of the two chromatographic methods was ascertained by application of the developed method to the laboratory-prepared mixtures containing different ratios of NIF and Imp-A, B, C, and D following the procedure stated in the *Linearity and range* section for each method. Also, specificity was confirmed by calculating system suitability testing parameters, such as capacity factor, resolution, and selectivity factor for the separated peaks.

(e) *Sensitivity.*—Sensitivity of the method was established with respect to LOD and LOQ for the NIF impurities. The LOD and LOQ were established by the slope method using the lower part of the calibration curves and the slope of the regression equations as mentioned below:

$$\text{LOQ} = 3.3 \times \frac{\text{SD of the response}}{\text{Slope of the calibration curve}}$$

**Table 1.** Gradient elution program of the HPLC method

AU15

Time, min	Mobile phase		Flow rate, mL/min
	A, % <sup>a</sup>	B, % <sup>b</sup>	
0–4	60	40	1.5
4–6	60→40	40→60	1.5→2
6–10	40	60	2
10–11	40→60	60→40	2→1.5
11–13	60	40	1.5

<sup>a</sup> A = 0.1% aqueous solution of sodium lauryl sulfate.

<sup>b</sup> B = Acetonitrile.

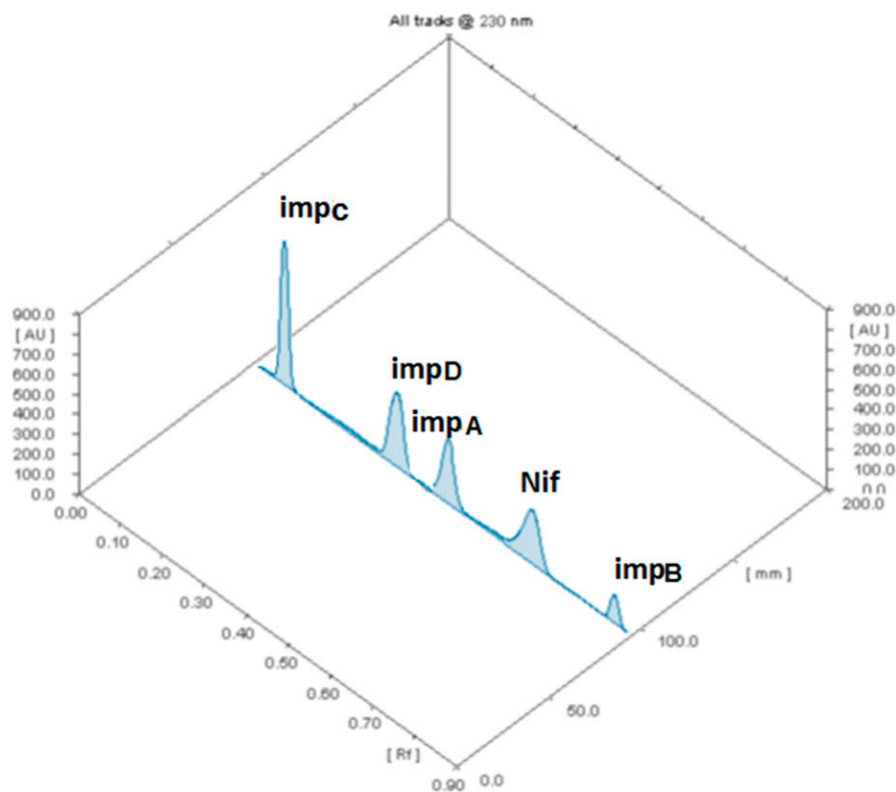


Figure 4. TLC chromatogram of a mixture of NIF and its impurities using ethyl acetate–acetone–methanol–ammonia (85 + 25 + 5 + 0.5, v/v/v/v) as the mobile phase.

$$LOQ = 10 \times \frac{SD \text{ of the response}}{\text{Slope of the calibration curve}}$$

(f) *Robustness.*—Robustness is the capacity of a method to remain unchanged with small changes in method parameters, e.g., changes in ethyl acetate ( $\pm 0.1\%$ ) and ammonia solution ( $\pm 0.01\%$ ) percentages and saturation time ( $\pm 5$  min) for the TLC method and changes in acetonitrile ( $\pm 1\%$ ) and mobile phase flow rate ( $\pm 0.1$  mL/min). The effect of these changes on  $R_f$  and  $R_f$  values were recorded and expressed as RSD.

(g) *System suitability testing parameters.*—Parameters such as resolution ( $R_s$ ), peak asymmetry, and selectivity factors ( $\alpha$ ) were calculated to test the overall system performance.

(h) *Application to the pharmaceutical formulation.*—The content of 20 Nifunal capsules were emptied and mixed well. An amount of the emptied capsules equivalent to 100 mg NIF was accurately weighed and transferred into a 100 mL amber

glass volumetric flask, 75 mL absolute ethanol added, and the prepared solution ultrasonicated for about 30 min. The solution was cooled well, diluted to volume with absolute ethanol to get 1000  $\mu\text{g/mL}$  stock solution, and then filtered. For the TLC method, a volume equivalent to 40  $\mu\text{g}/\text{band}$  was transferred from the sample solution (1000  $\mu\text{g/mL}$ ) and applied six times to the TLC plate. For the HPLC method, a concentration equivalent to 40  $\mu\text{g/mL}$  was prepared and 20  $\mu\text{L}$  injections made six times. Instructions stated in the *Linearity and range* section for each method were followed, and the concentrations of NIF were calculated from the corresponding regression equations.

## Results and Discussion

Impurity can arise during the manufacturing process and storage of drug substances. The criteria for acceptance of

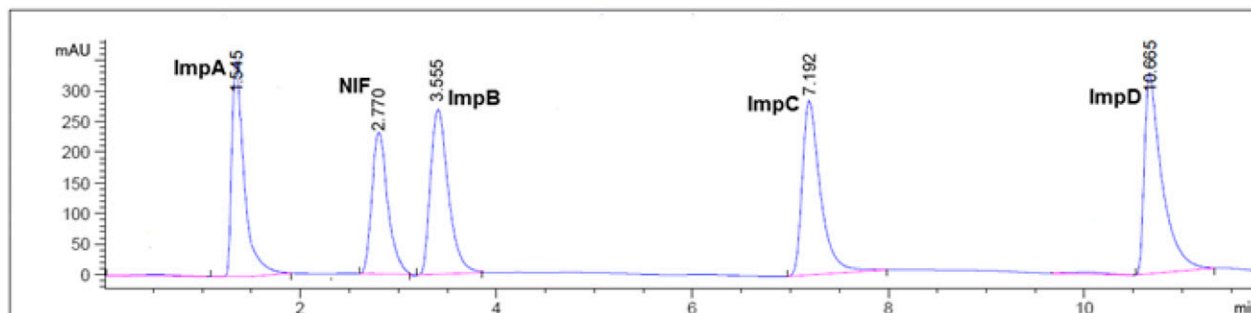


Figure 5. HPLC chromatogram of a mixture of NIF and its impurities using a 0.1% aqueous solution of sodium lauryl sulfate–acetonitrile as the mobile phase in a gradient program.



genotoxic and biohazardous impurities up to certain limits are based on pharmaceutical and clinical studies or known safety data (35). The use of chromatography to determine drug-related impurities has become established within industrial pharmaceutical analysis laboratories.

As per BP (1) and toxicological study (35) specifications, each of the NIF impurities must not exceed 0.15% of the NIF sample. Hence, in this work, we developed sensitive and robust TLC-densitometric and RP-HPLC methods to detect and quantify NIF and its genotoxic synthesized impurities.

### Method Developments and Optimization

#### Preparation of NIF impurities and structural elucidation.—

All the target compounds were prepared according to the synthetic pathways outlined in schemes I and II. The synthesis of the new hydrazone derivative (Imp-A<sub>2</sub>) was synthesized by refluxing Imp-B<sub>2</sub> and hydrazine hydrate in ethanol according to a previously reported method (36), with good yield. Moreover, it took a long time to prepare the 1,1-diacetates preparation from aldehydes and acetic anhydride, which depended on using Lewis acids or protonics as catalysts. Several catalysts have been reported for this reaction, e.g., sulfuric or phosphoric acids (37, 38), FeCl<sub>3</sub> (39), and PCl<sub>3</sub> (40), among others. From that, point Imp-C<sub>2</sub> was obtained through the reaction of furfural with acetic anhydride in an acidic medium at a very cold temperature (scheme II). However symmetrical azines readily synthesize, starting with excess aldehyde or ketone (41, 42). On the other hand, novel and selective methods for the synthesis of azines have been reported (43). So, in this work, we used semicarbazide HCl in an acidic medium to forward the reaction from the general intermediate, Imp-C<sub>2</sub> to obtain the required compound, Imp-D<sub>2</sub> which was produced with good yield.

The separated solid was filtered, dried, and crystallized to give the pure compounds. Physical and spectral data are listed below.

**Imp-A<sub>2</sub> white solid.**—Yield 76%; mp: 67–69°C; IR (KBr) 3452 (OH), 3251 (NH<sub>2</sub>), 3200 (NH), 3043 (CH aromatic), 1680 (C=O) cm<sup>-1</sup>; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, δ): 9.21 (s, D<sub>2</sub>O exch, <sup>1</sup>H, NH), 8.13 (d, 2H, ArH), 7.11 (d, 2H, ArH), 6.12 (s, <sup>1</sup>H, OH), 4.33 (s, D<sub>2</sub>O exch, 2H, NH<sub>2</sub>). Electron ionization MS (EIMS; *m/z*) 152 (M<sup>+</sup> 1, 18.88%), 138 (M<sup>+</sup>, 100%). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>): δ 114.00, 124.17, 130.01, 166.91, 168.60. Anal. calc. for C<sub>7</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub>: C, 55.26; H, 5.30; N, 18.41. Found: C, 55.41; H, 5.79; N, 18.63.

**Imp-C<sub>2</sub> buff solid.**—Yield 80%; mp: 90–95°C; IR (KBr) 3023 (CH aromatic), 2924 (CH aliphatic) 1730 (C=O) cm<sup>-1</sup>; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, δ): 8.12 (s, <sup>1</sup>H, CH), 7.62 (d, <sup>1</sup>H, H-C<sub>4</sub> furan), 7.13 (d, <sup>1</sup>H, H-C<sub>3</sub> furan), 2, 85(s, 6H, CH<sub>3</sub>). EIMS (*m/z*) 243.2 (M<sup>+</sup>1, 17.88%), 141 (M<sup>+</sup>, 100%). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ 20.11, 72.02, 105.21, 112.03, 117.90, 156.09, 164.01. Anal. calc. for C<sub>9</sub>H<sub>9</sub>NO<sub>7</sub>: C, 44.45; H, 3.73; N, 5.76. Found: C, 44.67; H, 3.79; N, 5.96.

**Imp-D<sub>2</sub> brown solid.**—Yield 70%; mp 236–238°C; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, δ): 8.33 (s, <sup>1</sup>H, CH), 7.21 (d, <sup>1</sup>H, H-C<sub>4</sub> furan), 6.52 (d, <sup>1</sup>H, H-C<sub>3</sub> furan). EIMS (*m/z*) 278 (M<sup>+</sup>1, 20.058%), 141.03 (M<sup>+</sup>, 100%). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ 113.01, 117.03, 153.21, 156.11, 165.43. Anal. calc. for C<sub>10</sub>H<sub>6</sub>N<sub>4</sub>O<sub>6</sub>: C, 43.18; H, 2.17; N, 20.14. Found: C, 42.91; H, 2.69; N, 20.31.

**TLC-densitometric method.**—The developed TLC-densitometric method depends on the difference in R<sub>f</sub> values of Imp-C<sub>2</sub> (R<sub>f</sub> = 0.08), Imp-D<sub>2</sub> (R<sub>f</sub> = 0.34), Imp-A<sub>2</sub> (R<sub>f</sub> = 0.46), NIF (R<sub>f</sub> = 0.67), and Imp-B<sub>2</sub> (R<sub>f</sub> = 0.86; Figure 4).

Table 2. Regression and analytical parameters of the proposed methods for the determination of NIF and its four impurities

Parameters	TLC				HPLC				
	NIF	Imp-A	Imp-B	Imp-D	NIF	Imp-A	Imp-B	Imp-C	Imp-D
Range	10–100 µg/band		0.10–1.00 µg/band		10.00–100.00 µg/mL		0.01–0.15 µg/mL		
Slope ± SE	107.24 ± 0.26	56804.00 ± 161.76	45949.00 ± 122.37	20092.00 ± 66.14	452.99 ± 1.17	2975.70 ± 7.76	1577.50 ± 4.37	584.41 ± 1.48	2292.80 ± 7.14
Intercept ± SE	1048.90 ± 16.24	11442.00 ± 99.34	822.62 ± 75.15	485.12 ± 43.04	-820.98 ± 75.77	15.08 ± 0.62	7.34 ± 0.37	11.06 ± 0.11	9.95 ± 0.63
Correlation coefficient	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
Accuracy, mean %	100.50	100.20	98.97	99.95	100.15	99.50	100.35	100.60	99.75
Precision, SD									
Repeatability	0.235	0.163	0.258	0.162	0.623	0.277	0.575	0.246	0.198
Intermediate precision	0.484	0.452	0.598	0.369	0.784	0.465	0.741	0.514	0.457
LOD <sup>a</sup>	— <sup>b</sup>	0.0280	0.0270	0.0240	—	0.0032	0.0029	0.0028	0.0031
LOQ <sup>c</sup>	—	0.0840	0.0800	0.0730	—	0.0096	0.0087	0.0085	0.0093

<sup>a</sup> LOD = (SD of the response/slope) × 3.3.

<sup>b</sup> — = XXX.

<sup>c</sup> LOQ = (SD of the response/slope) × 10.

<sup>d</sup> Represented by µg/band for TLC and µg/mL for HPLC.

**Table 3. Results of analysis of NIF in Nifunal capsules and results of the statistical analysis compared with the reported HPLC method**

Items	NIF		
	TLC method	HPLC method	Reported method <sup>a</sup>
Nifunal capsules, Batch No. 731302: found ± SD, % <sup>b</sup>	99.80 ± 0.771	100.12 ± 0.622	100.38 ± 0.372
N	5	5	5
Standard addition <sup>c</sup>	100.73 ± 0.824	100.13 ± 0.499	
Student's <i>t</i> -test (2.365) <sup>d</sup>	0.659	0.0423	— <sup>e</sup>
<i>F</i> -value (6.388) <sup>d</sup>	4.296	2.796	—

<sup>a</sup> The reported method (12) for the determination of NIF on a Zorbax SB-C18 column (4.6 × 250 mm, 5 μm) with gradient elution of the mobile phase composed of 0.05 M phosphoric acid–methanol.

<sup>b</sup> Average of five determinations.

<sup>c</sup> Average of three determinations.

<sup>d</sup> The values in the parentheses correspond to the theoretical value at the df; *P* = 0.05.

<sup>e</sup> — = XXX.

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Factors affecting the method performance were tested to obtain maximum chromatographic separation, such as the used developing system, scanning wavelength, and band and slit dimensions.

The striking structural similarity between the five compounds (making nearly no difference between their properties and their separation) became the big challenge to be achieved. In this aspect, our target was to adjust the polarity and the medium pH to the optimum point to provide the satisfactory result.

In the first trials, chloroform–methanol was tested, but NIF and Imp-A<sub>1</sub> and -B<sub>1</sub> eluted near the front line. Polarity was then decreased by using chloroform–acetone, but with the same results. An ethyl acetate–acetone mixture was then tested, which resulted in Imp-C<sub>1</sub> remaining on the base line and a tailed peak for Imp-D<sub>1</sub>. Depending on the results of the previous trials, we concluded that methanol was important for elution of Imp-C<sub>2</sub> whereas an ethyl acetate–acetone mixture was suitable for NIF and Imp-A<sub>2</sub> and -B<sub>2</sub>. Hence, a mixture of ethyl

acetate–acetone–methanol was tested, in which the five studied components were separated, but with tailed peaks. Changing the developing system pH was then tried by using either acetic acid or an ammonia solution, in which adding the ammonia solution to the developing system was essential for good resolution and symmetric peaks. Finally, satisfactory separation of the five components was observed upon using ethyl acetate–acetone–methanol–ammonia (85 + 25 + 5 + 0.5, v/v/v/v).

Also different scanning wavelengths (207, 230, 240, 254, and 360 nm) were tested, but the best sensitivity was obtained with UV-scanning at 230 nm.

The band dimensions and slit dimensions of the scanning light-beam were also tested and optimized, in which the optimum was 4 mm for band dimension, 8.9 mm for interspace between bands, and 3 × 0.45 mm for the slit dimensions.

*RP-HPLC method.*—Firstly, isocratic elution with different mobile phases was tested, such as water–methanol, water–methanol (pH 2.5–4) with either phosphoric or formic

**Table 4. Determination of NIF and its impurities in laboratory-prepared mixtures by the proposed methods**

NIF–A–B–C–D mixture	NIF found, % <sup>a</sup>	Imp-A found, % <sup>a</sup>	Imp-B found, % <sup>a</sup>	Imp-C found, % <sup>a</sup>	Imp-D found, % <sup>a</sup>
TLC					
100 + 0.5 + 0.5 + 0.5 + 0.5	99.88	99.40	100.20	99.00	99.60
100 + 0.2 + 0.2 + 0.2 + 0.2	99.25	100.50	99.50	99.25	100.30
60 + 0.6 + 0.6 + 0.6 + 0.6	100.03	100.17	98.33	100.17	100.33
20 + 1 + 1 + 1 + 1	102.50	102.00	100.10	98.00	101.00
50 + 0.4 + 0.4 + 0.4 + 0.4	99.96	100.13	99.50	100.75	100.25
Mean ± SD	100.32 ± 1.255	100.44 ± 0.961	99.53 ± 0.743	99.43 ± 1.066	100.30 ± 0.496
HPLC					
100 + 0.05 + 0.05 + 0.05 + 0.05	98.88	99.60	98.20	99.40	100.20
100 + 0.02 + 0.02 + 0.02 + 0.02	99.55	101.50	99.20	99.50	100.00
60 + 0.06 + 0.06 + 0.06 + 0.06	98.75	100.17	97.50	99.00	100.67
20 + 0.1 + 0.1 + 0.1 + 0.1	98.45	101.00	100.70	97.00	100.00
50 + 0.04 + 0.04 + 0.04 + 0.04	99.76	99.00	100.00	98.25	101.25
Mean ± SD	99.92 ± 0.942	100.25 ± 1.015	99.08 ± 1.299	98.63 ± 1.035	100.42 ± 0.536

<sup>a</sup> Average of three determinations.

<sup>b</sup> RSD change in response.

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**Table 5. Experimental results of robustness for the determination of NIF and Imp-A, -B, -C, and -D by the proposed TLC-densitometric and RP-HPLC methods**

Parameters	RSD <sup>a</sup>				
	NIF	Imp-A	Imp-B	Imp-C	Imp-D
TLC-densitometric method					
Ethyl acetate ±0.1, %	0.4525	0.3910	0.4452	0.5684	0.9262
Saturation time ±5, min	0.2561	0.3581	0.346	0.4550	0.2151
Ammonia ±0.01, mL	0.0458	0.1042	0.0853	0.1332	0.0723
RP-HPLC method					
Acetonitrile ±1, %	0.4581	0.5255	0.3021	0.7410	0.625
Flow rate ±0.1, mL/min	0.9250	1.209	0.8952	0.7582	1.108

<sup>a</sup> Change in response.

acid, water–acetonitrile, and water–acetonitrile (pH 2.5–4) with either phosphoric or formic acid. In all the tested systems, bad resolution among the studied components was observed. An aqueous solution of 0.1% sodium lauryl sulfate–acetonitrile was then tried in which the components were well separated, but after a very long time (>20 min). In a trial to decrease the analysis time, the percent acetonitrile was increased, but, unfortunately, NIF and Imp-A<sub>2</sub> eluted together. The last trial was to test using a gradient elution instead of an isocratic one.

Gradient elution was investigated to improve the resolution of the five cited components and decrease the time of analysis. For optimization of the elution program, several trials were carried out. Details of the used program are given in Table 1.

To effect of pH on separation was checked by changing mobile phase pH values (2.5–7). It was found that the separation efficiency was not affected by the change in mobile phase pH. The mobile phase was delivered at different rates (1, 1.5, and 2 mL/min), the optimum mobile phase flow rate was 1.5 mL/min for the first 4 min, increased to 2 mL/min until the end of the separation process, and then readjusted again to reach 1.5 mL/min.

Several wavelengths were tested (220, 245, 254, and 280 nm); the most suitable wavelength for detection was 220 nm, at which high sensitivity of the impurities with minimum detector noise was obtained (Figure 5).

**Table 6. System suitability testing parameters of the developed TLC-densitometric method**

Parameters	Imp-C	Imp-D	Imp-A	NIF	Imp-B
Symmetry factor	1.07	1.12	1.00	0.96	1.09
Resolution, R <sub>s</sub>	5.35	2.05	2.68		3.14
Capacity factor, k'	0.92	0.66	0.54	0.33	0.12
Selectivity, α	4.40	1.636	1.41		1.29

Upon using the optimum chromatographic conditions, satisfactory separation of the five proposed components was obtained and Imp-A<sub>2</sub>, NIF, and Imp-B, C and D were eluted at 1.545, 2.77, 3.555, 7.192, and 10.665 min, respectively, as shown in Figure 5.

*Method validation.*—Validation of the proposed method was performed according to ICH guidelines (22).

The linearity of the proposed methods was evaluated, and linearity was evident in the range of 10–100 µg/band for NIF and 0.1–10 µg/band for each impurity (Imp-A, B, C, and D) in the TLC densitometric method and in the range of 10–100 µg/mL for NIF and 0.01–0.15 µg/mL for each Imp-A, B, C, and D in the RP-HPLC method. Regression and analytical parameters are shown in Table 2.

Good percentage recoveries were obtained when testing method accuracy, and the results are given in Table 2. Accuracy was further assessed by applying the standard addition technique on Nifunal capsules for which good results were obtained, revealing the good accuracy of the proposed methods and proving that excipients did not interfere (Table 3). The proposed methods provided acceptable intra- and interday variation, indicating their acceptable precision, and that they are suitable for the QC of the suggested components. Good SD values were obtained (Table 2). Specificity of the proposed methods was evident from the TLC and HPLC chromatograms in Figures 4 and 5, respectively. Also, specificity of the methods was proven from the good recovery percentages obtained when they were applied for the determination of NIF and Imp-A, B, C, and D laboratory-prepared mixtures (Table 4). Moreover, the good results obtained when these methods were applied for analysis of Nifunal capsules (Table 3) confirmed that there was no interference from excipients. Low values of LOD and LOQ, as shown in

**Table 7. System suitability testing parameters of the HPLC method**

Parameters	Obtained value				
	Imp-A	NIF	Imp-B	Imp-C	Imp-D
Resolution, R <sub>s</sub>	3.50		1.96		8.68
Selectivity, α	2.96		1.38		1.54
Tailing factor, T	1.67			2.28	1.68
Capacity factor, K'	1.21	1.14	1.32	1.5	14.24
Column efficiency, n	424.40	2.96	4.07	9.27	
HETP <sup>a</sup>	0.059	1364.10	1263.80	5172.5	11374.10
		0.018	0.019	0.005	0.002

<sup>b</sup> HETP = Height equivalent to the theoretical plate (cm/plate).

Table 2, proved the high sensitivity of the developed methods, and that the developed methods met BP specifications (1) for Imp-A, B, C, and D detection limits.

The methods were found to be robust, and deliberate small changes in the studied factors did not lead to significant changes in  $R_f$  or  $R_f$  values or the area or symmetry of the peaks (Table 5). When system suitability testing parameters were evaluated, acceptable values were obtained (Tables 6 and 7).

Finally, when the statistical comparison of the results obtained by the proposed methods and the reported method (12) were carried out, the values of the calculated  $t$ - and  $F$ -values were found to be less than the tabulated ones, which revealed that there was no significant difference with respect to both accuracy and precision between the two proposed methods and the reported one.

**Conclusions**

In this work, NIF and Imp-A, C, and D were successfully synthesized with good yield and simple preparation methods.

The suggested TLC-densitometric method provides good resolution between the studied components within a short analysis time and was less money consuming. The RP-HPLC method is more sensitive, robust, accurate, and specific, but needs higher-cost instruments and chemicals. Moreover, the suggested methods have advantages of being more selective than the reported one (12). The developed methods were successfully applied for the quantification of NIF and its four toxic impurities, so they can be considered the first developed stability-indicating methods for the determination of NIF and its reported related impurities, which is very important for the safety assessment of NIF preparations.

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