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# A SENSITIVE ANALYTICAL (RP-HPLC-PDA, UV/VIS) METHOD FOR THE DETERMINATION OF NEWLY SYNTHESIZED N-ISONICOTINOYL-N´-(3-FLUOROBENZAL)HYDRAZONE (SH2) IN AQUEOUS PHASE

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**ABSTRACT.** The purpose of the present study was to develop a simple, rapid and reproducible analytical method for the determination of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone (SH2) - a synthesized by us halogenated isoniazid derivative with high tuberculostatic activity, in aqueous phase, on the basis of RP-HPLC-PDA and UV/VIS spectrophotometric investigations. Despite of the high linearity ($R^2$ 0.9984) of the UV/VIS spectrophotometric method applied, the significantly higher LOQ and LOD values indicated its unsuitability for detection and quantification of low N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone concentrations (< 10 µg/mL). The proposed RP-HPLC-PDA method with mobile phase ACN/phosphate buffer (60:40, v/v) offered short retention time (3.1 min), high precision (RSD 3.50 %) and linearity ($R^2$ 0.9898). It characterized with satisfactory LOD (0.346 µg/mL) and LOQ (1.05 µg/mL) values and allowed the qualitative detection of SH2 E/Z-isomer.

***Keywords:*** *N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone, RP-HPLC-PDA, UV/VIS, aqueous phase*

## INTRODUCTION

The rise in drug-resistant strains of *Mycobacterium tuberculosis* is a major threat to human health and highlights the need for new therapeutic strategies [1].

Despite its strong antibacterial effect, sometimes isoniazid (rimifon, INH), a widely used medication (xenobiotic) with a confirmed tuberculostatic activity, is proved to be ineffective due to the rapid appearance of drug resistance of

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tuberculosis bacteria [2]. These two problems – the toxicity and the resistance, motivate the necessity of synthesis of new tuberculostatics: more active, overcoming the resistance and with a lower toxicity. Particularly good results in the study of antituberculosis activity and toxicity are obtained for the family of isonicotinoyl hydrazones – structural analogues of isoniazid [1,3,4]. The blockade of the active hydrazide group (–$HNNH_2$) results in a significant decrease in the toxicity of isonicotinoyl hydrazones *vs* that of INH [5-7].

In experiments of ours, we synthesized and evaluated the tuberculostatic activity of new xenobiotics, not described in the literature – structural analogues of INH [8] against a standard *Mycobacterium tuberculosis* H37Rv-London strain, that is highly virulent, causes a generalized tuberculosis and is used in international studies. The results of the screening showed that all newly synthesized xenobiotics had a highly inhibiting activity against tuberculosis bacteria [9].

The study of Potuckova et al. (2014) highlighted important structure-activity relationships and provides insights into the further development of arylhydrazone iron chelators with more potent and selective anti-neoplastic effects [10]. Analysis of new chelators were performed using an Ascentis C18 chromatographic column (1063 mm, 3 mm) protected with a guard column with the same sorbent (Sigma-Aldrich). The mobile phase was composed of 1 mM EDTA in 5 mM phosphate buffer and methanol in different ratios. The column oven was set at 25$^{o}$C and the autosampler at 5$^{o}$C. A flow rate of 0.3 mL/min and injection volume of 20 mL were used. The linearity, precision and accuracy of the methods were examined by the analysis of plasma samples spiked with different amounts of the chelators. Selectivity was confirmed by an analysis of blank plasma samples. All evaluated parameters reached acceptable values [10].

It is accepted by some scientists that UV/VIS spectrophotometric methods are less time-consuming in terms of preparation and analysis of samples and are more cost effective than chromatographic methods. A new analytical method for the quantification of isonicotinoyl lactosyl hydrazone (INH-Lac) in oral solid dosage forms by UV-spectrophotometry was developed and validated by Cordoba-Diaz et al. (2009) [11]. The influence of several direct compression excipients on the specificity of the proposed analytical method were evaluated. The applied methodology showed a good repeatability as well as good accuracy. The results obtained from the assay of isoniazid tablets demonstrated that the proposed method constitutes a clear alternative to chromatographic methods and to the official titration method. It would be of interest for the routine quality control of oral dosage forms containing isoniazid and lactose and for stability studies [11]. In the study of Georgieva and Yaneva (2015) subjected to encapsulation of rimifon on natural and acid-modified zeolites, rimifon concentrations before and after encapsulation were determined by a UV/VIS spectrophotometric method. The standard curve was linear over the range of the tested concentrations [12]. A RP-HPLC-PDA analytical

methodology for the determination of the veterinary antibiotic tylosine in aqueous phase, which characterized with short analysis time, high precision, high linearity and satisfactory LOD and LOQ values, was developed [13].

Previous investigations of the scientific team proved the superoxide scavenging, radical scavenging and tuberculostatic activities of substituted isonicotynoylhydrazones [9,14]. However, no analytical methods for the determination of halogenated isonicotynoylhydrazones in aqueous medium have been reported so far, which provoked the present research.

The aim of this study was to develop a simple, rapid and reproducible analytical method for the determination of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone – a synthesized by the authors halogenated isoniazid derivative with high tuberculostatic activity, in aqueous phase, on the basis of RP-HPLC-PDA and UV/VIS spectrophotometric investigations.

## RESULTS AND DISCUSSION

### UV/VIS spectrophotometric analyses

The UV/VIS spectra of SH2 in acidic aqueous solutions (pH 2.4) (Fig. 1) displayed maximum absorbance peaks in the UV region at λ 250 nm for the entire concentration range 10 – 100 μg/mL. The pH of all solutions was adjusted to pH 2.4.

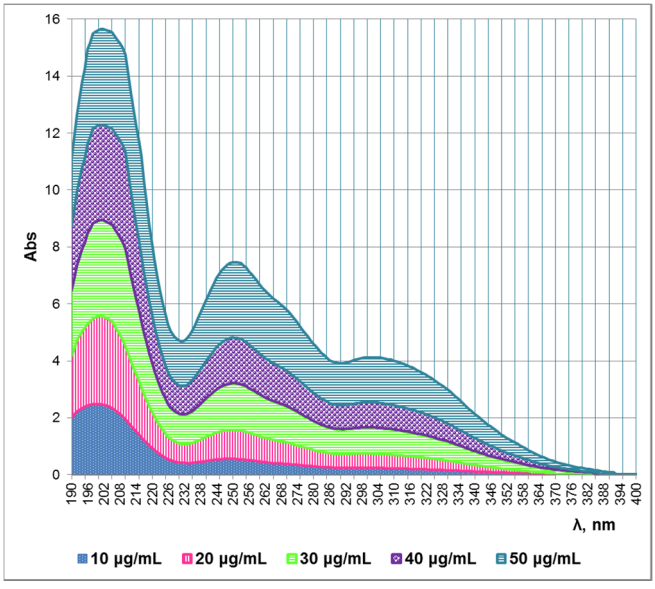



**Figure 1.** UV/VIS spectra of SH2.

The obtained standard curve for SH2 (Fig. 2) was linear over the tested range of initial concentrations $C_o$ 10 - 100 μg/mL.

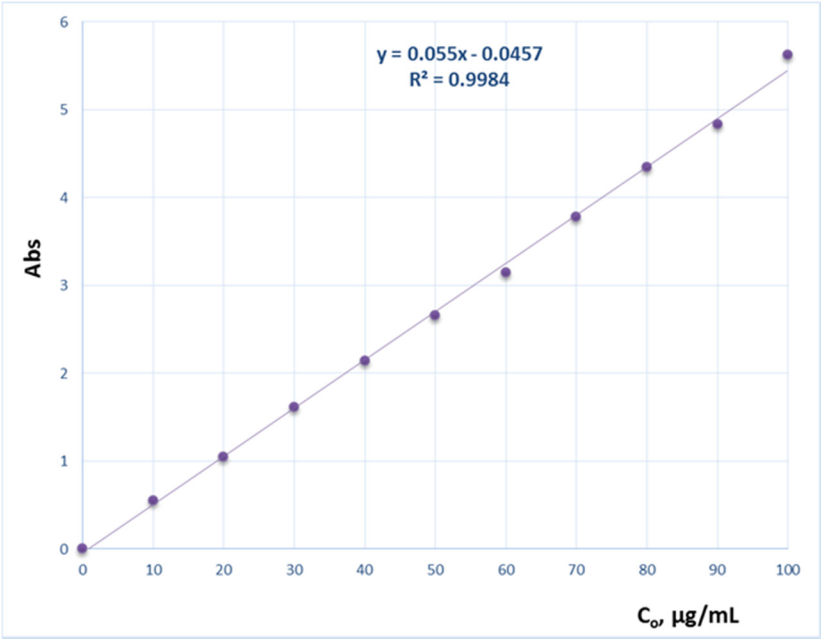



**Figure 2.** UV/VIS calibration curve of SH2 at λ 250 nm.

The obtained linear equation (Eq. 1) characterized with a slope a = 0.055 and an intercept b = -0.0457.

$$y = 0.055.x - 0.0457 \quad (1)$$

The high value of the calculated correlation coefficient - $R^2$ 0.9984, and the well resolved UV absorption peaks of the heterocyclic compound proved the applicability of this method for determination of SH2 concentrations in aqueous medium in the range $C_o$ 10 - 100 μg/mL.

**HPLC method development**

The development and optimization of a sensitive and rapid HPLC method for SH2 determination in aqueous phase included the performance of several preliminary experiments testing different HPLC conditions: mobile phases, temperatures and wavelengths.

### *HPLC Method I*

The first experimental series were conducted with ten SH2 standard solutions with concentrations 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL. The used mobile phase consisted of phosphate buffer (pH 5.0) and methanol (90:10, v/v). Optimal performance was obtained at a flowrate 0.8 mL/min. The effect of temperature on the separation process was studied in the range 18 - 30°C. Satisfactory peak resolution and optimum analyses time were established at 30°C, at wavelength λ 254 nm. SH2 was successfully detected within 3.35 min in the solution with $C_o$ 10 µg/mL (Fig. 3). However a mode of reduction of the detection time from 3.35 min to 3.1 min with an increase in the initial SH2 concentration within the range $C_o$ 10-100 µg/mL was observed (Fig. 3). The base lines of the chromatograms characterized with signal noise in the time range 1.9-2.5 min and unstable base line, especially in the lower concentration range. Besides, the characteristic peak for $C_o$ 100 µg/mL contained a curvature, which could affect the accuracy of the quantitative determination.

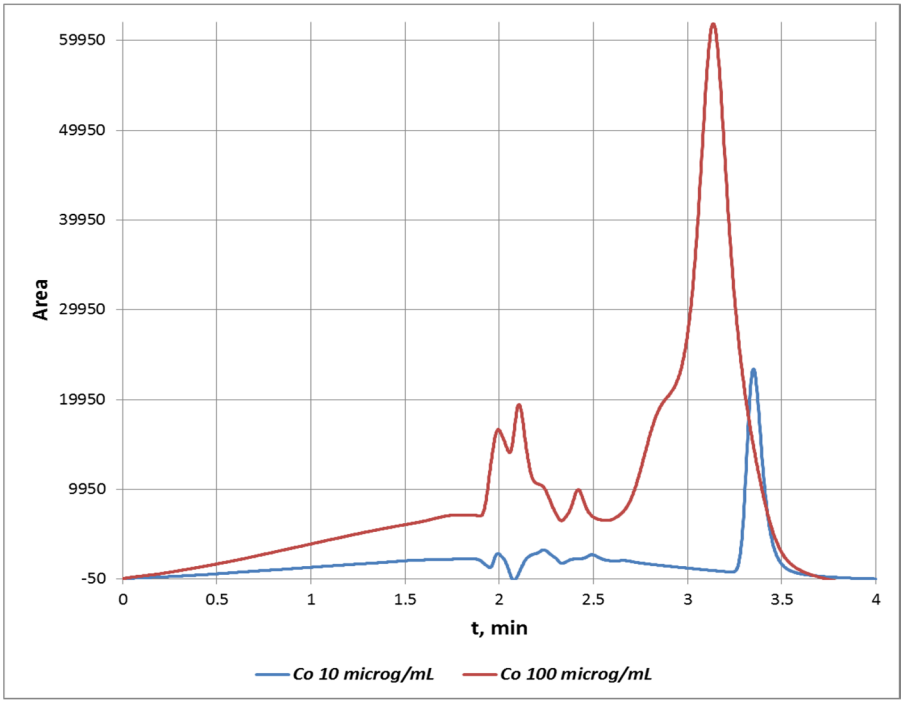



**Figure 3.** HPLC spectra of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone at λ 254 nm, mobile phase: phosphate buffer (pH 5.0)/methanol (90:10, v/v).

The standard calibration curve based on the spectral peak areas is presented in Fig. 4.

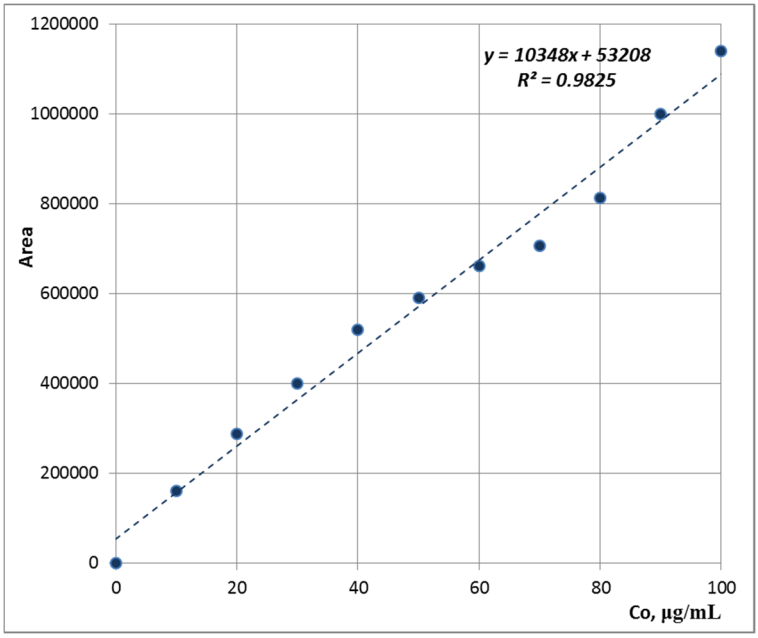



**Figure 4**. HPLC calibration curve of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone at λ 254 nm (mobile phase: phosphate buffer (pH 5.0)/methanol (90:10, v/v).

It characterized with a reasonable correlation coefficient $R^2$ 0.9825 and the following linear equation (2):

$$y = 10348.x + 53208 \quad (2)$$

### *HPLC Method II*

The second experimental series were conducted with the same ten SH2 standard solutions in the concentration range $C_o$ 10 – 100 µg/mL. The used mobile phase consisted of ACN and $H_3PO_4$ (0.1M) (60:40, v/v). Optimal performance was obtained at a flowrate 0.8 mL/min. The effect of temperature on the separation process was studied in the range 18 - 30°C. Satisfactory peak resolution and optimum analyses time were established at 30°C, at wavelength λ 300 nm. SH2 was successfully detected within 3.1 min (Fig. 5).

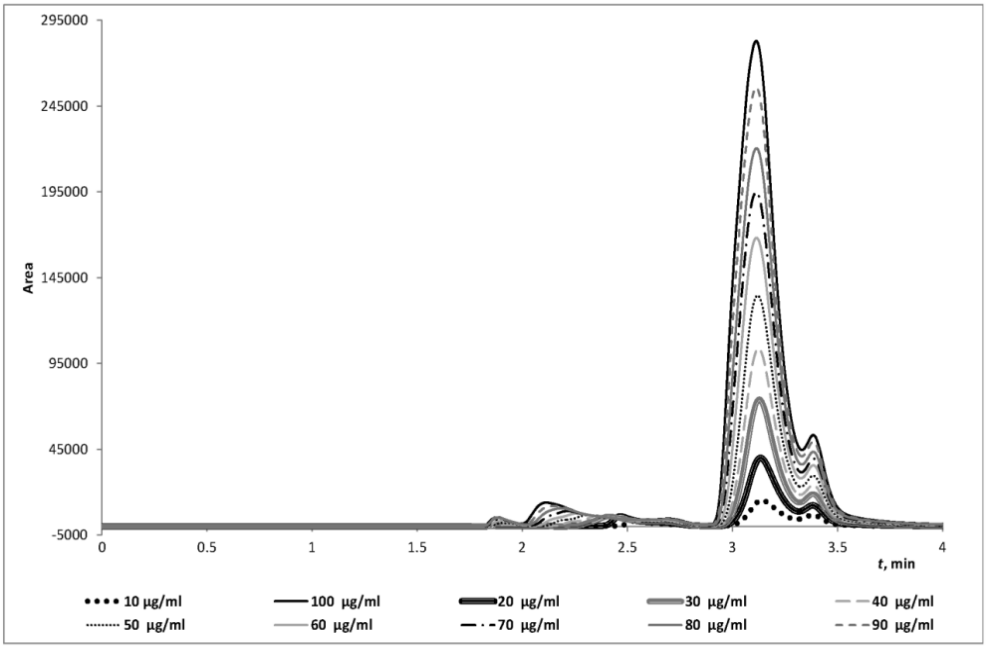
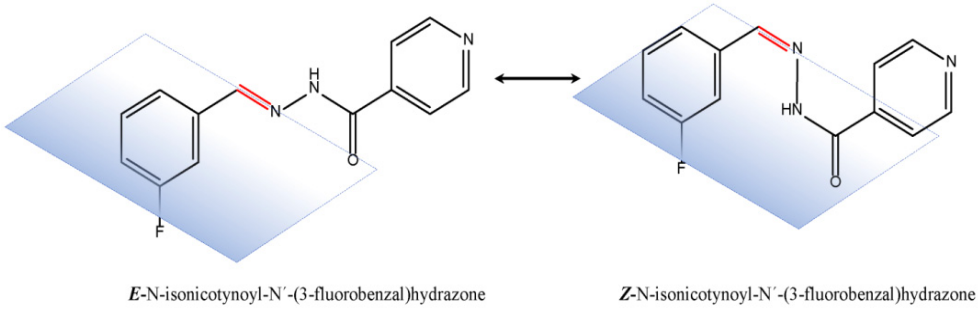



**Figure 5.** HPLC of *N*-isonicotynoyl-*N´*-(3-fluorobenzal)hydrazone detection at λ 300 nm, mobile phase: ACN/phosphate buffer (60:40, v/v), pH 2.4.

The obtained HPLC chromatograms did not contain any interference peaks, which could influence the quantitative results. However, the peaks obtained by this method were split, which could be due to the presence of E/Z-isomer of SH2 (Fig. 6), and consequently a sign for better separation of the main product. Besides, the base line is straight and stable with lower signal noise.




***E*-N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone** ***Z*-N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone**

**Figure 6.** Structural formulas of E/Z-isomers of SH2.

The obtained standard calibration curve at these HPLC conditions is presented in Fig. 7.

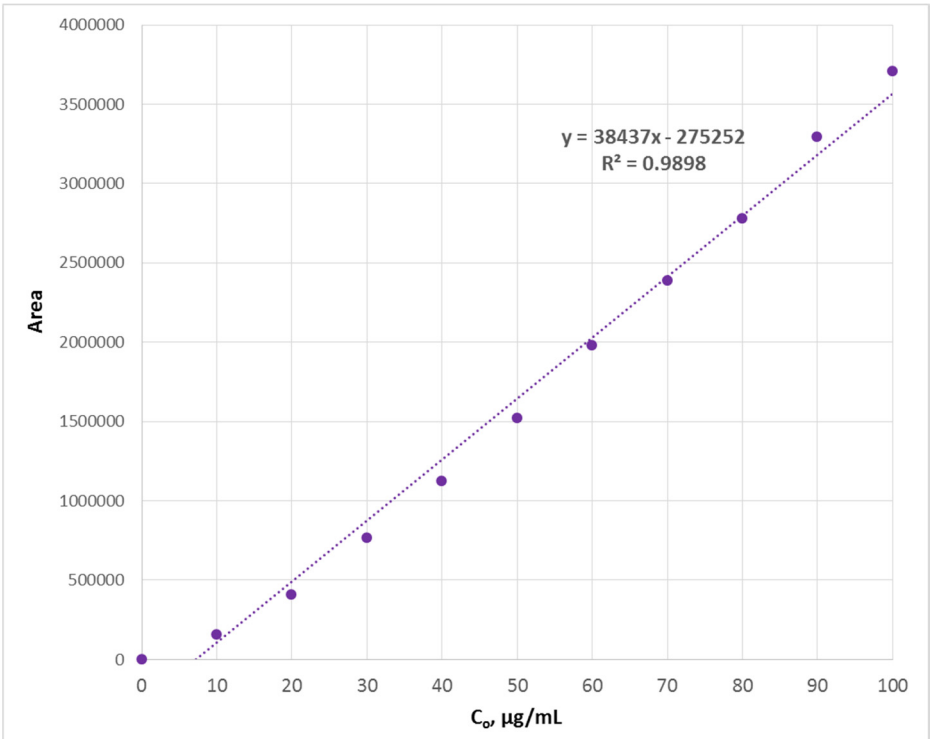



**Figure 7.** HPLC calibration curve of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone at λ 290 nm (mobile phase: ACN/$H_3PO_4$ (60:40, v/v).

The correlation regression coefficient in this case was higher ($R^2$ 0.9898) when compared to that obtained by HPLC Method I and lower than the value of the UV/VIS method. The linear equation (Eq. 3) characterized with a greater slope a = 38437 and with a negative intercept b = -275252.

$$y = 38437.x - 275252 \quad (3)$$

**Accuracy and effectiveness of the applied analytical methods**

To assess the applicability of the three methods investigated, the RSD, LOD and LOQ were determined based on the obtained in the recent study experimental data.

The values of LOD and LOQ were estimated according to the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) based on the standard deviation of the response and the slope of the calibration curve of the analyte [15]. The values of these parameters are presented **in** Table 1.

**Table 1.** Values of *RSD* %, *LOD* (μg/mL) and *LOQ* (μg/mL) for the UV/VIS and HPLC methods

| **Method** | ***RSD*, %** | ***LOD*, μg/mL** | ***LOQ*, μg/mL** |
|---|---|---|---|
| UV/VIS Method | **2.12** | 3.31 | 10.04 |
| HPLC Method I | 3.77 | 0.682 | 2.54 |
| HPLC Method II | 3.50 | **0.346** | **1.05** |

The comparative analyses of the obtained experimental results revealed that the developed and applied UV/VIS spectrophotometric method characterized with the highest correlation coefficient and lowest *RSD* value. However, the determined *LOD* and *LOQ* values were the highest. Thus, it could be suitable for analyses of aqueous samples with higher heterocyclic compound concentrations. According to the spectra presented in Fig. 1 solutions with SH2 concentrations lower than 10 μg/mL characterized with not well-resolved spectral peaks, i.e. the accuracy of the method below this limit would be unsatisfactory. However, the main advantage of this method is its cost-effectiveness and rapidity.

Regarding the developed HPLC methodologies, and based on the data from Table 1, it could be concluded that undoubtedly HPLC Method II displayed the highest accuracy and efficiency as the determined *RSD, LOD* and *LOQ* values were the lowest. The comparative estimation of the HPLC spectral data of SH2 obtained by both liquid-chromatographic methods (Fig. 2, 3) revealed that the spectral peaks of Method II characterized with approximately 3 times greater area. Besides, they were significantly more pronounced in the entire tested concentration range. The presence of a secondary small peak at the bottom of the main one could be attributed to the detection of an isomer molecule, which is a sign for better separation of the biologically active compound(s), as well as for the higher accuracy of the method. The proportional increase of the secondary peak area could be used for the quantitative analyses of the detected isomer by a proper modification of the current analytical method in future investigations of the present scientific team. The latter observations are supported by the significantly lower *LOD* and *LOQ* values. Another main advantage of Method II is the registered short retention time.

## CONCLUSIONS

A RP-HPLC-PDA analytical methodology appropriate for the quantitative determination of the novel drug candidate N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone in aqueous phase, and the qualitative analyses of its isomer was developed in the present study. Desirable chromatographic separation was achieved on a $C_{18}$ column employing a mixture of ACN and 0.1M $H_3PO_4$ (60:40; v/v) as the mobile phase. The obtained HPLC chromatograms were well pronounced and did not contain any interference peaks, which could influence the quantitative results. The applied method offered short analysis time (3.1 min), high precision (*RSD* 3.5%) and high linearity ($R^2$ 0.9898). It characterized with satisfactory *LOD* and *LOQ* values. The simple and rapid method developed enhances the capabilities for the accurate and selective determination of SH2 in concentrations even below 10 μg/mL in aqueous phase, as well as detection of its E/Z isomer.

## EXPERIMENTAL SECTION

### Chemicals

Isonicotynoylhydrazide (Bristol-Myers Squibb Co.), 3-fluorobenzaldehyde, acetonitrile (ACN, ≥99.8%), orthophosphoric acid ($H_3PO_4$ 85%), methanol (≥ 99.9%), ethanol (p.a. ≥ 99.8%), and $Na_2HPO_4.2H_2O$ (HPLC, ≥98.5%) HPLC grade, were obtained from Sigma-Aldrich.

### Synthesis of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone SH2

N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone was synthesized according to our previously reported procedure [8,9]. In brief: isonicotynoylhydrazide 2.74 g (0.02 mol) was mixed with 3-fluorobenzaldehyde in 50 mL absolute ethanol. The reaction mixture was heated to reflux for 4 hours. After cooling of the filtrate, white crystals crystallized, which after recrystallization from absolute ethanol had a melting temperature of 201.5-203°C. The yield was 86%.

Standard stock solutions (100 μg/mL) were prepared by dissolving the appropriate amount of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone in *Milli-Q* water acidified with $H_3PO_4$ at *pH* 2.4. Working standard solutions (10 – 100 μg/mL) were prepared daily.

### Physicochemical characteristics of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone

The software package CS Chem 3D ultra was used to calculate the basic molecular characteristic of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone. Elemental analysis and testing of the organic compound were conducted for identification and quantification of elements. The molecular formula of the compound is presented in Fig. 8.

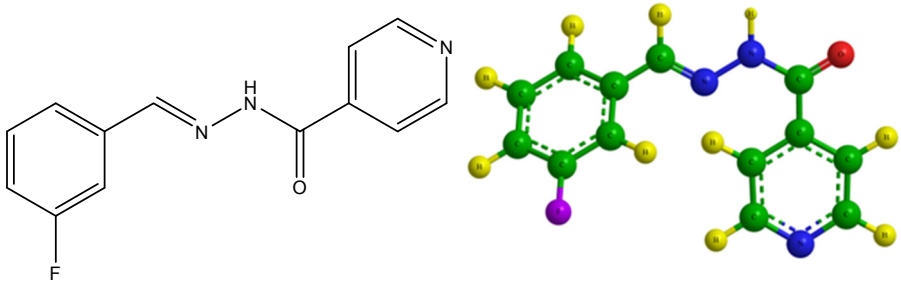



**Figure 8.** Molecular formula of SH2.

The physicochemical, molecular properties and elemental analyses data of SH2 are displayed in Table 2.

### UV/VIS spectrophotometric analyses

N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone concentrations were measured with UV-VIS spectrophotometer DR 5000 Hach Lange (Germany), supplied with 10 mm quartz cells. All spectra were recorded in the UV region at $\lambda$ 250 nm with 2 nm slit width, 900 nm $min^{-1}$ scan speed and very high smoothing.

**Table 2.** Physicochemical characteristics of N-isonicotynoyl-N´-(3-fluorobenzal)hydrazone

| | |
|---|---|
| Molecular formula | $C_{13}H_{10}FN_3O$ |
| UIPAC name | 3-fluorobenzaldehyde isonicotynoylhydrazone |
| Molecular mass, g/mol | 243.236 |
| Molecular ovality | 1.42697 |
| Connolly Accessible Area, $Å^2$ | 440.753 |

| | | | |
|---|---|---|---|
| Connolly Molecular Area, Å$^2$ | 219.941 | | |
| Connolly Solvent Excluded Volume, Å$^2$ | 179.932 | | |
| Mass-to-charge ratio, *m/z* | 243.08 (100.0%), 244.08 (15.2%), 245.09 (1.1%) | | |
| Elemental analyses: | | | |
| calculated (%): | C 64.19 | H 4.14 | N 17.28 |
| determined (%): | C 64.03 | H 4.01 | N 17.57 |

### HPLC system and conditions

RP-HPLC system comprising of a Hypersil BDS $C_{18}$ (5 μM, 4.6 x 150 mm) column, Surveyor LC Pump Plus, PDA detector, and Surveyor Autosampler Plus (Thermo Fisher Scientific) was used. The tested mobile phases consisted of a mixture of ACN/0.1M $H_3PO_4$ (60:40, v/v) and phosphate buffer (*pH* 5)/methanol (90:10, v/v). The samples were monitored at 290 nm and 254 nm, respectively. The buffer pH was adjusted to 2.4 with $H_3PO_4$. The volume injected into the HPLC column was 20 μl.

All UV/VIS spectrophotometric and HPLC analyses were made in triplicate.

The experimental data was analyzed by regression analyses and determination of the corresponding correlation coefficients ($R^2$) and relative standard deviation (*RSD*, %).

The efficiency and accuracy of the developed UV/VIS and HPLC methods was estimated based on the calculated limit of detection (*LOD*) and limit of quantification (*LOQ*).

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## REFERENCES


1. S. Ellis, D.S. Kalinowski, L. Leotta, M.L.H. Huang, P. Jelfs, V. Sintchenko, D.R. Richardson, J.A. Triccas, *Mol. Pharmacol*., **2014**, *85*, 269.
2. C.E. Cade, A.C. Dlouhy, K.F. Medzihradszky, S.P. Salas-Castillo, R.A. Ghiladi, *Protein Sci*., **2010**, *19* (3), 458.
3. D. Sriram, P. Yogeeswari, K. Madhu, *Bioorganic & Medicinal Chemistry Letters*, **2005**, *15*, 4502.
4. B. Moksharagni, K.D. Kumar, S. Chandrasekhar, K.H. Reddy, *Int. J. Pharm. Bio. Sci*., **2015**, *6* (3), 11.
5. S. Rollas, S.G. Küçükgüzel, *Molecules*, **2007**, *12*, 1910.
6. M. Hermes-Lima, M. S. Gonçalves, R. G. Andrade Jr., *Molec. Cell. Biochem*., **2001**, *228*, 73.
7. A.Q. Mauricioa, G.K.B. Lopes, C.S. Gomes, R.G. Oliveira, A. Alonso, M. Hermes-Lima, *Biochimica et Biophysica Acta*, **2003**, *1620*, 15.
8. S. Varbanova, N. Georgieva, *Veterinary Science*, **1993**, *27*, 81.
9. N. Georgieva, V. Gadjeva, *Biochemistry (Moscow)*, **2002**, *67*, 588.
10. E. Potuckova, K. Hruskova, J. Bures, P. Kovarıkova, I.A. Spirkova, *PLoS ONE*, **2014**, *9* (11), 112059.
11. A. Córdoba-Díaz, M. Córdoba-Díaz, M., B. Elorza, *Spectroscopy*, **2009**, *23*, 201.
12. N. Georgieva, Z. Yaneva, Cogent Chemistry (2015), 1, 1-16.
13. Z. Yaneva, N. Georgieva, V. Koinarski, D. Petrova, Trakia Journal of Sciences, 13, Suppl. 2, 309-314, 2015.
14. N. Georgieva, Z. Yaneva, G. Nikolova, S. Simova, *Advances in Bioscience and Biotechnology*, **2012**, 3(7A), 1.
15. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guideline, *Validation of Analytical Procedures: Text and methodology*, Q2 (R1), p. 1-13 (2005).