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# Application of a RP-HPLC method for determination of chemical and physiological stability of two newly synthesized methoxy-benzoylhydrazone derivatives

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*Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday*

The study investigates the stability of novel aroylhydrazones containing susceptible to hydrolysis hydrazone group at conditions close to physiological. Two methoxy-derived hydrazones (3-methoxysalicylaldehyde benzoylhydrazone – 3M and 4-methoxysalicylaldehyde benzoylhydrazone – 4M) were dissolved in different buffer solutions (pH 2.0, 7.4 and 9.0) at 37 °C and aliquot samples were drawn at definite time intervals. Stability of the compounds was determined using precise, selective and validated RP-HPLC method. No changes in the structures were detected at pH= 7.4 and pH = 9.0, whereas an appearance of new peaks corresponding to the retention time of the supposed hydrolysis products was observed at pH 2.0. The results revealed chemical stability of the tested compounds at neutral and low alkali pH under physiological temperature and hydrolytic sensibility at strong acidic medium.

**Key words**: benzoylhydrazones; chemical stability; physiological stability; RP-HPLC

## INTRODUCTION

Benzoylhydrazones are currently widely investigated from the viewpoint of their application as bioactive compounds. Many studies have discovered an extensive variety of biological activities such as anti-inflammatory, anti-malarial, analgesic, anti-oxidative, antimicrobial, and antiproliferative activity [1–10]. Hence, aroylhydrazones seem to be promising drug candidates with potential to be used in the treatment of some human diseases. Recently, the synthesis of some new methoxy-salicylaldehyde benzoylhydrazones and evaluation of their antiproliferative effect on a wide spectrum of human tumor cell lines was reported [9-10]. The investigations demonstrated that the presence of methoxy group in salicylaldehyde results in derivatives with high antiproliferative and antioxidant activity [4-6, 9-10].

The pharmaceutical stability of any promising drug candidate plays an important role in the process of the novel drug development. Many factors, such as air, heat, light, moisture as well as the inherent chemical susceptibility of a substance to hydrolysis affect the stability of compounds. The stability of a compound synthesized as a potential medicinal agent is related to the pharmacokinetic behavior in the body and to the conditions for the formulation, storage, occurrence of toxic effects associated with degradation products and so on. Most of the compounds are fairly stable in the neutral pH value found in the intestine but can be unstable at the pH value found in the stomach [11]. Some aroylhydrazones also have been reported to be sensitive to hydrolysis in both acid and alkaline medium [12-13]. Thus in the development of new drugs early information of stability becomes essential for subsequent processes of optimization and selection of leading active structures and can prevent unnecessary costs on developing products that subsequently prove to be unstable. The aim of this study is to establish and apply a reversed phase liquid chromatography (RP-HPLC) method for investigation of the stability of recently synthesized benzoylhydrazone derivatives under physiological pH and temperature and for identification of their hydrolytic degradation products. The structures of the hydrazones are shown on Fig. 1.

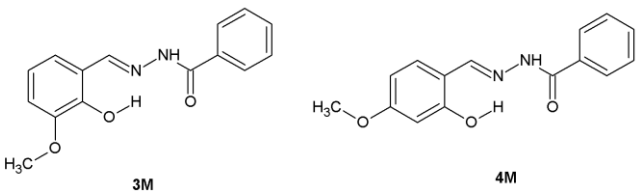


**Fig. 1.** Chemical structures of the investigated hydrazones **3M** (3-methoxysalicylaldehyde benzoylhydrazone) and **4M** (4-methoxysalicylaldehyde benzoylhydrazone).

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## EXPERIMENTAL

### *Chemicals and reagents*

3-methoxysalicylaldehyde, 4-methoxysalicyl-aldehyde and benzhydrazide used for the preparation of the 3-methoxysalicylaldehyde benzoylhydrazone (**3M**) and 4-methoxysalicylaldehyde benzoylhydrazone (**4M**) were purchased from Sigma-Aldrich and used without further purification. The investigated hydrazones were synthesized as already reported [9] by Schiff-base condensation in ethanol between 3-methoxysalicylaldehyde and benzhydrazide for **3M** and between 4-methoxysalicylaldehyde and benzhydrazide for **4M**. The structure and purity of the compounds **3M** and **4M** were confirmed by IR (Bruker Tensor 27 spectrophotometer), $^{1}H$ and $^{13}C$ NMR (Bruker Avance DRX 250 spectrophotometer) spectroscopy. The melting points were measured using a Buchi 535 apparatus. The necessary components used for the preparation of the mobile phase and the buffers were of analytical grade, whereas potassium dihydrogen phosphate dihydrate (Sigma-Aldrich,), orthophosphoric acid (Merck) and methanol (Sigma-Aldrich) were of gradient grade.

### *Preparation of the sample solutions*

Due to the poor solubility of the analyzed structures in water, methanol-buffer solutions were prepared at the necessary relevant ratio in order to obtain the desired pH values. A 10 mg sample of the model compounds was weighed and dissolved in the corresponding mixture of methanol and buffer with respective pH (2.0 or 7.4 or 9.0). The obtained solutions were thermostated and stirred in a micro reactor at 37 °C for a total time of 1440 min (24 hours). Aliquot samples of 20 μL of the analyzed solutions of **3M** and **4M** were taken at definite time intervals (15, 30, 60, 120, 240, 480 and 1440 min) and injected into the RP-HPLC system.

### *Instrumentation and chromatographic conditions*

Chromatographic separation was performed on a modular HPLC system LC-10A Shimadzu (Japan) which consisted of a LC-10A pump, solvent degasser DGU-3A, Rheodyne injector with 20 μL loop, column oven CTO-10A, SPD-M10A UV detector with fixed wavelength and communication bus module CBM-10A. The analysis was achieved with a Luna 5u CB 100A C18 (250 mm x 4.6 mm), 5 μm particle size column used as a stationary phase. The components were eluted isocratically with a mixture of methanol and phosphate buffer (0.5 M $KH_2PO_4$, pH 4.0 adjusted with orthophosphoric acid) 65:35 v/v as the mobile phase at flow rate of 1.0 mL/ min. The mobile phase was filtered through a 0.45 μm membrane filter and degassed. Detection was carried out by absorbance at 240 nm. The analysis was carried out at ambient column temperature and injection volume was 20 μL.

### *Preparation of the mobile phase buffer*

6.80 g of potassium dihydrogen phosphate dehydrate ($KH_2PO_4.2H_2O$) was dissolved in 1L of ultrapure water. An orthophosphoric acid solution (6 %) was used to adjust the pH to 4.0 (±0.05). The mobile phase buffer was filtered through a membrane filter (0.20 $\mu$m) using a Millipore glass filter holder. The mobile phase buffer was used immediately after preparation or stored in the refrigerator in closed borosilicate glass bottles for a maximum of 24 hours.

### *Preparation of the buffers 2.0, 7.4 and 9.0*

The buffers were prepared according to a procedure enlisted in European Pharmacopoeia 7.0 [14].

### *Validation procedure*

The developed RP-HPLC method for the analysis of aroylhydrazones was tested with respect to following validation parameters: precision, linearity, accuracy and selectivity.

*Precision*: Precision of the method was tested by performing six independent sample solutions from each of the evaluated hydrazones. Each sample was injected three times. The final results are reported as relative standard deviations (RSD %).

*Linearity*: Linearity was determined within the range of 25-200 μg/mL for both the hydrazones **3M** and **4M**. Calibration curves were created using 6 points covered 6 different concentrations of the hydrazones over the tested concentration range (25, 50, 75, 100, 150, 200). Linear regression was used to process the calibration data.

*Accuracy*: The solutions for injection were prepared using a placebo and stock solution of the tested structures. Six solutions were prepared from each of the two compounds. Each solution was injected onto the column three times. Accuracy is reported as a parameter recovery with relative standard deviations.

*Selectivity*: The selectivity was determined by comparing the chromatograms for the solutions of the tested **3M** and **4M** hydrazones with the solutions of the initial corresponding benzhydrazide and 3-methoxysalicylaldehyde (initial for **3M**) and 4-methoxysalicylaldehyde (initial for **4M**), alone and in mixture.

## RESULTS AND DISCUSSION

In an attempt to determine the chemical stability and the stability at close to physiological conditions of the synthesized benzoylhydrazone derivatives **3M** and **4M** wide range of pH was chosen. The analyzed compounds were investigated for hydrolytic decomposition under physiological temperature of 37 °C and at pH 2.0, 7.4 and 9.0, namely the physiological pH in the stomach, blood plasma and intestines, respectively. Based on the chemical structure of the evaluated compounds the most probable change is the cleavage of the characteristic hydrazone group -CH=N-. Thus as referent substances were chosen the corresponding initial benzhydrazide and 3-methoxy- or 4-methoxysalicylaldehyde. For identification of the possible degradation and formation of new products an RP-HPLC method was developed and validated.

### *Validation of the developed RP-HPLC analytical procedure*

The method was validated according to ICH Q2 (R1) guidelines [15]. The precision, linearity, accuracy and selectivity of the system were evaluated during the method validation. The obtained parameters for the two analyzed hydrazones are shown on Table 1.

*Precision:* The calculated RSD values for **3M** and **4M** for the assessment of the precision are 1-1.5 %, confirming that the method is precise.

**Table 1.** Validation parameters for compounds **3M** and **4M.**

| | 3M | 4M | Criterion |
|---|---|---|---|
| Repeatability $t_R$ (% RSD)* | 0.15 | 0.25 | $X < 1\%$ |
| Resolution* | 1.9 | 1.7 | $R_{ij} > 1.5$ |
| Precision (% RSD)# | 1.5 | 1.0 | $X < 5\%$ |
| Linearity (correlation coefficient)§ | 0.9992 | 0.9989 | R > 0.9990 |
| Accuracy (%)# | 100.2 | 99.98 | **X** = 100 ± 5% |
| Selectivity | No interference | No interference | No interference |

* Six injections.
\# Two samples, three injections of each solution
§ At 25, 50, 75, 100, 150 and 200 μg/mL concentration level.
% RSD: Relative standard deviation in %

*Linearity:* The correlation coefficients of linearity are 0.9992 for **3M** and 0.9989 for **4M**. The values indicate good correlation between the peak areas and the range of concentrations studied (Fig. 2).

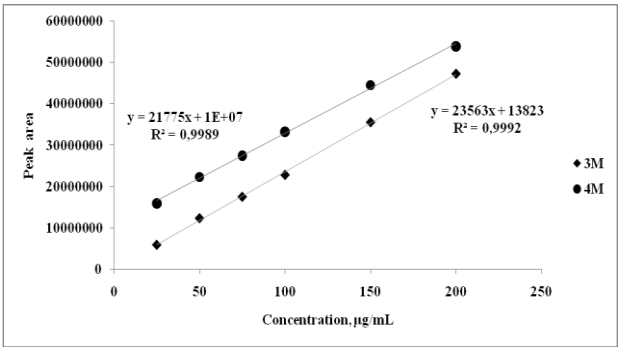



**Fig. 2.** Linearity of the developed RP-HPLC method.

*Accuracy:* The method was found to be accurate with recoveries of 99.98%–100.2%

*Selectivity:* The selectivity of the method is illustrated on the chromatograms on Fig. 3.

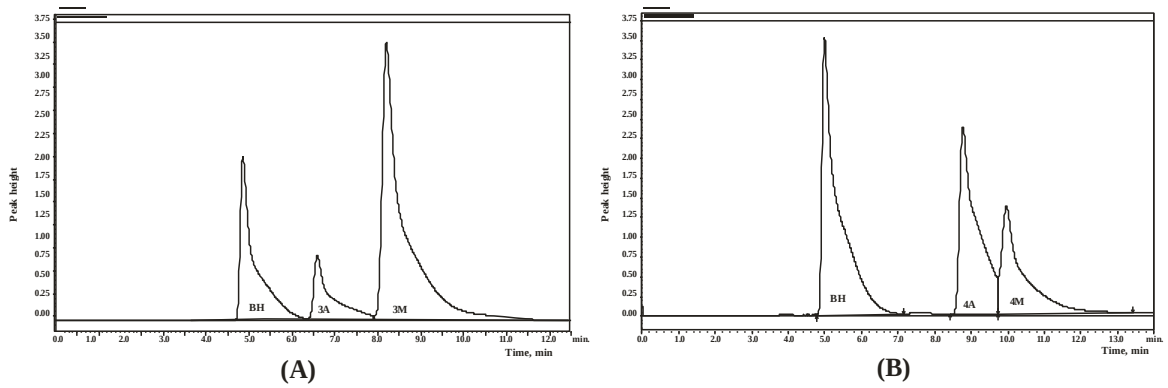



**Fig. 3.** Chromatogram of a model mixture of the tested **3M** and the corresponding benzhydrazide and 3-methoxysalicylaldehyde (A) and **4M** and the corresponding benzhydrazide and 4-methoxysalicylaldehyde (B).

It is evident that under the proposed chromatographic conditions each of the tested hydrazones (**3M** and **4M**) is completely separated from initial corresponding benzhydrazide (BH) and methoxy-salicylaldehyde (3A and 4A, respectively). No interferences were observed which indicates that the method is selective and could be used for their simultaneous identification.

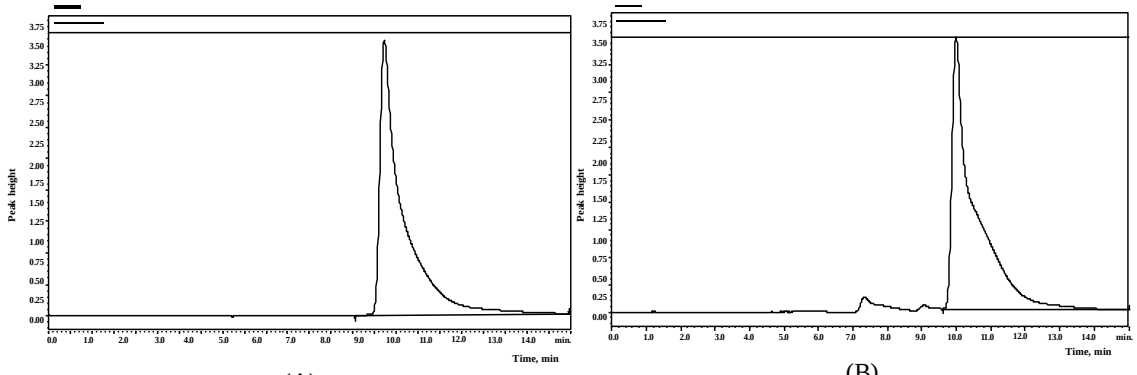
*Limit of quantitation and limit of detection:* The limit of quantitation (LOQ) and limit of detection (LOD) were calculated from the standard deviations and slopes of the responses using a signal-to-noise ratio. The LOQs for **3M** and **4M** were found to be 1.0 μg/mL and 0.8 μg/mL, while the LODs were 0.2 μg/mL and 0.1 μg/mL, respectively.

### *Chemical stability*

By definition, the chemical stability is the tendency of a substance to resist change or decomposition due to internal reaction, or due to the action of air, humidity, heat, light, pressure, etc. All compounds presented in this paper have been stored for 6 months at room temperature with access of air and light. It was determined that the compounds kept their physical and chemical properties unchanged under these conditions. Thus the tested compounds may be considered as chemically stable.

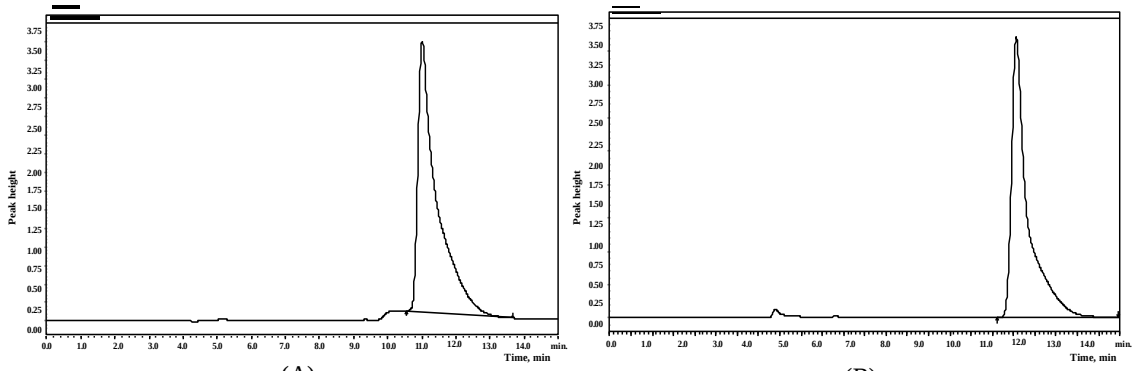
### *Physiological stability*

An important factor influencing the performance of the molecules in the organism is their hydrolytic stability at physiological conditions, such as: body temperature of 37 °C and physiological pH of 2.0 (in stomach), 7.4 (in blood plasma) and 9.0 (in intestine) [16]. The processed according to the above described procedure samples were injected into the RP-HPLC system and the corresponding chromatograms were obtained. The stability of **3M** and **4M** was firstly studied in alkaline solutions. For both of hydrazones no new peaks were observed at pH 7.4 and 9.0 for the tested period of 1440 min (24 h). This leads to conclusion that no hydrolysis of the hydrazones exists at these conditions. The analyzed **3M** and **4M** hydrazones are stable in alkaline medium under physiological temperature. The resulted chromatograms of the conducted stability studies at a temperature of 37 °C and pH=7.4 (blood plasma) and pH 9.0 (intestine) are presented on Figs. 4 and 5.




(A) (B)

**Fig. 4.** Representative chromatograms of the analyzed **3M** at pH 7.4 (A) and 9.0 (B).




(A) (B)

**Fig. 5.** Representative chromatograms of the analyzed **4M** at pH 7.4 (A) and 9.0 (B).

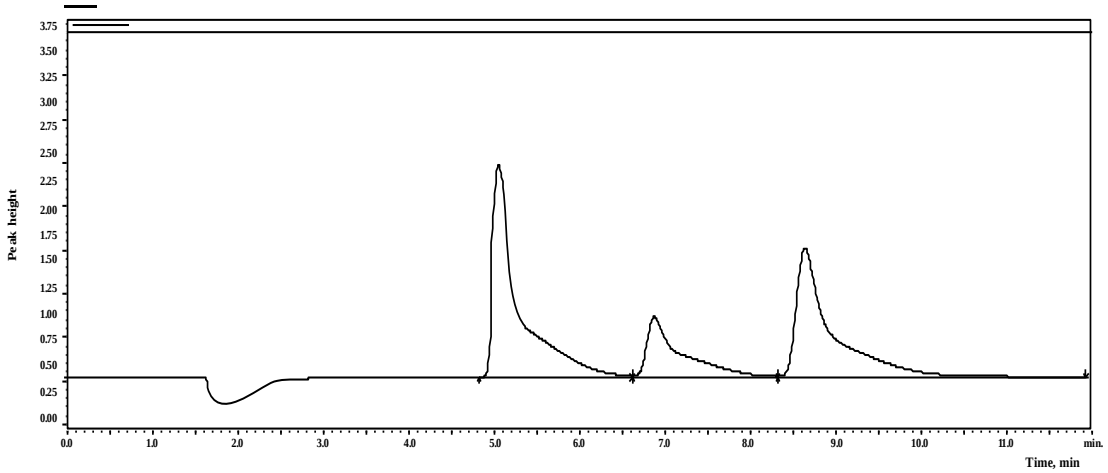



**Fig. 6.** Representative chromatogram of the analyzed **3M** at pH 2.0 at 30 minute.

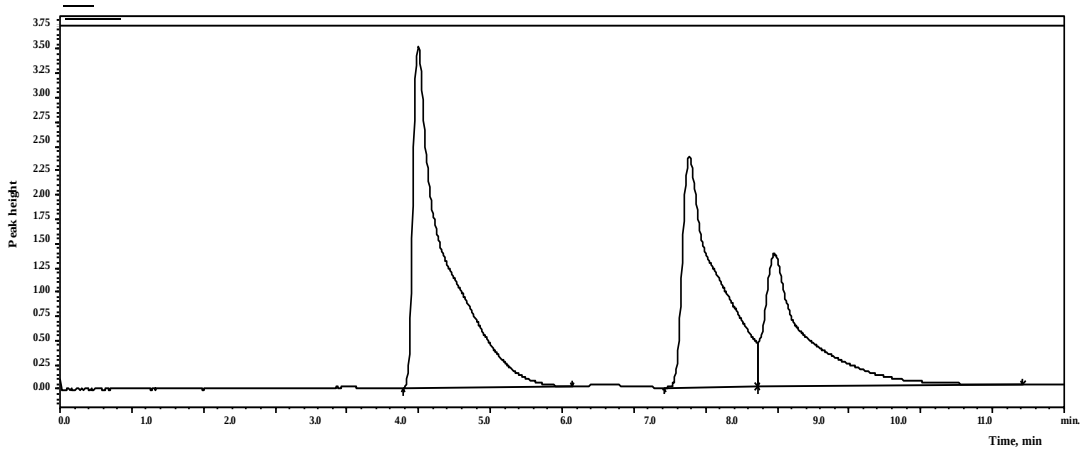



**Fig. 7.** Representative chromatogram of the analyzed **4M** at pH 2.0 at 30 minute.

The stability of **3M** and **4M** was also studied in the strong acidic medium (pH 2.0). At the 30$^{th}$ min of incubation at these conditions hydrolytic decomposition of the hydrazones was determined. An appearance of two new peaks due to the degradation products was observed. Both hydrazones hydrolyzed to corresponding initial benzhydrazide and 3-methoxy- or 4-methoxysalicylaldehyde. The resulted chromatograms of the conducted stability studies for **3M** and **4M** at a temperature of 37 °C and pH=2.0 (stomach) are presented on Figs. 6 and 7.

In addition a kinetic study of the established degradation for both analyzed products was performed. The corresponding time dependence curves for degree of degradation of the compounds at pH = 2 and temperature of 37 °C was drawn and presented on Fig. 8. The graphical dependency reveals a fast hydrolysis under the evaluated conditions for a period of 24 hours. The obtained results correspond to a first degree polynomial dependency of the degradation with $R^2$ of 0.925 and $R^2$ of 0.939, for the analyzed product **3M** and **4M**, respectively.

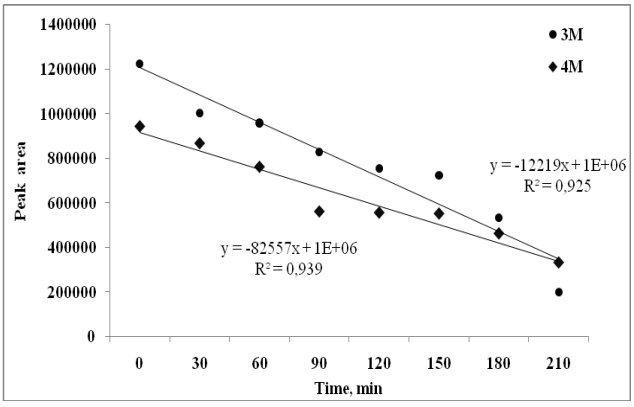



**Fig. 8.** Time dependent degradation of **3M** and **4M** at pH 2.0 and 37 °C.

## CONCLUSION

An isocratic RP-HPLC method for determination of the chemical stability and stability under close to physiological conditions of two benzoylhydrazones was developed and validated. The proposed method was found to be accurate, precise, reproducible and specific. The results indicate that the tested compounds are stable at moderate and low alkali pH and physiological temperature of 37 °C, but they are susceptible to hydrolysis in strong acidic media of the stomach. The decomposition of the **3M** and **4M** hydrazones proceeds through the hydrolysis of hydrazone bond. The products of this reaction have been detected in chromatograms – the corresponding initial benzhydrazide and methoxy-salicylaldehydes, used in the synthesis

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# ПРИЛОЖЕНИЕ НА RP-HPLC МЕТОД ЗА ОПРЕДЕЛЯНЕ НА ХИМИЧНАТА И ФИЗИОЛОГИЧНАТА СТАБИЛНОСТ НА ДВА НОВОСИНТЕЗИРАНИ МЕТОКСИПРОИЗВОДНИ БЕНЗОИЛХИДРАЗОНА

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(Резюме)

Проучването изследва стабилността на нови ароилхидразони, съдържащи хидразонова група, чувствителна към хидролиза, при условия близки до физиологичните. Два метокси-производни хидразона (3-метоксисалицилалдехид бензоилхидразон – 3М и 4-метоксисалицилалдехид бензоилхидразон – 4М) бяха разтворени в различни буферни разтвори (pH 2.0, 7.4 и 9.0) при 37 °C и аликвотни проби от тях бяха изтегляни на определени интервали от време. Стабилността на съединенията беше определена чрез използването на точен, селективен и валидиран RP-HPLC метод. Не бяха установени промени в структурите при pH = 7.4 и pH = 9.0, докато при pH = 2.0 хидразоните се хидролизират и се наблюдава появата на нови пикове, съответстващи на времето на задържане на хидролизните продукти. Резултатите показват химична стабилност на тестваните съединения при неутрално и нискоалкално pH и хидролизна чувствителност в силно киселинна среда.