Development and validation of RP-HPLC method for stability evaluation of model hydrazone, containing a pyrrole ring

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Abstract

RP-HPLC method with UV detection was developed and validated for determination of the chemical stability and stability in close to physiological conditions of a model pyrrole hydrazone ethyl 5-(4-bromophenyl)-1-(1-(2-(4-hydroxy-3-methoxybenzylidene) hydrazineyl)-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate (D₅d), containing susceptible to hydrolysis hydrazone group. The evaluated substance was subjected to the influence of a variety of pH, representing the main physiological values of 37°C and corresponding pH values in the stomach (pH 2.0), blood (pH 7.4) and small intestine (pH 9.0). Chemical stability in a highly alkaline medium with a pH of 13.0 was also evaluated. The hydrazone I tested was found to be stable at pH 7.4 and pH 9.0 and 37 ° C and hydrolyzed under strong acidic (pH 2.0) and highly alkaline media (pH 13.0) and at the same temperature. The products of hydrolysis were identified to be the initial hydrazide and aldehyde, pointing the hydrazone group as most liable.

Keywords

pyrrole-hydrazide; pyrrole-hydrazone; stability; RP-HPLC, validation

Introduction

Novel pyrrole containing hydrazones were synthesized and extensively investigated from the view point of novel drug development. Many previous studies (in vitro and in vivo) have indicated a wide variety of their remarkable biological properties such as anticancer (Alsante et al. 2001, Kovarikova et al. 2008) antioxidant, anti-tuberculosis, (Richardson et al. 1989, Kovarikova, et al. 2006, Vladimirova et al. 2016) anti-inflammatory (Svirskis et al. 2011) antidepressant (Tzankova et al. 2019a), antihypertensive, (Shaalan and Belal 2010, Grosjean et al. 1999) antimicrobial (Georgieva et al. 2012, Tzankova et al. 2018, 2019b, Kalia and Ronald 2008, Kovaříková et al. 2006) and anticonvulsants (Love 1963, Xia et al. 2008, Alqasoumi et al. 2009, Mohareb et al. 2010, Georgieva et al. 2012, Lessigiarska et al. 2012, El-Tombary and El-Hawash 2014, Kareem et al. 2015, Puskullu et al. 2016, Tzankova et al. 2018) activity. These hydrazones seem to be promising drug candidates with potential to be used in the treatment of several human pathologies (Bijev and Georgieva 2010, Navakoski et al. 2011, Kajal et al. 2014, Georgieva et al. 2017).

The process of novel drug development includes stability assessment of any promising drug candidate. Environmental factors, such as heat, light, moisture as well as the inherent chemical susceptibility of compounds to hydroly-
sis or oxidation play an essential role in drugs stability. Stability tests are used to define storage and handling conditions. Using of extreme external conditions in the stability studies (stress tests) helps revealing and identifying the likely degradation products (Ajani et al. 2010, de Oliveira et al. 2011, Leal et al. 2012, Yurttaş et al. 2013, Vaigunda et al. 2017) Several analytical methods like UV-VIS and HPLC (Dimmock et al. 2000) are reported in the literature for the identification and quantification of degradation of different pharmaceutical substances in various media.

The HPLC method with diode array detection (DAD) is widely used for determination of stability of pharmaceutical compounds. The (2,4-dinitrophenyl) hydrazones of carbonyls are separated by liquid chromatography and detected by ultraviolet spectroscopy (diode array detector).

### Selection of a model compound

From all previously synthesized compounds for the current study were used the initial hydrazide are ethyl 5-(4-bromophenyl)-1-(1-hydrazinyl-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrole-3-carboxylate (D_5) bearing the hydrolytically susceptible hydrazide group the corresponding aldehyde 4-hydroxy-3-methoxybenzaldehyde (d) and the product of their interaction - the hydrazone (E)-ethyl 5-(4-bromophenyl)-1-(1-(2-(4-hydroxy-3-methoxybenzylidene)hydrazinyl)-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrole-3-carboxylate (D_5d), bearing the hydrolytically susceptible hydrazone group. Some previous investigations with analogous hydrazides have shown that the followed CONHNH₂ group and the ester group at 3rd position in the pyrrole ring are stable at the evaluated physiological condition. Thus of an interest to our investigation to hydrolysis was subjected only compound D_5d.

### Experimental part

#### Chemicals and reagents

The model compounds were synthesized by Paal-Knorr condensation and characterized as previously (Tzankova et al. 2019a; Tzankova et al. 2019b) All the chemicals and reagents used as starting materials were purchased from Merck (Darmstadt, Germany).

The necessary products for preparation of the mobile phase and buffers were obtained from Sigma-Aldrich (Steinheim, Germany).

#### Chromatographic system and conditions

The chromatographic system UltiMate Dionex 3000 SD, Chromeleon 7.2 SR3 Systems, Thermo Fisher Scientific Inc was applied. The analysis was achieved on an analytical column 250 mm x 4.6 mm (Purospher’ STAR, RP-18, 5 μm) protected with a guard column (Hibar®, RT 125-4). The column oven was conditioned at 25°C. The mixture of an acetonitrile, phosphate buffer pH 4.0 and methanol in a ratio 60:30:10 (v/v/v) was used as a mobile phase. The flow rate was 1.0 ml/min and the detector was set up to 272 nm. The injection volume was 20 μl with analysis time of 15 minutes.

#### Preparation of buffer component of mobile phase

For the identification and quantification of degradation of pharmaceutical substances in various media the HPLC method with diode array detection (DAD) is widely used for determination of stability of pharmaceutical compounds. The (2,4-dinitrophenyl) hydrazones of carbonyls are separated by liquid chromatography and detected by ultraviolet spectroscopy (diode array detector).

### Preparation of buffers included in the stability evaluation

#### Preparation of buffer pH 2.0

Measured amount of 6.57 g of sodium hydrogen phosphate dihydrate and 3.01 g of potassium dihydrogen phosphate dihydrate were dissolved in 1000 ml of ultrapure water. Glacial acetic acid was used to adjust the pH to 4.0. The mobile phase buffer was filtered through a membrane filter (0.20 μm) 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 buffer pH 7.4

Measured amount of 2.38 g of sodium hydrogen phosphate dihydrate, 0.19 g of potassium dihydrogen phosphate dihydrate and 8.0 g NaCl were dissolved in 1000 ml distilled water.

#### Preparation of buffer pH 9.0

Solution A – Measured amount of 6.18 g boric acid was dissolved in 0.1 mol/l KCl. The obtained mixture was diluted up to 1000 ml with the same solvent.

Solution B – 0.1 mol/l NaOH.

Buffer pH 9.0 was prepared, where 1000 ml of solution A are mixed with 420.0 ml of solution B.

#### Preparation of buffer pH 13.0

A standard buffer with pH 13.0 (KCl/ NaOH) was used.

#### Preparation of samples for analysis for determination of stability at pH = 2.0, 7.4, 9.0 and 13.0

Measured amount of 0.0010 g sample of the analyzed compound was weighed and dissolved in 5 ml acetonitrile. The obtained mixture was diluted up to 50 ml with the corresponding buffers of pH 2.0, pH 7.4, pH 9.0 and pH 13.0. The obtained solutions were stirred at 37°C for a total time of 240 min. An aliquot 20 μl samples were drawn and injected into the HPLC system at the defined time intervals.

#### Mobile phase composition

Mobile phase, consisting of CH₃CN/phosphate buffer pH 4.0/ CH₃OH = 60/30/10 (v/v/v) was prepared and applied.
**Method development**

In order to achieve good separation and proper chromatographic conditions for stability assessment, we have endeavored to obtain chromatographic conditions which would allow the determination of as many peaks as possible from the set of test samples. The most common separation conditions include solvent type, mobile phase composition and pH of the medium, column type and column temperature. The experimental conditions for stability analysis were achieved by planned / systematic study of parameters including the pH of the medium, components included in the mobile phase and ratios, gradient, flow rate, temperature, sample volume, injection volume and solvent type.

**Method validation**

The developed RP-HPLC method was tested with respect to following validation parameters: specificity, precision, linearity, accuracy and selectivity.

**Specificity and selectivity**

Specificity of the chromatographic analytical procedure is the ability to measure the analyte response in the presence of all potential sample components such as the starting materials, intermediates in the synthesis, and inactive ingredients in the formulated products, and the degradation products. The selectivity of the method was evaluated by comparing the solution of the model compound (D_5d) with the solution of the corresponding hydrazone, aldehyde and used mobile phase alone and in mixture.

**Precision**

Precision is expressed as the standard deviation of the analytical results when the analysis is carried out in a laboratory by an operator using equipment over a relatively short time span. For precision of the method six independent sample solutions from evaluated hydrazone were used. Each sample was tested in triplicate. The final results are reported as relative standard deviations (RSD %).

**Linearity**

The linearity of an analytical method can be defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a given range. The linearity of the method was determined in a concentration range of 5 - 40 μg/mL for hydrazone D_5d. An eight points covering the eight different concentrations of the analyzed compound over the evaluated concentration range were used to create the calibration curve. For processing the calibration data was used linear regression.

**Accuracy**

Accuracy is the closeness of the analytical results obtained by the analyses to the true values, and usually presented as a percent of nominal. The analyzed solutions were prepared using a placebo and stock solution of the tested structure. Accuracy is reported as a parameter recovery with relative standard deviations calculated from the analysis of six solutions prepared from the compound. Each solution was tested in triplicate.

The aim of this study is to develop, validate and apply a simple RP-HPLC method for stability determination of pyrrole-containing derivatives.

**Results and discussion**

**Specificity and stability indicating study**

Specificity is the ability of the analytical method to measure the active ingredient response in the presence of other excipients and its potential degradants. Forced degradation was carried out to evaluate the specificity and stability-indicating properties of the method, by exposing samples of the substance to stress conditions of hydrolysis, oxidation, photodegradation, and thermal degradation.

Stress testing of the substance was performed to induce force degradation and determine degradation pathways and help evaluate the stability of the compound and also validate specificity of the analytical procedures.

**Selection of a model compound.**

There are a number of data confirming our suggestion that the hydrazone hydrolysis is connected mainly with cleavage of the C=N double bond from the hydrazone group and release of the parental hydrazide and corresponding carbonyl compound (aldehyde or ketone)

A previously developed and applied by us UV/VIS method for preliminary evaluation of the stability of this group of compounds determined that liable to hydrolysis is expected to be the corresponding hydrazone group in the tested model compounds.

Thus in order to evaluate and identify the products of hydrolysis from previously synthesized by us pyrrole containing hydrazones, as model compound was selected compound D_5d. The structure of the model hydrazone is presented on Figure 1.

![Figure 1. Structure of the selected model compound.](image-url)
Its stability was determined at different pH (2.0, 7.4, 9.0 and 13.0), close to physiological and stressful conditions of pH 13.0 and at two temperatures – (25 °C and elevated (37 °C).

Method development and optimization

With regard to the physical and chemical properties of the analytes and the information obtained from the literature, analytical method was developed to select a preliminary reversed phase RP-HPLC chromatographic conditions, including detection wavelength, mobile phase, stationary phase, and sample preparation procedure. For that, series of trials were performed, such as different compositions of mobile phase and different types of stationary phase and column lengths, with different pH values and buffering agents.

The performed experiments demonstrated that including acetonitrile as component of the mobile leads to improvement in the separation and formation of well developed Gaussian type peaks. Different compositions of solvents were analyzed, containing methanol, acetonitrile, buffer with pH of 2.0, 7.4 and 4.0 in various relations. As most suitable for achieving good separation and suitable peak forms was determined to be a mobile phase, consisting of CH₃CN/phosphate buffer pH 4.0/CH₃OH in 60/30/10 (v/v/v). In addition the flow rate, the temperature and the wavelength were also varied as follows: the flow rate was evaluated in the range of 0.9 to 1.2 ml/min. The flow rate of 1.0 ml/min was found to be most appropriate. A temperature of 25°C was found to be suitable for the good separation. The preliminary UV/VIS method developed by us identified 272 nm as adequate wavelength.

Thus the performed analysis determined the following chromatographic conditions, which were further applied for identification and determination of stability of the tested hydrazone D_5d:

- Mobile phase: CH₃CN/phosphate buffer pH 4.0/CH₃OH in 60/30/10 (v/v/v)
- Isocratic flow with flow rate of 1.0 ml/min
- Column temperature of 25°C
- Wavelength of 272 nm

Standard solutions of the analyzed hydrazone (D_5d) and the expected initial hydrazide (D_5) and aldehyde (d) were analyzed by the developed method for identification of the corresponding retention times. Under the discussed chromatographic conditions was found that the retention times for the tested hydrazone (D_5d), the initial hydrazide (D_5) and aldehyde (d) are as follows: tᵣ = 6.800 (Figure 2), tᵣ = 4.387 (Figure 3) and tᵣ = 1.387 (Figure 4), respectively.

The obtained under these conditions separation is presented on Figure 5.

![Figure 2. Chromatogram of standard solution of the analyzed hydrazone D-5d (tᵣ = 6.110).](image)

![Figure 3. Chromatogram of standard solution of the hydrazide D-5 (tᵣ = 4.380) as possible degradation product.](image)

![Figure 4. Chromatogram of standard solution of the aldehyde d (tᵣ = 1.283) as possible degradation product.](image)

![Figure 5. Chromatogram of the separated mixture of the analyzed hydrazone D-5d (tᵣ = 6.800) and its possible degradation products – the hydrazide D-5 (tᵣ = 4.387) and the corresponding aldehyde d (tᵣ = 1.387).](image)
**Method validation**

**Specificity**

No significant interfering peaks (peak area >0.1%) was observed at the retention time of the analyzed hydrazone and mixtures in blank solution. In addition, no evidence of co-elution was noted using peak purity analysis for the tested compounds and mixtures.

**Linearity**

The linearity of the method was observed in the concentration range of 5 μg/mL to 40 μg/mL for D_5d demonstrating its suitability for analysis. The goodness of fit evaluated through the correlation coefficient $r^2$ was found to be 0.9980, indicating a linear relationship between the concentration of analyte and area under the peak, as shown on **Figure 6**.

![Figure 6. Linearity of the developed RP-HPLC D_5d stability indicating method.](image)

**Accuracy**

The accuracy of an analytical procedure expresses the closeness of results obtained by that method to the true value. The results of accuracy testing showed that the method is accurate within the acceptable limits. The percentage recovery and RSD were calculated for all active ingredients; all the results are within limits. Acceptable accuracy was within the range of 99.98% to 100.4% recovery and not more than 2.0% RSD, as demonstrated in **Table 1**.

| Compound | Spiked level (μg/mL) | Replicate number | Recovery (μg/mL) | % mean recovery | % RSD  |
|----------|----------------------|------------------|------------------|----------------|--------|
| D_5d     | 5                    | 1                | 5.05             | 100.4          | 0.72   |
|          | 2                    | 4.98             |                  |                |        |
|          | 3                    | 5.03             |                  |                |        |
|          | 20                   | 1                | 19.99            | 100.2          | 0.16   |
|          | 2                    | 20.04            |                  |                |        |
|          | 3                    | 19.98            |                  |                |        |
|          | 40                   | 1                | 40.07            | 99.98          | 0.23   |
|          | 2                    | 39.89            |                  |                |        |
|          | 3                    | 40.02            |                  |                |        |

**Precision**

Precision of an analytical method is defined as “the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions,” and it is normally expressed as the relative standard deviation. The results of repeatability and intermediate-precision testing showed that the method is precise within the acceptable limits. The RSD was calculated and was found that the results are within limits. Precision was not more than 2.0% RSD, as demonstrated in **Table 2**.

**Table 2.** Evaluation of the accuracy of the method developed in this study.

| Intermediate precision (ruggedness) | Recovery (μg/mL) |
|-------------------------------------|------------------|
| Replicate number                    |                  |
| 1                                   | 19.99            |
| 2                                   | 20.01            |
| 3                                   | 20.03            |
| 4                                   | 19.98            |
| 5                                   | 20.04            |
| 6                                   | 19.87            |
| Mean recovery                       | 19.99            |
| % RSD                               | 0.31             |

**Limit of Detection and Limit of Quantification (LOD and LOQ)**

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, while the limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision. The method showed a LOD of 0.20 μg/mL and a LOQ of 0.40 μg/mL.

**Chemical stability**

Chemical stability is defined as the possibility of a substance to sustain to change or decomposition as a result of any internal reaction, action of air, humidity, heat, light, pressure, etc. The evaluated model compound has been stored for 6 months at room temperature with access of air and light. No change in the physical and chemical properties of the investigated compound was observed under these conditions; thus, it may be considered as chemically stable, when stored.

In addition the chemical stability was investigated at strong alkali media of pH 13.0 and at two temperatures: room temperature and temperature of 37°C.

**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) (Georgieva et al. 2012).

The selected model compound was subjected to analysis following the requirements for physiological stability.
evaluation. The tested D_5d was dissolved in the corresponding buffers, mimicking physiological conditions and stirred at 37°C for 240 min.

For both types of stability evaluation four solutions were prepared according to the above mentioned recipe. The obtained chromatograms for the tested D_5d for the physiological conditions were recorded and are presented on Figure 7 and Figure 8 respectively.

In the performed investigations an appearance of new peaks, corresponding to the retention times of the initial hydrazide and the corresponding aldehyde are observed in the chromatogram of D_5d in acidic (pH 2.0) (Figure 7) media. This led us to conclude that this substance is susceptible to hydrolysis at these conditions and as a result are formed the initial reagents.

No additional peaks appeared in the chromatograms of the analysis performed in the neutral buffer of pH = 7.4 and alkali buffer of pH 9.0 (Figure 8) and temperature of 37°C for the evaluated period of time, which led us to conclude that the structure is stable at these conditions.(33–35).

In addition the chemical stability was also evaluated in the same manner by following the same procedure, using buffer of pH = 13.0. The corresponding chromatogram is presented on Figure 9.

![Figure 7. Chromatograms indicating the behavior of D_5d in the presence of buffer with pH 2.0 and at 37°C at 0th min (A) and at 30th min (B).](image)

![Figure 8. Chromatograms indicating the behavior of D_5d in the presence of buffer with pH 9.0 and at 37°C at 0th min (A) and at 210th min (B).](image)

![Figure 9. Chromatograms indicating the behavior of D_5d in the presence of buffer with pH 13.0 and at 37°C at 0th min (A) and at 30th min (B).](image)
The chromatogram shows appearance of new peaks at the retention times of the initial hydrazide and the corresponding aldehyde. This shows that the structure is liable to hydrolysis at strong alkali media. The result is cleavage of the hydrolytically liable hydrazone group, as suggested previously.

Conclusion

A fast, simple, accurate, precise, and linear stability indicating HPLC method has been developed and validated for the simultaneous analysis of pyrrole hydrazones and its degradation products. The method is stability indicating and reliable to detect and quantify any potential degradation in the tested compounds during stability studies and can be used for routine quality control analysis. The method is robust enough to reproduce accurate and precise results under different chromatographic conditions. The method indicates that the tested model hydrazone $D_{5d}$ is sensitive to hydrolytic decomposition in aqueous media and pH values of 2.0 and 9.0 and temperature of 37°C, resulting in the splitting of the hydrazone bond and formation of the initial hydrazide and corresponding aldehyde. In addition a liability at strong alkali media (pH 13.0) is also observed, witnessing for sensibility under these conditions.

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