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Research Article

Development and validation of an RP-HPLC method for analysis of 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid and its impurities under different pH

Vania Maslarska[1], Stanislav Bozhanov[1], Stanislava Vladimirova[2], Lily Peikova[3], Diana Tzankova[3], Maya Georgieva[3]

1 *Department of Chemistry, Faculty of Pharmacy, Medical University, Sofia, Bulgaria*
2 *Department of Organic Synthesis and Fuels, University of Chemical Technology and Metallurgy, Sofia, Bulgaria*
3 *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University, Sofia, Bulgaria*

Corresponding author: Vania Maslarska (vmaslarska@mail.bg)

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Abstract

A simple, fast and selective stability indicating RP-HPLC method was applied for following the degradation and appearance of impurities of previously synthesized 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid. The chromatographic separation was achieved on a C18 column (150×4 mm i.d., 5 µm) using a mobile phase consisting of Acetonitrile: Phosphate buffer, pH=3, (50:50% v/v) with isocratic elution at a flow rate of 1.0 mL min^{-1} and temperature of the column of 30 °C applying a UV/VIS detector at 225 nm. The method was validated according to the ICH guidelines. A process related impurity was determined at pH 9.0 corresponding to ethyl 2-acetyl-4-(4-chlorophenyl)-4-oxobutanoate. No change in the structure was detected at pH = 7.4.

Keywords

degradation, impurities, N-pyrrolylcarboxylic acid, RP-HPLC, validation

Introduction

Organic and pharmaceutical chemists are usually attracted by novel structures and potent biological activity of pyrrole derivatives, which led to the synthesis of several representatives of this class of compounds, underlined with various pharmacological effects, like antiproliferative activity (Lewis 2014; Hargrove et al. 2015; Philchenkov et al. 2015; Rohena et al. 2016; Desplat et al. 2017), non-steroidal anti-inflammatory effect (Vladimirova and Bijev 2014; Danalev et al. 2016; Fatahala et al. 2017), antitubercular effect (Poce et al. 2013; Joshi et al. 2014; Rawat et al. 2017; Venditti et al. 2017) and many more. Lately, some pyrrole esters have been also evaluated as products with potential herbicide effects (Vladimirova et al. 2014). Few analytical methods have been reported in the literature for the determination of pyrrole including its degradation products (Vladimirova et al. 2016; Christ et al. 2016; Cuccia et al.

2017). In recent years, studies related to identification, synthesis and characterization of process and degradation related impurities, including stereoisomers of pyrrole, have been reported (Georgieva et al. 2011; Liu et al. 2016). The stability of a compound synthesized as a potential biologically active agent is related to its pharmacokinetic behavior in the body and the conditions for the formulation, storage, occurrence of toxic effects associated with degradation products etc. Most 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 (Zajac et al. 2010). The preliminary information on the stability and purity of newly synthesized compounds is essential in the development of new drugs. Thus the determination of the stability at different pH conditions is of particular importance.

It is known, that potential degradation products are formed through elevated temperatures, by applying acidic, basic, and oxidative conditions, and through photolysis (ICH Guidelines Q1 R2 2003). Several high-performance liquid chromatography (HPLC) methods are reported in the literature for the identification and quantification of degradation of different pharmaceutical substances in various media (El-Gindy 2005; Chaudhari et al. 2007; Hadad 2008; Gatti et al. 2010; Wagieh et al. 2010; Svirskis et al. 2011).

The aim of this study is the development and validation of a suitable RP-HPLC method for analysis of previously synthesized 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid and its process related impurities under different pH.

Materials and methods

Chemicals and reagents

The necessary products for preparation of the mobile phase and used buffers are of analytical grade, whereas potassium dihydrogen phosphate dihydrate (Sigma-Aldrich, Steinheim, Germany), orthophosphoric acid (Merck, Darmstadt, Germany) and acetonitrile (ACN) gradient grade (Sigma-Aldrich, Steinheim, Germany) were used. HPLC grade Acetonitrile and Methanol were procured from Merck Ltd.

Chromatographic system and conditions

A Shimadzu HPLC system consisting of the following components was utilized: pump LC – 20AD, vacuum degasser unit DGU – 20 A_5 and a UV/VIS detector SPD – 20 A. Separation was carried out on a LiChrosorb C 18 column (150 × 4 mm, particle size 5 µm) under reversed phase partition chromatographic conditions. The equipment was controlled by a PC using chromatographic software. The mobile phase was a 50:50% v/v, Acetonitrile: Phosphate buffer (0.136 g potassium dihydrogen phosphate in 1000 ml water, adjusted with ortho-phosphoric acid, pH = 3.0±0.1). The mobile phase was filtered through a 0.45 µm membrane filter and degassed by using a sonicator for about 10 min before use. The sample solutions were also filtered using 0.45 µm membrane filters. The mobile phase was delivered isocratically at a flow rate of 1.0 ml/min. The column was maintained at a temperature of 30 °C. The injection volume was 20 µl and the total run time was 20 minutes. The detection was carried out at 225 nm.

Preparation of the stock solution

Accurately weighed quantity of 10 mg 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl) propanoic acid (Compound 1a) was transferred in 100 ml volumetric flask, dissolved with a mixture of water and methanol (50:50) to volume, and mix. The concentration was 100 µg/ml. From the stock solution by further dilutions were prepared standard solutions within the concentration range 6.25 – 50.00 µg/ml.

Samples preparation

Compound 1a (2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid) was accurately weighed and transferred into a 100 ml volumetric flask. Approximately 30 ml of a mixture of water and methanol (50:50) was added and the mixture was sonicated for 1 minute. Then the mixture was diluted to volume with a mixture of water and methanol (50:50). From the stock solution by further dilutions with buffer solutions (pH = 7.4 and pH = 9.00) were prepared the required working solutions. The solutions were thermostated at 37 °C for a total time 1440 min.

Solutions of the parent compounds (25 µg/ml) were also prepared in mixture of water and methanol (50:50).

Preparation of buffer solutions

27.22 g of potassium dihydrogen phosphate dihydrate was dissolved in 1000 ml of deionized water. 50.00 ml of the solution was mixed with 39.10 ml sodium hydroxide (0.2 mol/l) and 100 ml deionized water in a suitable container. The solution was adjusted to pH 7.4 (±0.02) and the final volume made up to 200.0 ml. 50.00 ml of the solution was mixed with 20.80 ml sodium hydroxide (0.2 mol/l) and 100 ml deionized water in a suitable container. The solution was adjusted to pH 9.0 (±0.02) and the final volume made up to 200.0 ml.

Results and discussion

Synthesis of 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl) propanoic acid

The structure of the analyzed molecule is presented on Fig. 1.

The synthesis of the analyzed structure is described elsewhere (Vladimirova et al. 2014) and presented on Fig. 2:

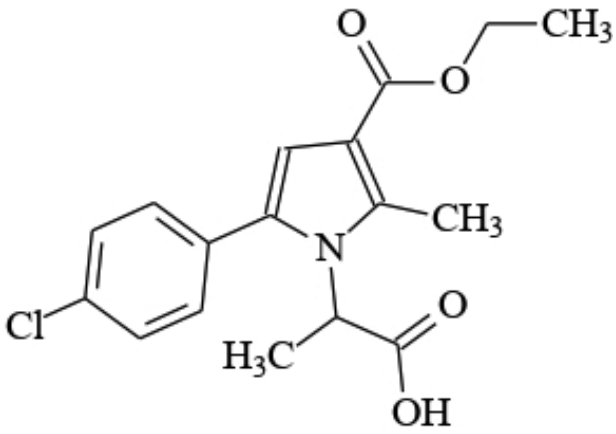

Figure 1. Structure of the analyzed compound (1a).

During the chemical synthesis, it was observed an appearance of an impurity, eluting together with the final product. In an attempt to identify the possible mechanism by which this impurity is formed, the conditions, under which this impurity would appear and to follow the stability of the obtained 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid we optimized a high-performance liquid chromatography method.

The method was applied to separate and selectively detect and identify all process-related impurities and degradation products under different pH conditions at a temperature of 37 °C.

Development of the used RP-HPLC method

For following the degradation and appearance of impurities of previously synthesized 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl) propanoic acid a simple, fast and selective RP-HPLC method was developed and applied. The presented method was validated in terms of precision, linearity, accuracy, selectivity, limits of detection, and quantification.

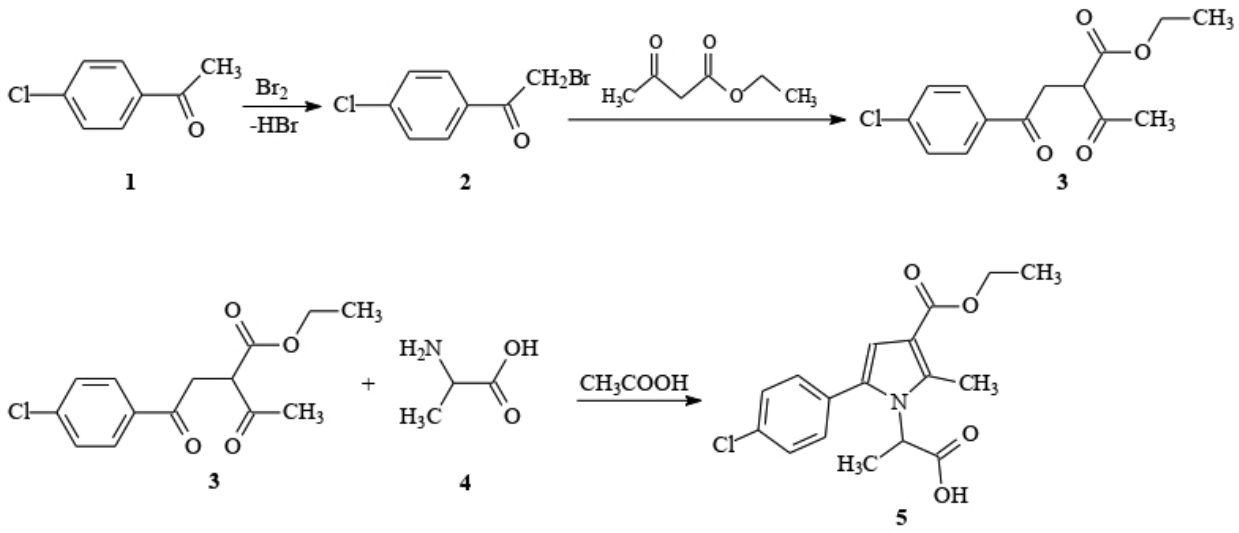

Figure 2. Synthesis of the analyzed 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid (5) (compound 1a).

Validation of a HPLC analytical procedure

The method was validated according to ICH guidelines (ICH Guidelines Q2 R1 2005). The system suitability (i.e., repeatability of retention times and areas, number of theoretical plates, and resolution, Table 1), precision, linearity, accuracy and selectivity were evaluated during method validation. The parameters accuracy, precision, and selectivity were performed and evaluated for the analyzed product.

Table 1. Validation parameters for compound 1a.

	1a
Retention time, min	8.14
Number of theoretical plates	21333.5
Resolution,	5.112

System suitability test (SST)

Limit of detection and quantification

The detection limit (LOD) is the lowest amount of analyte in the sample, which can be detected but not necessarily quantified as an exact value. The quantification limit (LOQ) is the lowest amount of analyte in the sample, which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ are calculated as given in Table 2.

Table 2. Linearity Results, Limit of Detection (LOD) and Limit of Quantification (LOQ).

Compounds	r^2	Calibration curve equation	LOQ, ng	LOD, ng
Compound 1a	0.999	Y = 70212.1X+1431.7	80	30

Selectivity

The selectivity studies were performed against solvents used. The placebo solution was injected twice according to the parameters stated under the developed method. It was found that there was no interference between the analyte and placebo solutions (Fig. 3a, b).

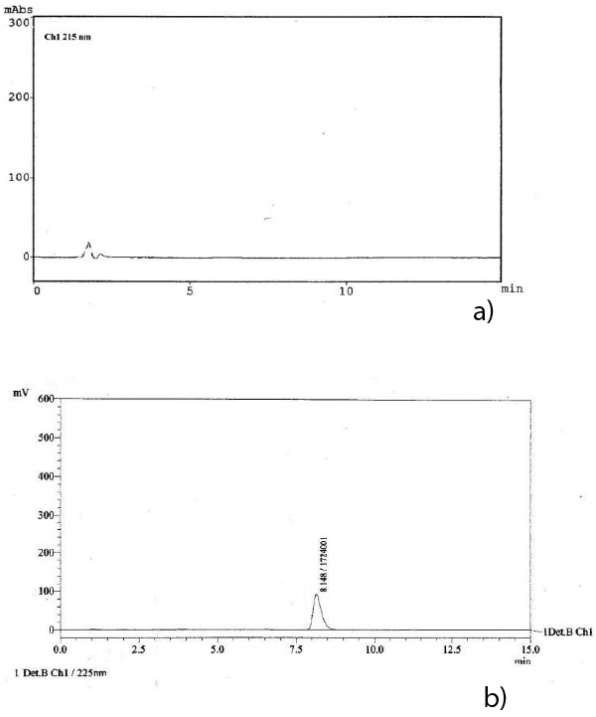

Figure 3. a. Placebo chromatogram **b.** chromatogram of compound 1a.

Linearity

Table 2 presents the equation of the regression line, correlation coefficient (r^2) values of the slope and intercept between the peak areas and concentrations of 6.25–50.0 µg/ml with $r^2 = 0.999$ (Fig. 4). The calibration curve equation shows a good linearity curve which means that the linearity test is validated.

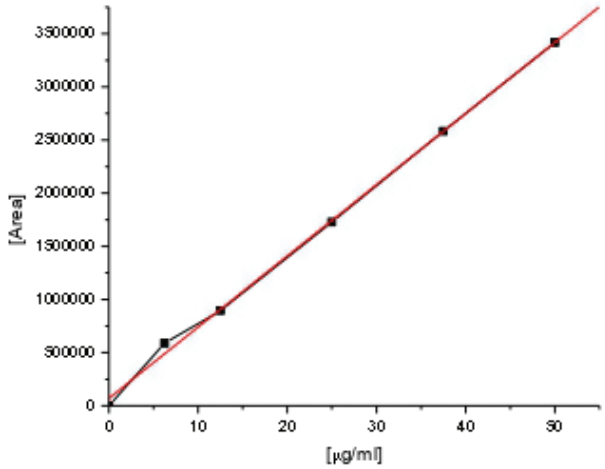

Figure 4. Linearity of Compound 1a.

Precision

For determination of the precision, the standard solution (25 µg/ml) was consecutively injected 6 times according to the above method. Assay % and RSD % values obtained are within the range of 98 – 102% (±2). The data given in Table 3 show a precise and valid method of analysis.

Table 3. Results of formulation and recovery studies.

Amount (µg/ml)	Taken	Found ±S.D.*	Percentage Recovery*
Compound 1a	25.0	24.8 ± 1.168	100.1_5 ± 1.179

*Average of 6 determinations.

Accuracy

The accuracy test was applied at three different levels of concentrations with triple injecting for each sample (Table 4), the % of recovery equation is:

% Accuracy = [(recovered amount / actual amount) X 100]

Table 4. Accuracy of compound 1a.

Parameters	Taken, %	Taken (µg/ml)	Found (µg/ml)	Found, %	Recovery, %
			12.18	48.72	97.44
	50.00	12.50	12.53	50.12	100.2
			12.42	49.68	99.36
			25.32	101.3	101.3
	100.0	25.00	24.79	99.16	99.16
			25.11	100.4	100.4
			37.43	149.7	99.80
	150.0	37.50	37.62	150.5	100.3
			37.22	148.9	99.27
Mean					**99.69**
SD					**±1.082**
% RSD					**1.086**

Identification of process related impurities

The tested 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid was dissolved in water and methanol (50:50) and thermostated for a period of 1440 min at 37 °C. No new peaks appeared on the obtained chromatogram. Thus we considered that the product is completely purified from all possible process impurities.

Determination of degradation at different pH

An important factor influencing the performance of the molecules in the organism is their hydrolytic stability at physiological conditions, such as: a body temperature of 37 °C and physiological pH of 7.4 (blood plasma) and 9.0 (intestine) (Kong and Singh 2008).

The fully substituted pyrrole, as well as the connected with it methyl groups, are stable at moderate temperatures and in a wide range of pH values. The stability of the obtained structure will be determined by the stability of

the identified as potential "vulnerable" group, positioned on 3rd place in the pyrrole cycle ester group (Jordanov et al. 1994).

In order to determine the chemical stability and the stability of the ester group, positioned on 3rd place at different pH of 7.4 (blood plasma) and 9.0 (intestine) and temperature of 37°C the analyzed structure was thermostated and stirred in a micro reactor at 37 °C for a total time of 1440 min. Aliquot samples of 0.5 ml of the analyzed solutions were taken at definite time intervals (15, 30, 60, 120, 240, 480 and 1440 min) and diluted to 1.5 ml with the corresponding mobile phase in a way that the concentration is in the range of 0.1 mg/ml and a 20 µl sample was injected. The corresponding chromatograms were obtained.

Stability determination of 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid at pH 7.4 (blood plasma) and temperature of 37 °C

A 20 mg sample of the tested compound was weighed and dissolved in buffer pH 7.4. The obtained solution was stirred in a micro reactor at 37 °C for a total time of 1440 min. At the determined above time intervals, a 20 µl sample was drawn and injected into the apparatus. The obtained chromatogram is presented on Fig. 5.

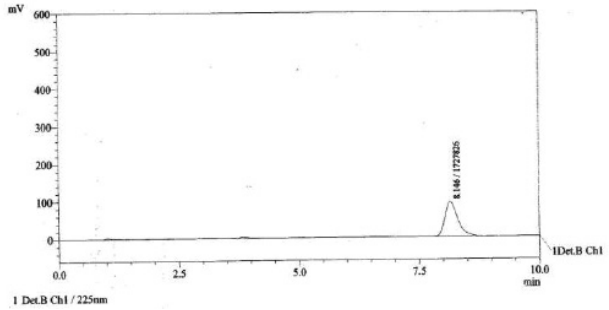

Figure 5. Chromatogram of the analyzed compound 5.

The performed analysis showed stability of the tested N-pyrrolylcarbocilyc acid and no impurities were detected under the discussed conditions. Thus 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid may be considered stable at this media.

Stability determination of compound 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl)propanoic acid at pH 9.0 (intestine) and temperature of 37 °C

A 20 mg sample of the tested compound was weighed and dissolved in buffer pH 9.0. The obtained solution was stirred in a micro reactor at 37°C for a total time of 1440 min. At the determined above time intervals a 20 µl sample was drawn and injected into the apparatus. Representative chromatograms,

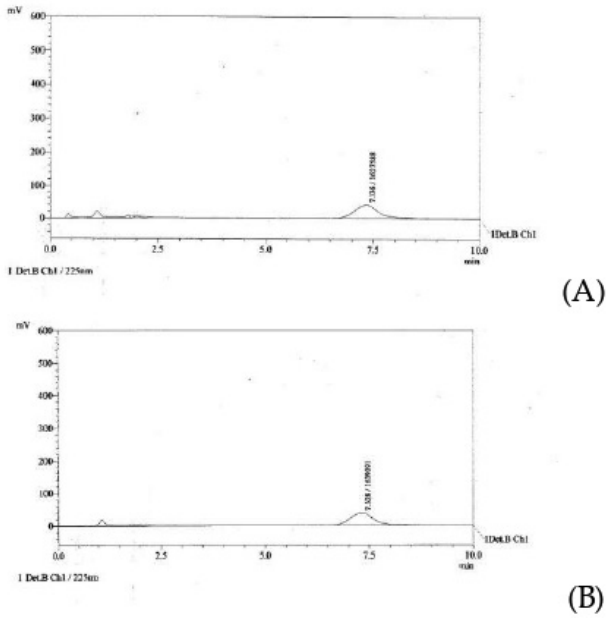

Figure 6. Chromatogram of the analyzed compound 5 at 0 min (**A**) and at 30 min (**B**).

showing the behavior of the analyzed structure at 0 min and 30 min of the incubation are presented on Fig. 6.

The performed analysis showed the appearance of a new peak with retention time (t_R) of 7.33 min at 0 min of the analysis. The new substance stayed stable through the whole time of analysis (1440 min.), keeping the peak area constant.

For identification of this substance, the initial products, used in the synthesis were analyzed by the developed RP-HPLC method. The analysis showed, that the obtained during the incubation peak with t_R of 7.33 min, corresponds with the initial ethyl 2-acetyl-4-(4-chlorophenyl)-4-oxobutanoate (Fig. 7).

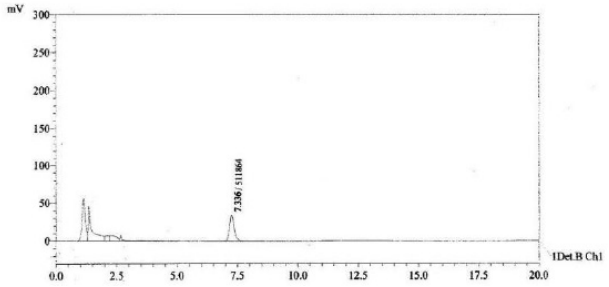

Figure 7. Chromatogram of the initial substance 3.

This lead to the conclusion, that at the low alkali media of pH 9.0 and temperature of 37°C the analyzed N-pyrrolylcarbocilyc acid degrades with cleavage of the pyrrole cycle and release of the initial ethyl 2-acetyl-4-(4-chlorophenyl)-4-oxobutanoate, identified as a process impurity.

On the other hand, the expected hydrolysis of the ester group at 3rd position of the N-pyrrolylcarbocilyc acid did not occur. Thus this group may be considered stable under the applied conditions.

Conclusion

In conclusion, a suitable RP-HPLC method was used and validated for the determination of process related impurities and degradation products of 2-(5-(4-chlorophenyl)-3-(ethoxycarbonyl)-2-methyl-1H-pyrrol-1-yl) propanoic acid under different pH. The proposed method was found to be accurate, precise, reproducible and specific. The results indicate that the tested compound is stable at moderate pH and temperatures. A process related impurity was determined at pH 9.0 corresponding to ethyl 2-acetyl-4-(4-chlorophenyl)-4-oxobutanoate.

Conflict of interest

The authors declared no conflict of interest.

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