Research Article

Development of new HPLC method for identification of metabolic degradation of N-pyrrolylhydrazide hydrazones with determined MAO- B activity in cellular cultures

Alexandrina Mateeva[1], Lily Peikova[1], Magdalena Kondeva-Burdina[2], Maya Georgieva[1]

1 *Department of Pharmaceutical chemistry, Faculty of Pharmacy, Medical University – Sofia, 2 Dunav str., 1000, Sofia, Bulgaria*
2 *Department of Pharmacology, toxicology and pharmacotherapy, Faculty of Pharmacy, Medical University – Sofia, 2 Dunav str., 1000, Sofia, Bulgaria*

Corresponding author: Maya Georgieva (maya.bg77@gmail.com)

Abstract

In this research, a new rapid PR- HPLC method was developed for the determination of metabolites in isolated rat hapatocytes. The chromatographic parameters, including the stationary and mobile phases, outlet pressure, temperature and flow rate, were optimized. The method identified two initial from the synthesis molecules in higher concentration and one new unidentified structure as products of the hepatocytic processing of the evaluated analyte. The results identified as first step of metabolism the hydrolysis of the hydrazone group. Further investigations should be aimed into determining the next metabolic transformations, predicted by the *in silico* application of the web server SMARTCyp.

Introduction

Drug-metabolism studies have major role in the medicinal chemistry considering the huge potential for lead optimization, detection of toxic metabolites, excretion route identification and rate of drug clearance (Zhang et al. 2021). The utilization of high-performance liquid chromatography (HPLC) as analytical strategy for metabolite identification and quantification has been significantly preferred, since the latter comprises high selectivity, rapid analysis and accessible use (Castro-Perez 2007). Essentially, the detection of compound metabolites is of great importance, considering that one of the major causes of failure in the development stages of drug candidate, have been attributed to drug metabolism and pharmacokinetic issues (Hsieh 2007). Therefore, the liquid chromatography, coupled with different detectors, such as mass spectrometer and fluorometer, is being rapidly adopted across all areas of drug discovery (Han et al. 2019).

Metabolism of drugs include complex of reactions, which biotransformed the initial molecule to structurally different derivatives (metabolites) during the first passage through the liver (Zhang and Tang 2018; Schaffner 1975). The cells engaged with this bio transformational process are liver hepatocytes.
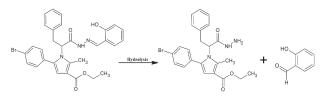
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Hepatocytes, the major parenchymal cells in the liver, play pivotal roles in metabolism, detoxification, and protein synthesis. Hepatocytes also activate innate immunity against invading microorganisms by secreting innate immunity proteins (Ma et al. 2020). Although morphologically quite similar, hepatocytes accomplish very different metabolic roles according to their specific position along the lobular portocentral axis. The segregation of hepatocytes into discrete functional areas is referred to as 'Liver Zonation' and is the basis for the partition of the hepatic lobule into three metabolic zones: periportal Zone 1 consisting of 6–8 hepatocyte layers that receive blood enriched in oxygen and nutrients and control glycogen metabolism, amino acid utilization and ammonia detoxification; intermediate perivenous Zone 2 consisting of 6–10 hepatocyte layers with a major role in xenobiotic metabolism; and perivenous Zone 3 formed by 2–3 hepatocyte layers that surround the central veins and perform biotransformation reactions, glutamine synthesis and glycolysis (Jungermann and Katz 1989).

In vitro hepatocyte models represent very useful systems in both fundamental research and various application areas. Primary hepatocytes appear as the closest model for the liver *in vivo*. *In vitro* hepatocyte models have brought a substantial contribution to the understanding of the biochemistry, physiology, and cell biology of the normal and diseased liver and in various application domains such as xenobiotic metabolism and toxicity, virology, parasitology, and more generally cell therapies (Guguen-Guillouzo and Guillouzo 2010).

The application of this model for preliminary metabolic identification of newly synthesized biologically active molecules seems quite reasonable and time and cost spearing.

Recently, a pyrrole-based aryl hydrazide-hydrazone with eminent antioxidant capacity has been reported (Tzankova et al. 2019a, 2020). The presence of the hydrzide-hydrazone fragment in its structure is pointing to a possible susceptibility to initial hydrolysis as shown in Scheme 1. (Tzankova et al. 2019b). In addition, the appearance of a pyrrolic ring, could lead to deactivation through ring oxidation and subsequent rearrangement (Assandri et al. 1987).

In this point of view, the tentative information on possible metabolic transformation of the targeted pyrrole based hydrazide-hydrazone molecule will be essential for the further development of this class of structures as core for biologically active molecules.

Scheme 1. Hydrolysis of evaluated pyrrole hydrazide-hydrazone in different pH media.

This defined the aim of the present work pointed to the development of a sensitive and reliable analytical method for determination of hydrazide-hydrazone metabolites of pyrrole based molecules in isolated rat hepatocytes and subsequent, validation of the method according to ICH guidelines.

Materials and methods

Reagents and chemicals

All of the solvents used in the current work were purchased from Sigma Aldrich and are with European pharmacopoeia purity. Potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate and orthophosphoric acid were obtained from Sigma-Aldrich and are of analytical grade. The evaluated hydrazide-hydrazone derivative was synthesized as described in a recent work by Tzankova et al. (Tzankova et al. 2020)

Animals and experimental procedure

A male Wistar rats with body weights around 200 g were used. The latter were housed in plexiglass cages at room temperature in 12/12 light/dark cycle. The animals were purchased from the National Breeding Centre, Sofia, Bulgaria and all performed procedures were approved by the Institutional Animal Care Committee with accordance with European Union Guidelines for animal experimentation.

Sodium pentobarbital (0.2 mL/100 g) was employed for the anesthesia of the rats. A modified method described by Fau et al. (Fau et al. 1992) was used for *in situ* liver perfusion and cell isolation. After portal catheterization, the liver was perfused with 100 mL HEPES buffer (pH = 7.85), containing 10 mM HEPES, 142 mM NaCl, 7 mM KCl, 5 mM glucose and 0.6 mM EDTA (pH = 7.85), followed by 200 ml HEPES buffer (pH = 7.85), with a final addition of 200 ml HEPES buffer containing collagenase type IV (50 mg/200 mL) and 7 mM $CaCl_2$ (pH = 7.85).

The liver was excised, minced into small pieces and hepatocytes were dispersed in 60 mL Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35), containing 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mMKCl 5 mM $NaHCO_3$, 4.5 mM glucose and 1% bovine serum albumin. After the initial filtration, the hepatocytes were centrifuged at 500 g for 1 minute and washed three times with KRB buffer. Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%), which was calculated to be 89% in average (Fau et al. 1992). Cells were diluted with a KRB, to make a suspension of about 3×10^6 hepatocytes/mL. Incubations were carried out in 25 mL Erlenmeyer flasks with each containing 3 mL of the cell suspension (i.e. 9×10^6 hepatocytes). The latter procedures were performed in a 5% CO_2 + 95% O_2 atmosphere.

Sample preparation

The hepatocytes were incubated at KRB buffer with 50 μM hydrazide- hydrazone compound for 2 h. Subsequently, the obtained cell emulsion was precipitated with 1 mL HPLC grade methanol and centrifugated for 15 min at 14 000 rpm. The supernatant was double filtered through PVDF sterile syringe filters (through 0.47 μm, and through 0.22 μm).

Instrumentation and chromatographic conditions

The applied chromatography system was constructed out of UltiMateDionex 3000 SD pump connected to UltiMateDionex DAD 3000 detector. Separation of compounds was performed on a 15 × 0.46 cm, 5 μm particle size, Purospher C18 Column protected by a C18 guard column (SecurityGuard HPLC Cartridge System; Phenomenex). The former was conditioned at 25 °C in a column oven. Data were recorded and evaluated by Chromeleon 7 software.

The mobile phase consisted of acetonitrile: buffer pH 3.5: methanol in ratio 57/38/5 (v/v/v). The mobile phase buffer was prepared according to the European Pharmacopea and filtered through a membrane filter (0.20 μm) using a Millipore glass filter holder. The flow rate was set to 0.8 mL/min with injection volume of 10 μL.

Validation parameters

The specificity is defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the developed technique was confirmed by checking of test solutions and the special solvent chromatograms (ICH Guidlines Q2 R1 2005).

Linearity

The linearity of the analytical method is based on the direct proportional dependence of the analytical signal on the concentration (amount) of the analyte in the sample within the analytical area of the method (ICH Q2 R1 2005). In order to prove the linearity of a method, the correlation coefficient must be above 0.99.

System suitability

To assess the system suitability, retention time of six injections of the standard solution, tailing factor and theoretical plates were used.

Sensitivity

Limit of Detection (LOD) is the smallest amount of analyte in a sample which can be identified, however not necessarily accurately quantified. Limit of Quantification (LOQ) is the minimum amount of a substance in a sample that can be quantified with the appropriate precision and accuracy. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were resoluted using calibration curve method by applying the following equals: $LOD = 3.3 \times Sa/b$ and $LOQ = 10 \times Sa/b$ (ICH Q2 R1 2005).

Precision

The ICH Q2 R1 Guideline defines the precision procedure as the closeness of results between a series of measurements for a sample taken from the same homogeneous solution under the specified conditions.

Accuracy

The accuracy of an analytical procedure expresses the closeness of the results obtained by the method to the true value (Borman and Elder 2017). To evaluate the accuracy of the proposed method, percentage recovery and %RSD values should be ranging from 98.0% to 102.0%, and not more than 2.0%, respectively.

Results and discussion

Development of the method

The stability of the targeted pyrrole hydrazone was evaluated through an RP-HPLC method developed and applied by Tzankova et al. (Tzankova et al. 2019b). This pointed our attention to employ in this study also a reversed phase HPLC method. In addition a column with geometric parameters 15 × 0.46 cm, 5 μm particle size, Purospher C18 Column with Phenomenex SecurityGuard C18 column was selected. The composition of the mobile phase was based on the analyte properties and blank solution content, using as a starting point the mobile phase described in Tzankova et.al. (Tzankova et al. 2019b), where for the hydrazone compound was assigned a peak with retention time of 6.110 min and for its hydrolysis product aldehyde – a retention time of 1.283 min. The method was found to be not applicable for our investigation, which required a reduction of the organic solvents - acetonitrile and methanol and increase of the volume of the pH 3.5 buffer. The performed changes determined as most suitable parameters for achieving good separation and suitable peak forms to be: mobile phase - CH_3CN/phosphate buffer pH 3.5/ CH_3OH: 57/38/5 (v/v/v). The flow rate, the temperature and the wavelength were also altered. 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 most adequate wavelength.

Validation of the method

Specificity of the method was evaluated by injecting $20 \mu l$ solutions of standard, sample, and blank separately. The

blank solution does not give interfering peaks at the retention time of analyzing compound.

Linearity was studied by analysis of standard solutions of the hydrazide- hydrazone compound at different concentrations using methanol as a solvent. The obtained data was analyzed after conducting 5 different levels of dilution (35, 70, 105, 140, 175 µg/mL). Thereafter, the results were used to build a calibration curve, with a subsequent calculation of the linear equation and the correlation coefficient. The aforementioned parameters are given in Table 1.

Table 1. Calibration data for hydrazide- hydrazone compound.

Compound	Calibration range (µg/mL)	Linear equation	Correlation coefficient
5a	35–175	y = 1.673×+ 13.082	0.9904

The obtained results confirm the linearity of the constructed method.

System suitability data was evaluated based on the chromatogram of the hydrazone solution. **Precision** was assessed by analyzing six samples with concentration of 70 µg/mL and the Relative standard deviation was calculated. Results are summarized in Table 2.

Table 2. System suitability of the developed RP/HPLC method.

Acceptance criteria	Limits	Result
Retention time (min) ± SD	-	24.652 ± 0.0059
Tailing factor	≤ 2	0.95
Theoretical Plates	≥ 850	5702
RSD of peak areas	≤ 2	0.581
LOD (µg/mL)	-	9.23
LOQ (µg/mL)	-	27.30

*LOD- Limit of detection; LOQ - limit of quantitation

The **accuracy** of the method was determined by recovery studies applying three concentration levels (50%, 100%, and 150%) and three samples from each concentration were injected. The obtained results demonstrated percentage recovery in the range of 99.9–100.8% at all three levels (Table 3). The percentage recovery and the %RSD values were within the accepted limits which affirmed the applicability of the method.

Table 3. Recovery data of the proposed HPLC method.

% Spiked level	Replicate number	Spiked level (µg/mL)	Recovery (µg/mL)	% Recovery
50	1	35	34.98	99.9
	2		35.12	100.3
	3		35.16	100.5
100	1	70	70.02	100.0
	2		70.58	100.8
	3		70.14	100.2
150	1	105	105.00	100.0
	2		104.87	99.9
	3		105.05	100.0
Mean (% of recovery)		98.0 – 102.0		100.192
% RSD		Max 2.0		0.304494

Evaluation and identification of the metabolism of the evaluated pyrrole hydrazide-hydrazone, incubated in rat hepatocyte suspension

As most appropriate compound for the preliminary evaluation of the metabolic transformations in isolated rat hepatocyte suspension was selected (E)-ethyl 5-(4-bromophenyl)-1-(1-(2-(2-hydroxybenzylidene)hydrazinyl)-1-oxo–3–phenylpropan-2-yl)–2–methyl–1H-pyrrole-3-carboxylate (Figure 1).

The developed RP-HPLC method was applied for determination of the initial hydrazide and salicyl aldehyde, which were hydrolytical products and possible metabolites. The identified retention times of the tested compounds are 5.74 min for the N-pyrrolylhydrazide and 2.10 min for the aldehyde.
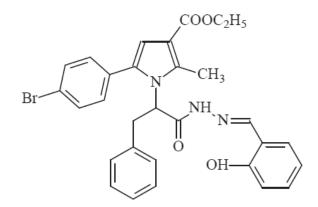

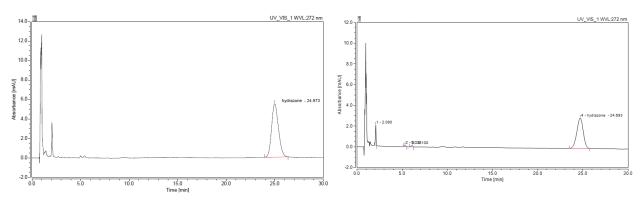**Figure 1.** Structure of the evaluated pyrrole hydazide-hydrazone.

Figure 2. Chromatograms demonstrating the hepatocytic metabolism of analyte at 30th min (**A**) and at 60th min (**B**).

For determination of the possible hydrazone`s metabolites the developed and optimized RP-HPLC-DAD method was applied. In the performed investigation after 30 min incubation in isolated rat hepatocytes, three new peaks appeared (Figure 2A). Two of the detected peaks corresponded to the retention times of the identified in the previous analysis salicyl aldehyde (2.08 min) and initial N-pyrrolylhydrazide (5.75 min) (Figure 2A).

As demonstrated in Figure 2 B, the concertation of studied molecule dropped to 38 μg while the area of the other peaks was increased in the 60th minute mark. After 2 hours of incubation, an appearance of new peak with retention time of 9.14 was detected (Figure 3).

The retention time of the new peak is not ascribable to any of the known and used as references molecules. This leads to the conclusion of possible formation of new unidentified metabolic product.

In silico prediction of metabolic pathways

In addition it was of interest to try to predict the possible structure of the unidentified metabolic derivative. For this purpose a virtual preliminary metabolic evaluation with the online server SMARTCyp (Rydberg et al. 2010) was done. The results identified 14 possible metabolites – 11 hydroxylated molecules and 3 epoxide derivates as presented in Table 4.

The performed RP-HPLC evaluation and *in silico* prediction determined that the metabolic transformation of the evaluated (E)-ethyl 5-(4-bromophenyl)-1-(1-(2-(2-hy-
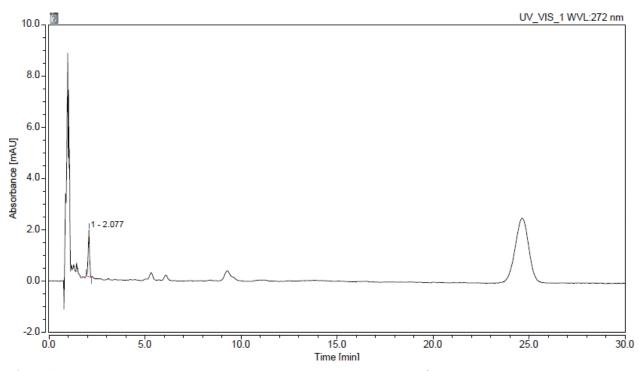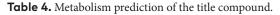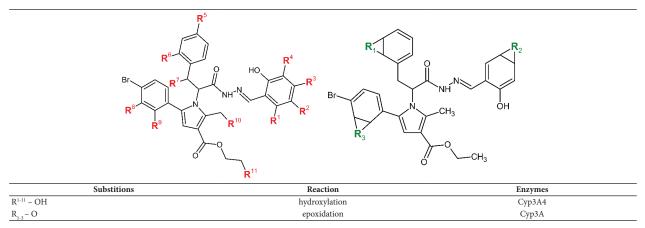

Figure 3. Chromatogram demonstrating the hepatocytic metabolism of analyte at 120th min.

Table 4. Metabolism prediction of the title compound.

Substitions	Reaction	Enzymes
R^{1-11} – OH	hydroxylation	Cyp3A4
R_{1-3} – O	epoxidation	Cyp3A

droxybenzylidene) hydrazinyl)- 1 oxo–3–phenyl-propan-2-yl) –2–methyl–1H-pyrrole-3-carboxylate is associated with hydrolytic degradation of the newly formed hydrazide-hydrazone group and additional hydroxylation or epoxidation in the presence of hepatocyte cells. The identification of the newly detected peak at 9.14 min will be a subject of additional research.

Conclusion

New optimized sensitive RP-HPLC method for identification of possible metabolic degradation of (E)-ethyl 5-(4-bromophenyl)-1-(1-(2-(2-hydroxybenzylidene)hydrazinyl)-1-oxo-3–phenylpropan-2-yl)–2–methyl–1H-pyrrole-3-carboxylate was developed and validated. The method was applied for tracking the metabolic changes of the evaluated pyrrole derivative. The method identified two initial from the synthesis molecules in higher concentration and one new unidentified structure as products of the hepatocytic processing of the evaluated analyte. The results identified as first step of metabolism the hydrolysis of the hydrazone group. Further investigations should be aimed into determining the next metabolic transformations, predicted by the *in silico* application of the web server SMARTCyp (Rydberg et al. 2010).

Acknowledgements

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