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Research Article

Optimization and validation of RP-HPLC method for evaluation of pyrrole -containing hydrazones in isolated rat synaptosomes

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Abstract

In the current study, RP-HPLC method for evaluation of 2 pyrrol-based hydrazones in isolated rat synaptosomes was optimized and validated according to ICH guidelines. The synaptosomes were obtained by multiple centrifugations with Percoll reagent and the selected 2 N-pyrollyl hydrazide-hydrazones were incubated for 2 hours at 37 °C. Subsequently, the purified fraction through protein precipitation was analyzed by an UltiMateDionex 3000 DAD system with Purospher STAR C18 (4.6 x 12.5 cm, 5 µm) column. The mobile phase, consisting of acetonitrile: phosphate buffer pH 3.5: methanol in ratio 42/36/22 (v/v/v), was eluted isocratically with 0.8 mL/min flow rate. Afterwards, the novel and rapid method was applied effectively for identification of biotransformation in isolated rat brain synaptosomes. The analysis results indicated an absence of new peaks and persistent sample concentration which determined the stability of the analyzed ethyl 5-(4-bromophenyl)-1-(2-(2-(2-hydroxybenzylidene) hydrazinyl)-2- oxoethyl)-2-methyl- 1H-pyrrole-3-carboxylate (**11b**) and ethyl 5-(4-bromophenyl)-1-(3-(2-(2-hydroxybenzylidene)hydrazinyl)-3-oxopropyl)-2-methyl-1H-pyrrole-3-carboxylate (**12b**) and pointed these structures as promising.

Keywords

Biotransformation, N-pyrrolyl hydrazide-hydrazones, Rat synaptosomes, RP-HPLC method

Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder, which is characterized by uncontrollable movements, such as shaking, stiffness, and difficulty with balance and coordination (Poewe et al. 2017). The prevalence and incidence of this illness in Europe are evaluated at approximately 108–257/100 000 and 11–19/100 000 per year, respectively (Balestrino and Schapira 2019). Therefore, the discovery and development of new small molecules is essential for the drug's therapy.

Monoamine oxidase B (MAO-B) is identified as the main target in the treatment of PD, comforting the interest towards this enzyme (Dezsi and Vecsei 2017).

In recent years, many studies have demonstrated that hydrazone derivatives have MAO-B inhibitory effects (Abid et al. 2017; Can et al. 2017; Evren et al. 2022). Moreover, a series of N-pyrollyl hydrazide-hydrazones

with promising MAO-B inhibitory activity, neuroprotective properties and good blood-brain barrier permeability were synthesized (Bijev and Georgieva 2010; Georgieva et al. 2012).

Biotransformation is the third step of the pharmacokinetic drug's journey through the body after oral administration. The liver is considered the major metabolic organ for endogenous and exogenous molecules. On the other hand, the available literary survey indicated that the enzymes in the brain might play a key role in the drug metabolism, which can lead to modified drug response and the risk of developing adverse drug reactions (Agúndez et al. 2014). The main metabolic path in synaptosomes biotransformation of xenobiotics is related to the attack from MAO enzyme complex (Bai and Witzmann 2007).

Therefore, the aim of this study is to gain a preliminary information on possible biotransformation processes affecting the two target N-pyrollyl hydrazide- hydrazones (ethyl 5-(4-bromophenyl)-1-(2-(2- (2-hydroxybenzylidene) hydrazinyl)-2- oxoethyl)-2-methyl-1H-pyrrole-3-carboxylate (**11b**) and ethyl 5-(4-bromophenyl)-1-(3-(2-(2-hydroxybenzylidene)hydrazinyl)-3-oxopropyl)-2-methyl-1H-pyrrole-3-carboxylate (**12b**)) (Fig. 1) with identified promising MAO-B inhibitory activity, neuroprotective properties and good blood-brain barrier permeability.

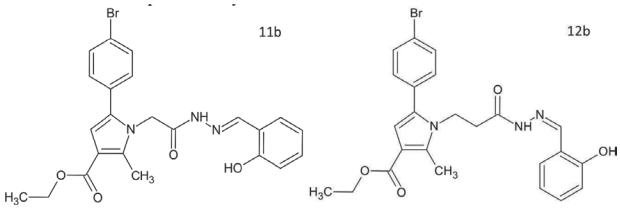


Figure 1. Structures of the selected compounds 11b and 12b.

Materials and methods

Reagents and chemicals

All reagents for chromatographic procedure and sample preparation were purchased from Merck Darmstadt, Germany. The methanol and acetonitrile used in analyses were HPLC gradient grade and were obtained from Fisher Chemical, UK. The phosphate buffer was prepared by dissolving 5.04 g Na_2HPO_4 and 3.01 g KH_2PO_4 in 1 L distilled water. To adjust the pH to 3.5, the orthophosphoric acid was used. The selected molecules 11b and 12b used in this study were previously synthesized and described in works of Bijev and Georgieva (2010) and Georgieva et al. (2012).

Animal experiments and chromatographic conditions

Synaptosomes were prepared from the brains of adult male Wistar rats through multiple, subcellular fractionation using a Percoll gradient, as previously described in work from Taupin et al. 2014 Thereafter, the isolated subcellular fraction was incubated for 2 hours in an appropriate buffer with 200 µL of 1 mM solutions of selected compounds. The procedure for sample purification included a rapid and simple protein precipitation using HPLC grade methanol followed by centrifugation at 14.000 rpm and filtration. The samples were initially analyzed using UltiMateDionex 3000 equipped with a diode array detector set up at 279 nm. The data was processed by Chromeleon 7.2 software. The analytes were separated on a Purospher STAR RP-18 (4.6 × 12.5 cm, particles size 5 µm) column with 0.8 mL/min flow rate of the mobile phase, which consisted of acetonitrile: phosphate buffer pH 3.5: methanol in ratio 42/36/22 (v/v/v).

Method validation

Method validation was carried out according to the requirements of the ICH guideline. The parameter linearity was evaluated using a calculated correlation coefficient, which value must be greater than 0.99. To estimate the repeatability of the method, relative standard deviation (RSD≤2) was generated from peak areas of 6 samples at 100% working concentration. The validation proceeded with an assessment test for accuracy. This necessitated triplicate analysis of solutions at three concentration levels (50, 100 and 150%) with subsequent determination of the Recovery %. The limits of detection (LOD) ant quantitation (LOQ) were calculated based on the method of the standard deviation of a response and the slope using the following equations:

$$LOQ = \frac{10\alpha_b}{S} \qquad LOD = \frac{3\alpha_b}{S}$$

where σ_b- the standard deviation of the response; S- the slope of the calibration curve

Results and discussion

Optimization of the chromatographic conditions

Our previous study (Mateeva et al. 2022) involved development and application of RP-HPLC method for possible hepatic metabolism evaluation of the pyrrole-containing hydrazide-hydrazone with similar structures (Tzankova et al. 2020). The separation was performed on a column Purospher C18 (15 × 0.46 cm, 5 µm) conditioned at 25 °C. The elution was isocratic with a mobile phase of acetonitrile: buffer pH 3.5: methanol in ratio 57/38/5 (v/v/v). This pointed our attention to utilize a shorter column (12.5 cm) of Purospher, Phenomenex with the same mobile phase. The results demonstrated retention times of

about 5 minutes for both hydrazones. Due to more polar properties of the expected metabolites, we concluded that the method was not suitable for our investigation. Thus, the experiments continued with a change in the volume ratios of the mobile phase. Several diverse combinations were applied in order to achieve the optimal retention times and good peak's shape of the **11b** and **12b**. This was accomplished through a mobile phase, which consisted of acetonitrile: buffer pH 3.5: methanol in ratio 42/36/22 (v/v/v). The temperature in the column compartment was also switched to 20 °C. The recording of UV/VIS spectra of the selected molecules revealed the most adequate wavelength at 279 nm.

The above mentioned changes in chromatographic parameters let to further validation of the method.

Method validation

Validation was prepared according to ICH guideline Q2 (R2) and the parameters as specificity, linearity, range, accuracy, precision and sensitivity were established.

The specificity of the method was evaluated through analysis of the blank solution containing isolated synaptosomes. The obtained chromatogram (Fig. 2) of the purified and pre-treated biological sample did not reveal any interfering peaks ascribable to the protein contents.

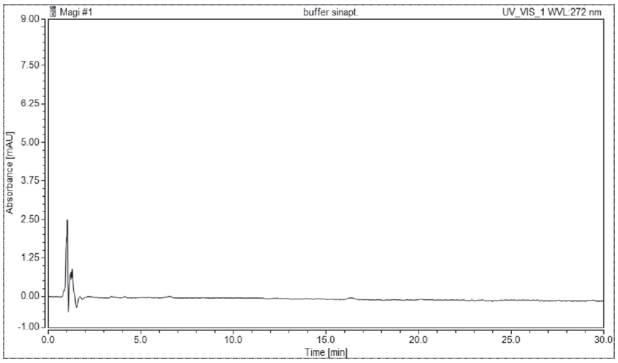


Figure 2. Chromatogram of the pre-treated biological media.

The lack of peaks with retention times close to, or equal to the ones of the analyzed samples determined the procedure as suitable for subsequent investigation.

To estimate the validation parameters linearity and range, six working solutions of each hydrazone with linearly increasing concentration were analyzed. The results were used to generate the calibration curves by plotting the peak area against the concentrations of **11b** and **12b**. Subsequently, the linear equation and correlation coefficient were calculated. The results are summarized in Table 1 and demonstrated good correlation in the investigated range.

Table 1. Results from linearity study.

	Linear equation	Correlation coefficient
11b	y=0.3871x+0.1118	0.9996
12b	y= 1..014x+0.2836	0.9994

Sensitivity was also evaluated by calculating the Limit of Detection (LOD) and Limit of Quantification (LOQ) using the aforementioned equations. The values of the latter parameters are 0.8825 and 2.6743 μM for 11b and 1.1022 and 3.0402 μM for 12b, respectively.

Precision of the method was estimated via calculation of the RSD. The obtained results of the latter, with values 0.5326 for 11b and 0.4952 for 12b, proved acceptable repeatability of the method. Accuracy was established by triplicate analyzing three different levels (50,100 and 150%) for both hydrazones. The results are presented in Table 2.

Table 2. Recovery data of the current method.

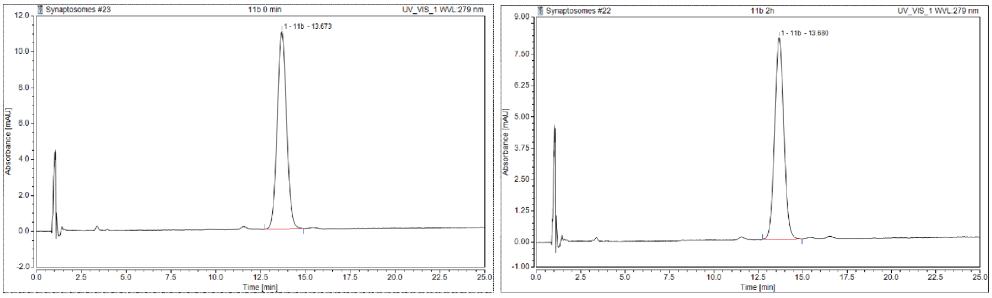
	Level, (%)	Spiked level, μM	Recovery, μM	Recovery, (%)
11b	50%	7.5	7.47	99.60
			7.54	100.53
			7.51	100.13
	100%	15	14.98	99.87
			14.92	99.47
			14.87	99.13
	150%	22.5	22.41	99.60
			22.36	99.38
			22.37	99.42
12b	50 %	7.5	7.55	100.67
			7.61	101.47
			7.43	99.07
	100%	15	15.00	100.00
			14.92	99.47
			14.94	99.60
	150%	22.5	22.49	99.96
			22.51	100.04
			22.37	99.87

Application of the method to evaluate the compounds' biotransformation in isolated rat synaptosomes

In an attempt to identify the possible appearance of biotransformation and the kinetics of the process, the selected molecules were incubated for 2 hours in isolated rat brain synaptosomes and aliquot samples were collected every 30 min. The drawn quantities were subjected to the purification process indicated in the Materials and Methods section. Prior to analysis, the supernatant was double filtered through PVDF sterile syringe filters (through 0.47 μm, and through 0.22 μm) and a volume of 10 μL was injected into the chromatographic system.

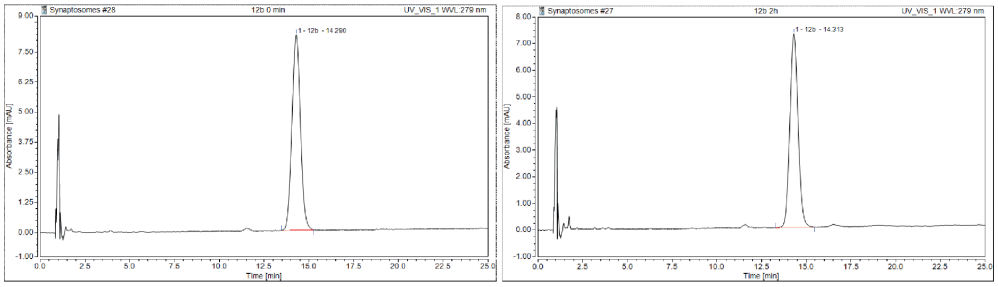
The obtained chromatograms (Figs 3, 4) disclosed negligible decrease in concentration of the analytes and absence of new peaks.

The observed chromatographic behavior of the tested hydrazones is believed to be due to the lack of a target group for the monoamine oxidase - the main enzyme in isolated subcellular culture.



(A) (B)

Figure 3. Chromatograms of compound **11b** at 0 (**A**) and 120th (**B**) minute.



(A) (B)

Figure 4. Chromatograms of compound **12b** at 0 (**A**) and 120th (**B**) minute.

Conclusion

In the current research, the rapid RP-HPLC method was successfully applied for detection of possible metabolites in isolated rat brain synaptosomes. The analysis results indicated an absence of new peaks and persistent sample concentration which determined the stability of the analyzed ethyl 5-(4-bromophenyl)-1-(2-(2- (2-hydroxybenzylidene) hydrazinyl)-2- oxoethyl)-2-methyl-1H-pyrrole-3-carboxylate (**11b**) and ethyl 5-(4-bromophenyl)-1-(3-(2-(2- hydroxybenzylidene)hydrazinyl)-3-oxopropyl)-2-methyl-1H-pyrrole-3-carboxylate (**12b**) and pointed these structures as stable.

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