DETERMINATION OF HYDROLYTIC STABILITY AND ACUTE TOXICITY OF PREVIOUSLY SYNTHESIZED PYRROLE-BASED HYDRAZONES

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Summary: Appropriate pyrrole derivative, containing susceptible to hydrolysis hydrazone group, was chosen to represent a series of inhibitors of *M. tuberculosis*. The model compound was incubated at 37°C in mixtures of dioxane : buffer (pH of 1.2, 7.0 and 9.0) and aliquot samples were drawn at definite time intervals. The absorbance at 282 nm was measured to determine possible hydrolytic cleavage of the hydrazone group. No change was detected at pH=7.0, whereas a decrease in the absorption was observed at pH = 1.2 and pH=9.0. The results witness for a chemical stability of the model compound at moderate pH and temperatures, but for a sensibility to acidic or low alkali media.

The acute intraperitoneal and per oral toxicities were also investigated, and the corresponding Indexes of Resorbtion (IR) were calculated for a selected group of representative compounds. The evaluated structures were proved to be 7-10 times less toxic than the reference Isoniazid. For most of the structures the calculated IR was 50%, but there were some with higher IR up to 60%.

Key words: Pyrole hydrazones, Hydrolysis, Stability, Acute toxicity

ОПРЕДЕЛЯНЕ НА ХИДРОЛИЗНАТА СТАБИЛНОСТ И ОСТРАТА ТОКСИЧНОСТ НА ПИРОЛ-СЪДЪРЖАЩИ ХИДРАЗОНИ

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Резюме: Подходящи производни на пирола, съдържащи чувствителната на хидролиза хидразнова група бяха подбрани като представители на серия продукти с инхибираща активност спрямо *M. tuberculosis*. Моделно съединение бе инкубирано при 37°C в смес от диоксан: буфер (с pH 1.2, 7.0 и 9.0) и аликвотни проби бяха отпипетирани на определени времеви интервали. Абсорбция при 282 nm бе измерена с цел измерване на вероятно хидролитично разкъсване на хидразоновата група. Не бе наблюдавана промяна в абсорбцията в буфер с pH=7.0, докато в абсорбцията на разтвори с буфер pH = 1.2 и pH=9.0 бе наблюдавано намаляване в абсорбцията. Тези резултати са доказателство за стабилността на моделното съединение при умерени стойности на pH и температурата, но за неговата чувствителност към кисела (pH=1.2) или слабо алкална (pH=9.0) среда.

В допълнение бе изследвана острата интраперитонеална и пер-орална токсичност и бе изчислен съответният Индекс на Резорбция (ИР) на подбрана група представителни съединения. Бе установено, че разглежданите структури са 7-10 пъти по-слабо токсични от Isoniazid, използван като сравнително вещество. За повечето от разглежданите структури бе изчислен ИР 50%, но за някои от тях ИР е установен за по-висок – 60%.

Ключови думи: Pyrole hydrazones, Hydrolysis, Stability, Acute toxicity

Introduction

Acccording to the 13th annual tuberculosis report of the World Health Organization (WHO) published on World TB Day, March 24, 2009 — there were an estimated 9.27 million new cases of tuberculosis worldwide in 2007 [6]. This figures and the progressive worsening of resistance of TB bacilli to the current pharmacotherapy has raised the efforts in developing new anti-tuberculosis agents, which are less susceptible to resistance and highly effective against HIV-TB co-infection [16].

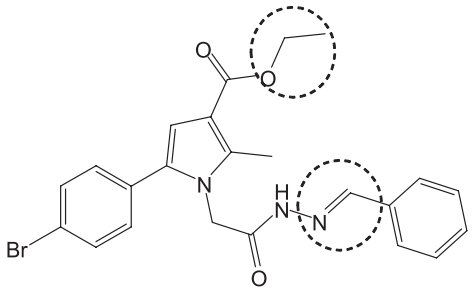
Motivated to combine the active principles of pyrrole- and hydrazone moieties, we have synthesized consecutively several series of hydrazone derivatives of pyrrole for evaluation as potential tuberculostatics [1, 4, 5]. The encouraging results obtained from these investigations were used as a reliable starting platform in further design and synthesis of potential anti-tubercular agents.

For optimal performance, biologically active substances such as pharmaceuticals, agrochemicals, and also flavours or fragrances have to be efficiently delivered to their target site and usually released at a well-defined rate [13]. Another important factor influencing this performance is their hydrolytic stability at physiological conditions, such as: body temperature of 37°C and physiological pH of 1.2 (in stomach), 7.4 (in blood plasma) and 8.5 – 9.0 (in small intestine) [11].

One of the main parameters determining the application of a biologically active compound as a therapeutic agent is its acute toxicity. Since up to this moment there are no available data for the toxicity of the pyrrole-based hydrazones proposed as antibacterials it was of interest to evaluate the acute toxicity of this class of compounds.

Selection of a model compound.

Previously synthesized hydrazones contain pyrrole cycle, bearing a variety of functional groups as ethoxy-carbonyle, acetyl, carboxamide etc. It is a well known fact, that the fully substituted pyrrole cycle, as well as the connected with it methyl groups are stable at moderate temperatures and in wide range of pH values. Thus as potential "vulnerable" groups susceptible to hydrolysis may be considered the ester and to lower extend the amide group [9]. However, as common conjugates hydrazones possess also the labile to hydrolysis hydrazone group [10]. Previous investigations with analogous compounds have already proved the ester and amide groups considered above to be stable at the modeled physiological conditions [17]. Therefore, the hydrazone group stands out as the only substantial moiety likely to be susceptible to hydrolysis. Thus, the following model compound bearing two hydrolytically susceptible groups (hydrazone and ester) was selected:



Ethyl 1-(2-(2-benzylidenehydrazinyl)-2-oxoethyl)-5-(4-bromophenyl)-2-methyl-1H-pyrrole-3-carboxylate

The model structure was in compliance with the ease of UV/VIS changes measurements.

Selection of model structures for toxicological analysis.

The acute toxicity of pyrrole-based hydrazones has not been investigated before. In order to prove the influence of peculiar structural elements upon the toxicity, a series of representative model structures was selected comprising diverse halogens (Hal) at position 4 and diverse length of the "spacer" n between the pyrrole N-atom and the attached carbonyl residue:

- hydrazone **01d** [2]
  (with no halogen substituent and "spacer" n = 1);
- hydrazones **7a** and **7b** [4]
  (Hal = Br and "spacer" n = 1);
- hydrazones **8a** and **8b** [7]
  (Hal = Br and "spacer" n = 2);
- hydrazones **1a**, **1b** and **1d** [2]
  (Hal = Cl and "spacer" n = 1);
- hydrazones **2a** and **2b** [3]
  (Hal = Cl and "spacer" n = 2);
- hydrazone **12a** [4]

(Hal = Cl and "spacer" n = 3) and

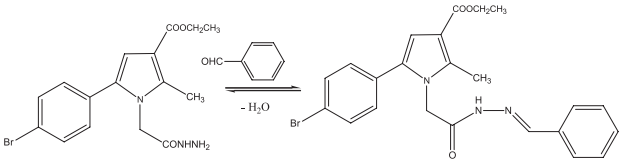
- hydrazone **4d** [1] (Hal = F and "spacer" n = 1).

Materials and Methods

Hydrolytic stability investigations

Synthesis of the model structure

For our investigations the model hydrazone was synthesized through condensation of the relevant hydrazyde (ethyl 5-(4-bromophenyl)-1-(2-hydrazinyl-2-oxoethyl)-2-methyl-1H-pyrrole-3-carboxylate) and benzaldehyde, reacting as a respective carbonyl partner, according to the following scheme:



Scheme 1. *Synthetic scheme of the model hydrazone structure.*

The synthesis was performed in acetic acid medium at heating an equimolar ratio of the participating reagents. The target product was isolated in water, and then purified by recrystallization in ethanol.

Hydrolytic stability evaluation

In order to overcome the poor solubility of the hydrazones in water, a dioxane : water solution 8:2 was prepared. For the purpose of this study, the necessary dioxane: buffer solutions were prepared at the same relevant ratio, in order to obtain the desired pH values, close to physiological. The appropriate amounts of the model compound were weighed and dissolved in the corresponding mixture of dioxane: buffer 1.2, 7.0 and 9.0 in a way that the concentration is in the range of 5.10^{-6} mol/l. The obtained solutions were stirred at 37°C for a total time of 720 min. Aliquot samples of 2 ml of the analyzed solutions were taken at definite time intervals (0, 65, 170, 275, 445 and 720 min) and the corresponding UV/VIS spectra were recorded. The absorbance at 282 nm was detected as a measure of the expected structural change due to a possible hydrolytic cleavage of the hydrazone group (as mentioned above).

Pharmacological tests

Animals

Male albino mice, line H, 25-30g b.w. (Institute of Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria) were used for acute intraperitoneal and per oral toxicity studies. The animals were housed individually, water and food being supplied *ab libitum*; animal room temperature 22°C ± 3°C; humidity 30%; lighting schedule 12 h light/dark cycle. Prior to administration, animals fasted for one day.

All the experimental procedures were conducted according to the NIH Guidelines of the Care and Use of Laboratory animals (USA) and approved by Ethical Committee for Care and Management of Laboratory Animals at Faculty of Pharmacy – MU, Sofia, Bulgaria.

Acute intraperitoneal toxicity

The compounds were suspended using Tween 80. Acute intraperitoneal toxicity studies were performed according to the OECD Guideline 425 "Up and Down procedure" with computer program calculations [15].

Acute per oral toxicity

The compounds were suspended in distilled water, using 2-3 drops of Tween 80. The solution was administered via per oral route in 0.1 – 0.2 ml of 10g animal body weight. Acute per oral toxicity studies were performed according to the OECD Guideline 425 "Up and Down procedure" with computer program calculations [15] and presented as mg/kg body weight (b.w.).

The corresponding Index of Resorbtion was calculated as the ratio:

$$IR = LD50\ i.p. / LD50\ p.os^{*}\ 100\ (\%).$$

Results

Hydrolytic stability study

Three solutions were prepared according to the above mentioned procedure: **Solution 1** with solvent dioxane: hydrochloric acid buffer giving a pH value of 1.2 – close to the physiological pH in the stomach; **Solution 2** with solvent dioxane: phosphate buffer

giving a pH value of 7.4 – equal to the physiological pH in the blood plasma and **Solution 3** with solvent dioxane: citrate buffer giving a pH value of 9.0 - close to the physiological pH in the small intestines. The obtained values for the corresponding absorbance at 282 nm were recorded are given in **Table 1**, **Table 2** and **Table 3** respectively.

Aliquot №	Time interval (min)	Absorbance at 282 nm
1	0	0.351
2	65	0.268
3	170	0.260
4	275	0.218
5	445	0.244
6	720	0.223

Table 1. *Measured absorbance at 282 nm for the Solution 1 at the cited time intervals.*

Aliquot №	Time interval (min)	Absorbance at 282 nm
1	0	0.290
2	65	0.281
3	170	0.322
4	275	0.282
5	445	0.295
6	720	0.285

Table 2. *Measured absorbance at 282 nm for the Solution 2 at the cited time intervals.*

Aliquot №	Time interval (min)	Absorbance at 282 nm
1	0	0.480
2	65	0.365
3	170	0.371
4	275	0.367
5	445	0.361
6	720	0.372

Table 3. *Measured absorbance at 282 nm for the Solution 3 at the cited time intervals.*

The corresponding UV /VIS spectra were obtained, for the above mentioned solutions. On the following figure (**Figure 1**) a representative part of the spectra of **Solution 1** and **Solution 2** is given, in order to present the graphical appearance.

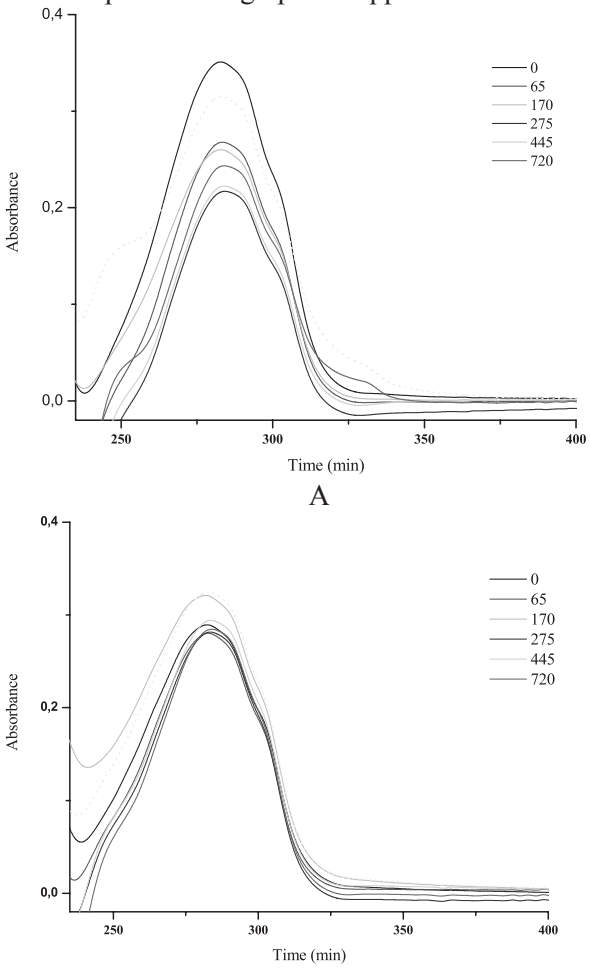


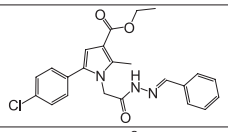
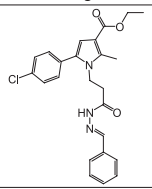
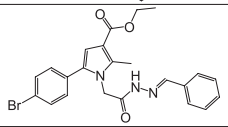
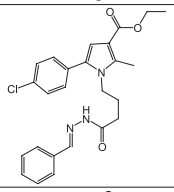
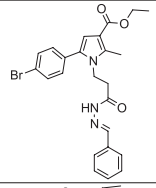
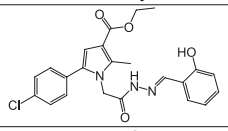
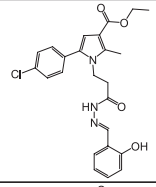
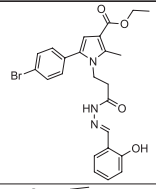
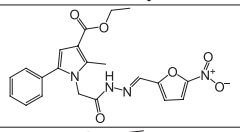
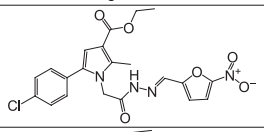
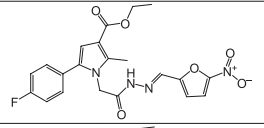
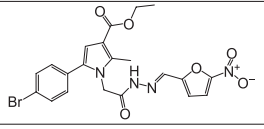
Fig. 1 *The corresponding UV/VIS spectra recorded for the relative aliquots of the model compound at 37°C and pH=1.2 (A) and pH=7.0 (B) at the specified time intervals.*

Pharmacological study

Adult male albino mice, line H (18-22g) were used for acute intaperitoneral and per oral toxicity evaluation. Isoniazide was applied as reference substance.

The registered results (in the form of LD_{50} mg/kg b.w. values after intraperitoneal (i.p.) and per oral (p.os.) administration) are presented in Table 4, along with the corresponding values for the calculated Index of Resorbtion (IR) for some of the investigated compounds.

Table 4.
Acute toxicity (LD_{50}) of the selected compounds after intraperitoneal and per oral administration on mice.

№	ID	Chemical structure	LD50 i.p. (mg/kg)	LD50 p.os.(mg/kg)	IR (%)
1.	1a		>1500	-	-
2.	2a		510	1000	51
3.	7a		750	-	-
4.	12a		1200	-	-
5.	8a		730	-	-
6.	1b		760	1500	50.6
7.	2b		1200	2000	60
8.	8b		1020	-	-
9.	01d		>1500	2500	60
10.	1d		1249	-	-
11.	4d		740	>1500	49.3
12.	7d		1500	>2500	60
INH			151	176	85.8

The obtained results for the toxicity of the evaluated pyrrole containing hydrazones were compared to the toxicity of **Isoniazid (INH)**, used as a reference compound.

Discussion

Hydrolytic analysis.

A number of data show, that the hydrazone hydrolysis is connected mainly with cleavage of the C=N double bond from the hydrazone group leaving the parental hydrazide and the relevant carbonyl compound (aldehyde or ketone) [17, 12, 14].

As shown in the literature [10] the most probable mechanism for hydrazone is consistent of $C^1=N^1$-NH-CO-R hydrolysis that entails protonation of N^1. The protonated species would be highly susceptible to hydrolysis, because of the enhanced electrophilisity of C^1. The result is a hydrolytical cleavage of the hydrazone C=N double bond leading to the parental hydrazide and the corresponding benzaldehyde.

The decrease in the measured absorbance at 282 nm, observed in the spectra of the model compound for **Solution 1** and **Solution 3** shows, that hydrolysis occurs in the first 65 min of the incubation at the corresponding pH values (1.2 for **Solution 1** and 9.0 for **Solution 3**). On the other hand, no visible decrease in the absorbance was observed in **Solution 2** (pH = 7.4) measurements, which indicates lack of hydrolysis at this conditions. This observation may be presented with the following graph (**Fig. 2**):

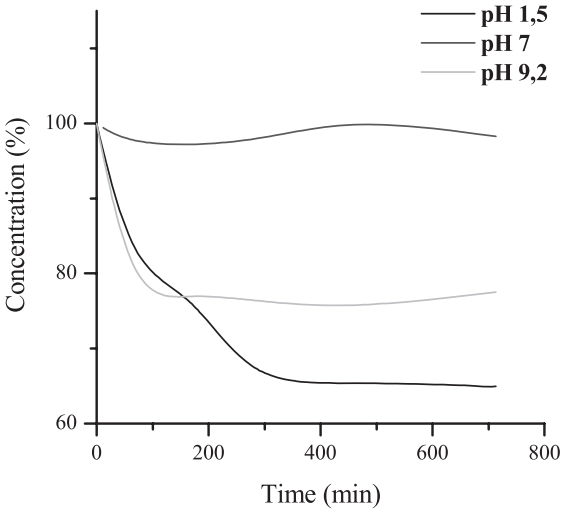


Fig. 2 *Time dependence of the measured absorption at 282 nm for the model compound solution in Series 1 (pH=1.2), Series 2 (pH=7.4) and Series 3 (pH=9.0).*

Toxicological analysis

The presented results determined the tested structures to be 7-10 times less toxic then Isoniazid, used as a reference. According to the determined scale for toxicity, LD_{50} of Hodge and Sterner [8] the products may be categorized as low toxic (between 500 and 5000 mg/kg body weight). This is extremely favorable reference as for the evaluated products so as for the entire class of compounds in which they are included. The low toxicity of the pyrrole containing hydrazones thus defines them as perspective structures for further pharmacological and toxicological investigations.

From the values of the calculated Index of Resorbtion (IR) may be concluded, that most of the compounds are resorbed approximately 50%. For some of them (**2b, 01d, 7d**) the resorbtion is higher – 60%. This may be due to their poor solubility in water. Thus the calculated IR and the obtained values for per oral toxicity are a premise for the further application of the products in higher doses with no toxic reactions originating from that.

Structure – toxicity relationships

a) Effect of the 4-halogen substituent

From the results presented above was established, that the nature of the halogen atom has a significant influence on the toxicity of the compounds. As seen from the graph given below for compounds containing Br as a substituent in the aromatic ring (**7d**) the lowest toxicity is observed, followed by Cl – substituted (**1d** and **4d**) compounds. Derivatives containing F atom as halogen substituent at position 4 in the aromatic ring (**01d**) were found to be most toxic (**Fig. 3**).

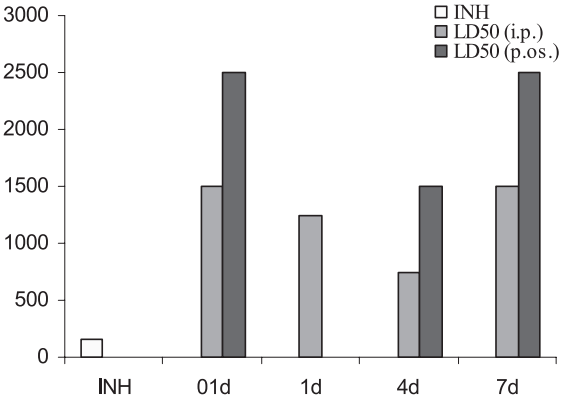


Fig. 3 *Influence of the halogen substituent on the toxicity.*

b) Effect of the "spacer" n

The length of the "spacer"n has no indicative influence on the toxicity.

Conclusion

Pyrrole hydrazones represented by the investigated model may be considered stable at physiological pH close to the neutral value of 7.0. A hydrolytic decomposition due to a splitting of the hydrazone bond was found to occur both in acid (pH =1.2) and alkaline (pH=9.0) media and should be taken into account in future optimizations within the group.

According to the evaluation results performed on the selected representative compounds, pyrrole-based hydrazones could be considered as low toxic, whereat the nature of 4-halogen substituent increases the toxicity in the sequence Br < Cl < F. The typical Index of Resorption was proved about 50%. This defines the compounds as very prospective for further pharmacological and toxicological investigations.

The results of the study could be useful at further design and optimization of lead compounds in the search of new tuberculostatics.

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