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NEW STRATEGIES FOR THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF *NITROFURAL* AND ITS NEW DERIVATIVES

DISSERTATION

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EXTENDED ABSTRACT

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The dissertation was reported on and discussed at a meeting of the Department of "Pharmaceutical Chemistry" at the Medical University of Varna in the presence of an expanded council which came to a decision for its public defense.

The experimental work on the dissertation was performed mostly at the Medical University - Varna. Certain studies were conducted in collaboration with the Bulgarian Academy of Sciences.

The dissertation covers 110 pages, 72 figures, 6 tables, 10 diagrams, and 3 appendices. The bibliography includes 240 titles.

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I. INTRODUCTION

Over the last few decades, polymorphism has become increasingly important in global pharmaceutical production. Today, in pharmaceutical practice, the concept of polymorphism, is the knowledge that, polymorphism is the most crucial factor determining the biological effect and ultimate therapeutic efficacy of many drugs. Polymorphism, in the hands of those synthesizing crystals, can be seen as the primary tool for fine tuning of the therapeutic benefits of a number of drugs. Polymorphism can also be a factor in reducing or completely eliminating a number of undesired side effects.

Targeted polymorphic synthesis is an important technological element of the general pharmaceutical production; an element with which even the necessary medicinal benefit/manifestation can be met.

Polymorphic analysis is rationally included into the content of the modern Pharmacopoeia as a mandatory and irrevocable element; as a part of the general analytical workshop.

Qualitative pharmacopoeial analysis is not less important. It aims at reaching a higher level of certainty in the identification of each pharmacopoeial representative. Today, generally the new analytical reactions used are strictly specific and sought after; namely those are reactions leading to the appearance of visual/perceptible changes unique to the tested pharmacopoeial analytes.

Of no less importance is the need for the emergence of new, more active and selective drugs. The necessity for the synthesis of new anti-infectives is closely related to the ongoing manifestation of bacterial resistance.

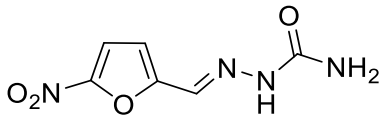
II. THEORETICAL PART

One of the significant achievements in the field of chemistry of anti-infective drugs is related to the discovery of 5-nitrofuran derivatives. Given their broad antibacterial spectrum, relatively low toxicity, and their action against resistant bacterial strains, they successfully compete with the most widely used antibiotics at the moment - penicillins, cephalosporins, macrolides and others. The broad-spectrum microbiological profile, on the one hand, and the low cost, on the other, define this drug class as extremely valuable for human and veterinary medical practice.

It is accepted that 5-nitrofuran derivatives belong to the group of anti-infective substances due to the fact that the first synthesized representative - *Nitrofural* (1946), continues to be used today as an effective antiseptic/anti-infective agent.

Nitrofural is a broad-spectrum anti-infective drug; representative of the contemporary *European Pharmacopoeia* [1,2].

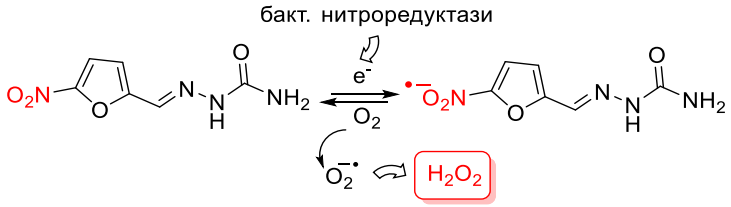
Nitrofural



Its bactericidal action affects the development of *Gram*-positive and *Gram*-negative flora, including *Staphylococcus spp., Streptococcus spp., Shigella dysenteriae, Shigella flexneri, Shigella boydii, Shigella sonnei, Escherichia coli, Clostridium perfringens, Salstri*. etc.

It also has antiprotozoal and antiparasitic effects [1,3]. It has also been shown to inhibit the activity of fungal flora.

The clinical efficacy of *Nitrofural* is related to its tendency to form, *in vivo*, hydrogen peroxide (Scheme 1) [4].



Scheme 1. Equation illustrating the interaction of *Nitrofural* with bacterial nitroreductases.

Although the mechanism of the bactericidal action of nitrofuran derivatives is still not understood fully, today it is considered that the partial reduction of their -NO_2 groups from bacterial nitroreductases (specialized enzymes) is essential for their activity [5,6].

Nitrofural has been used to treat superficial wounds covering all parts of the body. In practice, almost all types of injuries are treated with it - military, surgical, accidental, pathological lesions, etc.

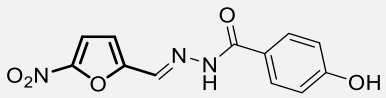
It is the drug of choice in the treatment of infections caused by *antibiotic*- and *sulphonamide*-resistant bacteria. In addition, no bacterial resistance to *Nitrofural* has been reported to date.

Data on *Nitrofural* synthesis.

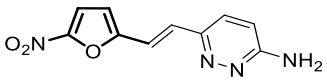
For the first time in 1943-1944, two USA scientists (M. Dodd and W. Stillman) reported that, the introduction of a nitro group at the 5th position of the furan nucleus produces a strong bacteriostatic and bactericidal effect onto the compounds thus synthesized [7].

Subsequently, the first official drug representative of that group was obtained - *Nitrofural*. Its undoubted benefits when treating life-threatening infections were the reasons that lead to the introduction of new 2-substituted-5-nitrofuran drugs. In this regard, over the years a number of 5-nitrofuran drugs have been launched on the pharmaceutical market - *Nifuroxazid*, *Nifurprazin*, *Nifurtimox*, *Nifurtoinol*, *Nifurzid*, *Nitrofurantoin*, *Furazolidone*, *Furazidin* and others.

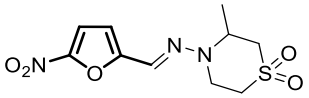
Nifuroxazide



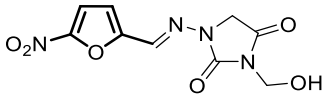
Nifurprazine



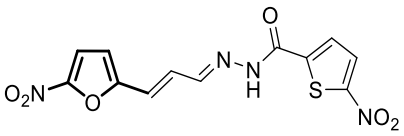
Nifurtimox



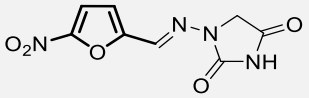
Nifurtoinol



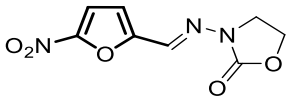
Nifurzide



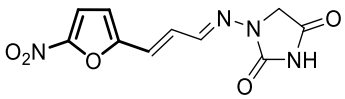
Nitrofurantoin



Furazolidone



Furazidine



Some of them are currently obsolete, while others are still present in the European Pharmacopoeia (*Nitrofurantoin*, *Nifuroxazide* and, of course, *Nitrofural*, discussed here). *Nifurtimox* in the form of oral tablets (Lampit® Bayer) is currently being used to treat African trypanosomiasis.

Nitrofural synthesis is carried out by the following method (Figure 1) [8]:

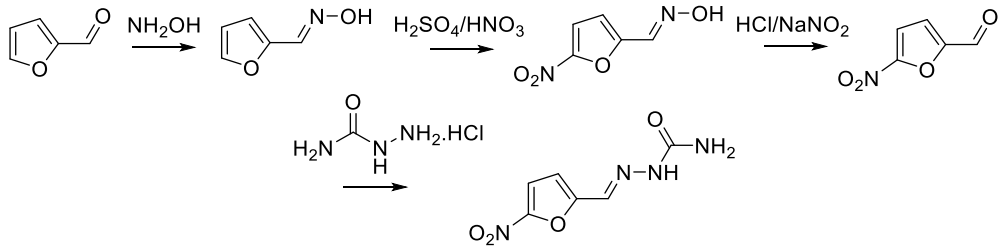


Figure 1. Synthesis of *Nitrofural*.

According to above method, *Nitrofural* after recrystallization, melts at 240 °C - a temperature higher than the expected (probably associated with the synthesis of its unknown polymorphic form). In practice, however, it is customary to use *Nitrofural* products with a melting point about 227 ÷ 232 °C which is lower than specified.

In bulk, *Nitrofural* is a yellow or yellow-brown crystalline powder that darkens an exposure to light. Its water solubility (1: 4200) extremely low. The compound is slightly soluble in 96% ethanol (1:590) and propylene glycol (1:35); and dissolves well in dimethylformamide and dimethyl sulfoxide. It is practically insoluble in chloroform and diethyl ether and dissolves partly in fats. *Nitrofural* melts with decomposition at 236 ÷ 240 °C [9]. pKa 10.0. Log P (octanol/water) 0.2.

***Nitrofural* Analysis.**

According to the European (including British) Pharmacopoeia [2], the identification of the pharmacopoeial substance *Nitrofural* is performed by the methods of:

• UV spectroscopy (UV),

• Infrared spectroscopy (IR),

• Thin Layer Chromatography (TLC),

• High Performance Liquid Chromatography (HPLC) and

• Classical qualitative analysis.

The photosensitivity of *Nitrofural* is the reason why its UV (qualitative and quantitative) analysis is performed in a place protected from bright light.

The presence of concomitant impurities is detected by HPLC. The following two expected impurities are commonly observed: 1,2-bis[(5-nitrofuran-2-yl)methylidene]diazane and the starting reagent for the synthesis of *Nitrofural*, (5-nitrofuran-2-yl)methylene diacetate (Figure 2).

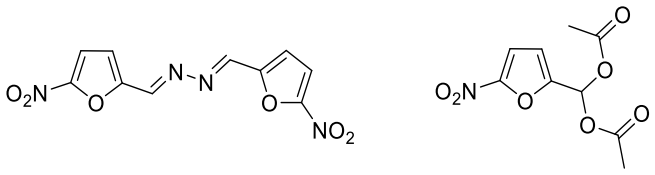


Figure 2. Expected *Nitrofural* impurities.

The overall *Nitrofural* analysis is complemented by tests for: sulphated ash (maximum 0.1%), drying losses (not to exceed 0.5%), and determination of the hydrogen index of its aqueous solution (pH 5.0 to 7.0) [2].

Qualitative pharmacopoeial *Nitrofural* analysis is performed in dimethylformamide medium and in the presence of potassium ethanolate (Figure 3).

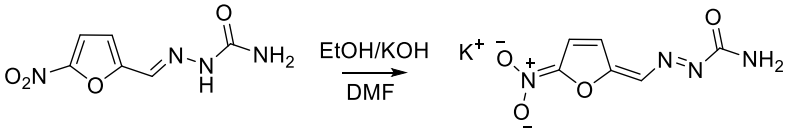


Figure 3. Pharmacopoeial *Nitrofural* analysis.

The resulting product is violet-red.

However, from an analytical point of view, each drug substance must be subjected to unquestionable quality control/analysis, which aims at a full confirmation of its chemical

structure as a whole or of its structural functional groups. Qualitative pharmaceutical analysis aims at proving the identity of medicinal substances with the so-called "acceptable" level of certainty.

In this connection, the analytical (colour) reactions to the less common hydrazone (RCH=N-NR'R'') structural residue should also be investigated.

The differences in the electrochemical behaviour of the three pharmacopoeial nitrofuran representatives, on the other hand, should reveal the possibility of their qualitative analytical differentiation.

Crystal structure. Polymorphism.

The crystal structure is a type of arrangement of the constituent particles (atoms or more generally molecules) of any solid, in which there is a repeatability of a particular model.

Polymorphism is the ability of some substances to form different crystal structures by changing some parameters of the crystallization medium (temperature, pressure, etc.). Polymorphic modifications are crystal-structural modifications.

There is a separate point (5.9) in the content of the European Pharmacopoeia (Edition 10.0) set aside for polymorphism, stating the following [2]:

"Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state; it is the ability of a compound in the solid state to exist in different crystalline forms having the same chemical composition. Substances that exist in a non-crystalline solid state are said to be amorphous."

The influence of polymorphism on the solubility and dissolution rate of organic drugs and their bioavailability.

Individual polymorphs (including solvates) have different lattice energies. The differences also lead to measurable deviations in a number of other properties, such as density, colour, hardness, refractive index, conductivity, melting point, enthalpy of fusion, etc.

Polymorphs also have different solubilities, solubility rates, inclusive.

If the difference in solubility between individual polymorphs is large enough, it may be a prerequisite for the low bioavailability of a number of sparingly soluble drug agents [10].

As the rate of drug dissolution is one of the most important and basic characteristics of drugs, it will have a great impact on the specifics of their therapeutic use/action.

That is why, there is a statement in the content of the European Pharmacopoeia, item 5.9., which unambiguously explains the meaning and significance of polymorphism in medical and pharmaceutical practice:

„The identity of chemical composition implies that all crystalline and amorphous forms of a given species have the same chemical behaviour in solution or as a melt; in contrast, their physico-chemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state."

Methods for preparing organic crystalline (including polymorphic) forms.

In most cases, the drugs are administered orally in the form of solid, dosage forms - tablets, capsules, etc., in which composition the active substance is dispersed in the inert matrix in the form of solid (mono)crystalline powder. The technological selection of solid (mono)crystalline (active) substances, over other types of solid organic forms such as amorphous solids or their "solid" conjugates (or solutions), is based on their tendency to be much more structurally stable and even to yield higher reproducibility in the context of their purification from accompanying "related" substances.

Crystallization, *i.e.* the formation and growth of crystals, even polymorphs, is possible only in saturated solutions. Saturation can be established as a result of changes in temperature and pressure of the medium; by evaporation of the solvent or co-solvent, or as a result of chemical interactions and other effects. In general, according to the conditions and methodology of crystal formation, crystallization is (Figure 4) [11,12]:

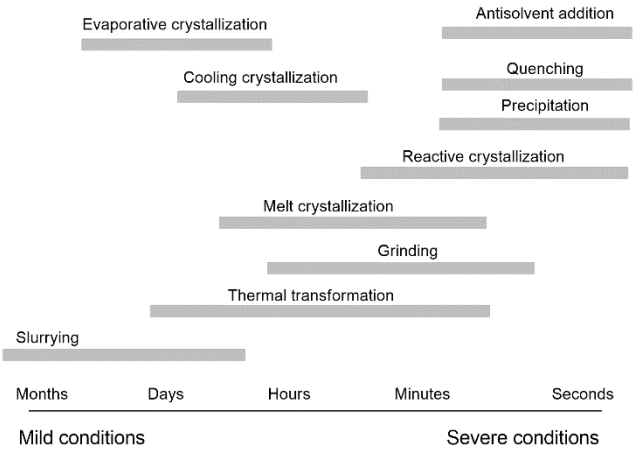


Figure 4. Strategies/methods for crystallization of organic substances in the so-called soft and hard conditions.

Polymorphic analysis - thermo- and spectroanalytical techniques, crystallographic methods.

The following techniques can be used in polymorphic analysis (according to European Pharmacopoeia 10.0):

– *X-ray diffraction of powders (2.9.33)*;

– *X-ray diffraction of single crystals*;

– *thermal analysis (2.2.34) (differential scanning calorimetry, thermogravimetry, thermomicroscopy)*;

– *microcalorimetry*;

– *moisture absorption analysis*;

– *optical and electronic microscopy*;

– *solid-state nuclear magnetic resonance*;

– *infrared absorption spectrophotometry (2.2.24)*;

– *Raman spectrometry (2.2.48)*;

– *measurement of solubility and intrinsic dissolution rate*;

– *density measurement*.

There are additional notes explaining the specificity of the method and its methodological features, a short guide, inclusive for some of the methods mentioned in the European Pharmacopoeia 10.0.

Often, these techniques complement each other and it is necessary to use several of them.

Vibration spectroscopy.

In addition to crystallographic techniques, molecular spectroscopy methods are extremely important for the characterization of various drug polymorphs and solvatomorphs [13]. The sensitivity of infrared spectroscopy to subtle changes in the crystal structure (supramolecular organization) is the reason that, has led to its rational application in polymorphic analysis (alone or in combination with Raman spectroscopy).

A minimum amount of analyte is required to record the infrared spectrum. The spectrum is obtained in a short period of time. The method is non-destructive; i.e. the sample remains unchanged and can be further analysed using other techniques.

Infrared absorption spectroscopy is an extremely useful technique for the complete characterization of solid and liquid pharmacopoeial substances.

The vibrational energy of solid organic forms can be further assessed using Raman spectroscopy. In general, however, these types of spectrometers are extremely expensive and are usually handled by highly qualified personnel. Modern Raman microscopes are more convenient and practical to work with, especially for the purposes of polymorphic analysis. Unfortunately, their cost is significantly higher than that of classic Raman appliances.

Sample preparation - methods for recording FTIR spectra of solid (crystalline) organic substances. Sample preparation in polymorphic FTIR analysis.

IR spectra can be obtained of gaseous, liquid, and solid samples. The physical state and the nature of work (type of research) are the factors that largely determine the choice of technique for (sample) preparation, which will predetermine the high level of effective interaction of the studied sample with the applied IR radiation. Today, in the course of work with modern FTIR spectrometers, many *sample preparation* approaches are applied, each of which, with its peculiarities, allows the recording of specific (specific to the analyte) vibrational spectra. For the purposes of polymorphic and solvatomorphic analysis, of all the applied IR techniques, only a small part of them are rationally used.

The classical tablet method is probably the least suitable in the polymorphic analysis due to the fact that, the test sample can be transformed (polymorphically) as a result of the applied pressure (several tons) on it. In this case, the possibility of an ion exchange reaction (exchange of ions from the matrix (KBr or KCl) with those of the test sample) is not excluded either [14].

The use of mineral oils in which the analyte is suspended largely overcomes some of the problems highlighted above, but also causes new ones related to the absorption properties of the mineral oil itself.

The recording of diffuse reflectance spectra does not address any of the above methodological shortcomings [15,16], but introduces a new one related to the need to dilute the test sample with "anhydride" KBr to concentrations 1.0 to 5.0 wt. %.

The most useful and effective method for studying organic polymorphs (including solvatomorphs) is that of attenuated total reflectance (ATR) [16,17]. When using ATR technique, infrared radiation is transmitted through a crystal at an angle less than the so-called 'critical angle', forcing the latter to suffer a total, but within the crystal volume, full internal reflection. With each such reflection, the conducted electromagnetic radiation penetrates a short distance from the surface of the reflector crystal, creating the so-called 'evanescent field' with which the analyte 'gets' into a physical contact (Figure 5). The energy of the reflected reflection decreases at those frequencies at which the changes in the vibrational states of the test substance take place.

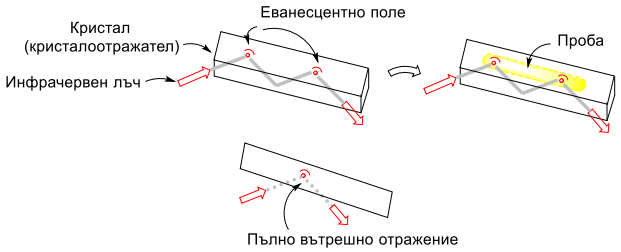


Figure 5. Scheme presenting the principle of total internal reflection method (ATR).

The advantage of the ATR technique is that, it does not require any significant sample preparation, due to the fact that, it is sufficient to "apply" the test analyte to the surface of the used reflector with the minimum pressure (insignificant pressure) required to provide the desired optical connection between the analyte and the evanescent field. The evanescent field does not propagate throughout the analyte; it usually penetrates to a depth of 5.0 to 10 μm.

Application of IR absorption spectroscopy in polymorphic pharmaceutical analysis.

The advantage of the ATR method over that of mineral oil dispersion can be illustrated by the example of FTIR study of one of the polymorphs of *Mepivacaine hydrochloride* [18].

FTIR-ATR spectroscopy is even used to determine salt-induced crystallization of metastable polymorphs (as in the case of *Flufenamic acid* [19]).

The method of FTIR-ATR spectroscopy is also used to characterize *Fluconazole* products obtained by the supercritical antisolvation method [20].

FTIR spectroscopy was also used to study and determine the composition of the amorphous salt formed by the co-precipitation of *Cimetidine* and *Diflunisal* [21] (Figure 6).

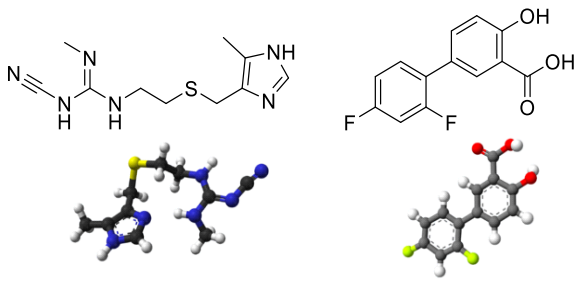


Figure 6. Illustration showing the molecular structures of the drugs *Cimetidine* and *Diflunisal*.

On the other hand, FTIR spectroscopy fails to be always useful. In the case of analyzing the individual crystal hydrate forms of *Diclofenac sodium*, the recorded FTIR spectra turned out to be identical, due to the fact that the expected changes fall within the instrumental resolution [22].

The importance of polymorphism in pharmaceutical practice.

In 1999, it was reported that one third of all pharmacopoeial agents (808 substances) could form various hydrates (crystal hydrates) [23]. The presence of a significant number of drug solvates, cocrystals, salts, was also reported [24].

Although the effects of polymorphism have long been known, in the chemistry of medicinal substances this term has been considered relevant since the mid-1960s, when the Norvir® (Ritonavir) case highlighted polymorphism as a serious threat to the pharmaceutical industry [25].

Only one crystalline form was identified during the development of Norvir®. However, in 1998, several batches of capsules failed the solubility test due to the emergence of a new polymorphic form of ritonavir (designated Form II); associated with its large-scale production [26,27]. As a result, the drug was withdrawn from the pharmaceutical market and the company ceased operations for one year [28]. To solve this problem, the manufacturer (Abbott Laboratories) had to spend hundreds of millions of dollars, and its estimated sales loss in 1998 alone was $ 250 million.

The reformulation of Norvir® took approximately one year. In this case, the impact on the standard of living of these patients caused by the polymorphism of the drug they needed emphasizes, in general, the serious effects of drug polymorphism on public health.

A similar situation was reported with Rotigotine (a transdermal drug used to treat the signs and symptoms of Parkinson's disease) - originally licensed as an active drug substance that did not show polymorphism (Schwarz Pharma, 2006).

However, in 2008, Rotigotine was removed from the pharmaceutical market due to its less soluble and unknown polymorphic form, which did not show dermal absorption and did not affect (eliminate or reduce) the symptoms of Parkinson's disease.

Pharmacopoeial agents with proven polymorphism are not limited to the above examples (Table 1).

Table 1. Pharmacopoeial substances (with different pharmacological affiliation) exhibiting polymorphism.

Active substance	Number of known polymorphs	Stable (preferred) form*
Acyclovir	6	I
Acetylsalicylic Acid	2	I
Ibuprofen	2	I
Acetaminophen	6	I
Albendazole	2	II
Mebendazole	3	A
Loratadine	2	‡
Ferrous sulfate	3	‡
Folic acid	1	Dihydrate
Diazepam	2	‡
Amiodarone hydrochloride	1	‡
Digoxin	3	Amorphous
Verapamil hydrochloride	1	‡
Amoxicillin	1	Trihydrate
Azithromycin	3	Dihydrate
Benzylpenicillin	1	I
Cephalexin (hydrochloride or sodium salt)	8	IV (monohydrate)
Ciprofloxacin	3	II (hydrate)
Doxycycline	2	‡
Erythromycin	4	Dihydrate
Sulfamethoxazole	4	III (hemihydrate)
Sulfasalazine	2	‡
Trimethoprim	1	‡
Carbamazepine	6	Dihydrate
Phenytoin	1	I
Phenobarbital	13	A
Amitriptyline hydrochloride	1	I
Fluoxetine hydrochloride	1	I
Glibenclamide	1	I
Metformin hydrochloride	2	A
Metoclopramide hydrochloride	3	‡

Active substance	Number of known polymorphs	Stable (preferred) form*
Clonazepam	1	I
Ketoconazole	2	I (enantiomer +)
Fluconazole	4	I
Miconazole Nitrate	3	‡
Promethazine hydrochloride	2	‡
Atenolol	2	I
Captopril	2	I (B)
Enalapril maleate	2	II
Losartan	5	I
Methyldopa	1	‡
Nifedipine	3	A= I= α
Propranolol hydrochloride	3	II
Simvastatin	3	I
Biperiden	1	I
Carbidopa	‡	‡
Levodopa	2	I
Benserazide hydrochloride	‡	‡
Metronidazole	1	‡
Chlorpromazine	2	Anhydrous
Haloperidol	1	I
Omeprazole	1	‡
Ranitidine hydrochloride	4	II
Oseltamivir phosphate	1	‡
Salbutamol sulfate	3	I
Allopurinol	1	I
Ethinyl estradiol	1	Hemihydrate
Levonorgestrel	2	‡
Norethisterone	1	‡
Furosemide	3	I
Hydrochlorothiazide	2	‡
Prednisone	1	‡
Azathioprine	2	‡

**: room temperature; ‡: not presented.*

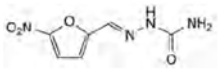
Nitrofural - IR spectral features, polymorphism.

According to the European (including the British) Pharmacopoeia, the identification of the pharmacopoeial substance *Nitrofural* is also performed using IR spectroscopy.

It is noted in the mentioned pharmacopoeial text that, the comparative analysis is performed using a relevant reference material - Chemical Reference Substance (CRS). However, the possibility of *Nitrofural* polymorphism is not considered (Figure 7).

NITROFURAL

Nitrofuralum



$C_6H_6N_4O_4$ M_r 198.1
[59-87-0]

DEFINITION

2-[(5-Nitrofuran-2-yl)methylene]diazanecarboxamide.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: yellow or brownish-yellow, crystalline powder.

Solubility: very slightly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Ultraviolet and visible absorption spectrophotometry (2.2.25). *Carry out the test protected from bright light.*
Test solution. Use the solution prepared for the assay.
Spectral range: 220-400 nm.
Absorption maxima: at 260 nm and 375 nm.
Absorbance ratio: A_{375}/A_{260} = 1.15 to 1.30.

B. Infrared absorption spectrophotometry (2.2.24).
Comparison: *nitrofural CRS.*

C. Thin-layer chromatography (2.2.27).
Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

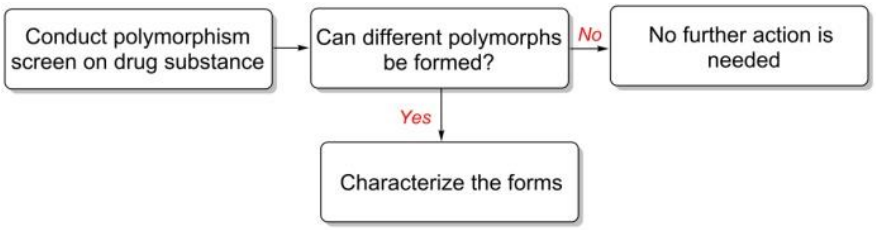
Figure 7. Excerpt from the respective contents of *Nitrofural* monograph [2]

Taking into account the data noted in the scientific sources concerning the IR analysis of this semicarbazone (*Nitrofural*), we came across several features that are inherent in its various polymorphic states, namely:

- the official deposit of IR data on the absorbability of the available carbonyl group at different frequencies;

- the presence of a recently published work proving the existence of three different *Nitrofural* polymorphs.

According to the standard Q6A [29] approved by The International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) [29], when testing any new drug substance (or preparing a new solid dosage form), it is necessary to run screening to prove that, the active ingredient can form different polymorphic forms. In general, it was announced in course of the conference debates as follows (Scheme 2):



Scheme 2. Illustration of the algorithm of ICH solutions related to the recommendations for monitoring and control of polymorphic drugs.

In the presence of clear evidence for the existence of different *Nitrofural* polymorphic forms, it is necessary to present a strategy for their analysis using pharmacopoeially validated instrumental methods.

The defined strategy should be validated (officially by agencies, in the model presented here or another model) and adopted officially in the subsequent edition of European Pharmacopoeia.

Although in Bulgaria *Nitrofural* is available in the form of a medicinal solution (ear drops) in other countries, it continues to be used in solid form intended for oral use. Oral use is also common in the tropics, where *Nitrofural* is used as a successful drug for treatment of trypanosomiasis.

Its bioavailability, in these cases, should be influenced by polymorphism, as well as by the size of its crystallites. Separately, it should be noted that, with the ‘internal use’ of larger *Nitrofural* crystals, the side effect of the drug on the gastrointestinal mucosa is significantly reduced [30].

In conclusion, it should be noted that, one of our tasks is to have a form justified fully and meeting the international pharmacopoeial standards.

III. AIMS AND TASKS

The main goals of this dissertation are:

- to study, mainly by infrared spectroscopy, the polymorphic features of the pharmacopoeial representative *Nitrofural* and to analyze the possibilities for the simultaneous growth of several of its polymorphs over As_2Se_3;

- to explore the possibilities for introduction of new and specific qualitative reactions into the identification analysis of *Nitrofural*.

- to synthesize new *Nitrofural* derivatives with the precursor *Nitrofural* itself.

To achieve the above goals, the following tasks have been set:

- to synthesize different polymorphic forms of the drug substance *Nitrofural*;

- to realize *Nitrofural* polymorphs synthesis on the surface of a As_2Se_3 substrate;

- to perform an FTIR analysis of *Nitrofural* in solution and evaluate the effect that the crystal lattice has on the absorbency of its C=O functional group;

- to synthesize *Nitrofural* crystals in the presence of NaCl;

- to synthesize *Nitrofural* cocrystals with citric acid;

- to investigate the potential of Gibbs reagent in the *Nitrofural* identification analysis;

- to study the electrochemical potential of the NO_2 group of *Nitrofural* in the qualitative *Nitrofural* analysis;

- to synthesize new 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole derivatives from *Nitrofural* and determine their antibacterial behavior

and

- to investigate the potential phototoxic effect of *Nitrofural*.

IV. EXPERIMENTAL PART

All details about the materials and methods used can be found in the presented publications related to the dissertation.

V. RESULTS AND DISCUSSION

Synthesis and spectral analysis of various polymorphic *Nitrofural* forms.
The synthesis of all *Nitrofural* polymorphic forms was performed by solvent/co-solvent evaporation methods.

In one case, a technique was used to "slow down" *Nitrofural* crystallization using a NaCl agent. It is known that, the added salt increases the solubility of the studied semicarbazone analyte.

The inorganic salt used is biocompatible and is often used in the preparation of infusion solutions.

Its presence in the synthetic solution should delay the crystallization of the *Nitrofural* analyte (i.e. change the point of *Nitrofural* saturation) and also change the dielectric constant of the solvent used (in this case, bidistilled and deionized water). The absence of occluded (included in the *Nitrofural* crystal lattice) NaCl was demonstrated using the AgCl test.

In another case, the crystal synthesis was run in the presence of a capping agent, citric acid. It is known that the added organic acid may be a prerequisite for the synthesis of *Nitrofural* cocrystal or serve as a major and atypical initiator in the process of its crystals formation.

In the condensed (solid) state, semicarbazones form stable intermolecular aggregates. The presence of their highly intense stretching C=O vibrations is registered in a significantly wide IR frequency range (1650 to 1760 cm^{-1}).

For the oscillations of the *Nitrofural* carbonyl functional, several different absolute values are presented, characteristic, most likely for its different polymorphic forms:

The Japanese spectral database [31], for the *Nitrofural* C=O functional, indicated a high intensity band at 1717 cm^{-1} when the spectrum was taken as a KBr tablet, and a medium intensity band at 1713 cm^{-1} when the sample was dispersed in nujol.

In the well-known for pharmacists analytical edition "Clarke's Analysis of Drugs and Poisons" [9] the absorbance of C=O functional group of *Nitrofural* is indicated as a band with a maximum at 1718 cm^{-1} (nujol).

In his work, Tze Wang presents the characteristic frequencies of C=O vibrations for several furan-substituted semicarbazones, incl. and the drug agent discussed herein. For *Nitrofural*, the author established an absorption maximum at 1708 cm^{-1} [32].

In Belikov's textbook [30] an absorption band with a maximum at 1724 cm^{-1} is indicated for the vibrations of this functional group.

In 2016, a team of Polish scientists was able to synthesize and structurally characterize two new *Nitrofural* polymorphs, denoted as β and γ [33]. The definition of "new" was introduced

because the first *Nitrofural* polymorph, which we now designate as α, was obtained more than 22 years ago [34].

Unfortunately, no method for the synthesis of α-*Nitrofural* polymorph has been presented in the literature yet. Separately, in a significant number of our experiments, we also failed to reproduce the synthesis of this polymorph. Therefore, we decided to conduct an additional experiment in which the synthesis of α-*Nitrofural* polymorph to be carried out directly on the surface of a chalcogenide (IR permeable) As_2Se_3 glass. Saturated DMSO solution of *Nitrofural* was used for the purpose. The latter was evaporated to dryness with a stream of warm air. The spectrum of the sample thus obtained is shown in Figure 8.

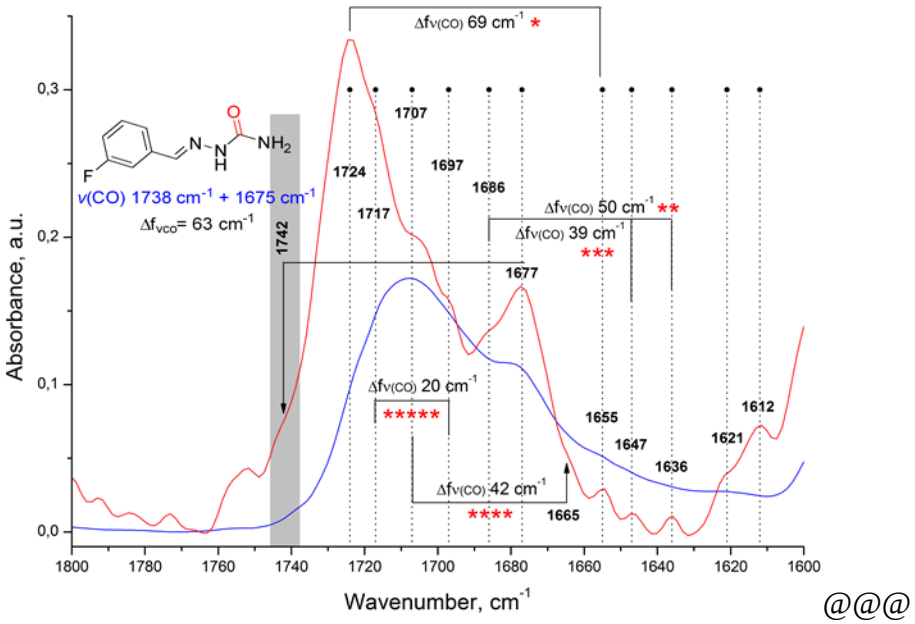


Figure 8. Illustration showing the FTIR spectrum of the *Nitrofural* sample deposited over As2Se3 (red line) and the ATR-FTIR spectrum of the γ-*Nitrofural* polymorph (blue line) in combination with those recorded by Kolb and co-authors, characteristic normal and abnormal CO vibrations of several benzyl-substituted semicarbazones.

The presence of a significantly high-intensity and complex band with clearly formed shoulders and lower-intensity additional bands in the studied spectral range, with maxima at 1724, 1717, 1707, 1697, 1686, 1677, 1665, 1655, 1647, and 1636 cm^{-1}, is reported in the newly obtained spectrum.

The performed FTIR analysis unequivocally proves the presence of several different *Nitrofural* polymorphs, one of which is the established and spectrally characterized already γ-*Nitrofural* polymorph.

If the carbonyl functional group in β- and γ-*Nitrofural* polymorphs is characterized by absorption bands with abnormally high frequencies falling in the close subregion (from 1710 to 1700 cm^{-1}), then for the same residues in the composition of the other polymorphs (α and unknown) we should expect sorption maxima in the 1710÷1730 cm^{-1} range.

Indeed, the presence of a hyperconjugation effect should be expected only in the case of *syn-anti-anti-anti* configurational *Nitrofural* isomers.

Despite the established geometric (configurational) similarity between the molecules of β- and γ-*Nitrofural* polymorphs, the appearance of two distinct polymorphs is attributed to the small differences in their molecular conformation (Figure 9).

Analogous conformational isomers within the established α-*Nitrofural* polymorph may be a prerequisite for the appearance of conformational mimicry, reflected in the close but still distinctive crystalline supramolecular polymorphic architecture or the so-called conformational polymorphism.

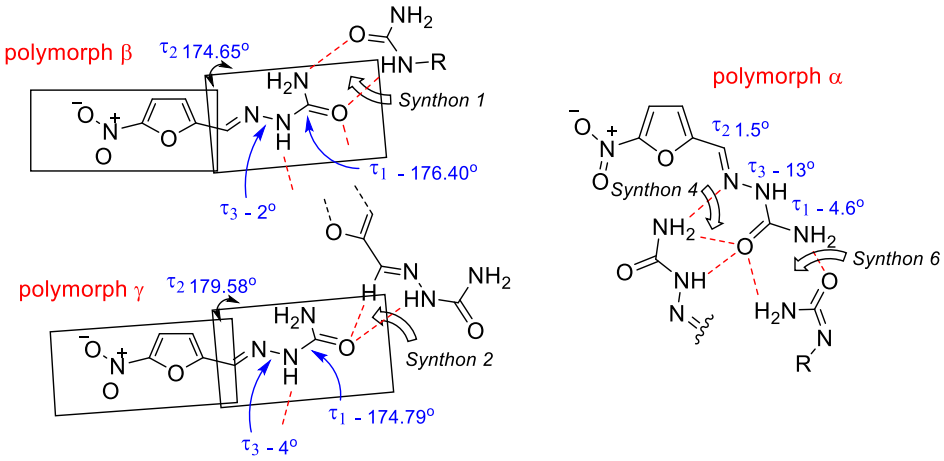


Figure 9. Illustration showing some of the more significant structural-geometric factors of the three *Nitrofural* polymorphs.

Furthermore, Sigma-Aldrich offers a *Nitrofural* standard for "spectral identification" and liquid chromatography - *Nitrofural* for peak identification; European Pharmacopoeia (EP) Reference Standard.

Vijay Narayan and co-authors determined viewing the same standard, that, the absorbance of the considered C=O functional falls in the abnormal IR spectral range, with a maximum at 1728 (1726) cm^{-1} [35].

The authors also encountered a significant discrepancy between the experimentally reported value and the value for IR abs. of the functional in question (1764 cm^{-1}) established by them using computational methods.

Indeed, it is not reasonable to consider the semicarbazone residue in an isolated field, but in a cluster of several, spatially translated *Nitrofural* molecules.

The effects of hyperconjugation are also not taken into account in the quantum chemical calculations. In addition, the performed computational analysis, which consists of finding a more energy-stable molecular (conformational) state, may be a prerequisite for further "hiding" of some spatial effects.

In conclusion, noteworthy is that, the combined results of DSC and XRD analysis actually argue on the existence of a new *Nitrofural* polymorph. The fact that, the initial evidence leading to the detection of the unregistered (new) *Nitrofural* polymorph came from FTIR analysis should not be ignored.

γ-Nitrofural metastability.

We found that, significant deviations in C=O absorption profile of γ-*Nitrofural* polymorph obtained were observed in the process of the repeated IR analyses, namely:

Initially, the presence of a broad absorption band with a pronounced extremum at 1677 cm^{-1} and lower-intensity overlapping bands superimposed on its left shoulder, with distinct extremes (occupying the spectral field from 1690 to 1725 cm^{-1}) were observed (Figure 10).

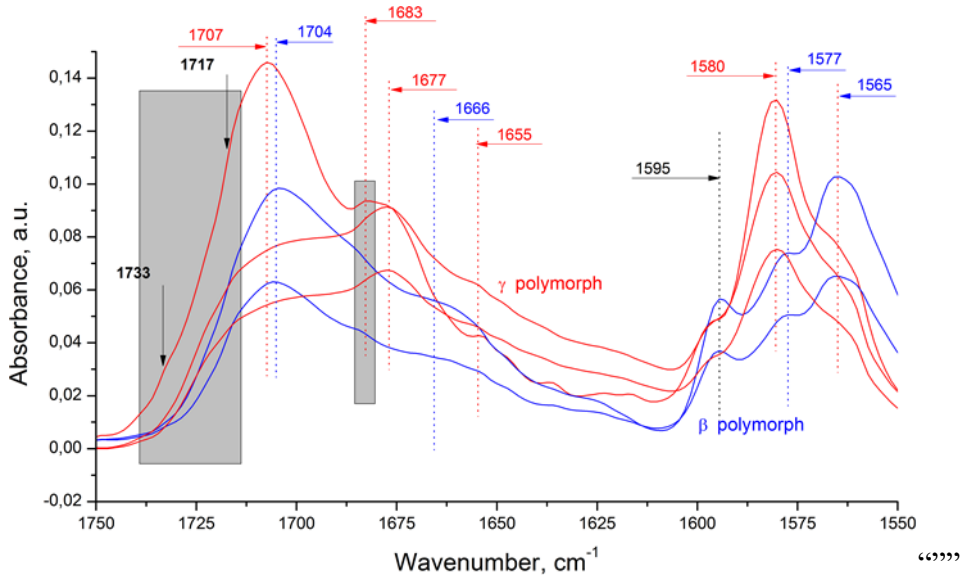


Figure 10. ATR-FTIR spectra of β- (highlighted in blue) and γ- (reflected in red) *Nitrofural* polymorphs presented in the range 1550 to 1750 cm^{-1}. The evolution of the phase transformation from a metastable to γ form is accompanied by an increase in the total absorbability of the sample.

Under the conditions of applied pressure on the analyzed sample (with the built-in ATR piston for pressing the sample to the diamond crystal) an atypical change in the IR pattern was registered – in general, the appearance of a high-intensity band with a maximum at 1707 cm^{-1} and of a lower-intensity one with a maximum at 1683 cm^{-1} was recorded (Figure 10).

there is an obvious similarity in the left absorption arm of the newly formed band in the spectrum recorded last. The presence in its composition of low-intensity bands with maxima at 1717 and 1733 cm^{-1} should be a prerequisite for the appearance of the registered absorption asymmetry.

In this case, it can be said that, several conformational *Nitrofural* polymorphs are present in the sample studied.

As a precondition for the reported changes in the profile of C=O vibrations we can indicate the occurred polymorphic changes in the studied sample at the imposed minimum pressure on it. The phase changes in the structure of the γ-*Nitrofural* sample, under the influence of "soft" physical contacts, we can, with good reason, attribute to the metastability of the polymorphic sample thus obtained.

As seen from the presented figure, significant differences in the absorbance of the two polymorphs are also observed in the 1600 ÷ 1550 cm^{-1} range.

***Nitrofural* samples obtained in the presence of NaCl.**

The appearance of a series of highly intense, overlapping bands with maxima at 1733, 1716, 1705, 1698, 1683, 1674, 1662, 1654, 1647, 1637 and 1624 cm^{-1} was recorded in the spectra of all *Nitrofural* samples obtained in the presence of NaCl (Fig. 11).

On the basis of the above facts, it can be assumed that, several different polymorphs are present in the composition of the samples thus obtained, in semi-equivalent mass percentages.

The presence of different NaCl content in the crystallization solution did not cause any changes in the composition (in the profile of all recorded spectra) of those samples.

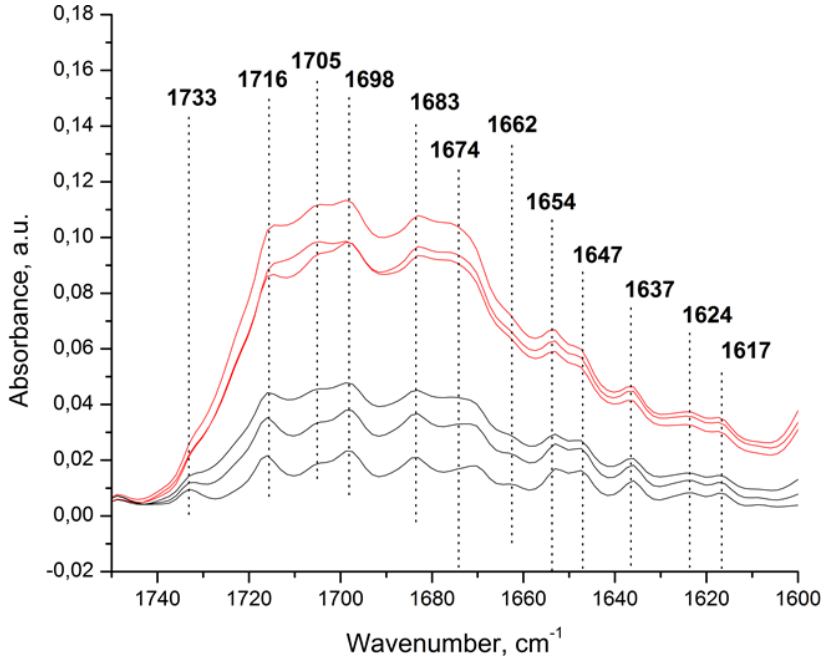


Figure 11. ATR-FTIR spectra of *Nitrofural* samples (range 1625 to 1750 cm^{-1}) obtained in the presence of various amounts of NaCl.

***Nitrofural* samples obtained in the presence of citric acid.**

Polymorphic metastability in those *Nitrofural* samples obtained in the presence of citric acid was also reported (Fig. 12).

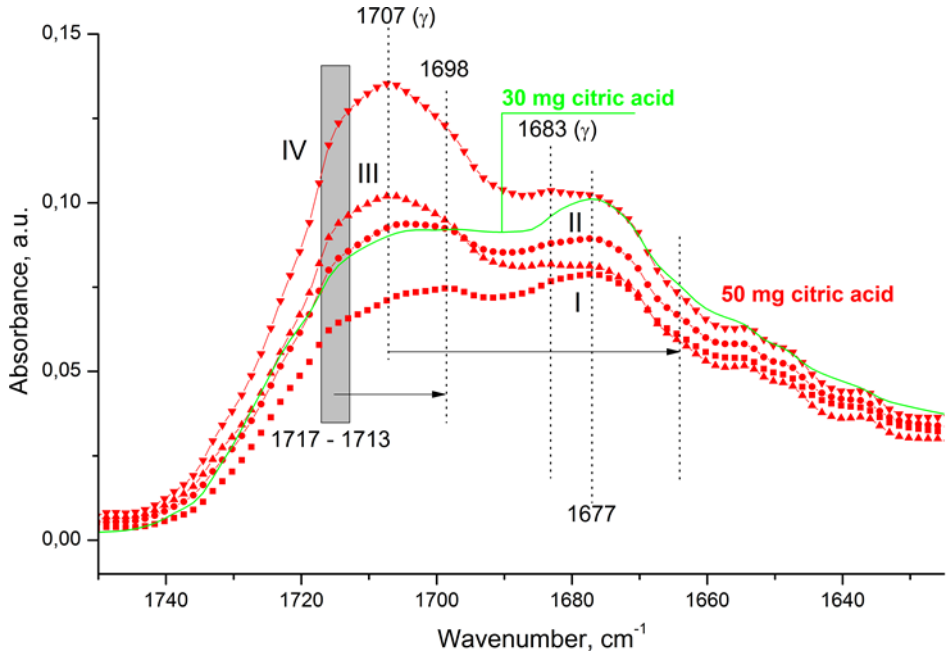


Figure 12. ATR-FTIR spectra of *Nitrofural* crystals (in the range 1625 to 1750 cm^{-1}) obtained in the presence of 30 (green line) and 50 mg (red with symbol lines) of citric acid.

As seen from the presented figure, the profile of the FTIR spectrum of the first analyzed sample, denoted by Roman numeral I, shows the presence of bands with maxima at 1677 and 1698 cm^{-1}. Lower intensity and overlapping bands with peaks at 1683, 1707 and 1717 cm^{-1} were also recorded in the spectrum. After application of the first and second pressures on the sample, the intensity of the initially recorded bands decreases, while the reported arms - increase their intensity. Upon application of the third pressure, the appearance of new bands with maxima at 1707 and 1683 cm^{-1} appeared in the spectrum of the analyzed sample (spectrum IV).

In this case, a polymorphic transition from an unknown, metastable *Nitrofural* conformational state to a significantly more stable one, γ, is most likely to be registered.

Qualitative *Nitrofural* analysis.

Analysis of the analytical behavior of Gibbs reagent (2,6-dichloroquinone-4-chloroimide) versus *Nitrofural*.

Gibbs reagent (Gibbs) exhibits the ability to bind (covalently) with phenols [36-39], thiols [40,41], nitroxils [39], amines [42,43], as well as with certain aliphatic and aromatic hydrazides, forming with them colored products.

Therefore, tour aim was to establish the potential of Gibbs reagent in the qualitative semicarbazone analysis. For the analysis intended we used the only pharmacopoeial semicarbazone representative - *Nitrofural*.

In the preliminary tests, we found the intactness of the two reactants in solution (AB, Fig. 13). The inertness of the reagent to the studied semicarbazone was maintained even in the presence of dilute HCl acid (ABD, Fig. 13).

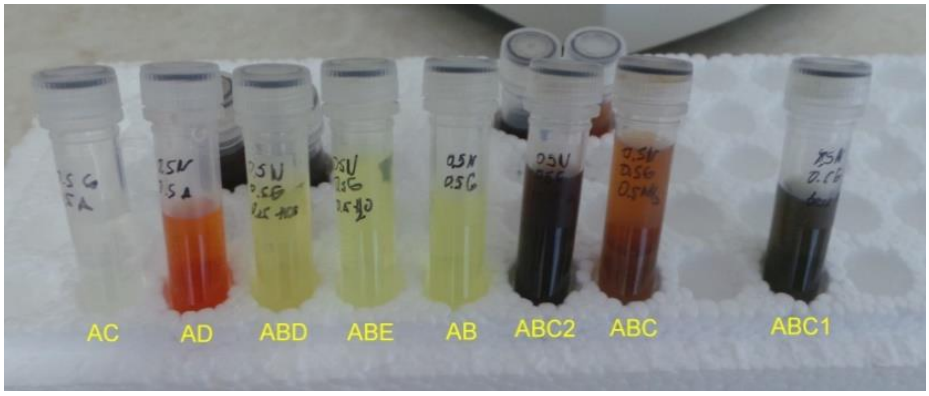
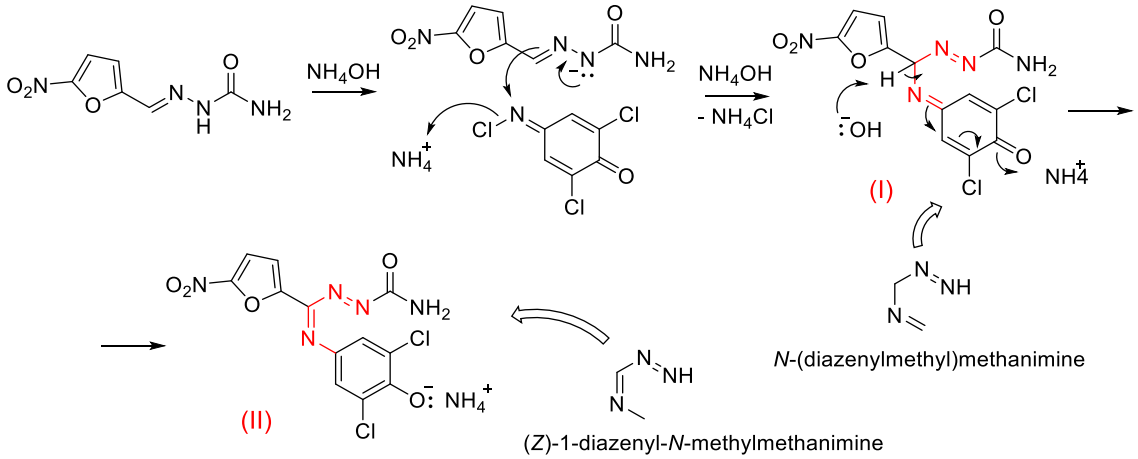


Figure 13. Photograph illustrating the differences in appearance of all reaction products.

In the presence of an insignificant amount of ammonia water, the visible (color) interaction between the tested substances was reported (ABC1, Fig. 13); interaction, expressed in the appearance of a characteristic, oil-green product. The presence of this compound is associated with the interaction between the easily dehalogenated chloroimide and the conjugated (α-azadelocalized) *Nitrofural* carbanion (Scheme 3). The latter was likely to be formed, *in situ*, as a result of the interaction between NH_4OH and the weakly acidic *Nitrofural* NH center tested.



Scheme 3. Presumed mechanism of the reaction between *Nitrofural* and Gibbs reagent.

It is logical to assume that in the course of this interaction an (intermediate) unstable N-(diazenylmethyl)methanimine (I) was initially formed, which spontaneously isomerizes (prototropically) to the much more stable 1-diazenyl-N-methylmethanimine (II). The participation of ammonia water in this interaction, at first glance, seems illogical, due to the fact that ammonia does not exhibit the properties of strong bases (active proton acceptors). However, its catalytic action seems justified given the weakly acidic reaction of *Nitrofural* [2] (Figure 14).

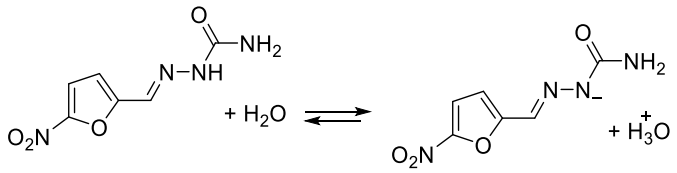


Figure 14. The dissociative behavior of *Nitrofural* in H_2O.

Additional evidence of the presence of 1-diazenyl-N-methylmethanimine product in the ABC1 mixture was obtained by UV-VIS spectral analysis (Fig. 15).

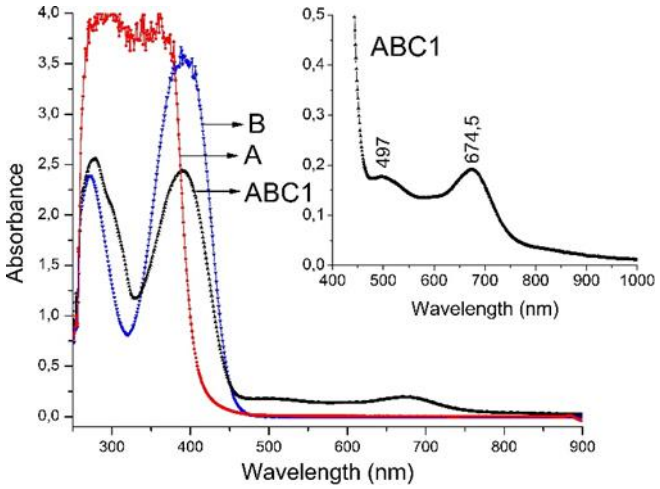


Figure 15. UV-VIS spectra of Gibbs reagent (A), *Nitrofural* (B) and their interaction product in ammonia medium (ABC1).

The presented figure shows that, in the 250 to 450 nm range, the spectra of the *Nitrofural* sample and the obtained colour reaction product (ABC1) have a similar analytical profile (Fig. 15). However, in the 450 to 800 nm range, in the ABC1 spectrum, the presence of two low-intensity bands with maxima at 497 and 675 nm are seen. The reported above similarities in the shortwave UV region can be entirely attributed to the excess of in the composition of the ABC1 sample. The yield of the colour product will be minimal and equivalent to the promoter introduced in the traces - ammonia.

A proof of the successful incorporation of a chromogenic element into the *Nitrofural* molecular backbone is found in the low-intensity band with a maximum at 675 nm. Conjugated chromophore systems generally absorb extensively in this UV region, as the molecule of the expected reaction product - ABC1 in the case (compound II in Scheme 3).

The presented method can also be used as experimental evidence, revealing the possibility of interaction of semicarbazones with Gibbs reagent.

Analysis of electrochemical behaviour of *Nitrofural* in the presence of sodium aminopentacyanoferrate ($Na_3[Fe(CN)_5NH_3]$).

Microanalysis or the so-called 'spot-test' analysis of aromatic and heterocyclic nitro compounds has been the subject of a constant analytical interest. There is literature on this particularly original electrochemical method with the help of which a number of much more complex aromatic nitro compounds can be analysed [44]. The electrochemical method presented by Feigl is unique both in terms of its experimental design and in terms of its group specificity. In addition, the presented methodology can be adapted easily and applied in both qualitative and quantitative pharmaceutical analysis.

To evaluate the potential of this electrochemical method in the field of pharmaceutical analysis, we conducted a series of analytical tests studying *Nitrofural*. We also aimed at determining the specificity of the test in relation to the test analyte, by also analyzing other nitroaromatic compounds.

Indeed, in the presence of a constant electric field generated by a 9V battery, we were able to record a positive *Nitrofural* electrochemical analytical course in the presence of $Na_3[Fe(CN)_5NH_3]$ salt. Electrochemical *Nitrofural* reduction resulted in the formation of two clearly colored zones around the two working electrodes, namely: a red colored zone around the anode surface and a blue-green colored one around the cathode surface (Fig. 16).

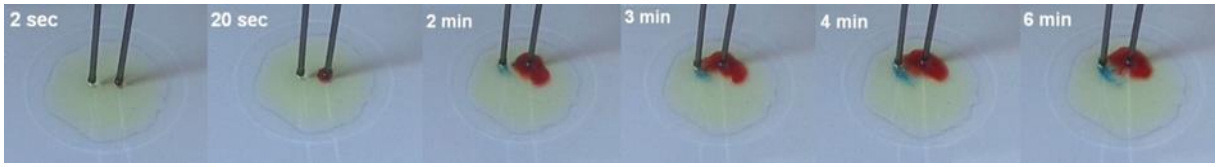


Figure 16. Photograph illustrating the appearance of two different colour zones around the working electrodes.

The appearance of a red-coloured product was associated by the rapid electrolysis and localized pH changes in the composition of the reaction medium; changes that had led to the formation of a violet-red nitro-quinoid *Nitrofual* form (Figure 17).

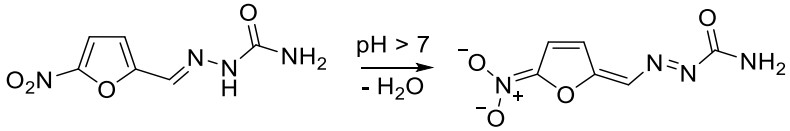


Figure 17. Presumed chemical behaviour of *Nitofural* in an alkaline environment.

In this regard, the electrochemical manifestations of the following aromatic nitro compounds were also established - *Nitrofurantoin*, *Chloramphenicol*, *Metronidazole*, 4-nitrotoluene, ethyl 4-nitrobenzoate, 4-nitrobenzoic acid, and 4-nitrobenzaldehyde. No *Nitrofural*-like result was reported in any of the electrochemical tests performed. The electrochemical changes that occur in the *Nitrofural* sample at the cathode surface are associated with the inorganic $Na_3[Fe(CN)_5NH_3]$ complex introduced into the reaction medium. It is known that, this inorganic reagent exhibits strictly specific reactivity to the reduced (*in situ*) NO_2 forms - nitroso- and hydroxylamino groups [44,45]. The resulting $Na_3[Fe(CN)_5R]$ complexes have a characteristic colour - emerald green (Fig. 18).

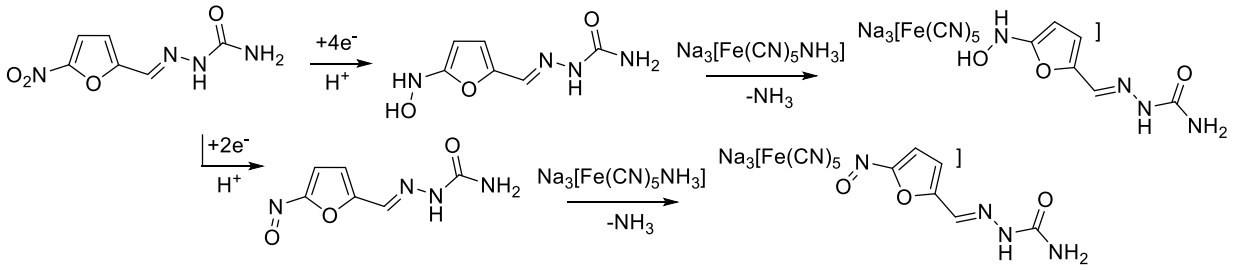


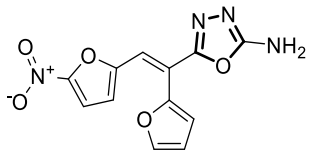
Figure 18. Probable mechanism of the interaction of $Na_3[Fe(CN)_5NH_3]$ reagent with the reduced forms of *Nitrofural*.

Synthesis of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole derivatives.

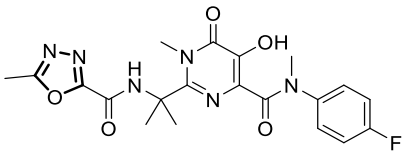
We tried to synthesize the title heterocycle system by three different methods intending to introduce a 1,3,4-oxadiazol-2-yl substitute to the "bactericidal" 5-nitrofural moiety.

The antibacterial agent *Furamizole* should be mentioned as a derivative of 1,3,4-oxadiazole. This heterocycle can also be found in the molecular compositions of the drugs *Raltegravir* (antiviral agent), *Nesapidil* (antiarrhythmic agent), as well as in the US Food and Drug Administration (FDA) approved anti-cancer agent *Zibotentan*. The antihypertensive agent *Thiodazosin* also has a 1,3,4-oxadiazole residue.

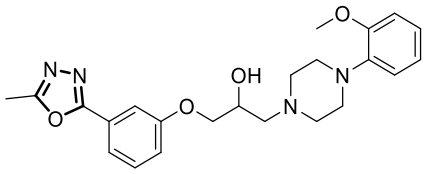
Furamizole



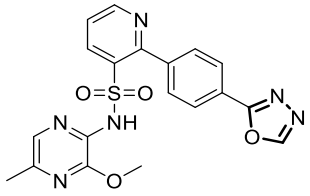
Raltegravir



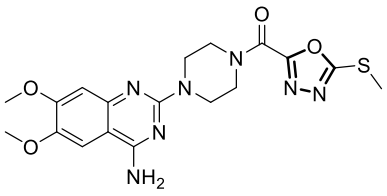
Nesapidil



Zibotentan

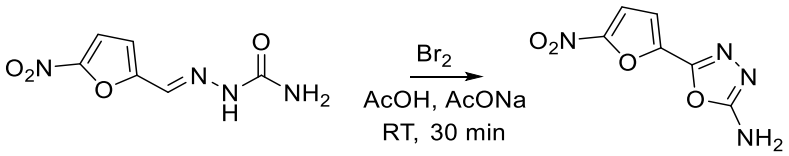


Tiodazosin



The presence of a 1,3,4-oxadiazole ring has been shown to affect significantly the pharmacokinetic properties of drugs. In medical chemistry, this hetero ring is used mainly as a bioisostere of a number of C=O containing functional groups - carboxylic acids, esters, amides, carbohydrazides and others. The oxadiazole ring is also used as a specific pharmacophore capable of binding to certain types of ligands. In many cases, it also acts as a planar aromatic linker, ensuring the correct biological orientation of the compounds containing it [46].

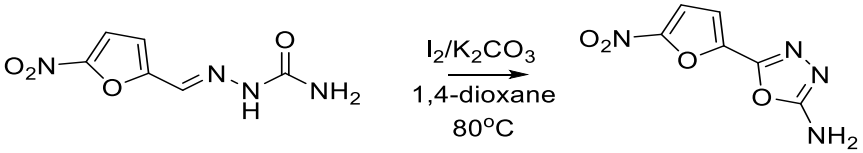
We use three approaches to the synthesis of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine. In one of them, we applied the strategy presented by H. Rajak and co-authors [47] (Scheme 4). It is a strategy the authors implemented successfully in the synthesis of a series of 2,5-disubstituted 1,3,4-oxadiazoles.



Scheme 4. Synthesis of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine involving elemental Br_2.

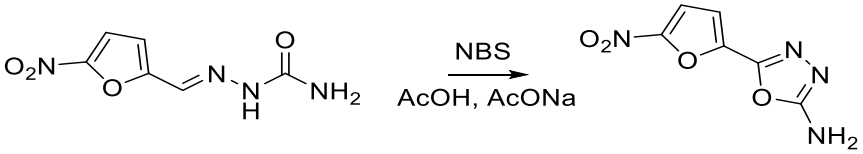
Surprisingly, however, our synthesis using the method, yielded a crude product containing only the unreacted *Nitrofural*.

The presence of the desired 1,3,4-oxadiazole was also not detected in the composition of the reaction product obtained by the second method (Scheme 5).



Scheme 5. Synthesis of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine with the participation of the reagent pair I_2/K_2CO_3.

The best result was achieved using the third approach (Scheme 6).



Scheme 6. Synthesis of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine involving NBS (N-bromosuccinimide).

The composition of the obtained new (crude) product was determined again by ^{1}H NMR spectroscopy (Fig. 19).

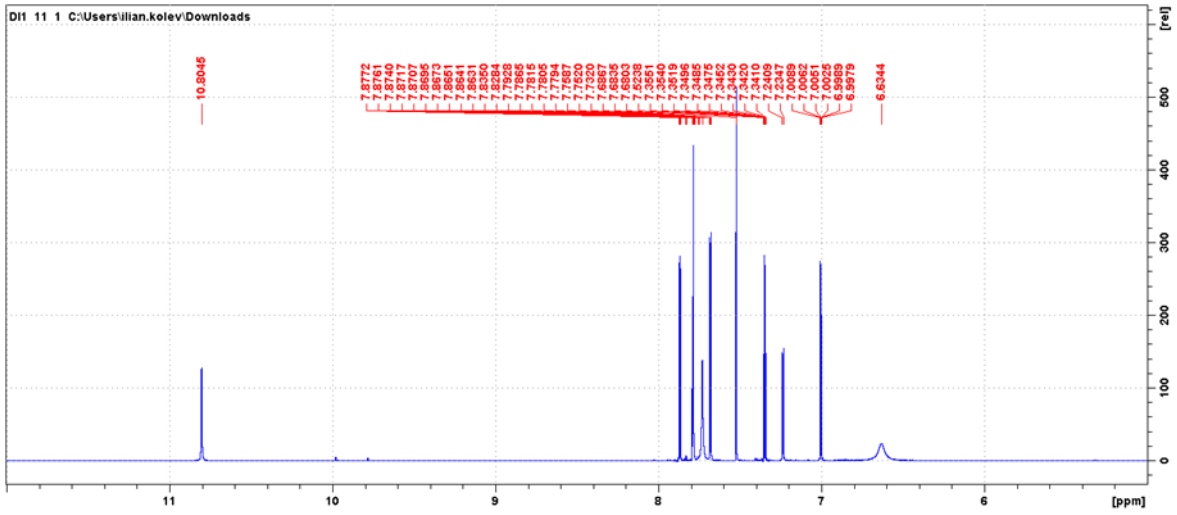
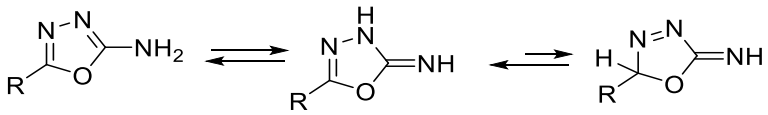


Figure 19. ^{1}H NMR spectrum of the crude reaction product obtained with NBS.

The quantitative ratio between unreacted *Nitrofural* and 1,3,4-oxadiazole produced was re-established by the ratio of the integral intensities of their signals. The established percentage of oxadiazole in the final product reached 65%.

The latter was isolated in a pure form by column chromatography; at using silica gel and eluent - methanol, acetonitrile. Approximately 200 mg of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine were isolated at a consumption of more than 3 liters of solvent. This, of course, is a shortcoming that needs to be overcome in the future by choosing a much more efficient and productive mobile phase.

The product thus obtained was subjected to additional spectral analysis. The ^{1}H NMR analysis presented an intriguing result. The presence of four signals was reported in the recorded spectrum at 7.68 (d), 7.53 (s), 7.00 (d) and 3.49 (s) ppm, respectively (Figure 20). Undoubtedly, the doublet signals are associated with the two furan protons, and the other two (singlet) for the 1,3,4-oxadiazol-2-amine residue. In this case, however, the presence of only one singlet signal is expected for this molecular fragment. Most likely, in this case, the simultaneous presence of at least one of the two expected 1,3,4-oxadiazole-2-amine prototropic forms is detected (Scheme 7).



Scheme 7. Probable prototropic equilibrium exhibited by the 2-amino-1,3,4-oxadaziazole residue.

Tautomerism in 1,3,4-oxadiazol-2-amines is well known and described in several sources [48].

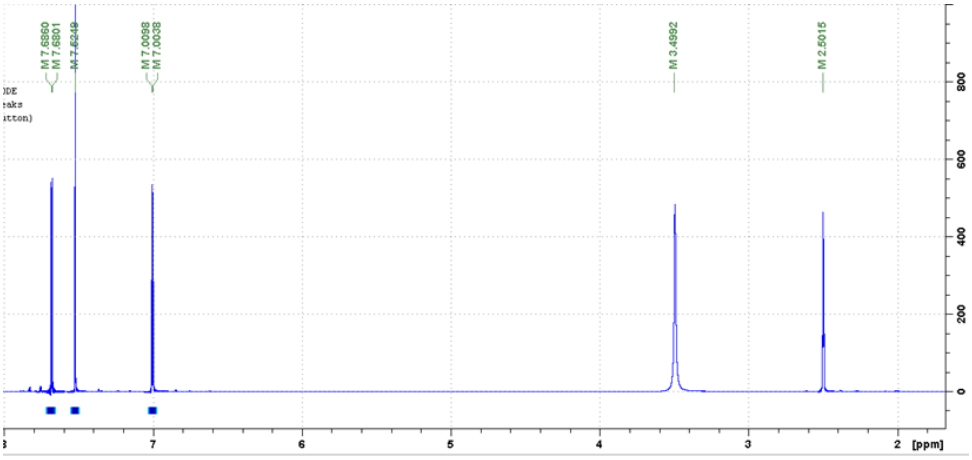


Figure 20. ^{1}H NMR spectrum (in DMSO-d6) 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine.

For the same reason, a smaller number of carbon signals were again reported in the ^{13}C NMR spectrum (Figure 21).

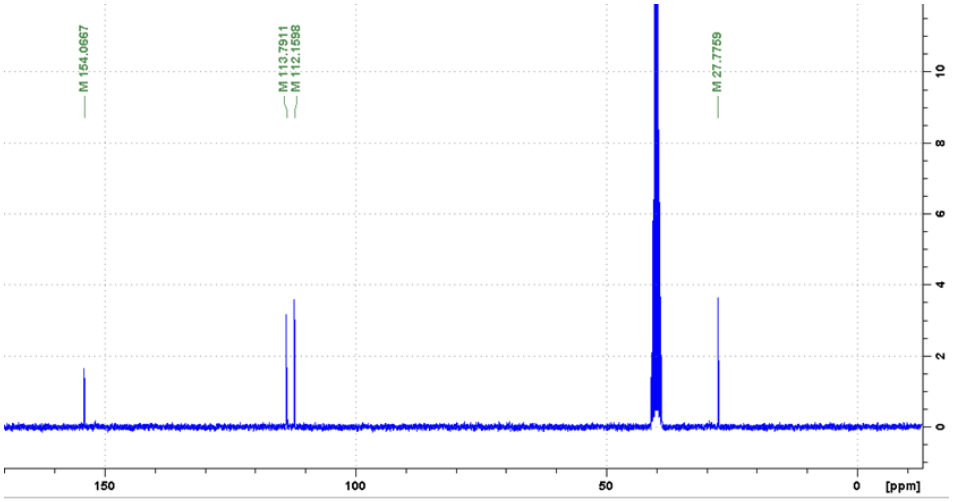


Figure 21. 5-(5-Nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine ^{13}C NMR spectrum (in DMSO-d6).

In order to obtain more detailed information about the product thus obtained, we performed an additional heteronuclear correlation analysis (Fig. 22).

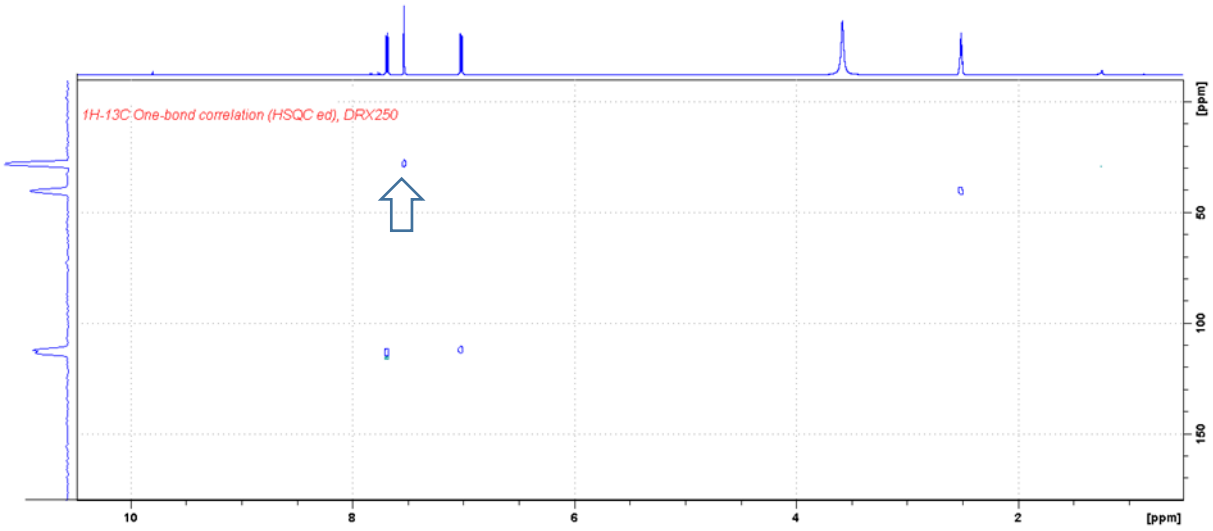
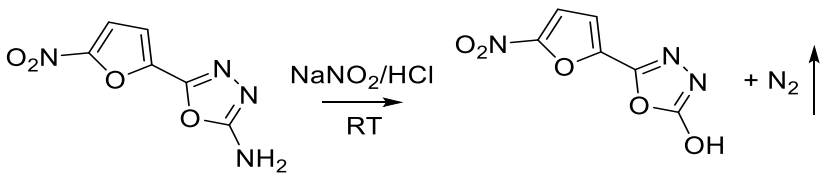


Figure 22. 2D ^{1}H, ^{13}C-HSQC spectrum (in DMSO-d6) of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine.

The established correlation between the singleton proton signal at 7.53 ppm and the strong localized carbon signal at 27.8 ppm remains completely inexplicable in this case.

In order to determine the presence of a primary aromatic amino group in the composition of the putative product, we also performed a qualitative analytical reaction (Scheme 8).



Scheme 8. *In situ* deazonation reaction of the obtained diazonium salt.

As soon as the introduction of $NaNO_2$ into the acidified aqueous solution of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine took place, a sudden onset of the effervescent effect

(denitrogenation reaction) observed. The reported manifestation unambiguously indicates the presence of a primary aromatic (1,3,4-oxadiazole) amino group in the composition of the studied product (Scheme 8).

The reaction product was also analysed by FTIR (Fig. 23) and HPLC.

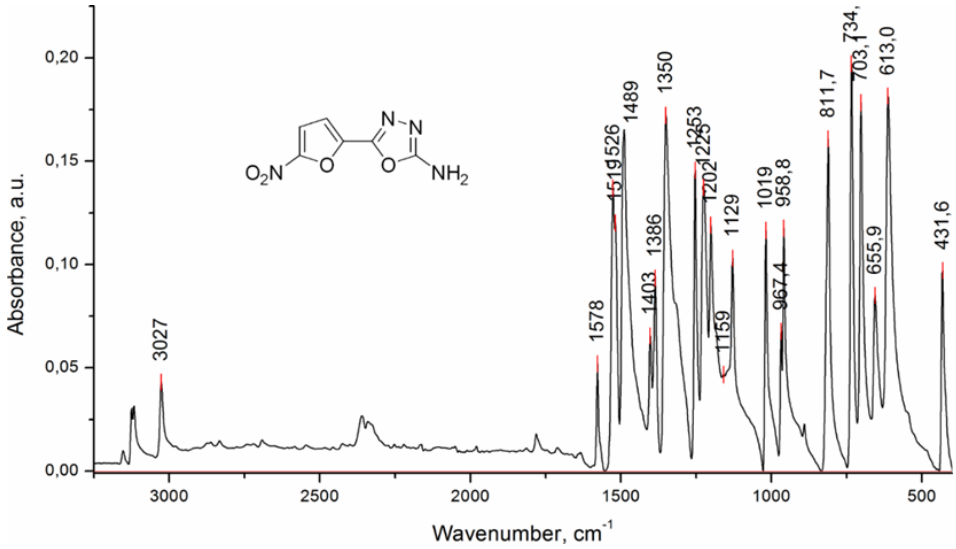


Figure 23. ATR-FTIR spectrum of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine.

The presence of the absorption band characteristic of the *Nitrofural* carbonyl functional group is not detected in the spectrum. Its absence should also be taken as evidence of the successful intramolecular cyclization of the semicarbazone residue. The presence of two medium to very intense bands at 1578, 1526 and 1489 cm^{-1} is associated with the stretching C=N vibrations of the 1,3,4-oxadiazole ring. Separately, symmetric and asymmetric stretching vibrations characteristic of the NO_2 group are also observed in the spectrum (1519, 1350 cm^{-1}). The presence of intermolecular NH… .O hydrogen contacts can be established by the absorption halo available in the range 1600 ÷ 3200 cm^{-1}.

The presence of only one component was found in the composition of the homogeneous aqueous-acetonitrile solution of the tested product (Fig. 24). The difference in retention times of the two components (*Nitrofural* precursor and 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine product) can be used as further evidence of the stated significant structural changes in the precursor used. The pharmacopoeial HPLC method for *Nitrofural* has been exploited for the purpose [2].

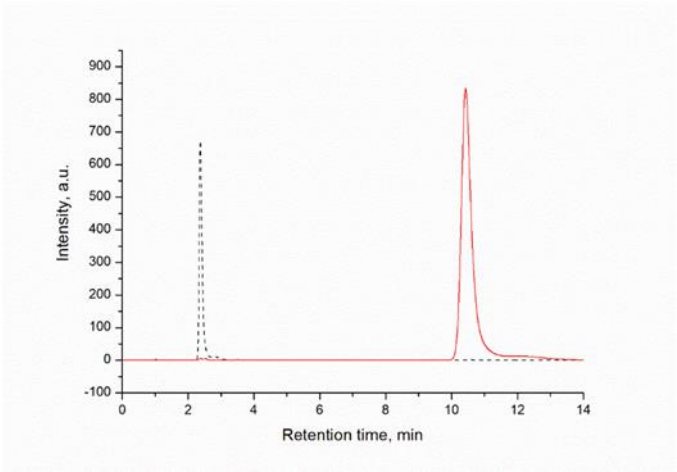
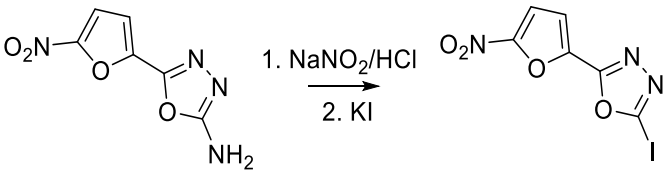


Figure 24. Chromatograms of *Nitrofural* (dashed line) and 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine (solid line).

The obtained intermediate diazonium salt was also utilized for the synthesis of 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole (Scheme 9). The reaction was performed in a two-phase (aqueous-chloroform) medium and in the presence of KI. The low water solubility of the obtained product, as well as the structural instability of the furan ring in a strongly acidic medium, is the reason that imposed the choice of this experimental methodology.



Scheme 9. Synthesis of 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole according to the Sandmeier method.

The reaction precursor used (5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine) was used in a minimal amount (35 mg). The desired product was obtained in 70% yield. The presence of covalently bound iodine was detected by FTIR analysis (Fig. 25). Separately, in an attempt to determine the melting point of the product, the release of violet vapors was observed, the presence of which we again associated with the covalently bound iodine.

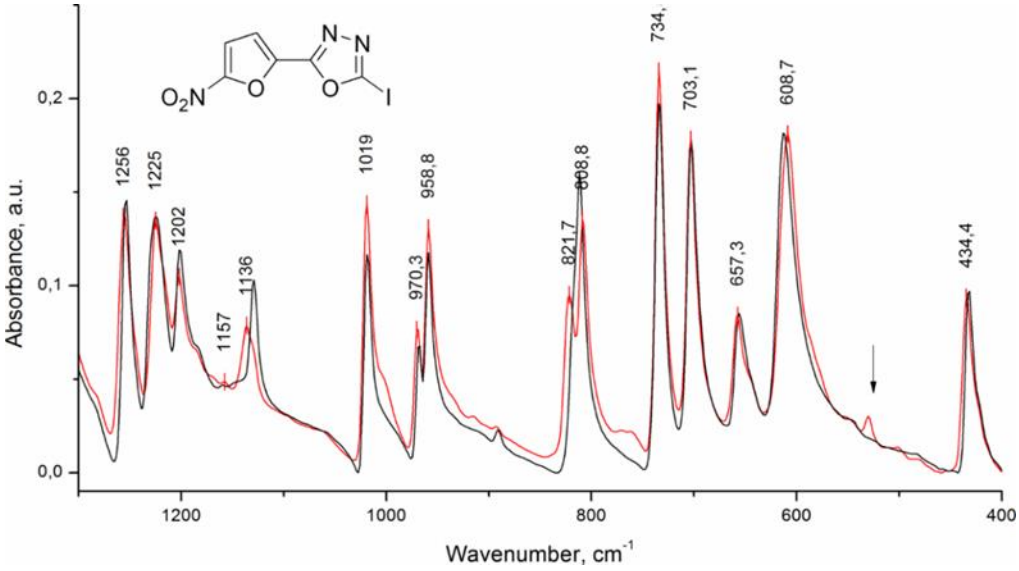
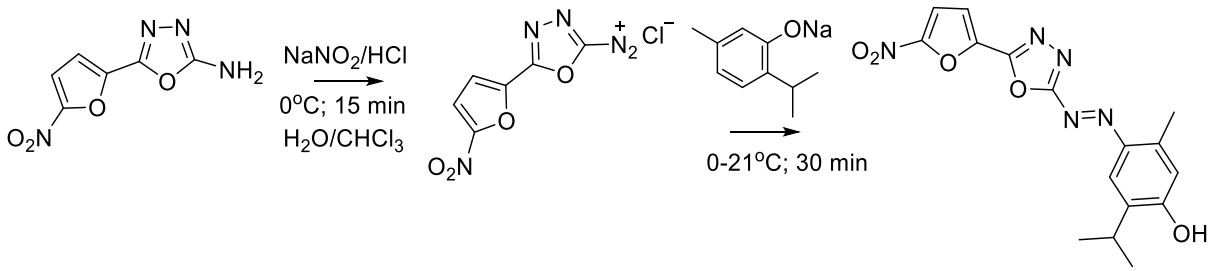


Figure 25. ATR-FTIR spectra of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine (black line) and 2-iodo-5-(5-nitrofuran-2-yl))-1,3,4-oxadiazole (red line).

The spectrum is presented in the fingerprint area, where the vibration of the C-I bond (indicated by a black arrow in the spectrum) are also located. Separately, the presence of iodine should affect most ring vibrations to a negligible degree. Therefore, a slight shift of most bands to lower frequencies was registered in the spectrum of 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole. The reason for the appearance of a new band at 822 cm^{-1} could not be determined correctly.

5-(5-Nitrofuran-2-yl)-1,3,4-oxadiazole-2-diazonium chloride was also used for the synthesis of 5-methyl-2-isopropyl-4-((5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl)diazenyl)phenol (Scheme 10). The reaction was carried out again in the two-phase $H_2O/CHCl_3$ system. Thymol was introduced in the form of Na^+ salt. As soon as it was added to the reaction medium, the the aqueous medium turned yellow-orange and a predominant yellow coloration of the chloroform layer was observed. After termination of the reaction, the chloroform layer was separated and evaporated to dryness *in vacuo*. Surprisingly, however, the reaction product formed a green amorphous mass. Given the small amount of reaction product obtained, its analysis was performed only on an ATR-FTIR spectrometer.



Scheme 10. Synthesis of 2-isopropyl-5-methyl-4-((5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl) diazenyl)phenol.

The ATR-FTIR spectrum of the product thus obtained is shown in Figure 26. The appearance of adsorption bands characteristic of both the starting 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine and for the included thymol was recorded in the spectrum. It is interesting to note that,

the presence of a band of medium intensity at 1440 cm^{-1}, characteristic of the *trans*-N=N- group in an aromatic encirclement, is also observed in the spectrum. As evidence of the successfully performed coupling reaction, we can use the observed significant displacements of the vibrations characteristic for thymol aromatic C=C bonds from 1620 and 1588 cm^{-1} (for thymol) to 1639 and 1605 cm^{-1} (for the reaction product). Those vibrations are particularly sensitive to changes in the aromatic (thymol) substitution.

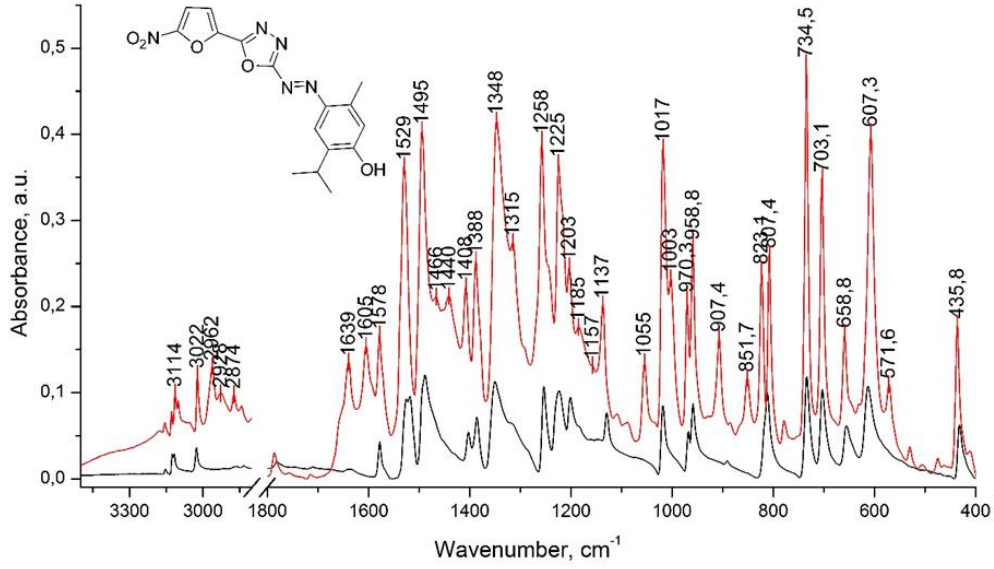


Figure 26. ATR-FTIR spectra of 2-isopropyl-5-methyl-4-((5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl) diazenyl) phenol (red line) and 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole (black line).

Additional confirmation of the formed azo contact between the two reaction participants can be found in the color (dark green) of the obtained reaction product; coloring, which is characteristic of most azo dyes.

In order to obtain more detailed information on the behavior of the two compounds newly obtained, we subjected them to additional microbiological studies.

Evaluation of the antibacterial activity of 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole and 2-isopropyl-5-methyl-4-((5-(5-nitrofuran-2-)yl)-1,3,4-oxadiazol-2-yl)diazenyl) phenol.

The microorganisms selected for the study cause some of the more common purulent-septic diseases and nosocomial infections - *E. coli* и *S. aureus*.

Solutions of test substances at known, progressively increasing concentrations were used to determine antimicrobial susceptibility. The results of the studies are presented in Tables 2 and 3.

Table 2. Determined antibacterial activity of *Nitrofural* (Nf), 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole (IODA) and 2-isopropyl-5-methyl-4-(5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl)diazenyl)phenol (OXA) versus *S. aureus*.

Tested substance	Concentration, µg/ml						
	8	16	32	64	128	256	512
Nitrofural	–	–	–	–	–	+	+
OXA	–	–	–	±	+	+	+
IODA	–	±	+	+	+	+	+

+ present zones of inhibition; - absent zones of inhibition; ± low activity

Table 3. Determined antibacterial activity of *Nitrofural* (Nf), 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole (IODA) and 2-isopropyl-5-methyl-4-(5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl)diazenyl)phenol (OXA) versus *E. coli*.

Tested substance	Concentration, µg/ml						
	8	16	32	64	128	256	512
Nitrofural	+	+	+	+	+	+	+
OXA	–	±	+	+	+	+	+
IODA	+	+	+	+	+	+	+

+ present zones of inhibition; - absent zones of inhibition; ± low activity

The test compounds exhibit pronounced antimicrobial activity as revealed by the reported areas of suppressed bacterial growth. We used the appearance of the so-called ‘sterile spots’ as real evidence of the suppressed microbial growth of the three studied substances.

Zones of inhibited *E. coli* growth were reported at all concentrations of newly derived 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole. Compared to *S. aureus*, the test iodine derivative is active at concentrations of ≥ 16 µg/ml. As the concentration of the iodinated product increased, the areas of the formed sterile spots over both bacterial cultures increased progressively. Smaller sterile spots were observed after treatment with 2-isopropyl-5-methyl-4-((5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl)diazenyl)phenol) over *E. coli* inoculated media. Suppressed growth of *E. coli* crops with this substance is reported at concentrations of ≥16 µg/ml.

The presented microbiological results will be used as a basis for our subsequent studies with the compounds and their derivatives presented here.

However, we found that the two reaction products obtained exhibited almost comparable to that of *Nitrofural* antibacterial activity. ATR-FTIR studies have also shown the successful synthesis of the two new 1,3,4-oxadiazole derivatives.

In order to determine the methodology for working with newly synthesized substances which are DMSO-soluble, we performed a preliminary photo- and cytotoxic study on *Nitrofural* and on the other two 5-nitrofuran pharmacopoeial agents - *Nitrofurantoin* and *Nifuroxazide*.

Determination of photo- and citotoxicity of *Nitrofural, Nitrofurantoin* and *Nifuroxazide.*

The mouse cell line used (BALB/3T3) aims at creating a real model of healthy tissue. Separately, it is also used as a standard (according to the World Health Organization) for phototoxic testing.

The results from the phototoxic tests performed on the embedded cell line were graphically presented (Fig. 27).

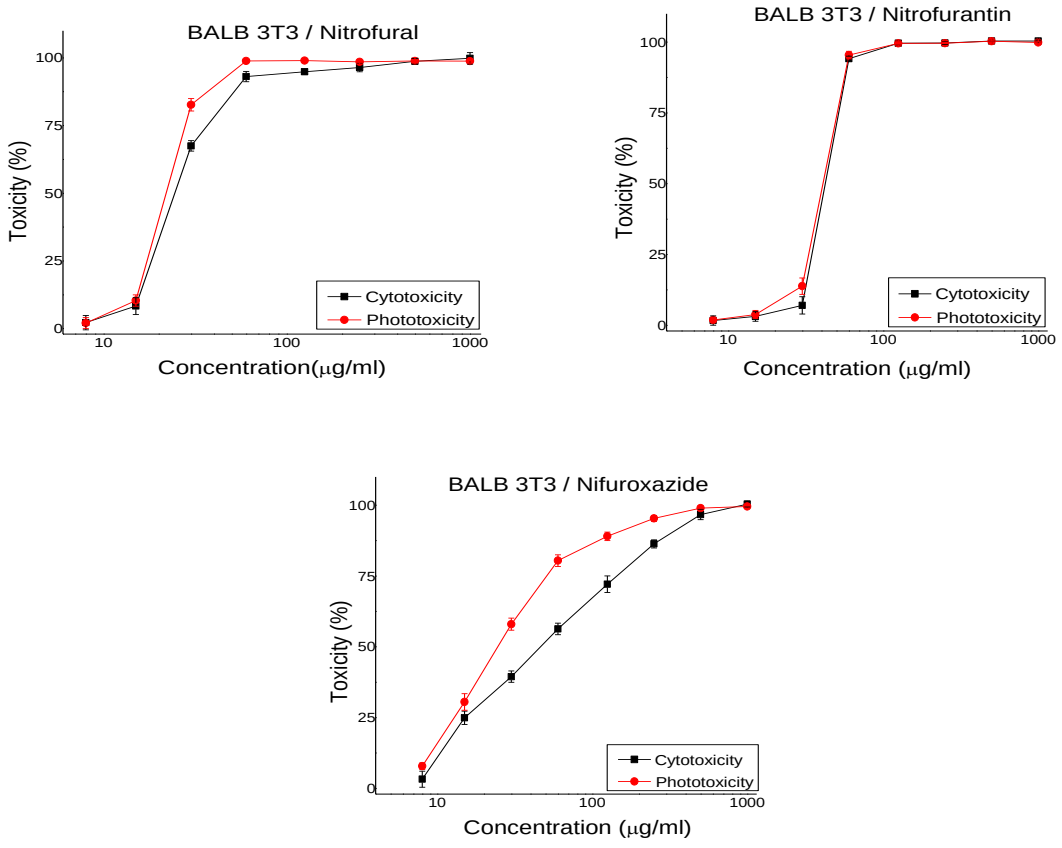


Figure 27. Photo- and cytotoxicity of the tested compounds against BALB/3T3 cell line A31.

The observed toxic effects are of the dose-dependent type (according to the sigmoidal dose-toxicity curve). Almost all of the three toxic effects of both drugs were observed. Minimal deviations were recorded only for *Nifuroxazide*, where weakly predominant phototoxic effects were observed at concentrations higher than 25 μg/mL.

The IC_{50} values for cytotoxicity and phototoxicity of the three substances were also calculated from the obtained sigmoidal curves (Table 4). Subsequently, the so-called PIF (Photo-irritancy factor) factor was calculated:

$$PIF = \text{Cytotoxicity } IC_{50} / \text{Phototoxicity } IC_{50}$$

This factor is indicative of phototoxicity.

Table 4. IC_{50} and PIF factor values.

Cell line	**Compounds**	**IC_{50} of Mean ± SD (µg/ml)**		**PIF***
		Cytotoxicity	Phototoxicity	
BALB 3T3	*Nitrofural*	25.57±0.5	23.24±0.33	1,1
	Nitrofurantoin	44.85±0.59	43.31±0.86	1,04
	Nifuroxazide	48.91±2.9	25.67±0.86	1,9

* PIF (Photo-irritancy factor): PIF < 2, does not exhibit phototoxicity; PIF ≥ 2 и < 5, probable phototoxicity; PIF ≥ 5 exhibited phototoxicity.

The calculated PIF values for all three compounds, fall within the photoinert zone. In reality, only the third analyte approaches the hypothetical phototoxic zone, but without "passing over" the 2.0 threshold.

VI. CONCLUSIONS

1. The research reported involves a new method for the spontaneous synthesis of several *Nitrofural* polymorphs on chalcogenide (As_2Se_3) glass from high-boiling and highly polar solvent DMSO.

2. The synthesis of a new, unknown *Nitrofural* polymorph was performed under the conditions of solvent shock evaporation (DMSO).

3. The synthesis of hitherto unknown metastable *Nitrofural* forms was realized.

4. A new, specific, qualitative electrochemical method for the identification analysis of *Nitrofural* was presented.

5. Gibbs reagent was used for the first time in *Nitrofural* (semicarbazone) qualitative analysis.

6. The synthesis was realized and the microbiological manifestations of two new 2-(5-nitrofuran-2-yl)-1,3,4-oxadiazole derivatives - 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole and 2-isopropyl-5-methyl-4-(5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl)diazenyl)phenol were established.

7. *Nitrofural* photo- and cytotoxicity was determined.

Plan for future actions complementing the current study

- To synthesize the established new *Nitrofural* polymorph and perform detailed X-ray crystal-structural analysis on it.
- To carry out research on the solubility of individual *Nitrofural* polymorphs and monitor their microbiological behavior.
- To demonstrate the crystal structure of 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-amine.
- To analyze in detail the structural features of the newly obtained 2-iodo-5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole and 2-isopropyl-5-methyl-4-((5-(5-nitrofuran-2-yl)-1,3,4-oxadiazol-2-yl)diazenyl)phenol products.
- To analyze in detail the biological behavior of the newly obtained 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole derivatives.

VII. CONTRIBUTIONS

1. The influence of *Nitrofural* polymorphism on the specific spectral (IR) behavior of the semicarbazone C=O functional group has been established.

2. The existence of a metastable *Nitrofural* form has been proven.

3. An innovative approach to the synthesis and spectral co-presence of several (including new) *Nitrofural* polymorphic forms that can be realized spectrally has been suggested.

4. Two new qualitative methods for the *Nitrofural* identification analysis have been introduced.

5. The possibilities for the synthesis of new 5-(5-nitrofuran-2-yl)-1,3,4-oxadiazole derivatives with a precursor *Nitrofural* itself have been investigated.

VIII. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

With Impact Factor:

1. Iliyan N. Kolev, Sonya Y. Ivanova, Anna K. Amova, Gergana E. Alexieva, Vesselin L. Strashilov. 2021. A new FTIR-based technique in the polymorphic analysis of Nitrofural. *Journal of Molecular Structure*. 1233: 130098. https://doi.org/10.1016/j.molstruc.2021.130098.

In refereed editions:

2. Sonya Ivanova, Svetlana Georgieva, Iliyan Kolev. 2021. NITROFURAL – ADVANTAGES AND DISADVANTAGES. *MANAGEMENT AND EDUCATION*. 17(5): 148-152.

3. Iliyan Nikolov Kolev, Sonya J Ivanova. 2018. The unusual behavior of the Gibbs reagent versus Nitrofural. *Scripta Scientifica Pharmaceutica*. 5(1): 14-19.

4. Iliyan Nikolov Kolev, Sonya J Ivanova, Mihail K Marinov. 2018. Electrochemical detection of Nitrofural in the presence of sodium amminepentacyanoferrate (II). *Scripta Scientifica Pharmaceutica*. 5(2): 34-39.

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