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Liquid-phase Extraction and Electrophoretic Determination of Heterocyclic Thioamides in Human Body Fluid

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Abstract- The work is devoted to the selection of optimal conditions for electrophoretic determination of heterocyclic thioamides which showed chemical activity to molecular iodine. It gives a reason to believe that the thioamides can be considered in the future as the basis for the development of antithyroid drugs. Due to the high sensitivity, the method of CE is versatile and successfully applied in toxicology and for monitoring pharmaceuticals in body fluids and tissues. The developed CE-technique of determination of *2-thioxo-5-(3,4,5-trimethoxy benzylidene)thiazolidin-4-one* (**I**), *5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione potassium salt* (**II**) and *4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione* (**III**) by aqueous capillary electrophoresis allows to estimate the content of compounds in the range of 3.10-25 μg/mL for (**I**)**,** 2.64-159 μg/mL for **(II)** and 1.45-12.80 μg/mL for (**III**). The technique was tested in analysis of urine. Selected conditions of the liquid-phase extraction are suitable for determination of compounds **(II)** and (**III)** in the urine**.** The optimal conditions of liquid-phase extraction which allow to extract approximately 74-97% of studied compounds were determined.

Keywords- Capillary electrophoresis; Thioamides; Urine sample; Liquid-phase extraction

1. INTRODUCTION

In recent years, capillary electrophoresis (CE) has attracted noticeable attention due to its widespread use as an efficient separation tool of complex analytes mixtures. It allows to separate a wide spectrum of compounds such as ions, small molecules, proteins. Moreover, CE can be conducted in various formats such as micellar electrokinetic capillary chromatography (MEKC) [1,2], capillary zone electrophoresis (CZE) in aqueous and nonaqueous CE (NACE) media [3,4], capillary isotachophoresis (CITP) [5,6], capillary electrochromatography (CEC) [7,8] etc.

One of the general trends in the field of analysis by CE is biomolecular research [9,10]. Undoubtedly, the development of separation techniques is important for selective and sensitive analysis of proteins and peptides, metabolites in biological fluids, DNA and pharmaceuticals. According to the studies that are carried out in specific areas and connected with definite molecular targets, all instrumental advances in CE are aimed at improvement sensitivity of analysis by combining with mass spectrometry method or using microfluidic electrophoresis platforms. New strategies such as gel electrophoresis, dielectrophoresis, and field (electric) flow fractionation for biochemical analysis of biomacromolecules, small molecule and bioparticles using microfluidic platform were developed by authors [11].

The review [12] exemplifies the versatility of CE for proteomics and metabolomics research. It encompasses the latest advances by separation of proteins due to sieving of protein ladder utilizing physical gel and sodium dodecyl sulfate. Likewise, CE has been used in biotherapeutics to identify enzyme inhibitors [13]. Utilization of a multi-dimensional separation platform which consists of size exclusion chromatography (SEC), reversed phase liquid chromatography (RPLC) and CE showed increasing of separating capacity of proteins and proteoforms in the E. coli proteome [14]. The most common and available classical detection scheme in CE is photometric method, although electrochemical, fluorescence and mass spectrometry methods are also widespread. It should be noted that mass spectrometry is quite often combined with separation methods. Additionally, it was found that performance of CE-MS is comparable with LC-MS and it has already given the opportunity to apply CE-MS in metabolomics research [15] and develop the database for the mobility of various analytes [16]. In work [17] the sheathless CE-ESI-MS/MS method that enables the characterization of the primary structure of antibody-drug conjugates was detailed.

CE as described above is successfully used in toxicology, diagnostics, anti-doping testing in sports for monitoring pharmaceuticals and drugs in body fluids and tissues. W. Thormann in the review [18] considers the general principles of CE for analysis of licit and illicit drugs in biological specimens. The key aspect in determination of drugs/pharmaceuticals is sample preparation. And the main objective is isolation of the analyte from interfering compounds in biological matrix. To achieve this purpose liquid-phase microextraction (LPME) based on

extractant drop-, plug-, film- and microflow formation [19,20] and micro-solid-phase extraction [21,22] are exploited.

Currently, mercapto-substituted of N, S-containing heterocycles - thioamides have been extensively used by chemists to design new drug candidates [23-25]. It is believed that the presence of thioamide moiety in a molecule of heterocycle determines its antithyroid properties with respect to the excessive production of thyroid hormones, which include iodine molecules. Due to the presence of a sulfur atom, thioamides are able to capture the active forms of iodine, performing a kind of antioxidant function and also inhibit thyroid hormone synthesis by forming complexes with iodine [26].

The correlation revealed between the antithyroid activity of heteroaromatic thioamides *in vivo* and the stability of charge transfer complex with the iodine [27] allows one to purposefully search for new-generation thyreostats.

Unequivocally, for potential drug candidates is necessary to develop simple, selective and high accuracy techniques of determination. Authors proposed MEKC and NACE methods to determine derivatives of mercaptoquinolines [28]. Application of this methods allowed to reduce time of determination of 8-mercaptoquinoline and stabilize it in capillary. Earlier studies of thioamides: *2-thioxo-5-(3,4,5-trimethoxybenzylidene) thiazolidin-4-one* (**I**)*, 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione potassium salt* (**II**) and *4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione* (**III**) performed in model conditions have revealed their thyreostatic activity to molecular iodine [25,29,30]. The obtained values of the stability of their charge transfer complexes with iodine (lgβ=4.33–**I**, lgβ=7.91-**II** and lgβ=3.47-**III**) are promising to consider this thioamides as perspective drug candidates. As a part of our program aimed at creating of database for mobility of potential pharmaceutical candidates, the development of quantitative technique of electrophoretic determination (**I**, **II**, **III**) is of great interest.

The main objective of this work is to develop capillary electrophoretic technique for determination of **I**, **II** and **III** and test it on the samples of urine.

2. EXPERIMENTAL

2.1. Reagents

A borate buffer solution (pH 9,18), *2-thioxo-5-(3,4,5-trimethoxybenzylidene) thiazolidin-4-one* (**I**), *5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione potassium salt* (Alfa Aesar) (**II**) and *4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione* (**III**). Compound **I** was **s**ynthesized by using microwave irradiation for cyclization of 2-thioxo- 1,3-thiazolidin-4-one with 3,4,5-trimethoxybenzaldehyde [31]. Compound **II** was obtained in several stages by converting $PhNHNH_2$ to the acid $PhN_2C_2S_3H$ using N, N-dimethylformamide, pyridine, CS_2 and aqueous 5% Na_2CO_3. At the final stage $PhN_2C_2S_3H$ was dissolved in hot EtOH of KOH. Compound **III** was prepared by the reaction of furan-2-carboxylic acid hydrazide with mixture of CS_2 and EtOH

in alkaline medium [32]. The addition of hydrazide hydrate in the solution gave *4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione.* The compounds were used without additional purification. As an extractant ethyl acetate was used.

2.2. Instrumentation

The electrophoretic determination was performed using a Kapel-103 R CZE system with UV-detector. Data acquisition and processing were performed using an IBM PC with Multichrom software.

2.3. Preparation of solutions

To select the optimal conditions for quantitative determination of investigated thioamides the fundamental characteristics such as constants of protolytic equilibrium were estimated by potentiometric titration in an aqua-ethanol medium (1:1) using pH-meter with multi-point calibration.

The constants of protolytic equilibrium for the compounds are equal pK=7.06, pK_{BH^+} =2.51- **(I)**; pK_{BH^+} =2,72 – **(II)**; pK=8,14, pK_{BH^+}=2.25 – **(III)**, respectively. ^{1}H NMR spectra of the considered compounds were taken from [29-31]. A 25 mM borate buffer with pH=9,18 was prepared by dissolving of accurate weight in twice-deionized water.

The stock standard solutions of compounds **I, II** and **III** with concentrations 1×10^{-4} M were prepared in ethanol solution. Similar concentrations for compounds **II** and **III** were prepared in twice-deionized water for analysis in urine. Water solution of compound **I** wasn't prepared by reason of insolubility in this medium.

The working ethanol solutions used for constructing of calibration curves by diluting of the stock solutions.

To test developed technique of CE the fresh sample of human urine was taken. Analyzed samples of urine were prepared by addition of water solutions of compounds **II** and **III** to the urine. Extraction of thioamides from matrix of urine was performed by liquid-phase extraction with three portions of ethyl acetate (5 ml) from 5 ml of urine. The obtained extracts were evaporated at room temperature and dissolved in 5 ml of ethanol. All electrolyte solutions were filtered through a 0.2 μm membrane filter.

2.4. Capillary electrophoresis

Separation was performed on unmodified quartz capillary with an inner diameter of 75 μm and a length of 60 cm (effective length of 50 cm). The samples were ejected hydrodynamically at a pressure of 3 kPa for 5 secs for thioamides **II** and **III** and for 10 secs for **I**. The working voltage was +15 and +20 kV. Photometric detection at 253.7 was used. Since analytes relate to various classes of heterocycles and can be used as individual components of future

pharmaceuticals we decided to analyze them separately from each other. The capillary was rinsed with 1.0 M hydrochloric acid, water, 0.5 M sodium hydroxide and water for 10 min, and then equilibrated with the carrier electrolyte for 10 min at the beginning of each day. Between all electrophoretic separations the capillary was rinsed for 10 min with the carrier electrolyte.

3. RESULTS AND DISCUSSION

The objects of research **I, II** and **III** are presented in Fig. 1. The tautomeric equilibrium «thioamide-thioiminol» can occur in the solutions of heteroaromatic thioamides [33,34]. The individual feature of ^{1}H NMR of the solutions of I-III indicate that the thioamides are in thione form.

^{1}H NMR of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione potassium salt (250 MHz, DMSO): δ=7.37-7.54 (m 5H); ^{1}H NMR of 2-thioxo-5-(3,4,5-trimethoxy benzylidene) thiazolidine-4-one (500 MHZ, DMSO-D_6): δ=3.74 (s, 3H, O-CH_3), 3.83 (d, 6H, O-CH_3), 6.88 (s, 2H, Ar-H), 7.58 (s, 1H, =CH-), 13.82 (s, 1H); ^{1}H NMR of 4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione (600 MHz, DMSO): δ = δ 5.78 (s, 2H), 6.67-7.88 (m 3H), 13.71 (s, 1H).

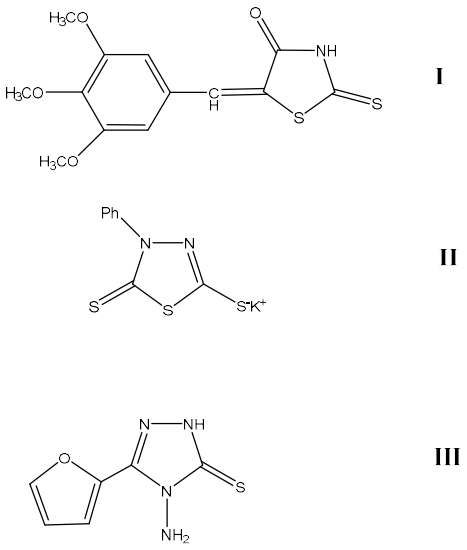

Fig. 1. *2-thioxo-5-(3,4,5-trimethoxybenzylidene) thiazolidin-4-one*-(**I**), *5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione potassium salt*-(**II**), *4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione*-(**III**)

The values of protolytic constants of the considered compounds indicate that the compounds have various protolytic properties. The protolytic properties and the stability of ethanol solutions of studied compounds in capillary didn't require the application of NACE or MEKC method. Since the compounds **I** and **III** are ampholites that are protonated at pH<2 and ionization of NH-proton in thioamides starts at pH ≥7.3, therefore, optimal conditions for **I** and

III determination can be selected due to the transformation them into anionic forms. The compound **II** is initially present in solution in anionic form. The UV-vis absorption spectra of ethanol solutions of compounds were recorded using a Cary 50 spectrometer (Varian) in the wavelength region of 200-800 nm at 22^{0}C. It was found that the position of peaks at absorption curves allows to perform the direct detection of all analytes at 253.7 nm (Fig. 2).

Since studied compounds had to be transferred to anionic form, various types of back ground electrolytes with pH >7 were tested for this aim. Using borate buffer solution with pH 9.18 gave the narrowest chromatographic peaks (Fig. 3).

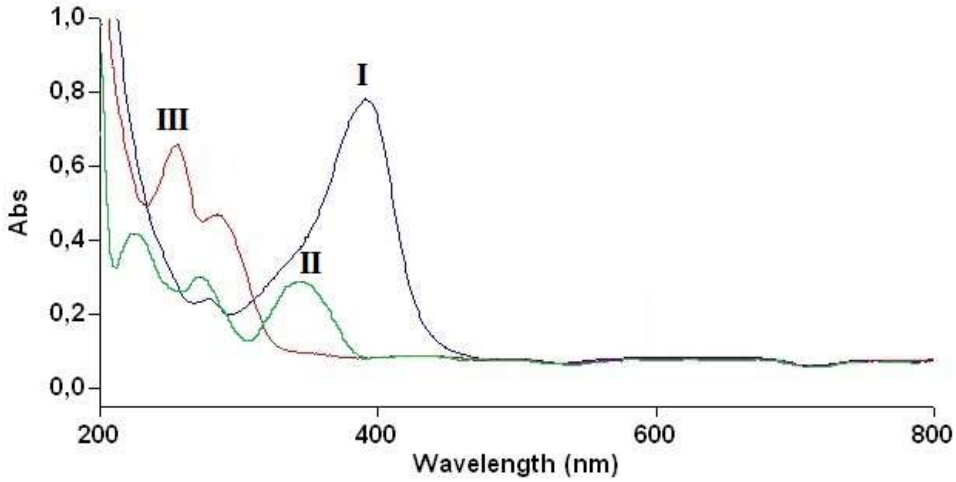

Fig. 2. UV-vis absorption spectra of ethanol solutions containing 15.50 μg/mL – **(I)**, 20 μg/mL – **(II)** and 7.30 μg/mL – **(III)**

Table 1. Performance characteristics of the procedure for the determination of the compounds **I**, **II**, **III** by CZE (n=5, P=0.95)

Compound	**LOD** (μg/mL)	**RSD (%)**	**t_R(min)**	Calibration graph parameters $S = a \cdot C + b$		
				a	***b***	***ρ***
I	1.49	3.95	8.45	1.54	5.49	0.990
II	1.1	3.66	9.90	2.38	11.48	0.998
III	1.18	2.88	9.10	1.78	4.081	0.996

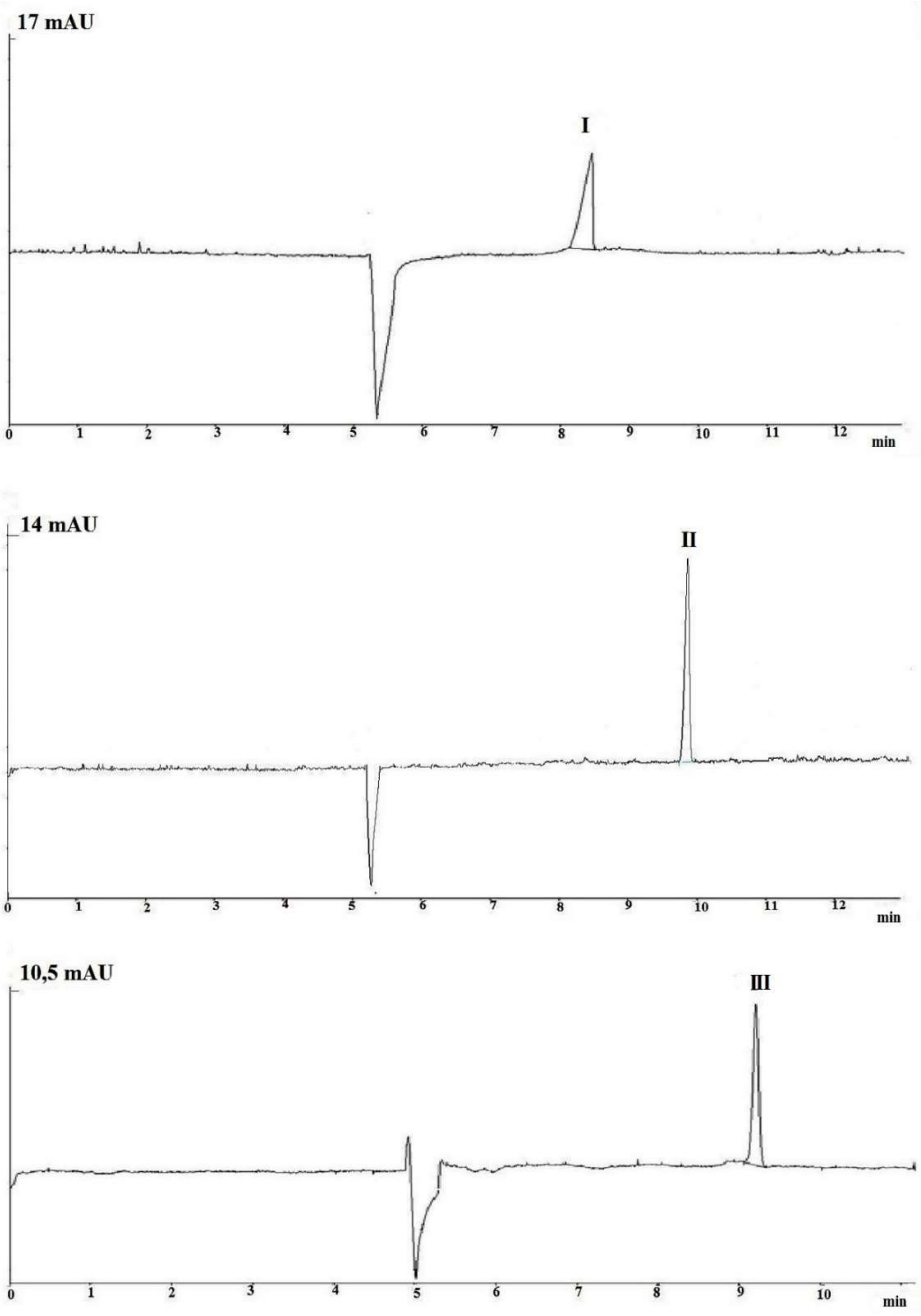

Fig. 3. Electrophoregrams of compounds (**I**) – 15.50 µg/mL**,** (**II**)- 20 µg/mL**,** (**III**)- 7.30 µg/mL. Background electrolyte – borate buffer pH = 9.18

The quantitative determination of considered analytes was performed by the external standard method with the use of linear dependence of area (*S*) of the peaks on concentration (*C*). This type of quantitative assessment is widely used and it has already been applied in our previous work [28]. Valid calibrations were constructed in the ranges of 3.10-25 µg/mL for (**I**),

2.64-159 μg/mL for **(II)** and 1.45-12.68 μg/mL for (**III)**, respectively. All performance characteristics of the determination procedure are exemplified in Table 1.

The analogue compounds based on thiazole, 1,3,4- thiadiazole and 1,2,4-triazole were determined by CZE [35]. Based on the values of LOD obtained under the same conditions of determination, we believe that the choice of borate buffer for the determination of majority of ampholytes based on mercapto-derivatives of thiazole, 1,3,4- thiadiazole and 1,2,4-triazole is optimal.

The thyreostats are excreted by kidneys with the urine in unchanged form, thus the technique of CZE determination of the thioamides was tested in analysis of urine. Since the compounds haven't passed any biological trials determination in biological fluid was performed *in vitro* through the treatment of urine by various quantity of studied compounds. It is important to emphasize that the compound **I** can not be determined in urine by reason of insolubility in aqueous medium. Authors in work [36] used diethyl ether for extraction of thyreostats from urine but this solvent is quite soluble (~4%) in water and difficult to blow to complete dryness. That's why ethyl acetate was chosen as the widespread and powerful volatile solvent for extraction of the compounds from urine.

Table 2. Percent recovery data for **II** and **III** in the urine samples by ethyl acetate

Sample (№)	**Spike level (μg/mL)**	**Observed (μg/mL)**	**SD**	**Recovery (%)**
Urine (II)	Low 2.64	1.99	0.18	75.38±0,30
	Med 13.22	11.38	0.23	86.08±0,39
	High 132.22	122.41	0.84	92.58±1,42
Urine (III)	Low 3.62	2.68	0.70	74.03±1.17
	Med 7.24	7.04	0.36	97.23±0,61
	High 14.50	12.52	0.73	86.34 ±1.59

The antithyroid preparations are prescribed to treat in high concentration, for instance propylthiouracil about 100-600 mg. Therefore, it was taken quite high concentrations of the thioamides for analysis. The results of quantity determination of thioamides **II** and **III** were performed by spike-recovery method. Analytical parameters evaluated for the two thiones are summarized in Table 2. Means of extraction recovery (n=3 at each level of concentrations) ranging from 75% to 93% for **II** and from 74% to 97% for **III**, respectively. Undoubtedly, quite high recovery values demonstrate approximately complete recovery of thioamides from the urine and chromatograms (Fig. 4) their isolation from coextraction of matrix components for all spikes tested.

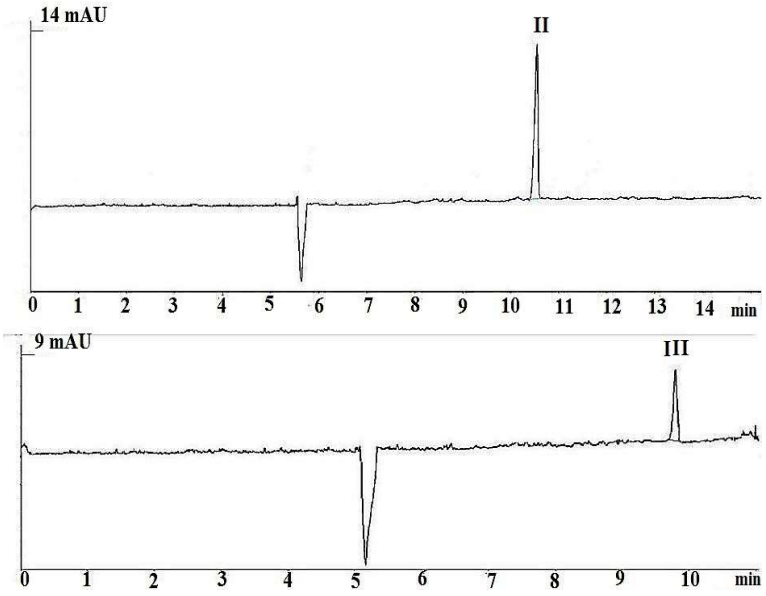

Fig. 4. Electrophoregrams of samples of urine with spiked concentrations: 13,22 μg/m -(II) and 7,24 μg/mL-(III)

The technique of extraction of thyreostats from urine in [36] is time-consuming and requires preliminary derivatisation of samples by 3-iodobenzylbromide in basic medium. Applying the developed technique (borate buffer) and ethyl acetate as extractant it can be achieved quite high percent of recovery of **II** and **III** without preliminary derivatisation. Thus, the optimal conditions for the quantitative extraction of studied compounds were determined.

4. CONCLUSION

The selected conditions (borate buffer pH=9.18) for determination of *2-thioxo-5-(3,4,5-trimethoxybenzylidene) thiazolidin-4-one* (**I**), *5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione potassium salt* (**II**) and *4-amino-5-(furan-2-yl)-4H-1,2,4-triazole-3-thione* (**III**) by capillary electrophoresis with photometric detection is notable for accuracy, rapidity and sensitivity. The proposed procedure is inexpensive, simple in performing and can be applied in monitoring of pharmaceutical preparations based on mercapto-derivatives of thiazole, 1,3,4- thiadiazole and 1,2,4-triazole. The technique was tested in analysis of urine. We believe that to test compound **I** *in vivo* it should be preliminary derivatized for improving solubility in aqua medium.

Using ethyl acetate as an extractant for extraction of thioamides a fairly high percent recovery can be achieved. This extractant can be proposed as an alternative replacement for diethyl ether which is used for monitoring of thyreostatic drugs in routine experiments. In addition, selection of ethyl acetate as the extractant allows to avoid not quite correct data of

extraction, isolate studied compounds from interfering compounds of biological matrix, thus giving undeniable advantages for monitoring pharmaceuticals in body fluids and tissues.

The identified mechanism of interaction of studied thioamides with molecular iodine allows to consider this thioamides as perspective compounds which possess thyreostatic action. The determination of the thioamides in biological matrix by physiochemical methods is a final stage in terms of analytical study of the compounds. In turn, it gives an opportunity for further tests to evaluate the biological effect of this compounds.

Conflict of interest

The authors have declared no conflict of interest.

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