A novel kinetic cholinesterase-inhibition based method for quantification of biperiden in pharmaceutical preparations

Vladan R. Djurić[1], Violeta D. Mitić[2], Nebojša R. Deletić[1], Marija D. Ilić[3], Bratislav M. Ćirković[1] and Vesna P. Stankov-Jovanović[2]

[1]University of Priština – Kosovska Mitrovica, Faculty of Agriculture, Kopaonička bb, 38219 Lešak, Serbia
[2]University of Niš, Faculty of Science and Mathematics, Department of Chemistry, Višegradska 3, 18000 Niš, Serbia
[3]Veterinary Specialized Institute "Niš", Laboratory Sector, Laboratory for Analytical Chemistry, Dimitrija Tucovića 175, 18106 Niš, Serbia

Abstract

Biperiden, an antiparkinsonian anticholinergic drug, has been found to inhibit enzymatic hydrolysis of butyrylthiocholine iodide, which is catalyzed by serum cholinesterase. By measuring the difference in the basic and inhibitory hydrolysis reaction rates, in the presence of biperiden as an inhibitor, it is possible to develop a kinetic method for its determination. Both systems, enzyme-substrate-chromogen and enzyme-substrate-chromogen-inhibitor, were characterized by biochemical parameters (K_M = 0.326 – 0.330 mmol dm^{-3}; V_{max} = 40 - - 99 µmol dm^{-3} min^{-1}), while inhibition was defined as non-competitive with the constant of inhibition K_i = 6.142 µmol dm^{-3}. The reaction conditions have been optimized followed by determination of the calibration curve, the corresponding equation and the limits of detection and quantification yielding 3.84 and 12.80 nmol dm^{-3}, respectively. Using the calibration chart, it is possible to determine biperiden in different samples in the concentration range of 0.035–35.940 µmol dm^{-3}. Influence of a number of substances, found in the sample, on the reaction rate was also examined. The optimized method was applied for determination of biperiden in pharmaceutical preparations. Accuracy of the method was tested using the standard addition method. The proposed method has good sensitivity, selectivity, it is simple and fast, and above all easily accessible, and thus applicable in biochemical and pharmaceutical laboratories.

Keywords: enzyme; hydrolysis; inhibition; butyrylthiocholine iodide; reaction rate; optimization.

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

1. INTRODUCTION

Biperiden, akineton or mendilex (1-(2-bicyclo[2.2.1]hept-5-enyl)-1-phenyl-3--(piperidin-1-yl)propan-1-ol, BIP) (Fig. 1) is an antiparkinsonic belonging to the group of anticholinergic drugs. It exerts its effect by blocking acetylcholine receptors, with a greater affinity for those of muscarinic type, which are activators of nerve cells. Thereby it restores the balance of dopamine and acetylcholine, which is impaired by the Parkinson's disease. Biperiden is not only used in the therapy of this disease but also to combat the symptoms of parkinsonism (tremor) that occur in antipsychotic therapy.

It is also used as an antidote for poisoning by organophosphorus-type nerve poisons, such as sarin, soman and tabun, as well as by some insecticides (malathion), that are potent acetylcholinesterase inhibitors [1].
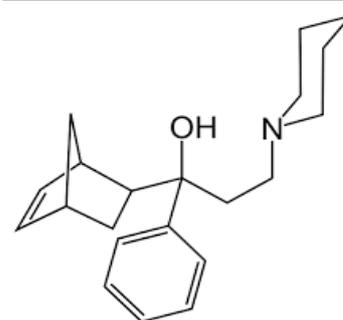

Figure 1. Structural formula of BIP

Various methods have been developed and tested for determination of BIP in samples of different origins, whether in pharmaceutical formulations, to monitor the active substance content and quality of preparations, or in physiological fluids. Application of matrix-assisted laser desorption ionization (MALDI) – quadrupole time-of-flight (QTOF) – tandem mass spectrometry (MS/MS) to the quantification of BIP in human whole blood was reported [2]. The limit of detection was 0.001 µmol dm^{-3} with a determination range of 0.003–0.3 µmol dm^{-3} using 20 µL of whole blood, while the concentrations of BIP in the whole blood samples were 0.005 and 0.056 µmol dm^{-3}. In another study, a gas chromatographic method, using a moving precolumn sample injector and a nitrogen-phosphorus detector, was presented for the quantitative analysis of BIP in rabbit plasma [3]. The processed standard curves were linear over the concentration range 0.004-0.16 µmol dm^{-3}, with the assay sensitivity of 0.002 µmol dm^{-3} [3]. Another sensitive and rapid method for determination of BIP in plasma and urine has been developed using solid phase extraction and gas chromatography–mass spectrometry (GC–MS), and the established determination range was 0.016–0.96 µmol dm^{-3} in plasma and 0.032–2.56 µmol dm^{-3} in urine, while limits of detection were 0.006 and 0.003 µmol/L, respectively [4]. Determination of BIP in human serum by liquid chromatography – electrospray ionization/mass spectrometry (ESI/MS), with an on-line sample clean-up procedure used, showed the limit of detection of 0.003 µmol dm^{-3} and determination range of 0.035–1.92 µmol dm^{-3} [5]. Sensitive disposable sensors have been constructed for potentiometric determination of BIP hydrochloride in dosage formulations and biological fluids, based on a multi-walled carbon nanotubes – poly-vinylchloride (MWNTs–PVC) composite in the presence of dibenzo 24-crown-8- ether as a molecular receptor [6]. The method had the sensitivity range of 1-10000 µmol dm^{-3}, with the limit of detection of 0.1 µmol dm^{-3} [6]. An interesting approach was based on using disposable pipette extraction with reversed-phase sorbent for the fast and simple GC determination of BIP in human urine, which gave detection limits of 0.90–1.36 µmol dm^{-3} (within-day and between-day), quantification limits of 2.75–11.58 µmol/L (within-day and between-day), while the determination range was between 1.1 and 16.2 µmol dm^{-3} [7]. In order to improve selectivity and stability-indicating properties of BIP determination In bulk form and pharmaceutical dosage forms, a high performance liquid chromatographic assay procedure was developed and validated for BIP [8]. In the mentioned study, the liquid chromatographic separation was achieved isocratically on a symmetry C8 column (150 mm x 3.9 mm i.d., 5 µm particle size) using a mobile phase containing methanol-buffer (50:50, v/v, pH 2.50) at a flow rate of 1 cm^3 min^{-1} and UV detection at 205 nm. The detection limit of the method was 0.096 µmol dm^{-3}, the quantification limit was 0.288 µmol dm^{-3}, while the determination range was 1.44–72 µmol dm^{-3} [8]. An original capillary electrophoretic method has been developed and applied for the enantioselective analysis of BIP in pharmaceutical formulations, using a modified cyclodextrin as the chiral selector, and the achieved limit of detection was 1 µmol dm^{-3}, the quantification limit was 2.88 µmol dm^{-3}, while the determination range was 2.88–100 µmol dm^{-3} [9]. Simple and rapid spectrophotometric procedures, based on the reaction between BIP and picric acid (I), alizarin (II), bromothymol blue (III) and chlorophenol red (IV) producing ion-associates, which can be measured at the optimum wavelengths (426, 410, 400, and 413 nm for reagents I-IV, respectively), were established for quantification of BIP hydrochloride in pure solutions and pharmaceutical preparations, and the observed determination range was between 171 and 478.3 µmol dm^{-3} [10].

Working on serum cholinesterase inhibition processes [11], and bearing in mind that BIP is a heterocyclic tertiary amine and a tertiary alcohol that binds to plasma proteins by up to 60 %, and not only M_1 type, but also M_2, M_3 and M_4 type antagonist of muscarinic receptors with inhibition constants of 0.480 to 10.70 nmol dm^{-3} [12], the idea of developing a kinetic method based on possible inhibition of this enzyme has emerged. The aim was to develop a highly sensitive and selective analytical method that could be performed quickly using relatively inexpensive instruments such as spectrophotometers so to be available for use in a large number of laboratories. High specificity of enzymatic reactions allowed using pooled human serum as a source of cholinesterase. To the authors' best knowledge, literature data on enzymatic inhibition-based methods for the determination of BIP do not exist. A modified Ellman's method was applied to monitor the reaction rate [13]. The biochemical parameters of reaction and inhibition were also determined. The proposed method has been tested in accordance with all important analytical requirements.

2. EXPERIMENTAL

All chemicals used were *p.a.* purity. Biperiden hydrochloride (BIP, purity >99 %) was provided by Biocrick Biotech Co., Ltd. (Sichuan, PRC). The stock solution was made at the concentration of 10 mg cm^{-3} BIP in deionized water, while the working solutions were prepared daily by diluting the stock solution.

Buthyrylthiocholine iodide (BUTC, purity >99 %) as well as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Merck KGaA (Darmstadt, Germany). The BUTC stock solution was at the concentration of 133.33 mmol dm^{-3}, while the DTNB stock solution was obtained by dissolving exactly 0.0792 g in 5 cm^3 of phosphate buffer with 60 mg of NaHCO$_3$ added, and then diluted to 10 cm^3 with deionized water. The NaHCO$_3$, KH$_2$PO$_4$, Na$_2$HPO$_4$ buffer components were acquired from Merck KGaA (Darmstadt, Germany).

Deionized water (conductivity <1 µS) was used to prepare all the solutions.

All concentrations of the presented components were the initial concentrations in the reaction mixture at time zero before mixing.

For spectrophotometric measurements, a Konelab 20 analyzer (Thermo Fisher Scientific, Waltham, MA, USA) was used, with 7 mm flow-through thermostated cells at the wavelength λ = 405 nm.

For preparing pooled serum (enzyme source), ten healthy volunteers (ages 18–65, both sexes) with the written consent, donated blood at the General Hospital of Pirot, Serbia. Each of the volunteers had neither a recent nor previous history of significant medical disorders, nor had they abused drugs, alcohol, and tobacco. Also, none of them took any medication in the last month before the blood was given.

Serum (5 cm^3) from each donor was collected in vacuum tubes and centrifuged for 10 min at 3000 rpm, after which the serum supernatants were pooled, mixed and used as a source of serum cholinesterase. Serum cholinesterase hydrolyses butyrylthiocholine to thiocholine, and it reacts with DTNB to form a colored product (5-thio-2-nitrobenzoic acid). The reaction rate is determined based on color development measured at 405 nm.

Solutions of biperiden hydrochloride of different concentrations (10 µL) were mixed with serum (10 µL), which was previously diluted with phosphate buffer in a ratio of 1:9 v/v, and 160 µl of phosphate buffer (preincubated at 37 °C for 10 min). After that, 10 µL of DTNB was added and left for 60 s. As a last component of the reaction mixture, the BUTC substrate solution (10 µL) was added. The same procedure was applied when samples were analyzed. All kinetic data were interpreted by the tangent method and represented as average of five replicates.

For preparing sample solutions, 50 tablets of biperiden hydrochloride (Mendilex 2 mg tablets – Alkaloid AD, Skopje, Northern Macedonia; Akineton 2 mg tablets – DESMA GmbH, Wiesbaden, Germany) were ground into powder. The mass of the powder obtained, equivalent to 100 mg of biperiden hydrochloride, was accurately measured and transferred to a 100 cm^3 calibration flask. After adding about 60 cm^3 of deionized water, the contents were shaken, then filtered through a quantitative filter paper and supplemented with water to the line.

Volume of 20 injections of biperiden lactate (Akineton 5 mg cm^{-3} – DESMA GmbH, Wiesbaden, Germany) was quantitatively transferred into a 100 cm^3 flask and supplemented to the line with deionized water. The content of the flask that is 100 mg biperiden lactate was considered as equivalent to 86.67 mg biperiden hydrochloride and used to determine the precision and accuracy of the proposed method.

3. RESULTS AND DISCUSSION

In order to determine the optimal operating conditions of the proposed method, the main parameters affecting its performance were examined. All parameters were optimized by the invariant method. For determination of kinetic biochemical parameters, dependence of the reaction rate on the substrate (butyrylthiocholine iodide) concentration was estimated in the range 0.208–6.650 mmol dm^{-3}. The Lineweaver-Burke, Eadie-Hofstee, and Hanes linearization methods resulted in the following values of the Michaelis-Menten's constant: 0.330, 0.305 and 0.326 mmol dm^{-3}, respectively, while the values of the maximum reaction rate, V_{max}, were 42.29, 40.00 and 42.38 mmol dm^{-3} min^{-1}, respectively (Supplementary material Figures D-1, D-2 and D-3).

The kinetic data obtained allow us to conclude that BIP and serum cholinesterase interact in a manner of non-competitive inhibition (Fig. 2), attributable to the existence of a nonpolar BIP structure that corresponds via π–π bonds

of the benzene and 2-bicyclohept-5-ene rings to the aromatic amino acid residues in the outer skeleton of the enzyme, and to the piperidine ring, due to the presence of nitrogen, cation–π and hydrophobic contacts with the same amino acids, which would indirectly lead to a shift in the active center of the cholinesterase and decrease its activity. The inhibition constant, calculated from the function of inhibition reaction rate depending on different concentrations of BIP, according to the Dixon's method, was K_i=6.142 µmol/L.

Different concentrations of BIP affect the relative activity of serum cholinesterase as shown in Figure 3. Relative activity of the enzyme is presented as the initial reaction rate ratio of inhibited reaction to the uninhibited one, multiplied by 100.
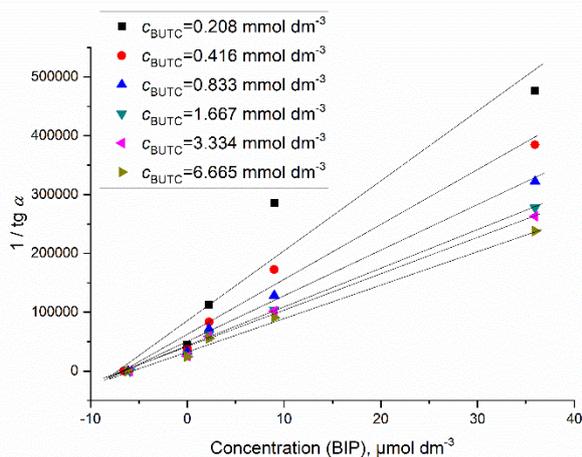

Figure 2. Dixon's diagram of the initial enzymatic reaction rate dependence on the concentration of BIP
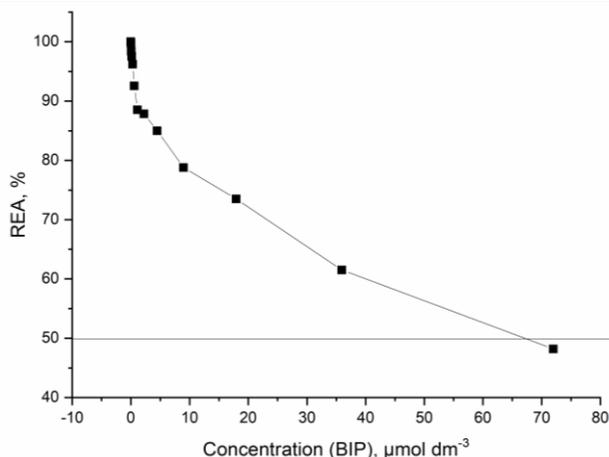

Figure 3. Relative activity of serum cholinesterase as affected by BIP concentration

The half maximal inhibitory concentration (IC$_{50}$) value was estimated by extrapolation as 69.4 µmol dm^{-3} and marked on Figure 2. An interval of the relative enzyme activity greater than 50.0 % was found to be suitable for development of a method for determining BIP.

The reaction rate dependence on the pH value of the reaction mixture, with different concentrations of inhibitors and without any, was established, and the pH value 7.6 was chosen as optimal because at this pH the difference between basic and inhibited reaction rates was greatest (Supplementary material Figure D-4).

Six concentrations of BUTC substrate (listed on figure 2) were used to estimate the reaction rate for reactions without and with different concentrations of BIP. The substrate concentration of 1.667 mmol/L was chosen as optimal because this value gave the maximum difference between rates of basic and inhibitory reactions (Supplementary material Figure D-5).

For constructing the calibration line, under the optimal conditions (pH 7.6; c_{buffer} = 100 mmol; c_{BUTC} = 1.667 mmol dm^{-3}), a differential variant of the tangent method was applied [14]. A linear relationship between the slope (tg α) and BIP concentration was established, allowing determination of BIP in range of 0.035–0.562 µmol dm^{-3} (in the probe).

The obtained equation for the calibration line is:

tg α = (-0.0288669 ± 0.0022133)c + (0.2263087 ± 0.0006404)   (1)

where c is expressed as µmol dm^{-3}, R^2=0.97689 (Fig. 4).

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the 3 SDB/m and 10 SDB/m formulas (where SDB was standard deviation of the blank signal, while m had a defined value when the cross section of the calibration graph was zero or practically zero) [15] and were 3.84 and 12.80 nmol dm^{-3}, respectively.

Five replicate blank experiments and four BIP concentrations were performed to evaluate the precision and accuracy of the proposed procedure, and the results are shown in Table 1.

By applying the calibration line, it is possible to determine BIP concentrations in different samples in a range of 0.035–35.940 µmol dm^{-3}.
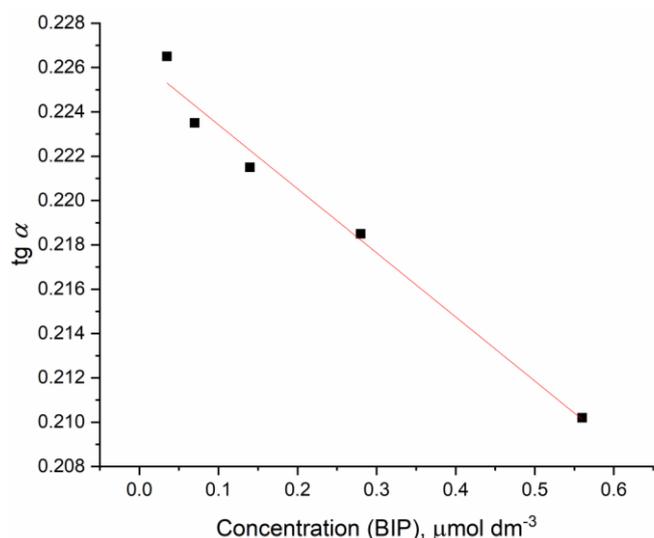
Figure 4. Calibration line for BIP determination

Table 1 Precision (shown as RSD) and accuracy (shown as recovery) of the proposed method; concentrations represent averages of five repetitions ± standard deviation

c_{BIP} (in probe) / μmol dm^{-3}		RSD, %	Recovery, %
Measured	Found±SD		
0.070	0.073±0.007	9.58	104.28
0.140	0.131±0.004	3.05	93.57
0.280	0.277±0.005	2.05	98.93
2.240	2.325±0.141	6.06	103.79
8.980	9.741±0.519	5.33	108.47
35.940	35.335±1.279	3.61	98.31

When the determination range of the proposed method is compared with the methods presented in the Introduction section dealing with BIP determination in biological samples, one can see either it is at the upper limit of sensitivity [2-5] or the method is more sensitive for the same order of magnitude [6,7]. On the other hand, when compared with the methods for BIP determination in pharmaceutical formulations [8,9], the proposed procedure is capable to determine BIP in concentrations lower by 30 to 100 times, while in regard to the described spectro-photometric method [10], it is even 5000-fold more sensitive.

In order to examine the effect of possible interferences, the influence of the most common filler substances (calcium carboxymethylcellulose, gelatin, glycerol, lactose, magnesium carbonate, magnesium stearate, methylhydroxypropyl-cellulose and TiO_2) on the inhibitor reaction rate was examined. Potential interfering substances were taken in excess and prepared in the same manner as pharmaceutical samples. With regard to the 2SD (twice standard deviation) criterion [16] at a constant BIP concentration in a probe of 0.25 μmol dm^{-3}, none of the potential interferences showed a measurable effect on the inhibitor reaction rate, though they were added in the double ratio than the one existing in pharmaceutical formulations.

The proposed method was applied for the determination of BIP in tablets of different manufacturers. The results were confirmed by the standard addition method (Table 2).

Table 2 Precision (RSD) and accuracy (standard addition method) of the BIP assay in tablets or injections. The presented results are the average of five repeated experiments for each concentration of BIP

c_{BIP} / (mg/tablet or injection)				RSD, %	Recovery, %
Sample	Declared[a]	Found[b]	Found[c]		
Mendilex	2	1.91±0.18	1.96±0.23	9.42	95.50
Akineton	2	1.94±0.14	2.07±0.17	7.21	97.01
Akineton	5	4.81±0.28	4.92±0.24	5.82	96.22

[a]declared by the manufacturer; [b]calibration line; [c]standard addition method.

All presented results, namely sensitivity, precision and accuracy, qualify this method to be placed within the most reliable methods for determination of BIP. Having in mind its rapidity and simplicity of performance, as well as utilization of relatively simple equipment and cheap reagents, the proposed method is one of the most applicable methods, affordable for every relevant laboratory.

4. CONCLUSION

Biperiden has been shown to reduce serum cholinesterase activity and kinetic biochemical parameters have been established. Based on these, a non-competitive type of inhibition was recognized. Spectrophotometric determination of a compound based on competitive and non-competitive inhibition of serum cholinesterase is not a novelty by itself, but confirmation and characterization of this inhibition enabled development of a new kinetic method for determination of BIP. This method for BIP quantification has been successfully applied for quality monitoring of pharmaceutical preparations.

Analytical parameters of the proposed method are comparable to the most sensitive methods described in literature. What emphasizes the proposed procedure certainly is short duration of analysis and simplicity of the procedure, as well as excellent sensitivity and precision. The equipment used in this procedure is available in any clinical and chemical laboratory. All the above including the low cost of reagents and equipment, allow wide applicability of the method.

REFERENCES

[1] Shih TM, McDonough JH. Efficacy of biperiden and atropine as anticonvulsant treatment for organophosphorus nerve agent intoxication. *Arch Toxicol*. 2000; 74:165–172.

[2] Minakata K, Yamagishi I, Nozawa H, Hasegawa K, Suzuki M, Gonmori K, Suzuki O. Quantitation of biperiden in whole blood by MALDI-QTOF tandem mass spectrometry, and estimation of new metabolites in urine of deceased subjects treated with biperiden antemortem. *Forensic Toxicol*. 2017; 35:86–93.

[3] Yokogawa K, Nakashima E, Ichimura F, Yamana T. Simultaneous microdetermination of biperiden, haloperidol, and trihexyphenidyl in plasma and its application to pharmacokinetic analysis after concomitant intravenous administration of the drugs to rabbits. *Chem Pharm Bull (Tokyo)*. 1985; 33:4581–4586.

[4] Hadidi KA. Development of a screening method for the most commonly abused anticholinergic drugs in Jordan; trihexyphenidyl, procyclidine and biperiden. *Leg Med (Tokyo)*. 2004; 6:233–241.

[5] Čápka V, Xu Y. Simultaneous determination of enantiomers of structurally related anticholinergic analogs in human serum by liquid chromatography–electrospray ionization mass spectrometry with on-line sample cleanup. *J Chrom B: Biomed Sci Appl*. 2001; 762:181–192.

[6] Khaled E, Hassan HNA, Ahmed MA, El-Attar RO. Crown ether/carbon nanotubes based biperiden disposable potentiometric sensor. *Electoanalysis*. 2017; 29:975–982.

[7] Samanidou V, Stathatos C, Njau S, Kovatsi L. Disposable pipette extraction for the simultaneous determination of biperiden and three antipsychotic drugs in human urine by GC–nitrogen phosphorus detection. *Bioanalysis*. 2013; 5:21–29.

[8] Mohammadi A, Mehramizi A, Aghaee Moghaddam F, Erfani Jabarian L, Pourfarzib M, Kashani HN. Development and validation of a stability-indicating high performance liquid chromatographic (HPLC) assay for biperiden in bulk form and pharmaceutical dosage forms. *J Chrom B: Biomed Sci Appl*. 2007; 854:152–157.

[9] Mandrioli R, Musenga A, Lasaponara SS, Saracino MA, Fanali S, Raggi MA. Enantioseparation and quality control of biperiden in pharmaceutical formulations by capillary electrophoresis. *Anal Chim Acta*. 2006; 560:57–63.

[10] Issa FAA, EL-Helbawy SM, Abed EL-Magied AM. Simultaneous determination of some anticholinergic drugs (biperiden and chlorphenoxamine) in various dosage forms by spectrophotometry. *International Journal of Chemical and Analytical Science*. 2012; 3:1296–1300.

[11] Stankov-Jovanović VP, Nikolić-Mandić SD, Mandić LjM, Mitić VD. Cholinesterase inhibition based determination of pancuronium bromide in biological samples. *Anal Bioanal Chem*. 2006; 385:1462–1469.

[12] www.bindingdb.org/bind/chemsearch/marvin/MolStructure.jsp?monomerid=50240680 (accessed March 27, 2020)

[13] Stankov-Jovanović VP, Nikolić-Mandić SD, Mandić LjM, Mitić VD. A modification of the kinetic determination of pancuronium bromide based on its inhibitory effect on cholinesterase. *J Clin Lab Anal*. 2007; 21:124–131.

[14] Yatsimirskii KB (Ed.) *Kinetic Methods of Analysis*. 2nd ed. Moscow, USSR: Khimiya; 1967; p.55–56.

[15] Miller JC, Miller JN. *Statistics for Analytical Chemistry*. New York, NY: John Wiley & Sons; 1984.

[16] Kirkbright GF. Development and publication of new spectro-photometric methods of analysis. *Talanta*. 1966; 13:1–14.

SAŽETAK

Nova kinetička metoda bazirana na inhibiciji holinesteraze za kvantifikaciju biperidena u farmaceutskim preparatima

Vladan R. Đurić[1], Violeta D. Mitić[2], Nebojša R. Deletić[1], Marija D. Ilić[3], Bratislav M. Ćirković[1] i
Vesna P. Stankov-Jovanović[2]

[1]*Univerzitet u Prištini – Kosovska Mitrovica, Poljoprivredni fakultet, Kopaonička bb, 38219 Lešak, Srbija*
[2]*Univerzitet u Nišu, Prirodno-matematički fakultet, Departman za hemiju, Višegradska 3, 18000 Niš, Srbija*
[3]*Veterinarski specijalistički institut ''Niš'', Sektor za laboratorijska ispitivanja, Laboratorija za analitičku hemiju, Dimitrija Tucovića 175, 18106 Niš, Srbija*

(Naučni rad)

Utvrđeno je da biperiden, koji pripada grupi antiparkinsoničkih i antiholinergičkih lekova, inhibira enzimsku hidrolizu butiriltioholin-jodida, koju katalizuje serumska holinesteraza. Merenje razlike između brzine reakcije bazne i inhibitorne hidrolize, u prisustvu biperidena kao inhibitora, omogućava razvoj kinetičke metode za određivanje ove supstance. Za oba sistema, enzim-supstrat-hromogen i enzim-supstrat-hromogen-inhibitor, definisani su biohemijski parametri (K_M = 0.326 – 0.330 mmol dm^{-3}; V_{max} = 40 – 42,99 µmol/Lmin), a utvrđeno je da je inhibicija beskonkurentna sa konstantom inhibicije od K_i = 6,142 µmol dm^{-3}. Uslovi reakcije su optimizovani. Konstruisane su kalibracione krive i razvijena je odgovarajuća jednačina, a izračunate su i granice detekcije (3,84 nmol dm^{-3}) i kvantifikacije (12,80 nmol dm^{-3}). Uz korišćenje kalibracionog grafikona, moguće je odrediti koncentraciju biperidena u različitim uzorcima u granicama od 0,035 - 35,940 µmol dm^{-3}. Ispitivan je i uticaj niza supstanci, koje se mogu naći u uzorku, na brzinu reakcije. Optimizovana metoda je primenjena za određivanje biperidena u farmaceutskim preparatima. Tačnost ove metode je proveravana standardnom adicionom metodom. Predložena metoda pokazuje dobru osetljivost, selektivnost, jednostavna je i brza i nadasve dostupna, pa se lako može primeniti u raznim laboratorijama.

Ključne reči: enzim; hidroliza; inhibicija; butiriltioholin-jodid; brzina reakcije; optimizacija.