Enzymatic kinetic method for determination of propranolol hydrochloride in pharmaceuticals based on its inhibitory effect on cholinesterase

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Abstract

Propranolol, a widely used beta-blocker, inhibits the hydrolysis reaction of enzyme cholinesterase. Measurements of the difference in rate of hydrolysis rate between uninhibited and inhibited reactions allow the development of a kinetic method for its determination. Both systems, enzyme–substrate–chromogen and enzyme–substrate–chromogen–inhibitor, were characterized through biochemical kinetic parameters (K_M, 0.326–0.330 mmol/L; V_{max} , 40.0–43.0 μmol/Lmin). The inhibition type was recognized as competitive and the inhibition constant, *Ki*, was determined to be 22.60 μmol/L. The detection and quantification limits were calculated as 0.004 and 0.0136 μmol/L, respectively. Accuracy and precision of proposed methods were tested. The proposed method showed good sensitivity, selectivity, simplicity and rapidity, thus it is convenient for clinical applications.

Keywords: propranolol; cholinesterase inhibition; kinetic method; pharmaceuticals.

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Propranolol ((*t*)-isopropylamino-3-(1-naphthyloxy propan-2-ol) hydrochloride, PPH) is a widely prescribed drug in treatment of hypertension, angina pectoris, cardiac arrhythmia and hyperthyroidism. It belongs to a group of non-selective β-blockers, expressed with moderate intrinsic sympathetic mimetic activity. The relationship between the propranolol concentration in patients' blood and its pharmacological action is not always possible to establish, so that the dosage of the drug is determined by heart rate following and blood pressure measurements [1]. Regarding that fact, the determination of propranolol amounts in patients' blood is herein not of essential significance, but still, it is very important to monitor the quality of pharmacological preparations of propranolol hydrochloride, its content in effluents and finally in forensic samples.

A variety of methods was developed and validated for propranolol determination in samples of different origin. In the following review of the existing methods for propranolol determination, special attention will be devoted to the methods applied in pharmaceutical preparations and effluents (Table 1).

Numerous spectrophotometric methods give the opportunity to determine propranolol in pharmaceutical samples in various ranges, that can be generalized as from 0 to 20 mmol/L, while the other methods offer even more wide interval of determination (from 0 to 96.4 mmol/L [33]). Some of the methods are applied in nanomolar concentration intervals, though their low operational range or sensitivity is not really of substantial importance, having in mind the macroscopic nature of pharmaceutical or environmental samples. The main issue can be availability of the proposed assay, considering instruments and reagent, time spare and of course reliability. The high selectivity, short analysis time, simplicity are desirable as well as wide operational range.

Working on cholinesterase inhibition analytical studies [35,36], and taking into consideration the presence of amino group in the molecule of propranolol (Figure 1) and literature data [37], the idea of developing an enzyme inhibition based assay emerged. The inhibiting effect of propranolol on serum cholinesterase was applied to a new kinetic enzyme method for its determination. Kinetic methods of analysis have the general advantage of combining high sensitivity, good selectivity with relatively simple, rapid procedures and inexpensive instruments, such as spectrophotometers. High specificity of the enzymatic reactions enabled usage pooled human serum as a source of cholinesterase. Modified Ellman's method [36] was applied for reaction rate monitoring. In that way, biochemical reaction and inhibition parameters were determined in common clinical biochemical laboratory conditions, without engagement of expensive instruments and reagents or using complex procedures. The proposed method was vali-

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Table 1. A review of the methods for propranolol determination in the pharmaceuticals

Method	Range of determination, μmol/L	Sample	Reference
Spectrophotometric determinations			
With $Ce(SO_4)_2$	0–16.90	Bulk drug, tablets	[2]
With metavanadate	0–13.52	Tablets	[3]
Bromate/bromide mixture, methyl orange	1.69–169.03	Tablets	[4]
$Hg(SCN)_2$, Fe^{3+} as indicator	33.81–169.03	Bulk drug, tablets	[5]
Nitration of propranolol with uranyl nitrate and thorium nitrate in H_2SO_4	6.76–108.18	Pure form, pharmaceutical preparation	[6]
Propranolol oxidation by known excess of *N*-bromosuccinimide	1.69–42.26	Dosage forms	[7]
Reaction of propranolol with sigma-acceptor iodine and pi-acceptors	13.52–405.68	Pure form, pharmaceutical formulation	[8]
With diazotized 4-amino-3,5-dinitrobenzoic acid	3.38–27.05	Tablets	[9]
Excess of N-bromosuccinimide +dye celestine blue	0.676–16.903	Pharmaceutical formulation	[10]
KNO_3 + H_2SO_4 (+ alkaline NaOH and urea)	1.08–405.68	Pharmaceuticals	[11]
$NaNO_2$ + K_2SO_4	6.76–169.03	Dosage forms	[12]
Dissolution in isopropanol, addition of Ag_2O + 10% CH_3CHO and 10% chloranil	16.90–202.84	Bulk tablets	[13]
With brom thymol blue	3.00–25.00	Bulk tablets	[14]
With 2,4 dinitro-1-fluorobenzene	16.90–101.42	Tablets	[15]
Complex formation with Cu(II) and Co(II)	20–20000	Dosage forms	[16]
Other methods			
Differential pulse polarography	0.50–50.00	Tablets	[17]
Cyclic voltametry and pulse voltametry	1–20	Spiked water samples	[18]
Phosphorimetric	0–1.69	Pharmaceutical preparations	[19]
AAS (chelation with Cu(II) ion)	0–135.23	Pharmaceutical preparations	[20]
Indirect AAS method	4.54–346.98	Water solutions, tablets	[21]
Spectrofluorimetry	0.08–0.68	Tablets, injections	[22]
Spectrofluorimetry	0.023–0.771	Pharmaceutical formulations	[23]
Spectrofluorimetry	0.077–3.856	Urine, pharmaceutical preparations	[24]
SIA with fluorescence detection	0–15.423	Tablets	[25]
Chemiluminometry	77.115–578.363	Pharmaceutical preparations	[26]
FIA with fluorescence detection	0–0.001	Pharmaceutical preparations, urine	[27]
FIA with chemiluminiscence detection	3.855–77.100	Pharmaceutical formulations	[28]
FIA with chemiluminiscence detection	3.855–67.462	Pharmaceutical preparations	[29]
Kinetic-potentiometric	100–1000	Commercial formulations	[30]
Miccelar stabilized room temperature phosphorescence	0.096–1.536	Commercial formulations, urine	[31]
Potentiometric sensors	0.1–10000	Dosage forms	[32]
LC	3.856–96.400	Pharmaceutical formulations	[33]
SPE-LC-ESI/MS	0.00068–0.02197	Hospital effluent wastewaters	[34]

dated in accordance with modern analytical demands (selectivity, sensitivity, linearity, accuracy, precision etc.).

EXPERIMENTAL

All used chemicals and reagents were p.a. grade. Propranolol hydrochloride (PPH, purity > 99%) was kindly provided by Galenika AD, Belgrade; stock solution was of 10 mg/mL in deionized water. Working solutions were prepared daily, by dilution of the stock solution. Buthyrylthiocholine iodide (BUTC, purity > 99%), 5,5'--dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma, Germany. BUTC stock solution was of 133.33 mmol/L. DTNB stock solution was prepared by

dissolving the exact weighted mass (0.0792 g) in 5 mL of phosphate buffer pH 7.4, followed by addition of 60 mg of $NaHCO_3$, and dilution to 10 mL in the volumetric flask. $NaHCO_3$, KH_2PO_4, Na_2HPO_4, used for the buffers preparation, were from Merck, Germany.

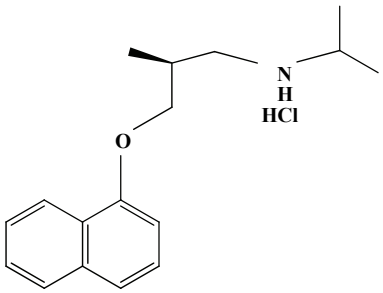

Figure 1. Structural formula of propranolol hydrochloride.

For solutions preparation and for final dishes washing, deionized water (conductivity < 1μS, MicroMed high purity water system, TKA Wasseraufbereitungssysteme GmbH) was used.

All concentrations described here are the initial concentrations in the reaction mixture at time zero after mixing.

A Konelab 20 analyzer (Thermo Scientific), with flow thermostated cells, length 7 mm, λ = 405 nm was used for spectrophotometric measurements. The main advantage of the Konelab 20 analyzer is its use of very small volumes of reagents (the volume of the measuring probe is 200 μL), though the possibility of performing about 40 analyses per hour is not negligible either.

Ten healthy volunteers (18–65 years old from both sexes) donated their blood with written consent, which was used for serum preparation in the Pirot General Hospital. Each of the volunteers had neither recent nor past history of significant medical disorder, as well as drug, cigarette or alcohol abuse. For at least a month before blood donation, none of the volunteers had been taking any medication. A 5 mL blood sample was collected from each donator in a vacutainer tube, centrifuged for 10 min at 3000 rpm, after which the serum supernatants were collected, mixed and used for the enzyme assay. The hydrolysis reaction of butyrylthiocholine to thiocholine is catalyzed by serum cholinesterase; the product reacts with DTNB forming colored compound (5-thio-2-nitrobenzoic acid); the reaction rate is determined from the increase of color, measured at 405 nm, in 6 cycles of 28 s and thermostated at 310 K.

Solutions of propranolol (10 μL) were mixed with serum diluted with the phosphate buffer in ratio 1:9, v/v (10 μL), the phosphate buffer solution (160 μL) and preincubated 10 min (at 310 K); DTNB solution (10 μL) was added and left for 60 s; substrate solution (BUTC, 10 μL) was added. The same procedure was applied for analysis of samples. The obtained kinetic data were interpreted by tangent method. Each kinetic result is the average of five determinations.

Twenty tablets of every pharmaceutical preparation was taken and ground into a fine powder. Mass of the powder equivalent to 100 mg of PPH was exactly weighed and transferred into a 100 mL calibrated flask. About 60 ml of water was added and the content was vigorously shaken. The contents were filtered using a quantitative filter paper into the 100 mL volumetric flask and diluted to the mark. An appropriate volume of the stock solution was used for analysis.

RESULTS AND DISCUSSION

The main parameters affecting the performance of the proposed method were studied to determine the optimal working configuration. These parameters were optimized by the invariant method, *i.e*., each parameter was optimized by setting the other parameters to be constant. To determine the kinetic biochemical parameters, the dependence of reaction rate on substrate (butyrylthiocholine) concentration was estimated for the range 0.208–6.650 mmol/L. The methods of Lineweaver-Burk, Eadie–Hofstee and Hanes linearization, resulted with the similar values for Michaelis-Menten's constant (0.330, 0.305 and 0.326 mmol/L, respectively) and also for V_{max} (42.29, 40.00 and 42.38 μmol/L, respectively). The obtained kinetic data enable conclusion that the PPH and serum cholinesterase interact in the competitive inhibition manner. Once the mechanism of inhibition was established, the inhibition constant was calculated from the function of the inhibition reaction rate depending of different PPH concentrations. Dixon's method was applied for inhibition constant determination, which was found to be 2.71 μmol/L.

Different PPH concentrations affect relative enzyme activity of the serum cholinesterase in the manner shown in Figure 2. At PPH concentration of 169.03 μmol/L, the enzyme is almost completely inhibited. The IC_{50} value was determined as 22.60 μmol/L. The interval of relative enzyme activity higher than 48.35% was applied for the development of method for PPH determination.

The dependences of reaction rates on pH for reaction without/with inhibitor are shown in Figure 3. The pH 7.6 was chosen as optimal, because that pH value gave the greatest difference for basic and inhibitor reaction rates.

Six substrate (butyrylthiocholine iodide) concentrations (0.208, 0.416, 0.833, 1.667, 3.334 and 6.665 mmol/L) were used for the estimation of the reaction rates for reaction without/with different PPH concentrations (Figure 4). The substrate concentration 1.667 mmol/L was chosen as optimal, because that value

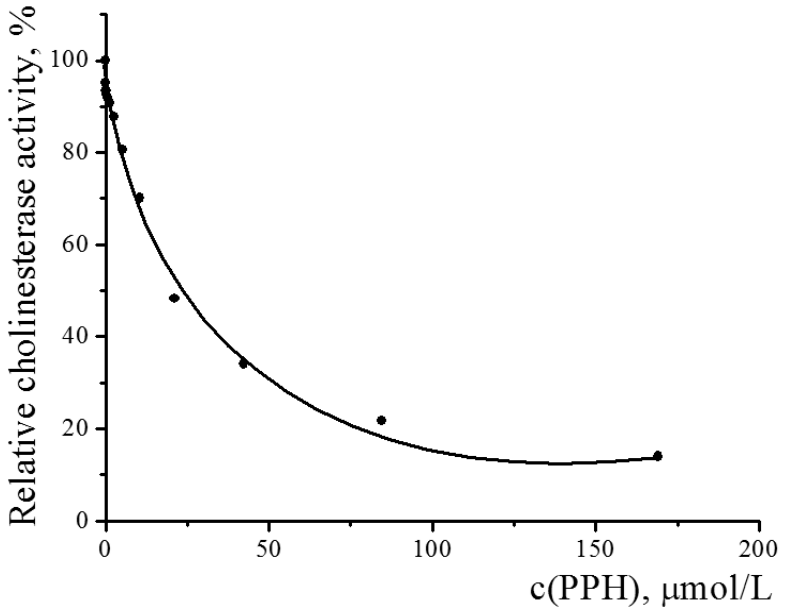

Figure 2. Function of relative activity of serum cholinesterase on concentration of propranolol (PPH).

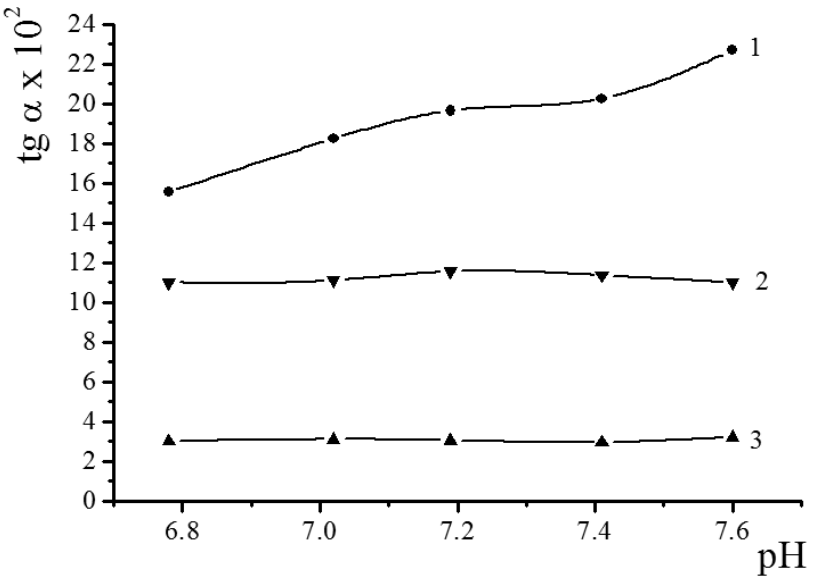

Figure 3. The dependence of the reaction rate in absence of PPH (1) and in presence of PPH (2,3) on pH. Conditions: c(BUTC) = 1.667 mmol/L; c(phosphate buffer) = 100 mmol/L; 2-c(PPH) = 2.637 μmol/L; 3-c(PPH) = 21.129 μmol/L); T = 310 K.

gave the maximal difference in the basic and inhibitor reaction rates.

The differential variant of tangent method [38] was applied for the construction of calibration graph, under optimal conditions (pH 7.6; *c*(buffer) = 100 mmol; *c*(butyrylthioholine iodide) = 1.667 mmol/L; *T* = 310 K). The linear relationship between the slope (tan α) and PPH concentrations in the range 0.082–21.120 μmol/L (in probe) was established. The equation used for the calibaration graph was:

$$\tan \alpha \times 100 = (21.2853 \pm 0.0871) - (0.4937 \pm 0.0107)c \quad (1)$$

where *c* is expressed in μmol/L and *R* = –0.99836.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated applying formulas $3SD_B/m$ and $10SD_B/m$ respectively (where SD_B is standard deviation of the blank signal and m has a definite value when the intercept of the calibration graph is zero or virtually zero [39] and they were 0.004 and 0.0136 μmol/L, respectively. Five replicate experiments for the blank and three concentrations of PPH were conducted for precision and accuracy estimation of the proposed method and the results are shown in Table 2.

By applying the calibration graph, it is possible to determine propranolol from different samples in the range 1.65–422.40 μmol/L.

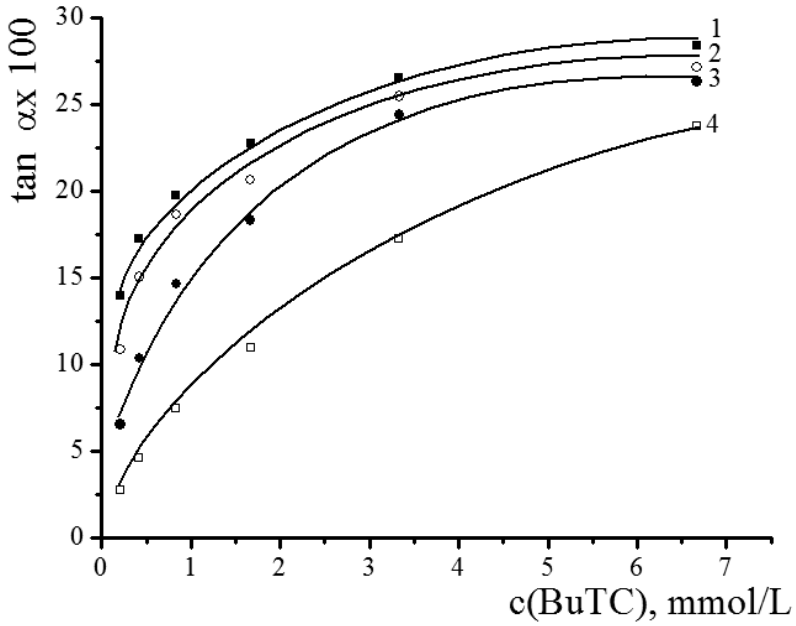

Figure 4. The dependence of the reaction rate without (1) and with PPH at different concentrations (2–1.318, 3–5.282 and 4–21.129 μmol/L) on buthyrylthiocholine concentration; c(buffer) = 100 mmol/L, pH 7.6; T = 310 K.

Table 2. Precision (presented as RSD %) and accuracy (presented as Recovery %) of the proposed method; the concentrations present averages of five determinations ± standard deviation (x ± SD)

c(PPH) in probe (measured), μmol/L	*c*(PPH) in the initial solution(measured), μmol/L	*c*(PPH) in probe (found), μmol/L, $x \pm SD$	*c*(PPH) in the initial solution (found), mmol/L, $x \pm SD$	*RSD* / %	Recovery, %
0.659	13.180	0.666±0.023	13.328±0.462	3.47	101.06
2.637	52.740	2.668±0.083	53.352±1.655	3.10	101.17
10.565	211.300	10.697±0.15	211.300±3.093	1.45	101.46

In order to examine the impact of possible interferences, the influence of the most common filling substances (calciumcarboxymethylcellulose, carmine, gelatin, glycerol, lactose, magnesium carbonate, magnesium stearate, methylhydroxypropylcellulose and TiO_2) was tested on the inhibitor reaction rate. The potential interfering substances were taken in excess and prepared in the same way as pharmaceutical samples. Regarding the 2SD criteria [40] at constant propranolol concentration in the probe of 10.56 μmol/L, none of the potential interferences showed measurable influence on the inhibitor reaction rate, in the double ratio that exists in pharmaceutical formulations.

The proposed method was applied for PPH determination in tablets of different origin. Results were validated by standard addition method (Table 3).

CONCLUSION

The known fact that propranolol inhibits human serum cholinesterase activity was confirmed and kinetic biochemical parameters were established. On that basis, the competitive type of inhibition was proposed, which can be justified by the existence of charge depolarization in the propranolol hydrochloride molecule, which can be very well fitted in the active site of the enzyme very similar to the acylcholine esters. Con-

Table 3. Precision (RSD) and accuracy (standard addition method) test of PPH determination in tablets. Results are expressed as average of five replicate (x ± SD) experiments for different PPH concentrations

Sample	*c*(PPH)[a], mg/tablet	Found *c*(PPH)[b], mg/tablet	*RSD* / %	Found c(PPH)[c], mg/tablet	Mean value of recovery, %
Propranolol tablets	40	39.89±0.60	1.50	40.21±0.56	100.15
Inderal tablets	10	9.96±0.14	1.16	9.98±0.11	99.85
Inderal tablets	40	39.77±0.60	1.52	39.86±1.17	99.64
Inderal tablets	80	79.75±1.25	1.57	79.87±0.88	99.84

[a]Labeled by manufacturer; [b]calibration graph; [c]standard addition method

firming and characterizing the inhibition enabled the possibility of development of a new enzymatic kinetic assay for propranolol determination.

Methods for quantification of propranolol are crucial in determining the quality of pharmacological preparations. The proposed method, with its broad determination interval, excellent sensitivity, simplicity, reproducibility, precision, accuracy and selectivity, satisfies all the requirements for practical application. In addition, the equipment used for its implementation is available to any clinical laboratory, which greatly contributes to the availability of the method.

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IZVOD

ENZIMSKA KINETIČKA METODA ZA ODREĐIVANJE PROPRANOLOL-HIDROHLORIDA U FARMACEUTSKIM PREPARATIMA ZASNOVANA NA NJEGOVOM INHIBITORSKOM DELOVANJU NA HOLINESTERAZU

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(Naučni rad)

Za propranolol, često propisivani neselektivni beta blokator, utvrđeno je da inhibira reakciju enzimske hidrolize butiriltioholin-jodida, koja je katalizovana serumskom holinesterazom. Merenjem razlike u brzini osnovne i inhibitorske reakcije hidrolize u prisustvu propranolola kao inhibitora, moguće je razviti kinetičku metodu za određivanje propranolola. Oba sistema, enzim–supstrat–hromogen kao i enzim–supstrat–hromogen–inhibitor okarakterisani su biohemijskim kinetičkim parametrima (K_M, 0,326–0,330 mmol/L; V_{max}, 40–42,99 μmol/L min), inhibicija je definisana kao kompetitivna i određena je konstanta inhibicije 22,60 μmol/L. Da bi se u potpunosti iskoristile sve mogućnosti predložene metode u pogledu osetljivosti, tačnosti, preciznosti i selektivnosti, optimizovani su reakcioni uslovi. Konstruisana je kalibraciona prava, izračunata odgovarajuća jednačina i određeni granica detekcije i kvantifikacije, i to 0,004 i 0,0136 μmol/L, redom. Tačnost i preciznost predložene metode su ispitane za tri koncentracije propranolola u oblasti kalibracione prave (0,082–21,120 μmol/L) u pet ponavljanja. Takođe, ispitan je uticaj većeg broja supstanci koje se mogu naći u uzorku na brzinu reakcije. Optimizovana metoda je primenjena za određivanje propranolola u farmaceutskim preparatima. Tačnost predložene metode je ispitana primenom metode standardnog dodatka. Predložena metoda ima dobru osetljivost, selektivnost, jednostavna je i brza, i nadasve lako dostupna, i na taj način primenljiva u velikom broju laboratorija.

Ključne reči: Propranolol • Holinesteraza • Kinetička metoda • Farmaceutski preparati