A Modification of the Kinetic Determination of Pancuronium Bromide Based on Its Inhibitory Effect on Cholinesterase

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A modification of the existing spectrophotometric kinetic method for the determination of pancuronium bromide (PCBr), based on pooled human serum cholinesterase (ChE, EC 3.1.1.8 acylcholine acylhydrolase) inhibition, was developed. Butyrylthiocholine iodide (concentration 1.667 mmol/L) was used as substrate and determination was performed at pH 7.6. Essential basic kinetic parameters were also determined: Michaelis-Menten's constant $K_M = 0.33$ mmol/L, maximal reaction rate $V_{max} = 42.29$ μ mol/L min, inhibition constant $K_I = 0.34$ μ mol/L, and $IC_{50} = 0.235$ μ mol/L. Linear dependence between the reaction rate and the inhibitor concentration exists in PCBr concentration range 8.29–265.28 nmol/L, which corresponds to the real sample concentrations from 0.166 to 5.306 μ mol/L. The

method detection limit was established to be 1.86 nmol/L and the quantification limit was 6.18 nmol/L. Precision of the method was tested for three pancuronium concentrations (16.58, 99.48, and 198.96 nmol/L). The relative standard deviation (RSD) was in the range 0.78–5.13%. Accuracy was examined by the standard addition method. The influence of substances usually present in serum and urine on the reaction rate was determined. The method developed was applied for PCBr determination in spiked serum and urine samples and in the urine taken during surgery. The method was proven to have good sensitivity, accuracy, and precision and can be considered suitable for clinical practice. *J. Clin. Lab. Anal.* 21:124–131, 2007. © 2007 Wiley-Liss, Inc.

Key words: cholinesterase inhibition; pancuronium bromide; buthyrylthiocholine; kinetic determination

INTRODUCTION

Nondepolarizing muscle relaxants are widely administered in clinical anesthesia, and among them pancuronium bromide (PCBr) is designed as a first-line agent in 2–4-hr surgical procedures (1). PCBr is partly hydrolyzed in the liver to the active 3-desacetyl derivate and its elimination half-life is 100–140 minutes. The major amount of PCBr is excreted via urine in the unchanged form. In patients with renal insufficiency or with hepatic dysfunction, the pharmacokinetic parameters of PCBr are widely modified (2). Therefore, determination of PCBr in such cases as well as during and after surgery is essential.

There is also a great interest for PCBr methods development, from a forensic point of view. PCBr is registered as the cause in many murder (3–6) and suicide (7–9) cases.

Many methods for PCBr determination have been reported; techniques and methods such as: high perfor-

mance liquid chromatography (HPLC) with ultraviolet (UV) detector (10) and fluorimetric detector (9), gas chromatography (GC) with nitrogen sensitive detector (11), liquid chromatography-electron spray ionization mass spectrometry/mass spectrometry (LC-ESI-MS (MS)) (12–14), fluorimetric and spectrophotometric methods (15–17), electroanalytical methods employing membrane ion-selective electrodes (18), and a modified carbon paste electrode (19). In spite of the fact that many different methods have been developed, only some of them are applicable for the determination of PCBr concentration in serum and urine during surgery, and even these did not satisfy all of the important demands of clinical and toxicological practice (accuracy, repro-

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ductivity, and precision, as well as the duration and the cost of the analysis). Some of the methods require a complex sample preparation procedure or the use of expensive equipment usually not available in clinical laboratories. For these reasons, the spectrophotometric kinetic method was developed (20), based on the inhibition of the serum cholinesterase by PCBr. Serum cholinesterase catalyzes the hydrolysis of benzoylcholine. The catalytic activity is determined from the rate of quinone imine formation by means of the choline oxidase and peroxidase-coupled reactions. To avoid expensive reagents usage, in this work the possible application of butyrylthiocholine iodide (BuTC) as the substrate and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (21) as the reagent, for PCBr determination by spectrophotometric kinetic method was investigated.

MATERIALS AND METHODS

Chemicals and Reagents

All used chemicals and reagents were of pro analysis (p.a.) grade. Butyrylthiocholine iodide (purity >99%), DTNB, urea, creatinine, uric acid, hippuric acid, and D-glucose were obtained from Sigma (Germany), and Sephadex LH-20 from Amersham (Sweden). Pancuronium bromide was acquired from Pavulon injections (Organon Technica, Holland), which contain 2 mg/mL of PCBr. Methanol, acetonitrile, KH_2PO_4 , Na_2HPO_4 , ethylenediaminetetraacetic acid (EDTA) EDTA-2Na and F_3CCOOH were purchased from Merck (Germany), and bilirubin from Chemica (Zagreb, Croatia).

The concentration of BuTC and DTNB stock solutions was 33.34 mmol/L and 4.76 mmol/L, respectively.

All used equipment and dishes were finally washed by deionized water (conductivity <1 μS).

Apparatus

All absorbance measurements are performed on a Perkin Elmer spectrophotometer, type $\lambda 15$, with cylindrical thermostatted cells and on a Konelab 20 analyzer, with flow thermostatted cells, length 7 mm ($\lambda = 405 \text{ nm}$). The advantage of the Konelab 20 analyzer, besides a significant reduction in reagent consumption (200 μL) compared to the Perkin Elmer spectrophotometer, was the possibility of performing up to 20 analyses per hr.

Cholinesterase Source for the Assay and Spiked Samples (Pooled Serum)

A total of 10 healthy volunteers (18–65 years old from both sexes), from the Pirot General Hospital, donated blood with written consent. According to the questionnaire, none of them had serious medical disorders, nor are or have been drug, cigarette, or alcohol abusers.

At least a month before the blood donation, none of them had been taking any medication. From all participants, a 5-mL blood sample was collected in a Vacutainer tube, centrifuged at 3,000 rpm for 10 min and the serum supernatant was collected and used as the source of the enzyme for the assay and for the spiked sample preparations.

Procedure

Serum cholinesterase catalyzes the hydrolysis of butyrylthiocholine to thiocholine, which reacts with chromogen DTNB. The reaction rate is determined from the rate of 5-thio-2-nitro benzoic acid formation, measured at 405 nm, in six cycles of 28 sec.

PCBr solutions (10 μL) were mixed with 10 μL of the pooled serum (diluted with the phosphate buffer in ratio 1:9, vol/vol), and the phosphate buffer solution (160 μL). These were preincubated for 10 minutes (at 310°K) when a DTNB solution (10 μL) was added. After being allowed to stand 60 sec, finally the substrate solution (BuTC, 10 μL) was added. Assays for the biological samples were performed in the same manner, only instead of the PCBr solution; the same volume of the sample was taken.

Urine Source for Preparation of Spiked Samples

Urine was collected from five healthy individuals, mixed, centrifuged at 3,000 rpm, and diluted with deionized water in the ratio 1:10 (vol/vol). The urine samples were conserved by the addition of 1 mL of HCl (concentrate) to 100 mL of the sample.

Sample Preparation

Preparation of spiked serum samples

To 1 mL of the pooled serum 50 μL of the PCBr solution variable in concentration (9.84, 23.32, 47.15, and 93.28 $\mu\text{mol/L}$) was added and allowed to stand for 5 min at 310°K. Then, 0.1 mL of concentrated hydrochloric acid was added to precipitate the cholinesterase (other proteins were precipitated as well), and this resulted in the release of PCBr. By mixing 1.05 mL of PCBr serum solution with 0.1 mL of hydrochloric acid, a final acid concentration of 1.04 mol/L was obtained. After protein precipitation, pH of supernatant was measured and the value of 3.90 was registered. Detailed calculations, taking in consideration the volumes of protein-free solutions of PCBr (10 μL) and phosphate buffer solution (160 μL) had no affect on the pH of the final solution. Also, reaction rates for the blank solution and for the blank deproteinized serum solutions were in good compliance, which confirmed our previous conclusion.

The supernatant of the centrifuged samples was analyzed for PCBr content, applying the proposed method.

Preparation of spiked urine samples

Four spiked urine samples were prepared by mixing 1 mL of urine with PCBr solutions: 50 μL of 10 $\mu\text{mol/L}$; 80 μL of 20 $\mu\text{mol/L}$; 80 μL of 40 $\mu\text{mol/L}$, and 80 μL of 60 $\mu\text{mol/L}$, respectively. Samples were allowed to stand for 5 min at 310°K, and then 0.1 mL of concentrated hydrochloric acid was added. PCBr content was analyzed in the supernatant applying the proposed method. A real urine sample taken in a sterile vessel during a surgery (hip replacement) was treated in the same way.

Preparation of spiked serum and urine samples for gel chromatography

To 1 mL of the pooled serum 20 μL of the solution containing a different PCBr concentration (10, 20, 40, and 80 $\mu\text{mol/L}$) was added. After 5 min, the samples were applied to a Sephadex column, and eluted. The procedure was repeated five times for all serum samples.

Urine samples were prepared by adding 20 μL PCBr solutions of different concentrations (10.70, 34.15, 68.3, and 102.45 $\mu\text{mol/L}$) to 1 mL of diluted urine, and after 5 min the samples were chromatographed. The procedure was repeated 5 times for all urine samples.

Chromatographic Isolation of PCBr From Serum and Urine Samples

To separate PCBr from the matrix (cholinesterase and other serum or urine proteins, eventually present, as well as low molecular weight molecules that may interfere with the determination), gel chromatography on a Sephadex LH-20 (dry particle diameter range, 18–111 μm) column (15 mm $\times$ 300 mm) was applied. The composition of the mobile phase was: solvent mixture methanol: acetonitrile: F_3CCOOH (74.9:25:0.1, vol/vol/vol) (20). The flow rate was 0.8 mL/min (10) and the fraction volume 1 mL. Thin layer chromatography (TLC) was utilized to determine which fractions to combine, using the same mobile phase. Urea, creatinine, uric acid, hippuric acid, D-glucose, and bilirubin were added in 20-fold higher concentration than the concentration of the spiked samples, to be detectable by TLC. Protein fractions were detected by ninhydrin solution (0.2% ninhydrin in acetone). All other components were visualized using H_2SO_4 (1:1, vol/vol). The PCBr containing fractions were pooled, the solvent mixture was evaporated to the dryness in vacuo ($T = 300^\circ\text{K}$) and the

dry residue was dissolved in 0.4 mL of deionized water in the case of serum and in 0.5 mL in the case of urine.

RESULTS

Optimization of Kinetics and Reaction Conditions

The dependence of the reaction rate on substrate concentration (butyrylthiocholine) in the range of 0.208 to 6.650 mmol/L was estimated. Applying Lineweaver-Burk's and Eadie-Hofstee's linearization, similar values for Michaelis-Menten's constant (0.33 and 0.30 mmol/L, respectively) and for V_{max} (42.29 and 40.00 $\mu\text{mol/L min}$, respectively) were obtained. The dependence of the inhibition reaction rate was followed for different PCBr concentrations in order to determine the inhibition constant. The inhibition constant was determined by the Dixon's method, and established to be $K_i = 0.34 \mu\text{mol/L}$.

The inhibition percentage of the serum cholinesterase in the presence of different PCBr concentrations is shown in Fig. 1. The enzyme is inhibited more than 90% at PCBr concentration of 2.130 $\mu\text{mol/L}$. The value of median inhibition concentration (IC_{50}) was 0.235 $\mu\text{mol/L}$. A percentage inhibition interval lower than 60% was used for the development of the method.

The influence of pH on the reaction rate in the absence and in the presence of PCBr was tested for pH values 6.80, 7.02, 7.19, 7.41, and 7.60. The dependence of reaction rates on pH is shown in Fig. 2. A pH of 7.6 was chosen as optimal having in mind the greatest reaction rate difference for the basic and inhibitor reactions.

The reaction rate dependence (both basic and inhibitor) on the substrate concentration was estimated for six BuTC concentrations (0.208, 0.416, 0.833, 1.667, 3.334, and 6.665 mmol/L), at different inhibitor (PCBr) concentrations (Fig. 3). The substrate concentration of

Fig. 1. Dependence of cholinesterase inhibition percentage on PCBr concentration.

Fig. 2. The dependence of the basic (curve 1) and inhibitor (curve 2) reaction rate on pH. Conditions: $c(\text{BuTC}) = 1.667 \text{ mmol/L}$; $c(\text{phosphate buffer}) = 100 \text{ mmol/L}$; curve 2: $c(\text{PCBr}) = 132.64 \text{ nmol/L}$; $T = 310^\circ\text{K}$.

Fig. 3. The dependence of the basic (curve 1) and inhibitor reaction rates at different PCBr concentrations (curve 2: $c(\text{PCBr}) = 8.29 \text{ nmol/L}$; curve 3: $c(\text{PCBr}) = 33.16 \text{ nmol/L}$; curve 4: $c(\text{PCBr}) = 66.32 \text{ nmol/L}$; curve 5: $c(\text{PCBr}) = 132.64 \text{ nmol/L}$) on butyrylthiocholine concentration; $c(\text{buffer}) = 100 \text{ mmol/L}$, $\text{pH} = 7.6$; $T = 310^\circ\text{K}$.

1.667 mmol/L was chosen as optimal, because that value gave the maximal difference in the basic and inhibitor reaction rates.

By applying the differential variant of the tangent method (22), a calibration graph (dependence of the reaction rate on PCBr concentration) was established under the optimal conditions ($\text{pH} = 7.6$; $c(\text{buffer}) = 100 \text{ mmol}$; $c(\text{butyrylthiocholine iodide}) = 1.667 \text{ mmol/L}$; $T = 310^\circ\text{K}$). There is a linear relationship between $\tan \alpha$ and PCBr concentration in the range $8.29\text{--}265.28 \text{ nmol/L}$ (in probe), which corresponds to the real sample concentrations from 0.166 to

$5.306 \mu\text{mol/L}$. The equation for the calibration graph is:

$$\tan \times 100 = (22.100 \pm 0.885 - (0.0469 \pm 0.00006)c$$

c is expressed in nmol/L , $R = -0.9990$.

The detection limit (LD) was calculated as $3 \text{ SD}_B/m$, where SD_B is the standard deviation of the blank signal and m has a definite value when the intercept of the calibration graph is zero or virtually zero (23) and it was determined to be 1.86 nmol/L . The quantification limit (LQ) was calculated as $10 \text{ SD}_B/m$, and it was established to be 6.18 nmol/L .

Validation of the Method

The precision and accuracy of the method were tested in five replicate experiments for three concentrations of PCBr. Results are shown in Table 1.

Interference Study

Some substances usually present in the serum and urine could cause a possible interference in the established method and therefore there was a need for their evaluation. Each substance was added to the reaction system substrate-enzyme-PCBr in the same manner as it was performed for the inhibitor reaction. Concentrations of potential interfering substances were increased, until they caused measurable influence on the inhibitor reaction rate (Table 2). The interfering degree was estimated by 2 SD criteria (24) at the constant concentration of PCBr of 99.5 nmol/L . It is considered that the foreign substance does not interfere if the change in the inhibitor reaction rate is less than 5% . The interference on the rate for nine substances and a mixture of the dominant components was from -2.5% to $+2.35\%$.

PCBr Content Determination in the Serum Samples

Determination of PCBr in the patient's serum cannot be performed without a pretreatment of the samples. One way of preparation of the serum samples was the denaturation of the cholinesterase in the serum by an acid, and PCBr determination from the supernatant. The other way was PCBr isolation from the serum samples by gel chromatography. The pooled serum portions were spiked with a known amount of PCBr and the obtained spiked samples were further treated in both ways. The PCBr content was analyzed, applying the proposed method and the results are given in Table 3. PCBr content in the serum samples, applying the described method, can be determined in the concentration range from 0.428 to $4.056 \mu\text{mol/L}$.

TABLE 1. Precision and accuracy of the method

c(PCBr) ^a measured (nmol/L)	c(PCBr) ^b measured (μmol/L)	c(PCBr) ^a found ($\bar{X} \pm SD$ nmol/L)	c(PCBr) ^b found ($\bar{X} \pm SD$ μmol/L)	RSD (%)	Recovery (%)
16.6	0.332	16.6 ± 0.8	0.331 ± 0.017	5.13	99.70
99.5	1.990	99.7 ± 1.9	1.989 ± 0.038	1.91	99.95
199.0	3.979	199.5 ± 1.6	3.979 ± 0.031	0.78	100.00

^ac(PCBr) in the probe; the PCBr solution was diluted 20 times (to 10 μL of diluted serum cholinesterase source, 10 μL of the PCBr solution, 10 μL of substrate, 10 μL of DTNB, and 160 μL of phosphate buffer were added).

^bc(PCBr) in the solution.

TABLE 2. The influence of possible interfering compounds on the determination of PCBr (99.5 nmol/L in probe)

Spiked material	Mol ratio PCBr:compound	Interference (%)
Creatinine	1:10 ⁴	+2.35
Urea	1:10 ⁵	−2.50
Uric acid	1:10 ⁴	+2.23
Hipuric acid	1:10 ⁴	−0.88
Mix creatinine:urea:uric acid:hippuric acid	1:10 ⁴ :10 ⁵ :10 ⁴ :10 ⁴	+0.26
D-glucose	1:10 ⁵	−0.85
Bilirubin	1:10 ²	0
EDTA-2Na	1:10 ⁵	0
Sodium oxalate	1:10 ⁴	−0.45
Sodium citrate	1:10 ⁵	+0.38

TABLE 3. Determination of PCBr in spiked serum samples after two ways of sample preparation*

Sample	c(PCBr) measured		c(PCBr) found	RSD (%)
	In spiked serum sample (μmol/L) ^a	In probe (nmol/L) ^b	In spiked serum sample (μmol/L)	
Serum 1 (a)	0.428	21.4	0.431 ± 0.037	8.59
Serum 2 (a)	1.014	50.7	1.011 ± 0.051	5.08
Serum 3 (a)	2.050	102.5	2.020 ± 0.065	3.22
Serum 4 (a)	4.056	202.8	4.055 ± 0.041	1.01
Serum 1 (b)	0.500	25.0	0.469 ± 0.034	7.19
Serum 2 (b)	1.000	50.0	0.971 ± 0.041	4.53
Serum 3 (b)	2.000	100.0	1.962 ± 0.055	2.80
Serum 4 (b)	4.000	200.0	3.991 ± 0.041	1.01

*Results are given as the mean value ± SD of five replicate experiments for different PCBr concentrations in the spiked serum samples. Precision of the assay is expressed as percent RSD.

^aSpiked samples of the pooled serum were prepared as described in section Sample Preparation.

^bTo 10 μL of the spiked sample, 10 μL of diluted serum (cholinesterase source), 10 μL of substrate, 10 μL of DTNB, and 160 μL of phosphate buffer were added.

(a), denaturation of proteins and centrifugation; (b), gel-chromatography.

The precision of PCBr determination in the serum was examined by five replicate measurements for each of four different concentrations of PCBr (Table 3). Relative standard deviations were from 1.01 to 8.59% (denaturation of proteins and centrifugation) and from 1.01 to 7.19% (gel chromatography). Thus, the applied method has good precision that is independent of sample preparation method.

A standard addition method was applied to estimate the accuracy of the proposed method (considering both methods of the sample preparation) (25) and the results are shown in Table 4. The recovery values for HCl treated samples are slightly higher than those for the gel-chromatographed samples, but all the results had values close to 100%. Regardless of the interfering substances eliminating procedure both methods had very good accuracy and precision. As the gel chromatography treatment of samples required longer time (undesirable for clinical practice) the HCl precipitation elimination of interfering substances is recommended.

PCBr Content Determination in the Urine Samples

Urinary PCBr content was also determined by the proposed method by the two ways of eliminating the interfering substances: addition of HCl and gel-chromatographic separation. Results are given in Table 5. The precision of PCBr determination in urine, treated in both ways, was similar (relative standard deviation (RSDs) range from 1.48 to 9.52% for HCl treated samples and from 1.55 to 8.32% for the gel chromatographed samples). These two procedures of interfering substances elimination were applied also for PCBr content analysis of urine sample taken during the hip replacement surgery (real sample). The obtained results were in a good accordance (12.37 ± 0.38 and 11.30 ± 0.34 μmol/L for the HCl treated sample and for the gel chromatographed sample, respectively).

The standard addition method was used to examine the accuracy of the proposed method for urinary PCBr determination. The results are given in Table 6.

The values obtained for the recoveries show that PCBr determination in urine is very accurate. There is no

TABLE 4. The accuracy test of PCBr determination in the serum model samples by the standard addition method*

Sample	c(PCBr) ($\mu\text{mol/L}$) ^a	Added PCBr (nmol) to 1mL of the sample	Found c(PCBr) ($\mu\text{mol/L}$)	Recovery (%)	Mean value of recovery (%)
Serum 1 (a)	0.428	0.4	0.427 ± 0.035	106.05	103.53
		0.9	0.451 ± 0.032	105.46	
		1.7	0.432 ± 0.028	101.03	
		3.5	0.435 ± 0.026	101.58	
Serum 2 (a)	1.014	0.5	1.050 ± 0.042	103.55	102.33
		1.0	1.035 ± 0.040	102.08	
		2.0	1.28 ± 0.039	101.36	
		4.1	1.037 ± 0.038	102.33	
Serum 3 (a)	2.270	0.8	2.300 ± 0.065	101.34	101.27
		1.6	2.280 ± 0.063	100.50	
		3.2	2.298 ± 0.066	101.96	
Serum 1 (b)	0.428	0.4	0.417 ± 0.028	97.40	96.96
		0.9	0.427 ± 0.027	99.68	
		1.7	0.409 ± 0.027	95.45	
		3.5	0.408 ± 0.025	95.31	
Serum 2 (b)	1.014	0.5	1.023 ± 0.042	100.91	99.71
		1.0	1.002 ± 0.041	98.78	
		2.0	1.000 ± 0.040	98.62	
		4.1	1.019 ± 0.040	100.52	
Serum 3 (b)	2.270	0.8	2.270 ± 0.052	100.02	99.84
		1.6	2.259 ± 0.053	99.53	
		2.4	2.270 ± 0.053	99.98	

*Results are expressed as the average of three replicate ($\bar{X} \pm \text{SD}$) experiments for different PCB concentrations in the serum samples.

^aThe serum samples were prepared as described in the section Sample Preparation.

(a), denaturation of proteins by HCl and centrifugation; (b), gel-chromatography.

TABLE 5. The determination of PCBr in urine samples after two ways of sample preparation*

Sample	c(PCBr) measured		c(PCBr) found	RSD (%)
	In spiked diluted urine sample ($\mu\text{mol/L}$) ^a	In probe (nmol/L) ^b	In spiked diluted urine sample ($\mu\text{mol/L}$)	
Urine 1 (a)	0.435	21.7	0.473 ± 0.045	9.52
Urine 2 (a)	1.356	67.8	1.443 ± 0.070	4.85
Urine 3 (a)	2.712	135.6	2.810 ± 0.070	2.48
Urine 4 (a)	4.068	203.4	4.119 ± 0.061	1.48
Urine R (a)	—	—	1.130 ± 0.038	2.98
Urine 1 (b)	0.428	21.4	0.405 ± 0.034	8.32
Urine 2 (b)	1.366	68.3	1.241 ± 0.060	4.83
Urine 3 (b)	2.732	136.6	2.802 ± 0.068	2.44
Urine 4 (b)	4.098	204.9	4.175 ± 0.065	1.55
Urine R (b)	—	—	1.237 ± 0.034	2.72

*Results are expressed as the mean value $\pm$ SD of five replicate experiments for different concentrations of PCBr in urine model samples. Precision of the assay is expressed as percent RSD.

^aSpiked urine samples were prepared as described in section Sample Preparation.

^bTo 10 μL of the spiked urine sample, 10 μL of diluted serum (cholinesterase source), 10 μL of substrate, 10 μL of DTNB, and 160 μL of phosphate buffer were added.

Urine R, real urine sample taken during hip replacement surgery; (a), addition of HCl and centrifugation; (b), gel-chromatography.

significant difference between the results obtained with two ways of separation of PCBr. As gel-chromatography is a time-demanding technique, the precipitation of proteins by HCl, centrifugation and PCBr determination from the supernatant is proposed as a more practical one.

DISCUSSION

The determination of the nondepolarizing myorelaxant PCBr in serum and urine during and after surgery gives a very important pharmacokinetic insight and enables an excellent anesthetic dose control as well as additionally clarifying the question of PCBr concentration in possible murder and suicide cases.

By the development of the novel kinetic method for PCBr determination (20), based on cholinesterase inhibition (benzoylcholine used as the hydrolysis substrate), the application of expensive equipment (HPLC (9,10), GC (11), and LC-ESI-MS (12–14)) could be avoided, as well as previously necessary complex sample preparation procedures. To replace the coupled reactions for the detection of the hydrolysis product (oxidation by cholinoxidase and peroxidase), the present work deals with the application of a more convenient and cheaper butyrylthicholine iodide as the substrate.

TABLE 6. The accuracy test of PCBr determination in the urine model samples by the standard addition method*

Sample	c(PCBr) in sample (μmol/L) ^a	Added PCBr in probe (nmol)	Found c(PCBr) (μmol/L)	Recovery (%)	Mean value of recovery (%)
Urine 1 (a)	0.435	0.4	0.459 ± 0.045	105.52	106.57
		0.9	0.475 ± 0.044	109.20	
		1.7	0.456 ± 0.044	104.83	
		3.5	0.458 ± 0.042	105.29	
Urine 2 (a)	1.356	0.5	1.474 ± 0.115	108.70	106.22
		0.9	1.456 ± 0.110	107.37	
		1.4	1.401 ± 0.106	103.32	
		2.0	1.430 ± 0.102	105.46	
Urine 3 (a)	2.712	0.4	2.734 ± 0.102	100.81	100.91
		0.9	2.747 ± 0.100	101.29	
		1.7	2.728 ± 0.086	100.59	
Urine R (a)	1.130	0.9	1.185 ± 0.055	104.87	106.75
		1.7	1.214 ± 0.052	107.43	
		3.5	1.220 ± 0.051	107.96	
Urine 1 (b)	0.428	0.4	0.416 ± 0.036	97.20	98.44
		0.9	0.427 ± 0.085	99.77	
		1.7	0.417 ± 0.030	97.43	
		3.5	0.426 ± 0.029	99.53	
Urine 2 (b)	1.366	0.5	1.358 ± 0.086	99.41	99.15
		1.0	1.356 ± 0.082	99.27	
		2.0	1.362 ± 0.083	99.71	
		4.1	1.341 ± 0.085	98.17	
Urine 3 (b)	2.732	0.8	2.740 ± 0.105	100.29	100.07
		1.6	2.708 ± 0.102	99.12	
		2.4	2.754 ± 0.099	100.81	
Urine R (b)	1.237	0.9	1.216 ± 0.045	98.30	97.41
		1.7	1.198 ± 0.044	96.95	
		3.5	1.201 ± 0.043	97.09	

*Results are expressed as the average of three replicate ($\bar{X} \pm SD$) experiments for different PCBr concentrations in the urine samples.

^aThe urine sample were prepared as described in section Sample Preparation.

Urine R, real urine sample taken during a hip replacement surgery; (a), denaturation by HCl and centrifugation; (b), gel-chromatography.

TABLE 7. An overview of the kinetic and analytical parameters of the spectrophotometric kinetic method based on cholinesterase inhibition by PCBr applying two different substrates

Kinetic and analytical parameters	Substrate	
	Butyrylthiocholine iodide	Benzoylcholine chloride*
K_M (mmol/L min)	0.33	0.40
V_{max} (μmol/L min)	42.3	52.2
K_i (μmol/L)	0.34	0.56
IC_{50} (μmol/L)	0.235	1.31
Linear range (nmol/L)	8.29–265.28	8.20–68.25
LD (nmol/L)	1.86	2.01
LQ (nmol/L)	6.18	6.67
RSD (%)	0.78–5.13	0.15–7.45

*Results from reference 20.

The modified method with butyrylthiocholine iodide was optimized and validated for application in the serum and urine samples, using two ways of removal of the

interfering substances (gel-chromatography and denaturation of proteins with HCl followed by centrifugation). Treatment with HCl, due to its better accuracy, significant time reduction, and analysis cost reduction, is recommended.

The kinetic and analytical parameters, obtained by applying both substrates benzoylcholine and butyrylthiocholine, are presented in Table 7. Besides the fact that the analytical parameters (LD, LQ, precision, and accuracy) obtained for both substrates are good and have almost the same values, the following advantages were achieved using butyrylthiocholine:

- wider linear determination range (convenient for clinical practice)
- shorter duration of the analysis (makes possible an easier full automation of the analysis)
- higher cholinesterase sensitivity to PCBr presence (lower IC_{50})
- application of a more economic chromogen for the detection of the hydrolysis product (DTNB).

Therefore, the modified method for the PCBr determination based on of cholinesterase inhibition and using butyrylthiocholine as the substrate is recommended for clinical and forensic practice.

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