Journal of Chromatography B, 911 (2012) 162–169

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A highly sensitive capillary electrophoresis method using *p*-nitrophenyl 5′-thymidine monophosphate as a substrate for the monitoring of nucleotide pyrophosphatase/phosphodiesterase activities

Sang-Yong Lee[a], Sébastien A. Lévesque[b,c,d], Jean Sévigny[b,c], Christa E. Müller[a,*]

[a] PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany
[b] Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Québec, Canada
[c] Département de Microbiologie-Infectiologie et d'Immunologie, Faculté de Médecine, Université Laval, Québec, Canada
[d] Centre de Recherche en Endocrinologie Moléculaire et Oncologique et Génomique Humaine, Centre Hospitalier Universitaire de Québec, Québec, Canada

ARTICLE INFO

Article history:
Received 24 August 2012
Accepted 26 October 2012
Available online 7 November 2012

Keywords:
AOPCP
Benzoylbenzoyl-ATP
Capillary electrophoresis
Dial-ADP
p-Nitrophenyl 5′-thymidine monophosphate
NPP inhibitors
NPP substrates
Nucleotide pyrophosphatase
Polybrene

ABSTRACT

A highly sensitive capillary electrophoresis method has been developed to monitor the activity of nucleotide pyrophosphatases/phosphodiesterases (NPPs) and screen for NPP inhibitors. In this method, *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) was used as an artificial substrate, and separation of reaction products was performed on a dynamically coated capillary. We found that the optimal capillary electrophoresis (CE) conditions were as follows: fused-silica capillary (20 cm effective length × 75.5 μm (id)), electrokinetic injection for 60 s, 70 mM phosphate buffer containing polybrene 0.002%, pH 9.2, constant current of −80 μA, constant capillary temperature of 15 °C and detection at 400 nm. To allow precise quantification, 2-methyl-4,6-dinitrophenol (dinitrocresol) was applied as an internal standard. The limit of detection (LOD) and the limit of quantification (LOQ) were 137 and 415 nM, respectively. This new method was shown to be over 8-fold more sensitive than the conventional spectrophotometric assays and 16-fold more than the previously reported CE procedure, and the results (K_m values for NPP1 and NPP3, K_i values for standard inhibitors) obtained were in accordance with previous literature data. Therefore, this new method is an improvement of actual techniques and could be used as a quick and standard analytical technique for the identification and characterization of NPP inhibitors.

1. Introduction

Nucleotide pyrophosphatases/phosphodiesterases (NPPs; E.C. 3.1.4.1, E.C. 3.6.1.8, and 3.6.1.9) represent a group of conserved eukaryotic proteins that are expressed both as transmembrane ecto-enzymes and as soluble proteins in body fluids [1,2]. Three members of the NPP family hydrolyze nucleotides, namely, NPP1 (PC-1), NPP2 (autotaxin), and NPP3 (B10, gp130^{RB13-6}) [3,4]. NPP1–3 reveal a broad substrate specificity, being capable of hydrolyzing ATP, ADP, NAD^+, phosphoadenylate sulfate (PAPS), FAD, dinucleoside polyphosphates (e.g. AP_3A, AP_4A) and nucleotide sugars (e.g. UDP-glucose) to their nucleoside monophosphate derivatives [1–7]. NPP1-3 have been involved in various biological processes. A major function of NPP1 is in bone mineralization and soft-tissue calcification [8–10]. Moreover, NPP1 has also been linked to insulin resistance and type 2 diabetes [11–15]. NPP2 has been shown to promote cancer cell invasion, cell migration, lymphocyte trafficking and angiogenesis [1,16]. NPP3 expression is associated with carcinogenesis and metastasis of cancer cells and has been proposed as a tumor marker [1,10,17]. Additionally, it has been reported that this enzyme is expressed by basophils and mast cells where it might have a function in the activation process of these cells [4,18].

To allow for biochemical characterization and to discover subtype-selective inhibitors and activators of NPPs, a highly sensitive and reproducible screening method is required. In our laboratory, we previously developed CE-based enzyme assays with adenosine 5′-triphosphate (ATP) as a substrate for NPP1 and NPP3 [1]. However, the previously developed method has some drawbacks, including (i) moderate sensitivity, (ii) partial decomposition of the substrate ATP upon termination of the reaction by heating, and therefore (iii) some problems with reproducibility especially when the assays were performed by different persons. Therefore, there was a strong need for the development of a new CE method to monitor the nucleotide pyrophosphatase/phosphodiesterase reactions.

* Corresponding author at: Pharmazeutisches Institut, Pharmazeutische Chemie I, An der Immenburg 4, D-53121 Bonn, Germany. Tel.: +49 228 73 2301; fax: +49 228 73 2567.
E-mail address: christa.mueller@uni-bonn.de (C.E. Müller).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.10.044

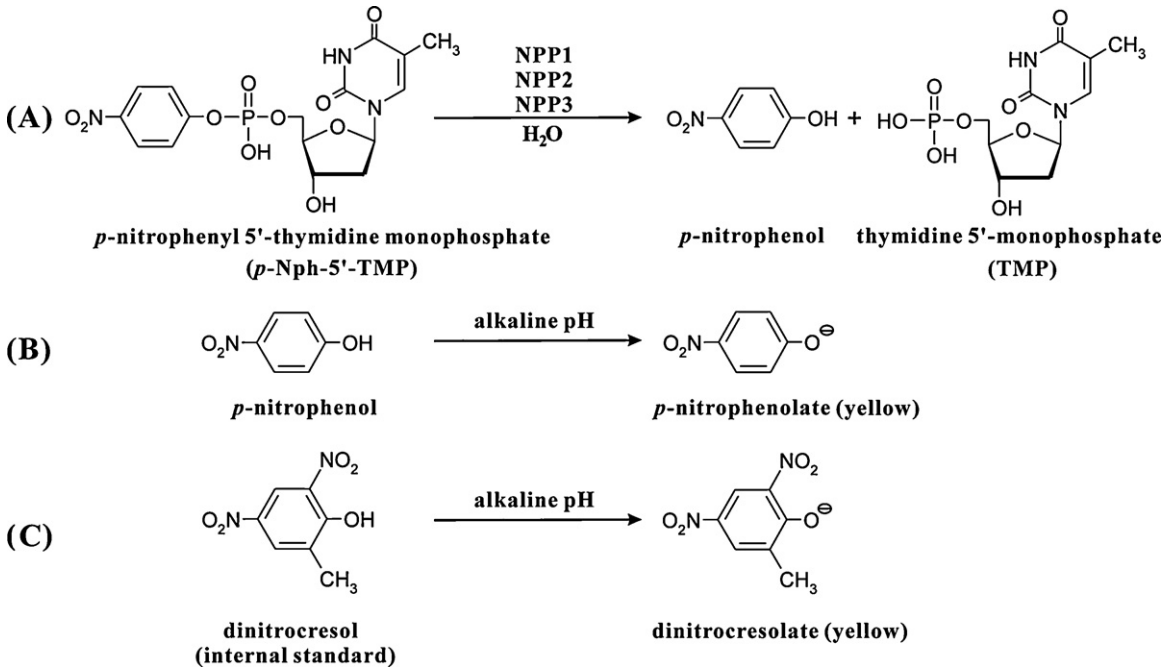

Fig. 1. Hydrolysis of *p*-nitrophenyl 5′-thymidine monophosphate by NPP1-3 (A) and formation of yellow *p*-nitrophenolate (B), dinitrocresolate (C) at alkaline pH.

To date, several methods have been developed for the quantitative determination of NPP activity. There are radioactive methods applying [γ-^{32}P]-labeled substrates applying thin-layer chromatography for the separation of the radioactive products [2,19], and fluorimetric enzyme assays using fluorescent etheno-adenine nucleotides as substrates [20,21]. However, the most frequently used techniques for measuring NPP activities are spectrophotometric assays with the artificial substrate *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) [2,9,16,17,22–26]. During the enzyme reaction, this synthetic substrate is cleaved to thymidine 5′-monophosphate (TMP) and *p*-nitrophenol. The latter can be measured due to its absorption in the visible region upon deprotonation (Fig. 1). Although these routine methods are relatively quick and simple, they require large amounts of material (enzymes, substrates, and test compounds), and in addition, they have a limitation when the test compounds possess light absorption at the wavelength of absorption of *p*-nitrophenolate.

We have recently developed and applied a CE-UV/VIS method for monitoring alkaline phosphatase using *p*-nitrophenylphosphate as a substrate and detecting the enzymatic reaction product *p*-nitrophenolate [27]. This method was further developed using an immobilized enzyme microreactor in a fused-silica capillary [28], resulting in a limit of detection of 2.13 μM for *p*-nitrophenol. Very recently, the development of an indirect fluorescence detection method of various nitrophenols by micellar electrokinetic chromatography (MEKC) with an in-column fiber optics LED-IF (light emitting diode-induced fluorescence) detection system has been reported [29]. However the detection limit of 16 μM for *p*-nitrophenol, the same product which we obtain in the NPP-catalyzed hydrolysis of *p*-Nph-5′-TMP, was fairly high.

All of the described CE methods for the detection of *p*-nitrophenolate are not sufficiently sensitive to be applied to the monitoring of NPP reactions using *p*-nitrophenyl 5′-thymidine monophosphate as a substrate. Thus, the aim of the present study was to develop a sensitive and efficient CE method applicable for the determination of *p*-nitrophenolate in routine assays. To validate the new CE method, different inhibitors of NPP1 and NPP3 have been tested and compared with the current literature.

2. Materials and methods

2.1. Materials

p-Nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP), thymidine 5′-monophosphate (TMP), adenosine 5′-diphosphate-2′,3′-dialdehyde (dialADP), 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (bzATP), 2-methyl-4,6-dinitrophenol (dinitrocresol), uridine 5′-diphosphate (UDP), and hexadimethrine bromide (polybrene) were obtained from Sigma (Steinheim, Germany). Magnesium chloride, calcium chloride, *p*-nitrophenol, sodium tetraborate decahydrate, zinc chloride and Tris (Trizma base) were also from Sigma. Disodium hydrogen phosphate was purchased from Carl Roth (Karlsruhe, Germany). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was from Applichem (Darmstadt, Germany). Adenosine 5′-(α,β-methylene)diphosphate (AOPCP) was obtained from Biolog Life Science Institute (Bremen, Germany). DMEM/F-12, lipofectamine and fetal bovine serum (FBS) were purchased from Invitrogen (Burlington, ON, Canada). Plasmids encoding human NPP1, and NPP3, respectively, were obtained from Dr. Goding and Dr. Sano, respectively [30].

2.2. Transfection and membrane preparation of human NPP1 and NPP3

Human NPPs were transfected transiently in human embryonic kidney (HEK293T) cells with a plasmid encoding either NPP1 or NPP3 and cell membranes were prepared as previously described [30].

2.3. CE instrumentation

All experiments were carried out using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a DAD detection system. Data collection and peak area analysis were performed by 32 Karat software obtained from Beckman Coulter (Fullerton, CA, USA). The capillary temperature was kept constant at 15 °C, and the temperature of the sample storing unit was adjusted to 25 °C. The electrophoretic separations were

carried out using eCAP uncoated fused-silica capillaries of 30 cm total length (20 cm effective length) × 75.5 μm (id) × 363.7 μm (od) obtained form Polymicro Technologies (Kehl, Germany). The following conditions were applied: λ_{max} = 400 nm, separation current = −80 μA, running buffer 70 mM phosphate buffer containing polybrene 0.002%, pH 9.2, electrokinetic injection (−6 kV, 60 s). The capillary was washed with 0.1 N NaOH aq. for 3 min (20 psi) and subsequently with running buffer for 3 min (20 psi) before each injection.

2.4. Preparation of test solutions for method validation

p-Nitrophenol and dinitrocresol were dissolved in deionized water to obtain 10.0 mM stock solutions. These were further diluted to obtain 1.0 mM solutions in assay buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 8.5). The 1.0 mM stock solutions were further diluted in the same buffer as required for method validation.

The proposed CE method was validated for various parameters, viz. linearity, accuracy, precision, LOD, LOQ and robustness as recommended [31–34].

2.4.1. Linearity

The linearity of the method was assessed by processing (in triplicate) a nine-point calibration curve (0.1, 0.3, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 μM) for *p*-nitrophenol containing 0.5 μM dinitrocresol as an internal standard (IS) in assay buffer. Calibration curves were obtained by plotting the ratios of peak areas of analyte and IS against the analyte concentrations.

2.4.2. Precision and accuracy

The precision and accuracy values were determined at the following concentrations of *p*-nitrophenol: 0.4 (at LOQ), 1.0, 25.0 and 50.0 μM in the presence of 0.5 μM of dinitrocresol as IS; each concentration was measured six times. Accuracy was determined by calculating the ratios of the predicted concentrations and the spiked values. Relative standard deviation (RSD) was calculated.

2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated from the calibration curve, using the values determined for the standard deviation of the *y*-intercept (σ) and the slope (S) (LOD 3.3 σ/S, LOQ 10 σ/S).

2.4.4. Robustness (with respect to sample stability)

The stability of the substrate (*p*-Nph-5′-TMP) and the product (*p*-nitrophenol) in assay buffer was investigated at different time points at room temperature. For this experiment, two different stock solutions were prepared (test solution 1: 25.0 μM *p*-Nph-5′-TMP, 0.5 μM dinitrocresol; test solution 2: 20.0 μM *p*-nitrophenol, 0.5 μM dinitrocresol). Samples of 100 μl of both solutions were drawn at 0–3 day(s), and analyzed; each analysis was repeated three times.

2.5. Comparison with previously published methods

The new CE method was compared with both, the published spectrophotometric procedures [2,9,16,17,22–26], and the previously reported CE method [28], with regard to LOD, LOQ, accuracy and precision. For this purpose, the preparation of stock and test solutions of *p*-nitrophenol and the determination of the evaluation parameters were carried out as described in Section 2.4. We used the same Tris buffer (5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50 mM Tris–HCl, pH 9.5) that was employed in the previously described CE method [28]. For reasons of comparability and simplification we also used the same buffer for the colorimetric assays, since Tris buffer has been typically used for the described spectrophotometric assays, although the composition may have varied in the different studies [2,9,16,17,22–26].

2.5.1. Instrumentation for spectrophotometric assays

Spectrophotometric measurements were carried out on a BMG PheraStar FS (BMG LABTECH GmbH, Ortenberg, Germany) microplate reader with a wavelength range of 220–1000 nm and a spectral bandwidth of 2.0 nm. The absorbance of each test solution was measured at 400 nm in a clear 96-well microplate (Greiner-Bio-One GmbH, Frickenhausen, Germany).

2.5.2. Instrumentation for CE method performed in accordance with published procedure

The experiments were performed on a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a DAD detection system. Data collection and peak area analysis were performed by 32 Karat software obtained from Beckman Coulter (Fullerton, CA, USA). The electrophoretic separations were carried out using eCAP fused-silica capillaries of 30 cm total length (20 cm effective length) × 75.5 μm (id) × 363.7 μm (od) obtained form Polymicro Technologies (Kehl, Germany). The separation was performed using an applied voltage of −15 kV. Analytes were detected using direct UV absorbance at 322 nm. The capillary temperature was kept constant at 37 °C and the temperature of the storing unit was adjusted to 25 °C. The running buffer consisted of 50 mM borate buffer (pH 9.5) and polybrene 0.0025%. Samples were introduced into the capillary by electrokinetic injection (−6 kV, 60 s). Between separations, the capillary was washed with 0.1 N aq. NaOH solution for 2 min (20 psi) and subsequently with running buffer for 2 min (20 psi) before each injection.

2.6. Determination of Michaelis–Menten constant (K_m) and maximum velocity (V_{max})

For the determination of the kinetic parameters (K_m and V_{max}) of nucleotide pyrophosphatase/phosphodiesterase 1 and 3 (NPP1, NPP3) by CE, different substrate concentrations of *p*-Nph-5′-TMP were used, 1, 10, 20, 50, 100, 200, 500, and 1000 μM. The reaction buffer containing 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 8.5, was preincubated with approximately 2.4 μg NPP1 or 2.6 μg NPP3 membrane preparation per tube at 37 °C for 10 min. The enzyme reactions were initiated by the addition of *p*-Nph-5′-TMP solutions in a final volume of 100 μl and then incubated at 37 °C for 60 min and subsequently stopped by heating at 90 °C for 2 min. Finally, 50 μl of the reaction mixture were transferred into mini-CE vials containing 50 μl of the internal standard dinitrocresol (final concentration 0.5 μM). Controls to correct for non-enzymatic substrate hydrolysis were measured in the absence of enzyme as described above. Each analysis was performed in triplicate in three separate experiments.

2.7. Investigation of NPP inhibitors

The inhibitory effect of AOPCP, dialADP and bzATP on the enzymatic activity of NPPs was tested in reaction buffer containing 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 8.5, and 500 μM *p*-Nph-5′-TMP, with a range of inhibitor concentrations of 0.3–1000 μM. Incubation and separation conditions remained the same as described in Section 2.6. Negative controls were performed in the absence of enzyme. Each analysis was carried out three times in three separate experiments. The Cheng–Prusoff equation was used to calculate K_i values from IC_{50} values determined by nonlinear curve fitting using the program Prism 4.0 (GraphPad software, San Diego, CA, USA) [35].

2.8. Investigation of NPP substrate properties of nucleotide derivatives and analogs

Nucleotide analogs (AOPCP, dialADP and bzATP, 500 μM) were incubated at 37 °C for 60 min in a reaction mixture containing membrane preparation expressing either NPP1 (2.4 μg) or NPP3 (2.6 μg), which had already been preincubated at 37 °C for 10 min. The enzyme reaction was stopped by heating at 90 °C for 2 min. An aliquot (10 μl) of the reaction mixture was taken and diluted with 90 μl of water containing the internal standard UDP (final concentration 10 μM). The diluted reaction mixture was transferred into mini-CE vials for measurement. Injection of all solutions was done three times each, and three separate experiments were performed.

The CE operating conditions were altered for the detection of the nucleotide derivatives and analogs as compared to the detection of nitrophenolate. The following conditions were applied: eCap fused-silica capillary (60 cm [50 cm effective length] × 75.5 μm (id) × 363.7 μm (od)), λ_{max} = 260 nm, separation current = −120 μA, running buffer 70 mM phosphate buffer (polybrene 0.002%, pH 6.5), electrokinetic injection −6 kV, 60 s. The relative hydrolysis of compounds was calculated in percent by comparing the peak area of the sample with the peak area of the control in the absence of enzymes (=100%).

3. Results and discussion

3.1. Development of CE method

Capillary electrophoresis (CE) is a fast, low-cost and powerful separation technique that has been highly useful for genomics and proteomics investigations [36–38]. Recently, CE methods with a dynamic coating of silica-fused capillaries have gained considerable interest because of a significant improvement of detection sensitivity [1,28]. The use of the polycationic polymer polybrene as a dynamic coating material has provided an effective stacking of sample zones due to its sweeping effect [1,39]. Conventional CE techniques with UV detection typically show detection limits in the range of about 1–10 μM [1,27,40]. Due to the preconcentration effect by polycationic polymers the detection limits of analytes could be improved to the nanomolar range [1,41]. This concentration effect has been shown to work well for negatively charged analytes, including nucleotides like ATP, ADP or AMP [1,42]. *p*-Nitrophenol forms a negatively charged phenolate anion (Fig. 1), and therefore, the sweeping effect using polybrene should be applicable for the detection of *p*-nitrophenol [28].

In the current work, we therefore employed the CE separation technique using a dynamically coated capillary to monitor the hydrolysis of *p*-Nph-5′-TMP as a substrate of NPPs. *p*-nitrophenolate shows its absorption maximum (λ_{max}) at 400 nm, in the visible range. Although the λ_{max} of dinitrocresol (internal standard) was below 400 nm, its absorbance at 400 nm was high enough for peak detection. Thus, a detection wavelength of 400 nm was selected for the analysis.

Moreover, the choice of a suitable buffer is very important for the separation of analytes by CE. In CE, there are some established combinations of running and reaction buffers, e.g. phosphate–HEPES, phosphate–Tris, borate–HEPES and borate–Tris buffers. An important finding in our study was that some buffer combinations were more advantageous than others for dynamically coated capillary systems with polybrene. The method sensitivity was increased according to the following rank order: phosphate–Tris (very bad) < borate–HEPES (bad) < borate–Tris (satisfactory) ≪ phosphate–HEPES (very good). Thus, the buffer combination phosphate–HEPES was chosen for the subsequent assay development.

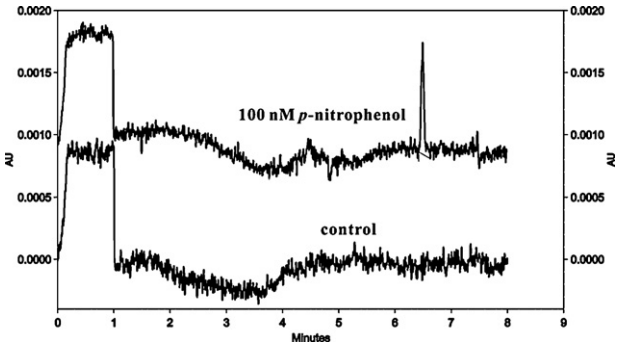

Fig. 2. Typical electropherograms of 100 nM *p*-nitrophenol (concentration near the limit of detection) and control (reaction buffer) measurements. The separation conditions were: 70 mM phosphate buffer at pH 9.2, 0.002% polybrene, fused-silica capillary 30 cm (20 cm to the detector), 75.5 μm (id), −80 μA, 15 °C, detection at 400 nm.

The ionic strength of the running buffer is also an important factor to control the separation efficiency and migration times [27,43]. Initially, a 50 mM concentration of running phosphate buffer was selected; however the internal standard did not separate from *p*-nitrophenol under these conditions. For an adequate separation between both peaks, a higher concentration of 70 mM phosphate buffer was found to be optimal. At the same time, a short capillary length (30 cm [20 cm effective length]) was used to further reduce the analysis time, which was necessary due to the migration-prolonging effect of using a high buffer concentration.

Usually, pH values of phosphate buffers between 2.5 and 8.0 have been suggested in CE [1,40,44–47]. However, an alkaline pH value was required for the formation of *p*-nitrophenolate. But such a high pH value disturbed the dynamic coating system. Therefore, a slightly alkaline pH of 9.2 turned out to be a good compromise.

The last critical factor affecting reproducibility of migration time is the temperature of the capillary. Recently, it has been found that a high capillary temperature in a dynamic coating system with polybrene negatively influenced the reproducibility of migration time (relative standard deviation (RSD) of migration time over 4.0%). Thus, a low capillary temperature at 15 °C was selected for all subsequent measurements, which led to a 2–3-fold reduction in the RSD (%) of the migration time (the %RSD of migration times for *p*-nitrophenol and dinitrocresol in the current study was typically below 1.9%). The final, optimized parameters were as follows: reaction buffer consisting of 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH 8.5), 70 mM phosphate buffer containing 0.002% polybrene (pH 9.2) as the running buffer, a fused-silica capillary (20 cm effective length), a constant capillary temperature of 15 °C and detection at 400 nm.

3.2. Method validation

The newly developed CE method was validated following ICH and FDA guidelines [31,32]. An overview of the quantitative parameters of the method validation is provided in Table 1. A strictly linear correlation between *p*-nitrophenol concentration and the peak-area ratio of *p*-nitrophenol to IS was observed: a correlation coefficient (R^2) of 0.9978 for *p*-nitrophenol was calculated for a concentration range from 0.1 to 50.0 μM. The determined equation was $y = 304.4\,x + 49.0$ and the relative standard deviation (RSD) of the slope was 2.75%. The LOD and LOQ of *p*-nitrophenol were 137 and 415 nM, respectively, demonstrating high sensitivity of the method. Fig. 2 shows that the newly developed CE method allows the detection of a clear, narrow peak of *p*-nitrophenol close to the detection limit (*p*-nitrophenol at 100 nM). Accuracy and precision, as a degree of repeatability, were established across the

Table 1
Results of method validation with new CE method and comparison with conventional methods for the determination of *p*-nitrophenolate.

Parameters	Method			
	Colorimetric assay	Capillary electrophoresis assay (previous method)	Capillary electrophoresis assay (new method)	Acceptable range[a]
Regression equation ($n = 3$)	$y = 0.0059\,x + 0.04517$	$y = 342.7\,x + 268.5$	$y = 304.4\,x + 49.0$	
R^2	0.9971	0.9936	0.9978	
LOD (nM)	1119	2235	137	
LOQ (nM)	3390	6773	415	
Accuracy ($n = 6$, recovery %)				
At LOQ, 0.4 μM	n.a.[b]	n.a.[b]	87.8	80–110%
Low, 1.0 μM	n.a.[b]	n.a.[b]	93.6	80–110%
Medium, 25.0 μM	93.5	94.5	97.5	80–110%
High, 50.0 μM	100.5	93.7	100.2	80–110%
Precision ($n = 6$, RSD[c] %)				
At LOQ, 0.4 μM	n.a.[b]	n.a.[b]	9.0	15%
Low, 1.0 μM	n.a.[b]	n.a.[b]	6.0	11%
Medium, 25.0 μM	4.8	1.8	4.0	7.3%
High, 50.0 μM	3.9	1.3	3.8	7.3%

[a] Acceptable range according to AOAC Peer Verified Methods [33,34].
[b] n.a., not possible to analyze.
[c] RSD, relative standard deviation.

linearity range. Precision was high below 15% with a maximum RSD of 9.0%, and the accuracy ranged from 87.8% to 100.2%. Those obtained values were acceptable for the measured low analyte concentrations [33,34]. Furthermore, we investigated the robustness regarding the stability of both, substrate (*p*-Nph-5′-TMP) and product (*p*-nitrophenol), in reaction buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 8.5) at the room temperature (25 °C). Our results showed that the product concentration was constant over the whole test period of three days; however the substrate was stable only for about one day in reaction buffer, and during the observation period of 72 h the amount of *p*-Nph-5′-TMP was decreased from 99.2 ± 0.4% to 91.6 ± 1.3%. Thus, the CE measurements were subsequently completed within one day.

3.3. Comparison of the new CE method with previously published methods

In order to directly compare the newly developed CE method with the previously published CE-based [28] and colorimetric methods [2,9,16,17,22–26], we used those methods for the determination of nitrophenolate and compared them with our new method. Table 1 summarizes the results of the comparison of the different methods with regard to LOD, LOQ, accuracy and precision. In comparison not only with conventional colorimetric assays but also with the formerly described CE method for nitrophenolate detection [28], these results show that the sensitivity of the method has been significantly improved. The detection limit for *p*-nitrophenol was 8-fold lower with the new method than with spectrophotometric assays and 16-fold lower than with the previous CE procedure. Furthermore, this study demonstrated that the newly developed CE technique allows the quantification of the analyte down to the nanomolar concentration range. In contrast, both

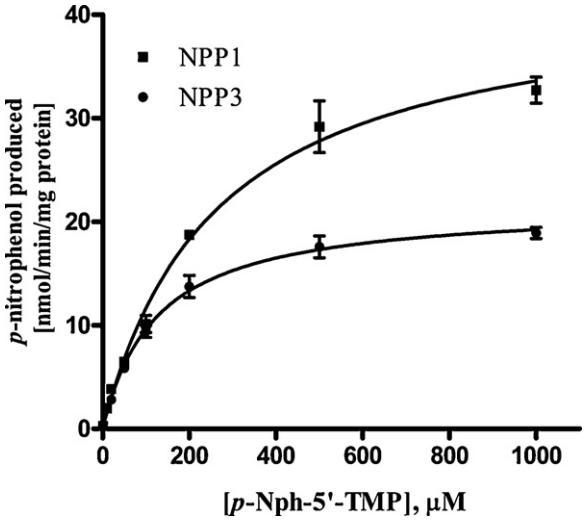

Fig. 3. Michaelis–Menten plots of the hydrolysis of *p*-Nph-5′-TMP by human NPP1 (■) and NPP3 (●). Data points represent mean ± SEM from three separate experiments each run in triplicate. The determined kinetic parameters were: NPP1, $K_m = 281 \pm 25$ μM, $V_{max} = 42.5 \pm 2.7$ nmol/min/mg protein; NPP3, $K_m = 133 \pm 6$ μM, $V_{max} = 21.5 \pm 1.0$ nmol/min/mg protein.

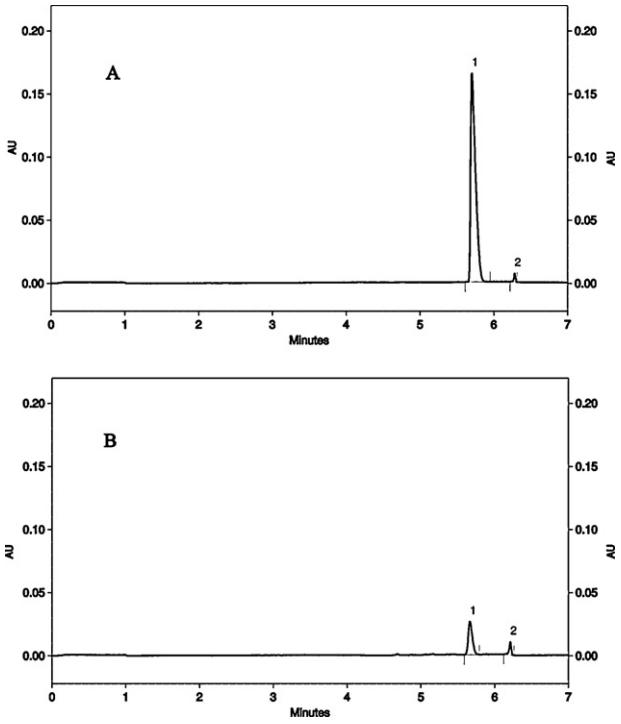

Fig. 4. Typical electropherograms of NPP1 enzymatic reaction as control without inhibitor (A) and inhibition assay in the presence of the inhibitor AOPCP at 1 mM (B). The concentration of *p*-Nph-5′-TMP was 500 μM. The separation conditions were: 70 mM phosphate buffer at pH 9.2, 0.002% polybrene, fused-silica capillary 30 cm (20 cm to the detector), 75.5 μm (id), −80 μA, 15 °C, detection at 400 nm (peak 1: *p*-nitrophenol, peak 2: dinitrocresol as internal standard).

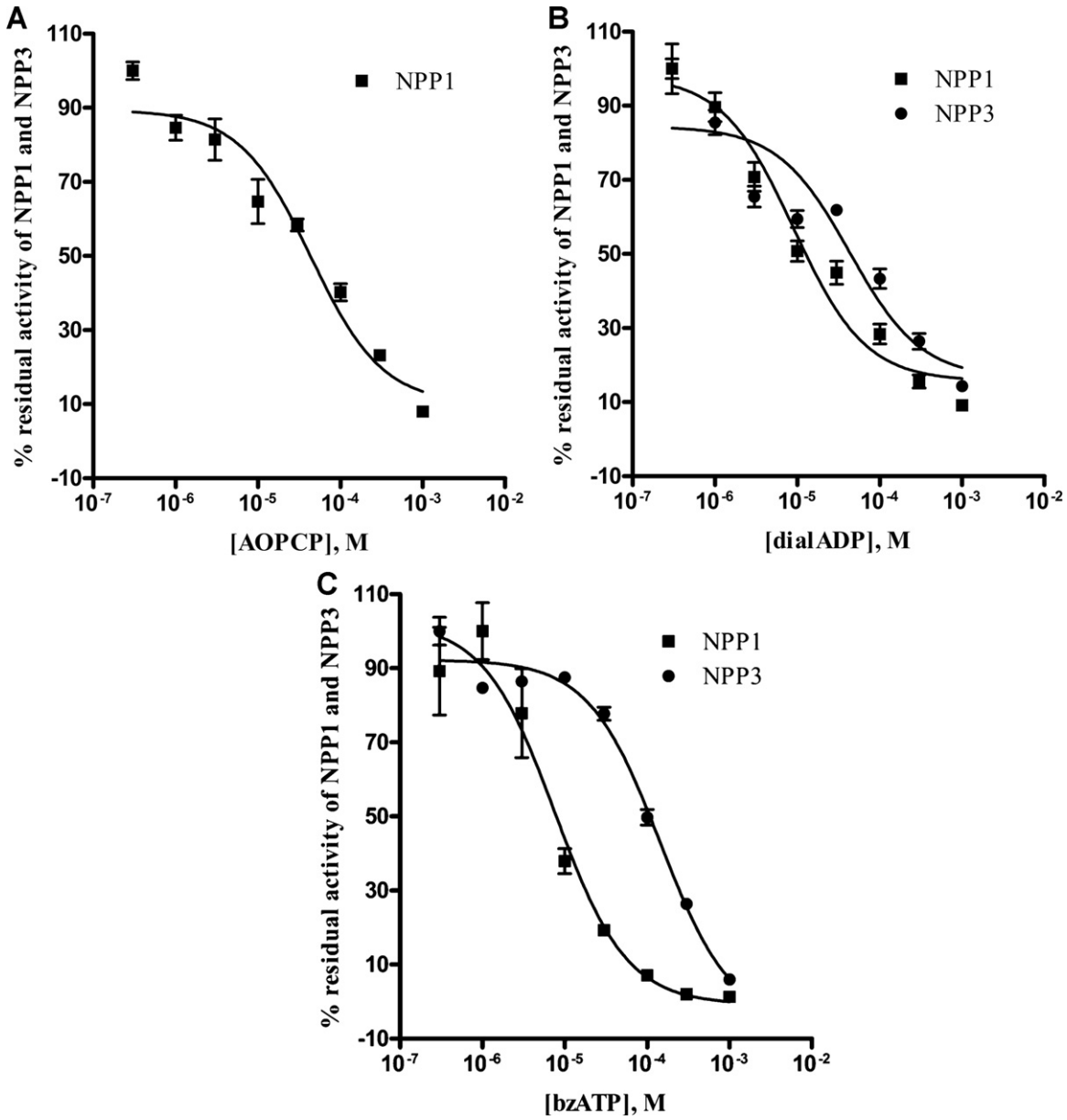

Fig. 5. Concentration-dependent inhibition of human NPP1 (■) and NPP3 (●) by adenosine 5′-(α,β-methylene)diphosphate (AOPCP), adenosine 5′-diphosphate-2′,3′-dialdehyde (dialADP), and 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (bzATP) determined by capillary electrophoresis. A substrate concentration of 500 μM *p*-Nph-5′-TMP, a reaction buffer consisting of 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES, pH 8.5, and various concentrations of AOPCP, dialADP and bzATP were used. Data points represent means ± SEM from three separate experiments, each run in triplicate.

previous methods did not allow to determine the accuracy and the precision in an analyte concentration of lower than 1.0 μM. The lowest concentration for the colorimetric method and for the earlier CE procedure, at which an acceptable accuracy with a recovery range of 80–110% and a precision RSD of less than 11% could be obtained, were around 4.0 μM, and 7.0 μM, respectively.

3.4. Determination of kinetic parameters of NPP1 and NPP3

For the determination of the Michaelis–Menten constants (K_m) and maximal velocity (V_{max}) of both, human NPP1 and NPP3, eight concentrations of *p*-Nph-5′-TMP in the range of 1–1000 μM were used (Fig. 3). The enzyme velocity was determined by measuring the peak areas of the products of the enzymatic reaction. Evaluated Michaelis–Menten values (K_m) of 281 ± 25 and 133 ± 6 μM were obtained for NPP1 and NPP3, respectively. Calculated V_{max} values were 42.5 ± 2.7 and 21.5 ± 1.0 nmol/min/mg protein for NPP1 and NPP3, respectively. A direct comparison with K_m and V_{max} values of both human NPP subtypes for the same substrate (*p*-Nph-5′-TMP) is not possible, because such data have not been described so far. Reported K_m values for NPPs from human [48], rat [17,22,49,50], mouse [51] and cow [52] were between 50 and 300 μM and therefore in very good agreement with the values determined in the present study.

3.5. Enzyme inhibition assay

The newly developed method was subsequently used to measure NPP inhibition. Inhibition of NPPs was determined for concentrations ranging from 0.3 to 1000 μM of inhibitors in the presence of a fixed amount of *p*-Nph-5′-TMP (500 μM).

Table 2
K_i values at NPP1 and NPP3 obtained for AOPCP, dialADP and bzATP ($n = 3$).

	AOPCP	dialADP	bzATP
NPP1			
$IC_{50} \pm SEM^a$ (μM)	44.0 ± 8.6	9.09 ± 1.00	7.87 ± 2.18
$K_i \pm SEM^a$ (μM)	16.5 ± 3.2	3.42 ± 0.38	2.96 ± 0.82
NPP3			
$IC_{50} \pm SEM^a$ (μM)	n.d.[c]	43.1 ± 13.3	146.3 ± 16.0
$K_i \pm SEM^a$ (μM)	n.d.[c]	9.45 ± 2.92	32.1 ± 3.5
Literature[b]			
$K_i \pm SEM^a$ (μM)	9.6 ± 0.8	9.8 ± 1.3[d]	20.5 ± 2.2

[a] SEM, standard error of the mean.
[b] Literature values from rat NPPs of unspecified subtype [49].
[c] n.d., not determined due to low inhibition (32%) at 1 mM concentration.
[d] K_i value for dialATP at rat NPPs.

We analyzed the potency of the nucleotide analogs and derivatives, adenosine 5′-(α,β-methylene)diphosphate (AOPCP), adenosine 5′-diphosphate-2′,3′-dialdehyde (dialADP), and 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (bzATP) to inhibit nucleotide phosphodiesterase activity of NPP1 and NPP3 (see Table 2). All those standard inhibitors were found to be competitive inhibitors of both NPP subtypes (data not shown). Fig. 4A shows a typical electropherogram for the NPP1 with 500 μM of *p*-Nph-5′-TMP as substrate, where less than 5% of substrate was converted to the product *p*-nitrophenolate. The second electropherogram in Fig. 4B shows a typical NPP inhibition; in this case the inhibitor AOPCP (1 mM) had been added. One could see that in the latter electropherogram, the peak area for the product (*p*-nitrophenolate) was significantly decreased as compared to the control assay. Clearly narrow peaks were observed on a quite straight baseline, and concurrently, the main peak was completely separated from the IS peak. Substrate *p*-Nph-5′-TMP and product TMP were not detected since those nucleotides do not absorb at 400 nm. The product *p*-nitrophenolate migrated faster than the internal standard dinitrocresolate due to its lower molecular weight. The obtained concentration-inhibition curves for AOPCP, dialADP, and bzATP at NPP1 and 3 are presented in Fig. 5. The ATP derivative was 10-fold more potent to block the human NPP1 ($K_i = 2.96 \pm 0.82$ μM) compared to human NPP3 ($K_i = 32.1 \pm 3.5$ μM). The most potent inhibitor of the compounds tested was dialADP; it blocked the activity of both, NPP1 ($K_i = 3.42 \pm 0.38$ μM) and NPP3 ($K_i = 9.45 \pm 2.92$ μM). AOPCP was found to exhibit a moderate inhibitory activity on NPP1 ($K_i = 16.5 \pm 3.2$ μM) but hardly any effect on NPP3. The calculated K_i values of the ADP and ATP analogs and derivatives were similar to published values determined at NPPs present in rat serum [49].

3.6. Investigation of hydrolysis of nucleotide derivatives and analogs by NPPs

In order to investigate whether the applied enzyme inhibitors, an analog (AOPCP) and a derivative (dialADP) of ADP, and a derivative of ATP (bzATP), act as alternative substrates or as true inhibitors, hydrolysis of these nucleotides was investigated in the presence of NPP1, and NPP3, respectively. CE operation conditions were optimized for the separation and detection of each of the nucleotide analogs and derivatives (see Section 2.8). The ADP analog AOPCP was neither metabolized by NPP1 nor by NPP3, while the ADP derivative dialADP was hydrolyzed by both subtypes. It was a better substrate of NPP1 (87 ± 2% hydrolysis) than of NPP3 (40 ± 2%). The ATP derivative bzATP was somewhat more slowly hydrolyzed by both enzymes, NPP1 (27 ± 6%) and NPP3 (27 ± 3%). The electropherogram (Fig. 6) shows a typical hydrolysis experiment of nucleotide derivatives and analogs, in which the inhibitor bzATP was hydrolyzed by NPP1. In that electropherogram, the peak size for bzATP (peak 3) was slightly smaller as compared with the control assay, while the peak area of UDP (peak 2 as IS) was virtually the same in Fig. 6A and B. The peak area of ATP (peak 1) was an impurity of bzATP. ATP and UDP showed strait peaks in the electropherogram, but the peak of bzATP was broad and included a shoulder because this compound is a mixture of two isomers, which could be not be perfectly separated by the applied operating conditions. With respect to the results of the present study, we confirmed that AOPCP is a true NPP inhibitor consistent with published data [49], while dialADP and bzATP act as alternative substrates of NPP1 and NPP3.

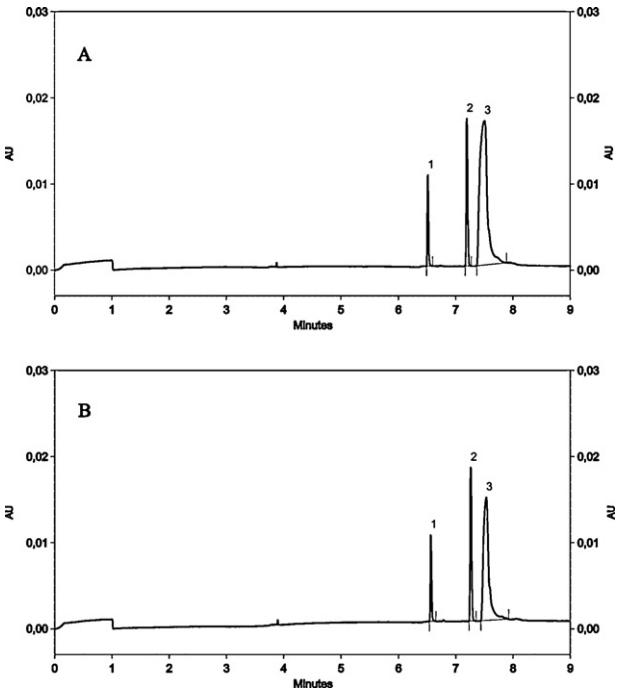

Fig. 6. Typical electropherograms of the hydrolysis assay of 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (bzATP, 500 μM) by NPP1: (A) control without enzyme, and (B) hydrolysis in the presence of enzyme. The separation conditions were: 70 mM phosphate buffer at pH 6.5, 0.002% polybrene, fused-silica capillary 60 cm (50 cm to the detector), 75.5 μm (id), −120 μA, 15 °C, detection at 260 nm (peak 1: ATP, peak 2: UDP (IS), peak 3: bzATP).

4. Conclusions

In conclusion, we have developed a highly sensitive capillary electrophoresis method for the screening of potential inhibitors and substrates of nucleoside pyrophosphatases/phosphodiesterases (NPPs). *p*-Nitrophenyl 5′-thymidine monophosphate was used as an artificial substrate forming *p*-nitrophenol upon NPP hydrolysis, which is converted to the intensely yellow-colored *p*-nitrophenolate at alkaline pH. By applying dynamic coating with the cationic polymer polybrene on the negatively charged silica-fused capillary walls, combined with an optimized selection of running and reaction buffer, and a short capillary length, very low limits of detection (LOD) and quantification (LOQ) in the nanomolar range were achieved. Thus the sensitivity of the new CE method is much higher, not only than that of our previously reported CE method [27,28], but also than that achieved with conventional spectrophotometric assays [2,9,16,17,22–26]. The CE method was further validated following ICH and FDA guidelines [31,32] and the results of the validation confirm its high suitability according to all evaluation criteria. Furthermore, this method has been successfully applied to Michaelis–Menten kinetic analysis and subsequent enzyme inhibition tests. In the future, this technique will be used as a routine procedure for the screening of compound libraries and

the confirmation of hit compounds in order to develop selective inhibitors for this pharmacologically important class of enzymes.

Acknowledgements

J. S. was supported by grants from the Canadian Institutes of Health Research (CIHR). Finished in 2007 and J.S. of a Senior Scholarship from the FRSQ.

References

[1] J. Iqbal, S.A. Lévesque, J. Sévigny, C.E. Müller, Electrophoresis 29 (2008) 3685.
[2] A. Cimpean, C. Stefan, R. Gijsbers, W. Stalmans, M. Bollen, Biochem. J. 381 (2004) 71.
[3] H. Zimmermann, M. Zebisch, N. Sträter, Purinergic Signal. 8 (2012) 437.
[4] C. Stefan, S. Jansen, M. Bollen, Trends Biochem. Sci. 30 (2005) 542.
[5] M. Bollen, R. Gijsbers, H. Ceulemans, W. Stalmans, C. Stefan, Crit. Rev. Biochem. Mol. 35 (2000) 393.
[6] H. Zimmermann, Naunyn Schmiedebergs Arch. Pharmacol. 362 (2000) 299.
[7] P. Vollmayer, T. Clair, J.W. Goding, K. Sano, J. Servos, H. Zimmermann, Eur. J. Biochem. 270 (2003) 2971.
[8] R. Gijsbers, H. Ceulemans, M. Bollen, Biochem. J. 371 (2003) 321.
[9] R. Terkeltaub, Purinergic Signal. 2 (2006) 371.
[10] J.W. Goding, B. Grobben, H. Slegers, Biochim. Biophys. Acta 1638 (2003) 1.
[11] N. Grarup, S.A. Urhammer, J. Ek, A. Albrechtsen, C. Glümer, K. Borch-Johnsen, T. Jorgensen, T. Hansen, O. Pedersen, Diabetologia 49 (2006) 2097.
[12] J.F. Youngren, Cell. Mol. Life Sci. 64 (2007) 873.
[13] T. Saarela, M. Hiltunen, S. Helisalmi, S. Heinonen, M. Laakso, Gynecol. Obstet. Invest. 61 (2006) 124.
[14] C.-N. Chin, Q. Dallas-Yang, F. Liu, T. Ho, K. Ellsworth, P. Fischer, T. Natasha, C. Ireland, P. Lu, C. Li, I.-M. Wang, W. Strohl, J.P. Berger, Z. An, B.B. Zhang, G. Jiang, Eur. J. Pharmacol. 606 (2009) 17.
[15] T. Zhao, Z. Liu, D. Zhang, Y. Liu, Y. Yang, D. Zhou, Z. Chen, L. Yu, Z. Zhang, G. Feng, L. He, H. Xu, J. Hum. Genet. 56 (2011) 12.
[16] S. Eliahu, J. Lecka, G. Reiser, M. Haas, F. Bigonnesse, S.A. Lévesque, J. Pelletier, J. Sévigny, B. Fischer, J. Med. Chem. 53 (2010) 8485.
[17] A. Buffon, E.A. Casali, V.V. Cardoso, L.F. Zerbini, S.C. Robson, J.J.F. Sarkis, M.R. Wink, Life Sci. 86 (2010) 435.
[18] S. Chirumbolo, M. Brizzi, R. Ortolani, A. Vella, P. Bellavite, Inflamm. Res. 58 (2009) 755.
[19] I. Aerts, B. Grobben, X.V. Ostade, H. Slegers, Eur. J. Pharmacol. 654 (2011) 1.
[20] A.C. Asensio, C.R. Rodríguez-Ferrer, A. Castañeyra-Perdomo, S. Oaknin, P. Rotllán, Neurochem. Int. 50 (2007) 581.
[21] S.M. Joseph, M.A. Pifer, R.J. Przybylski, G.R. Dubyak, Br. J. Pharmacol. 142 (2004) 1002.
[22] S.L. Henz, C.R. Fürstenau, R.A. Chiarelli, J.J.F. Sarkis, Arch. Oral Biol. 52 (2007) 916.
[23] C.R. Fürstenau, D.D.S. Trentin, M.L.M. Barreto-Chaves, J.J.F. Sarkis, Platelets 17 (2006) 84.
[24] A.B. Hoeglund, A.L. Howard, I.W. Wanjala, T.C.T. Pham, A.L. Parrill, D.L. Baker, Bioorg. Med. Chem. 18 (2010) 769.
[25] L.A. van Meeteren, V. Brinkmann, J.S. Saulnier-Blache, K.R. Lynch, W.H. Moolenaar, Cancer Lett. 266 (2008) 203.
[26] C.B. Petersen, A.-B. Nygård, B. Viuff, M. Fredholm, B. Aasted, J. Salomonsen, Dev. Comp. Immunol. 31 (2007) 618.
[27] J. Iqbal, D. Jirovsky, S.-Y. Lee, H. Zimmermann, C.E. Müller, Anal. Biochem. 373 (2008) 129.
[28] J. Iqbal, Anal. Biochem. 414 (2011) 226.
[29] F. Huo, H. Yuan, X. Yang, D. Xiao, M.M.F. Choi, Anal. Sci. 27 (2011) 879.
[30] S.A. Lévesque, É.G. Lavoie, J. Lecka, F. Bigonnesse, J. Sévigny, Br. J. Pharmacol. 152 (2007) 141.
[31] ICH Harmonised Tripartite Guideline, Q2(R1), Validation of Analytical Procedures: Text and Methodology, 2005.
[32] FDA Guidance for Industry, VICH GL2, Validation of Analytical Procedures: Methodology, 1999.
[33] I. Taverniers, M.D. Loose, E.V. Bockstaele, Trends Analyt. Chem. 23 (2004) 535.
[34] AOAC International, Method Validation Programs (OMA/PVM Department), including Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis, 2000.
[35] Y. Cheng, W.H. Prusoff, Biochem. Pharmacol. 22 (1973) 3099.
[36] Z.A. Al-Othman, I. Ali, J. Liq. Chromatogr. Relat. Technol. 34 (2011) 1295.
[37] I. Ali, H.Y. Aboul-Enein, V.K. Gupta, Chromatographia 69 (2009) S13.
[38] I. Ali, H.Y. Aboul-Enein, V.K. Gupta, Anal. Lett. 39 (2006) 2345.
[39] H. Lodén, A. Amini, Electrophoresis 28 (2007) 1548.
[40] H. Wätzig, M. Degenhardt, A. Kunkel, Electrophoresis 19 (1998) 2695.
[41] Z. Malá, A. Šlampová, P. Gebauer, P. Boček, Electrophoresis 30 (2009) 215.
[42] X.-J. Chen, F.-Q. Yang, Y.-T. Wang, S.-P. Li, Electrophoresis 31 (2010) 2092.
[43] J. Iqbal, L. Scapozza, G. Folkers, C.E. Müller, J. Chromatogr. B 846 (2007) 281.
[44] Y.J. Yao, K.C. Loh, M.C.M. Chung, S.F.Y. Li, Electrophoresis 16 (1995) 647.
[45] M. Kaulich, R. Qurishi, C.E. Müller, Cell. Mol. Neurobiol. 23 (2003) 349.
[46] X. Huang, M.J. Gordon, R.N. Zare, Anal. Chem. 60 (1988) 1837.
[47] J.P. Quirino, S. Terabe, J. Chromatogr. A 902 (2000) 119.
[48] J. Lüthje, A. Ogilvie, Eur. J. Biochem. 149 (1985) 119.
[49] D. Laketa, I. Bjelobaba, J. Savic, I. Lavrnja, M. Stojiljkovic, L. Rakic, N. Nedeljkovic, Mol. Cell. Biochem. 339 (2010) 99.
[50] B. Rücker, M.E. Almeida, T.A. Libermann, L.F. Zerbini, M.R. Wink, J.J.F Sarkis, Mol. Cell. Biochem. 306 (2007) 247.
[51] W.H. Evans, D.O. Hood, J.W. Gurd, Biochem. J. 135 (1973) 819.
[52] S.J. Kelly, D.E. Dardinger, L.G. Butler, Biochemistry 14 (1975) 4983.