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Study of enzyme kinetics of phenol sulfotransferase by electrophoretically mediated microanalysis

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Abstract

Electrophoretically mediated microanalysis (EMMA) was applied for the study of the kinetic parameters of the enzymatic reaction of phenol sulfotransferase SULT1A1 isoenzyme with 4-nitrophenol as a substrate. The SULT1A1 activity was determined by the quantitation of the product, 4-nitrophenyl sulfate, at 274 nm by using different injection and separation steps. This new approach solved the problem of the presence of the very strong inhibitor, adenosine 3′,5′-bisphosphate (PAP), in the co-substrate solution (adenosine 3′-phosphate 5′-phosphosulfate, PAPS) which is unstable at room temperature. The inhibitor PAP was electrophoretically separated from the co-substrate PAPS before the injection of enzyme and substrate inside the capillary (and thus before their in-capillary encountering). With the developed in-capillary SULT1A1 activity assay an average Michaelis constant (K_m) for 4-nitrophenol was calculated to be 0.84 μM, a value which is consistent with a previously reported value. Strong substrate inhibition (above a 4-nitrophenol concentration of 2.5 μM) was observed, and this is also in accordance with literature values.

Keywords: Kinetic studies; Electrophoretically mediated microanalysis; Enzymes; Phenol sulfotransferase

1. Introduction

Sulfation, or more precisely sulfonation, represents a major pathway for the biotransformation/detoxication of drugs and xenobiotics, as well as endogenous compounds such as cholesterol, catecholamines, steroid and thyroid hormones, and bile acid [1]. The responsible enzymes are called the sulfotransferases (STs). Two general classes of these enzymes exist in tissue fractions: the cytosolic enzymes that are important in drug metabolism, and the membrane bound enzymes that are involved in the sulfonation of glycoproteins and glycosaminoglycans [2]. Cytosolic sulfotransferases play an important role in the second-phase metabolism of xenochemicals, and are also involved in the inactivation of endogenous signal molecules such as neurotransmitters [3]. The family of cytosolic sulfotransferases catalyzes the sulfonation reaction involving the transfer of an electrophilic sulfonate group from a biologically active form of inorganic sulfate, adenosine 3′-phosphate 5′-phosphosulfate (PAPS), to a nucleophilic acceptor substrate [4–8]. Structural analogues of the sulfate donor PAPS are effective inhibitors of these enzymes [9–12].

Spectrophotometric assays [13–15] and LC-based methods with UV detection [16,17] have been reported for assaying sulfotransferase activity. However, the most common assay of sulfotransferase activity involves the monitoring of the transfer of radioisotopic sulfate from [^{35}S] PAPS to the reaction product [1]. The separation of [^{35}S] PAPS from the sulfated reaction products can be performed by a precipitation reaction with barium salts [17] or by use of thin layer chromatography [18] or LC techniques [2]. Alternatively, radioactively labeled sulfate acceptors (substrates) have been used to assay ST activity [18,19]. In general, the use of

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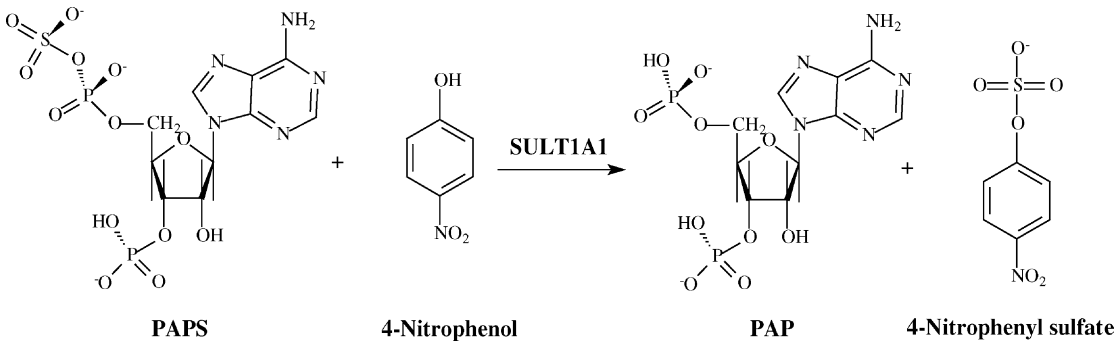

Fig. 1. The sulfonation reaction catalyzed by phenol sulfotransferase.

radioactively labeled PAPS or radioactive sulfate acceptor provides assays with a high degree of sensitivity [1].

Capillary electrophoresis is a powerful and relatively new analytical tool, characterized by high resolution separations, short analysis times and low sample load. This implies that the capillary, the separation tool, can also be used as a small reaction vessel. In this manner, all the different assay steps (i.e. reaction, separation, quantitation) can be combined in one automated, microscale assay.

CE systems have been successfully applied for on-line enzyme-catalyzed reactions by a methodology known as electrophoretically mediated microanalysis (EMMA), firstly described by Bao and Regnier [20]. EMMA utilizes the different electrophoretic mobilities of enzyme, substrate, and product to initiate reaction inside the capillary and to separate the components from each other for final in-capillary quantitation. There are basically two ways to mix reaction components [21–23] in a capillary under electrophoretic conditions. The first one is the continuous format of EMMA ("long contact mode"). In this format the capillary is initially filled with one of the reactants while the second analyte is introduced. The second one is the plug–plug format of EMMA (transient format or "short contact mode"). In contrast, this format is based on a plug–plug interaction. One of the advantages of the plug–plug format of EMMA is the electrophoresis process prior to the contact of enzyme and substrate inside the capillary. This process permits the separation of potential interfering substances, which may be advantageous when assaying enzyme activity in crude biological samples [24]. EMMA methodology has been used for different biochemical systems: enzyme activity assays [21,25–27], kinetic studies with the determination of Michaelis constants [28–31], or the study of inhibitors and inhibition constants [32–34].

In this work we used the isoenzyme SULT1A1 that is responsible for the sulfonation of small phenolic substrates such as 4-nitrophenol (Fig. 1). The purpose of this study was to determine whether it is possible to assay SULT1A1 activity with 4-nitrophenol as a model substrate by capillary electrophoresis, more precisely by using the EMMA methodology.

2. Materials and methods

2.1. Materials and reagents

Phenol sulfotransferase SULT1A1*2 cytosolic extract (human, recombinant) 60 μl, 4.4 mg prot./ml, 15766 units/mg prot. (one unit conjugates one picomole of sulfate to 4-nitrophenol at pH 6.5 at 37 °C), 4-nitrophenol (pNP), 4-nitrophenyl sulfate (pNPS), adenosine 3′,5′-bisphosphate (PAP), bovine serum albumin (BSA), dithiothreitol (DTT) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma (St. Louis, MO, USA). Adenosine 3′-phosphate 5′-phosphosulfate (PAPS, purity ≥80% by LC) was purchased from Calbiochem (San Diego, CA, USA) and cholic acid (sodium salt) was obtained from Acros Organics (Geel, Belgium). In the off-line mode the background electrolyte consisted of a 150 mM HEPES buffer of pH 6.5 (adjusted with 1 M NaOH at 37 °C). In the in-line mode the background electrolyte consisted of a 150 mM HEPES buffer (pH 6.5 at 37 °C) with 20 mM cholic acid (sodium salt), which was freshly prepared each day. All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through 0.2 μm nylon filters (Alltech, Lokeren, Belgium).

Solutions of SULT1A1 were prepared in 25 mM potassium phosphate buffer, pH 6.5 (at 37 °C), that contained 1.5 mg/ml BSA, 10 mM DTT, and 8 mM $MgCl_2$. The final SULT1A1 solution contained 8.32 U/μl and was stored at −70 °C. Before use, the SULT1A1 solution was thawed rapidly at 37 °C (water bath) and stored on ice. Solutions of the substrate 4-nitrophenol (0.05–20 μM) and of the co-substrate PAPS (115 μM), were prepared in 25 mM potassium phosphate buffer, pH 6.5 (at 37 °C) and were kept in the thermostated storage room of the CE instrument at 5 °C in order to prevent degradation of the PAPS and the enzyme during the analyses.

2.2. CE instrumentation

All experiments were carried out on a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA). On-line detection was performed at 260 and 274 nm with

a diode array detection system. Data collection and peak area analysis were performed by P/ACE MDQ 32 Karat software (Beckman Coulter, version 5.0). Calculation of the Michaelis constant was done by means of SigmaPlot 2001 software (version 7.101). Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 cm (21 cm from the injection side to the detector) ×75 μm i.d. were used. The capillary was thermostated by liquid cooling at 37 °C and the sample tray was thermostated at 5 °C.

3. Results and discussion

3.1. Strategy for the development of a CE-based assay

The human recombinant phenol sulfotransferase (PST) enzyme was studied. Therefore, the thermostable sulfotransferase SULT1A1 that conjugates sulfate to simple phenols such as pNP was purchased. This expensive human recombinant enzyme has only become recently available on the market. The co-substrate PAPS is also expensive, and unstable at room temperature. Therefore, commercial PAPS always contains a certain amount of PAP. The reaction product PAP is a competitive inhibitor [10,11] of the sulfonation reaction, having a K_i of 0.4 μM [12] and should be avoided in the reaction mixture. Firstly, separation conditions were established for the model substrate pNP and the reaction products pNPS and PAP. Secondly, the reaction was carried out outside the capillary in an off-line study. Finally, the previously developed separation step was combined with the enzymatic reaction step in order to develop an in-line method.

3.2. Off-line mode

The reaction mixture contained 50 μl SULT1A1 solution (8.32 U/μl), 100 μl of pNP solution (1 mM), 250 μl of PAPS solution (115 μM) and 600 μl of a 25 mM potassium phosphate buffer (pH 6.5 at 37 °C) that contained 8 mM $MgCl_2$, 1.5 mg/ml BSA and 10 mM DTT. The electrophoresis ran at 5 kV.

The human recombinant SULT1A1 enzyme was incubated at 37 °C with the substrate pNP and the corrected peak area of pNPS was determined at 274 nm. The mixture was directly injected into the capillary by a pressure injection (0.3 p.s.i., 5 s; 1 p.s.i. = 6894.76 Pa). A linear relationship was found when the reaction product was determined at regular time intervals (up to 100 min). A correlation coefficient (r) of 0.9914 was determined for the following regression equation: $y = 2.023x + 177$ with y the corrected peak area of pNPS and x the time expressed in minutes. Unfortunately, there was no direct relationship between the quantity of SULT1A1 and the enzyme activity and moreover, the results were not repeatable (data not shown). A possible explanation is the formation of PAP that strongly inhibits the reaction. PAP is a reaction product, and moreover, commercial PAPS typically contains significant amounts of PAP and other impurities [19] since PAPS is very unstable at room temperature. Therefore, a certain amount of PAP can not be excluded from the reaction mixture.

3.3. In-line mode

3.3.1. Preseparation of PAP from commercial PAPS

One of the advantages of transient engagement EMMA is the electrophoresis process prior to the contact of enzyme and substrate inside the capillary. This process permits the separation of potential interfering substances, which may be advantageous when assaying enzyme activity in crude biological samples [24]. This means that the inhibitor PAP can be electrophoretically separated from the co-substrate PAPS before the injection of enzyme and substrate inside the capillary (and thus before their in-capillary encountering).

PAP and PAPS are well separated from each other in a 150 mM HEPES buffer of pH 6.5 (37 °C) that contained 20 mM cholic acid (sodium salt). The electrophoretic mobility of PAP was determined to be -3.300×10^{-4} $cm^2\ V^{-1}\ s^{-1}$ and that of PAPS -4.029×10^{-4} $cm^2\ V^{-1}\ s^{-1}$. This difference in electrophoretic mobility was determined to be large enough to allow a fast separation of PAP from the PAPS co-substrate, e.g. during 1 min at 15 kV.

3.3.2. In-capillary assay based on EMMA with PAP preseparation step

The co-substrate PAPS was used at a 115 μM concentration. The solutions of SULT1A1, PAPS and pNP were kept in the thermostated storage room of the CE instrument at 5 °C in order to prevent degradation of the PAPS and the enzyme during the analyses. The electrophoresis ran at 10 kV. According to our calculations, about 37 nl of the SULT1A1 solution (8.32 U/μl) was injected in each EMMA analysis. The activity of the SULT1A1 enzyme was determined by measuring the corrected peak area of the product pNPS at 274 nm.

First, the co-substrate PAPS was injected in the capillary by a pressure injection (0.5 p.s.i., 5 s). Then, a 15 kV electric field was applied during 1 min in order to separate the inhibitor PAP from the co-substrate PAPS. Subsequently, the substrate pNP and the enzyme were injected by pressure injections (0.3 p.s.i., 5 s). The injection order of enzyme and substrate was determined experimentally. Then, the electrophoresis ran at 5 kV (1 min) in order to mix the enzyme, substrate and the co-substrate PAPS inside the capillary. In a next step, the voltage was turned off, e.g. for 2 min, in order to let enzyme and substrate react in the absence of the electric field (i.e. zero-potential amplification). Again, this in-capillary incubation protocol was determined experimentally. Finally, the electrophoresis ran at 10 kV in order to sweep the reaction product to the detector for on-line quantitation. The product passes the detector within 4 min. After each injection step, the electrodes (and the capillary ends) were dipped into water to prevent carry over of sample. An

Table 1
Different steps of the EMMA-based reaction for PST activity

Steps	Plugs	Pressure p_i (p.s.i.)	Time t_i (s)	Voltage (kV)
(1) Injection of PAPS	PAPS	0.5	5	/
(2) Preseparation PAP from PAPS	/	/	60	15
(3) Injection of S	pNP	0.3	5	/
(4) Injection of E	SULT1A1	0.3	5	/
(5) Mixing of the compounds and subsequent reaction of E and S	/	/	60	5
(6) Incubation	/	/	120	0
(7) Separation	/	/	300	10

overview of the different steps of the EMMA assay for recombinant PST activity is provided in Table 1. An electropherogram after in-capillary reaction of the SULT1A1 enzyme and its substrate pNP is shown in Fig. 2.

3.3.3. Repeatability of the EMMA-based assay and quantitation of pNP

The within-day repeatability of the in-capillary formation and the subsequent analysis and quantitation of the reaction product pNPS was determined for 12 consecutive analyses of the SULT1A1 solution (8.32 U/μl). A substrate concentration of 20 μM was used and a 115 μM concentration of PAPS. The R.S.D. value determined for the corrected peak area of pNPS was 5.2% ($n = 12$) and the R.S.D. of the peak migration time was 2.2% ($n = 12$). This R.S.D. value is not only affected by the injections of the enzyme and substrate solutions, but also by the enzymatic reaction itself.

The determination of SULT1A1 activity was achieved by measuring the corrected peak area of the product pNPS generated during the in-capillary reaction. Therefore, a strict linear correlation between the pNPS concentration and its corrected peak area is required. The background electrolyte consisted of 150 mM HEPES (pH 6.5 at 37 °C) with 20 mM cholic acid (sodium salt) and a 10 kV potential was applied. A stock solution of pNPS was prepared in a 25 mM potassium phosphate buffer (pH 6.5) and a series was made by diluting the stock solution with the same buffer over a concentration range between 0.78 μM and 12.5 μM. In the regression equation, $y = 0.591x + 0.373$, y represents the corrected peak area and x represents the concentration of pNPS in μM. A correlation coefficient (r) of 0.9932 was observed. The limit of detection (LOD) was found to be 0.05 μM. According to our calculations, 37.2 nl was injected into the capillary (0.3 p.s.i., 5 s), which corresponds to an injected amount of 1.9×10^{-15} mol of pNPS.

3.3.4. Effect of the amount of SULT1A1 enzyme on product formation

The injection of different concentrations of the SULT1A1 enzyme was impossible due to the high cost of the recombinant enzyme. Thus, in order to measure the effect of the amount of enzyme on the reaction, the extent of the sulfation reaction was controlled by injecting longer or shorter enzyme plugs. Fig. 5 (Section 3.3.6) shows an overlay of six electropherograms obtained after in-capillary reaction between pNP and varying amounts of SULT1A1.

As shown in Fig. 3, the sulfonation reaction inside the capillary was proportional to the injection time of SULT1A1.

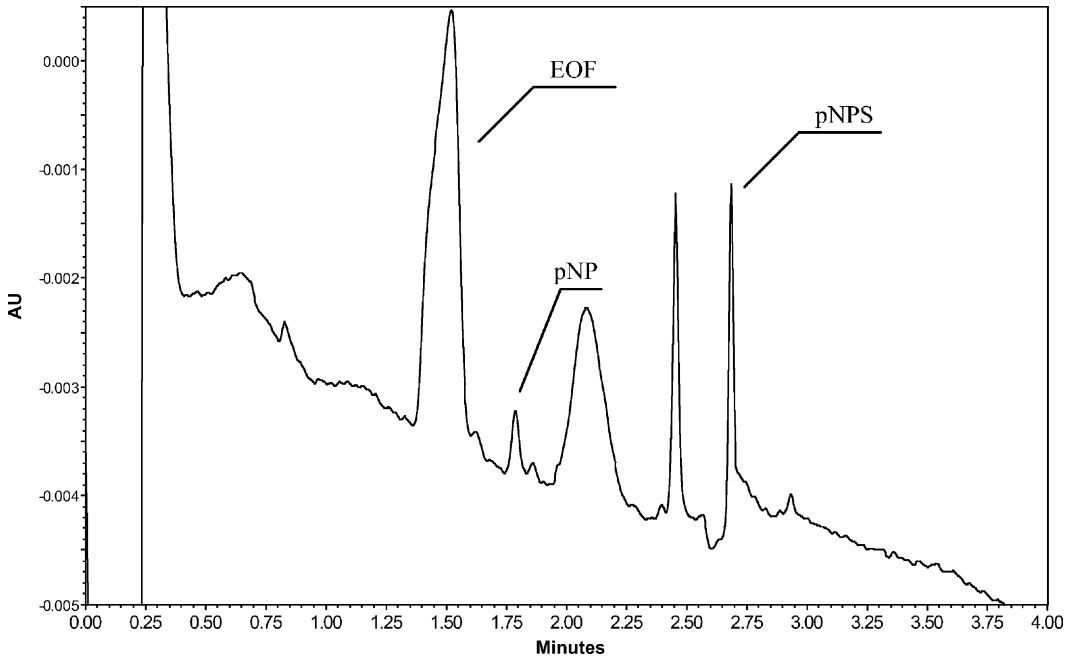

Fig. 2. Electropherogram after in-capillary reaction between the SULT1A1 enzyme and pNP. Incubation at zero potential during 2 min. Concentration of SULT1A1: 8.32 U/μl, pNPS: 115 μM and pNP: 20 μM. CE conditions: run buffer: 150 mM HEPES (pH 6.5) with 20 mM sodium cholate; voltage: 10 kV (45 μA); detection at 274 nm.

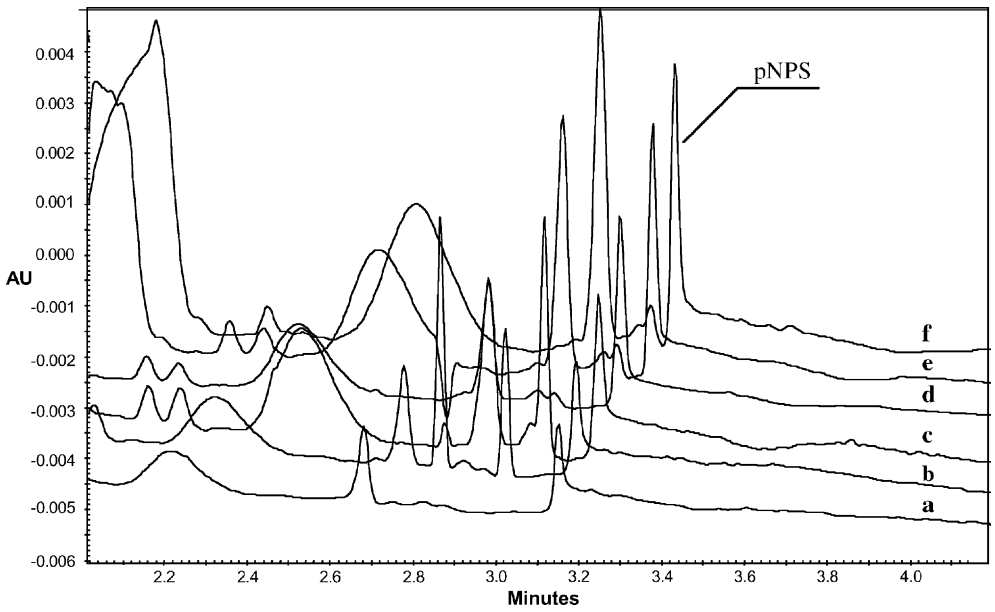

Fig. 3. Overlay of six electropherograms after in-capillary reaction of the SULT1A1 enzyme with increasing enzyme injection times of 2, 3, 4, 5, 6 and 7 s (a, b, c, d, e and f, respectively) for an injection pressure of 0.3 p.s.i. Incubation at zero potential during 2 min. Concentration of SULT1A1: 8.32 U/μl, concentration of pNPS: 115 μM and concentration of pNP: 20 μM. CE conditions: 150 mM HEPES (pH 6.5) with 20 mM sodium cholate; voltage: 10 kV (45 μA); detection at 274 nm.

The injection time of the enzyme plug was increased (injection pressure at 0.3 p.s.i.) from 2 to 7 s and after each run, the amount of pNPS generated during the in-capillary encountering of enzyme and substrate was determined. A linear relationship was found: a correlation coefficient (r) of 0.9984 for the regression equation $y = 285x + 116$ with y the corrected peak area of pNPS and x the injection time of the enzyme plug in seconds.

3.3.5. *Effect of incubation time on the in-capillary SULT1A1 reaction*

According to Michaelis–Menten kinetics, we assume that what is being measured is the initial rate of product formation (v), in such a way that products have not significantly accumulated [35]. Therefore, we expect the amount of product to be linear with time. Fig. 4 shows an overlay of four electropherograms, obtained after in-capillary reaction

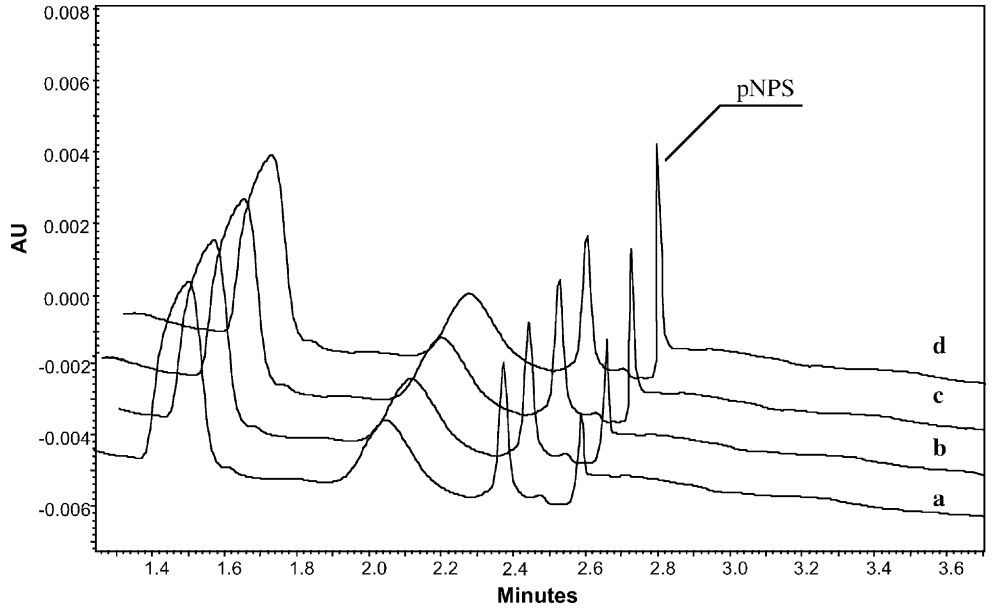

Fig. 4. Overlay of four electropherograms after in-capillary reaction of the SULT1A1 enzyme with increasing in-capillary incubation times of 0.5, 1, 2 and 3 min (a, b, c and d, respectively). Concentration of SULT1A1: 8.32 U/μl, concentration of pNPS: 115 μM and concentration of pNP: 0.25 μM. CE conditions: run buffer: 150 mM HEPES (pH 6.5) with 20 mM sodium cholate, voltage: 10 kV (current 45 μA); detection at 274 nm.

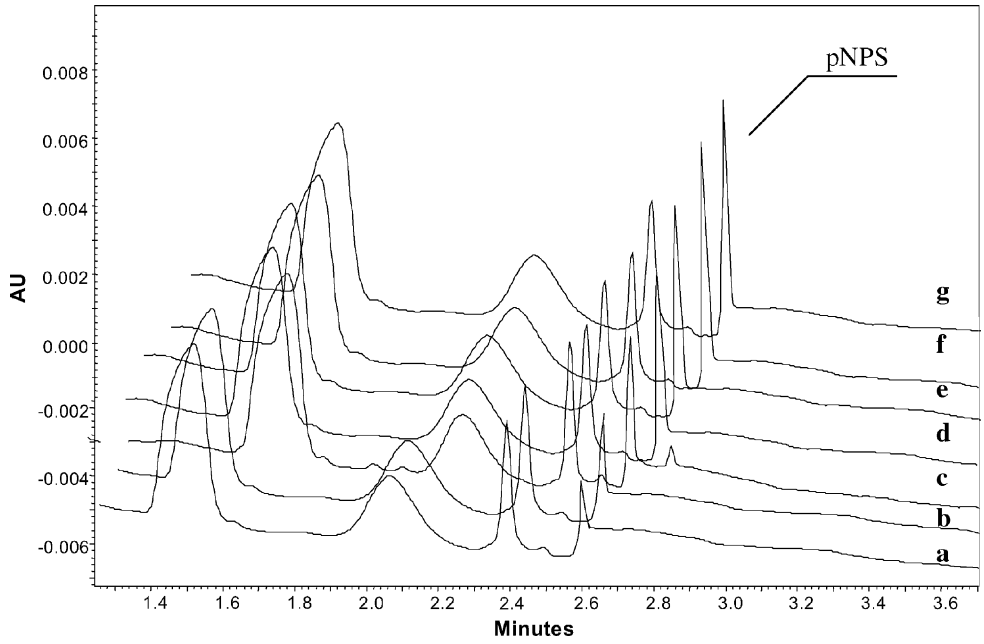

Fig. 5. Overlay of seven electropherograms after in-capillary reaction between SULT1A1 and pNP. Incubation at zero potential during 2 min. Concentration of SULT1A1: 8.32 U/μl, concentration of pNPS: 115 μM and concentration of pNP: 0.05, 0.10, 0.25, 0.50, 1.00, 1.50 and 2.50 μM (a, b, c, d, e, f, g, respectively). CE conditions: see Fig. 5.

during varying times of incubation at zero potential: 0.5, 1, 2 and 3 min.

The corrected peak areas of pNPS were plotted against incubation time and subjected to linear regression analysis. A correlation coefficient (r) of 0.9957 was found, for the regression equation $y = 394x + 275$ with y the corrected peak area of pNPS and x the incubation time in minutes.

3.3.6. Determination of the Michaelis constant

In order to investigate the effect of the pNP concentration on the activity of SULT1A1, seven different pNP concentrations were used, ranging from 0.05 μM to 2.50 μM. The SULT1A1 solution contained 8.32 U/μl and a PAPS solution of 115 μM was used. Fig. 5 shows an overlay of seven electropherograms, in which the substrate concentration was varied from run to run.

The Michaelis–Menten plot that corresponds to the electropherograms in Fig. 4 is shown in Fig. 6A. This line is a fit of the data to the Michaelis–Menten equation. The insert (Fig. 6B) shows the enzyme activity over a substrate concentration range from 0.05 up to 10 μM. Clearly, above a pNP concentration of 2.5 μM, substrate inhibition occurs. This is in agreement with literature findings [8].

The Michaelis–Menten constant for the pNP sulfation was computed from the Lineweaver–Burk plot or the double reciprocal plot. The K_m value for SULT1A1 (8.32 U/μl) was

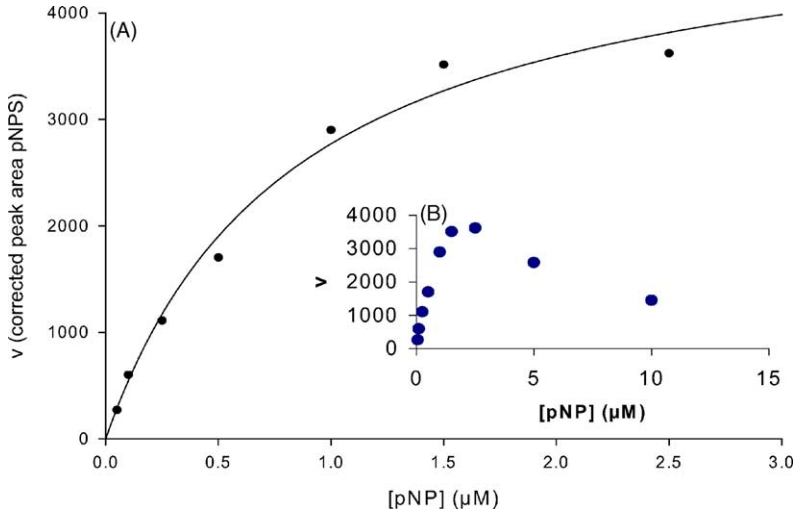

Fig. 6. (A) The Michaelis–Menten plot for the sulfonation of pNP by SULT1A1. The pNP concentration was varied between 0.05 μM and 2.50 μM. Concentration of SULT1A1: 8.32 U/μl and concentration of pNPS: 115 μM. (B) Plot identical to (A) except for two extra pNP concentrations.

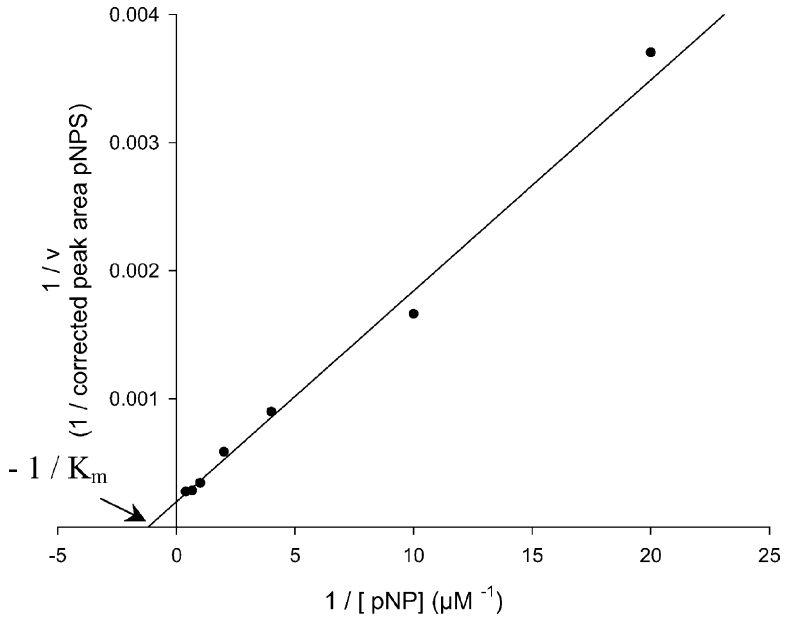

Fig. 7. Lineweaver–Burk plot for a SULT1A1 concentration of 8.32 U/μl. The pNP concentration was varied between 0.05 μM and 2.50 μM, and concentration of pNPS: 115 μM.

determined to be 0.84 ± 0.04 μM. This value corresponds to literature values for the SULT1A1 isoenzyme. Gamage et al. reported a K_m value of 1.0 ± 0.2 μM for the recombinant SULT1A1 enzyme, determined with the substrate pNP [8]. The computed Lineweaver Burk plot of these data is shown in Fig. 7.

4. Conclusion

A new method based on electrophoretically mediated microanalysis (EMMA) was developed for SULT1A1 assay. The kinetic study yielded a K_m value of 0.84 ± 0.04 μM, a value consistent with literature findings. The strong substrate inhibition observed (above a 4-nitrophenol concentration of 2.5 μM) is also in accordance with literature values. Due to the small path length, concentrations in the nanomolar range or lower cannot be determined with CE–UV. In this plug–plug EMMA method, the electrophoresis process prior to the in-capillary contact of enzyme and substrate permitted the preseparation of the inhibitor PAP from the sulfate donor PAPS. Compared to spectrophotometric assays, the EMMA method is rapid, automated and requires only small amounts of the expensive reagents (nanoliter injection volumes). Moreover, the EMMA assay does not need expensive radiolabeled compounds and their disposal.

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