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Research Article

# Kinetic study of cytochrome P450 by capillary electrophoretically mediated microanalysis

An electrophoretically mediated microanalysis method for the determination of CYP3A4 activity using testosterone and nifedipine as substrates was developed. Initially, the enzymatic reaction was performed off-line and the samples were subsequently injected into the capillary by pressure. The CYP3A4 activity was determined by quantitation of the reactant cofactor, NADPH. To further optimize, speed-up and miniaturize the enzyme assay, the enzymatic reaction was performed directly in the capillary, prior to separation and quantitation of the product cofactor, NADP, employing the plug–plug mode of electrophoretically mediated microanalysis. An amplification step was introduced by means of an on-capillary incubation of 15 min, in order to accumulate enough reaction product to detect spectrophotometrically at 260 nm. This setup resulted in a fully automated assay, which can be carried out in less than 35 min. Using the Lineweaver–Burk equation, the Michaelis constants ($K_m$) for the oxidation of testosterone and nifedipine by CYP3A4 were calculated to be $58.6 \pm 8.3$ and $19.1 \pm 2.4\,\mu M$, respectively, which are consistent with off-line assay and previously reported values.

**Keywords:**
CE / Cytochrome P450 enzyme / Electrophoretically mediated microanalysis / NADP / NADPH
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## 1 Introduction

Cytochrome P450 (CYP) enzymes constitute a superfamily of hemoproteins that are responsible for the metabolism of endogenous compounds and the detoxification of xenobiotic molecules. CYP3A4 is one of the most important forms in humans, and mediates the metabolism of around 70% of therapeutic drugs and endogenous compounds. The monooxygenase function of CYPs involves a number of steps but the end reaction is the transfer of one oxygen atom to the substrate (SH) that has a site for oxidation as shown below, where NADPH is needed as a cofactor to donate two electrons to the heme iron of CYPs.

$$NADPH + H^+ + O_2 + SH \overset{CYPs}{\rightarrow} NADP^+ + H_2O + SOH$$

Here, we have focused on the depletion of NADPH and the production of NADP by CYPs as a means to indirectly measure the oxidation of the substrate. In addition, it was taken into consideration that the CYPs, because of their non-specific properties, also consume NADPH in non-productive reactions as shown below

$$NADPH + H^+ + O_2 \overset{CYPs}{\rightarrow} NADP^+ + H_2O_2$$

$$2NADPH + 2H^+ + O_2 \overset{CYPs}{\rightarrow} 2NADP^+ + 2H_2O$$

Therefore, by monitoring the relative depletion of NADPH, or the relative production of NADP, we should be able to determine the metabolic stability of compounds toward CYP-mediated metabolism in a high-throughput format [1].

CE, viewed as an alternative and a complement to liquid chromatography, has recently emerged as a powerful and versatile separation tool for the study of minute quantities of samples. Enzyme assays based on CE have been developed to take advantage of this high-efficiency separation technique, its short analysis time, extremely low sample volume requirements, high sensitivity and ability to utilize several detection methods, such as UV spectrophotometry, laser-induced fluorescence, mass spectrometry, electrochemical detection and chemical luminescence.

Electrophoretically mediated microanalysis (EMMA), first described by Bao and Regnier [2], has been successfully used for on-line enzyme assays. In this technique, the capillary is used as a microbioreactor as well as for the separation of substrates and products. In general, there are two ways to mix the reaction components in a

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**Abbreviations: CYP,** cytochrome P450; **EMMA,** electrophoretically mediated microanalysis

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capillary under electrophoretic conditions. One is the continuous mode of EMMA (long contact mode), in which the capillary is initially completely filled with one of the reactants while the second reactant is introduced as a plug or continuously from the inlet vial. The product continuously forms during the electrophoretic mixing of enzyme and substrate. The other is the plug–plug mode of EMMA (transient format or short contact mode), in which substrate and enzyme are introduced into the capillary as distinct plugs. The reactant with the lower mobility is first injected into the capillary followed directly by the injection of the second, higher mobility reactant. Upon application of an electric potential, these zones mix with each other due to differences in their electrophoretic mobilities and the reaction proceeds during the mixing process. If necessary, the potential can be turned off ( = zero-potential amplification) once the reactant zones have overlapped to allow an increased product yield for reactions. The latter mode is advantageous since fewer amounts of reactants (only a plug) are required as opposed to filling of the capillary and buffer reservoirs. It is worth mentioning that partial filling and at-inlet EMMA have been proposed as alternatives to the classical plug–plug mode [3, 4]. In the partial filling technique, part of the capillary is filled with the optimum buffer for the enzymatic reaction, whereas the rest of the capillary is filled with the background electrolye (BGE) optimal for the separation of substrate and product. In the at-inlet technique, the enzyme and substrate solutions are introduced into the inlet of a capillary either by tandem mode (*i.e.* enzyme solution – substrate solution) or sandwich mode (*i.e.* enzyme solution – substrate solution – enzyme solution). The consecutively injected enzyme QJ;and substrate plugs are mixed by simple diffusion and allowed to react for a given time at the inlet part QJ;of the capillary. Basic principles and applications of the different modes of EMMA have been recently exhaustively reviewed [5–8]. In the present study, our aim is to apply QJ;the plug–plug mode of EMMA to the kinetic study of recombinant human CYP3A4 action on testosterone or nifedipine, based upon relative rates of NADPH depletion or NADP formation.

## 2 Materials and methods

### 2.1 Chemicals

NADPH, NADP and nifedipine were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Testosterone, sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Fluka (Buchs, Switzerland). NaOH was purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Recombinant human CYP3A4, co-expressed with human P450 reductase and human cytochrome b5 in baculovirus–insect cell was purchased from GENTEST (Erembodegem, Belgium).

### 2.2 Instrumentation

All experiments were carried out on an Agilent 3D-CE system (Agilent Technologies, Waldbronn, Germany) with the anode at the injection side and the cathode at the detection side. On-line detection was performed with the diode array detector at 260 nm. Data collection and peak area analysis were performed by Agilent 3D-CE ChemStation. The capillary used was a 48.5 cm × 75 μm id uncoated fused-silica column (Polymicro Technologies, Phoenix, AZ, USA), with a capillary-to-detector distance of 40 cm. The capillary was thermostated by air cooling at 37°C, unless specified otherwise.

### 2.3 CE conditions

The BGE consisted of 25 mM sodium phosphate buffer at pH 6.6 and was prepared by adding 25 mM sodium dihydrogen phosphate to 25 mM disodium hydrogen phosphate under continuous stirring, until pH 6.6 was reached. The pH of the solutions was measured with a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland). The electrophoresis was carried out at 18 kV. The typical running current was 80 μA. Before use, a new capillary was treated with 1 M NaOH, 0.1 M NaOH and Milli-Q water (Millipore, Milford, MA, USA) for 10 min each at 50°C. At the beginning of each day, the capillary was conditioned by a wash cycle starting with a 5-min rinse with 0.1 M NaOH, followed by a 5-min rinse with Milli-Q water and a 10-min rinse with running buffer. Between runs, the capillary was rinsed with acetonitrile, Milli-Q water, 0.1 M NaOH, Milli-Q water and running buffer for 3, 1, 1, 1 and 3 min, respectively. At the end of each day, the capillary was rinsed with Milli-Q water for 5 min. All solutions were prepared with Milli-Q water and filtered through 0.2 μm RC membrane filters (Schleicher-Schuell MicroScience GmbH, Dassel, Germany).

## 3 Results and discussion

### 3.1 Off-line approach

In a preliminary study, an off-line (precapillary) method for analyzing the enzymatic reaction of CYP3A4 was developed. A similar setup as used in our previous drug metabolism-screening studies was adopted in this work; however, slight modifications were performed. Owing to the main purpose of this work – kinetic study of recombinant human CYP3A4, based upon relative rates of NADPH depletion or NADP formation – testosterone and nifedipine were chosen as preferred substrates because they are suitable probes to assess CYP3A4 activity. Moreover, to reduce the degradation rate of NADPH as much as possible, EDTA was not included in the incubation mixture [1]. Incubation mixtures contained 4 nM CYP3A4, 20 μM NADPH and one of the

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probe substrates (testosterone: 6.25, 12.5, 25, 50, 100, 200 μM; nifedipine: 3.125, 6.25, 12.5, 25, 50, 100 μM) in 10 mM sodium phosphate buffer at pH 7.4. The buffer was prepared by adding 10 mM sodium dihydrogen phosphate to 10 mM disodium hydrogen phosphate under continuous stirring, until pH 7.4 was reached. Each concentration point was analyzed in triplicate. Probe substrates were first dissolved in methanol and then diluted with 10 mM sodium phosphate buffer at pH 7.4. The final concentration of methanol in the incubation mixture was under 1% v/v. Before use, the enzyme was thawed rapidly at 37°C and stored on ice. NADPH, testosterone and nifedipine solutions were used shortly after preparation. Besides, since nifedipine is extremely photosensitive [9], solutions were always protected from light. The reaction mixture, in a final volume of 100 μL, was preincubated for 5 min at 37°C in an Eppendorf Thermomixer (Hamburg, Germany) before the reaction was initiated by adding NADPH, and then incubated for 20 min. The incubation was stopped by adding 100 μL of ice-cold methanol (final composition 1:1, aqueous/methanol). Samples were analyzed by a CE method previously developed for drug metabolism-screening studies, using an ARGOS 250B fluorescence detector (Flux Instruments, Basel, Switzerland) [1]. The separation conditions were as follows: capillary, 80.5 cm (75 μm id, 64 cm effective length); 25 mM sodium phosphate buffer (pH 8.8); 28 kV (80 μA) applied voltage; fluorescence detection, Exc: 310 nm, Em: 418 nm; capillary temperature: 25°C. For both testosterone and nifedipine, the CYP3A4 activity was determined by monitoring the depletion of NADPH, and the reactions proceeded linearly with time up to 20 min (correlation coefficients at least 0.9817). The depletion of NADPH was corrected for the contribution of the blank (incubation without testosterone and nifedipine) (Fig. 1). With the Lineweaver–Burk equation, the Michaelis–Menten parameters $K_m$ and $V_{max}$ for the oxidation of testosterone and nifedipine were calculated, based upon relative rates of NADPH depletion. The data are presented in Table 1. The $K_m$ values were consistent with previously reported values (testosterone: range from 52 to 94 μM; nifedipine: range from 5.1 to 47 μM) (http://www.fda.gov/cder/Guidance/6695dft.htm). To further optimize, speed-up and miniaturize the enzyme assay, in the next round of experiments, the main effort went into development of a method with which the enzymatic reaction could be performed directly within the capillary.

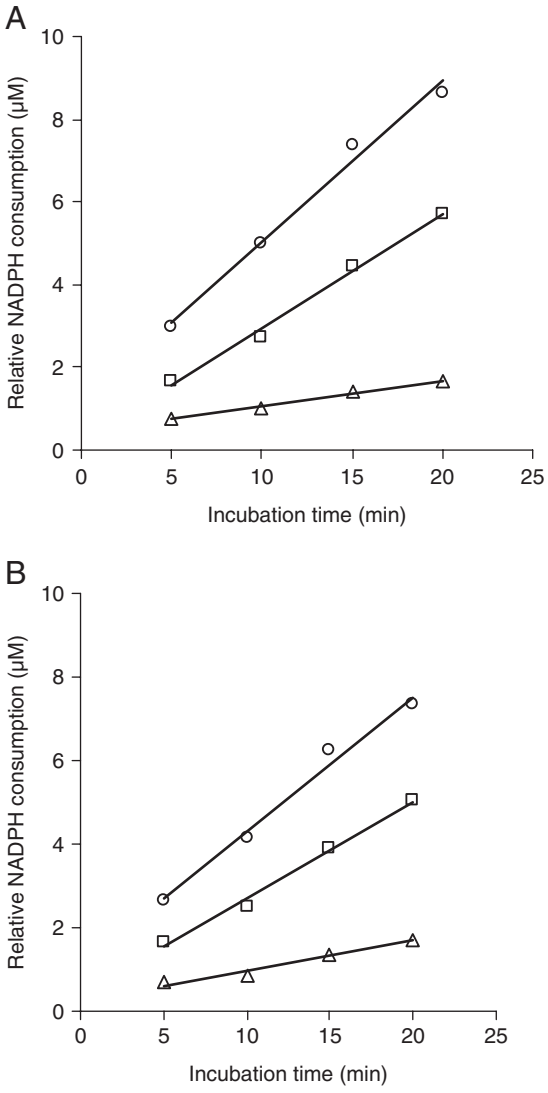


**Figure 1.** Depletion of NADPH with time in the incubation mixture containing 4 nM CYP3A4 and (A) testosterone at 6.25 μM (triangles), 50 μM (squares), 200 μM (circles); (B) nifedipine at 3.125 μM (triangles), 25 μM (squares), 100 μM (circles). The data points were fitted to a linear function. For incubation and CE conditions, see Section 3.1.

**Table 1.** Michaelis–Menten parameters $K_m$ and $V_{max}$ for standard CYP3A4 substrates obtained with off-line and on-line CE methods

| | Testosterone | | Nifedipine | |
|---|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (nmol/min/nmol) | $K_m$ (μM) | $V_{max}$ (nmol/min/nmol) |
| Off-line CE method | 51.5 ± 4.9 | 151.7 ± 5.6 | 13.9 ± 0.6 | 129.8 ± 1.8 |
| On-line CE method | 58.6 ± 8.3 | 159.6 ± 9.1 | 19.1 ± 2.4 | 139.4 ± 6.2 |

## 3.2 On-line approach: EMMA

### 3.2.1 Plug–plug reaction and zero-potential amplification

To succeed in developing an EMMA-based method for kinetic study of CYP3A4, electrophoretic conditions are required that permit both enzymatic reaction and subsequent separation and detection to be performed entirely on-capillary. Based on the off-line separation conditions, initially three items were modified to perform enzymatic reactions on-line. First, the capillary temperature was

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modified from 25 to 37°C to increase the reaction rate. Second, the at-inlet technique was applied which allowed the successively introduced enzyme and substrate plugs to react for an adequate period of time in the capillary inlet part. Third, the partial filling technique was used to ensure the enzymatic reaction in a suitable incubation buffer. However, the enzymatic reaction rate was still much lower compared with the off-line approach. Therefore, in the following work, a great deal of investigation was undertaken to maximize the effectiveness of on-line mixing of reactants and to optimize separation conditions.

With the intention of mixing the substrate, enzyme and cofactor electrophoretically instead of by simple diffusion in the capillary, migration times of all reactants must be determined to calculate their apparent mobilities. UV detection was applied instead of fluorescence detection since none of the reactants except NADPH showed fluorescence. Suitable separation conditions were then investigated with a mixture of 20 nM deactivated CYP3A4, 100 μM NADPH, 100 μM NADP and 200 μM testosterone (or 100 μM nifedipine). Different capillary lengths (48.5, 64.5 and 80.5 cm), BGE pHs (from 6 to 9) and voltages (14, 16, 18, 20, 22 kV) were tested. The migration order was testosterone (or nifedipine), CYP3A4, NADP and NADPH. Testosterone and nifedipine came together with the EOF. In all instances, all peaks were baseline separated. However, the most favorable choice was the method comprising a 48.5-cm capillary, BGE pH 6.6 and 18 kV applied voltage because it provided shorter analysis time and higher sensitivities. It is worth mentioning that the peak height of NADP and NADPH is very sensitive to BGE pH, and even small changes in pH lead to noticeable and important changes in the peak height of NADP and NADPH as shown in Fig. 2. The NADPH peak reaches the highest peak height at pH 6.6. On the other hand, pH values affect the peak height of NADP differently. The peak height of NADP gradually increases with the pH from 6.0 to 7.0. In conclusion, BGE pH 6.6 was finally selected.

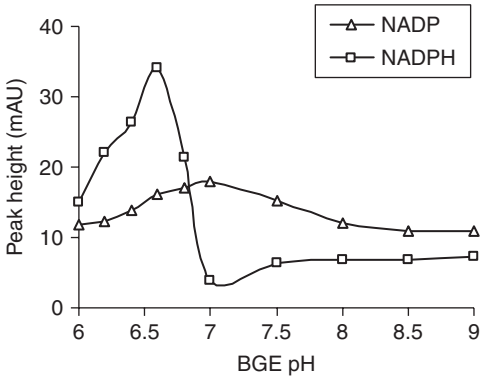


**Figure 2.** Effect of BGE pH on the peak height of NADP and NADPH. CE conditions – BGE: 25 mM sodium phosphate buffer at pH from 6.0 to 9.0; applied voltage: 18 kV; UV 260 nm; capillary temperature: 37°C.

An important consideration when using the plug–plug EMMA technique is to ensure accurate reactant plug overlap. Accurate plug overlap is important because the greatest analytical response will be observed when the amount of time the mixing potential is applied results in complete overlap of the reactant plugs. Two parameters, the linear length of the reactant plugs and the apparent mobilities of the reactants in each plug, must be considered to determine the appropriate time a given potential should be applied to ensure accurate plug overlap. The initial studies utilized the rinsing procedure, 0.1 M NaOH, Milli-Q water and running buffer for 1, 1 and 3 min, respectively. With this rinsing procedure, the run-to-run variation of migration times (apparent mobilities) was too large to perform repeatable on-capillary CYP3A4 activity assay. This was expected since proteins tend to be adsorbed onto the capillary surface (negatively charged surface), producing variations in migration behavior. In order to overcome this problem, different rinsing procedures were tested. Different combinations of washing steps including SDS, methanol, acetonitrile, $H_3PO_4$, NaOH and Milli-Q water were tried. Only one rinsing procedure could successfully maintain the repeatability of the electropherograms, and was therefore chosen: 3 min with acetonitrile, 1 min with Milli-Q water, 1 min with 0.1 M NaOH, 1 min with Milli-Q water, and 3 min with the running buffer.

In the plug–plug reaction approach, a plug of slowly migrating NADPH is hydrodynamically injected into the capillary followed by the injections of faster migrating enzyme, and substrate. After each injection step, the capillary ends were dipped into water to prevent carryover of the sample. Upon the application of 5 kV potential, the three plugs will interpenetrate. Knowledge of the difference in electrophoretic mobilities and injected plug lengths (see Table 2) allows calculating the time at which the three plugs largely overlap each other. Harmon *et al.* [10] derived a general equation, neglecting the effects of longitudinal diffusion:

$$t_{\text{merge}} = \delta/(\mu_1 - \mu_2)E$$

**Table 2.** Injection parameters, plug lengths and mobilities for CYP3A4 activity assay by EMMA

| Injection order and compounds | Pressure, $P_i$ (mbar) | Time, $t_i$ (s) | Length, $l_i$ (cm) | $\mu_{app}$ ($cm^2 V^{-1} s^{-1}$) | $\mu_{EOF}$ ($cm^2 V^{-1} s^{-1}$) |
|---|---|---|---|---|---|
| (1) NADPH | 50 | 4 | 1.046 | $3.508 \times 10^{-4}$ | |
| (2) CYP3A4 | 50 | 4 | 1.046 | $5.162 \times 10^{-4}$ | |
| (3) 25 mM phosphate buffer (pH 7.4) | 25 | 4 | 0.523 | | $7.712 \times 10^{-4}$ |
| (4) Testosterone or nifedipine | 50 | 4 | 1.046 | $7.712 \times 10^{-4}$ | |

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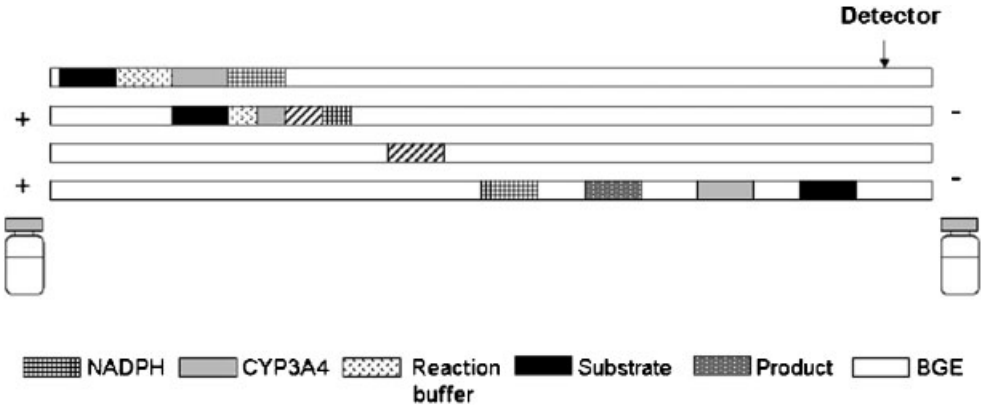


**Figure 3.** Schematic illustration of CYP3A4 activity assay by EMMA using the plug–plug mode. (i) The NADPH solution is introduced into the capillary by pressure injection, followed by CYP3A4 solution, an intermediate buffer plug and substrate solution; (ii) the voltage is turned on and the NADPH, CYP3A4 and substrate plug start to migrate, the latter one being faster than the former one; (iii) the voltage is turned off when the three plugs have merged and the products are generated; (iv) after a certain incubation time, the different compounds are swept to the detector upon application of the voltage.

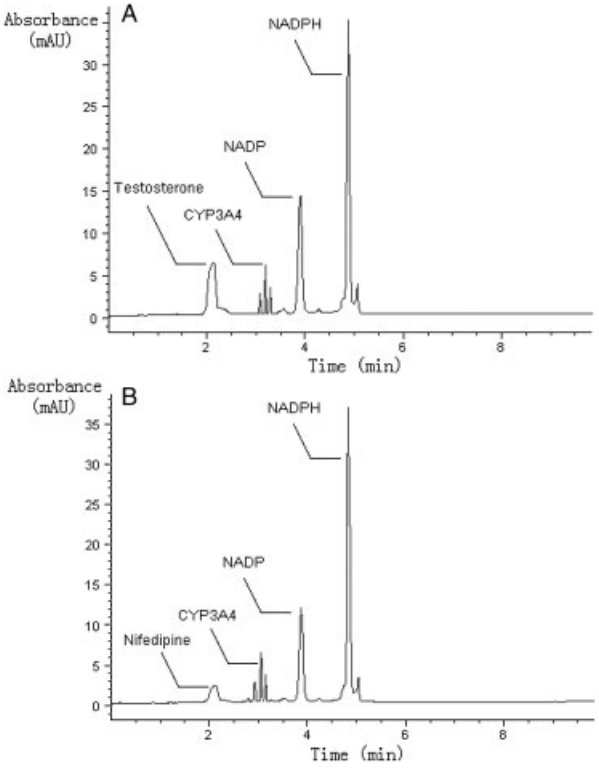


**Figure 4.** Electropherograms after on-capillary reactions. Enzymatic reaction conditions – NADPH: 200 μM; CYP3A4: 20 nM; (A) testosterone: 200 μM or (B) nifedipine: 100 μM; on-capillary incubation: 15 min. Injection parameters and order of injection: see Table 2. CE conditions – BGE: 25 mM sodium phosphate buffer at pH 6.6; applied voltage: 18 kV; UV 260 nm; capillary temperature: 37°C.

where $(\mu_1 - \mu_2)$ is the difference in electrophoretic mobility between the two analytes and $\delta$ is a specific distance within the capillary. For the enzyme and NADPH, $\delta$ could be defined as the distance between the leading edge of the enzyme plug and the leading edge of the NADPH plug, resulting in a distance of 1.046 cm. The time at which two zones merge ($t_{merge1}$) could be calculated to be 61.3 s. For the substrate and enzyme, δ could be defined as the distance between the leading edge of the substrate plug and the leading edge of the enzyme plug, resulting in a distance of 1.569 cm. The time at which two zones merge ($t_{merge2}$) could be calculated to be 59.7 s. The point where the three zones merge can be estimated as the mean of $t_{merge1}$ and $t_{merge2}$, to be 60.5 s. Turning off the voltage at this point allows the enzymatic reaction to continue in the absence of an electric field. This built-in amplification step was necessary to accumulate enough product to detect spectrophotometrically. A schematic overview of the different assay steps is given in Fig. 3. Figure 4 shows the electropherograms after on-capillary reaction between NADPH, CYP3A4 and substrate with a 15-min incubation period. The metabolites of testosterone and nifedipine migrate together with the EOF.

### 3.2.2 CYP3A4 activity assay

#### 3.2.2.1 Linearity of the on-line CE method

For all enzyme concentrations tested (in the range from 5 to 20 nM), the on-line reaction proceeded linearly with time up to 30 min (correlation coefficient was at least 0.9828). As shown in Fig. 5A, for a CYP3A4 concentration of 20 nM, the on-capillary reaction proceeded linearly with time for both substrates, with correlation coefficients of 0.9965 and 0.9988 for testosterone and nifedipine, respectively. Under the circumstances of 15 min on-line incubation, linear calibration plots were obtained with different CYP3A4 concentrations for both substrates. The correlation coefficients were 0.9968 and 0.9967 for testosterone and nifedipine, respectively (Fig. 5B). Finally, an incubation time of 15 min and an enzyme concentration of 20 nM were chosen as

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a compromise between short analysis time and enough reaction product to detect for the following kinetic analysis

#### 3.2.2.2 CYP3A4 kinetics

For many enzyme-catalyzed reactions, the relation between initial reaction velocity ($V$) and substrate concentration [$S$] can be described by the Michaelis–Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where $V_{max}$ is the maximum reaction velocity and $K_m$ is the Michaelis constant, the substrate concentration at half the maximum velocity. By inversion of this equation, the Lineweaver–Burk equation is obtained, which describes a linear relation between $1/V$ and $1/[S]$

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

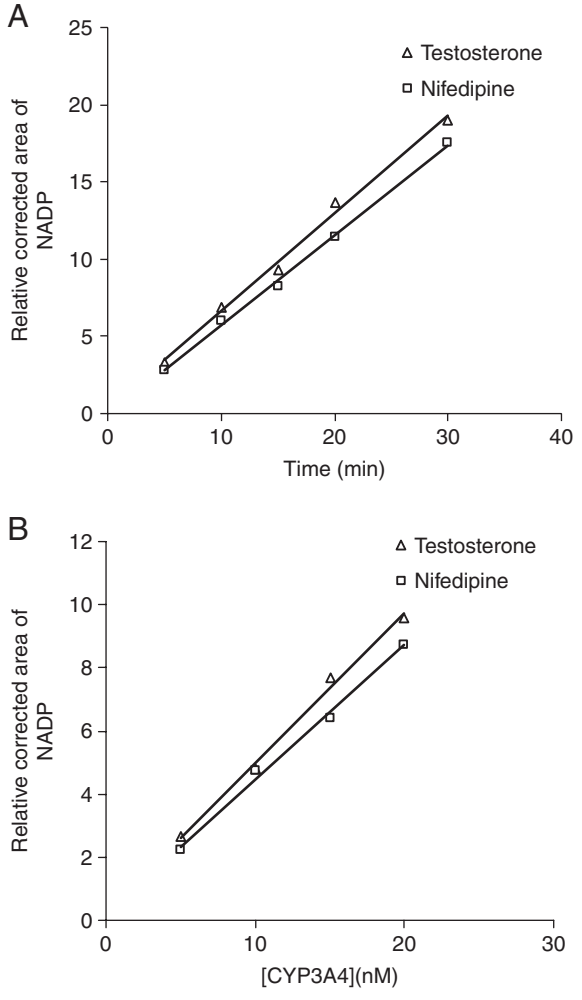


**Figure 5.** Linearity of the on-line CYP3A4 activity assay (A) with progress of time; (B) as a function of enzyme concentration. The formation of NADP is corrected for the contribution of the blank (incubation without testosterone or nifedipine). Enzymatic reaction conditions: see Fig. 4. Injection parameters and order of injection: see Table 2. CE conditions: see Fig. 4.

With the on-line CYP3A4 activity assay and the Lineweaver–Burk equation, the Michaelis–Menten parameters for the oxidation of testosterone and nifedipine were determined, based upon relative rates of NADP formation (see Table 1). The Michaelis–Menten plots are presented in Fig. 6. No significant differences were observed between on-line and off-line reactions for $K_m$ and $V_{max}$ values for testosterone and nifedipine

#### 3.2.2.3 Quantitation of NADP: Validation

In this on-line approach, quantitation of CYP3A4 activity was achieved by measuring the corrected peak area of NADP generated during the enzymatic reaction. Therefore, a strict linear correlation between NADP concentration and its corrected peak area is necessary. A stock solution of NADP was prepared by dissolving the compound in 10 mM sodium phosphate buffer at pH 7.4. A series of standards of NADP was obtained from the stock solution by dilution with the same buffer to cover a concentration range from 1.5 to 200 μM. The calibration curve was obtained by plotting the corrected peak area of the NADP peak against its

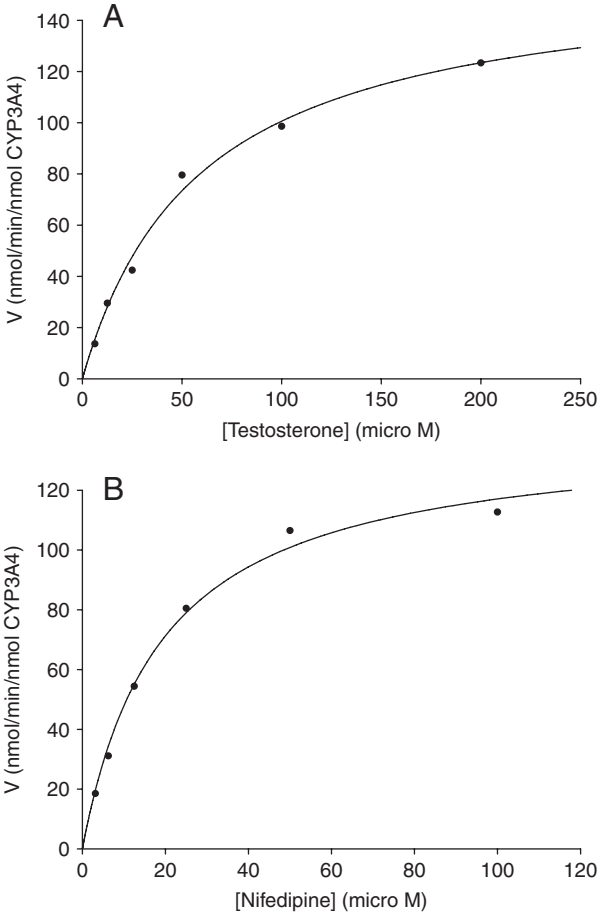


**Figure 6.** Michaelis–Menten plots for the oxidation of (A) testosterone and (B) nifedipine obtained from on-line CE experiments. Enzymatic reaction conditions: see Fig. 4. Injection parameters and order of injection: see Table 2. CE conditions: see Fig. 4.

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concentration. A correlation coefficient of 0.9997 ($n = 3$) was calculated, the regression equation being $y = 0.253x + 0.15$. In the equation, $y$ stands for the corrected peak area and $x$ represents the concentration in μM. The lower control of 1.5 μM showed an RSD of 2.2% ($n = 6$) for the corrected area and an RSD of 0.6% for the migration time. The higher control of 200 μM showed an RSD of 1.9% ($n = 6$) for the corrected area and an RSD of 0.3% for the migration time. The LOD and LOQ of NADP were found to be 0.5 and 1.5 μM. According to our calculations, 46.2 nL was injected into the capillary (50 mbar, 4 s), which corresponds to the injected amount of 17.7 and 53.0 pg.

## 4 Conclusions

In conclusion, we have developed an EMMA method for the determination of CYP3A4 activity using the substrates, testosterone and nifedipine. The CYP3A4 activity has been determined by quantitation of reactant and product cofactors, NADPH and NADP. The on-line approach uses capillary as the reaction vessel with the dramatic reduction in consumption of enzyme and substrate and combines the required assay steps in a fully automatic assay. Our method should be theoretically applicable to every compound that migrates with the EOF, on condition, of course, that the enzymatic reaction product does not co-migrate with NADP or NADPH.

*The authors have declared no conflict of interest.*

## 5 References

[1] Zhang, J., Lou, Y., Hoogmartens, J., Van Schepdael, A., *Electrophoresis* 2006, *27*, 4827–4835.

[2] Bao, J. M., Regnier, F. E., *J. Chromatogr.* 1992, *608*, 217–224.

[3] Van Dyck, S., Van Schepdael, A., Hoogmartens, J., *Electrophoresis* 2001, *22*, 1436–1442.

[4] Van Dyck, S., Vissers, S., Van Schepdael, A., Hoogmartens, J., *J. Chromatogr. A* 2003, *986*, 303–311.

[5] Van Dyck, S., Kaale, E., Nováková, S., Glatz, Z. *et al.*, *Electrophoresis* 2003, *24*, 3868–3878.

[6] Nováková, S., Van Dyck, S., Van Schepdael, A., Hoogmartens, J., Glatz, Z., *J. Chromatogr. A* 2004, *1032*, 173–184.

[7] Zhang, J., Hoogmartens, J., Van Schepdael, A., *Electrophoresis* 2006, *27*, 25–43.

[8] Zhang, J., Hoogmartens, J., Van Schepdael, A., *Electrophoresis* 2008, *29*, 56–65.

[9] Laik Ali, S., in: Florey, K. (Ed.), *Analytical Profiles of Drug Substances*, vol. 18, Academic Press, London, 1989, pp. 221–288.

[10] Harmon, B. J., Patterson, D. H., Regnier, F. E., *Anal. Chem.* 1993, *65*, 2655–2662.

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