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Kinetic study of CYP3A4 activity on verapamil by capillary electrophoresis

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Abstract

The use of capillary electrophoresis (CE) for the determination of CYP3A4 activity with verapamil as a substrate was investigated. CYP3A4 activity was determined by the quantitation of the product, norverapamil, based on separation by CE. The separation conditions were as follows: capillary, 80.5 cm (75 μm i.d., 72 cm effective length); 50 mM sodium phosphate buffer (pH 8.8); 20 kV (100 μA) applied voltage; UV detection at 200 nm; capillary temperature, 25 °C. With the developed CYP3A4 activity assay and the Lineweaver–Burk equation, the Michaelis–Menten parameters K_m and V_{max} for formation of norverapamil from verapamil in the presence of CYP3A4 were determined and were 22.8 ± 2.5 μM and 7.67 ± 0.26 pmol/min/pmol (or 983 pmol/min/mg) CYP3A4, respectively.

Keywords: Kinetic study; Michaelis–Menten analysis; Enzyme; CYP3A4

1. Introduction

The cytochrome P450 (CYP) enzyme system, a very large group of enzymes encoded by the P450 gene superfamily, is one of the widely studied topics in drug development [1–3]. CYP enzymes participate in the metabolism of a variety of naturally occurring and foreign compounds, by reactions requiring NADPH and O_2. The diversity of reactions catalyzed and the extensive substrate specificity, render CYP enzymes one of the most versatile known catalysts. There are more than 57 human cytochrome P450 enzymes, out of which only five, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are the major metabolizing enzymes. The sole fact that CYP3A4 is responsible for the majority of drug metabolism, being also the most abundant form in human liver, makes CYP3A4 the most important CYP enzyme [4–8].

Oxidative metabolism involves the conversion of a substance into a more polar species by the insertion or incorporation of atmospheric oxygen into the molecule. CYP enzymes mediate a number of different biochemical oxidative reactions, such as aliphatic hydroxylation, aromatic hydroxylation, *N*-dealkylation, *O*-dealkylation, *S*-dealkylation, etc. The final result of each of these biotransformations is to produce a polar metabolite that can be eliminated in urine or feces. Often the final step is conjugation of the metabolite at a polar site with a moiety, such as glucuronic acid (i.e. phase II metabolism). The resulting conjugated product is water soluble and can be eliminated in the urine [8].

Many studies have focused on the determination of CYP activity by substrate assays. However, most of them used liquid chromatography (LC) as analytical technique. Only few studies used capillary electrophoresis (CE) for the drug metabolism investigations. These studies focused on the analysis of incubation mixtures of drugs or model substances (coumarine, phenol, quinidine, mephenytoin, methadone, methaqualone, pyrazoloacridine, itraconazole) with human liver microsomes or cDNA-expressed human CYPs, by looking at the formation of metabolites [9–18]. Verapamil belongs to the pharmacological class of calcium channel blockers. It is widely used for the treatment of cardiovascular diseases

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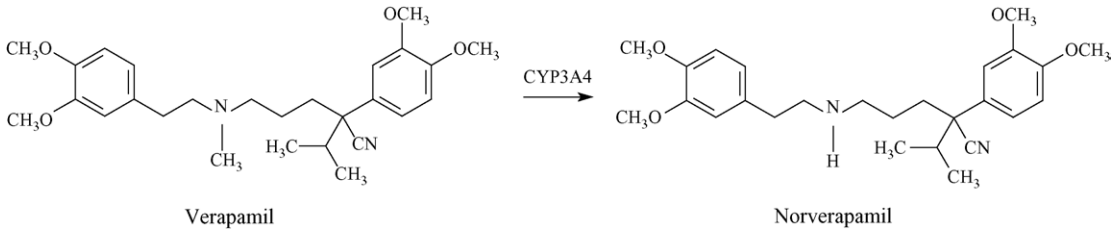


Fig. 1. Conversion of verapamil to norverapamil.

like hypertension, angina pectoris and cardiac arrhythmias [19]. The substance is a tertiary amine with one asymmetric carbon and it can thus exist in two enantiomeric forms. However, verapamil is administered as a racemate despite documentation of stereogenic differences in pharmacological potency as well as in pharmacokinetics [20,21]. The drug undergoes extensive and variable hepatic metabolism in the human body. The major metabolic pathways are *N*-demethylation, *N*-dealkylation, and *O*-demethylation. CYP3A4 plays a major role in verapamil *N*-demethylation (Fig. 1). Table 1 summarizes articles dealing with the kinetic study of verapamil *N*-demethylation by CYP3A4. In this study, the metabolism of verapamil to norverapamil was investigated with cDNA-expressed human CYP3A4 by using CE.

2. Materials and methods

2.1. CE 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 (DAD) at 200 nm. Data collection and peak area analysis were performed by Agilent 3D-CE ChemStation. The capillary used was a 80.5 cm × 75 μm i.d. uncoated fused silica column (Polymicro Technologies, Phoenix, AZ, USA), with a capillary-to-detector distance of 72 cm. The capillary was thermostated by air-cooling at 25°C.

2.2. CE conditions

The separation buffer consisted of 50 mM sodium phosphate buffer (Acros Organics, Geel, Belgium) at pH 8.8 and was prepared by adding 50 mM sodium dihydrogen phosphate to 50 mM disodium hydrogen phosphate under continuous stirring, until pH 8.8 was reached. The pH of the solutions was adjusted with a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland). The electrophoresis was carried out at 20 kV. Typical running current was 100 μA. Before use, a new capillary was treated with 1 M NaOH (BDH Laboratory Supplies, Poole, UK) for 2 h: a rinsing step of 10 min, followed by a waiting period, where the capillary was left to stand into contact with the NaOH solution. At the beginning of each day, the capillary was conditioned by a wash cycle starting with a 10 min rinse with 0.1 M NaOH, followed by a 5 min rinse with Milli-Q water (Millipore, Milford, MA, USA) and a 10 min rinse with running buffer. Between runs, the capillary was rinsed with 0.1 M NaOH, Milli-Q water and running buffer for 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 nylon filters (Alltech, Lokeren, Belgium).

2.3. Quantitative analysis of norverapamil

A standard solution of norverapamil (Sigma, Steinheim, Germany) was prepared by dissolving the compound in 50% (v/v) MeOH in water. Suitable dilutions were made to prepare samples of a series of desired concentrations from the standard solution with the same solvent. The norverapamil solution was introduced into the capillary by a pressure injection (50 mbar, 10 s). After the analysis, the corrected peak area of norverapamil was plotted against its concentration.

2.4. Final incubation conditions

Verapamil was purchased from Fluka (Bornem, Belgium) and NADPH was purchased from Sigma (Steinheim,

Table 1
Michaelis–Menten parameters of verapamil *N*-demethylation by CYP3A4 (formation of norverapamil)

Quantitation method	K_m (μM)		V_{max} (pmol/min/mg)		Reference
	S-Verapamil	*R*-Verapamil	*S*-Verapamil	*R*-Verapamil	
LC	52.8 ± 41.3	63.8 ± 50.4	809 ± 611	817 ± 701	[22]
LC	117 ± 25	127 ± 32	7.9 ± 0.6[a]	6.9 ± 0.7[a]	[23]
LC–MS	4.85 ± 0.55	4.24 ± 1.33	995 ± 61	486 ± 105	[24]
LC–MS	21.8		3320[a]		[25]

[a] V_{max} expressed in pmol/min/pmol CYP3A4.

Germany). Verapamil and NADPH were dissolved in 10 mM sodium phosphate buffer pH 7.4.

Recombinant human CYP3A4, coexpressed with human P450 reductase and human cytochrome b5 in baculovirus–insect cell, was purchased from GENTEST (Erembodegem, Belgium).

CYP3A4 concentration was 200 pmol/ml in 10 mM sodium phosphate buffer pH 7.4. The latter 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. The incubation volume was 100 μl, in a water bath at 37 °C, with an assay stop time of 20 min via the addition of 100 μl of ice-cold MeOH (final composition 1:1, aqueous/MeOH). Samples were filtered through 4 mm filters with 0.45 μm PTFE membranes (PALL Gelman Laboratory, Portsmouth, UK) into 200 μl snap polypropylene vials (Agilent Technologies).

2.5. Protein linearity assay

Individual incubations were carried out with CYP3A4 and 200 μM verapamil (at near V_{max} condition) in 10 mM sodium phosphate buffer pH 7.4, which was pre-warmed for 5 min at 37 °C. The CYP3A4 concentrations used were 25, 50, 100, 150, and 200 pmol/ml. Each concentration point was analyzed in triplicate. The reaction was initiated by the addition of 1 mM NADPH, and was incubated at 37 °C with stop times of 30 min. Aliquots were quenched by addition to an equal volume of ice-cold methanol.

2.6. Time linearity assay

The conditions described above were repeated but with 200 pmol/ml CYP3A4. The stop times were 5, 10, 15, 20, 30, and 40 min. Each time point was analyzed in triplicate.

2.7. Determination of K_m and V_{max} for formation of norverapamil

Incubations were carried out with six substrate concentrations, 10, 20, 40, 60, 80 and 100 μM, and with stop times of 20 min, to determine the Michaelis–Menten parameters K_m and V_{max} of norverapamil formation. Each concentration point was analyzed in triplicate. Experimental conditions were as above.

3. Results and discussion

3.1. Separation of reaction mixture

The capillary electrophoretic conditions for the analysis of CYP3A4 reaction mixture were established by investigating different sodium phosphate buffer concentrations with different pH values. Phosphate buffer was chosen due to the sensitivity of norverapamil. The spectrum of norverapamil shows three absorption maxima in 50 mM phosphate buffer at pH 8.8: a first one at 200 nm, a second one at 230 nm, and a third one at 278 nm. The wavelength chosen for the enzyme assay was 200 nm. High sensitivity can often be realized by using low-UV detection wavelength. Detection at the low wavelength necessitates the use of minimally absorbing running buffers, since high background absorbance increases baseline noise and decreases signal-to-noise ratio. Phosphate and borate are useful in this respect. Many biological buffers such as *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 3-(cyclohexylamino)-1-propanesulfonic acid (CHAPS), and tris(hydroxymethyl)aminomethane (Tris) are inappropriate for use below about 215 nm. In addition, the incubation was performed in phosphate buffer medium and the use of additional buffer reagents would introduce other co-ions. Then, the sensitivity would be reduced. 50 mM sodium phosphate buffer at pH 8.8 provides the most satisfying separation of verapamil and norverapamil (Fig. 2). This buffer pH is close to the pK_a of verapamil and norverapamil (∼9), and provides the biggest changes in the selectivity versus pH because the change in charge with pH is most rapid at this point. At pH 8.8, verapamil and norverapamil were positively charged and they came before the electro-osmotic flow (EOF). While NADP and NADPH were negatively charged, since their pK_a values are 6–7, and they came after the EOF. Temperature is rarely used in systematic method development, maybe because the temperature can be varied within a rather small range. It can become troublesome to use CE below 20 °C due to condensation of water that can cause electrical breakdowns. CE methods using temperatures above 50 °C are rarely reported. However, since temperature is an influential parameter, considerable effort is needed to control it. It is sensible to limit the electrical power, even when working with a thermostated instrument, because it is not possible to have the whole capillary thermostated, due to the part that dips into the buffer and the part in the detector cell. A temperature of 37 °C was tried in the preliminary study so

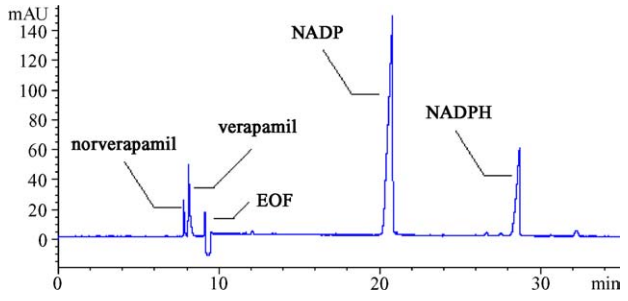


Fig. 2. Electropherogram after incubation. Incubation conditions: CYP3A4, 200 pmol/ml; verapamil, 100 μM; NADPH, 1 mM; stop time, 20 min. CE conditions: background electrolyte (BGE), 50 mM sodium phosphate buffer (pH 8.8); applied voltage, 20 kV; current, 100 μA; UV detection at 200 nm; capillary cassette temperature, 25 °C.

that we could perform reaction incubation in the capillary in the following work. But verapamil and norverapamil were not really separated. The best separation was obtained at 25 °C. Electrical current dropped from 120 to 100 μA, and electrical power decreased from 2.4 to 2.0 W (with 50 mM sodium phosphate buffer and 20 kV).

However, major variations in the migration times and some spikes occurred whenever the microsomal mixture was injected (migration times increased dramatically). In addition, the capillary gradually got clogged and the current dropped to zero. Acetonitrile and SDS, as well as NaOH, Milli-Q water and running buffer, were tried in the rinse procedure to remove the water insoluble compounds and proteins. This rinse procedure stabilized the current, but the EOF still decreased from run to run. This implies that the capillary wall changes with time, most likely because of the adsorption of compounds present in the CYP3A4 supersomes. These compounds are not removed even after extreme rinse conditions. Good repeatability of migration times and corrected peak areas were obtained when we added the filtration procedure before injection.

3.2. Quantitative analysis of norverapamil

The determination of CYP3A4 activity is achieved by measuring the corrected peak area of norverapamil formed during the enzymatic reaction. Therefore, a strict linear correlation between norverapamil concentration and the corrected peak area is necessary: a correlation coefficient (r) of 0.9998 ($n = 3$) for six norverapamil concentrations in the range from 1 to 100 μM was obtained. In the regression equation, $y = 0.579x + 0.05$, x represents the concentration of norverapamil in μM and y represents the corrected peak area of norverapamil (scaled arbitrarily). The lower control 1 μM showed a R.S.D. of 2.6% ($n = 6$) for the corrected area and a R.S.D. of 0.2% ($n = 6$) for the migration time. The higher control 100 μM showed a R.S.D. of 1.3% ($n = 6$) for the corrected area and a R.S.D. of 1.6% ($n = 6$) for the migration time. The limit of detection (LOD) corresponds to a signal-to-noise ratio of three and was found to be 0.3 μM of norverapamil. The limit of quantitation (LOQ) corresponds to a signal-to-noise ratio of 10 and was found to be 1 μM of norverapamil. According to our calculations, 53.1 nl was injected into the capillary (50 mbar, 10 s), which corresponds to an injected amount of 7.0 pg (LOD) or 23.4 pg (LOQ) of norverapamil.

3.3. Protein and time linearity assay

Incubation experiments were performed at five protein concentrations and metabolite production was linear with respect to CYP3A4 concentration up to at least 200 pmol/ml (Fig. 3) ($r = 0.9998$). Time linearity experiments were performed over 40 min. Reactions were linear for approximately 20 min (Fig. 4) ($r = 0.9925$).

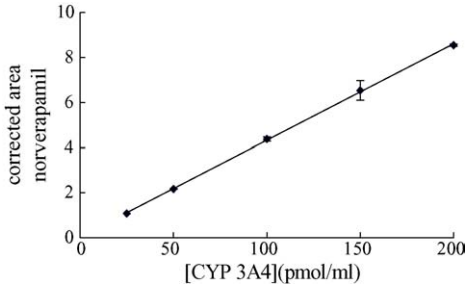


Fig. 3. Linearity of the off-line activity assay in function of enzyme concentration. Concentration of CYP3A4 varied from 25 to 200 pmol/ml; verapamil, 200 μM; NADPH, 1 mM; stop time, 30 min. CE conditions are as in Fig. 2.

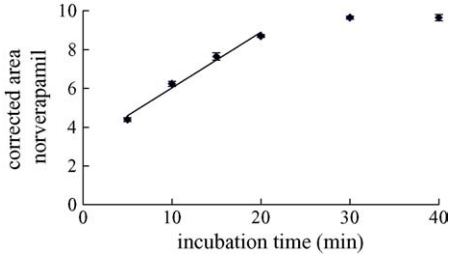


Fig. 4. Linearity of the off-line activity assay in function of time. Incubation times varied from 5 to 40 min; CYP3A4, 200 pmol/ml; verapamil, 200 μM; NADPH, 1 mM. CE conditions are as in Fig. 2.

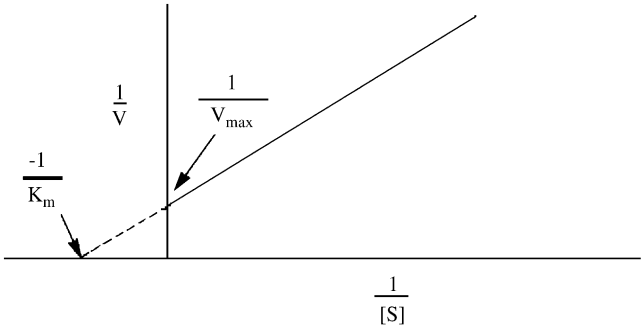
3.4. Determination of K_m and V_{max} parameters

For many enzyme-catalyzed reactions, the relation between initial reaction velocity (V) and substrate concentration [S], can be described by the Michaelis–Menten equation [26]:

$$V = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]}$$

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 $1/[\mathrm{S}]$.

$$\frac{1}{V} = \frac{K_{\mathrm{m}}}{V_{\max}}\frac{1}{[\mathrm{S}]} + \frac{1}{V_{\max}}.$$



A typical Lineweaver–Burk plot appears to the left. Note that V_{max} is derived from the y-intercept, and K_m can be derived either from the slope, or from extrapolating the line to the negative x-axis.

Enzyme action can be modulated by a number of factors. One of the most fundamental factors affecting enzyme activity is substrate concentration. To obtain accurate Michaelis–Menten parameters, substrate concentrations should span a range of at least $1/3K_m$ to $3K_m$ with at least six concentrations [27]. Enzyme activity is strongly affected by two additional factors, temperature and pH. Enzymes operate at an optimal temperature, and deviation from this temperature produces a reduction in activity. Most metabolic enzymes function with an optimal temperature, near body temperature, of 37 °C. Enzymes also operate at a pH optimum, and deviation from this pH produces a reduction in activity. In this work, baculovirus–insect cell expressed human CYP3A4 incubation was performed at 37 °C, pH 7.4 according to the manufacture's instructions. The K_m and V_{max} for the CYP3A4 reaction were estimated by linear regression from Lineweaver–Burk plots by using six verapamil concentrations ranging from 10 to 100 μM. Each concentration was analyzed three times. Fig. 5A shows the Michaelis–Menten plot obtained. The double reciprocal plot of these data is represented in Fig. 5B. The K_m and V_{max} were computed and were 22.8 ± 2.5 μM and 7.67 ± 0.26 pmol/min/pmol (or 983 pmol/min/mg) CYP3A4, respectively. Previously reported K_m values range from 4.24 to 127 μM [22–25]. The Michaelis constant for CYP3A4 with verapamil as a substrate cannot be compared exactly with the K_m values from other studies, since K_m values vary depending on the origin of the investigated CYP3A4 enzyme and the exact assay conditions. Nevertheless, the K_m of 21.8 μM reported by von Richter et al. [25], using liquid chromatography–mass spectrometry (LC–MS) as the supporting analytical technology, is similar to the K_m reported here.

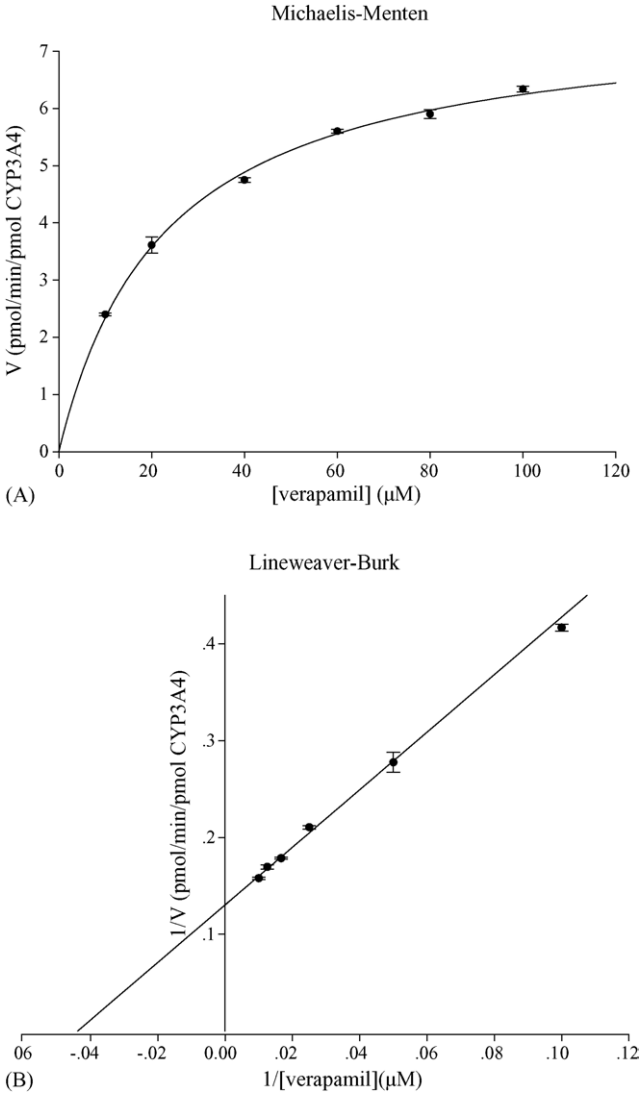


Fig. 5. (A) Michaelis–Menten plot: reaction velocity V plotted vs. verapamil concentration. (B) Corresponding linear Lineweaver–Burk plot, where $1/V$ is plotted vs. 1/[verapamil]. The correlation coefficient (r) of the double reciprocal plot (or Lineweaver–Burk plot) was calculated to be 0.9994. Concentration of CYP3A4, 200 pmol/ml; NADPH, 1 mM; stop time, 20 min. CE conditions are as in Fig. 2.

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