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Journal of Chromatography A, 1082 (2005) 235–239
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Kinetic study of cytochrome P450 3A4 activity on warfarin by capillary electrophoresis with fluorescence detection

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Received 24 January 2005; received in revised form 6 May 2005; accepted 24 May 2005

Abstract

The use of capillary electrophoresis (CE) for the determination of cytochrome P450 3A4 (CYP3A4) activity with *R*-warfarin as a substrate was investigated. CYP3A4 activity was determined by the quantitation of the product, 10-hydroxywarfarin, based on separation by CE. The separation conditions were as follows: capillary, 80.5 cm (75 μm i.d., 60 cm effective length); 50 mM sodium phosphate buffer (pH 6.5); 23 kV (90 μA) applied voltage; fluorescence detection, excitation wavelength, 310 nm, emission wavelength, 418 nm; capillary temperature, 37 °C. With the developed CYP3A4 activity assay and the Lineweaver–Burk equation, the Michaelis–Menten parameters K_m and V_{max} for formation of 10-hydroxywarfarin from *R*-warfarin in the presence of CYP3A4 were calculated to be 166 ± 12 μM and 713 ± 14 pmol/min/nmol (or 91.4 pmol/min/mg) CYP3A4, respectively.

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

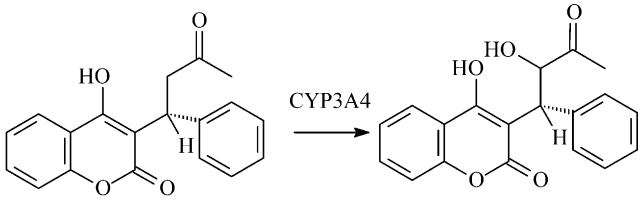
1. Introduction

Cytochrome P450s are the most important enzymes involved in the oxidative metabolism of drugs and, therefore, are of considerable interest in both discovery and development in the pharmaceutical industry [1–5]. 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.

$$NADPH + H^+ + O_2 + SH \xrightarrow{CYP450} NADP^+ + H_2O + SOH$$

Although the list of human CYP genes currently stands at 57 [2], a relatively small subset is involved in the metabolism of drugs. Approximately 90% of oxidative drug metabolism can be attributed to CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 [1,6]. Considerable emphasis is placed on the roles of these CYPs in the pharmaceutical industry, in terms of assays for contributions to metabolism of new drugs and inhibition. CYP3A4 is the human enzyme known to be involved in the metabolism of the largest number of medications [6–8].

Warfarin is an anticoagulant which acts by inhibiting Vitamin K-dependent coagulation factors. It occurs as a racemic mixture of the *R* and *S* enantiomers that are differentially metabolized by human CYPs to a series of monohydroxylated metabolites. 10-hydroxylation of *R*-warfarin has been demonstrated to be an intermediate affinity reaction catalyzed by CYP3A4 [9].



R-WARFARIN 10-HYDROXYWARFARIN

CYP3A4 is the predominant CYP in human liver and this, together with its relatively high capacity for 10-hydroxylation

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0021-9673/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.05.071

of *R*-warfarin, implies that it is an important participant in human *R*-warfarin metabolism. This drug also offers the advantage of native fluorescence with an excitation maximum of 310 nm, which makes it ideal for use with sensitive fluorescence detection [10,11].

Capillary electrophoresis (CE) is a relatively new separation technique. The mechanisms responsible for separation in CE are different from those in chromatography, and thus can offer orthogonal, complementary analyses. As a result of its versatility, high separation efficiency, minimal sample requirements, speed of analysis and low consumable expense, CE is likely to play an increasingly significant role in drug metabolism investigations [12–17]. In this work, a kinetic analysis of CYP3A4 activity on *R*-warfarin was performed 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 detection side. On-line detection was performed with the ARGOS 250B fluorescence detector (Flux Instruments, Basel, Switzerland) at an excitation wavelength of 310 nm and an emission wavelength of 418 nm. The photomultiplier tube (PMT) was maintained at 750 V. Data collection and peak area analysis were performed by an electronic integrator HP 3393A (Hewlett-Packard, Avondale, PA, USA). 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 60 cm. The capillary was thermostated by air cooling at 37 °C.

2.2. CE conditions

The separation buffer consisted of 50 mM sodium phosphate buffer at pH 6.5 and was prepared by adding 50 mM disodium hydrogen phosphate (Acros Organics, Geel, Belgium) to 50 mM sodium dihydrogen phosphate (Acros) under continuous stirring, until pH 6.5 was reached. The electrophoresis was carried out at 23 kV. Typical running current was 90 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 10-hydroxywarfarin

A standard solution of 10-hydroxywarfarin (Ultrafine, Manchester, UK) 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 10-hydroxywarfarin solution was introduced into the capillary by a pressure injection (50 mbar, 10 s). After the analysis, the corrected peak area of 10-hydroxywarfarin was plotted against its concentration.

2.4. Final incubation conditions

R-warfarin was purchased from Ultrafine (Manchester, UK) and NADPH was purchased from Sigma (Steinheim, Germany). *R*-warfarin was dissolved in 4 mM NaOH and NADPH was dissolved in 10 mM sodium phosphate buffer pH 7.4.

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

CYP3A4 concentration was 200 pmol/ml in 10 mM sodium phosphate buffer pH 7.4. 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, Waldbronn, Germany).

2.5. Protein linearity assay

Individual incubations were carried out with CYP3A4 and 2 mM *R*-warfarin (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, 200 pmol/ml. Each concentration 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, 40 min. Each time point was analyzed in triplicate.

2.7. Determination of K_m and V_{max} for formation of 10-hydroxywarfarin

Incubations were carried out with six substrate concentrations, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM, and with stop times of 20 min, to determine the Michaelis–Menten parameters K_m and V_{max} for 10-hydroxywarfarin. Each concentration 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 CYP3A4 reaction mixture were established by investigating different buffer compositions and concentrations, and with different pH values. Simple buffers such as phosphate, Tris–HCl, Tris–H_3PO_4 and borate were tested, but no satisfactory separation between the substrate and the product was obtained at pH 7.4. This pH was chosen in the beginning since it is the optimum pH for the CYP3A4 enzymatic reaction. Then buffers with different pH values were tested. Phosphate buffer at pH 6.5 provides a most satisfying separation. 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

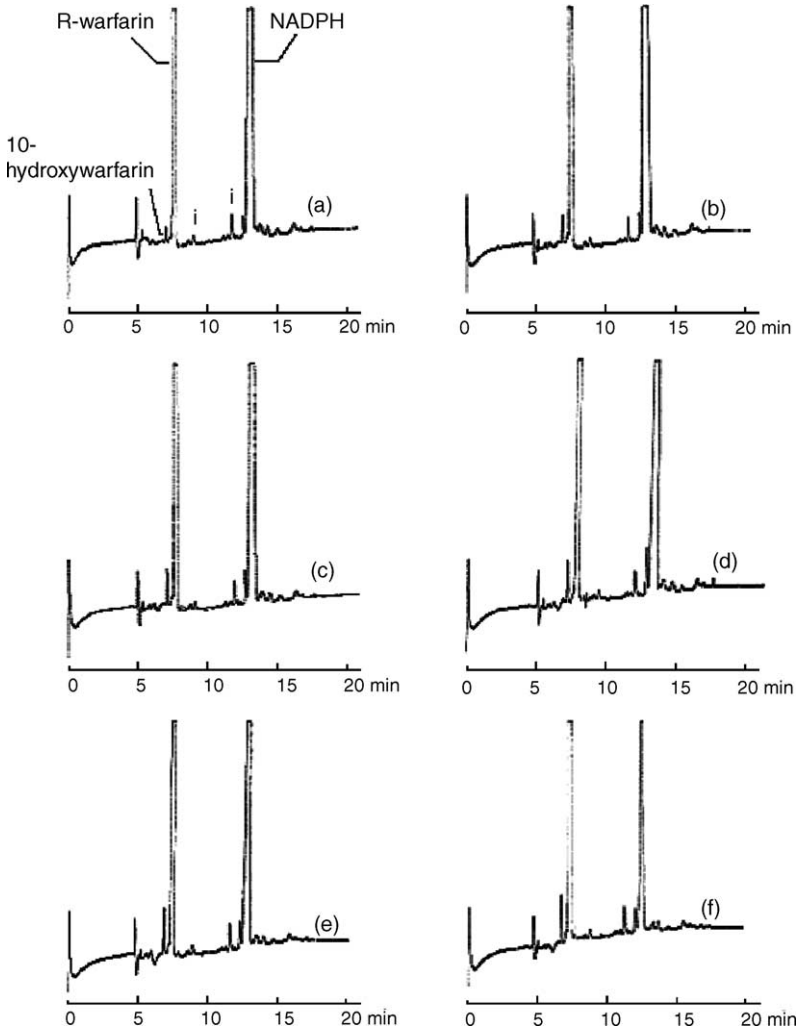

Fig. 1. Set of six electropherograms after incubation. Incubation conditions: CYP3A4: 200 pmol/ml; *R*-warfarin: 2 mM; NADPH: 1 mM; stop time: (a) 5 min, (b) 10 min, (c) 15 min, (d) 20 min, (e) 30 min, (f) 40 min. CE conditions: background electrolyte (BGE): 50 mM sodium phosphate buffer (pH 6.5); applied voltage: 23 kV; current: 90 μA; detection at Exc: 310 nm, Em: 418 nm, PMT: 750 V; capillary cassette temperature: 37°C. i: impurity present in *R*-warfarin.

times and corrected peak areas were obtained when we added the filtration procedure before injection. Fig. 1 shows a set of six electropherograms after increasing incubation times.

3.2. Quantitative analysis of 10-hydroxywarfarin

The determination of CYP3A4 activity is achieved by measuring the corrected peak area of 10-hydroxywarfarin formed during the enzymatic reaction. Therefore, a strict linear correlation between 10-hydroxywarfarin concentration and the corrected peak area is necessary: a correlation coefficient (r) of 0.9995 ($n = 3$) for six 10-hydroxywarfarin concentrations in the range from 0.15 μM to 5 μM was obtained. In the regression equation, $y = 6093x - 336$, x represents the concentration of 10-hydroxywarfarin in μM and y represents the corrected peak area of 10-hydroxywarfarin (scaled arbitrarily). The lower control 0.15 μM showed a RSD of 3.3% ($n = 6$) for the corrected area and a RSD of 1.4% ($n = 6$) for the migration time. The higher control 5 μM showed a RSD of 2.2% ($n = 6$) for the corrected area and a RSD of 0.6% ($n = 6$) for the migration time. The limit of detection (LOD) corresponds to a signal-to-noise ratio of 3 and was found to be 0.04 μM of 10-hydroxywarfarin. The limit of quantitation (LOQ) corresponds to a signal-to-noise ratio of 10 and was found to be 0.14 μM of 10-hydroxywarfarin. According to our calculations, 80.1 nl was injected into the capillary (50 mbar, 10 s), which corresponds to an injected amount of 1.04 pg (LOD) or 3.64 pg (LOQ) of 10-hydroxywarfarin. Since the kinetic results of the CYP3A4 reaction were obtained in a different matrix and after filtration through a PTFE membrane, we investigated the recovery of 10-hydroxywarfarin. Instead of *R*-warfarin, 2.5 μM 10-hydroxywarfarin was added to the incubation mixture that contained 200 pmol/ml CYP3A4, 1 mM NADPH in 10 mM sodium phosphate buffer, pH 7.4, followed by the addition of MeOH up to the final composition of 1:1, aqueous/MeOH, and filtration. The recovery was 100.8% compared with 12.5 μM 10-hydroxywarfarin in 50% (v/v) MeOH in water.

3.3. Protein and time linearity assay

Incubation experiments were performed at five protein concentrations in the range from 25 to 200 pmol/ml and metabolite production was linear with respect to CYP3A4 concentration up to at least 200 pmol/ml ($r = 0.9980$). In the regression equation, $y = 54x - 316$, x represents protein concentration in pmol/ml and y represents the corrected peak area of 10-hydroxywarfarin (scaled arbitrarily). Time linearity experiments were performed over 40 min. Reactions were linear for approximately 20 min ($r = 0.9837$). In the regression equation, $y = 338x + 1463$, x represents the incubation time and y represents the corrected peak area of 10-hydroxywarfarin (scaled arbitrarily).

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 [18]:

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

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 plot is obtained, which describes a linear relation between $1/v$ and $1/[S]$.

The K_{m} and $V_{\max}$ for the CYP3A4 reaction were estimated by linear regression from Lineweaver–Burk plots

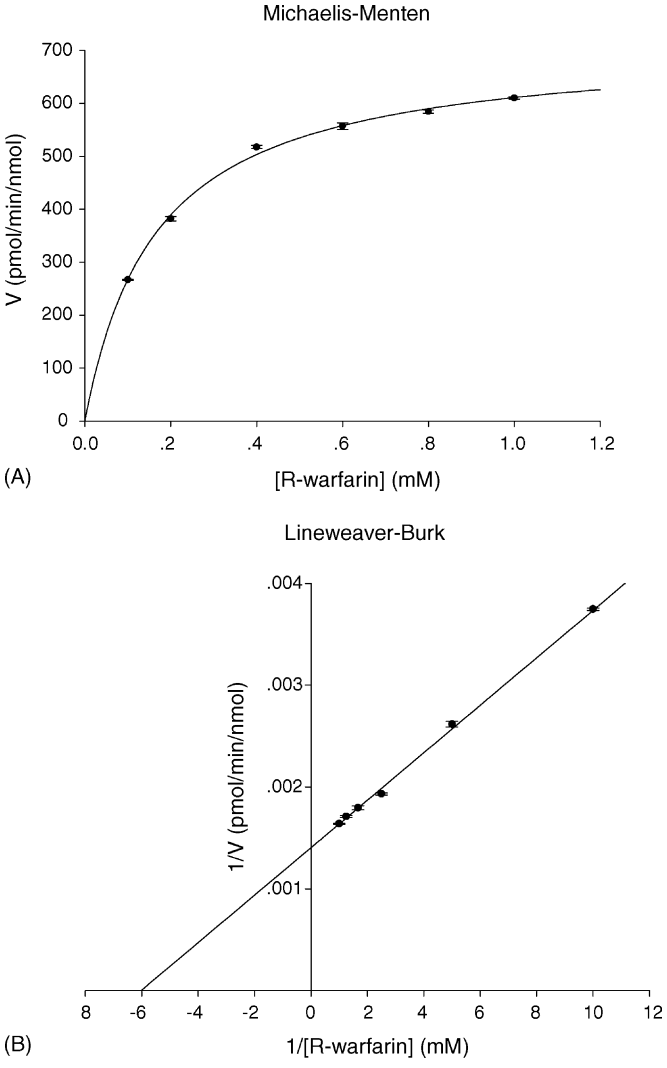

Fig. 2. (A) Michaelis–Menten plot: reaction velocity v plotted vs. *R*-warfarin concentration. (B) Corresponding linear Lineweaver–Burk plot where $1/v$ is plotted versus $1/[R\text{-warfarin}]$. The correlation coefficient (r) of the Lineweaver–Burk plot was calculated to be 0.9992. Concentration of CYP3A4: 200 pmol/ml; NADPH: 1 mM; stop time: 20 min. CE conditions are as in Fig. 1.

by using six *R*-warfarin concentrations ranging from 0.1 to 1 mM. Each concentration was analyzed in triplicate. Fig. 2A shows the Michaelis–Menten plot obtained. The double reciprocal plot of these data is represented in Fig. 2B. The K_m and V_{max} were computed and were 166 ± 12 μM and 713 ± 14 pmol/min/nmol CYP3A4 (or 91.4 pmol/min/mg), respectively. The results are quite in accordance with the previous studies by Ngui et al. [19] using liquid chromatography (LC) as the supporting analytical technology.

4. Conclusions

A CE method has been successfully developed and established for determination of warfarin and its CYP3A4 metabolite 10-hydroxywarfarin. Michaelis–Menten constants were determined and the results obtained were quite comparable to those from previous studies. The velocity of the reaction could also be monitored by the decrease of the co-enzyme NADPH. Our results show that CE with fluorescence detection has sensitivity, broad dynamic range and short separation time allowing the study of drug metabolism.

Acknowledgments

The Fund for Scientific Research (Flanders) is acknowledged for financial support. The authors would like to thank Ron Gilissen (Johnson & Johnson Pharmaceutical Research and Development) for fruitful discussions.

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