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A direct injection capillary electrophoretic technique for miniaturized high-throughput metabolic screening of the CYP 3A4 enzyme using quinidine as a probe

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Abstract

A capillary electrophoresis (CE) method has been developed for the determination of quinidine sulfate (QS) and (3*S*)-3-hydroxyquinidine (3-OHQ) by direct injection of microsomal incubation mixtures. 3-OHQ is the CYP 3A4 metabolite of QS and hence useful for metabolism screening studies. The method was validated analytically and tested for its effectiveness as a metabolic inhibition model. A linear calibration was found to provide the best fit for the standard curve with an r of 0.9966 and all residuals less than 12%. The percent relative standard deviations (RSDs) of the two controls, 2 and 8 μg/ml were 5.27 and 2.90% and the percent difference from normal (% DFN) were -12.58 and -0.31% respectively. The limit of quantitation (LOQ) in the incubation matrix was 0.5 μg/ml. 3-OHQ formation complied with Michaelis–Menten kinetics and the mean values $\pm$ S.D. of K_m and V_{max} were 36.98 ± 4.62 μg/ml and 321.39 ± 3.88 ng/mg/h respectively. Preliminary inhibition studies suggest that the method has adequate sensitivity to screen for high and medium inhibitors of the CYP 3A4 isozyme. The lack of sample preparation coupled with the small sample size capability of CE would enable the direct injection technique to aid in miniaturized high-throughput screening.

1. Introduction

Screening for drug metabolism is a very significant step in the process of developing a novel drug. It has primarily three important uses, the first is to find metabolically stable compounds. The rationale behind this approach is that compounds resistant to metabolism are likely to have low hepatic clearances in vivo and exhibit a potentially higher bioavailability. The second to predict drug–drug interactions, which is of particular relevance for the cytochrome (CYP) 3A family of enzymes and also for the polymorphically expressed CYP forms. The third is to study potential drug induction and inhibition of enzyme systems [1,2].

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In recent years drug metabolism has undergone a significant evolution as a result of advances in our understanding of different human drug-metabolizing systems and the development of analytical methods that can be coupled to automated handling systems. Two additional emerging areas in drug development, genomics and combinatorial chemistry have increased the throughput of drug discovery, leading to the development of high throughput screening (HTS) techniques [3]. HTS is a process by which a large number of compounds can be tested in an automated fashion for activity as inhibitors or activators. A technique should have the capability to separate complex mixtures, possess reduced time of analysis, low sample volume requirements and the feasibility for automation to be suitably adapted for HTS [4].

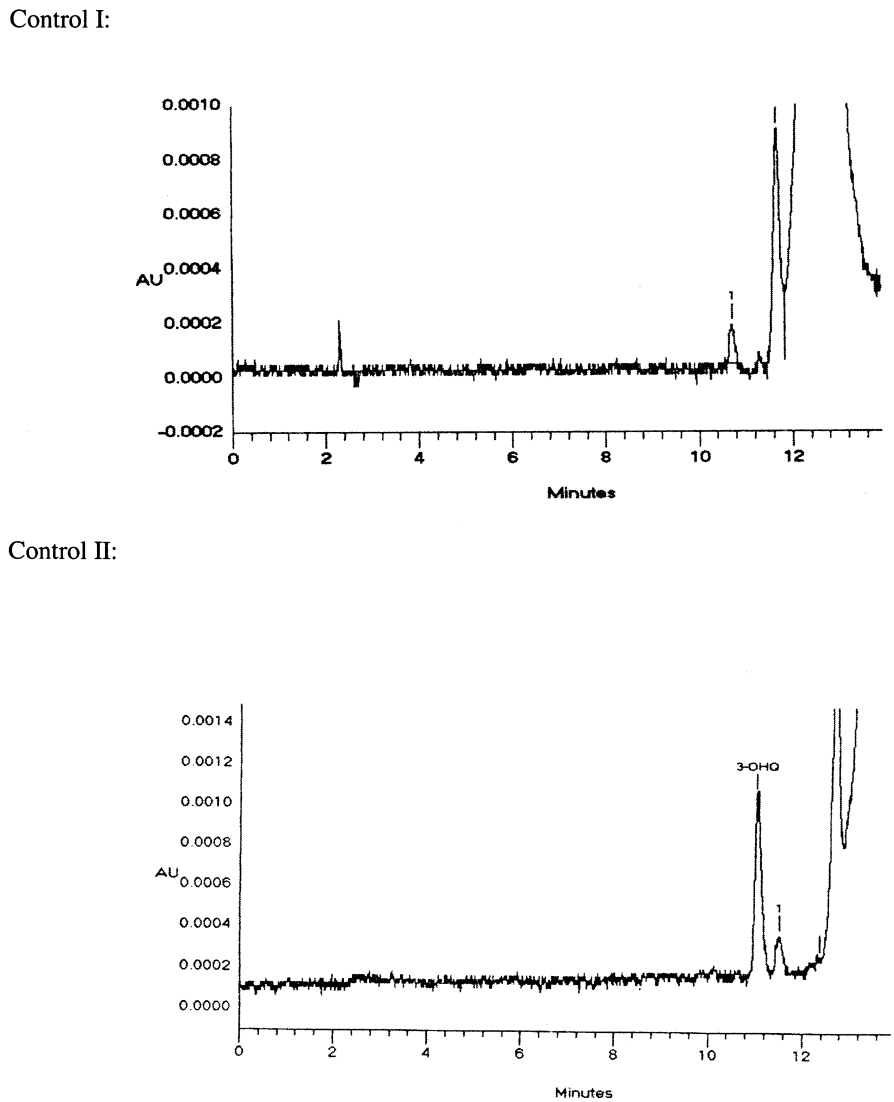

Fig. 1. Comparison of controls II and I to show selectivity for detection of 3-OHQ. Control I: 3 mg/ml of microsomal protein and 1 mM NADPH in phosphate buffer. Control II: control I spiked with 2 μg/ml of 3-OHQ.

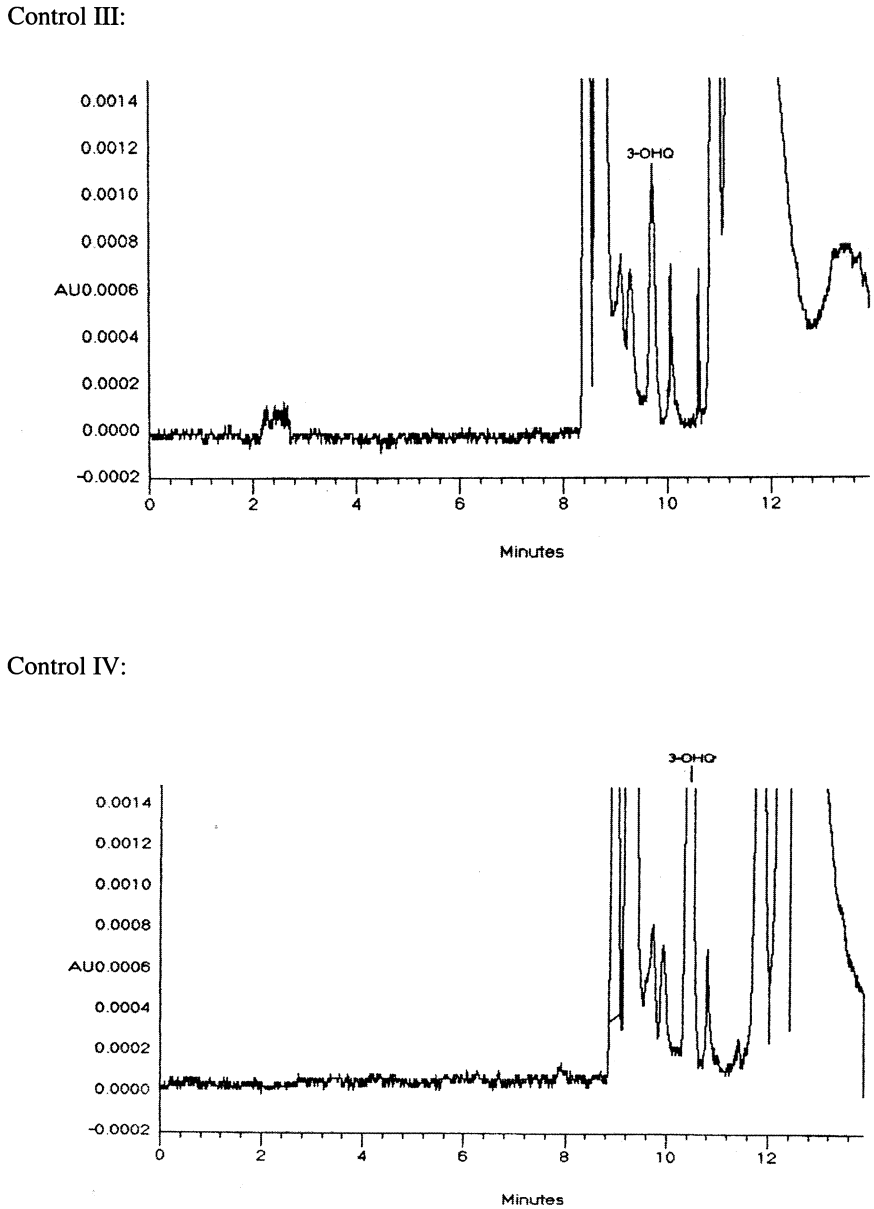

Fig. 2. Comparison of controls III and IV to show selectivity of 3-OHQ, on incubating QS at 37°C for 20 min. Control III: 150 μl of QS, 3 mg/ml of microsomal protein, 1 mM NADPH in phosphate buffer, incubated at 37°C for 20 min. Peak ~8–9 min is the probe drug QS. Control IV: control III spiked with 5 μg/ml of 3-OHQ.

Capillary electrophoresis (CE) a powerful analytical technique has all the inherent advantages to be useful for HTS [5,6]. A possible limitation of this technique would be its low concentration sensitivity as compared to HPLC but linking CE to more sensitive detectors (e.g. MS, LIF) or the introduction of membrane pre-concentration could alleviate this problem [7,8]. High-throughput applications using CE has been shown to be applicable for DNA sequencing [9] and rapid analysis of human serum proteins for routine use in a clinical setting [10].

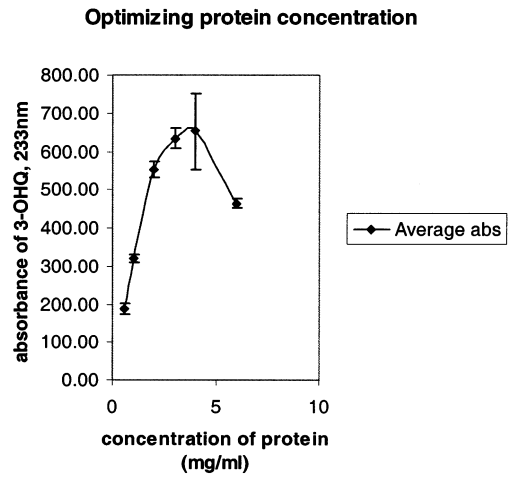

Fig. 3. Effect of different concentrations of microsomal protein content on the absorbance of 3-OHQ. Bars = S.D. of triplicate measurements.

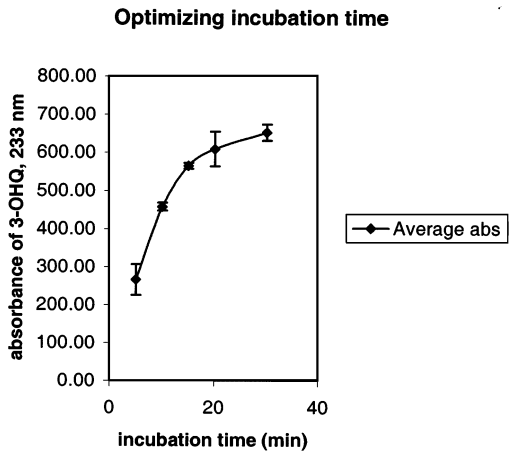

Fig. 4. Effect of incubation time on the absorbance of 3-OHQ. Bars = S.D. of triplicate measurements.

Table 1
Analytical validation of the direct injection method for the determination of 3-OHQ

Controls (μg/ml)	Mean ± S.D. (μg/ml)	RSD (%)	DFN (%)
2[a]	1.75 ± 0.09	5.27	−12.58
8[a]	7.98 ± 0.23	2.90	−0.31

[a] $n = 6$. LOQ = 0.5 μg/ml, $r^2 = 0.9931$, linear range = 0.5–10 μg/ml. DFN: difference from spiked concentration. RSD: relative standard deviation.

The goal of our research is to develop an analytical technique using CE, to allow a significant reduction in the volume needed for metabolism screening studies and to decrease analysis time. The technique will be validated as an analytical model and as a metabolism screening model using established inhibitors of the CYP 3A4 isozyme. Direct injection techniques have been used before to determine in-vitro metabolism using microsomes [11,12] but these methods have been used for non-specific CYP 450 probes hence cannot be used for screening studies. In this paper we show the feasibility of direct injection of microsomal incubation mixtures into the capillary and extend this technique to determine the Michaelis–Menten characteristics of our CYP 3 A4 probe drug and for preliminary screening of CYP 3A4 inhibitors. We propose an on-line CE method, equipped with LIF detection to carry out in-vitro metabolism studies and for use in high-throughput drug metabolism studies. Direct injection of the incubation mixtures into the capillary is the first step towards making on-line incubation feasible. This method also has the advantage of no sample preparation requirement since the incubation mixture is directly injected into the capillary. A similar approach in conventional techniques like HPLC would lead to clogging of the column with cell components and other insoluble materials present in the incubation mixtures. The CE separation buffer had to be favorable for conducting incubation studies on-line, therefore we chose phosphate buffer (100 mM, pH 7.4) as opposed to buffers used in micellar electrokinetic capillary chromatography.

The probe drug chosen for the in-vitro metabolism studies is quinidine sulfate (QS). The advantages with this drug are twofold: (1) it exhibits native fluorescence with an excitation wavelength maximum compatible with the emission line of a helium–cadmium laser [13,14] hence making it suitable for LIF detection and (2) it is metabolized by the CYP 3A4 isozyme to form (3*S*)-3-hydroxyquinidine (3-OHQ) [15]. This metabolite can be used as a biomarker for CYP 3A4 activity in liver microsomes and to study drug interactions with this isozyme in screening experiments. For the current study detection was

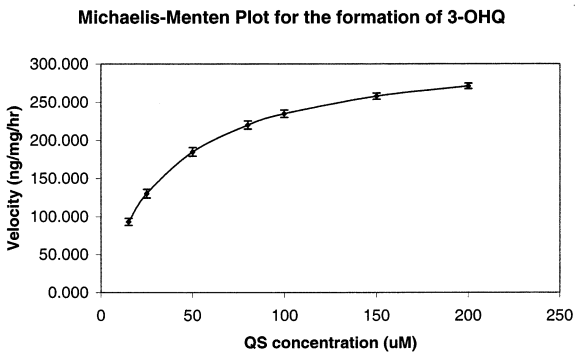

Fig. 5. Michaelis–Menten plot for the formation of 3-OHQ.

Table 2
Michaelis–Menten results for the formation of 3-OHQ

Plot	V_{max} (ng/mg/h)	K_m (μg/ml of QS)
(1)	324.95	33.66
(2)	317.25	35.03
(3)	321.98	42.25
Mean	321.39	36.98
S.D.	3.88	4.62

performed in the absorbance mode (233 nm) but QS gives us the option of using LIF to further lower our sensitivity.

2. Experimental

2.1. Materials and reagents

QS was a USP reference standard and purchased from USP (Rockville, MD). OHQ was kindly donated by Wyeth Ayerst Research (Princeton, NJ); ketoconazole was purchased from ICN Biomedicals Inc. (Aurora, OH), terfenadine and ethynyl estradiol were purchased from Sigma Chemicals (St. Louis, MO). Sodium hydroxide, potassium phosphate (monobase) and potassium phosphate (dibase) were all ACS grade chemicals and purchased from Sigma Chemicals (St. Louis, MO). Methanol was of analytical grade and purchased from Baxter, B & J Brand (Muskegon, MI). Rat liver microsomes used for the incubation studies were prepared in the laboratory [16] and their protein content was determined using a BCA-200 Protein Assay Kit purchased from Pierce (Rockford, IL). Approval of the institutional animal care committee was received prior to microsomal isolation. All solutions used in the experiments were prepared in deionized water distilled in the laboratory with a Corning Mega Pure automatic purification system (Corning, USA).

2.2. Apparatus

The CE system was a Dionex CES I system (Atlanta, GA) equipped with an absorbance detector (UV/VIS absorbance), separation was performed on a fused silica capillary, Polymicro Technologies Inc., (100 μm id × 48 cm length). A Dionex advanced computer interface controlled the CE apparatus and recorded the electropherograms. Initially the fused silica capillary was activated by treatment with 0.1 M sodium hydroxide

Table 3
Summary of inhibition results

Inhibitors	K_i[a] (μM)	Concentration used (μM)	% Inhibition (mean ± S.D.)
Ketoconazole	0.15	50[b]	100[c]
Terfenadine	10.1	50[b]	43.19 ± 22.99
Ethynyl estradiol	107.6	50[b]	47.48 ± 3.50

[a] K_i values reported in human liver microsomes [13].
[b] $n = 3$.
[c] 50 μM ketoconazole resulted in no visible peak for 3-OHQ, hence inhibition was assumed to be 100%.

for 15 min and then rinsed with distilled deionized water before introduction of the run buffer. The run buffer used in the analysis was phosphate buffer (100 mM, pH 7.4). The samples were injected by electrokinetic injection (6.5 kV for 5 s). The run potential was 6 kV and the run time was 14 min. The capillary was cleaned once every three samples by pressure injecting 0.1 M NaOH for 180 s. Detection was performed using the UV absorbance mode at 233 nm, which is the wavelength of maximum excitation for 3-OHQ. For the incubation studies, V-vials of 1.0 ml volume were purchased from Wheaten (Millville, NJ) and these were incubated in a Pierce Reacti-Therm, Heating/Stirring mobile (Rockford, IL). To determine the wavelength of maximum absorbance for QS and its metabolite, 3-OHQ a Shimadzu UV-1601PC, UV-Visible spectrophotometer (Columbia, MD) was used.

2.3. Methods

2.3.1. CE method for the analysis of QS and 3-OHQ

Standards of QS were prepared in phosphate buffer in concentrations ranging from 0.5 to 10 μg/ml and standards of 3-OHQ were prepared in 50% methanol and phosphate buffer in the same concentration range. Different experimental conditions including the concentration of the run buffer, run voltage and injection mode were optimized. It was finally decided that phosphate buffer (100 mM and pH 7.4), an electrokinetic injection of 6.5 kV for 5 s and a run voltage of 6 kV led to an optimum analysis. Using higher run voltages did lead to faster analysis times but the increased heat generation in the capillary lead to a breakdown of the electrosmotic flow due to bubble formation.

2.3.2. Selectivity on direct injection of incubation mixtures

The incubation mixture basically consists of rat liver microsomes, NADPH, QS and phosphate buffer (100 mM, pH 7.4) which also serves as the run buffer. In the CE method development for the analysis of QS and 3-OHQ we have shown that no components of the buffer interfere with the detection of the metabolite. For direct injection to be successful, two additional components had to be tested, rat liver microsomes and NADPH. To check for interference in the presence of microsomes and NADPH, a control was prepared by diluting 15 μl of microsomes in phosphate buffer and then adding NADPH to give a concentration of 1 mM NADPH (control I). The final volume of the mixture was 150 μl resulting in a protein content of 3 mg/ml. This mixture was then directly injected into the capillary. Then the control was spiked with 2 μg/ml, 3-OHQ (control II) and again injected directly into the capillary to check for interference. For further confirmation of selectivity, we decided to carry out an actual incubation and inject it directly into the capillary. A control was prepared containing 150 μg/ml of QS, 1 mM NADPH and 3mg/ml of protein from rat liver microsomes, this was incubated for 20 min at 37°C (control III) when the incubation was stopped by immersing the control in ice. The mixture was then directly injected into the capillary. The same control was later spiked with 5 μg/ml 3-OHQ (control IV) to confirm selectivity of the metabolite on performing incubation.

2.3.3. Optimization experiments

2.3.3.1. Optimization of the protein content required for incubation studies. Different protein concentrations ranging from 0.6 to 6 mg/ml were added to an incubation mixture containing 100 μg/ml of QS and 1 mM of NADPH. These mixtures were incubated at 37°C for 30 min. Incubations were terminated by storage in ice. The CE conditions were the same as previously described (Section 2.3.1).

2.3.3.2. Optimization of the incubation time. The optimal protein content was chosen and this was added to an incubation mixture containing 100 μg/ml of QS and 1 mM of NADPH. These mixtures were then incubated for different lengths of time ranging from 5 to 30 min to determine the optimal time. CE conditions as described previously (Section 2.3.1).

2.3.3.3. Effect of increased storage in ice after incubation. Incubations were to be terminated by storage in ice before injecting the sample into the capillary. To avoid any discrepancies in metabolite formation, an experiment was carried out to see if prolonged storage in ice led to an increase in metabolite formation. The incubation mixture used for this experiment contained 4.65 mg/ml of protein content, 1 mM NADPH and 100 μg/ml QS in phosphate buffer. These mixtures were incubated at 37°C for 30 min and then stored in ice for various lengths of time ranging from 5 to 95 min.

2.3.4. Validation of precision and accuracy for the determination of 3-OHQ

A standard curve of 3-OHQ was prepared by spiking different concentrations to a matrix containing 1 mM NADPH and 3 mg/ml microsomal protein content in phosphate buffer. The concentrations of 3-OHQ used were 0.5, 1.0, 3.0, 5.0, 7.0 and 10 μg/ml. Two controls of 3-OHQ were also prepared in concentrations of 2 and 8 μg/ml in the matrix. The CE procedure remained the same as described previously (Section 2.3.1). The standards were injected in duplicate and the controls were injected six times each to determine the accuracy and precision of the method.

2.3.5. Validation of the system as a metabolic inhibition model

2.3.5.1. Determination of the Vmax and Km for formation of 3-OHQ. Incubation mixtures were prepared with 3 mg/ml of microsomal protein and 1 mM NADPH and to this was added 15, 25, 50, 80, 100, 150, 200 and 300 μg/ml of QS. These incubation mixtures were then incubated for 37°C for 20 min to determine the Michaelis–Menten kinetics for formation of 3-OHQ. A calibration curve was also prepared for 3-OHQ in matrix with concentrations of 0.5, 0.8, 1.0, 2.0, 2.5, 3.0 μg/ml. Two controls were also prepared at concentrations of 0.6 and 1.5 μg/ml in matrix. Experiments used for the determination of V_{max} and K_M of 3-OHQ were performed in triplicate, the standards were injected only once and the controls were injected twice each.

2.3.5.2. Effectiveness of the method as a screening technique for CYP 3A4 drug interactions. For a method to effectively screen for drug interactions it should be capable of detecting high potency, medium potency and low potency inhibitors of a particular enzyme. Based on a previous study [15] of K_i values for the inhibition of 3-OHQ in human liver microsomes, we decided to choose ketoconazole, terfenadine and ethynyl estradiol in decreasing order of potency. A single point measurement was carried at a fixed concentration of the three inhibitors and compared to a control incubation of QS without inhibitors. Standards and controls of 3-OHQ were not run with this preliminary screening study. The absorbance of 3-OHQ peak was compared in each case between the control experiment and the inhibition experiment and percent inhibition was determined. Both the inhibition experiments and the control experiments were performed in triplicate. The fixed concentration chosen for each inhibitor had to be in the high micro molar range so as to exhibit inhibition for the weak inhibitor. Hence, we decided to choose a concentration of 50 μM. Stock solutions of the inhibitors were made at concentrations of 1 mM in 50% methanol/distilled deionized water and these were later diluted in phosphate buffer (pH 7.4, 100 mM) for use in the inhibition experiments. The final concentration of the organic was less than 2.5% in each incubation. The control in each case was an incubation containing 150 μg/ml of QS, 1 mM NADPH and 3 mg/ml of microsomal protein incubated for 20 min at 37°C. Inhibition experiments were performed by incubating 50 μM of the inhibitors, with 150 μg/ml of QS, 1 mM NADPH and 3 mg/ml of microsomal protein.

3. Results and discussion

3.1. Selectivity of 3-OHQ on direct injection of microsomal incubation mixtures

Fig. 1 is a comparison of controls II and I (as explained in Section 2.3.2). It is seen that 3-OHQ can be selectively detected in the presence of the incubation matrix. Fig. 2 is a comparison of

controls III and IV (as explained in Section 2.3.2) which shows that the peak of 3-OHQ can be analyzed by carrying out an incubation of QS.

3.2. Optimization experiments

3.2.1. Optimization of the protein content required for incubation studies

A plot of the concentration of protein versus absorbance is shown in Fig. 3. From this figure it can be seen that there is almost a linear increase in metabolite formation up to a protein concentration of 3 mg/ml. There is a only a marginal increase in 3-OHQ formation from 3 to 4 mg/ml of protein content and then there is a decrease in absorbance as the protein concentration is increased to 6 mg/ml. Hence 3 mg/ml was chosen to be optimum concentration.

3.2.2. Optimization of incubation time

The plot of incubation time in minutes versus absorbance can be found in Fig. 4. This plot shows a steady increase in 3-OHQ formation up to 20 min and only a marginal increase until the next time point. Based on this we chose 20 min as our optimal incubation time.

3.2.3. Effect of increased storage in ice after incubation

Triplicate incubations of QS were stored in ice for different lengths of time ranging from 5 min to 95 min. There was no significant difference in the absorbance of 3-OHQ upon increased storage showing that the concentration of 3-OHQ was stabilized by immersion in ice.

3.3. Validation of precision and accuracy for the determination of 3-OHQ

Various concentrations of 3-OHQ prepared in the incubation matrix were directly injected in to the CE. A linear fit was found to provide the best fit line for the standard curve with an r of 0.9966 and residuals less than 12%. The range of the assay was from 0.5 to 10 μg/ml. The percent RSDs of the two controls, 2 and 8 μg/ml were 5.27 and 2.90% and the percent errors were -12.58 and -0.31% respectively. The limit of quantitation had been set at 0.5 μg/ml, which represented the lowest point on the standard curve. Table 1 summarizes the analytical validation results.

3.4. Validation of the system as a metabolic inhibition model

3.4.1. Determination of the Vmax and Km for the formation of 3-OHQ

QS concentrations ranging from 15 to 300 μg/ml were incubated at 37°C for 20 min to determine Michaelis–Menten kinetics. The incubations were performed in triplicate. A calibration curve was again plotted for 3-OHQ in the incubation matrix over a concentration range of 0.5 to 3 μg/ml. Two controls were also prepared at 0.6 and 1.5 μg/ml. The controls were injected twice, the lower control had a percent error of 11.92% and the higher control had a percent error of 0.45%. The results from QS incubation experiments were fit to Michaelis–Menten plots using a non-linear curve-fitting program (Scientist). The three plots demonstrated an r of 0.999, 0.997 and 0.998 respectively. They have been condensed in Fig. 5 to a composite plot that includes error bars. The V_{max} and K_m for formation of 3-OHQ were computed for each set of data points and these are presented in Table 2. The average K_m values, represented as mean $\pm$ S.D. were 36.98 ± 4.62 μg/ml or 47.2 ± 5.89 μM of QS and the average V_{max} values were 321.39 ± 3.88 ng/mg/h. Each molecule of QS contains two molecules of quinidine, therefore the K_m in terms of quinidine is 94.4 ± 11.78μM. Previously, [13] K_m and V_{max} values have been reported for formation of 3-OHQ during incubation with microsomes from human liver and these values are 74.0 ± 4.3 μM and 65.8 ± 22.6 ng/mg/h respectively. This difference in K_m and V_{max} values can be attributed to the variance of the species used in these experiments [17].

3.4.2. Effectiveness of the method as a screening technique for CYP 3A4 drug interactions

The fixed concentration chosen for each inhibitor was 50 μM. At this concentration an incubation of ketoconazole showed no visible peak. Hence inhibition was assumed to be almost

complete. Terfenadine and ethynyl estradiol showed inhibitions of 43.19 and 47.48% when the absorbance of the 3-OHQ peaks were compared with the control. The results are tabulated in Table 3. The later two results were surprising as the K_i values reported for terfenadine and ethynyl estradiol in human liver microsomes show terfenadine to be ten times more potent than ethynyl estradiol. This can again possibly be attributed to the differential selectivity of CYP 450 inhibitors against probe substrates in human and rat liver microsomes [17]. Studies need to be carried out determine the K_i values of these compounds for inhibition of 3-OHQ in rat liver microsomes as these have not as yet been presented in the literature.

4. Conclusions

A CE method has been successfully developed and established for the determination of QS and its CYP 3A4 metabolite 3-OHQ. Our results show that the sensitivity achieved using absorbance detection is sufficient to calculate the V_{max} and K_m for formation of the metabolite in rat liver microsomes. In our preliminary screening studies it was discovered that the rank order of potencies for the inhibitors are not the same in human and rat liver microsomes. Ethynyl estradiol, which is a low potency inhibitor of the CYP 3A4 isozyme in human liver microsomes, behaves more like a medium inhibitor in rat liver microsomes. Hence it is necessary to carry out further studies with different CYP 3A4 inhibitors to establish the method as an useful for screen for low potency inhibitors. The probe drug chosen offers the unique advantage of having an excitation maximum compatible with a laser line (He–Cd laser, 325 nm) and hence LIF detection can be used to lower our concentration limits and decrease the amount of material needed for injection into the CE. The advantages of the direct injection technique used in this paper are twofold over the conventional HPLC methods used to determine and quantify metabolites. First, it involves no sample preparation and therefore increases the speed of analysis and ensures no metabolite is lost in the extraction steps. Second, this technique consumes very little reagents and probe drug. The incubation volume used in our experiments was 150 μl, but only a few nanolitres are needed for injection into the CE. Therefore, there is potential to further reduce the quantities of probe drug, microsomes and other reagents used by carrying out the incubation on-line coupled with LIF detection.

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