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Development and validation of an enzyme-linked immunosorbent assay for the quantification of cytochrome 3A4 in human liver microsomes

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ABSTRACT

Little is known about the influence of hepatic pathologies on cytochrome P450 (CYP) mediated drug metabolism in children. The determination of the abundance of the different isoforms in pediatric microsomes may provide valuable information on the mechanisms of possible changes in activity. Until now, western blotting was mostly used for abundance measurements, but this technique only provides semi-quantitative data. Therefore, this study aimed to develop and validate an indirect ELISA for the quantification of the most important CYP isoform, CYP3A4, in human liver microsomes, using commercially available reagents. Samples, calibrators and validation samples were diluted to a final concentration of 10 μg microsomal protein/ml. A polyclonal antibody raised against the full length human protein was used as primary antibody; horseradish peroxidase conjugated secondary antibodies for detection. The assay was validated for sensitivity, working range and calibration, accuracy and precision. Amounts of CYP3A4 between 2 and 300 pmol/mg microsomal protein could be quantified with a 5-parameter logistics function with $1/x$ weighting factor. Coefficients of variation of intra and inter assay variability were between 9.54 and 13.98% (16.34% at LLOQ), and between 10.51 and 14.55% (19.44% at LLOQ), respectively. The relative error (%RE) varied between −5.96 and 6.68% (11.53% at LLOQ), and the total error between 11.93 and 21.23% (30.97% at LLOQ). The cross-reactivity of the method with human CYP2E1 showed to have no significant effect on the accuracy of the results. Successful analysis of five samples from an ongoing study demonstrated the usefulness of the method.

1. Introduction

Cytochrome P450 (CYP) enzymes are a superfamily of enzymes involved in the metabolism of endogenous substrates, many drugs and other xenobiotics. The main CYP isoforms involved in drug metabolism are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. The presence of these isoforms has initially been determined by Shimada et al. [1]. The most abundant isoform is CYP3A4 (~30% of total CYP). This protein, composed of 503 amino acids [2], is responsible for the metabolism of over 50% of the drugs currently on the market [1]. CYP3A4 is one of the four members of the CYP3A subfamily. Apart from CYP3A4, CYP3A5 is the only member of the CYP3A family that was detected in significant concentrations in the liver [3], albeit in much lower concentrations than CYP3A4 [4]. Only very rare polymorphic sites of CYP3A4 were identified. This isoform is probably well preserved because of its role in the metabolism of many endogenous and environmental factors [4].

CYP-mediated drug metabolism has been shown to be impaired in adult patients with hepatic dysfunction [5]. Furthermore, the degree of impairment in adults correlates with the severeness of the hepatic dysfunction [6], as well as with the etiology of the disease [7]. In children, however, no similar investigation was performed up till now. The effect of pathology on drug metabolism can be investigated through phenotyping reactions in regard to CYP activity. Furthermore, the abundance of the relevant isoforms can be determined. CYPs are mainly located in the liver and 96% of the CYPs can be found in the subcellular fraction called microsomes [8]. As microsomes are easy to prepare and have excellent long term stability, they are a good choice to perform the characterizations mentioned before (phenotyping and abundance). Previous studies in adults showed a positive correlation between activity and abundance [1,9]. This relationship between enzyme activity and abundance may offer valuable information on the causes of possible changes in enzyme activity. A reduced abundance has been described in patients with liver cirrhosis, as clearly reviewed by Villeneuve et al. [6]. Post-translational mechanisms, on the other hand, may result in low activities but regular abundances of quantifiable CYP protein.

Abbreviations: CYP, Cytochrome P450; 5-PL, Five-parameter logistics function; HRP, Horseradish peroxidase; VS, Validation sample

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To determine CYP abundance in, e.g. microsomes, many studies have used the technique of western blotting. These methods, however, are time consuming and electrophoresis is relatively prone to technical complications. Also, quite large amounts of microsomal protein are necessary. Therefore, Snawder et al. developed an indirect enzyme linked immunosorbent assay (ELISA), using primary antibodies against CYPs in rats [9]. These methods for immunoquantification have a substantially higher throughput and are easier to perform than western blots. This is a clear advantage in studies with a larger number of samples. Barter et al. developed an indirect competitive ELISA for the quantification of human CYP3A4 and CYP3A5 [10], using primary antibodies against the C-terminus of the 3A4 enzyme [11]. Immunologically targeting only part of the protein might introduce bias in the correlation between activity and abundance as protein dysfunctions (e.g. truncation) in the non-targeted but active part of the protein would go undetected.

Therefore, the study described here aims to develop a sensitive indirect ELISA using commercially available primary antibodies raised against the full length human protein, in order to obtain supportive information for a pharmacokinetic study on the impact of disease on hepatic biotransformation in children. The method was thoroughly validated according to the recommendations of DeSilva et al. [12]. Furthermore, as a proof of concept, samples from the ongoing pharmacokinetic study were analyzed.

2. Materials and methods

2.1. Chemicals

Sodium carbonate and bicarbonate, sodium chloride, and Tween 20® were purchased from VWR (Leuven, Belgium), tris(hydroxy-methyl)aminomethane from Sigma-Aldrich (Buchs, Switserland), and hydrochloric acid from Acros Organics (Geel, Belgium). All chemicals were at least reagent grade.

2.2. Calibrators: recombinant CYP3A4

For the preparation of the calibrators and for certain validation aspects, microsomes prepared from insect cells infected with a virus engineered to express human CYP3A4 were used (2000 pmol CYP3A4/ml; 2.1 mg microsomal protein/ml BD Supersomes™, BD Gentest, Franklin Lakes, USA). As negative controls (blanks) for the analysis, Control Supersomes™ (BD Gentest) were used. These control supersomes were prepared from the same type of insect cells, but no human CYPs were expressed. After optimization of the protocol, the final composition and concentrations of the calibrators were 300, 150, 60, 30, 15, 7.5, 4 and 2 pmol rCYP3A4/mg protein in a pH 9.4 carbonate-bicarbonate plating buffer. Additional Control Supersomes™ were added to each calibrator, in order to obtain a final concentration of total microsomal protein of 10 μg/ml. The validation samples (VS), with final concentrations of 2, 5, 100, 200 and 300 pmol rCYP3A4/mg protein, were prepared similarly. For the cross-reactivity experiment, Supersomes™ of insect cells which expressed human CYP2E1 (BD Gentest) were used.

2.3. Patient samples: microsomes

Liver samples were collected from the diseased liver from children undergoing a liver transplantation (approved by the Ethics Committee of Ghent University Hospital, B67020084281). Microsomes were prepared following the method of Wilson et al. [8]. Protein content of the microsomal samples was determined by the method of Bradford [13].

2.4. Primary and secondary antibodies

For the indirect ELISA, two types of antibodies were necessary. The primary antibody to bind the antigen was a polyclonal antibody raised in rabbit against the full-length human CYP3A4 protein (MaxPab® antibody, Abnova, Taiwan). As the secondary antibody, a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was purchased from Thermo Scientific (Rockford, USA).

2.5. Development of indirect ELISA for CYP3A4 in human microsomes

Microsomal samples were diluted to a concentration of 10 μg of microsomal protein/ml using a carbonate-bicarbonate buffer pH 9.4. One hundred microliters of the pH 9.4 carbonate-bicarbonate plating buffer dilution of the calibrators or unknown samples were loaded on a black 96-well MaxiSorp® micro-titer plate (Nunc, Roskilde, Denmark). After an overnight incubation at 4 °C, the plating solution was removed. Subsequently, plates were washed 3 times by the addition of 300 μl wash buffer (Tris-buffered saline (TBS) with 0.05% (v/v) Tween 20®, pH 7.2), soaking during 2.5 min and aspiration of the buffer. Nonspecific binding sites were blocked with 300 μl/well of StartingBlock™ blocking buffer in TBS with Tween 20® (Thermo Scientific, Rockford, USA) and incubation for 1 h at room temperature (± 23 °C). After aspiration of the blocking buffer, plates were washed 3 times as described previously. The primary antibody was diluted in blocking buffer (1:3200), and 100 μl was added to each well, followed by incubation of the plates for 1 h at 37 °C. Primary antibody was aspirated and plates were washed as described previously. For the detection of the primary antibodies, 100 μl of secondary antibody dilution in blocking buffer (1:20,000) was added and plates were then incubated at room temperature for 1 h. The unbound secondary antibody was removed and plates were washed, and 100 μl of premixed HRP substrate (QuantaBlu™ Fluorogenic peroxidase substrate and peroxide, Thermo Scientific, Rockford, USA) was added to each well. After 30 min at 37 °C, the reaction was stopped with the stop solution from the QuantaBlu™ kit, and fluorescence was determined at an excitation wavelength of 320 nm, and emission at 405 nm (Ascent Fluoroscan, Thermo Scientific, Rockford, USA). Calibration curves were fitted and data were analyzed using the Masterplex® Readerfit 2010 software (Hitachi, San Francisco, CA, USA) and Microsoft® Excel (v 2007). The amounts of CYP3A4 were expressed in pmol/mg microsomal protein.

The final protocol was obtained after the optimization of several parameters. Optimal concentrations of primary and secondary antibodies were determined by a checkerboard titration experiment. In this experiment, samples with CYP3A4 at 3 concentration levels (0, 5, and 100 pmol/mg protein) were analyzed using the combination of 10 concentrations of primary antibody (2500, 1250, 625, 417, 312.5, 208, 156, 75, 40, and 20 ng/ml), with 4 concentrations of secondary antibody (25, 50, 75, and 100 ng/ml, according to the manufacturers guidelines). Furthermore, the optimal amount of protein to load in each well (0.5, 1 or 1.5 μg protein), the blocking buffer, incubation times and temperature were determined.

2.6. Validation of indirect ELISA for CYP3A4 in human microsomes

The method was validated for assay sensitivity, linearity of dilution, spiking recovery, calibration model and working range, intra and inter assay variability and precision, and cross-reactivity.

The calibration model was assessed as described by DeSilva et al. [12]. The curve was fitted for six independent assay runs. The appropriateness of the model was evaluated by analysis of the relative error (%RE) of the back-calculated calibration points (%RE=100*x* (calculated concentration − nominal concentration)/ nominal concentration). The %RE should be $\leq$20% ($\leq$25% at LLOQ) for $\geq$75% of the calibrators within a curve. Furthermore, the mean %RE and %CV calculated from all runs (n=6) should both be $\leq$15% for each calibrator, except at the LLOQ where both should be $\leq$20%.

Sensitivity of the method was determined by calculating the mean response of 10 blank samples plus 3 standard deviations. The concentration calculated with this response was defined as the lowest concentration that could be distinguished from a blank sample, and was used as the lower limit of the working range. The upper limit of the working range was defined based on literature information (based on naturally occurring CYP3A4 abundance in microsomes) and compliance with the validation criteria was tested. Linearity of dilution was determined by diluting a sample with a known concentration above the upper limit of the working range 1:2, 1:3, 1:4 and 1:6. The recovery (%) of the observed concentration to the expected concentration should be within the 85–115% interval. In order to evaluate matrix effects, a spiking recovery experiment was set up. Samples were prepared at five concentration levels in both dilution buffer (carbonate-bicarbonate, pH 9.4) and in blank matrix (blank supersomes, final protein concentration of 10 μg/ml). The recovery (%) of the observed concentration to the nominal concentration was calculated and evaluated against the 85–115% interval in both matrices.

Assessment of the intra assay and inter assay precision and accuracy was also performed according to the recommendations of DeSilva et al. [12]. Method precision and accuracy were estimated by the analysis of validation samples prepared in the sample matrix at five concentration levels: anticipated LLOQ, less than 3 times LLOQ, medium, high and anticipated upper limit of the working range. For each validation sample, at least 2 independent determinations were done each run, for a minimum of 6 runs. The statistical methods used for the evaluation of the repeated measurements of each validation sample were described by DeSilva et al. [12]. The coefficient of variation used to express the intra assay precision was calculated by dividing the pooled intra assay standard deviations of the calculated assay run means with the sample nominal concentration. The standard deviation needed for the calculation of the %CV for the inter assay precision was calculated by the method of analysis of variance (ANOVA). Method accuracy (%RE) was determined by the percent deviation of the weighted sample mean from the sample nominal concentration. The a priori set target limits were an intra assay and inter assay precision (%CV) and absolute value of the mean bias (%RE) $\leq$20% (25% at LLOQ). In addition, the total error of the method (=sum of %CV and absolute value of the %RE) should be $\leq$30% (40% at LLOQ).

The cross-reactivity was assessed following MacFarlane et al. [14], according to the equation:

[(observed concentration−control concentration)/

supplemented concentration]100,

with observed concentration=calculated CYP3A4 concentration, control concentration=calculated CYP3A4 concentration in a sample without supplementation of rCYP2E1, and supplemented concentration=concentration of rCYP2E1 supplemented. Also, the calculated mean concentrations from the samples with supplementation of recombinantly expressed CYP2E1 at 4 concentration levels (zero – low – expected physiologically – high) were compared with one-way ANOVA (using Microsoft Excel® 2007).

In-study validation was performed during each patient sample analysis run. Two determinations of validation samples at 3 concentration levels (less than 3 times LLOQ, medium and high) were measured. These results were evaluated with the 4:6:30 rule [12], i.e. 4 out of the 6 VS should have a total error below 30%, and the 2 samples not meeting this requirement should not be at the same concentration level. Furthermore, $\geq$75% of the standard points should have a %RE of $\leq \pm$15%.

2.7. Application of the method

Five patient samples from an ongoing pharmacokinetic study were analyzed with the validated method. After determination of the total protein content, the microsomal suspensions were diluted in the plating buffer to a final protein concentration of 10 μg/ml. For each of the samples, two wells were loaded with hundred μl of the dilution. The actual concentration of the samples was determined by the average concentration of the two wells.

3. Results

3.1. Validation

Based on six independent assay runs, a five-parameter logistics (5-PL) curve with a 1/*x* weighting factor was validated (Fig. 1). Eight calibrators within the range of 2 to 300 pmol CYP3A4/mg protein were used to fit the model. The %RE of the individual calibration points, and the intercurve mean %RE and %CV of each calibrator are shown in Table 1, and were all within the specified limits. The signal bigger than the mean signal of blank microsomes+3 SD corresponded with a concentration of 2 pmol/mg protein.

The results from the spiking recovery experiment for the evaluation of the matrix effects are shown in Table 2. Dilution of the stock solution of rCYP3A4 with plating buffer revealed bad recovery, whereas samples diluted in blank matrix and with a final protein concentration of 10 μg/ml, showed a recovery within the 85–115% limits.

Dilution of a sample with a known concentration of 450 pmol CYP3A4/mg protein, which is above the upper limit of the working range of 300 pmol CYP3A4/mg protein, resulted in concentrations with a recovery within the 85–115% limits (Table 3).

The results of the statistical analysis of the precision and accuracy at the 5 concentration levels of the validation samples are presented in Table 4. Intra assay and inter assay precision were between 9.54 and 16.34%, and 10.51 and 19.44%, respectively. The mean bias (%RE) was always $\leq \pm$11.53%. Furthermore, the total error of the method at the 5 concentration levels was always substantially below the required 30% ($\leq$40% at LLOQ).

The cross-reactivity with human CYP2E1 was assessed through the calculation of the percentage of cross-reactivity (Table 5). The % deviation from the nominal concentration value was always within the method (inter assay) precision interval. One can conclude that the deviation from nominal seen is due to normal variability and cross reactivity can be considered insignificant. This was confirmed by the ANOVA, which showed a p-value $>$ 0.05.

3.2. Application of the method

Table 6 depicts the results of the analysis of microsomes prepared from explanted liver tissue from five children with biliary atresia.

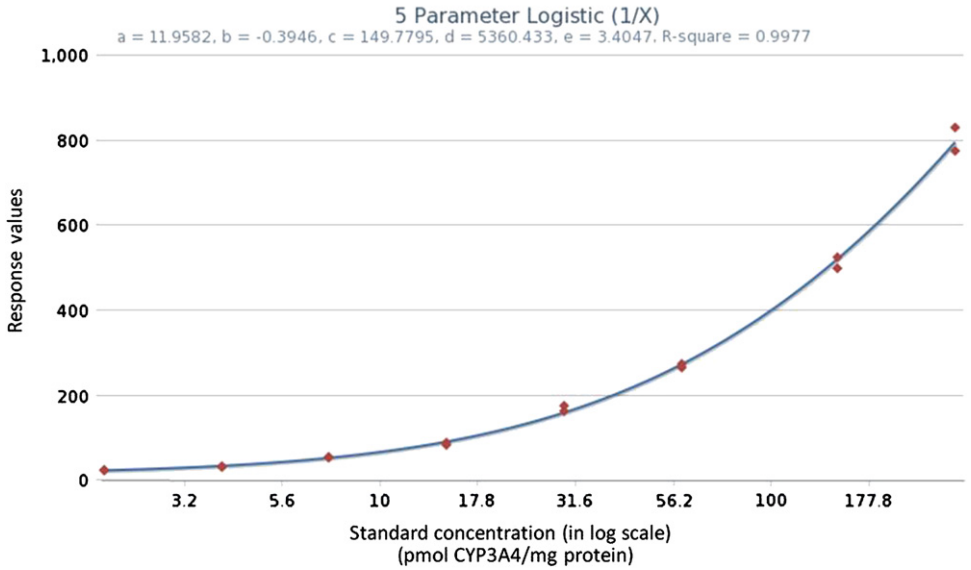

Fig. 1. Representative calibration curve (range 2–300 pmol CYP3A4/mg protein) from the indirect ELISA for human CYP3A4, calculated with a five-parameter logistics curve, with 1/*x* weighting factor.

Table 1
Validation of the standard curve: Relative error (%) of the back-calculated standard concentrations, mean relative error and coefficient of variation (%CV).

Batch run	Nominal concentration (pmol CYP3A4/mg protein)							
	2	4	7.5	15	30	60	150	300
1	6.00	−8.00	4.00	−6.67	7.93	−1.65	−2.50	1.67
2	5.50	−9.50	8.00	−6.60	4.17	−0.25	−1.90	1.77
3	−0.50	2.50	−0.53	−1.33	1.23	0.22	−0.93	1.16
4	6.00	−7.25	0.40	−0.53	2.27	0.70	−2.31	1.42
5	1.00	−3.75	3.47	−3.87	1.80	−1.75	0.23	0.13
6	−6.00	12.00	0.00	−6.53	3.03	2.80	−3.59	1.72
Mean %RE	4.25	−5.56	2.97	−3.78	3.90	−0.25	−1.91	1.51
%CV	3.05	5.78	3.77	3.44	2.84	1.02	0.71	0.27

Table 2
Spiking recovery experiment: Percent recovery of the observed concentration compared to the nominal concentration. Samples diluted in plating buffer showed very poor recovery at the lower concentrations.

Nominal concentration (pmol CYP3A4/mg protein)	Observed concentration (pmol CYP3A4/mg protein)		Recovery %	
	Diluted in blank matrix	Diluted in plating buffer	Diluted in blank matrix	Diluted in plating buffer
2	1.84	0.58	92.0	28.88
5	5.18	3.32	104	66.38
100	107	84.27	107	84.27
200	187	180.49	93.4	90.25
300	279	275.04	93.1	91.68

4. Discussion

An indirect ELISA was developed for the quantification of CYP3A4 in human liver microsomes. A fit-for-purpose optimization was performed in order to optimize all parameters in relation to the intended applications [15].

Table 3
Recovery of the diluted samples (in blank matrix, with a final protein concentration of 10 μg/ml), compared to the expected concentration of 450 pmol CYP3A4/mg protein.

Dilution Factor (DF)	Observed concentration (pmol CYP3A4/mg protein) *x* DF	Recovery %
2	426.2	94.7
3	436.5	97.0
4	431.6	95.9
6	433.3	96.3

Commercially available polyclonal antibodies against full length human protein were used for the primary detection of the antigen. This type of antibody was considered essential for this method, as the antigen, i.e. the microsomal vesicle membrane bound CYP3A4 enzyme, is being adsorbed to the wall of the microplate. As it is unpredictable which part or side of the enzyme would be exposed for antibody binding, it would be advantageous to add polyclonal antibodies to maximize the chance of antigen-antibody interaction. The down side of this approach could possibly be the fact that truncated and non-functional protein, e.g. due to pathological conditions, would most probably also be measured. In contrast, monoclonal antibodies targeted at the active part of the protein would not include truncated and non-functional protein in the abundance measure. We, however, think the use of monoclonal antibodies would introduce bias in the activity/abundance picture because abundance measurement then already includes an activity estimation. The optimal concentrations of primary and secondary antibody were determined by a checkerboard titration experiment (see methods). Based on outcome parameters such as a background signal as low as possible, combined with sufficient sensitivity for the low concentrations and no saturation for the higher concentration, a combination of 312.5 ng/ml of primary antibody, and 50 ng/ml of secondary antibody was further used. Incubation times and temperatures of the antibodies were also optimized, aiming for optimal sensitivity. Also, the amount of protein to be plated was tested. 0.5 μg protein per well showed insufficient sensitivity, as the response from the spiked sample containing 2 pmol/mg protein could not be distinguished from the blank

Table 4
Precision and accuracy assessment.

Characteristic	Statistic	Nominal concentration (pmol CYP3A4/mg protein)				
		2	5	100	200	300
# Results	*N*	18	18	18	18	18
Accuracy	Mean bias (%RE)	11.53	6.68	0.69	−1.41	−5.96
Precision	Intra assay (%CV)	16.34	13.98	10.27	11.62	9.54
	Inter assay (%CV)	19.44	14.55	11.24	11.62	10.51
Total error (Accuracy+Precision)	|Mean bias|+Inter assay %CV	30.97	21.23	11.93	13.03	16.47

Table 5
Cross-reactivity experiment with recombinant CYP2E1.

Supplemented concentration CYP2E1 (pmol/mg protein)	Mean observed concentration CYP3A4 (pmol/mg protein) ($\pm$SD)	% cross reactivity
0	125.7 ± 12.4	
20	124.5 ± 7.5	−5.9
140	140.5 ± 6.3	10.5
280	132.5 ± 8.5	2.4

Table 6
CYP3A4 content of microsomes prepared from explanted liver tissue from five children with biliary atresia.

Sex	Age at transplantation (months)	CYP3A4 abundance (pmol/mg protein)
M	4.7	54.32
M	7.3	4.14
F	8.9	133.36
F	9.4	6.55
F	9.5	18.76

samples. 1 μg was shown to be the optimal plating concentration, as a higher amount (1.5 μg protein per well) resulted in a significantly higher background signal. For reducing the non-specific binding, two types of commercially available blocking buffers were compared. The Starting Block buffer finally chosen provided an assay with highest precision.

The selection of the calibration model was achieved based on the %RE of the back-calculated concentrations. A visual inspection of the relationship between the response and the concentration already suggested the use of a 5-parameter logistics (5-PL) function, due to clear asymmetry (compared to a 4-parameter logistics function) of the curve (see Fig. 1). A comparison of the mean %RE in back-calculated concentrations from an unweighted and a weighted 5-PL model is depicted in Fig. 2. A weighting factor was shown to be necessary in order to obtain sufficient accuracy at the lower concentration level. The 5-PL function with $1/x$ weighting factor met all requirements of %RE and %CV to validate the calibration model (see Table 1).

The working range was selected based on previously determined mean concentrations of CYP3A4 in adults [9]. Nevertheless, as no information is present on this parameter in children (our research focus), nor on the effect of liver disease on the abundance in children, the possibility of lower or higher concentrations should be taken into account. The aspired lower limit of the working range was based on the mean abundance of CYP3A4 in adult liver (i.e. ± 100 pmol/mg protein). If liver disease would reduce the abundance with 90%, the remaining 10 pmol/mg protein should still be detectable. However, Snawder et al. reported a minimum of 19.7 pmol CYP3A4/mg protein in adult microsomes [9]. If the method should be able to detect a 90% reduction, an LOQ of 1.97 pmol/mg protein would then be required. Our validated lower limit of 2 pmol/mg protein meets this requirement. Achieving this LLOQ meant optimizing for sensitivity. For example, a clear increase in sensitivity, or decrease in background signal, was obtained by diluting the antibodies (both primary and secondary) in the commercially prepared blocking buffer, instead of in a 2% BSA solution in the wash buffer (TBS with 0.05% Tween 20®, pH 7.2). The results from the spiking recovery experiment (Table 2) show a clear influence of the matrix proteins on the response. If the microsomes used for the calibrator samples would be diluted in a buffer without additional proteins, a low concentration in a sample would be severely overestimated. This is probably due to non-specific binding of the antibodies. Therefore, all calibrators and validation samples should be diluted in blank matrix, in order to have an equal final microsomal protein content in all samples (10 μg/ml).

A dilution experiment was set up in order to define the approach for the determination of samples with a concentration above the upper limit of the working range (300 pmol/mg protein). A sample was spiked at a high CYP concentration, and was subsequently diluted 2, 3, 4 and 6 fold. Again, it was important to adjust final protein content using blank microsomes. Table 3 shows that the concentrations, calculated after multiplying the observed concentration with the dilution factor, all were within the 85–115% interval of the nominal concentration, indicating dilution of high dosed samples will not deteriorate the quantitative measurement results.

The variability of the results for the same sample analyzed under repeatability conditions and the intermediate precision are determined by the intra and inter assay precision, respectively. The coefficient of variation calculated to express these variabilities should not exceed 20% (25% at the LLOQ). The precision of this assay was evaluated at 5 concentration levels by performing 6 independent assay runs (with $n=3$ within each run). Furthermore, these 6 assay runs were used for the assessment of the assay's accuracy (expressed as %RE). The assay's total error, i.e. the sum of the absolute mean value of the %RE and the inter assay %CV, should be below 30% (40% at LLOQ). The results from these analyses are summarized in Table 4. Both precision (intra and inter assay) and accuracy, as well as the total error, showed results below these limits at all 5 concentrations.

The CYPs are a group of hemoproteins classified according to their homology in amino acid sequence. Consequently, isoforms from the same family have $\geq$40% homology, whereas members from the same subfamily show at least 55% similarity [16]. This homology may lead to cross-reactivity of the primary antibody. The ability of this assay to differentiate between CYP3A4 and other human CYPs was tested through the addition of CYP2E1 to a sample with a known concentration of CYP3A4. Even at a physiologically high abundance of CYP2E1, the method showed sufficient selectivity towards the detection of CYP3A4 (Table 5).

The method was tested through the analysis of samples from an ongoing PK study. The CYP3A4 abundance could be determined in all five samples, which proved the applicability of the method.

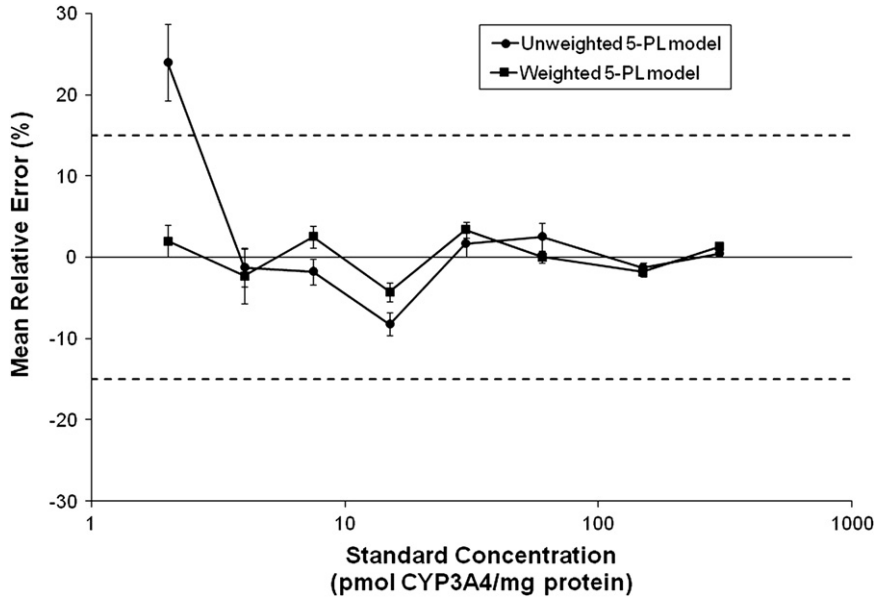

Fig. 2. Comparison of the mean (± standard error) percent relative errors in back-calculated concentrations from an unweighted and weighted 5-PL function. The weighted model showed a better fit than the unweighted model, which showed an unacceptable accuracy at the lower range of the curve.

5. Conclusion

An indirect ELISA was developed for the quantification of CYP3A4 in human liver microsomes. After optimization of several parameters, the method was successfully validated. Concentrations of CYP3A4 between 2 and 300 pmol/mg microsomal protein could be selectively determined with adequate accuracy and precision. All samples, including validation samples and dilutions of samples with a concentration above the working range, should have a concentration of total protein of 10 μg/ml, as proven by the spiking recovery experiment. This method will be applied to samples from a pharmacokinetic study investigating the impact of disease on the metabolic capacity of the liver in children. The analysis of five samples from the study showed that the method has adequate sensitivity for the intended application.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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References

[1] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui, F.P. Guengerich, J. Pharmacol. Exp. Ther. 270 (1994) 414–423.
[2] D.T. Molowa, E.G. Schuetz, S.A. Wrighton, P.B. Watkins, P. Kremers, G. Mendezpicon, G.A. Parker, P.S. Guzelian, Proc. Natl. Acad. Sci. USA 83 (1986) 5311–5315.
[3] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui, F.P. Guengerich, J. Pharmacol. Exp. Ther. 270 (1994) 414–423.
[4] M. Ingelman-Sundberg, Naunyn Schmiedebergs Arch. Pharmacol. 369 (2004) 89–104.
[5] R.K. Verbeeck, Eur. J. Clin. Pharmacol. 64 (2008) 1147–1161.
[6] J.P. Villeneuve, V. Pichette, Curr. Drug Metab. 5 (2004) 273–282.
[7] M.C. Bastien, F. Leblond, V. Pichette, J.P. Villeneuve, Can. J. Physiol. Pharmacol. 78 (2000) 912–919.
[8] Z.E. Wilson, A. Rostami-Hodjegan, J.L. Burn, A. Tooley, J. Boyle, S.W. Ellis, G.T. Tucker, B. J. Clin. Pharmacol. 56 (2003) 433–440.
[9] J.E. Snawder, J.C. Lipscomb, Regul. Toxicol. Pharmacol. 32 (2000) 200–209.
[10] Z. Barter, H. Perrett, K. Yeo, D. Allorge, M. Lennard, A. Rostami-Hodjegan, Biopharm. Drug Dispos. 31 (2010) 516–532.
[11] R.J. Edwards, D.A. Adams, P.S. Watts, D.S. Davies, A.R. Boobis, Biochem. Pharmacol. 56 (1998) 377–387.
[12] B. DeSilva, W. Smith, R. Weiner, M. Kelley, J.M. Smolec, B. Lee, M. Khan, R. Tacey, H. Hill, A. Celniker, Pharm. Res. 20 (2003) 1885–1900.
[13] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[14] G.D. MacFarlane, D.G. Scheller, D.L. Ersfeld, L.M. Shaw, R. Venkatarmanan, L. Sarkozi, R. Mullins, B.R. Fox, Clin. Chem. 45 (1999) 1449–1458.
[15] J.W. Lee, V. Devanarayan, Y.C. Barrett, R. Weiner, J. Allinson, S. Fountain, S. Keller, I. Weinryb, M. Green, L. Duan, J.A. Rogers, R. Millham, P.J. O'Brien, J. Sailstad, M. Khan, C. Ray, J.A. Wagner, Pharm. Res. 23 (2006) 312–328.
[16] D.R. Nelson, T. Kamataki, D.J. Waxman, F.P. Guengerich, R.W. Estabrook, R. Feyereisen, F.J. Gonzalez, M.J. Coon, I.C. Gunsalus, O. Gotoh, K. Okuda, D.W. Nebert, DNA Cell Biol. 12 (1993) 1–51.