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Quantification of cytochrome 2E1 in human liver microsomes using a validated indirect ELISA

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Abstract

CYP2E1 is an important cytochrome P450 isoform in many endogenous processes and in the metabolism of organic solvents, a number of drugs and pre-carcinogens. Information on the abundance of the enzyme may be valuable in various types of research in the field of toxicology and pharmacology. An indirect ELISA for the quantification of CYP2E1 in human liver microsomes was developed and successfully validated. All samples, including validation samples and calibrators, were diluted to a final concentration of microsomal protein of 10 µg/ml. Detection of the antigen was obtained through binding of a polyclonal antibody raised against the full length protein, followed by the addition of horseradish peroxidase conjugated secondary antibodies and enzymatic detection. A five-parameter logistics function with 1/x weighting was used for quantification within the concentration range of 4-256 pmol CYP2E1/mg microsomal protein. The method showed acceptable intra- and inter-assay precision, with calculated coefficients of variation of 6.3-15.2% and 11.3-21.0%, respectively. The relative error varied between -2.3 and 8.9%, and the total error between 16.0 and 27.2%. No significant cross reactivity with other abundant CYP isoforms was observed. The method was evaluated through the analysis of samples from a pharmacokinetic study, and the comparison with the CYP2E1 activity in those samples.

KEYWORDS:

Cytochrome P450; drug metabolism; human liver microsomes; Enzyme linked immunosorbent assay; validation

1. Introduction

Cytochrome P450 (CYP) enzymes are key enzymes in the metabolism of many endogenous compounds and numerous xenobiotics. One member of the CYP superfamily is the isoform CYP2E1, which has broad substrate specificity. Besides being the main enzyme in the biotransformation of organic solvents, it also metabolizes certain drugs and pre-carcinogens. CYP2E1 is reasonably conserved between mammalian species, probably due to its important endogenous role [1]. The enzyme is highly inducible by alcohol, and many of its substrates often are also inducers. Due to its important function in the biotransformation of xenobiotics, information on CYP2E1 may be needed in various types of studies in the fields of, amongst others, toxicology and pharmacokinetics. Changes in the enzyme in specific situations may occur on the level of mRNA expression, protein expression or enzyme activity. For example, Liddle et al investigated the effects of growth hormone on the mRNA expression, the protein level and the activity of CYP3A4 [2]. They observed a similar change in all three measures, and thus suggested that the changes occurred at a pretranslational level. The same factors were evaluated by George et al in patients with cirrhosis [3, 4]. For some isoforms (CYP1A2, 3A, and 2C), a good correlation was observed between the three measures, indicating pretranslational alterations in liver diseases. For CYP2E1 however, there was no strong correlation between the mRNA and protein levels, suggesting both pre- and posttranslational effects of the disease. These examples show the importance of abundance measurements in the characterization of enzymes in a specific situation.

The CYP enzymes are mainly located in the liver and 96% of these enzymes are present in the subcellular fraction called microsomes [5]. Microsomes are easy to prepare and have an excellent long term stability [6, 7]. Therefore, they are a good choice to perform abundance measurements. About 10% of the total CYP content of the liver consists of CYP2E1 [1, 8]. In adults, an average abundance of 50 pmol CYP2E1/mg microsomal protein was detected. Several techniques can be used to determine CYP abundance. Western blotting is the most widely used technique, but it has some disadvantages, such as the time consuming process and the susceptibility to technical difficulties. Moreover, it provides only semi-quantitative data. In order to overcome these disadvantages, Snawder et al developed an indirect ELISA for the quantification of several CYPs in rat liver microsomes [8]. This method has a higher throughput and is easier to perform than a western blot. However, the described method is applied to rat samples, not to human samples, and a primary antibody raised against only a part of the protein is used. Consequently, some proteins may be missed during analysis, as it is unpredictable which part of the protein is available for antigen-antibody interaction after adsorption of the microsomally embedded antigen to the microplate.

In this article, we report the development and validation of an indirect enzyme-linked immunosorbent assay for the quantification of CYP2E1 in human liver microsomes, using a polyclonal primary antibody raised against the full length protein.

2. Materials and methods

2.1. Chemicals

Tween 20®, sodium chloride, potassium chloride, and sodium carbonate and bicarbonate were purchased from VWR (Leuven, Belgium), hydrochloric acid from Acros Organics (Geel, Belgium), and tris(hydroxy-methyl)aminomethane, and chlorzoxazone from Sigma-Aldrich (Buchs, Switzerland). NADPH was obtained from Biopredic International (Rennes, France). All other chemicals were at least reagent grade.

2.2. Recombinant CYP enzymes

Microsomes prepared from insect cells infected with a virus engineered to express human CYP2E1 were used (2000 pmol rCYP2E1/ml, 8.4 mg microsomal protein/ml, BD Supersomes™, BD Gentest, Franklin Lakes, USA) to prepare calibrators and validation samples. Corresponding Control Supersomes™ (BD Gentest), i.e. microsomes prepared from the same type of insect cells but without expression of human CYP2E1, were used as negative controls (blanks) for the analysis. The final composition and concentrations of the calibrators were 256, 128, 64, 32, 16, 8, 4 and 2 pmol rCYP2E1/mg protein in a pH 9.4 carbonate-bicarbonate plating buffer. A final concentration of total microsomal protein of 10 µg/ml was obtained by adding Control Supersomes™, if necessary. The validation samples (VS), with final concentrations of 4, 10, 50, 130 and 256 pmol rCYP2E1/mg protein, were prepared similarly. For the cross-reactivity experiments, the Supermix Supersomes™ of BD Gentest were used. These were prepared from the same type of insect cells as described above, but which expressed human CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

2.3. Determination of CYP2E1 in human microsomes

Prior to plating one µg of microsomal protein/well in a black 96-well MaxiSorp® micro-titer plate (Nunc, Roskilde, Denmark), the microsomal samples were diluted to a concentration of 10 µg of microsomal protein/ml using a carbonate-bicarbonate buffer pH 9.4. Plates were incubated overnight at 4°C, after which the plating solution was removed. Subsequently, plates were washed 3 times by adding 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. Three hundred µl of StartingBlock™ blocking buffer in TBS with Tween 20® (Thermo Scientific, Rockford, USA) was added as a blocking agent, and plates were incubated for 1h at room temperature (±23°C). After aspiration of the blocking buffer, plates were washed 3 times as described previously. The primary antibody, i.e. a polyclonal antibody raised in rabbit against the full-length human CYP2E1 protein (MaxPab® antibody, Abnova, Taiwan), was diluted in blocking buffer (1:1600). Hundred µl was added to each well, followed by incubation of the plates for 1h at 37°C. Primary antibody was removed and plates were washed as described above. Subsequently, 100 µl of secondary antibody dilution in blocking buffer (1:10000; goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP) from Thermo Scientific (Rockford, USA)) was added and plates were incubated at for 1 h at 37°C. Unbound secondary antibody was removed, 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. The reaction was stopped after 60 min at 37°C 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). Curve fitting and data analysis was performed using the Masterplex® Readerfit 2010 software (Hitachi, San Francisco, CA, USA) and Microsoft® Excel (v 2007). The calculated amounts of CYP2E1 were expressed in pmol/mg microsomal protein.

The antibodies were stored and handled following manufacturer's recommendations in order to guarantee optimal stability. The primary antibody was stored in small aliquots at -80°C in order to avoid repeated freeze-thawing. The reconstitution solution of the secondary antibody contained glycerol in order to prolong long term stability at -20°C. During the experiments, all solutions were stored on ice.

2.4. Method validation

Assay sensitivity, calibration model, linearity of dilution, spiking recovery, working range, intra and inter assay variability and precision, and cross-reactivity were evaluated prior to analysis of study samples.

For the evaluation of the calibration model, a curve was fitted for each of six independent assay runs. The suitability of the model was evaluated by analysis of the relative error (%RE) of the back-calculated calibration points within each run (%RE = 100 x (calculated concentration - nominal concentration)/nominal concentration). The %RE should be ≤20% (≤25% at LLOQ) for ≥75% of the calibrators within a curve. Furthermore, the mean %RE and mean %CV calculated from all runs (n=6) should both be ≤15% for each calibrator, except at the LLOQ where both should be ≤20% [9].

The mean response of 10 blank samples plus 3 standard deviations was calculated to determine the assay sensitivity. The concentration corresponding 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 CYP2E1 abundance in microsomes) and tested to comply with validation criteria. Linearity of dilution was evaluated by diluting a sample with a known concentration (500 pmol CYP2E1/mg protein) above the upper limit of the working range 1:3, 1:4 and 1:6. The recovery (%) of the observed concentration to the expected concentration should be within the 85-115% interval. A spiking recovery experiment was used to assess matrix effects. Hereto, 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.

Intra assay and inter assay precision and accuracy were also evaluated according to the recommendations of and the statistical methods described by DeSilva et al [9]. The formulas used to perform this evaluation can be found at the end of the publication by De Silva et al. Validation samples were 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. Three independent determinations were done each run, for a minimum of 6 runs. The intra assay precision was estimated by the coefficient of variation, obtained after dividing the pooled intrabatch standard deviation of measured concentration values from the calculated 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 target limits were an intra assay and inter assay precision (%CV) and absolute value of the mean bias (%RE) ≤ 20% (25% at LLOQ). In addition, the total error of the method (= sum of %CV and absolute value of the %RE) should be ≤ 30% (40% at LLOQ).

Possible cross-reactivity with the most abundant CYP isoforms in liver microsomes was evaluated in 2 of the validation runs. In the first run, a mix of recombinantly expressed CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 ("Supermix") was added at 4 concentration levels (zero - low – intermediate – high) to a sample with a fixed concentration of CYP2E1. In order to cover the complete range of known important and abundant CYPs, cross-reactivity with recombinant CYP2A6 and CYP2B6 (Corning® Supersomes™, Corning BV Life Sciences EMEA, Amsterdam, The Netherlands) was assessed in the second run. CYP2A6 was added at 4 concentration levels (zero - low – intermediate (and physiologically expected) – high), whereas CYP2B6 was only added in a low and intermediate concentration, due to the high protein content of the supersomes and the consequent impossibility to prepare a sample with a protein concentration of 10 µg/ml. . The cross-reactivity was evaluated following MacFarlane et al [10], according to the equation:
[(observed concentration - control concentration)/ supplemented concentration] * 100,
with observed concentration = calculated CYP2E1 concentration of the supplemented sample, control concentration = calculated CYP2E1 concentration of the sample with no other CYP isoform(s) added, and supplemented concentration = concentration of the supplemented CYP(s). Additionally, the calculated mean concentrations were compared with one-way ANOVA (using Microsoft Excel® 2007).

During sample analysis, in-study validation was performed within each run through the measurement of two validation samples, each of 3 concentration levels (less than 3 times LLOQ, medium and high). In order to accept the run, these results should meet the 4:6:30 rule [9], 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, ≥75% of the calibration points should have a %RE of ≤ ±15%.

2.5. Proof of concept: application of the method and CYP activity determination

Liver tissue samples were taken from the explanted livers from children undergoing liver transplantation, after obtaining written informed consent from the parent(s), as approved by the Ethics Committee of Ghent University Hospital (B67020084281). Microsomes were prepared following the method of Wilson et al [5] and subsequently analyzed with the validated method. Total protein content was estimated with the method of Bradford et al [11], and the microsomal suspensions were diluted to a final protein concentration of 10 µg/ml in the plating buffer (carbonate-bicarbonate, pH 9.4). For each of the samples, two wells were loaded with 100 µl of the dilution, and the average concentration in those two wells was considered the actual concentration of the samples.

Furthermore, CYP2E1 activity was determined through incubation of the microsomes with chlorzoxazone (CZ). The reaction was initiated by the addition of NADPH to the reaction mixture containing the microsomes, KCl, phosphate buffer and CZ. After 15 min, reactions were stopped

using a mixture of formic acid, acetonitrile, and water (3:55:42 *(v:v:v)*), and the amount of hydroxy-chlorzoxazone (HCZ) was quantified using UPLC-MS/MS [12].

The relationship between the activity and abundance was evaluated graphically using locally weighted scatterplot smoothing (LOWESS). Moreover, the Pearson's correlation coefficient was calculated. All data analysis was performed using R® v.2.13 (R foundation for statistical computing, Vienna, Austria).

3. Results and discussion

Quantification of a specific CYP isoform in microsomes requires recombinant CYP enzymes (rCYP) for the preparation of calibrators. The rCYP2E1 enzymes used in this protocol were expressed in a baculovirus/insect cell system. This expression system has a high yield of functional CYP enzymes [13]. Moreover, it is capable to perform post-translational modifications, such as phosphorylation or O-linked glycosylation [14]. The obtained microsomes containing the enzyme are preferred over the purified enzyme, as purified rCYPs have a different conformation compared to the enzyme embedded in the endoplasmatic membrane [15].

The final protocol for the quantification of CYP2E1 in microsomes using indirect ELISA was obtained after the optimization of several parameters, such as primary and secondary antibody concentration, optimal amount of protein to load in each well, the choice of blocking buffer, incubation times and temperatures.

3.1. Choice of primary and secondary antibody

Cytochrome P450 enzymes are embedded in the membrane of the smooth endoplasmatic reticulum, and remain as such during the preparation of the microsomes. Upon adsorption of the antigen, i.e. the CYP enzyme, to the wall of the microplate, some parts will be available for interaction with the antibody. However, it is unpredictable which parts are exposed and thus available. Therefore, the use of a polyclonal antibody raised against the full length human protein would be favorable in order to increase the probability of antigen-antibody interaction.

3.2. Optimization of the protocol

Several parameters were optimized in order to achieve a background signal as low as possible, in combination with sufficient sensitivity. Previously published studies describe average concentrations of 50 pmol CYP2E1/mg protein, and a minimum amount of 11 pmol CYP2E1/mg protein [8]. Based on these observations, a sensitivity of about 5 pmol CYP2E1/mg protein was aimed for. As such, decreases of up to 50% in CYP2E1 abundance could still be detected.

The method for the evaluation of the assay sensitivity, i.e. the analysis of 10 blank samples, followed by the calculation of the concentration corresponding to the mean response plus 3 standard deviations, was used in order to determine the optimal conditions. Those conditions leading to the highest sensitivity were eventually chosen for validation. An assay sensitivity of 4 pmol CYP2E1/mg protein was obtained , thus allowing the detection of large decreases in CYP2E1 abundance

compared to healthy adult individuals. Comparison of the sensitivity with other published methods for the quantification of CYP2E1 is difficult, as often the method is described, but without the validation parameters. One study, published by Kornilayev et al [16], describes the method characteristics of the quantification of CYP2E1 tryptic peptides using both ELISA and western blot. The ELISA methods show comparable sensitivity to our method, whereas the sensitivity of the western blot method was 10 pmol CYP2E1/mg protein. The same issue arises when trying to compare the sensitivity of our method to previously published western blot methods: the validation parameters are seldom described. Moreover, due to the semi-quantitative nature of western blot methods, where often the amount of investigated protein is relatively expressed compared to a control sample or other sample, an absolute comparison of the sensitivity is difficult to perform.

In order to determine the optimal assay conditions, , the most favorable concentrations of primary and secondary antibody were determined by a checkerboard titration experiment. Samples containing 3 concentrations of CYP2E1 (0, 5 and 100 pmol/mg protein) were analyzed using different combinations of primary antibody concentration (156, 312, 468, 625, 938, 1250 ng/ml) and secondary antibody concentration (50, 75, and 100 ng/ml, according to the manufacturers guidelines). The combination of 625 ng/ml of primary antibody, and 100 ng/ml of secondary antibody was further used. Furthermore, in order to increase sensitivity by decreasing the background signal, the antibodies (both primary and secondary) were diluted in Starting Block buffer, a commercially prepared buffer, instead of in a 2% BSA solution in the wash buffer (TBS with 0.05% Tween 20®, pH 7.2). The dilution in the blocking buffer most likely decreased non-specific binding. The optimal amount of protein to load in each well was evaluated by comparing the signals after plating 0.5µg, 1µg and 1.5µg protein per well. One µg protein was chosen above 0.5 µg and 1.5 µg, which resulted in insufficient sensitivity and higher background signal, respectively. The incubation times and temperatures of the antibodies were also optimized, aiming for optimal sensitivity. Two types of commercially available blocking buffers were compared in order to aim for minimal non-specific binding. The highest precision was obtained by using the Starting Block buffer.

3.3. Validation

3.3.1. Calibration model

After the determination of the assay sensitivity as described above, the working range of the assay was selected based on previously determined (mean) concentrations of CYP2E1 in adults [8]. The lowest calibrator was determined to be 4 pmol CYP2E1/mg protein, the upper limit was chosen at 256 pmol CYP2E1/mg protein. Consequently, very low abundances, corresponding with a 50% reduction of the lowest reported concentration, as well as rather high abundances can be measured.

The calibration model was selected based on the %RE of the back-calculated concentrations. Due to the clear asymmetry (compared to a 4-parameter logistics function) of the curve, a 5-parameter logistics (5-PL) was suggested (see Figure 1). Evaluation of the mean %RE in back-calculated concentrations showed the necessity of a 1/x weighting factor, in order to obtain sufficient accuracy at the lower concentrations. This model, a 5-PL function with 1/x weighting factor, was validated based on six independent assay runs. All requirements of %RE and %CV were met (see Table 1).

(Figure 1)

(Table 1)

3.3.2. Spike and recovery

A clear influence of the matrix proteins on the response was demonstrated by the spiking recovery experiment. As depicted in Table 2, the samples of rCYP2E1 diluted in plating buffer showed a low recovery, outside the 85-115% limits. In contrast, the samples diluted in blank matrix, thus having a final protein content of 10µg/ml, showed a recovery within those limits. These results show that 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). If this would not be done, and the calibrator samples would be diluted in a buffer without additional proteins, a low concentration in a sample would be severely overestimated, probably due to non-specific binding of the antibodies.

(Table 2)

3.3.3. Dilution experiment

As CYP2E1 is a highly inducible enzyme, high amounts of CYP2E1 can be expected. Increasing the upper limit of the working range was not possible due to the total protein content of the calibrators, which could not exceed 10 µg/ml. Therefore, 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 (256 pmol CYP2E1/mg protein). The recoveries of the 3-fold, 4-fold, and 6-fold dilutions in blank matrix of a sample with a high CYP2E1 concentration are shown in Table 3. After multiplication of the observed concentration with the dilution factor, all samples were within the 85-115% interval of the nominal concentration. Thus, dilution of samples with a concentration above the upper limit of quantification will provide reliable quantitative measurement results.

(Table 3)

3.3.4. Accuracy and precision

The results of the accuracy and precision evaluation are depicted in Table 4. The intra and inter assay precisions determine the variability of the results for the same sample analyzed under repeatability conditions and the intermediate precision, respectively. The assay precision was evaluated at 5 concentration levels in 6 independent assay runs, with n=3 within each run. The coefficient of variation did not exceed 20% (25% at the LLOQ) for the intra assay precision (6.3 – 15.2%RSD), as well as for the intermediate precision (11.3 – 21.0%RSD), except for the 50 pmol CYP2E1 sample, where a minor deviation of the limit was seen. The assay accuracy (expressed as the %RE) was determined using the same 6 assay runs. The absolute value of the mean bias did not exceed the limit of 20% (25% at the LLOQ), indicating a good accuracy. Moreover, the assay's total error was below 27.2 % at all concentration levels.

(Table 4)

3.3.5. Cross reactivity

The homology in amino acid sequence between CYP isoforms from the same family (>40%) or subfamily (>55%) may lead to cross-reactivity of the primary antibody. The selectivity of the primary

antibody was tested through the addition of CYP isoforms (CYP2A6, CYP2B6, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4), selected based on their high abundance or high importance in drug metabolism [1]. Table 5 summarizes the results from the cross-reactivity experiments. The percentage of cross reactivity appears to be quite high at low supplemented concentrations of CYP2B6 (25%). The recovery, however, was within the 85-115% interval of the concentration of the non-supplemented CYP2E1 sample. At higher and physiologically more relevant concentration, much lower % cross-reactivity was calculated (13.5%). Consequently, cross-reactivity of the antibody with CYP2B6 was considered negligible. Moreover, the method showed sufficient selectivity toward the detection of CYP2E1 in the presence of other abundant CYP isoforms, as also shown in Table 5.

(Table 5)

3.4. Application of the method

The validated method for the quantitative determination of CYP2E1 was applied for the analysis of samples from an ongoing PK study. Additionally, the CYP2E1 activity was determined through the evaluation of the chlorzoxazone hydroxylase activity. As depicted in Figure 2, a positive correlation was observed between the two variables after logarithmic transformation, which is also reflected in the Pearson's correlation coefficient of 0.371. This implies that the studied pharmacokinetic situation does not alter the protein on the posttranslational level (Figure 2).

4. Conclusion

This article describes the development and full validation of an indirect ELISA for the quantification of CYP2E1 in human liver microsomes in a concentration range of 4 and 256 pmol CYP2E1/mg microsomal protein. The method was proven to be accurate and precise. The spiking recovery experiment showed the importance of an equal concentration of total protein in all samples, validation samples and calibrators. The polyclonal antibody against the full length protein showed acceptable cross-reactivity with the other abundant CYP isoforms. Analysis of samples from a pharmacokinetic study showed the suitability of this ELISA in the quantification of CYP2E1. We conclude that a valuable alternative to Western blot analysis has been presented, appropriate for use in various fields of research, e.g. toxicology and pharmacokinetics.

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Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1: Validation of the calibration model: %RE of the back-calculated concentrations was ≤20% (≤25% at LLOQ) for ≥75% of the calibrators within each batch run. The mean %RE and %CV calculated from all runs (n=6) were both ≤15% for each calibrator (≤20% at LLOQ).

	Nominal concentration (pmol CYP2E1/mg protein)						
	4.00	8.00	16.0	32.0	64.0	128	256
Batch run	% RE of the back-calculated concentrations						
1	-7.0	2.0	1.9	-3.0	1.7	-0.4	0.5
2	-8.8	4.5	-5.3	4.6	-0.2	-1.2	0.4
3	-10.0	8.3	-7.9	5.4	-2.9	1.0	0.8
4	-5.8	-2.4	2.8	1.0	-0.9	0.5	-0.4
5	-5.5	-2.8	-7.6	12.1	-3.8	-0.7	0.4
6	0.8	10.4	2.6	-1.9	-3.2	2.0	-0.7
Mean %RE	-6.0	3.3	-2.2	3.0	-1.5	0.2	0.2
%CV	4.0	5.2	5.3	5.4	2.1	1.2	0.5

Table 2: Spike-and-recovery experiment: samples diluted in plating buffer show poor recovery, all samples should have a total protein content of 10µg/ml (dilution in blank matrix) in order to obtain a good recovery.

Nominal concentration	Observed concentration (pmol CYP2E1/mg protein)		% recovery	
	Diluted in blank matrix	Diluted in plating buffer	Diluted in blank matrix	Diluted in plating buffer
4	3.68	2.93	91.9	28.9
10	11.6	10.1	116	66.4
50	52.9	47.9	106	84.3
100	101	90.0	101	90.3
130	131	122	101	91.7

Table 3: dilution of a sample with a concentration of CYP2E1 above the upper limit of quantification of 256 pmol CYP2E1/mg protein.

Dilution factor (DF)	Observed (pmol CYP2E1/mg protein) x DF	Recovery %
3	454	90.8
4	447	89.3
6	480	96.0

		Nominal concentration (pmol CYP2E1/mg protein)				
Characteristic	Statistic	4.00	10.0	50.0	130	256
# Results	N	18	18	18	18	16
Accuracy	Mean bias (%RE)	0.9	8.9	6.0	4.7	-16.4
Precision	Intra assay(%CV)	15.2	6.3	7.0	6.4	6.8
	Inter assay (%CV)	19.7	16.5	21.0	11.3	9.8
Total error (accuracy + precision)	|Mean bias| + Inter assay	20.5	25.4	27.2	16.0	26.2

Table 4: Accuracy and precision evaluation

Table 5: Cross-reactivity with CYP2A6, CYP2B6 and the BD Supermix®, containing human CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Cross-reactivity was calculated as:
[(observed concentration - control concentration)/ supplemented concentration] * 100,
with observed concentration = calculated CYP2E1 concentration of the supplemented sample,
control concentration = calculated CYP2E1 concentration of the sample with no other CYP isoform(s) added, and supplemented concentration = concentration of the supplemented CYP(s).

	Supplemented concentration (pmol CYP/mg protein)	mean observed concentration CYP2E1 (pmol/mg protein) (± SD)	% cross reactivity	% recovery
Run 1				
CYP2E1		52.7 ± 2.2		
Supermix	20	47.9 ± 2.4	-23.9	90.9
	50	47.6 ± 2.7	-10.1	90.4
	350	46.0 ± 3.1	-1.91	87.3
Run 2				
CYP2E1		53.1 ± 1.6		
CYP2A6	10	53.7 ± 2.5	5.39	101
	50	57.1 ± 4.8	7.96	107
	250	57.9 ± 2.0	1.91	109
CYP2B6	10	55.6 ± 2.3	25.0	105
	50	59.9 ± 4.2	13.5	113

Figures

Figure 1: Representative 5-PL calibration curve with 1/x weighting factor.

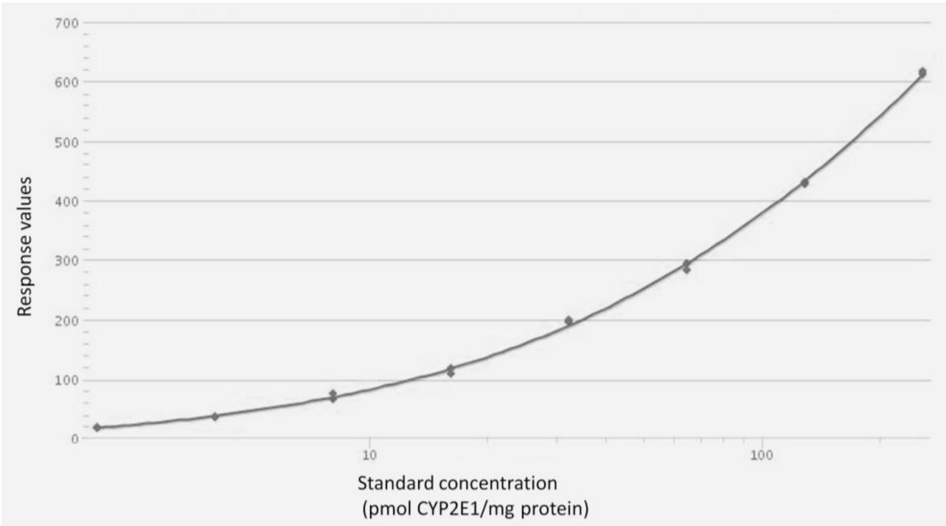

Figure 2: Correlation of CYP2E1 abundance as determined by ELISA with the chlorzoxazone hydroxylase activity: protein levels were positively correlated with CZ-OH activity.