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UPLC-MS/MS analysis of CYP1A-mediated ethoxyresorufin-O-deethylation activity in the rat kidney microsomes

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ABSTRACT

Ethoxyresorufin (ER)-O-deethylation (EROD) activity has been widely used to assess cytochrome P450 1A (CYP1A) activity. The kinetics of CYP1A activity have been well characterized in the liver microsomes. However, studies in kidney microsomes are limited due to the much lower EROD activity in this organ. Here, we developed and validated a sensitive UPLC-MS/MS assay for the characterization of the EROD activity in the rat kidney microsomes. In a 50 µL reaction mixture, rat kidney microsomes (0.25 mg/mL) were incubated with ER (0.1–5 µM) and NADPH (1 mM) for 10 min. Acidic solvents, such as trichloroacetic acid or formic acid, used for quenching of the metabolic reactions and precipitation of the proteins, unexpectedly caused a spontaneous formation of resorufin (RES) from ER. Therefore, the metabolic reactions were terminated by adding acetonitrile, containing a deuterated internal standard (IS). Chromatographic separation was achieved on a C18 UPLC column, and the MS/MS ion transitions were 213.9/185.9 for RES and 220.0/192.0 for IS. The assay was validated in the linear range of 0.5 nM to 75 nM of RES and had a lower limit of quantitation of 0.5 nM. The overall recoveries of RES (90%–99%) and IS (85%–103%) were relatively high, with minimal matrix effect. The assay was successfully applied to the estimation of the Michaelis-Menten (MM) kinetics of EROD activity in the rat kidney microsomes (n = 3), which showed a maximum velocity of 2.68 ± 0.17 pmol/min/mg and a MM constant of 1.72 ± 0.24 µM (mean ± SD). It is concluded that our sensitive and specific analytical method, coupled with the optimized microsomal incubation conditions, provides a robust platform for further investigations of the effects of xenobiotics, environmental factors, or pathophysiologic conditions on the kinetics of EROD activity in the kidney microsomes.
Keywords

UPLC-MS/MS

CYP1A

Ethoxyresorufin-O-deethylase

Kidney

Microsomes

Michaelis-Menten kinetics
1. Introduction

Cytochrome P450 (P450) enzymes are involved in the metabolism of xenobiotics and endogenous compounds. P450 enzymes are preferentially expressed in the liver but also occur in extrahepatic tissues such as the kidneys, lungs, and brain [1]. Although the P450 1A enzyme subfamily (CYP1A) is expressed in the kidneys, in addition to the liver, its enzymatic activity in the kidneys is relatively low [2, 3]. The kidney CYP1A activity is inducible by xenobiotics and environmental chemicals, such as phenobarbital [4], 3-methylcholanthrene [5, 6], pyridine [6], caffeine [7], and nicotine [3]. Additionally, CYP1A1 is one of the most active P450 enzymes in the metabolism of polycyclic aromatic hydrocarbons and activation of pro-carcinogens [8]. It has been reported that higher activity of CYP1A1 in the kidneys of patients with renal cell carcinoma is associated with the aggressiveness and metastatic nature of the renal tumors [9]. Others have also reported that the polymorphism of CYP1A1 has been strongly associated with renal cell carcinoma [8]. Despite the importance of kidney CYP1A1, published studies of the CYP1A1 activity in the kidneys are scarce [3, 5, 7, 10]. This may be due, at least in part, to a lack of sensitive analytical methods to quantitate the relatively low baseline enzymatic activity of CYP1A1 in the kidneys.

Alkoxyresorufin substrates have been traditionally used to probe the level of activity, inducibility, and substrate specificity of various P450 isozymes in both humans and animals [11-13]. The ethoxyresorufin-\textit{O}-deethylase (EROD) activity, where 7-ethoxyresorufin (ER) is metabolized to resorufin (RES) (Fig. 1), is widely used to assess CYP1A1/2 activity in humans and CYP1A1 activity in rats [13]. Resorufin is a highly fluorescent compound, hence the studies reported in the literature either employed spectrofluorometric or chromatographic techniques with the fluorescent detection for characterizing the EROD activity [14-16]. Although these methods
are sensitive enough to quantitate the relatively high EROD activity in the liver, their application to the measurement of the low activity of CYP1A in the kidneys is challenging.

A few studies have reported the EROD activity in the kidney microsomes from rodents based on the fluorometric assay of RES [3, 5, 7, 10]. To compensate for the low sensitivity of the assay and low kidney EROD activity, these methods require relatively high protein concentrations (100–500 µg), long incubation times (0.5–2 h), or relatively high substrate concentrations (5-6 µM) [5, 7, 10]. Therefore, a complete characterization of the kidney EROD activity, including estimation of its Michaelis-Menten (MM) parameters, is lacking in the literature because of the lack of sensitivity of the current fluorometric assays.

Previously [17], an HPLC-MS/MS method, coupled with a direct online guard cartridge extraction technique, was reported for the quantification of RES generated from the EROD activity in the rat and human liver microsomes. The lower limit of quantification (LLOQ) of the assay in the rat liver microsomes was 10 nM using 200 µL of rat hepatic microsomal protein (0.25 mg/mL). Additionally, the method utilized protein precipitation with 1 M formic acid (FA) for sample preparation [17]. In our initial attempt to apply this method to the rat kidney microsomes, we noticed that the addition of FA to the incubation mixture caused a spontaneous, non-P450-mediated conversion of ER to RES. Although this conversion might have been insignificant in the presence of relatively large hepatic EROD activity, it completely masked the low level of enzymatic production of the metabolite in our studies with the kidney microsomes.

We also found that the LLOQ of 10 nM reported before [17] was not sensitive enough for the characterization of the low EROD activity in the kidney microsomes. Therefore, we developed and validated a sensitive UPLC-MS/MS method for the characterization of the MM kinetics of EROD activity in the kidney microsomes. The method has several advantages over the earlier
published LC-MS/MS method, including lack of spontaneous production of the metabolite during the sample preparation, higher sensitivity (0.5 nM), and the use of a stable isotope of RES as an internal standard.

2. Materials and methods

2.1. Chemicals

Ethoxyresorufin (ER), resorufin (RES), resorufin-d₆, NADPH, and analytical grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade water, acetonitrile, and methanol were purchased from Fisher Scientific (Hampton, NH, USA). All other reagents and chemicals were of the highest grade and obtained from commercially available sources.

2.2. Instrumentation and chromatographic conditions

The UPLC system (Bruker Scientific LLC; Billerica, MA, USA) was equipped with a degasser, an integrated column oven, a binary pump, and an autosampler (CTC-PAL). Chromatographic separation was achieved through a Kinetex 1.7 µm C₁₈ (50 x 2.1 mm, 100 Å) column (Phenomenex Inc; Torrance, CA, USA), connected to a Phenomenex C₁₈ Security Guard ULTRA (2.1 mm) pre-column, which were maintained at 40°C. A gradient mobile phase consisting of a mixture of 5 mM ammonium acetate in water (A) and 0.05% formic acid in acetonitrile: methanol (B) (95:5) was delivered to the column at a flow rate of 0.4 mL/min. The gradient conditions were as follows: 0–0.5 min, 2% B; 0.5–1.7 min, linear gradient 2–98% B; 1.7–3.4 min, 98% B; 3.6 min, 2% B; 3.6–4 min, 2% B.

Quantitation was achieved in the positive ion mode by a Bruker EVOQ triple quadrupole mass spectrometer, equipped with ion spray interface, using a temperature of 400°C and ion spray voltage of 3000 V. The curtain gas, heated probe gas, and nebulizer gas flow were set at 30, 45 and 55 psi, respectively. Detection of the ions was performed in the multiple reaction monitoring
(MRM) mode, with the m/z transitions of 213.9 to 185.9 for RES and 220.0 to 192.0 for IS. Quadrupole Q1 and Q3 were set to unit resolutions. The dwell time was 100 msec. The instrument was controlled by the Bruker MSWS-8 software.

2.3. Preparation of calibration standards and samples

The primary stock solutions (1 mg/mL) of RES and IS were prepared in water and methanol, respectively. The RES stock solution was further diluted to a working stock solution of 200 nM in 100 mM Tris-HCl buffer (pH 7.4). For calibration curves, samples containing 0.25 mg/mL kidney microsomal protein in Tris-HCl buffer were spiked with the working stock solution of RES to give final concentrations of 0.0 (blank), 0.5, 1, 2, 5, 10, 20, 50, and 75 nM of the metabolite.

Calibration standards or metabolic incubation samples (50 μL) in a microcentrifuge tube were mixed with 100 μL of an ice-cold solution of IS (10 nM) in acetonitrile. Subsequently, tubes were vortex-mixed for 10 sec and centrifuged at 14,000 rpm for 10 minutes at 4°C to precipitate proteins. The resultant supernatants were transferred into autosampler vials, and a 5-μL aliquot was injected onto the column.

Calibration curves were constructed by plotting the analyte: IS area ratio versus the added concentrations of RES using a weight of 1/x, where x is the added concentration of RES.

2.4. Spontaneous, non-P450-mediated formation of resorufin from ethoxyresorufin by acidic protein precipitants

In our preliminary studies, we noticed that the use of acidic protein precipitants, such as trichloroacetic acid (TCA) or formic acid (FA), to stop the metabolic reactions and precipitate microsomal proteins resulted in the spontaneous formation of RES from ER. Therefore, the spontaneous formation of RES from ER was investigated in the presence of 30% TCA, 1 M FA,
or acetonitrile as protein precipitant solvents. In a final reaction volume of 100 µL in Tris buffer (pH 7.4), 10 µL of heat-inactivated (60°C for 30 minutes) [18] microsomes (0.25 mg/mL) were incubated at 37°C for 5 min with ER (1.0 µM) before the addition of NADPH (1 mM) or vehicle. Subsequently, 20 µL of 30% TCA or 1 M formic acid or 100 µL of acetonitrile were added for protein precipitation. The samples were then processed for measurement of their RES content. All these experiments were carried out in triplicate.

2.5. Method validation

Method validation was partially based on the published regulatory guidelines [19], with some modifications as described in detail in the following sections.

2.5.1. Accuracy and precision

The inter- and intra-run accuracy and precision of the method were evaluated in the quality control samples prepared in the rat kidney microsomes at the LLOQ (0.5 nM) and the middle (10 nM) and highest (75 nM) points in the resorufin calibration curves (n = 6 per each concentration). For each run, a different stock solution of RES was used. The accuracy was calculated by measured concentration × 100/nominal concentration. Precision was estimated as the percent of the relative standard deviation (RSD). The acceptable range for accuracy was set at 85–115% for the middle and high concentrations and at 80–120% for the LLOQ. The acceptable precision for the middle and high concentrations was 15%, and for the LLOQ was 20%.

2.5.2. Selectivity, Carryover, and Sensitivity

The selectivity of the assay for RES was investigated by injecting six blank kidney microsomal samples prepared from six different animals. Carryover studies were conducted by analyzing the highest RES standard in the calibration curves (75 nM), prepared in kidney microsomes, followed by the blank kidney microsomal calibrator and the lowest concentration of
RES in the calibration standard (n = 5). The sensitivity of the assay (LLOQ) was established as the lowest concentration in the calibration curve, which met the accuracy and precision criteria for LLOQ [19].

2.5.3. Recovery and matrix effect

The overall recovery of RES and IS after acetonitrile precipitation of kidney microsomal protein (0.25 mg/mL) and the effect of matrix on the signal intensity were determined in six replicates at three concentrations in the calibration curve (0.5, 10, and 75 nM). The overall recovery was estimated by the ratio of peak areas of RES or IS in the spiked microsomal samples over those in the neat standards (no microsomes), subjected to the sample preparation method. The overall recoveries were expressed as percentages. The matrix effect was evaluated by preparing blank matrix (microsomes) and blank neat samples, followed by protein precipitation and direct addition of RES or IS to the resultant supernatants. The matrix effect was expressed as matrix factor, calculated by the ratio of peak areas of RES or IS added to the microsomal supernatants over those added to the neat supernatants [20]. A matrix factor of 1 is an indication of no matrix effect, while matrix factors of below and above 1 are indications of ion suppression and enhancement, respectively.

2.5.4. Autosampler stability

The stability of RES and IS for 18 h in the autosampler (10°C) was determined by injecting the triplicate preparations of the processed samples at three RES concentrations of 0.5, 10, and 75 nM. The peak area counts of the analyte and IS obtained at the initial cycle were used as the reference to determine the stability at subsequent time points (4, 7, and 18 h).

2.6. Preparation of microsomes from rat kidney
Rat kidney microsomes were prepared by differential centrifugation method, as described below. Briefly, the frozen kidney samples from adult (8 to 12 weeks old), male Sprague-Dawley rats \( (n = 6) \) were weighed and homogenized in an ice-cold buffer (250 mM mannitol, 0.1 mM EDTA, 5 mM HEPES, pH 7.4) at a 1:10 ratio. The homogenate was centrifuged at 600 g for 5 min at 4°C to remove large debris and nuclei. The supernatant was collected, and the pellet washed with the homogenizing buffer and centrifuged again at 600 g for 5 min. The supernatants were combined and spun at 10,300 g for 10 min at 4°C to pellet mitochondria. The supernatant was collected and centrifuged at 110,000 g for 70 min to obtain microsomes. The pellet was washed and centrifuged again using a fresh homogenization buffer. The microsomal pellet was resuspended in 2 mL of storage buffer (250 mM Mannitol, 0.1 mM EDTA, 5 mM HEPES, 20% glycerol, 0.1 mM dithiothreitol, 22 µM butylated hydroxytoluene, and 0.1 mM phenylmethylsulfonylfluoride; pH 7.4) and stored at -80°C for later experiments. Total protein concentrations were estimated by the Bradford method using bovine serum albumin as standard. The animal studies were approved by the Chapman University Institutional Animal care and Use Committee.

2.7. Determination of the ethoxyresorufin O-deethylase activity

Stock solutions (1 mM) were prepared by dissolving the ER powder in acetonitrile: water (50:50) and were further diluted (50 µM) with Tris-HCl buffer. First, the linearity of the formation of RES with regard to the microsomal protein concentrations (0.1, 0.25, and 0.5 mg/mL) and incubation time (0–10 min) was investigated at a substrate concentration of 0.5 µM in triplicate. Subsequently, a protein concentration of 0.25 mg/mL and an incubation time of 10 min was set for the determination of EROD activity in the rat kidney microsomes. The MM experiments were conducted at ER concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2.5, and 5 µM. To determine the inter-
animal variability, the MM curves were constructed using kidney tissues obtained from three different animals. In a final volume of 50 μL of 100 mM Tris-HCl buffer (pH 7.4), microsomal protein (0.25 mg/mL) was pre-incubated at 37°C for 5 min with different concentrations of ER as the substrate. The reaction was initiated by the addition of 1 mM NADPH. After 10 min of incubation at 37°C, reactions were terminated by the addition of 100 μL of ice-cold acetonitrile containing 10 nM of IS. Samples were centrifuged and subjected to the LC-MS/MS analysis. The maximum velocity (Vmax) and MM constant (Km) parameters were obtained by nonlinear regression analysis (GraphPad Prism; La Jolla, CA, USA) of the metabolism rate-substrate concentration data using a one-enzyme MM model.

3. Results and Discussion

3.1. UPLC-MS/MS optimization

In addition to the HPLC-MS/MS method validated for the analysis of RES generated from ER in the rat and human liver microsomes [17], there are only very few other LC-MS/MS methods [21, 22], which quantitated RES in cocktail P450 assays in the liver microsomes. All of these methods used HPLC systems with internal standards that were chemically different from RES, such as phenacetin [17], dextrophan [21], or metoprolol [22]. The assay reported here uses a UPLC system along with a deuterated RES as IS. Compared with HPLC, UPLC results in sharper peaks and potentially higher sensitivities [23]. Additionally, the use of deuterated RES as an IS is preferred because a structurally different IS may show a different response to matrix effect or inter and intra-run changes to the MS detector response.

The previously validated HPLC-MS/MS report [17] showed that ER molecular ions present in the sample undergo source fragmentation during the ionization process, partially generating RES following the elution of ER from the column. Therefore, to distinguish between
the preformed RES in the sample and that generated from ER by the post-column source fragmentation, it is necessary to optimize the chromatographic conditions to separate the substrate (ER) from the metabolite (RES).

Figure 2 depicts chromatograms of blank (Fig. 2A) and the lowest (Fig. 2B) and the highest (Fig. 2C) standards in the calibration standards, in addition to the chromatograms of the metabolic incubation samples at the lowest (Fig. 2D) and the highest (Fig. 2E) concentrations of the substrate. For comparison, the chromatogram of IS is also shown (Fig. 2F). As demonstrated in Fig. 2, the authentic metabolite and IS eluted at ~1.9-1.95 min as sharp peaks with relatively low baseline noises (Figs. 2B-2F). However, the metabolic incubation samples contained an additional intense peak at ~2.25 min in the RES channel (Figs. 2D and 2E), which corresponds to the retention time of the substrate. This peak, which is very well separated from the authentic RES, is generated by the post-column source fragmentation of the substrate (ER) [17]. Although the separation of ER and RES was achieved in the previously validated HPLC-MS/MS assay in the rat liver microsomes [17], the potential of a post-column generation of RES from the substrate or separation of ER from RES was not addressed in the P450 cocktail assays [21, 22].

Following optimization of mass spectrometric conditions, MRM of the precursor/product ion at m/z of 213.9/185.9 and 220.0/192.0 in positive ion mode was used for the quantification of RES and IS (resorufin-d6), respectively (Fig. 2). Additionally, the peak of the substrate (ER), which was eluted at ~2.25 min, was also monitored at an m/z transition of 242.1/213.9 but was not quantitated.

3.2. Spontaneous (non-P450-mediated) formation of resorufin from ethoxyresorufin

As reported in the previous LC-MS/MS assay developed for the determination of EROD activity in liver microsomes [17], we initially used 1 M FA to terminate the enzymatic formation
of RES from ER and to precipitate the kidney microsomal proteins. However, we noticed an erratic and time-independent formation of RES, associated with an early burst, in our kidney microsomal samples. Therefore, we investigated the possibility of spontaneous, non-P450-mediated formation of RES from ER in the presence of different precipitating solvents (TCA, FA, and acetonitrile). Fig. 3 depicts representative chromatograms of RES spontaneously generated from ER (peak at ~2 min) in samples containing 1 µM of ER in the presence (Fig. 3, top panel) or absence (Fig. 3, bottom panel) of NADPH after treatment with TCA (Figs. 3A and 3D), FA (Figs. 3B and 3E), and acetonitrile (Figs. 3C and 3F). In the presence of NADPH (Fig. 3, top panel), both TCA (Fig. 3A) and FA (Fig. 3B) resulted in the substantial spontaneous formation of RES, with the TCA’s effect being larger. However, there was no spontaneous formation of RES in the presence of acetonitrile (Fig. 3C). In the absence of NADPH, the spontaneous formation of RES from ER was less for TCA (Fig. 3D) and was not observed for FA (Fig. 3E) and acetonitrile (Fig. 3F). These data indicate that the addition of acidic protein precipitants, such as TCA and FA, to the P450 metabolic incubation systems causes the spontaneous conversion of ER to RES. Whereas this spontaneous formation may not introduce a significant error in the estimation of EROD activity in samples with high EROD activity, it may result in substantial errors in the low EROD activity samples, such as the kidney microsomes. Indeed, using the FA method, we could not establish linearity between the EROD activity and the reaction time because, after an initial burst of substantial RES formation early, the activity barely increased with time. This was because, at a substrate concentration of 1 µM, the concentration of the FA-induced RES in the presence of inactivated kidney microsomes (15.4 ± 2.7 nM; Fig. 3B) was several-fold higher than the concentrations of the metabolically-generated RES over 10 min of incubation, which was determined using acetonitrile (see section
3.8). Therefore, we chose acetonitrile to terminate the enzymatic reaction and precipitate proteins in our studies of EROD activity in the kidney microsomes.

3.3. **Calibration curve**

The calibration standard curves were linear within the RES concentration range of 0.5–75 nM, with the coefficient of determination ($r^2$) values of $\geq 0.99$ ($n = 6$) for the inter- and intra-run experiments. The representative equation, calculated from the averages of the inter-day calibration curves, was $y = 0.1417x - 0.0095$ where $y$ and $x$ refer to the RES: IS peak area ratio and the concentration of RES, respectively. The back-calculated concentrations of calibrators for all the validation runs were between −12.1% to 13.1% of their nominal (theoretical) values for all the concentrations, including the lowest concentration on the calibration curve (−0.6% to 6.4%).

3.4. **Recovery and matrix effect**

The recovery of RES and IS from the kidney microsomal proteins after the protein precipitation method and the effects of the microsomal matrix on the signal intensity of RES and IS were tested using the absolute peak areas. As listed in Table 1, our protein precipitation method using acetonitrile resulted in very high recoveries for both RES (90.1%–99.4%) and IS (85.3%–103%) at all the tested concentrations. These recoveries are higher than the 89% and 55% recoveries reported for RES and phenacetin (IS) in a previous study that used FA for protein precipitation of liver microsomes [17].

Additionally, the matrix factor ranged from 0.901 to 0.945 for RES and 0.927 to 0.934 for IS, indicating a minimal effect of the microsomal matrix (< 10% ion suppression) on the signal intensity of the analytes in our method.
3.5. Accuracy and precision

Accuracy and precision data for intra- and inter-day experiments are presented in Table 2. Accuracy and precision values for both intra- and inter-day experiments were within the acceptable limits [19]. The accuracy values were between 96% and 105%, and the precision values (RSD) were ≤ 12% (Table 2).

3.6. Selectivity, Carryover, and Sensitivity

There were no detectable responses at the retention time of either RES or IS after injection of blank matrix samples from six different animals, indicating the selectivity of the assay for both the analyte and IS. Additionally, after injection of the highest concentration of RES in the calibration curve, no detectable peak could be found in a subsequent blank chromatogram for any of the five replicates, indicating no carryover. Based on the accuracy and precision values reported in Table 2, the lower limit of quantitation (LLOQ) of RES in our assay was set at 0.5 nM. Our LLOQ (0.5 nM) is substantially lower than 10 nM [17] or 23.4 nM [22], which were previously reported for the LC-MS/MS analysis of EROD activity in the liver microsomes, allowing us to measure the low EROD activity in the rat kidney microsomes.

3.7. Autosampler stability

Resorufin and IS in the samples containing 0.5, 10, or 75 nM of RES and 10 nM of IS were stable in the autosampler (10°C) for up to 18 h. The peak areas at 4, 7, and 18 h of storage in the autosampler fluctuated between 93.1% to 106% (RES) and 95.0% to 102% (IS) of the areas at time zero.

3.8. Optimization of ethoxyresorufin O-deethylation incubation conditions

Figure 4 depicts the EROD activity of kidney microsomes as a function of time and microsomal protein concentrations. Using a substrate concentration of 0.5 µM, the amount of
metabolite formed was linear with respect to the incubation time (up to 10 min) and microsomal proteins (0.1, 0.25, and 0.5 mg/mL) (Fig. 4). From these experiments, we chose a protein concentration of 0.25 mg/mL (12.5 μg protein for an incubation volume of 50 μL) and 10-min incubation time for our metabolic studies. The selection of the 0.25 mg/mL protein concentration was based on the desire to produce measurable quantities of the metabolite even at the very low substrate concentrations used in our MM studies without wasting microsomal proteins. Compared with our incubation method, the few available studies estimating the EROD activity in the rodent kidney microsomes, which used the spectrofluorometric method of analysis, required much higher protein amounts of 200 [5] or 100–500 [10] μg and long incubation times of 30 min [5] or 2 h [10]. The much lower protein amount and shorter incubation time used in our assay are due to the much higher sensitivity of our LC-MS/MS assay, compared with that of the spectrofluorometric method.

3.9. Estimation of ethoxyresorufin O-deethylation kinetics in the rat Kidney microsomes

The sensitivity and specificity of the UPLC-MS/MS method developed for RES allowed us to accurately characterize the MM kinetic parameters of EROD activity in the kidney microsomes for the first time. As demonstrated in Fig. 5, the EROD activity-substrate concentration profile showed a typical MM profile in the substrate concentration range of 0.1–5 μM, approaching a plateau around the substrate concentrations of 5 μM. Nonlinear regression analysis of data indicated that a single-enzyme MM model with a V_max of 2.68 ± 0.17 pmol/min/mg and a K_m of 1.72 ± 0.24 μM would adequately describe the experimental data (mean ± SD, n = 3 animals). Comparison of the EROD activity in our kidney microsomes with the literature data is challenging because of the limited available data in this organ [3, 5, 7, 10], which were all carried out at a single substrate concentration using the spectrofluorometric assay.
4. Conclusions

In conclusion, we report the development and validation of a highly sensitive and specific UPLC-MS/MS assay for the quantitation of RES in kidney microsomes. The assay was applied to the characterization of the MM kinetics of the relatively low baseline activity of EROD in the kidneys. The optimized microsomal incubation conditions, requiring a relatively low microsomal protein and short incubation time, coupled with the sensitive assay, provides a robust platform for further investigations of the effects of xenobiotics and environmental factors or pathophysiologic conditions on the kinetics of EROD activity in the kidney microsomes.

Acknowledgments

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Table 1

Overall recovery and matrix factor (mean ± SD, n = 6) for resorufin and internal standard (IS) in samples prepared in the rat kidney microsomes at the lower limit of quantitation (0.5 nM) and the middle (10 nM) and highest (75 nM) points in the resorufin calibration curves. The IS concentration was the same (10 nM) in all the samples.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Overall Recovery (%)</th>
<th>Matrix Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resorufin</td>
<td>IS</td>
</tr>
<tr>
<td>0.5</td>
<td>90.1 ± 12.6</td>
<td>85.9 ± 12.5</td>
</tr>
<tr>
<td>10</td>
<td>91.2 ± 14.8</td>
<td>85.3 ± 15.7</td>
</tr>
<tr>
<td>75</td>
<td>99.4 ± 15.9</td>
<td>103 ± 18.2</td>
</tr>
</tbody>
</table>
Table 2

Intra- and inter-run accuracy and precision (RSD) values for the quality control samples prepared in the rat kidney microsomes at the lower limit of quantitation (0.5 nM) and the middle (10 nM) and highest (75 nM) points in the resorufin calibration curves ($n = 6$).

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Intra-run</th>
<th>Inter-run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>0.5</td>
<td>101</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>95.9</td>
<td>4.6</td>
</tr>
<tr>
<td>75</td>
<td>105</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Observed concentration* × 100/theoretical concentration
LEGENDS TO FIGURES

**Fig. 1.** Schematic illustration of the conversion of 7-ethoxyresorufin to resorufin by CYP1A enzyme.

**Fig. 2.** Typical MRM chromatograms of resorufin (RES) in blank kidney microsomal matrix spiked with zero (A), 0.5 (B), or 75 (C) nM of RES or in metabolic incubation mixtures at the substrate (ethoxyresorufin) concentrations of 0.1 (D) and 5 (E) µM, with acetonitrile as the protein precipitating agent. The concentrations of resorufin were 0.75 and 4.23 nM in D and E, respectively. For metabolic incubations, rat kidney microsomes (0.25 mg/mL) were incubated with ethoxyresorufin for 10 min. For comparison, the chromatogram of the internal standard is also shown (F).

**Fig. 3.** Representative chromatograms of resorufin (RES) spontaneously generated from ER (peak at ~ 2 min) in samples containing 0.25 mg/mL of heat-inactivated kidney microsomes and 1 µM of ethoxyresorufin in the presence (top panel) or absence (bottom panel) of NADPH after treatment with TCA (A and D), FA (B and E), or acetonitrile (C and F). The concentrations of RES were 42.0, 18.5, and 10.8 nM in A, B, and D, respectively. The arrows point to the expected position of RES peak. The peak at ~2.2 min is the post-column source-generated RES form ER.

**Fig. 4.** Resorufin formation versus incubation time for kidney microsomal protein concentrations of 0.1, 0.25 and 0.5 mg/ml with 0.5 µM ethoxyresorufin. The values represent the mean ± SD of three replicates incubated at 37°C.

**Fig. 5.** Michaelis-Menten kinetics of ethoxyresorufin-O-deethylation in the rat kidney microsomes. Rat kidney microsomes (0.25 mg/mL) were incubated (37°C) with ethoxyresorufin concentrations of 0.1–5 µM and 1 mM NADPH for 10 min. The symbols and error bars represent
the mean and SD, respectively ($n = 3$ different kidneys), and the line represents the nonlinear regression fit of the experimental data to a single-enzyme Michaelis-Menten kinetic model.
Figure 1

7-Ethoxyresorufin → Resorufin

CYP1A
Figure 2
Figure 3
Figure 5

Metabolite Formation Rate (pmol/min/mg)

Ethoxyresorufin (µM)
Devaraj Chandrashekar: Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization, Project administration

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