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Short communication

Validation of a HPLC method for the determination of *p*-nitrophenol hydroxylase activity in rat hepatic microsomes

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Abstract

We report a HPLC-UV method for determination of *p*-nitrophenol (PNP) hydroxylation to 4-nitrocatechol (4NC) as a marker for CYP2E1 activity in rat hepatic microsomes. Proteins were precipitated by addition of 50 μL phosphoric acid (50%, v/v in water) to 500 μL microsomal suspensions. Following vortex mixing and centrifugation the supernatant (20 μL) was injected onto a Supelcosil® C_{18} column (150 mm × 4.6 mm, 5 μm), and mobile phase (22% acetonitrile, 0.1% trifluoroacetic acetic acid, 0.5% triethylamine) delivered at 1.0 mL/min produced resolved peaks for internal standard, 4NC, and PNP in <11 min. Calibration curves were linear ($r^2 = 0.999$) from 0.1 to 40 μM with intra- and inter-day precision <12% and accuracy >90%. The method's improved sensitivity (LOQ = 0.1 μM) and minimal sample processing allowed rapid monitoring of PNP hydroxylase activity in fetal, neonatal, juvenile, and adult rat livers.
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1. Introduction

Cytochrome P450 2E1 enzyme (CYP2E1) is an important member of the cytochrome P450 superfamily of heme-thiolate proteins responsible for the oxidative metabolism of numerous endogenous (i.e. steroids, fatty acids, vitamins, prostaglandins) and exogenous (i.e. drugs, carcinogens, and environmental toxins) chemicals [1,2]. CYP2E1 is mainly expressed in the liver and to a minor extent in the lung [3,4] and can bioactivate structurally diverse chemicals, including aromatic compounds, halogenated alkanes, and other low molecular weight compounds, to toxic metabolites [5–10]. Despite its toxicological significance details of CYP2E1's role during postnatal development remain limited. Our knowledge that developmental events alter a growing infant's susceptibility to toxic insults emphasizes a critical need to enhance our understanding of the molecular determinants of risk during maturation to adulthood [11]. Therefore, the determination of CYP2E1 activity during pre- and postnatal development is essential for appropriate assessments of infant exposure risks to environmental toxicants or therapeutic agents.

CYP2E1 mediates *p*-nitrophenol (PNP) hydroxylation to 4-nitrocatechol (4NC) (Fig. 1). This reaction is used as a marker of hepatic CYP2E1 activity in different animal species [12–14] because the method is simple and reaction components are inexpensive and commercially available as compared with other methods (e.g. chlorzoxazone hydroxylase activity).

Many laboratories have used spectrophotometric methods to assay PNP hydroxylase activity [15–17]. Analytical sensitivity and possible interference from other phenolic compounds, which have significant absorbance at 546 nm, limits the usefulness of these spectrophotometric methods. Their low analytical sensitivity requires higher microsomal protein concentrations to ensure quantifiable metabolite (4NC) levels for hepatic microsomal incubation studies. Consequently, these methods are not applicable when measuring CYP2E1 activity in extrahepatic tissues or hepatic tissues from young ages, where CYP2E1 activity is low and tissue sources are very limiting.

The literature reports several HPLC-UV detection methods for monitoring PNP hydroxylase activity [13,18,19]. These methods demonstrate improved sensitivity (nearly 20-fold) over the spectrophotometric methods, but they require laborious and time-consuming extraction and evaporation steps, which are potential sources of error. A more sensitive HPLC method has

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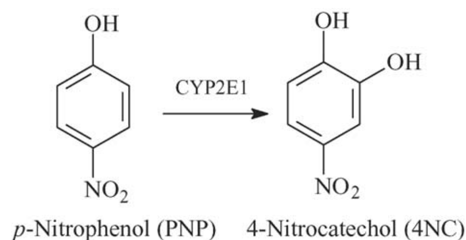

Fig. 1. CYP2E1-mediated PNP hydroxylation to 4NC.

been published [20], but this assay requires electrochemical detection (ECD), a technique not commonly available in many laboratories.

In this paper, we describe a validated, simple, rapid, and sensitive HPLC method for the assay of CYP2E1 enzyme-mediated PNP hydroxylase activity based on UV detection. Furthermore, we demonstrate the suitability of this method to monitor the ontogeny of CYP2E1 activity in male Sprague–Dawley rat hepatic microsomes at fetal, neonatal, juvenile, and adult ages.

2. Experimental

2.1. Chemicals and reagents

p-Nitrophenol, 4-nitrocatechol, salicylamide, and all chemicals used for microsomal preparation, determination of microsomal protein content, and enzyme assays were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ont., Canada). Acetonitrile HPLC grade was purchased from EMD chemicals (Darmstadt, Germany). A MilliQ Synthesis (Millipore, Bedford, MA, USA) water purification system provided purified deionized water. All other chemicals used were analytical grade.

2.2. Animals

Sprague–Dawley rats were obtained from Charles River Canada (St. Constant, PQ, Canada). Six pregnant rats received at 14-day pregnancy were allowed to acclimatize for 1 week. At day 21 of pregnancy, three pregnant rats were anesthetized with isoflurane to collect the fetuses. The remaining pregnant rats were allowed to give birth and one male pup from each dam was collected at postnatal ages 1, 3, 5, 7, and 10 days. Rats for the other age groups (2, 3, 9, 12, and 16 weeks) ($n = 3$) were obtained from Charles River Canada 1 week before the required age during which time rats were allowed to acclimatize. The animals received rat chow and water ad libitum throughout the acclimatization period. At the appropriate age, rats were anaesthetized with isoflurane and then killed by guillotine, their livers rapidly removed, rinsed in ice-cold 0.9% NaCl, flash frozen in liquid nitrogen, and stored at −80 °C until microsomal preparation. Short-term anesthesia with isoflurane does not affect CYP2E1 enzyme activity in adult rat hepatic microsomes [21].

All procedures described in this study were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan.

2.3. Preparation of hepatic microsomes

Hepatic microsomes were prepared as described previously [22]. Briefly, 0.5 g of liver was homogenized in 2 mL homogenization buffer (50 mM Tris buffer, 150 mM KCl, 0.1 mM dithiothreitol, 1 mM EDTA, 20% glycerol, and 0.1 mM phenylmethylsulfonylfluoride). The homogenate was centrifuged at $9000 \times g$ for 30 min in a Beckman L8-55 Ultracentrifuge (Palo Alto, CA, USA). The supernatant was carefully transferred to clean ultracentrifuge tubes and centrifuged at $100{,}000 \times g$ for 30 min. The pellet was washed in 2 mL of 150 mM KCl and centrifuged again at $100{,}000 \times g$ for 30 min. The pellet was resuspended in 2 mL of 0.25 M sucrose solution and 400 μL aliquots were transferred to cryogenic microcentrifuge tubes (1.5 mL). Microsomal suspensions were stored at −80 °C until use.

2.4. Determination of microsomal protein content

Microsomal protein concentrations were determined by the method of Lowry et al. [23] using bovine serum albumin as the standard. Analysis was carried out on an Agilent 8453E UV-visible spectrophotometer using Chemstation software (Palo Alto, CA, USA). Absorbance was measured at 750 nm.

2.5. Apparatus and HPLC conditions

The HPLC system consisted of Water's Model 600 solvent delivery system, Model 486 variable UV–vis detector, Model 717 Plus autosampler and a Millennium data module (Millipore-Waters, Milford, MA, USA). Absorbance was monitored at 250 nm (λ_{max} for 4NC). Chromatographic separation was carried out on a reverse phase C_{18} column (Supelcosil® 150 mm × 4.6 mm I.D., 5 μm particle size). The column was kept at 25 °C. The analytes were eluted under isocratic conditions using a mobile phase composed of 22% acetonitrile, 0.1% trifluoroacetic acetic acid, and 0.5% triethylamine delivered at 1.0 mL/min.

2.6. Preparation of stock and working solutions

Stock solutions of 4NC (1000 μM) and internal standard (IS), salicylamide (6 μg/mL) were prepared in the mobile phase and stored at 4 °C. Working solutions of 4NC (1–400 μM) were prepared by serial dilutions of the 1000 μM stock solution with mobile phase. These solutions were stored at 4 °C protected from light and were stable for at least 1 week.

2.7. Preparation of calibration standards and quality control (QC) samples

Known amounts of working solutions were added to heat-inactivated (55 °C for 5 min) rat hepatic microsomes and diluted with 50 mM phosphate buffer (pH 6.8) to achieve calibration standards of 0.1–40 μM 4NC in a total volume of 500 μL. Three

quality control samples at 0.5 μM (low), 5 μM (medium), and 20 μM (high) were prepared independent of those used for the calibration curves. These QC samples were prepared on the day of analysis in the same way as calibration standards.

2.8. Microsomal incubation

PNP hydroxylase activity was determined by quantification of 4NC formation rates in age specific rat hepatic microsomes. Preliminary experiments were conducted to determine linear metabolite formation kinetics with respect to time and microsomal protein concentration. Microsomal incubation mixtures consisted of PNP (200 μM), 0.4 mg/mL liver microsomal protein, 1 mM ascorbic acid, 2 mM $MgCl_2$, 1 mM NADPH, and 50 mM phosphate buffer, pH 6.8, in a final volume of 0.5 mL. After a preincubation period of 1 min at 37 °C, the reaction was started by addition of NADPH and incubated at 37 °C for 30 min in a shaking water bath. The reaction was terminated by addition of 50 μL ice-cold phosphoric acid and processed as indicated below. Metabolite formation rate was calculated by dividing the amount of the metabolite formed by the incubation time and microsomal protein content (nmol/(min mg)).

2.9. Sample preparation

To 500 μL calibration standards, QC samples, or microsomal incubation mixtures, 50 μL ice-cold phosphoric acid (50%, v/v in water), and 50 μL of the internal standard solution were added. The mixtures were vortex mixed for 20 s. After centrifugation at 12,000 × *g* in an Eppendorf microcentrifuge (Model 5417 C, Brinkmann instruments, Westbury, NY, USA) for 20 min, 20 μL of the supernatant was injected directly onto the analytical column for immediate HPLC analysis.

2.10. Method validation

Method validation procedures were performed according to FDA guidelines (www.fda.gov/cder/guidance/cmc3.pdf) to evaluate the suitability of the method for the quantitative determination of PNP hydroxylase activity in age-specific rat hepatic microsomes. Specificity was tested by analysis of four different rat hepatic microsomal preparations supplemented only with internal standard to ensure the absence of endogenous compounds with the same retention times as 4NC and PNP.

The linearity of the method was evaluated by processing a 10-point calibration curve range from 0.1 to 40 μM including a zero level spiked into blank hepatic microsomal suspensions on five different days. The peak height ratios between 4NC and the internal standard were plotted against the nominal concentration of 4NC. A linear least-squares regression analysis was conducted to determine slope, intercept, and coefficient of determination (r^2) to demonstrate linearity of the method.

The accuracy and precision of the proposed method were determined by analysis of the QC samples. The intra-day accuracy and precision were assessed from the results of six replicate analyses of QC samples (0.5, 5, and 20 μM) on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on six consecutive days. Precision is expressed as % relative standard deviation (R.S.D.), while accuracy (%) is expressed as [(calculated amount/predicted amount) × 100].

The limit of detection (LOD) was defined as the lowest detectable concentration, taking into consideration a signal-to-noise ratio of 3. Limit of quantification (LOQ) was determined at the lowest concentration at which the precision, expressed as %R.S.D., is less than 20% and accuracy, expressed as relative difference between the measured and true value, is less than 20%.

The recovery of 4NC was determined by comparison of peak heights obtained from injection of 20 μL aliquots of either 4NC standard (0.5, 5, and 20 μM) prepared in mobile phase or samples containing the same amount of 4NC after microsomal incubation without NADPH ($n = 6$) and processed as indicated above.

2.11. Kinetic and statistical analysis

Descriptive statistics (mean ± S.D.) was used to report 4NC formation rate from hepatic microsomes prepared within the same age group. A Student's *t*-test was used to determine whether slopes and intercepts of the calibration curves were significantly different from 0 using Prism 3.0 (GraphPad software, San Diego, CA, USA). The level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Method validation

Fig. 2 illustrates typical chromatograms from blank microsomal suspensions (Fig. 2A), a calibration standard at 10 μM (Fig. 2B), and a microsomal incubation at 200 μM of PNP (Fig. 2C). Fig. 2A demonstrates that the method is specific since rat hepatic microsomal suspensions have no endogenous peaks that co-elute with the analytes of interest. Fig. 2B and C indicate that under the chromatographic conditions used for the analysis, the retention times for IS, 4NC, and PNP were 4.4, 5.4, and 9.2 min, respectively.

4-Nitrocatechol limit of detection was 0.02 μM and the low limit of quantitation (LOQ) was 0.1 μM. The LOQ was calculated as the lowest 4NC concentration in the working solutions that could be measured routinely with acceptable accuracy (80–120%) and precision (R.S.D. < 20%).

The method was linear over 4NC concentration range of 0.1–40 μM. Coefficients of determination were greater than 0.999 and the relevant slope values were statistically different from 0 ($P < 0.001$) (Table 1). Although intercepts of the calibration curves were significantly different from 0 (Table 1), they did not affect the accuracy of the method [24,25]. Furthermore, a linear regression of the back-calculated concentrations versus the nominal concentrations produced a slope of unity and an intercept equal to 0.

Table 2 summarizes the results for intra- and inter-day accuracy and precision. During the course of method validation,

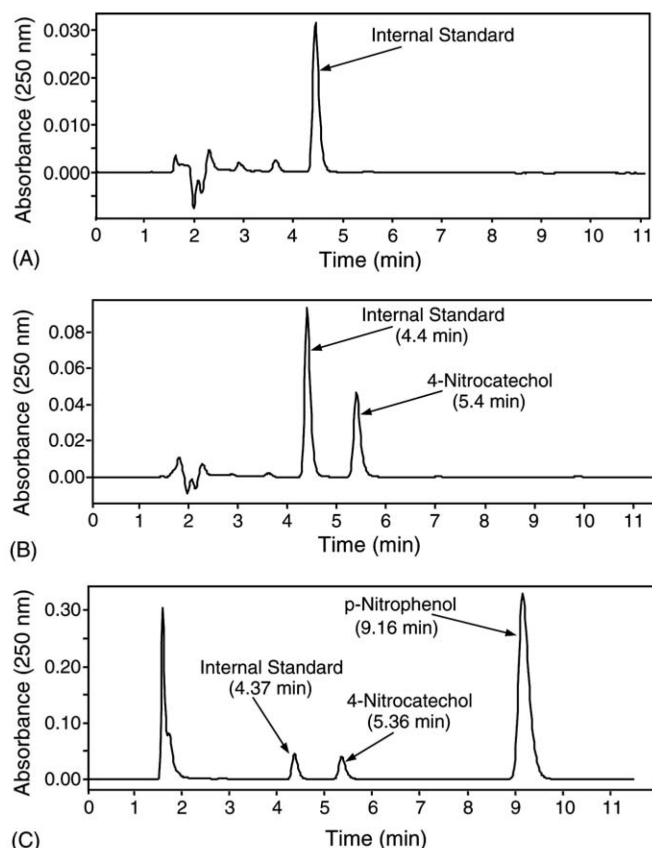

Fig. 2. Representative HPLC chromatograms. (A) Incubation of rat hepatic microsomes without PNP. (B) Standard working solution containing 4NC (10 μM) and internal standard. (C) Incubation of rat hepatic microsomes with PNP (200 μM).

Table 1
Linearity data for the determination of 4NC with the proposed HPLC method

Calibration curve	Slope	Intercept	r^2
1	0.1025	−0.0428	0.9997
2	0.1043	−0.0401	0.9989
3	0.1032	−0.0443	0.9997
4	0.1028	−0.0418	0.9997
5	0.1021	−0.0455	0.9995
Mean	0.10298	−0.0429	0.9995
S.D.	0.00084	0.002108	0.000346

S.D.: standard deviation of the mean.

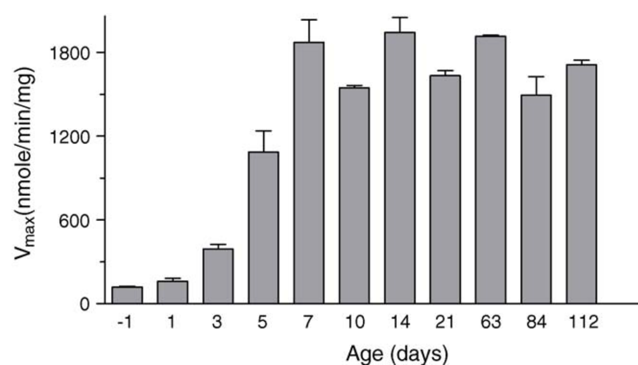

Fig. 3. Age-related changes in rat hepatic CYP2E1-mediated PNP hydroxylase maximal activities (V_{max}). Data are expressed as mean ± S.D. of three male rats; all fetal livers (−1 day) from each respective dam were pooled.

intra- and inter-day precisions were less than 12%. The accuracy of estimated 4NC concentrations ranged from 90 to 104%. These data show that our method is both accurate and precise in rat hepatic microsomal suspensions. The mean ± S.D. absolute recoveries of 4NC at 0.5, 5, and 20 μM were 103 ± 5%, 99 ± 3%, and 97 ± 2%, respectively. The mean (±S.D.) absolute recovery of the internal standard was 92 (±3)%. Our method, which involved only a protein precipitation step and no extraction procedures, provided good specificity and analytical recoveries.

3.2. Application of the HPLC-UV detection method to monitor age-dependent changes in hepatic CYP2E1-mediated PNP hydroxylase activity in male rats

A principal goal of our laboratory is to evaluate the ontogeny of CYP enzyme activity from gestational age through to adulthood to further our understanding of the molecular determinants of exposure risk in the developing infant. To meet this goal, we rely heavily on HPLC-UV detection. Such research requires analytical methods that are both sensitive (to detect and quantify enzyme activity especially in the very young) and simple (due to the high number of samples that require processing). 4NC formation rate was measured at a saturating PNP concentration (200 μM) in age-specific male rat hepatic microsomes to provide estimates of maximal enzyme activity (V_{max}) to assess age-dependent changes in CYP2E1 enzyme activity. Fig. 3 indicates hepatic PNP hydroxylase activity was detectable in fetal (−1 day) microsomes and increased more than 20-fold by days

Table 2
Intra-day ($n = 6$) and inter-day (six consecutive days) accuracy and precision values of 4NC determination by HPLC-UV detection in rat hepatic microsomal suspensions

Quality controls (QC)	Nominal concentration (μM)	Observed concentration (mean ± S.D., μM)	Accuracy (%)	Precision (CV%)
Intra-day accuracy and precision				
QC1	0.5	0.47 ± 0.05	94.9	11.1
QC2	5	5.17 ± 0.49	103	9.44
QC3	20	20.8 ± 1.04	104	4.98
Inter-day accuracy and precision				
QC1	0.5	0.40 ± 0.03	91.0	7.13
QC2	5	4.68 ± 0.24	93.6	5.06
QC3	20	20.5 ± 0.95	103	4.62

7–14 of age (about 110% of the adult value). PNP hydroxylase activity remained relatively constant and similar to the adult value after 21 days of age.

The simple sample processing procedures led to improved analytical sensitivity [15,16], which allowed the measurement of CYP2E1 activity in rat hepatic microsomes during late gestation and postnatal development. The total analytical run time was 10.5 min, which allowed sequential assay of multiple samples in a relatively short time period. The minimal sample preparation and short analytical run times offer an economical advantage to currently available methods [13,15,16,18,22] with respect to resources and operator time.

4. Conclusion

In this study we report a simple, rapid, and sensitive HPLC method for the quantification of 4NC in rat hepatic microsomal suspensions to serve as a marker of CYP2E1 activity. The proposed liquid chromatographic with UV detection method is specific, sensitive, accurate, and precise for the quantification of 4NC in rat hepatic microsomal suspensions. We have successfully applied this method to monitor the ontogeny of CYP2E1 enzyme-mediated PNP hydroxylase activity in male Sprague–Dawley rats at different developmental stages. This method can be used for in vitro microsomal studies concerned with the assessment of CYP2E1 enzyme activity in any mammalian species.

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