A Comparison of Calcium Aggregation and Ultracentrifugation Methods for the Preparation of Rat Brain Microsomes for Drug Metabolism Studies

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Short Title: Preparation of Brain Microsomes by Calcium Aggregation and Ultracentrifugation

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Abstract

Preparation of brain microsomes by the calcium chloride aggregation method has been suggested as an alternative to the ultracentrifugation method. However, the effects of the calcium chloride concentration on the quality of the microsomal fractions are not known. Brain microsomes were prepared from the adult rat brains using the high-speed ultracentrifugation and low-speed calcium chloride (10-100 mM) aggregation methods (n = 5-6 per group). The microsomal protein yield (spectrometry), cytochrome P450 reductase (CPR) activity (spectrometry), and the monooxygenase activities (UPLC-MS/MS) of CYP2D and CYP2E1 were determined in the obtained fractions. Increasing the concentrations of calcium chloride progressively increased the protein yield of the low-speed microsomal fractions. However, the increased yield was associated with a significant decrease in the activities of CPR, CYP2D, and CYP2E1. Additionally, the CYP2D and CYP2E1 activities were significantly correlated with the CPR activities of the fractions. In conclusion, when an ultracentrifuge is available, preparation of brain microsomes by the ultracentrifugation method might be preferable. However, the calcium aggregation method at a calcium chloride concentration of 10 mM is an acceptable alternative to the ultracentrifuge method.

Introduction

The cytochrome P450 (P450) enzymes are responsible for the metabolism of most xenobiotics and endogenous compounds. In addition to the liver, which is the main organ with the highest P450 content, P450 enzymes are also expressed in extra-hepatic tissues such as the brain [1]. The distribution of P450 enzymes in different brain regions is not uniform, with the highest P450 content found in the brain stem and cerebellum, while the striatum and hippocampus reportedly contain the lowest P450 content [2]. Although the total P450 content in the brain is only 0.5% - 2% of that in the liver [3, 4], brain P450 enzymes are concentrated in specific cells and important brain regions, such as the blood-brain barrier [5]. Therefore, brain P450 may significantly impact the pharmacologic activities or toxicity of the centrally-acting drugs and toxins [1, 3-5].

Cytochrome P450 enzymes are primarily associated with the endoplasmic reticulum as membrane-bound enzymes, which form microsomes during the homogenization of the tissues and cell break up. Traditionally, microsomes have been prepared by ultracentrifugation of the post-mitochondrial supernatant at ≥100,000 g for ≥ 1 hour. In 1971, Kamath et al. [6] reported a simplified method for the preparation of liver microsomes using aggregation of the post-mitochondrial fractions by adding 8 mM calcium chloride ($CaCl_2$), followed by a low-speed centrifugation. The method was later adapted to the preparation of brain microsomes [7-9] to improve the low yield of the relatively unstable brain microsomal proteins and preserve the enzymatic activity of the brain P450 by presumably reducing the preparation time. However, a side-by-side comparison of the two methods with regard to the effects of $CaCl_2$ concentration on the microsomal yield and P450 monooxygenase activities is lacking in the literature. Therefore, the aim of the present study was to determine the effects of various concentrations of calcium

chloride on the microsomal protein yield and P450 activities in comparison with the ultracentrifugation method.

Materials and Methods

Chemicals

Dextromethorphan (DXM), dextrorphan (DXT), DXT-d_3, chlorzoxazone (CZX), 6-hydroxychlorzoxazone (HCZX), and HCZX-d_2 were purchased from Cerilliant (Round Rock, TX). NADPH was purchased from Sigma Aldrich (St. Louis, MO). All other reagents and chemicals were obtained from commercial sources.

Preparation of Microsomes from Whole Rat Brain Using $CaCl_2$ with Low-Speed Centrifugation

The preparation of microsomes using $CaCl_2$ was based on minor modifications of published methods [7, 8]. The modifications consisted of the addition of a low-speed (1,000 g) centrifugation of brain homogenates to remove cell debris and mitochondrial removal centrifugation speed of 10,000 g [6] instead of 17,000 g. Frozen whole brains from adult (8 to 12-week old) male Sprague-Dawley rats were purchased from Innovative Research (Novi, Michigan, USA). Individual, whole brains (n = 20) were homogenized with Potter-Elvehjem homogenizer in 9 volume of Tris buffer (0.1 M, pH = 7.4), containing 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 22 μM butylated hydroxytoluene, 0.2 mM EDTA, 150 mM potassium chloride, and 10% (v/v) glycerol at PH 7.4 (Buffer A). The homogenate was centrifuged at 1,000 g for 15 min at 4°C to remove cell debris and nuclei. The supernatant was collected and centrifuged at 10,000 g for 30 minutes to remove mitochondria. The mitochondrial pellet was resuspended and spun again at 10,000 g for 30 minutes, and the supernatant was collected to retrieve any trapped microsomes. The supernatants from the two 10,000 g spins were combined, and $CaCl_2$ was added

to achieve a final concentration of 10, 25, 50, or 100 mM (n = 5 brains per concentration). After 30 minutes of incubation with $CaCl_2$ at 4°C, the samples were centrifuged at 39,000 g for 1 hour at 4°C. The pellet was collected and resuspended in buffer A and spun again at 39,000 g for 1 hour at 4°C. The microsomal pellet was resuspended in buffer A and stored at -80°C.

Preparation of Microsomes from Whole Rat Brain Using the Ultracentrifugation Method

To prepare microsomes using the ultracentrifugation method (n = 6), the homogenization and the removal of cell debris and mitochondria steps were similar to the procedure outlined above for the $CaCl_2$ method. However, instead of incubation with $CaCl_2$, the combined post-10,000 g supernatants were spun at 110,000 g for 1 hour at 4°C. The pellet was resuspended in buffer A and spun again at 110,000 g for 1 hour at 4°C. The pellet was collected, resuspended in buffer A, and stored at -80°C.

Protein Assay

Microsomal protein concentrations were estimated by the Bradford method using bovine serum albumin (Thermo Scientific, Waltham, MA) as the standard.

Cytochrome P450 Reductase Activity

Cytochrome P450 reductase (CPR) activity was determined based on the reduction of cytochrome c by the brain microsomes using an established spectrophotometric method [10].

Dextromethorphan-O-Demethylation Activity

In rats, CYP2D1-5 isoforms show significant homology and substrate overlap, including dextromethorphan-O-demethylation, with the human CYPD6 [11]. The rat brain CYP2D activity was measured based on dextrorphan (DXT) formation from dextromethorphan (DXM) at a substrate concentration of 200 µM and a reaction volume of 200 µL. Briefly, DXM in methanol was added to microcentrifuge tubes, and methanol was evaporated before adding 100 mM Tris

buffer (pH 7.4) and microsomal protein (0.2 mg/mL). The mixtures were vortex-mixed and pre-incubated at 37°C for 5 min. Subsequently, the reaction was started by the addition of NADPH (0.25 mM). After 30 min of incubation at 37°C, the reaction was terminated by the addition of 150 µl acetonitrile, containing 10 nM DXT-d_3 as an internal standard. The reaction mixtures were vortex-mixed, placed on ice for 5 min, centrifuged for 5 min, and the supernatant was stored at –80°C before analysis by UPLC/MS/MS.

Chlorzoxazone 6-Hydroxylation Activity

The CYP2E1 activity was measured based on the formation of 6-hydroxychlorzoxazone (HCZX) from chlorzoxazone (CZX). Briefly, the reaction mixture (200 µL) contained 750 µM CZX and 0.4 mg/mL microsomal protein in 100 mM Tris-HCl buffer (pH 7.4). The mixtures were vortex-mixed and pre-incubated at 37°C for 5 min. The reaction was then started by the addition of NADPH (0.25 mM). After 15 min of incubation at 37°C, the reaction was terminated by the addition of 150 µl acetonitrile, containing 10 nM HCZX–d_2 as an internal standard. The reaction mixtures were vortex-mixed, placed on ice for 5 min, centrifuged for 5 min, and the supernatant was stored at –80°C before analysis by UPLC/MS/MS.

UPLC-MS/MS Analysis

The concentrations of DXT in the CYP2D assay and HCZX in the CYP2E1 assay were quantitated by UPLC-MS/MS (Bruker EVOQ; Billerica, MA), based on the previously reported methods [12, 13] with some modifications. Briefly, the chromatographic separation was achieved for both analytes using a Phenomenex Kinetex 1.7 µm C18 (100 A, 100 x 2.1 mm) column, connected to a Phenomenex C18 SecurityGuard ULTRA (2.1 mm) pre-column and maintained at 40°C. A gradient system consisting of solvent A (5 mM ammonium formate: formic acid, 100:0.05) and solvent B (acetonitrile: methanol: formic acid, 95:5:0.05), and a flow rate of 0.2

mL/min were used in both assays. For the DXT assay, the gradient conditions were 0–0.5 min, 10% B; 0.5–7 min, linear gradient 10–90% B; 7–10 min, 90% B; 10 min, 10% B; 10–13 min, 10% B. For the HCZX assay, the gradient conditions were 0–0.5 min, 10% B; 0.5–4 min, linear gradient 10–90% B; 4–7 min, 90% B; 7 min, 10% B; 7–9 min, 10% B. Under these conditions, the retention times were 3.2 min for DXT and 3.75 min for HCZX.

The mass spectrometer ion source was operated in positive ion mode for DXT and negative ion mode for HCZX analyses. Selected reaction monitoring for the parent/fragment transitions (m/z) were 258→156.90 for DXT, 261→156.90 for DXT-d_3, 184→64.2 for HCZX, and 186→65.9 for HCZX-d_2. Calibration standards, which were prepared in the microsomal matrix, were linear in the range of 0.25 to 50 nM for DXT or HCZX.

Data Analysis

The microsomal yield was estimated as the total microsomal protein obtained per each g of brain tissue. The activities of CPR, CYP2D, and CYP2D1 were expressed as per mg of the microsomal proteins (i.e., specific activity). The statistical differences between the ultracentrifugation and the calcium aggregation methods containing different concentrations of $CaCl_2$ were analyzed by one-way ANOVA, followed by Bonferroni's post-hoc analysis of the individual means. In all cases, a p value of < 0.05 was considered significant. Data are presented as mean ± SD.

Results

The protein yields and CPR activities of the microsomal fractions after the ultracentrifugation method and the 39,000 g fraction after treatment with different concentrations of $CaCl_2$ are presented in Figure 1. Calcium aggregation method using a 10 mM concentration of $CaCl_2$ resulted in significantly less protein yield than the ultracentrifugation method (Fig. 1A). An

increase in the concentrations of $CaCl_2$ up to a concentration of 100 mM resulted in a progressive increase in the protein yield. The yield at 100 mM $CaCl_2$ (5.15 mg/g brain tissue) was 2.8 fold higher than that after the 10 mM $CaCl_2$ concentration (1.87 mg/g brain tissue) and 1.6 fold higher than that after the ultracentrifugation method (3.14 mg/g brain tissue) (Fig. 1A). In terms of CPR activity, the $CaCl_2$ aggregation fractions at all the tested concentrations contained significantly less CPR activity than that in the microsomal fraction obtained by the ultracentrifugation method (Fig. 1B).

The CYP2D and CYP2E1 enzymatic activities of the ultracentrifugation and calcium aggregation fractions are presented in Fig. 2. For both isoenzymes, there were no significant differences between the ultracentrifugation and the 10 mM calcium aggregation fractions in terms of their activities. However, the activities of both isoenzymes in the fractions obtained in the presence of 25–100 mM $CaCl_2$ were significantly lower than those obtained after ultracentrifugation.

The extent of correlations between CYP2D or CYP2E1 activities and the CPR activities are presented in Fig. 3. Both CYP2D (Fig. 3A) and CYP2E1 (Fig. 3B) activities were significantly ($p < 0.0001$) correlated with the CPR activities of the fractions. The coefficients of determination (r^2) values were 0.7695 and 0.5187 for the CYP2D and CYP2E1 activities, respectively, suggesting that 77% (CYP2D) and 52% (CYP2E1) of the variations in the activities of the isoenzymes obtained by different methods and concentrations of $CaCl_2$ are due to the changes in the CPR activities.

DISCUSSION

The brain contents of P450 enzymes are much lower than those in the liver [3, 4]. Additionally, it has been suggested that because of the high lipid content of the brain, brain P450

enzymes are more sensitive to lipid peroxidation and degradation during microsomal preparations [8]. Therefore, to maximize the yield of microsomal proteins obtained from the brain samples, previous studies [7-9] have suggested calcium aggregation as an alternative to ultracentrifugation. However, the effects of calcium chloride concentrations on the yield and/or the quality of the obtained fractions from the brain have not been studied. Our results presented here clearly show that although the calcium aggregation method may result in higher protein yields at higher concentrations of $CaCl_2$ (Fig. 1A), the increase in the protein yield is associated with reduced activity of CPR (Fig. 1B) as well as the monooxygenase activities of CYP2D (Fig. 2A) and CYP2E1 (Fig. 2B). It has been reported that calcium ions precipitate many proteins in a concentration-dependent manner [14]. Therefore, it is likely that the concentrations of $CaCl_2$ higher than 10 mM results in sedimentation of other cellular organelles and proteins, besides smooth endoplasmic reticulum.

The first study reporting the calcium aggregation method for the brain microsomes [9] used 8 mM of $CaCl_2$ after a 27,000 or 15,000 g centrifugation of a 20-25% rat brain homogenate. The average protein yield for the calcium aggregation methods was similar to or higher than that for the ultracentrifugation method. However, the P450 monooxygenase activities of the microsomal fractions were not determined in that study. Additionally, the authors reported that the protein yield in the calcium aggregation method was dependent on the concentration of the original homogenate; when a 15% brain homogenate was used, the yield was 3-4 times lower than that for the 25% homogenate [9]. In our studies, the protein yield in the 10 mM $CaCl_2$ method was 40% lower than that in the ultracentrifugation method, which might be due to the much lower concentration of brain homogenate (10%) in our studies.

Later studies by other groups indicated similar microsomal yields for both methods with either similar [7] or even higher [8] P450 enzymatic activities for the calcium aggregation method. However, these studies [7, 8] used different homogenization or storage buffers and ingredients for the ultracentrifugation and calcium aggregation methods, which might have affected the quality of the obtained fractions and the activity of the enzymes.

The original calcium aggregation method applied to liver microsomes [6] presented the method's speed as one of its advantages over the traditional ultracentrifugation method. However, the length of the calcium aggregation method applied to the brain microsomes was similar to or even slightly longer than that for the ultracentrifugation method [7, 8]. Similarly, in our studies, the length of the centrifugation steps for the two methods was the same, with the calcium aggregation method requiring an extra 30 min time for incubation with Ca_2Cl.

In conclusion, increasing the concentrations of $CaCl_2$ from 10 to 100 mM to prepare brain microsomes using the calcium aggregation method progressively increases the protein yield. However, the increased protein yield is associated with decreases in the CPR and monooxygenase activities of P450. Additionally, a side-by-side comparison of the calcium aggregation and the ultracentrifugation methods does not indicate any superiority for the calcium aggregation method when both the microsomal yield and monooxygenase activities are considered. Therefore, when an ultracentrifuge is available, preparation of brain microsomes by the ultracentrifugation method might be preferable. However, the calcium aggregation method at a $CaCl_2$ concentration of 8 or 10 mM is an acceptable alternative to the ultracentrifuge method.

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Statement of Ethics

The authors have no ethical conflicts to disclose. Because no human or animal research was conducted as part of these studies, the report did not require ethical committee approvals.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.M., B.N.D., and F.A. designed the studies. B.N.D. and F.A. carried out the experiments. RM, BND, and FA analyzed the data and provided the initial draft for the manuscript. R.M. wrote the final version of the manuscript. All the authors approved the final version of the manuscript.

Data Availability Statement

The raw data for Figures 1-3 are available from the corresponding authors by request.

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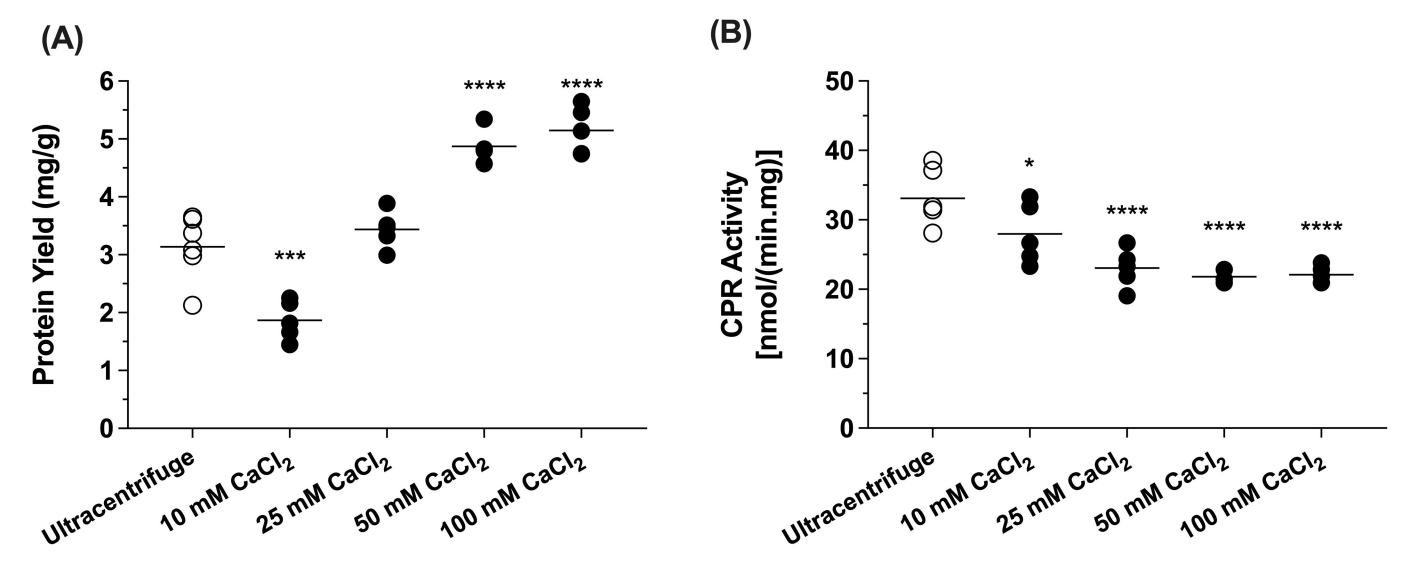
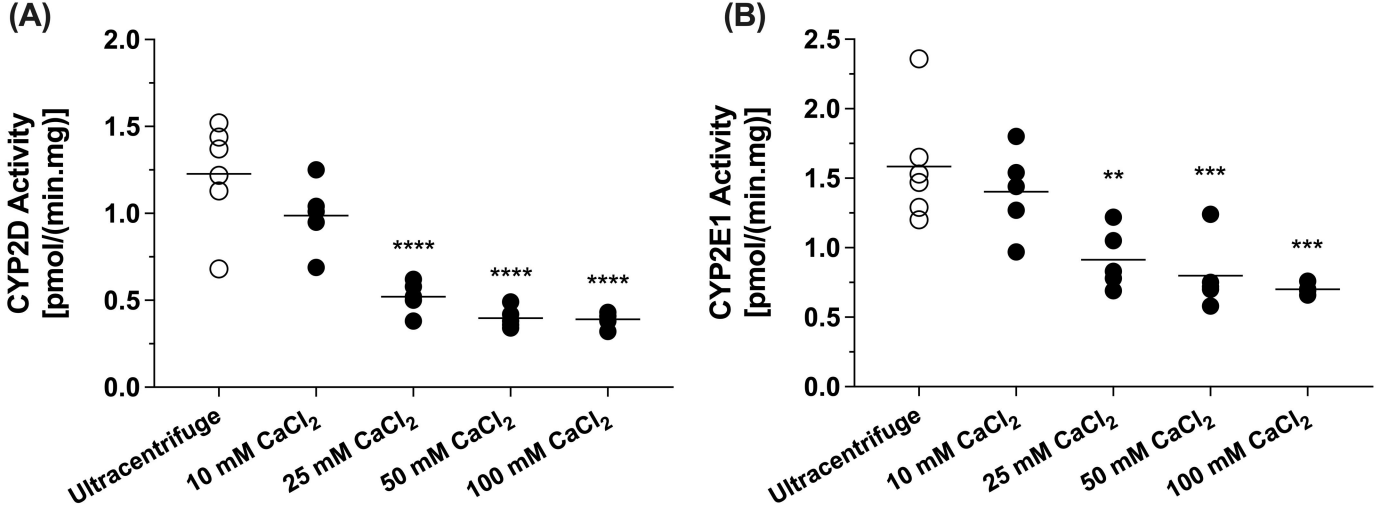
Legends to Figures

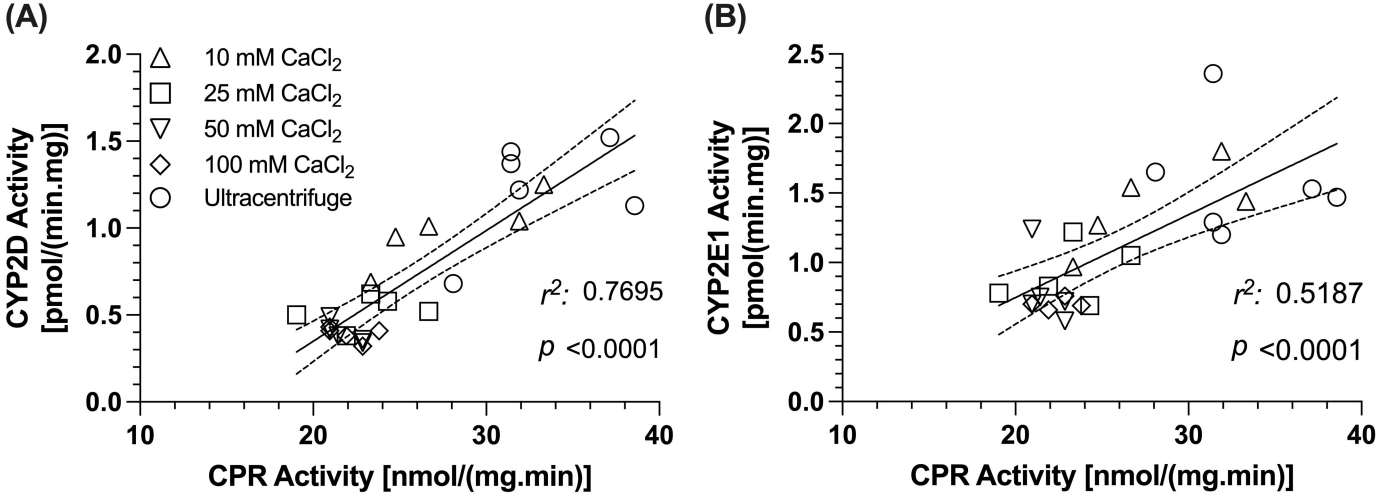
Fig. 1. Protein yield (A) and cytochrome P450 reductase (CPR) activities (B) of the brain microsomal fractions obtained by the ultracentrifugation method or calcium aggregation method using different concentrations (10-100 mM) of calcium chloride. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Fig. 2. CYP2D (A) and CYP2E1 (B) activities of brain microsomal fractions obtained by the ultracentrifugation method or calcium aggregation method using different concentrations (10-100 mM) of calcium chloride. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Fig. 3. The correlations between the CYP2D (A) or CYP2E1 (B) activities and cytochrome P450 reductase (CPR) activities of the brain microsomal fractions obtained by the ultracentrifugation method or calcium aggregation method using different concentrations (10-100 mM) of calcium chloride.


(A)
Protein Yield (mg/g)
0
1
2
3
4
5
6
***
****
****
Ultracentrifuge
10 mM CaCl2
25 mM CaCl2
50 mM CaCl2
100 mM CaCl2
(B)
CPR Activity [nmol/(min.mg)]
0
10
20
30
40
50
*
****
****
****
Ultracentrifuge
10 mM CaCl2
25 mM CaCl2
50 mM CaCl2
100 mM CaCl2


(A)
CYP2D Activity [pmol/(min.mg)]
2.0
1.5
1.0
0.5
0.0
****
****
****
Ultracentrifuge
10 mM CaCl2
25 mM CaCl2
50 mM CaCl2
100 mM CaCl2
(B)
CYP2E1 Activity [pmol/(min.mg)]
2.5
2.0
1.5
1.0
0.5
0.0
**
***
***
Ultracentrifuge
10 mM CaCl2
25 mM CaCl2
50 mM CaCl2
100 mM CaCl2


(A)
10 mM CaCl2
25 mM CaCl2
50 mM CaCl2
100 mM CaCl2
Ultracentrifuge
CYP2D Activity [pmol/(min.mg)]
CPR Activity [nmol/(mg.min)]
r2: 0.7695
p <0.0001
(B)
CYP2E1 Activity [pmol/(min.mg)]
CPR Activity [nmol/(mg.min)]
r2: 0.5187
p <0.0001