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Chromatographic assays of drug oxidation by human cytochrome P450 3A4

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Abstract

Cytochrome P450 enzymes (P450s) are heme-thiolate mono-oxygenases involved in the oxidation of many endogenous and exogenous substrates. Herein, we describe two protocols for measuring the activity of a key enzyme of drug metabolism, P450 3A4. In this protocol, the substrate is incubated with human liver microsomes, the reaction is quenched, and the substrates and products are extracted and subjected to liquid chromatography (LC) separation and detection. Oxidation of the calcium-channel blocker nifedipine is measured using UV-Vis spectroscopy in-line with high performance liquid chromatography (HPLC). 6 β -Hydroxytestosterone formation from testosterone is measured by HPLC coupled to mass spectrometry (MS). Both of these procedures are rapid, requiring 2 h or less, and can be used to confirm and measure P450 3A4 activity and can also be used as a guide for developing other assays for measuring P450 catalysis. The separation strategy described here is more rapid than many available methods, except when ultra-performance liquid chromatography (UPLC) is used.

INTRODUCTION

Cytochrome P450s (P450s) are heme-containing enzymes that are involved in the detoxication of many exogenous compounds, as well as the synthesis and metabolism of steroids, fatty acids, bile acids and eicosanoids. P450s are typically associated with hydroxylation reactions, but they have also been shown to catalyze many others, including reduction, desaturation, ring expansion and aromatization reactions¹. P450 3A4 has received much attention because, in part, of its importance in drug metabolism; it is involved in the metabolism of ~50% of the currently marketed therapeutics^{2,3}. P450 3A4 is the most abundant P450 in the liver and can catalyze reactions involving >1000 exogenous and endogenous compounds, including molecules as diverse as erythromycin, nifedipine, aflatoxin B₁, progesterone and testosterone. Variation among individuals in the expression of the *CYP3A4* gene, as well as dramatic effects of drug inducers and inhibitors, can lead to fast and slow metabolizing phenotypes and result in differences in drug response⁴⁻⁶. The ability of P450 3A4 to accommodate differently shaped substrates can be attributed at least, in part, to the relatively large active site, a feature shown in some of the most recent crystal structures of P450 3A4, which have either erythromycin, ketoconazole, metyrapone or progesterone (the latter at a peripheral site) bound⁷⁻⁹. P450 3A4 has also been shown to display cooperative kinetics in a substrate-selective manner¹⁰⁻¹³.

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AUTHOR CONTRIBUTIONS C.D.S. optimized the assays. C.D.S. wrote most of the paper, with the assistance of F.P.G. F.P.G. and Q.C. checked the protocols, and Q.C. helped with some of the mass spectrometry.

Rapid and sensitive assays to measure P450 3A4 catalysis are imperative for pharmaceutical research, characterizing new substrates, identifying possible mechanisms for catalysis, measuring cooperativity and characterizing mutant forms of enzymes and so on. Exploiting distinctive spectroscopic properties of substrates and products is a facile way to monitor product formation. UV–Vis spectroscopy (UV–Vis) is an inexpensive, accessible and sensitive tool that can be used to measure products of metabolism if the product has a UV–Vis spectrum differing from the substrate¹⁴. When UV–Vis is coupled to high performance liquid chromatography (HPLC), the UV–Vis scan profiles need not be different; absorbance of products and substrate are separated into discrete peaks that can then be quantified using an internal or external standard and peak area. When standards are available, products of P450 catalysis can be identified by overlaying UV–Vis scans of the product and the standard.

In addition to UV–Vis–HPLC, mass spectrometry (MS) is an obvious choice for studying the products of P450 catalysis because reactions generally involve a change in mass (e.g., addition of oxygen). Although identification and quantification can be possible using UV–Vis–HPLC, MS has proved to be superior in both sensitivity and specificity¹⁵. Further, it does not require any spectroscopic qualities of the substrates or products. First, separating the sample using LC further enhances sensitivity and allows the study of very complex samples. With the advent of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), compounds previously were only assayed with gas chromatography–MS (GC–MS) (which often requires chemical derivatization) are now often amenable for LC–MS study. Selective ion monitoring allows focusing on products of a chosen mass and has been successfully used in kinetic studies with P450s. The use of standards (either internal or external) allows for the quantification of product formation by integrating the signal.

In this protocol, two assays that highlight the use of UV–HPLC and HPLC–MS in monitoring product formation in P450 3A4 catalysis are presented. P450 3A4 was originally isolated because of its ability to oxidize the calcium channel blocking drug nifedipine to its inactive pyridine derivative (Fig. 1)¹⁶. Nifedipine oxidation has become a prototypic P450 3A4 reaction for study because product formation is easily measured by UV–HPLC¹⁷. Human liver microsomes are incubated in the presence of nifedipine, and after quenching the reaction with solvent, any remaining substrate and the pyridine derivative product are extracted with organic solvent, dried, and then separated and detected using UV absorbance in-line with HPLC. Baseline separation of product and substrate (A_{254}) can easily be achieved in <6 min using a simple water/methanol solvent system, even with conventional analytical columns. Product formation is optimum at pH 7.85 and is generally linear up to 20 min incubation times and over the range of 5–1,000 pmol P450 per ml (see below)¹⁷.

P450 3A4 (and other 3A family members, to varying extents) are capable of oxidizing testosterone at several positions, generating the 1 β -, 2 β -, 6 β - and 15 β -hydroxy products^{18–21}. The primary product of P450 3A4 testosterone oxidation is 6 β -hydroxytestosterone (Fig. 2) and, like nifedipine oxidation, this reaction has been used as a model for P450 3A4 kinetic studies. Human liver microsomes (or recombinant P450 3A4) are incubated with testosterone, and after quenching the reaction and extracting unreacted substrate and products with organic solvent, these compounds are separated using HPLC and detected with UV or MS. Well-devised LC programs are vital for separating the various products of oxidation in this reaction because they can have the same mass and fragmentation tendencies. Ionization of steroids can be difficult due to their lack of charge, but we have previously reported success with both ESI and, as reported here, APCI as ionization sources^{18,19}.

Both the procedures presented in this protocol can be completed within 1–2 h. Thus, this protocol can be used to quickly assess P450 3A4 activity. As working with purified P450 3A4 requires somewhat difficult lipid reconstitution, the assays described can also be used to confirm that the proteins required for an active system (P450 3A4, NADPH–P450 reductase and cytochrome *b*₅) have been incorporated into liposomes in a functional way²². Further, these protocols can be used as templates for developing other protocols for investigating different P450 3A4 substrates or other P450s. Plots of *v* versus *S* can easily be generated based on incubation reactions, described in these protocols, if a range of several substrate concentrations is used. A brief description of generating such a plot is described in Box 1.

Experimental design

The incubation procedure described here can be applied to most P450 activity measurement experiments using microsomes, although P450 concentration and incubation times may need to be optimized. To optimize time, various time points are taken (e.g., at a range of 5 min–1 h) at some saturating substrate concentration and the rates of product formation are measured. Product formation versus time is then plotted to determine the range of incubation times at which product formation is linear. Experiments should be designed within this range. The same procedure is followed when optimizing P450 concentrations, varying from 1–1,000 pmol of total P450, e.g., and plotting product formation versus P450 concentration. Regardless of the incubation time chosen, a 3–5 min pre-incubation step is required for the incubation mixture and the NADPH-generating system to equilibrate to the reaction temperature.

Although purified P450 can also be used for such studies, we have used microsomes in this example. Both nifedipine and testosterone are known P450 3A4 substrates, and using purified P450 3A4 requires the generation of a reconstituted membrane system, which is not a trivial process²². Extreme care should be taken in the preparation of human liver microsomes. Liver samples should be tested for HIV and hepatitis; however, extreme caution should be exercised. Safety precautions include wearing a disposable face mask, gloves and gown as well as goggles during the preparation of microsomes. Working in a fume hood on a disposable blotting pad is important in preventing inhalation of any aerosolized material and lessening the chance of fume hood contamination. All equipments should be properly disinfected and all waste should be labeled as biohazard and destroyed as appropriate. The procedures for obtaining both rat and human liver microsomes have been described in detail in previous work, and any precautions defined by the Institutional Review Board (IRB) should be followed^{17,23}. A spectral P450 assay can be used to determine the total P450 content¹⁷ (see also the protocol by Guengerich *et al.*²⁴).

In both the nifedipine and testosterone assays, methylene chloride is used to quench the incubation reactions. The organic solvent denatures the protein, effectively stopping catalysis and removing any associated substrate or product, and is used to extract the typically nonpolar substrate and products of P450 oxidation. The quench is followed by the addition of a salt solution to facilitate extraction into the organic phase (and makes the definition of the organic and aqueous layers clearer), and, in the case of nifedipine, a sodium carbonate buffer ensures that alkaline conditions are maintained to promote extraction of substrate and product into the organic layer. Centrifugation makes collection of the organic layer much easier and decreases the likelihood of water contamination; these spins do not need to be particularly fast, i.e., a $3 \times 10^3 g$ spin for 10 min should be sufficient for nearly all applications. If there is difficulty in avoiding trace amounts of water being collected with the organic layer, the volume of organic layer that is collected should either be decreased (this should be noted as it will affect any calculations used to determine the amount of product

formed), or the collected organic layer can be dried with anhydrous sodium sulfate and filtered.

The use of an NADPH-generating system is favored over simply adding NADPH to the incubation mixture because it ensures that NADPH is not the limiting reagent and also prevents the excessive buildup of NADP⁺, which is a competitive inhibitor of NADPH-P450 reductase. A key negative control is replacing the addition of the NADPH-generating system with water to an otherwise complete incubation mixture (i.e., containing microsomes, substrate and buffer). In this case, only the substrate should be observed in the LC chromatograms, helping to identify any product peaks. Any minor peaks found both in the negative control and test incubation can be used to determine the limit of detection for rates if a standard is available.

When using LC, the dried organic extract containing products and remaining substrate is redissolved in an organic solvent (or aqueous/organic mixture) before injection. Selection of the solvent will be determined by the polarity of the compounds (to ensure that they will fully dissolve). It is important to also select a solvent that has a UV cut-off below the monitored wavelength when using UV-HPLC (i.e., no absorbance at the wavelength used for product detection). Ideally, the compounds are dissolved in the initial solvent used for HPLC to reduce the 'injection peak' artifacts seen at the early time points in the chromatogram.

In the case of product formation measurement, compounds with either appropriate spectroscopic properties (UV-Vis) or the capability to ionize well (MS) are required for using these particular assays. Otherwise, tools not mentioned here (e.g., fluorescence coupled to HPLC or the use of GC-MS) may be useful. The use of ultra high performance liquid chromatography (UPLC) versus HPLC can reduce sample running time and solvent usage with little, if any, loss of resolution and sensitivity.

MATERIALS

REAGENTS

- Human liver microsomes (e.g., 500 pmol total P450 per incubation, i.e., ~1–2 mg protein) ● **CAUTION** Human liver microsomes are a biohazard. Use a fume hood and wear a gown, face mask, gloves and goggles during preparation. Ensure that handling follows the Institutional Review Board (IRB) protocol.¹⁷
- Potassium phosphate (Fisher, cat. no. P285 (monobasic) and P288 (dibasic))
- Glucose-6-phosphate (Sigma-Aldrich, cat. no. G7250)
- NADP⁺ (Sigma-Aldrich, cat. no. N0505)
- 10³ IU ml⁻¹ yeast glucose-6-phosphate dehydrogenase (Sigma-Aldrich, cat. no. G6378)
- Methanol (Fisher, cat. no. A452-4) ! **CAUTION** Flammable and toxic! Use goggles and work in a fume hood.
- Methylene chloride (Fisher, cat. no. D143-4) ! **CAUTION** Flammable and toxic! Use goggles and work in a fume hood.
- Acetonitrile (Fisher, cat. no. A998-4) ! **CAUTION** Flammable and toxic! Use goggles and work in a fume hood.
- Na₂CO₃ (Sigma-Aldrich, cat. no. S7795)

- NaCl (Fisher, cat. no. S641-212), prepared at 0.3 M in water
- Ammonium acetate (Sigma-Aldrich, cat. no. 32301)
- Nifedipine (Sigma Aldrich, cat. no. N7634)
- Testosterone (Steraloids, cat. no. A6950-000) ▲ **CRITICAL** Water for all buffers, incubations and so on should be purified using a Milli-Q (Millipore) system.

EQUIPMENT

- Glass 16 mm × 100 mm test tubes (Fisher, cat. no. 14-961-29)
- 8-ml amber glass vials (Wheaton Science Products, cat. no. 224984)
- Reacti-vials (5-ml amber and 3-ml clear) (Pierce, cat. nos. 13099 and 13222)
- Shaking water bath set at 37 °C (Amerex Instruments)
- Vortex device (Fisher Scientific)
- N₂ stream evaporating device (Organomation Associates)
- HPLC system (20 µl loop) capable of UV detection at 254 nm (e.g., Hitachi L-7100 pump, Hitachi; ThermoFinnigan SpectraPHORESIS UV3000 scanning UV detector, ThermoFinnigan)
- Octyldecylsilane (C₁₈) reversed-phase HPLC column (e.g., 6.2 mm × 80 mm, 3 µm, Agilent Technologies, for nifedipine assay; 4.6 mm × 250 mm, 5 µm, Phenomenex, for testosterone assay)
- APCI TSQ Quantum triple quadrupole mass spectrometer with Surveyor LC and autosampler (ThermoFinnigan) and Agilent 1100 VWD UV–Vis detector (Agilent) in-line

REAGENT SETUP

- Phosphate buffer: dissolve 25.9 g of monobasic potassium phosphate and 141.0 g dibasic potassium phosphate in 1000 ml H₂O to prepare a 1.0 M solution of pH 7.4. KOH can be used to prepare the 1 M pH 7.85 buffer. These buffers are stable for many months to a year if stored at 4 °C.
- Prepare glucose-6-phosphate at 0.1 M in water. If stored frozen as stocks at –20 °C, it remains stable for many months to a year.
- Prepare NADP⁺ at 10 mg ml^{–1} in water. If stored frozen as stocks at –20 °C, it remains stable for many months to a year.
- Prepare 10³ IU ml^{–1} yeast glucose-6-phosphate dehydrogenase at 1 mg ml^{–1} in 10 mM Tris-acetate buffer (pH 7.4) with 1 mM EDTA and 20% glycerol (vol/vol). If stored as stocks at –20 °C, it remains stable for several months.
- NADPH-generating system: combine 50 parts 0.1 M glucose-6-phosphate, 25 parts 10 mg ml^{–1} NADP⁺ and 1 part 1 mg ml^{–1} yeast glucose-6-phosphate dehydrogenase. ▲ **CRITICAL** This system must be prepared fresh daily and stored on ice until use.
- Prepare Na₂CO₃ at 1 M in 2 M NaCl buffer (pH 10.5). This buffer is stable for many months to a year if stored at 4 °C.
- Prepare NaCl at 0.3 M in water. This solution is stable for many months to a year if stored at 4 °C.

- Recrystallize nifedipine from ethanol in amber glass in the dark (m.p. 171 °C–174 °C) (heat in ethanol to dissolve, filter if necessary and allow to cool slowly, then recover crystals by filtration or centrifugation). Dissolve at 20 mM in methanol. Recrystallized nifedipine can be stored at room temperature (25 °C) in an amber vial for at least several months. If stored in solution at –20 °C in an amber glass Teflon-sealed vial to prevent evaporation, this solution will remain stable for at least a month.
- Prepare testosterone at 10 mM in methanol. If stored at –20 °C in a glass Teflon-sealed vial to prevent evaporation, this solution is stable for at least several months.
▲ CRITICAL All of the above buffers, microsomes, substrates and components of the NADPH-generating system should be stored on ice until use. However, the incubation reactions are carried out at 37 °C and must be equilibrated to reach 37 °C before initiating the reaction.

EQUIPMENT SETUP

- For the UV–HPLC analysis of nifedipine oxidation, set up an isocratic solvent system of 45% water and 55% methanol (vol/vol) at a flow rate of 2–4 ml min^{–1}. Monitor absorbance at 254 nm.
- For UV–HPLC–MS analysis of testosterone oxidation, prepare solvent A (95% water, 5% acetonitrile (vol/vol) and 10 mM CH₃CO₂NH₄) and solvent B (99% acetonitrile, 1% water (vol/vol) and 10 mM CH₃CO₂NH₄). The following gradient is used with a 0.8 ml min^{–1} flow rate: 78% A from 0–5.5 min, linear gradient to 64% A 5.5–12 min, hold at 64% A from 12–20 min, linear gradient to 20% A from 20–22 min and hold at 20% A for 26 min. Monitor absorbance at 244 nm. Then connect the Surveyor LC to the Agilent UV–Vis detector and then to the TSQ Quantum mass spectrometer using APCI operating in the positive ionization mode. Use the following APCI conditions: sheath gas pressure (N₂) 35 p.s.i, APCI vaporizer temperature 400 °C, capillary temperature 300 °C, tube lens offset 100 V and discharge current 11.0 μA. Note that if a different type of MS is used, the APCI conditions will have to be optimized.

PROCEDURE

Nifedipine and testosterone oxidation by human liver microsomes: Incubation • TIMING 1 h 1

1. Prepare an incubation solution of 0.1 M potassium phosphate buffer (pH 7.85 for nifedipine or pH 7.4 for testosterone incubations), human liver microsomes containing 5–1000 pmol of P450 (here, 500 pmol), and 200 μM nifedipine in methanol (1% organic solvent, vol/vol) or 100 μM testosterone in methanol (1% organic solvent vol/vol). Use an 8-ml amber glass vial for nifedipine incubations or a glass test tube for testosterone incubations. The final volume should be 425 μl.

! CAUTION Human liver microsomes are a biohazard. Use a fume hood and wear a gown, face mask, gloves and goggles during preparation. Ensure that handling follows the Institutional Review Board (IRB) protocol¹⁷.

▲ CRITICAL STEP In the case of nifedipine, Steps 1–8(A) need to be carried out in amber glass vials because nifedipine is light sensitive. Do not use aluminum foil, because this only serves as a mirror.

- 2 In separate vials pre-incubate the incubation solution and the NADPH-generating system in a shaking water bath (~50 r.p.m.) at 37 °C for 3 min.

Prepare a sufficient volume of NADPH-generating system to add 75 μ l to each incubation tube.

- 3 Add 75 μ l of the NADPH-generating system to the incubation solution to start the oxidation reaction.
- 4 After 10 min at 37 °C, quench the incubation solution reaction with 2 ml of methylene chloride and mix with a vortex device. If assaying nifedipine, add 100 μ l of 1 M Na₂CO₃ buffer (pH 10.5) containing 2.0 M NaCl. Mix with a vortex device. If assaying testosterone, add 1 ml of 0.3 M NaCl. Mix with a vortex device.
- 5 Centrifuge the incubation solution for 10 min at 3×10^3 g, room temperature, to separate the two layers.
- 6 Remove 1.5 ml of the bottom organic layer using a long-tipped glass pipette and bulb and transfer to an amber Reacti-vial, in the case of nifedipine, or a clear reacti-vial, in the case of testosterone.

▲ **CRITICAL STEP** Do not use plasticware.

- 7 Dry the organic layer under an N₂ stream without heating (this usually takes about 30 min or less, keep the vials in a water bath at room temperature because evaporation will cool the samples).

Detection of P4503A4 generated metabolites • TIMING 10 min–1 h

- 8 Detection of metabolites can be carried out using option A for nifedipine oxidation or option B for 6 β -hydroxylation of testosterone.

A. Detection of nifedipine oxidation

- i. Redissolve the dried nifedipine samples from Step 7 in 30 μ l of methanol, vortex to mix.
- ii. Inject 20 μ l into HPLC, monitor at A₂₅₄ for 6 min. ?

TROUBLESHOOTING

B. Detection of 6 β -hydroxylation of testosterone

- i. Redissolve the dried testosterone samples from Step 7 in 35 μ l of methanol, vortex to mix.
- ii. Inject 20 μ l into HPLC, monitor at A₂₄₄.
- iii. Run a total ion scan from m/z 200 to 1500. From this, extract scans of m/z 289 (singly positively charged testosterone) and m/z 305 (singly positively charged hydroxytestosterone products).

? TROUBLESHOOTING

● TIMING

Steps 1–7, Nifedipine and testosterone oxidation by human liver microsomes: 1–1.5 h

Step 8, Option A: Detection of nifedipine oxidation: 10 min

Step 8, Option B: Detection of 6 β -hydroxylation of testosterone: 1 h

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

Sample UV chromatograms for oxidation of nifedipine and testosterone by P450 3A4 are shown in Figures 3 and 4, respectively. In Figure 3a, a negative control is shown where the NADPH-generating system is omitted. The peak at the retention time (t_R) = 4.9 min is the substrate. In Figure 3b, the formation of the pyridine product is seen at t_R = 3.27 min. Figure 4a also shows a negative control, which lacks the NADPH-generating system and the substrate is seen at t_R = 25.0 min. In Figure 4b, generation of the primary product (6 β -hydroxytestosterone) is seen as a peak at t_R = 14.4 min. A total ion chromatogram (TIC) is shown in Figure 5a. Chromatograms monitoring m/z 305 (singly hydroxylated testosterone ($[M+H]^+$) products) and m/z 289 (testosterone ($[M+H]^+$) substrate) and are shown in Figure 5b,c, respectively. At least three singly hydroxylated products can be seen in Figure 5b, with the primary product t_R = 14.4 min, corresponding to 6 β -hydroxytestosterone. Typically 10% or less of substrate is turned over to product.

Internal or external product standards can be used to convert peak area to nmol of a product (examples of internal standards can include diazepam in the case of the nifedipine assays and deuterated testosterone for the testosterone assays)²⁵. After the amount of product is calculated (in nmol), this number is divided by the total P450 (in nmol) in the human liver microsomes sample and the time of incubation, generating the rate of oxidation in nmol product formed per min per nmol P450 (i.e., min^{-1}). Further, if several concentrations of substrate are used, oxidation rates can be plotted against substrate concentration to generate v versus S plots. An example of a procedure for producing these types of plots is shown in Box 1.

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BOX 1 | SAMPLE RATE CALCULATIONS FOR GENERATING V VERSUS S PLOTS

1. Obtain a product standard, make up a suitable dilution and run this on the HPLC system. Find the peak area and correlate this to the number of nmol. You will use this conversion factor for future calculations. Ideally, a standard curve can be generated using several dilutions of the standard.
2. Determine the area of the peak corresponding to the product from an experimental chromatogram and convert this to nmol of product using the conversion factor obtained in (1). Calculate the nmol of P450 used in the incubation reaction.
3. Apply dilution factors (this may include corrections for the volume removed from the total organic layer during the extraction step, the volume injected into the HPLC versus the volume in which the dried extract was redissolved).
4. Calculate rates:

$$\nu \left(\text{in } \text{min}^{-1} \right) = \frac{\frac{\text{nmol of product}}{\text{nmol of P450}}}{\text{min of incubation time}}$$

5. Plot each of these rates against the starting substrate concentration. Several substrate concentrations need to be selected, particularly at low concentrations. Typically, such curves yield hyperbolic fits, but other non-linear fit methods may be required (e.g., the use of sigmoidal fits in cases of positive cooperativity). In general, a wide range of substrate concentrations is chosen initially, and after plotting these data, a second experiment is conducted where the focus is on the range of very low concentrations to discern hyperbolic versus sigmoidal trends through saturation.

Figure 1.
Oxidation of nifedipine by P450 3A4.

Figure 2.
6β-Hydroxylation of testosterone by P450 3A4.

Figure 3.

UV-HPLC chromatogram of nifedipine oxidation by human liver microsomes, monitoring at A_{254} . Institution Review Board (IRB) permission was obtained for working with human liver. **(a)** Incubation of nifedipine and human liver microsomes without the NADPH-generating system. The nifedipine substrate is shown at $t_R=4.9$ min. **(b)** Incubation of nifedipine and human liver microsomes with the NADPH-generating system, showing the pyridine derivative product at $t_R=3.3$ min.

Figure 4.

UV-HPLC chromatogram of testosterone oxidation by human liver microsomes, monitoring at A_{244} . Institution Review Board (IRB) permission was obtained for working with human liver. **(a)** Incubation of testosterone and human liver microsomes without the NADPH-generating system, with the testosterone substrate shown at $t_R = 25.0$ min. **(b)** Incubation of testosterone and human liver microsomes with the NADPH-generating system, with the 6β -hydroxytestosterone product shown at $t_R = 14.4$ min.

Figure 5.

HPLC–MS chromatogram of testosterone oxidation by human liver microsomes. Institution Review Board (IRB) permission was obtained for working with human liver. **(a)** Total ion chromatogram. **(b)** Ion chromatogram of m/z 305, which corresponds to the positive ion singly hydroxylated testosterone products ($[M+H]^+$). The 6β -hydroxytestosterone product shown at $t_R = 14.4$ min. **(c)** Ion chromatogram of m/z 389, which corresponds to the positive ion testosterone substrate ($[M+H]^+$ at m/z 289), shown at $t_R = 25.1$ min.

TABLE 1

Troubleshooting guide for nifedipine and testosterone reactions.

Step	Problem	Possible reason	Solution
8A(ii) 8B(iii)	No formation of products	Inactive NADPH-generating system	Verify that the NADPH-generating system is working. Combine 100 μl of 1 M potassium phosphate (pH 7.4), 100 μl of 0.1 M glucose-6-phosphate, 50 μl of 10 mg ml^{-1} NADP ⁺ and 750 μl of water in a cuvette (glass or Pyrex). Monitor the A_{340} for several seconds. Add 2 μl of 1 mg ml^{-1} yeast glucose-6-phosphate and then monitor for ~1 min, which should result in a rapid (~1 min) increase in absorbance (to >1.0) as NADPH is generated (ϵ_{340} for NADPH = 6.22 $\text{mM}^{-1} \text{cm}^{-1}$)
8A(ii) 8B(iii)	Inconsistent amounts of product formation	Failure to pre-incubate NADPH-generating system or incubation solution	Pre-incubate both for 3 min at 37 °C