Development of Label-Free, Enzymatic CYP17A1 Assays Using the Agilent 6150 Single Quadrupole LC/MS System

Application Note

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Abstract

This application note describes the development of fast, label-free in vitro enzymatic assays for cytochrome P450, 17α-hydroxylase/C17,20-lyase (CYP17A1), which are adapted to a high throughput 96-well format. Enzymatic activity was characterized by monitoring the conversion of natural steroid substrates to products, and their subsequent detection using an Agilent 6150 Single Quadrupole LC/MS System. These highly sensitive assays are performed at physiologically relevant conditions and are suitable for screening potential CYP17A1 inhibitor candidates in drug discovery assays.

Introduction

In humans, CYP17A1 plays a key role in the biosynthesis of steroid hormones.[1] This 56 kDa steroidogenic cytochrome P450 (CYP) is located in the zona fasciculata and zona reticularis of the adrenal cortex (and the gonad tissues), and has dual functions: hydroxylation of pregnenolone or progesterone at the C17 position, and cleavage of the C17-C20 bond of 17 α-hydroxypregnenolone or 17 α-hydroxyprogesterone to form dehydroepiandrosterone and androstenedione respectively. CYP17A1 dysfunction has been associated with a number of diseases, including polycystic ovary syndrome,[5, 7, 8] Cushing's syndrome,[6] congenital adrenal hyperplasia,[4] and prostate cancer.[2]

Numerous pharmaceutical companies are engaged in discovery projects in order to identify potent and selective inhibitors of CYP17-hydroxylase and CYP17-lyase. This application note describes the development of two in vitro enzymatic assays that can be used to measure the potency of New Chemical Entities (NCEs) against CYP17A1. The assays described herein are typical enzymatic assays; however, after the NCEs were tested the results were analyzed by using an Agilent 6150 Single Quadrupole LC/MS System with an Agilent Jet Stream source. Both assays are suitable for a high throughput, 96-well format and yield sensitive results due the detection method.

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Experimental

The same general procedure was performed for both enzymatic assays. The enzyme was mixed with the substrate and distributed to a 96-well reaction plate that was maintained at 37 °C. NCEs (test articles) were added to the reaction wells and allowed to equilibrate. The reaction was initiated by the addition of cofactors and stopped 10 minutes later by the addition of acetonitrile. The stopped plate was centrifuged and the supernatant was transferred to another 96-well plate for analysis by LC/MS.

Analytical measurements were performed by comparing the response (peak area) of the product formed to the product response of an active control containing only microsomes, cofactors, and substrate. Calibration curves were not prepared for substrates or products. The response of the mass spectrometer was assumed to be linear over the range measured. The IC_{50} value for each test compound combination was determined by plotting measured responses of product against the concentration of the test compound and finding the concentration of test compound which corresponds with a 50 % reduction in product response. XLfit (IDBS) was used to fit the dose response curves to the data and report the IC_{50}.

CYP17-hydroxylase assay conditions

The enzyme source was rat testicular microsomes. The protein concentration in the final reaction mixture was 100 µg/mL. Progesterone was present at 1 µM in the final reaction mixture. NADPH regeneration solution was present at 1x in the final reaction mixture. Test articles were typically present at a final concentration between 0.012 and 200 µM final. The contribution of organic solvent to the reaction mixture from the addition of test articles was 1 %. Dilutions were prepared in potassium phosphate buffer, pH 7.4. After 10 minutes, the reactions were stopped by the addition of a 2-fold excess of acetonitrile. The reaction product, 17α-hydroxyprogesterone, was quantified by LC/MS.

Table 1. LC/MS conditions for hydroxylase and lyase assays.

LC Conditions	
Column	Zorbax Poroshell 120 EC C18 (2.1 x 30 mm) at 55 °C
Autosampler	Injection volume: 5 µL 1 s needle wash in the flush port (methanol) Draw and eject speed: 200 µL/min Equilibration: 1.0 s Sample flush out factor: 4.0 Temperature: not thermostatted Delay volume reduction: automatic Overlapped injection enabled at 0.20 min
Pump	Solvent A: water with 0.1 % formic acid Solvent B: methanol with 0.1 % formic acid Flow rate: 0.80 mL/min
MS Conditions	
Agilent 6150 Single Quadrupole LC/MS System	Ionization source: Agilent Jet Stream - ESI positive Drying gas flow rate: 12.0 L/min Nebulizer pressure: 50 psig Drying gas temperature: 350 °C Sheath gas temperature: 350 °C Sheath gas flow rate: 12 L/min Capillary voltage: 3000 V Nozzle voltage: 300 V LC: Agilent 1290 Infinity LC System
Signal Settings	Peak width: 0.020 min Cycle time: 0.12 s/cycle Fragmentor voltage: 70 V Gain: 1.0 Dwell: 24 ms for each m/z

Table 2. CYP17-hydroxylase assay specific LC/MS conditions.

Pump	Stop time: 0.72 min	
Timetable	Time (min)	% B
	Initial	60
	0.50	68
	0.60	68
	0.61	100
	0.70	100
	0.71	60
6150 Single Quad Mass Spectrometer	Signal Settings Signal 1: Substrate M+H: 315.2 M+Na: 337.2 Signal 2: Product M+H: 331.2 M+Na: 353.2	

Table 3. CYP17-lyase assay specific LC/MS conditions.

Pump	Stop time: 0.62 min	
Timetable	Time (min)	% B
	Initial	55
	0.50	62
	0.51	100
	0.60	100
	0.61	55
6150 Single Quad Mass Spectrometer	Signal Settings Signal 1: Substrate M+H: 331.2 M+Na: 353.2 Signal 2: Product M+H: 287.2 M+Na: 309.2	

CYP17-lyase assay conditions

The enzyme source was rat testicular microsomes. The protein concentration in the final reaction mixture was 250 µg/mL. The substrate (17α-hydroxyprogesterone) was present in the final reaction mixture at 1 µM. NADPH regeneration solution was present at 1x in the final reaction mixture. Test articles were typically present at a final concentration between 0.012 and 200 µM. The contribution of organic solvent to the reaction mixture from the addition of test articles was 1 %. Dilutions were prepared in potassium phosphate buffer, pH 7.4. After 10 minutes, reactions were stopped with the addition of a 2-fold excess of acetonitrile. The reaction product, androstenedione, was quantified by LC/MS.

Results and Discussion

CYP17-hydroxylase assay

Development of the hydroxylase assay was successful as shown in Figure 1, a full scale chromatogram with product and substrate peaks at 8 µM inhibitor concentration. The void volume elutes at 0.1 minutes as evidenced by the suppression in the background. The peak at 0.7 minutes corresponds to the step up to 100 % organic. Figure 2 illustrates a close up view of the substrate and product peak at the same inhibitor concentration, which is near the IC_{50}. The signal is clearly sufficient to monitor both the substrate depletion and product formation. In the substrate chromatogram an interference peak elutes at 0.6 minutes. Resolution of the interference from the substrate required extending the run time out to 0.7 minutes. The overall injection-to-injection time was 0.9 minutes.

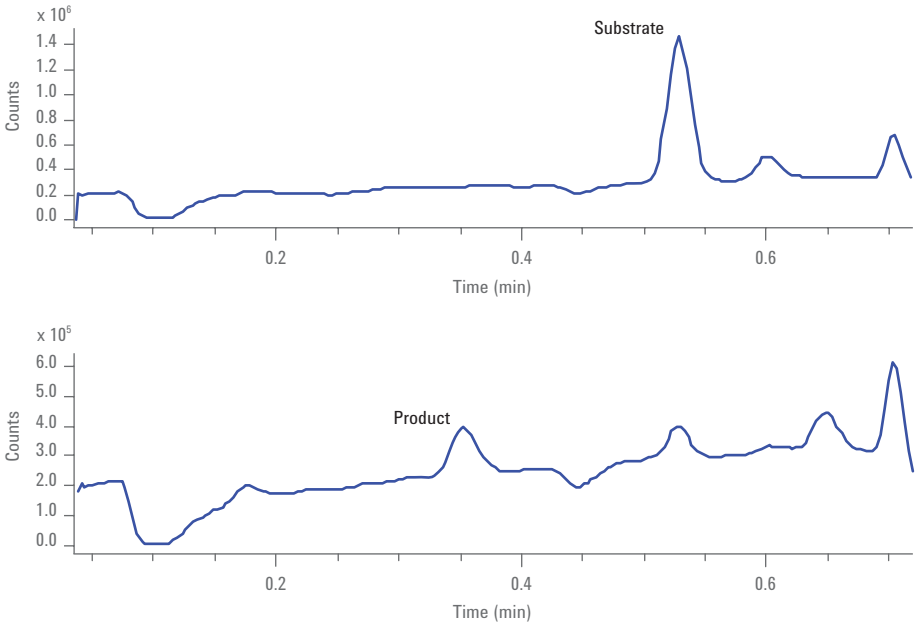

Figure 1. Full scale hydroxylase chromatogram at 8 µM inhibitor concentration, which is near the IC_{50}.

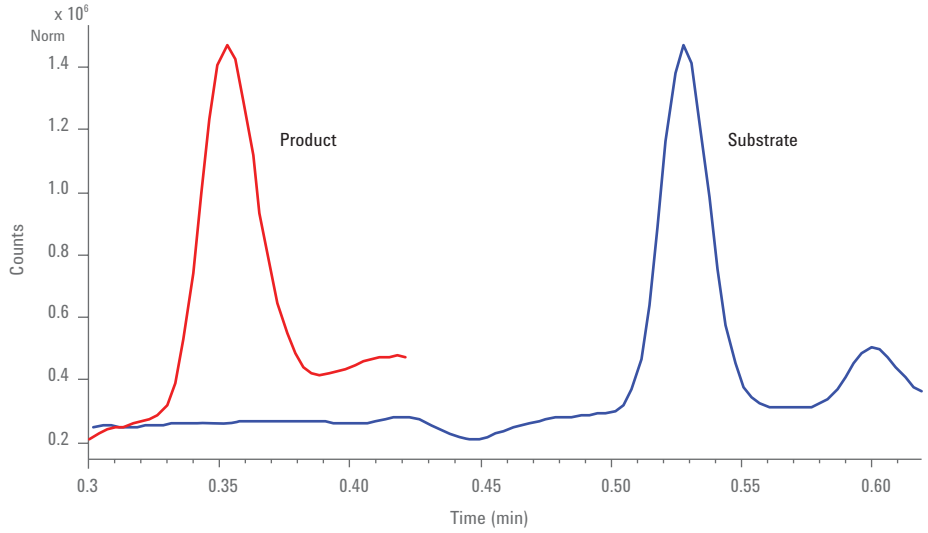

Figure 2. Hydroxylase assay with a close-up view of the peaks of interest; captured at an inhibitor concentration of 8 µM, which is near the IC_{50}.

CYP17-hydroxylase inhibition

As expected, an inverse relationship was found between product formation and inhibitor concentration. This is shown in Figure 3, an overlay of product chromatograms at differing inhibitor concentrations. Enzymatic assays are usually designed so that less than 10% of the substrate is consumed. Since there is usually little change in the substrate concentration, most detection methods only monitor formation of the product. However, in this hydroxylase assay an unusually large amount of the substrate is consumed. Figure 4 shows an overlay of the substrate chromatograms at differing inhibitor concentrations and demonstrates the parallel relationship between substrate concentration and inhibitor concentration.

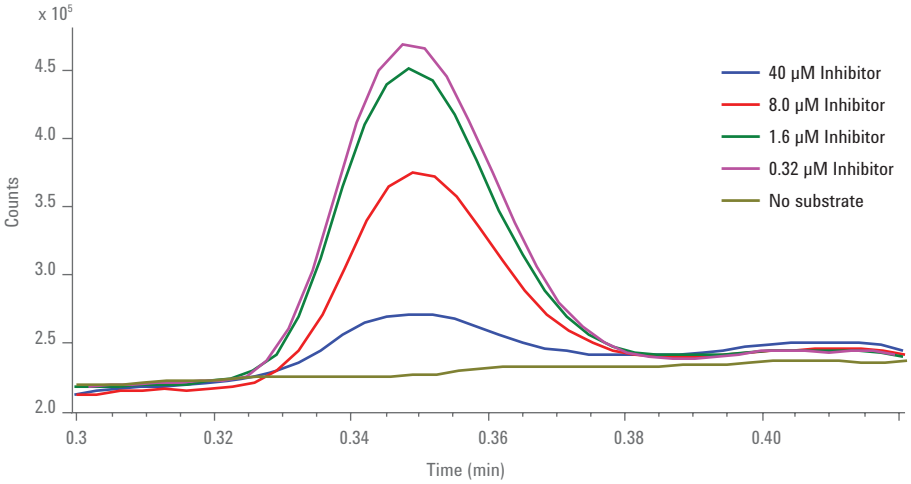

Figure 3. Hydroxylase product formation at inhibitor concentrations from 0.32 to 40 µM.

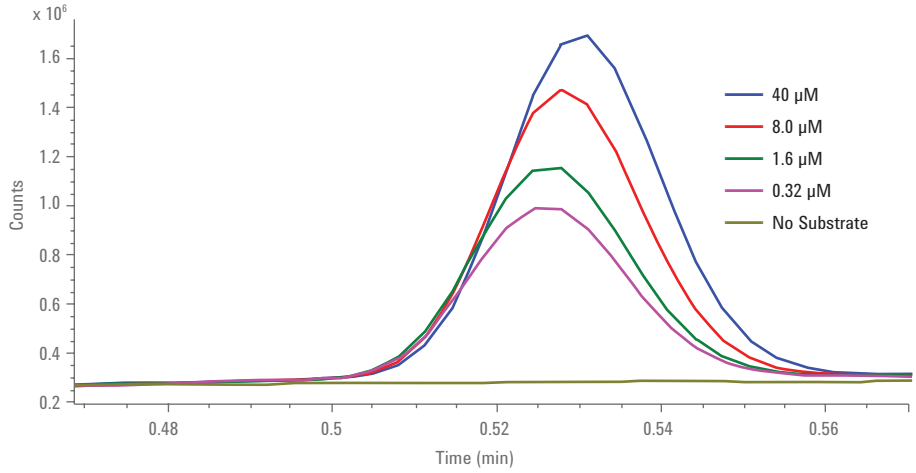

Figure 4. Hydroxylase substrate depletion at inhibitor concentrations ranging from 0.32 to 40 µM.

In order to demonstrate the utility of this method for evaluating NCEs, plots of normalized substrate and product concentration vs. inhibitor concentration were created from triplicate hydroxylase assay samples (Figures 5 and 6). The IC_{50} value calculated from the appearance of product is very close to that calculated from the disappearance of substrate.

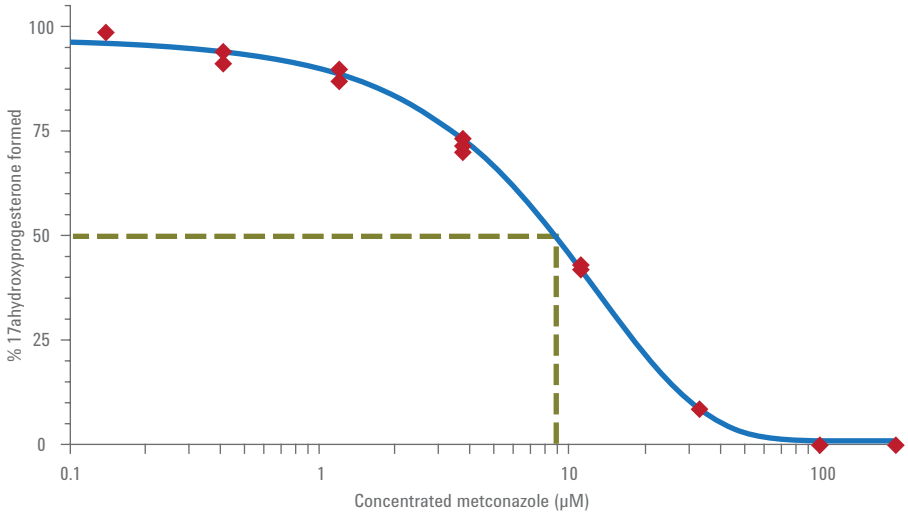

Figure 5. CYP17-hydroxylase IC_{50} plot for product formation yields an IC_{50} value of 8.8 µM.

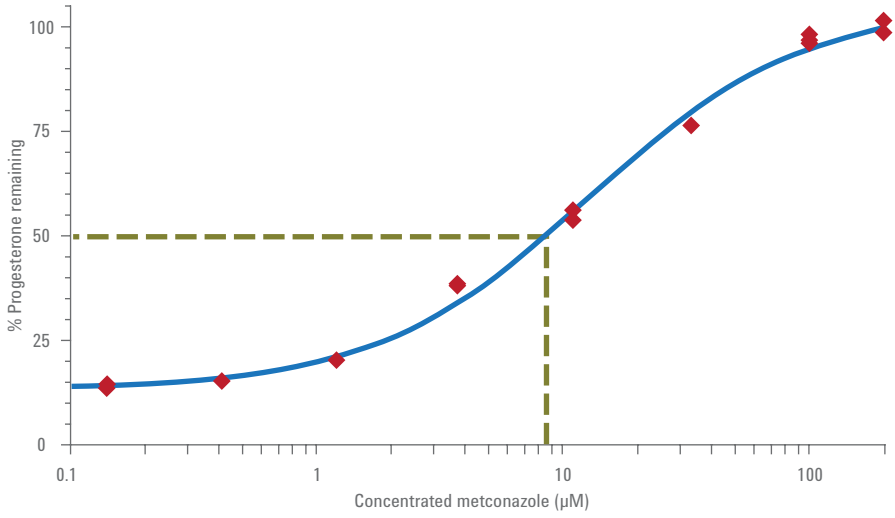

Figure 6. The CYP17A1-hydroxylase IC_{50} plot for substrate depletion yields an IC_{50} value of 8.4 µM.

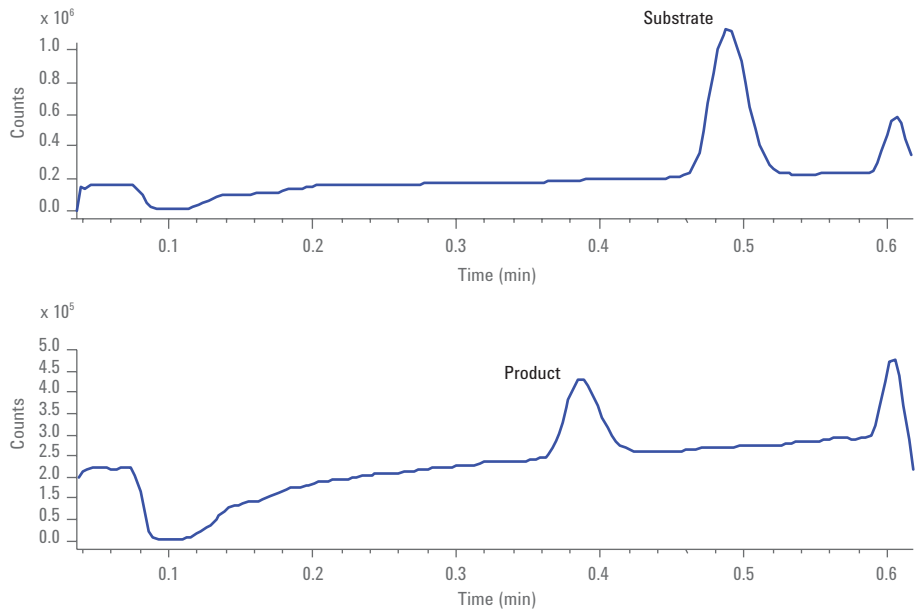

Figure 7. Full scale CYP17-lyase chromatogram at an inhibitor concentration of 1.6 µM, which is near the IC_{50}.

CYP17-lyase assay

Development of this assay was also successful, as shown in Figure 7 a representative complete chromatogram showing substrate and product at an inhibitor concentration of 1.6 µM. The void volume elutes at 0.1 minutes as evidenced by the suppression in the background. The peak at 0.6 minutes corresponds to the step up to 100% organic.

As in the hydroxylase assay, it is possible to monitor both substrate loss and product appearance. In Figure 8 a sample near the IC_{50} is shown which is clearly sufficient to monitor both substrate depletion and the product formation. In this case, there is very little interference in these chromatograms so the run time was shortened to 0.6 minutes and the overall injection-to-injection time was 0.8 minutes.

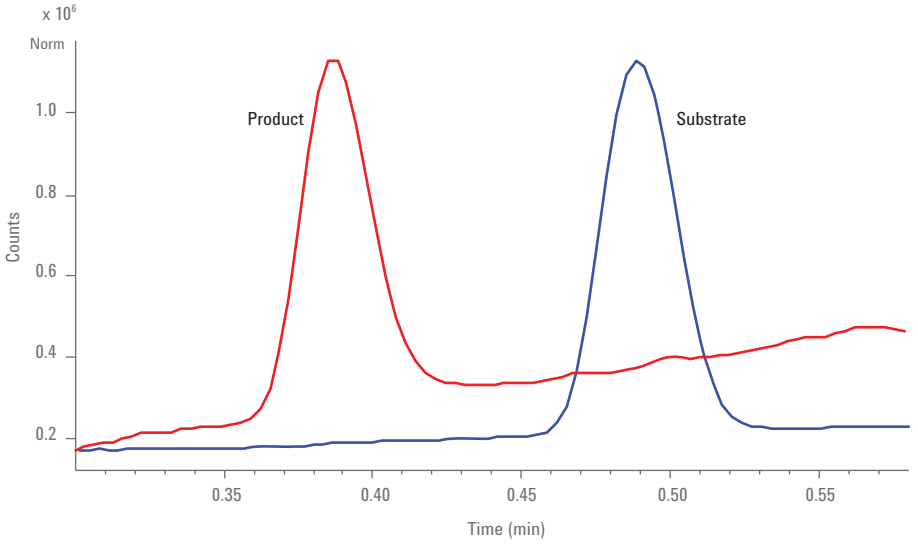

Figure 8. A close-up view of the CYP17-lyase peaks of interest at an inhibitor concentration of 1.6 µM, which is near the IC_{50}.

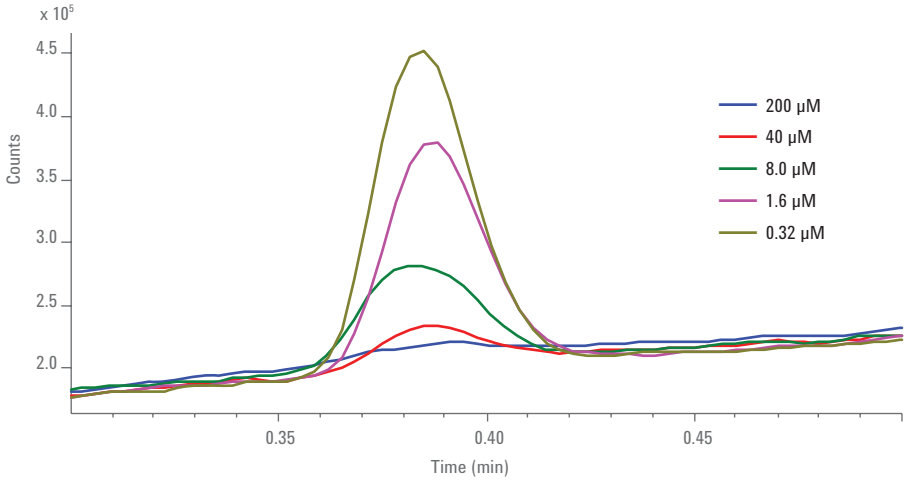

Figure 9. CYP17-lyase product formation at inhibitor concentrations ranging from 0.32 µM to 200 µM.

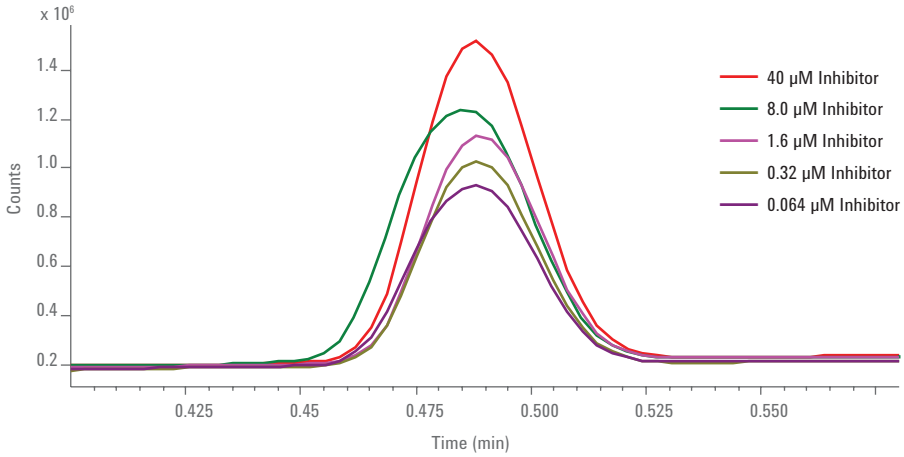

Figure 10. CYP17-lyase substrate depletion at inhibitor concentrations ranging from 0.064 to 40 µM.

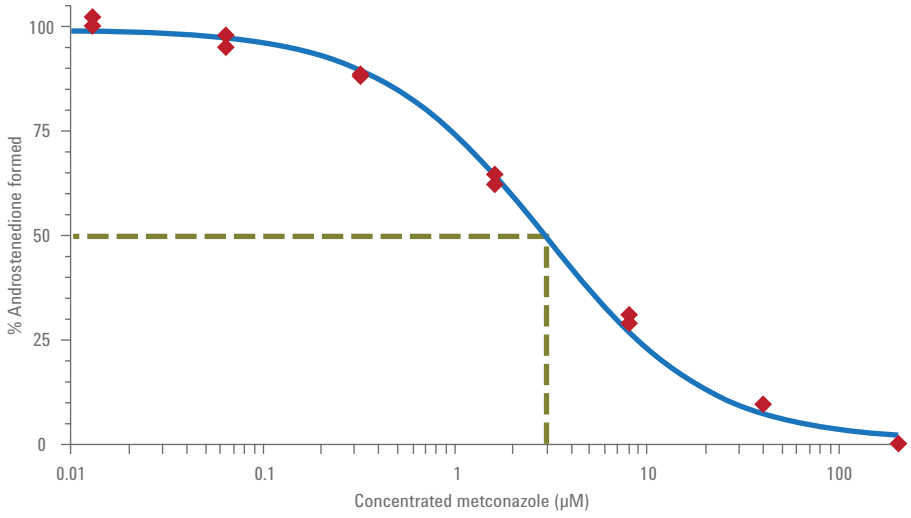

Figure 11. CYP17-lyase IC_{50} plot for product formation yields an IC_{50} value of 2.9 µM.

CYP17-lyase inhibition

Figure 9 shows an overlay of product chromatograms at differing inhibitor concentrations and demonstrates the inverse relationship between product formation and inhibitor concentration. In order to demonstrate the utility of this method for evaluating NCEs, Figure 11 shows the normalized product concentration vs. inhibitor concentration for the assay performed in triplicate. Figure 10 shows an overlay of the substrate chromatograms at differing inhibitor concentrations, and demonstrates the parallel relationship between substrate concentration and inhibitor concentration. In this assay about one third of the substrate is consumed. Measurement of inhibition from partial substrate depletion is not as accurate as measurement from product formation. Therefore, the IC_{50} based on substrate depletion was not calculated.

Conclusions

We have developed two label-free in vitro assays for CYP17A1, one measuring hydroxylase activity and the other lyase activity. These assays rely upon a sensitive detection system based on the Agilent 1290 Infinity LC System coupled to an Agilent 6150 Single Quadrupole MS System with an Agilent Jet Stream source. A complex, but readily available, enzyme source is used for these assays, which are performed at physiologically relevant conditions. Enzyme activity was characterized by monitoring the conversion of natural steroid substrates to products. It was possible to detect both substrate depletion and product formation as a means of monitoring enzymatic activity, and to show the inverse effect of inhibitor concentration on product formation. These assays allow the complete analysis of a 96-well plate in less than 1.5 hours, allowing for the rapid evaluation of NCEs in drug discovery assays.

References

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