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The development of a GLP protocol for the measurement of 17β-estradiol and testosterone in the H295R steroidogenesis assay, Test No 456

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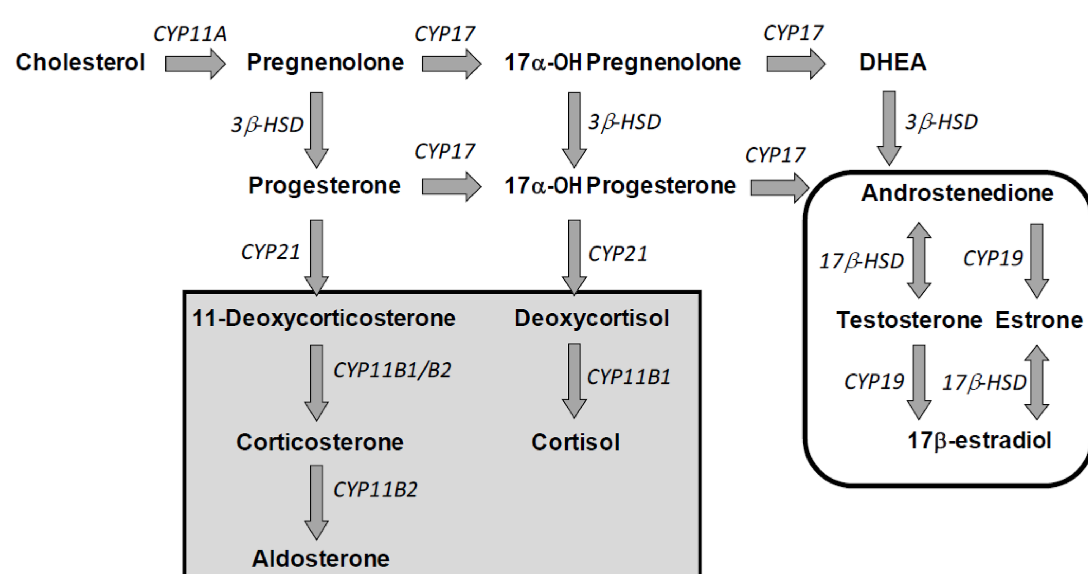
Regulators are concerned about the potential for environmental chemicals such as agrochemicals and their metabolites to perturb hormone systems. This has led to recommendations for the testing of potential endocrine disrupting chemicals[1]. The Steroidogenesis H295R assay is an *in vitro* cell model used to investigate compound effects on steroid hormone biosynthesis, specifically 17β-estradiol (E2) and testosterone (T). The human H295R adreno-carcinoma cell line expresses genes that encode for all the key enzymes for steroidogenesis and thus forms one of the required OECD *in vitro* tests (TG456) for the assessment of potential endocrine disrupting chemicals[2]. Although it is possible to assess hormone levels with ELISA we elected to perform the TG456 assay with LC-MS/MS hormone detection, avoiding the test item interference issues reported for immunoassay-based readouts.

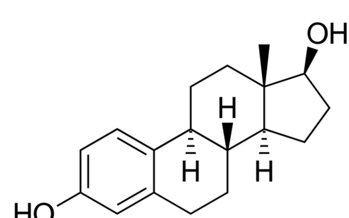
Introduction

Steroidogenesis is the production of steroid hormones from cholesterol

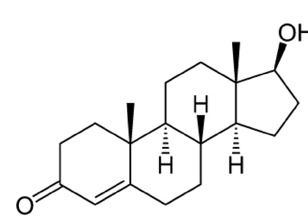


OECD 456 Figure 1: steroidogenic pathway in H295R cells

OECD Test No. 456 is concerned with the determination of the steroid hormones 17β-estradiol and testosterone within this pathway.



17β-Estradiol (272.38 g/mol)



Testosterone (288.42 g/mol)

We describe herein the implementation of a robust GLP bioanalytical method for the detection of testosterone and 17β-estradiol in the steroidogenesis assay, to LLOQ levels of 10 pg/mL for each hormone. This method has been used to correctly identify inducers and inhibitors of T and E2 production while remaining unresponsive to a negative chemical. We present assay performance with respect to the proficiency items and discuss the benefits of the optimised bioanalytical protocol.

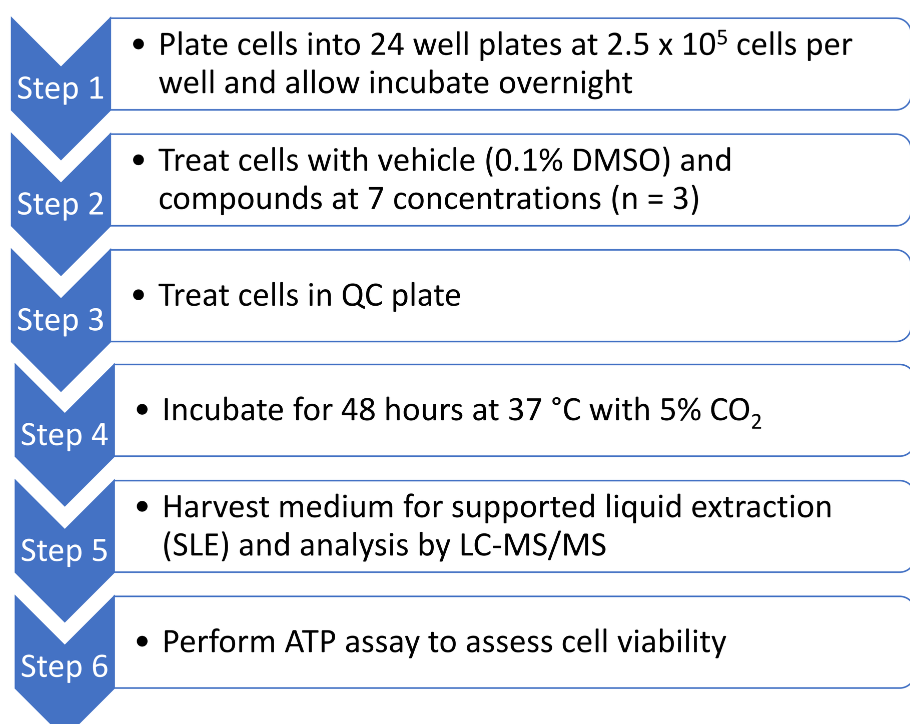
We tested both inducers and inhibitors of steroidogenesis and a negative chemical in the H295R human adreno-carcinoma cell line and measured T and E2 in the spent medium. Hormone concentrations were measured using supported liquid extraction (SLE) followed by LC-MS/MS. For each run of the steroidogenesis assay, a quality control (QAC) plate was run to verify the performance of the H295R cells under standard culture conditions. Intracellular ATP levels were used to assess cell viability.

Compounds

Compound	Effect on Steroidogenesis
Forskolin	Strong inducer of T and E2
Prochloraz	Strong inhibitor of T and E2
Atrazine	Moderate inducer of T and E2
Aminoglutethimide	Moderate inhibitor of T and E2
Bisphenol A	Weak inhibitor of T
Molinate*	Weak inducer of E2
Folic Acid	No effect

Methods

Treatment of H295R Cells

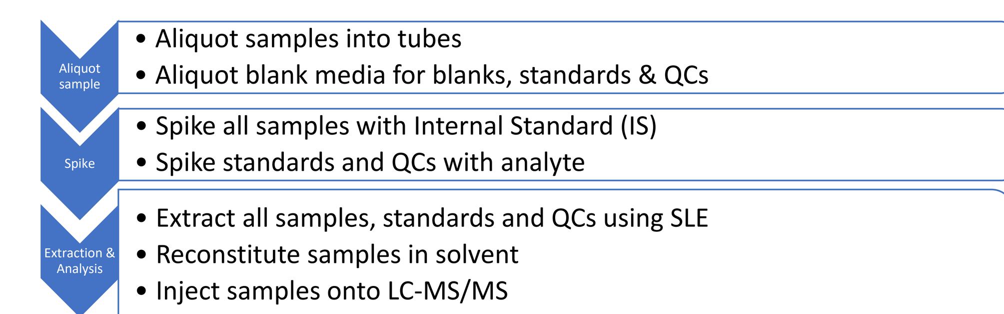


Acceptance criteria for the QC plate

Performance criterium	T	E2
Basal hormone production in the vehicle control	≥ 5.0 times the LOQ	≥ 2.5 times the LOQ
Induction (10 μM forskolin)	≥ 1.5 times the LOQ	≥ 7.5 times the LOQ
Inhibition (1 μM prochloraz)	≤ 0.5 times the LOQ	≤ 0.5 times the LOQ

Analysis of Hormone Levels in Medium

Supported Liquid Extraction[3]



LC-MS/MS

The method was developed using a Sciex Exion AD LC system coupled to a Sciex 6500+ triple quadruple MS. Reverse phase LC was used with a gradient of aqueous and organic mobile phases. Data were acquired and quantified using Analyst 1.6.3 software. Validation included precision, accuracy, selectivity, sensitivity, linearity, recovery and stability, delivering LLOQ levels of 10 pg/mL for each hormone. OECD Test No. 456 guidelines were used as a framework for this validation, and the assay satisfied the necessary criteria.[3]

Results

H295R Cell Viability

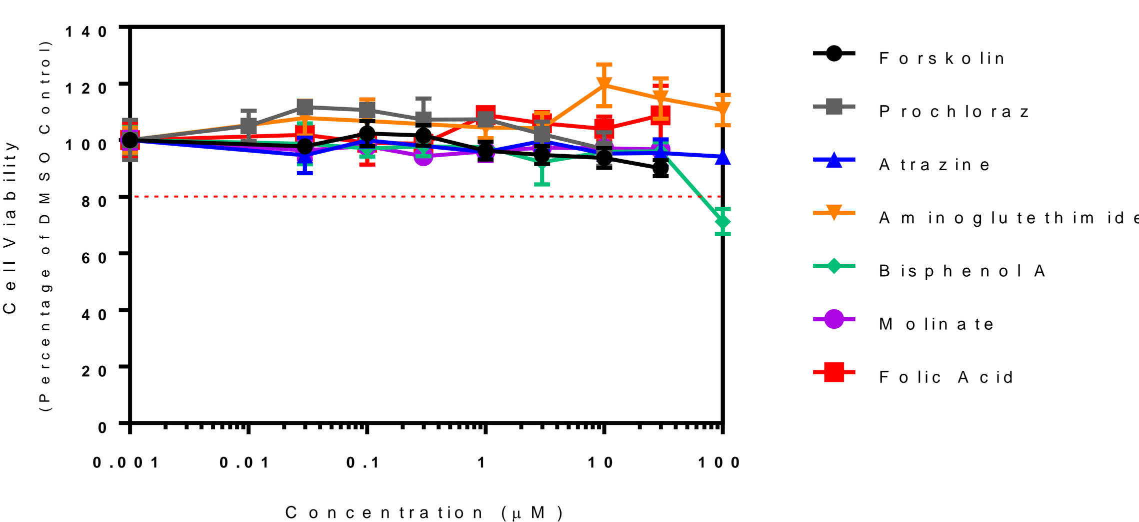


Figure 3: Effect of Compounds on intracellular ATP
Bisphenol A was cytotoxic at 100 μM (ATP depleted to < 80% of control) and was therefore used at a top concentration of 10 μM for the steroidogenesis assay. No other compound was cytotoxic.
Red dotted line indicates threshold of cytotoxicity (viability less than 80% of control).

Testosterone Levels in H295R Medium

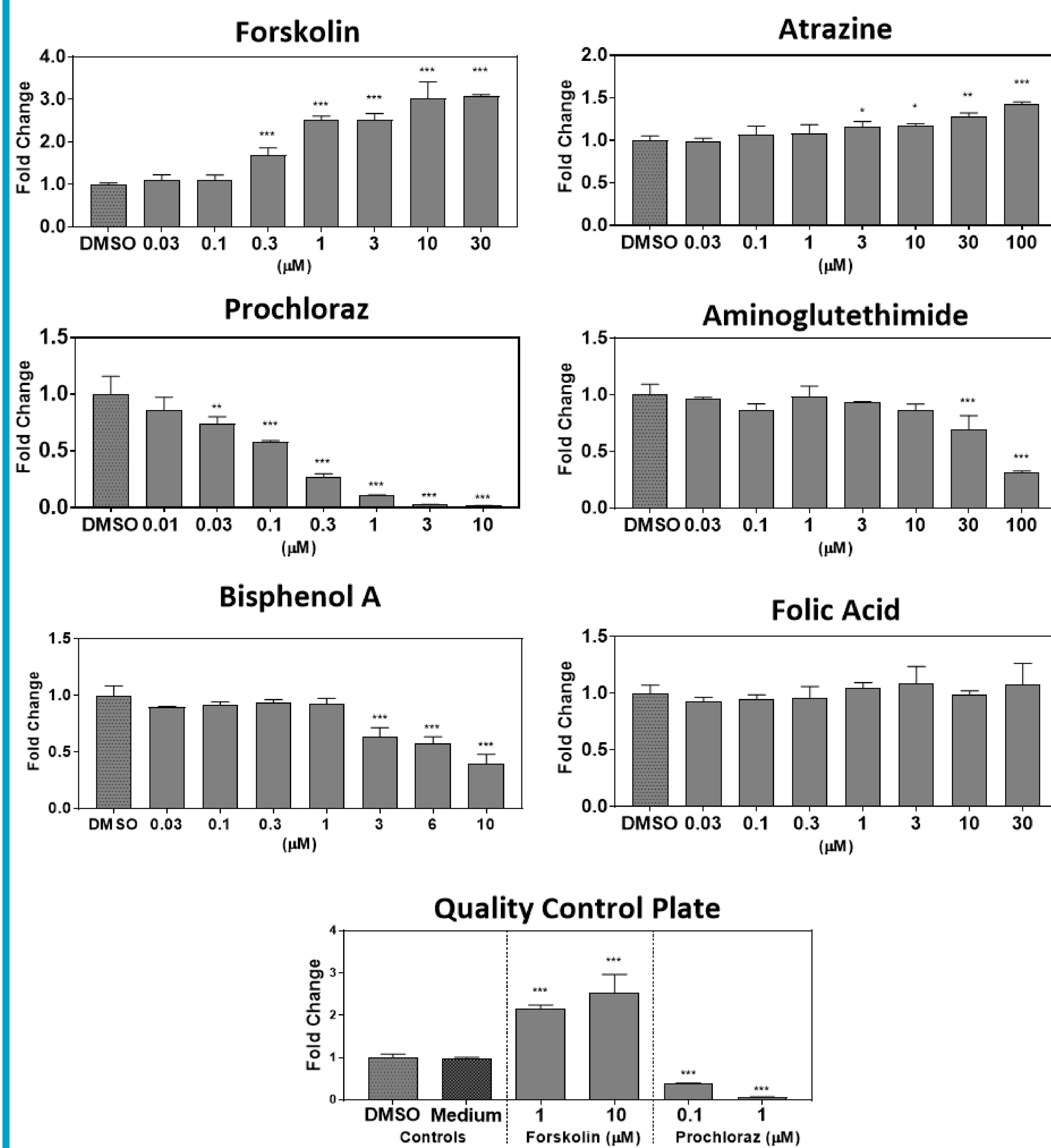


Figure 4: Representative fold changes in Testosterone production in H295R cells following 48 hours' exposure to known inducers (forskolin and atrazine) and inhibitors (prochloraz, aminoglutethimide and bisphenol) of steroidogenesis as well as a negative control (folic acid). A quality control plate was run in parallel that contained an inducer (forskolin) and an inhibitor (prochloraz) of testosterone production. Values are expressed as mean ± SD; n = 2 – 3 for each compound and n = 5 – 6 for 0.1% DMSO and medium on the quality control plate. Significantly different from 0.1% DMSO control by one way ANOVA and Dunnett's multiple comparison tests: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

17β-Estradiol Levels in H295R Medium

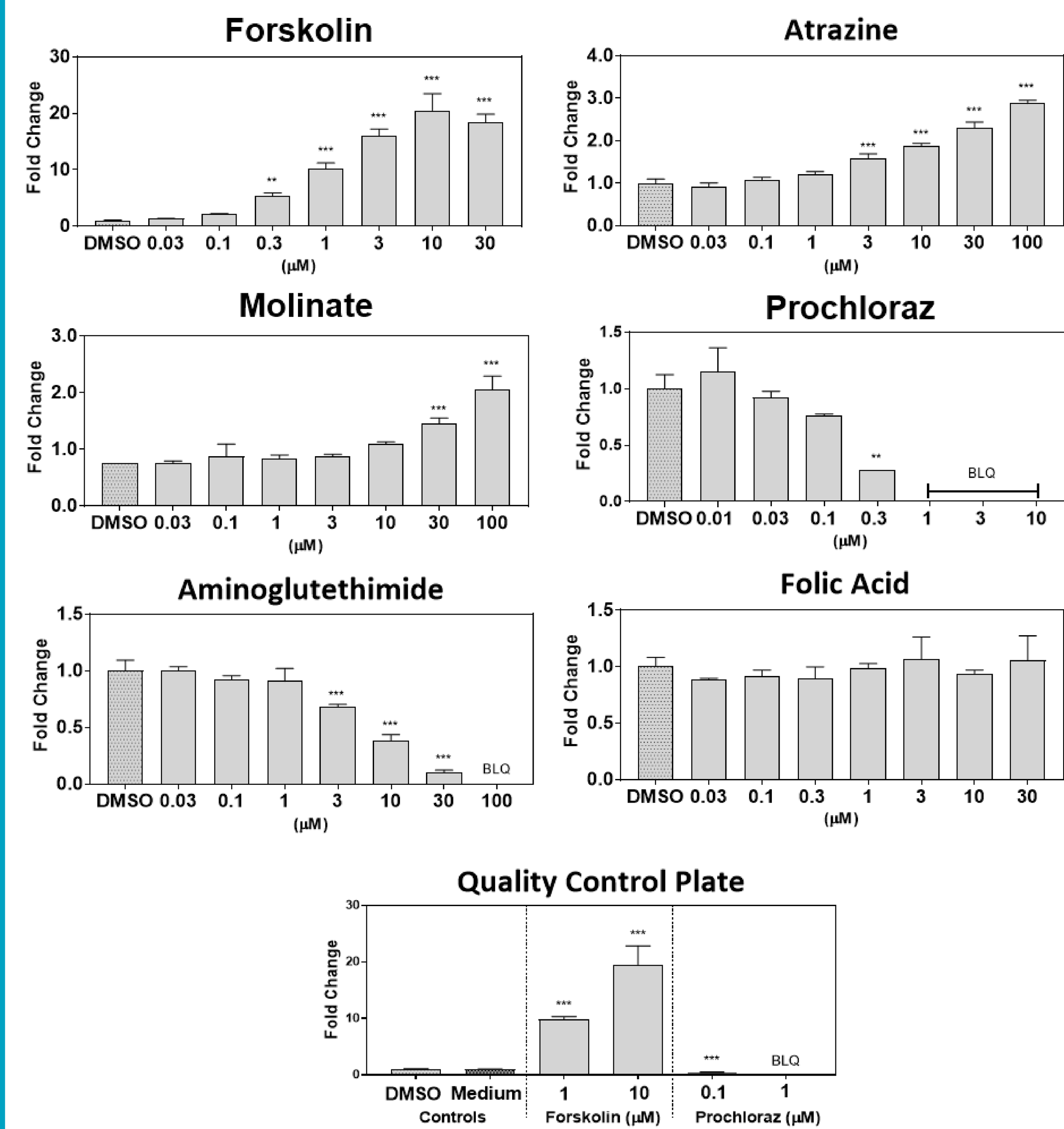


Figure 5: Representative fold changes in Estradiol production in H295R cells following 48 hours' exposure to known inducers (forskolin, atrazine and molinate) and inhibitors (prochloraz and aminoglutethimide) of steroidogenesis as well as a negative control (folic acid). A quality control plate was run in parallel that contained an inducer (forskolin) and an inhibitor (prochloraz) of estradiol production. Values are expressed as mean ± SD; n = 2 – 3 for each compound and n = 5 – 6 for 0.1% DMSO and medium on the quality control plate. Significantly different from 0.1% DMSO control by one way ANOVA and Dunnett's multiple comparison tests: ***P* < 0.01; ****P* < 0.001.

Compound	Testosterone		Estradiol	
	LOEC	Max Change	LOEC	Max Change
Forskolin	0.3 μM	3.07- fold	0.3 μM	20.48 - fold
Prochloraz	0.03 μM	0.02 - fold	0.3 μM	< 0.28 - fold
Atrazine	3 μM	1.42 - fold	3 μM	2.87 - fold
Aminoglutethimide	30 μM	0.32 - fold	3 μM	< 0.10 - fold
Bisphenol A	3 μM	0.40 - fold	N/A	N/A
Molinate*	N/A	N/A	30 μM	2.05 - fold
	NOEC	Max Change	NOEC	Max Change
Folic Acid	30 μM	N/A	30 μM	N/A

LOEC = lowest observed effective concentration. NOEC = no observed effect concentration

Summary

We have developed a method and validated an assay to measure 17β-estradiol and testosterone in medium from H295R cells. This assay uses SLE followed by LC-MS/MS to measure both hormones in a single sample, thus providing an efficient and reliable bioanalytical protocol. The assay is highly sensitive, detecting both estradiol and testosterone at levels as low as 10 pg/mL in spent medium from H295R cells.

Based on OECD TG456, compounds at non-cytotoxic concentrations were correctly identified as inducers- and inhibitors- of testosterone and estradiol production; H295R cells were unresponsive to a negative chemical. These results were confirmed in 2 independent steroidogenesis assays.

We have demonstrated that we have a robust, highly sensitive method for use in determining the effects of compounds on steroidogenesis.

References

1. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC)No 1107/2009. DOI https://doi.org/10.2903/j.efsa.2018.5311

2. Test No. 456: H295R Steroidogenesis Assay, https://www.oecd.org/env/test-no-456-h295r-steroidogenesis-assay-9789264122642-en.htm

3. McIntyre, A. *et al.* (5th RSC / DMDG / DMG New Perspectives in DMPK February 2022). The benefits of SLE in the development of a GLP protocol for the measurement of 17β-estradiol and testosterone in the H295R steroidogenesis assay, Test No 456. Poster available at: https://www.malvernpanalytical.com/en/learn/knowledge-center/posters/PT220420-CLS-GLP-protocol-estradiol-testosterone-assay

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* Currently undergoing validation

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