Steroidomic footprinting based on UHPLC qualitative and quantitative high-resolution MS for the evaluation of endocrine disrupting chemicals in H295R cells

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Introduction

The screening of endocrine disrupting chemicals potentially altering steroidogenesis in humans is a topic of growing importance; the main reason being the numerous pathologies related to endocrine disruption such as cancer, diabetes, obesity, and infertility. The development of efficient methods to screen chemicals and hence evaluate their potential effect on human steroidogenesis is pursued by regulatory agencies. This study aimed to assess the capabilities of high-resolution MS for the simultaneous untargeted detection of steroids and quantification of selected key steroids in human adrenal H295R cells culture media. This experimental setup was applied to the evaluation of triclocarban (TCC) toxicity, an antibacterial agent used in detergents, cosmetics and personal care products, and suspected to be an endocrine disruptor.

Materials and Methods

H295R cells were cultured according to the Organization for Economic Cooperation and Development (OECD) guidelines. Cells were, after a resting phase of 24 h, incubated 48 h with solvent control (DMSO), Abiraterone (10 µM), Trilostan (5 µM) or TCC at different concentrations (0.1, 0.25, 0.5, 1.0, 2,5, and 5.0 µM; n=5 for each condition). The culture media of the incubated cells were precipitated with perchloric acid supplemented with three internal standards (testosterone-d3, 17α-hydroxyprogesterone-d8 and cortisol-d4) and concentrated 20-

fold using SPE HLB cartridges before injection in a ultra-high performance liquid chromatography (UHPLC, Acquity H-Class) coupled with high-resolution MS operated with an ESI source operated either in positive or negative mode (QTOFMS, MaXis 3G). Gradient separation was performed in 15 min with a Kinetex C18 column (2.1 x 150 mm, 1.7 µm). Simultaneous quantification analyses were performed using standards spiked in surrogate matrix. Untargeted data pre-processing was performed using Progenesis QI software (Nonlinear Dynamics); Principal Component Analysis (PCA) and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) models were performed with the SIMCA-P software (version 13, Umetrics, Umeå, Sweden).

Results

Analysis of steroids represent a challenge due to the low concentration observed in cell culture media. It is for this reason that the sample preparation was aimed to concentrate steroids in the most generic manner. As the evaluation of untargeted analysis added to quantitative analysis in a single run was one of the aim of this work, the data acquisition was only first performed in TOFMS mode due to instrumental limitations in terms of duty cycle. After data acquisition and import into Progenesis QI software, a first data filtering was performed using a chemically-driven feature selection [1, 2] that filtered "steroid-like" compounds as a function of *m/z* using a tailored and curated library composed of HMDB [3] and Lipidmaps databases [4]. Pareto scaling was then applied to the reduced dataset acquired in positive mode and supervised OPLS modeling was carried out to highlight steroid changes, *i.e.* putative markers of endocrine disruption, due to the addition of different amounts of TCC in the cell culture medium. For that purpose, the concentration of TCC added in each sample was used as the response to predict (n = 5 for each group). A model with two latent variables (one predictive and one orthogonal) was obtained with high fit and prediction ability indices (R2Y=0.88, Q2Y=0.84) (Figure 1A). An S-Plot was used to summarize the contributions of all ion features to the predictive component related to TCC metabolic effects, in terms of covariance (*i.e.* amplitude, p[1]) and correlation (i.e. reliability, p(corr)[1]).

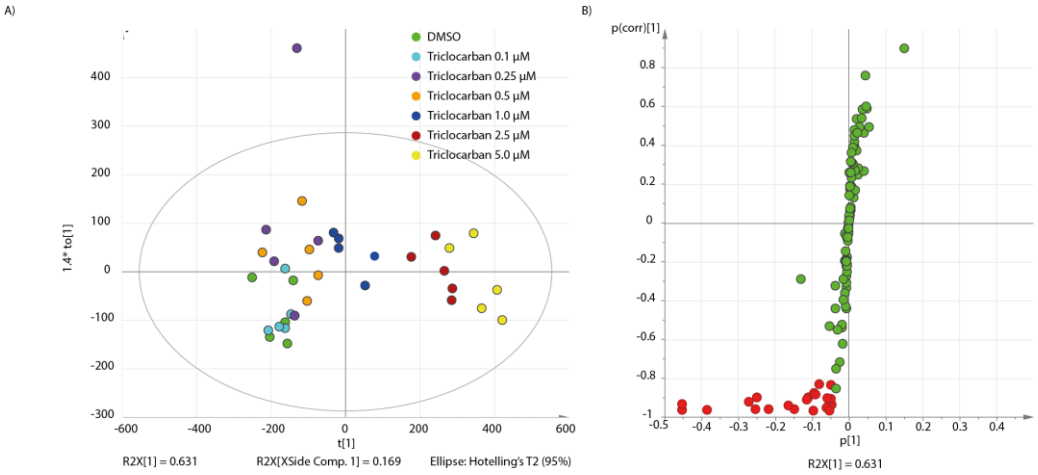

Figure 1: A) OPLS-DA with Pareto scaling for sample set using the steroid filter and B) S-Plot highlighting the 22 most predictive variables (in red)

A series of 22 features was hence selected on the basis of the S-plot, as the most promising steroid markers of TCC toxicity (Figure 1B). Among the 22 steroid-like features, eleven (50 %) were straightforwardly identified with authentic chemical standard comparison and quantified using the same dataset.

As often in metabolomics approaches, unknown compound identification remains the principal bottleneck. We emphasized here first the usefulness to reduce the chemical search space using concomitant strategies; i) the extraction of a subset of compounds (steroid-like compounds) based on matching high-resolution mass determination with curated databases represented a significant decrease of potential candidates of interest (from > 8,000 features to > 400 features); ii) the evaluation of the most relevant candidates able to predict a concentration of TCC in cells led to a decrease from > 400 variables to 22 features. However, definite identification of the remaining candidates can still be tedious due to the properties of steroids (*e.g.*, high number of isomers for a single elemental composition). The use of known endocrine disruptors (trilostan and abiraterone in these series of experiments) with known disrupted enzymatic reactions helps to reduce the number of possible identifications. For instance, it was possible to decrease from eleven possible identifications for a specific candidate (RT = 7.85 min, *m/z* 305.2114) down to five possible ones using the respective measured intensities of this unknown in trilostan and abiraterone experiments.

Using this analytical workflow, it was possible to hypothesize a perturbation of TCC in the steroidogenesis corresponding to upstream precursors of pregnenolone (Figure 3).

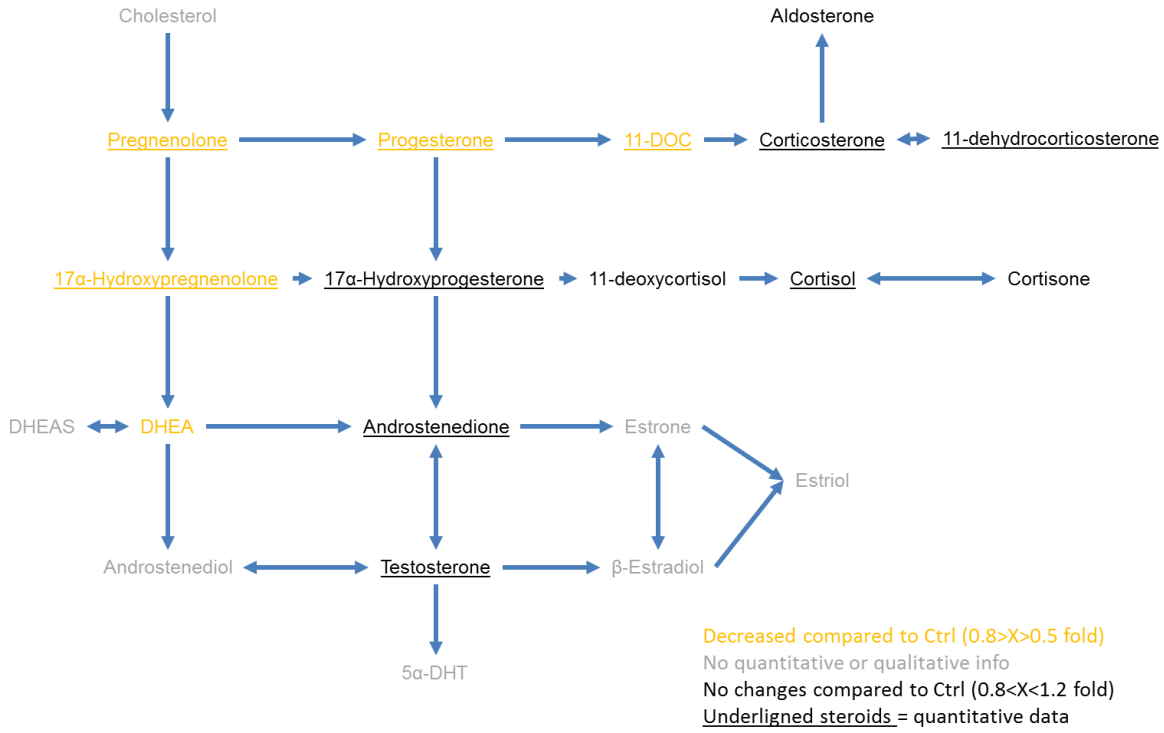

Figure 3: Modifications of steroidogenesis in cells exposed to TCC (0.5 µM; n=5) compared to DMSO control (n=5)

Conclusion

This study presents the development of an untargeted steroidomic footprinting strategy allowing the simultaneous acquisition of qualitative and quantitative data with UHPLC hyphenated to QTOFMS. In a single analysis, it was possible to quantify eleven steroids, and to evaluate and localize the disruption of steroidogenesis induced by TCC upstream the biosynthesis of pregnenolone. The developed workflow for H295R cellular model, which can be applied to other biological matrices, underlines the potency of extracting steroid-like features from untargeted raw data using automatic peak annotation based on exact mass measurement. The data mining strategy combining multivariate analysis was demonstrated as a relevant approach to extract and highlight biomarker candidates. The addition of biological information demonstrated also a potent way to increase the identification rate. Even if the definitive identification of the molecular actors remains critical to improve mechanistic understanding of EDCs, the developed method constitute a potent approach to screen and classify an important number of potential EDCs in a straightforward manner. Further developments in this area will be pursued to develop diverse models of perturbations of

steroidogenesis using known EDCs along with strategies to improve identification using retention time modeling and in depth study of steroid MS/MS fragmentation mechanisms.

References

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