P42. A MULTI-ANALYTE METHOD FOR THE QUANTIFICATION OF XENOBIOTIC-INDUCED THYROXINE METABOLISM

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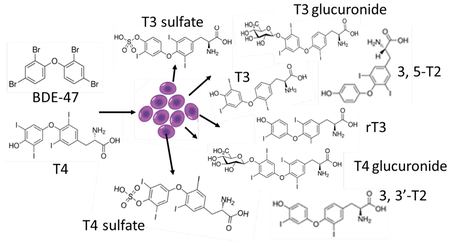
Abstract

Thyroid hormones (THs) are essential to normal fetal development and particularly to the development of the nervous system. Because of the importance of THs to the developing fetus, xenobiotics that disrupt TH homeostasis are of great concern. Exposure to polybrominated diphenyl ethers (PBDEs), a major class of brominated flame retardants, has been shown to decrease TH serum concentrations in rodents by inducing the hepatic metabolism of thyroxine (T4). TH metabolism is carried out by deiodinases, UDP-glucuronosyltransferases, and sulfotransferases in the liver; therefore, assessing the expression (activity or mRNA) changes of these enzymes in the presence of xenobiotics is key to predicting TH clearance. However, enzyme activity assays can be inactivated or inhibited by the test compound and mRNA levels does not always correlate with protein levels; therefore, metabolite quantitation for induction studies is considered a desired approach. Here, we developed a simple and efficient analytical method to identify and quantify TH metabolites in cell culture media. Using human sandwich cultured hepatocytes, we examined the effects of 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47), a major PBDE congener, on T4 metabolism. Human hepatocytes were treated with 0, 0.3, 3, or 30µM BDE 47 (0.1% DMSO final concentration) for 72 hours. After treatment with BDE 47, hepatocytes were incubated (24 hours) with 0.1µM T4 (median physiological concentration in humans). After 24 hours, media were collected and prepared for extraction through protein precipitation. Extracts were analyzed using electrospray ionization with positive ion scheduled multiple reaction monitoring (sMRM) on a Sciex 6500+ Qtrap LC/MS/MS. The metabolites analyzed include: T3, reverse T3, and T2 (deiodination), T4G and T3G (glucuronidation), and T4S and T3S (sulfation). In controls, T3 was the predominate metabolite found in media, followed by rT3, T4G, and T2. T3G, T4S, and T3S were not detected. Compared to controls, 30µM BDE 47 increased T4G and T3 formation by 12.4- and 2.5-fold, respectively. The results indicate not only the pathways for BDE 47-induced hepatic metabolism of T4, but also that deiodination is a favored pathway in human hepatocytes and xenobiotic-induced glucuronidation can play a key role in T4 clearance. These results highlight the utility of hepatocytes and this method as an in vitro screening tool to yield a mechanistic understanding of TH clearance and to evaluate potential TH disruptors. (This abstract of a proposed presentation, does not reflect USEPA policy.)

Objectives

- Develop an LC/MS/MS method with fast and efficient extraction protocol to identify and quantify thyroid hormone (TH) and TH metabolites in hepatocyte culture medium.
- Evaluate TH metabolism with primary human hepatocytes exposed to BDE-47 (2,2′4,4′-tetrabromodiphenyl).



Structures of BDE-47, thyroxine (T4), and thyroxine metabolites

Methods

LC/MS/MS

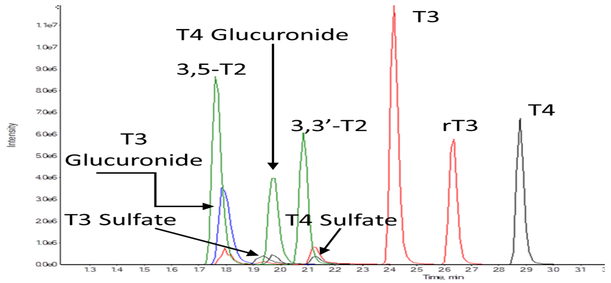
- Performed in positive ion scheduled multiple reaction monitoring (sMRM) with electrospray ionization (ESI) on a Sciex (Framingham, MA) Qtrap 6500+ equipped with a Restek (State College, PA) Raptor Biphenyl (100 x 2.1 mm, 2.7 µm) column.
- Sample injection volume was 10 µL and the flow rate was 0.500 mL/min. Mobile phase A: 0.1% Formic acid; B: Methanol:acetonitrile (50:50) in 0.1% formic acid.

Sample Preparation (Protein Precipitation)

- Vortex 250 µL hepatocyte media with internal standard mix (10 µL, 125 ng/mL) in 1 mL acetonitrile with 0.1% formic acid.
- Freeze (30 min,-20 °C). Centrifuge at 10,000 rpm (10 min). Evaporate supernatant under N_2 gas; resuspend in 250 µL initial mobile phase.

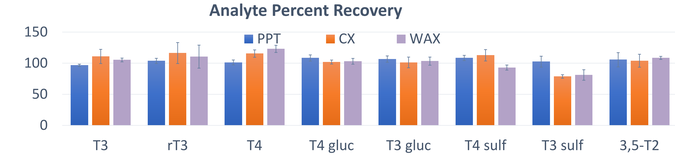
Hepatocyte Treatment

- Cryopreserved human hepatocytes (Lonza; Walkersville, MD) in sandwich-culture plated in 24-well plates.
- 72-hour incubation with control (0.1 %DMSO), or BDE-47 (0.3, 3, 30) followed by 24- hour incubation with 0.1uM thyroxine (T4).
- Media collected for metabolite analysis with LC/MS/MS



Extracted ion chromatogram (XIC) of standards (10 ng/mL) spiked into cell culture media

Results

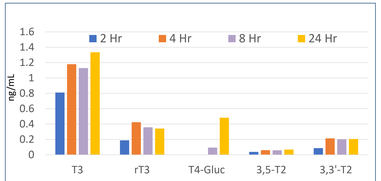


Accuracy: TH in hepatocyte media were prepared by protein precipitation (PPT), solid phase extraction using cation exchange (CX), or weak anion exchange (WAX) cartridges. Recoveries were comparable between methods. PPT was selected for further validation due to its simplicity and speed. Gluc:glucuronide; sulf:sulfate

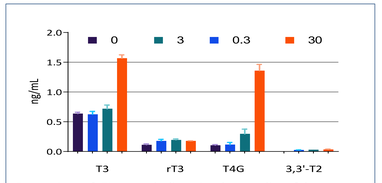
PPT-LC/MS/MS Method Sensitivity

Analyte	T3	rT3	T4	T4 gluc	T3 gluc	T4 sulf	T3 sulf	3,5-T2
MDL, ng/mL	0.01	0.01	0.02	0.01	0.01	0.2	0.2	0.02
LOQ, ng/mL	0.025	0.025	0.050	0.025	0.025	0.50	0.50	0.050

MDL: Method Detection Limit LOQ: Limit of Quantitation



TH metabolites formed following exposure to 0.1 µM T4 over 24 hr.



Formation of TH metabolites in the media of hepatocytes exposed to BDE-47 (0 to 30 µM) for 72 hours followed by a 24-hour exposure to T4 (0.1 µM).

Conclusions

- We developed and validated a PPT-LC/MS/MS method for determination of TH metabolites in hepatocyte media.
- The method is sensitive enough to observe changes to hepatocyte metabolism of 0.1 µM T4 after BDE-47 exposure.