

## Introduction

Membrane transporters play a key role in determining the exposure of liver, kidney, brain and other tissues to a variety of solutes, including nutrients, cellular by-products, environmental toxins, drugs, and other xenobiotics. Membrane transporters are also essential in the delivery and excretory processes of drugs and their metabolites. Oocyte expression systems have been used extensively for the study of membrane transporters because of the low background, high expression level and proper post-translational modifications. This system has been becoming very useful to study drug-transporter and drug-drug interactions. Drugs transported into oocytes can be analyzed directly by radioactivity or by electrophysiological method. However, not all of drugs are radiolabeled, and electrophysiological method can only be applied to these transporters that have transport processing with substrate-evoked currents. For these reasons, indirect assays has been generally used for oocyte uptake assay of non-radiolabeled drugs. In indirect uptake assay, the non-radiolabeled drugs are tested for their competition or inhibition effects on a radio-labeled model substrate. However, inhibitors are not necessarily substrates of the same transporter,<sup>1</sup> and a substrates may not compete with another substrate for the same transporters.<sup>2</sup> Reported here is a method of using liquid chromatography mass spectrometry (LC/MS) to directly study the uptake of non-radiolabeled substrates into oocytes. In this study, the uptake of estrone 3-sulfate mediated by human Organic Anion Transporting Polypeptide 2 (hOATP2, *SLC21A6*) expressed in *Xenopus laevis* oocytes was analyzed. hOATP2 is a liver specific transporter and localized to the sinusoidal membrane.<sup>3</sup> It has broad substrate selectivity, including conjugated and unconjugated bile salts, steroids and steroid conjugates, organic compounds and thyroid hormones, such as estrone 3-sulfate, bromosulphophthalein (BSP) and dehydroepiandro-sterone sulfate (DHEAS).<sup>3-6</sup> Under experimental conditions, estrone 3-sulfate uptaken into a single hOATP2-cRNA injected oocytes can be detected. Although only about 40% estrone 3-sulfate are recovered through the sample-processing, the time dependent uptake can be analyzed by using LC/MS, and results are comparable with that obtained by radioactivity. Results indicated that LC/MS can be used as an analytical method in oocyte uptake studies. These methods can detect substrates uptakes into oocytes, and can also be applied for time-dependent studies.

## Methods

### 1. Expression and Transport Assay in *Xenopus laevis* Oocytes

To obtain higher expression and function of the human transporters in oocyte, an oocyte expression vector was engineered by inserting *Xenopus*  $\beta$ -globin 5' and 3' untranslated regions into pBluescript II KS (+) (Stratagene).<sup>7,8</sup> Oocytes were harvested and digested with collagenase D (Boehringer Mannheim). Fifty nanoliters of hOATP2 cRNA (~0.4 ng/ml) was injected individually into defolliculated oocytes. Oocytes were incubated at 16°C for 3 to 7 days. Uptake assays were performed by incubating hOATP2 cDNA injected or uninjected oocytes with 100  $\mu$ l uptake buffer containing 2  $\mu$ M estrone 3-sulfate for 30, 60 and 120 minutes at room temperature. To compare the uptake assays by using LC/MS and radioactivity, uptake assays were also performed with 2  $\mu$ M <sup>3</sup>H-estrone 3-sulfate under the same conditions. Oocytes were washed with ice-cold water. For uptake assay by using radioactivity, oocytes were lysed individually in 10% SDS, and the amount of radiolabeled compounds transported into each oocyte was determined by liquid scintillation counting. For uptake assay by using LC/MS, samples were prepared following the **Uptake Assay by LC/MS** protocol.

### 2. Uptake Assay by LC/MS

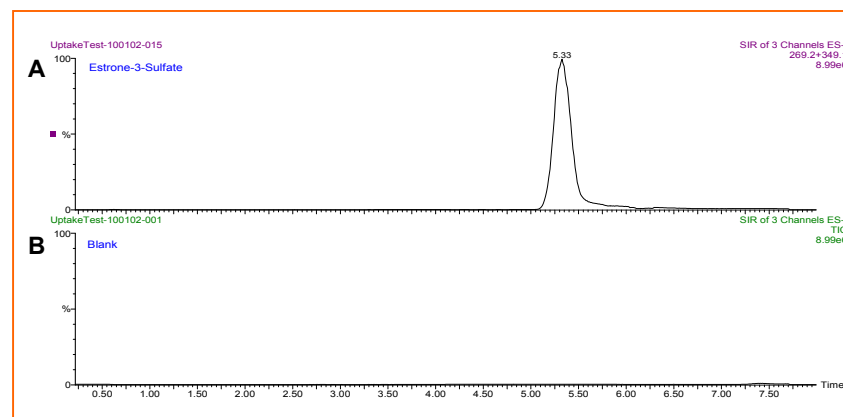
- Each oocyte is placed into a 1.5 ml eppendorf tube.
- Remove rinse water.
- Add 30  $\mu$ l of water.
- Sonicate for 5 minutes.
- Centrifuge for 5 minutes at 14,000 rpm.
- Transfer the supernatant to a clean tube.
- Add 30  $\mu$ l of 100% Methanol.
- Vortex briefly.
- Centrifuge for 5 minutes at 14,000 rpm.
- Transfer the supernatant to a clean tube.
- Inject 25  $\mu$ l of the supernatant onto a 2.1 x 50 mm C-18 column.
- Elute under a water and methanol gradient.
- Estrone 3-sulfate is detected using Single Ion Recording utilizing a cone voltage of 40 V.
- Both the mass to charge ratio of 349.1 and its fragment 269.2 are monitored for maximum sensitivity.

### 3. Statistics and Data Analysis

Group of eight cRNA-injected or water-injected oocytes were used for each experiment. Uptake values are expressed in mean  $\pm$  S.E.

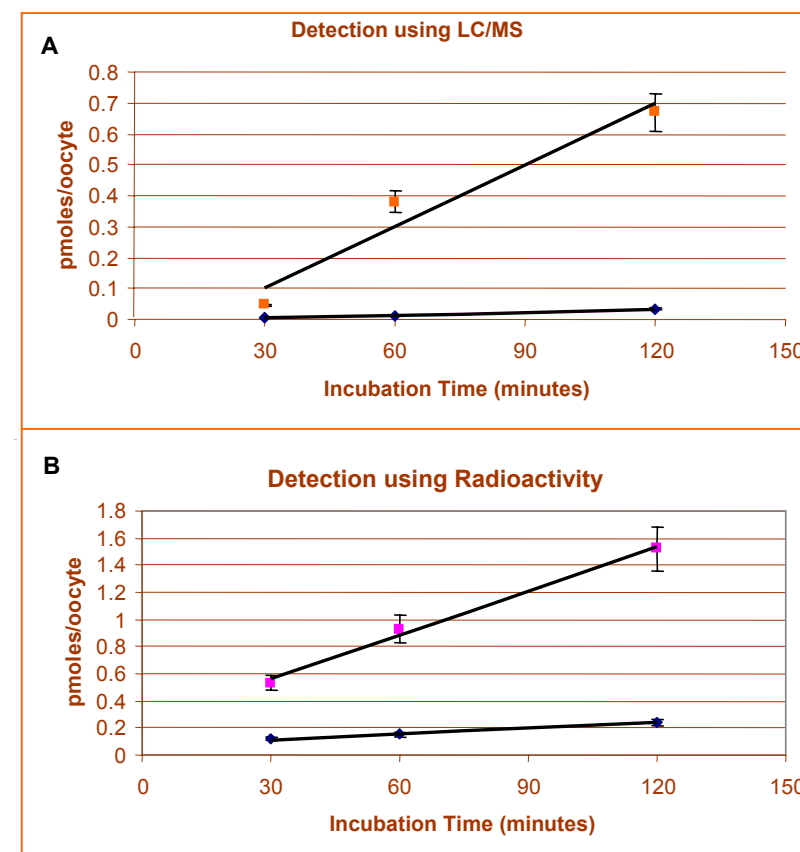
## Results

### 1 LC/MS analysis of estrone 3-sulfate uptake in a single oocyte



hOATP2 cRNA injected and water control oocytes were incubated with 2  $\mu$ M estrone 3-sulfate for 30, 60 or 120 minutes. Each oocyte was then sonicated, protein-precipitated, centrifuged, and estrone 3-sulfate in the supernatant was analyzed by LC/MS as described in the methods. (A) hOATP2 cRNA injected oocyte; (B) water control oocyte.

### 2 Uptake of estrone 3-sulfate mediated by hOATP2 as the function of incubation time



Five days after expression, uptake was carried out by incubating the cRNA-injected or water control oocytes with  $\mu$ M non-radiolabeled estrone 3-sulfate (top) or <sup>3</sup>H-estrone 3-sulfate (bottom) for 30, 60 and 120 minutes in transport buffer at pH 7.4 at room temperature. The amount of estrone 3-sulfate uptaken in each oocytes was analyzed by (A) LC/MS or (B) radioactivity. Squares: hOATP2 cRNA injected oocytes; Diamonds: water control oocytes. Values are expressed as mean  $\pm$  S.E. (n = 8).

## Conclusions

1. LC/MS can be used as an analytical method in oocyte uptake studies. Estrone 3-sulfate uptaken into a single hOATP2-cRNA injected oocytes can be detected with good signal/noise ratio.
2. Compared with uptake value obtained by radioactivity, only about 40% of Estrone 3-sulfate uptaken into each oocyte were recovered. Even though the time-dependent uptake of Estrone 3-Sulfate can be analyzed by LC/MS, the results are comparable with that obtained by radioactivity.
3. Future studies will focus on improving the recovery rate and applying LC/MS in kinetic studies and inhibition studies.

## References:

1. Baldwin, S.A., et al., *Mol Med Today* **5**:216 (1999).
2. Sugiyama, D., et al., *Drug Metab Dispos* **30**:220 (2002).
3. Kong, J., et al., *Am J Physiol Gastrointest Liver Physiol* **278**:G156 (2000).
4. Gao, B., et al., *J Pharmacol Exp Ther* **294**:73 (2000).
5. Tamai, I., et al., *Pharma Res* **18**:1262 (2001).
6. Abe, T., et al., *J Biol Chem* **274**:17159 (1999).
7. Jegla, T. and Salkoff, L., *J Neurosci* **17**:32 (1997).
8. Patient, R.K., Harris, R., Walmsley, M.E. and Williams, J.G., *J Biol Chem* **258**:8521 (1983).