Determination of estradiol metabolites in human liver microsome by high performance liquid chromatography-electrochemistry detector

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KEY WORDS high pressure liquid chromatography; liver microsome; estradiol

ABSTRACT

AIM: To constitute a method to determine the estradiol metabolites in human liver microsome in low concentration of estradiol. METHODS: Use high performance liquid chromatography after solvent extraction, evaporation, and reconstitution to separate the metabolites and use a electrochemistry detector to detect the metabolites. RESULTS: With a mobile phase of acetate acid buffer-acetonitrile (50:50, v/v, pH 4.5) at flow rate of 1.0 mL/min and a potential of +0.7 V vs Ag/AgCl, all six composition were well separated and satisfactorily detected. There are E2, 16α-OHE2, 2-OHE2, E1, and two unidentified composition. The minimum detectable amount is about 100 pg on column. This method is sensitive enough to detect E1 in a substrate concentration of 1 μmol/L. CONCLUSION: The method can be used to study the metabolism mechanism of estradiol in liver microsome.

INTRODUCTION

 Estradiol (E2) is one of the most abundant estrogen with important biological effects. A number of reports indicated that estradiol is closely related to breast cancer, atherosclerosis, and osteoporosis. More than 90% of estradiol in the bloodstream is protein bound, with sex hormone-binding globulin being the major serum estrogen-binding moiety. Estradiol is metabolized into estrone (E1), 2-hydroxyestradiol (2-OHE2), estradiol (E2), 16α-hydroxyestrone (16α-OHE1) and many other metabolites. E2 17β-hydroxy dehydrogenase reaction is catalyzed by 17β-hydroxy dehydrogenase (17β-HSD). Studies with purified cytochrome P450 isoenzyme from rat liver microsome also indicated that CPY2C11, CPY2C12, CPY2C13, and CPY2A2 have high activity for catalyzing the dehydrogenate reaction of E2 17β-hydroxy to form E1. CPY2C11 and CPY2C12 also have high activity for catalyzing the 2 and 16α-hydroxylation of E2. CYP2D family that is constituted in male and female rats also has high activity for the formation of 2 and 16α-hydroxy metabolites. CYP1A2 has high activity for the formation of 2-hydroxy metabolite. Study in hamster liver microsomes indicated that E2 2-hydroxylation catalyzed by CYP1A1 and CYP3A families. Other study also indicated that CYP1A2 and CYP3A4 were the most important enzymes in catalyzing E2 2-hydroxylation in cDNA expressed human cytochrome P450.

Studies in human liver microsome also found that CYP1A2 and CYP3A4 played important role in catalyzing E1 and E2 2-hydroxylation. All these studies base on a high substrate concentration (about 100 μmol/L). In order to study the E2 catalyzing mechanism in low substrate concentration, we constitute the high performance liquid chromatography-electrochemistry detector (HPLC-ESD) method, which is sensitive and easy in determining the metabolites of E2 in human liver microsome.

MATERIALS AND METHODS

Chemicals and reagents The standard estradiol, estrone, 2-hydroxyestradiol, 16α-estrone, estradiol, and stilbestrol were purchased from Sigma company. Sodium acetic, acetic acid, and chloroform were AR grade. Chloroform was redistilled before use. Acetonitrile and methanol were of HPLC grade. Acetic acid buffer was prepared by solving 6.8 g of sodium acetic,
edetic acid-2Na 100 mg, and 36 % acetic acid 100 mL in 1000 mL.

Collection of human liver sample Adult human liver tissue from renal transplant donors without known liver disease and patients who had undergone partial hepatectomy were collected in our liver bank. The Ethics Committee of Hunan Medical University approved the collection and utilization of human liver tissues. Candidate patients for liver sample collection were those who did not suffer from acute or chronic hepatitis or cirrhosis, and took no medications known to induce or inhibit cytochrome P450 activity. Portions of surgical liver "waste tissue" distant from disease-affected regions and which appeared visually normal was collected. After removal, the liver sample was immediately cut into small pieces, washed with ice-cold isotonic saline, rapidly frozen in liquid nitrogen for 30 min, and was then stored at −80 °C. Prior to use, all samples were confirmed as being normal histologically.

Preparation of microsomes Washed microsomes were prepared by differential centrifugation(14) and stored at −80 °C until required. Microsomal protein concentrations were determined by the method of Lowry et al(15).

Incubation condition Estradiol incubated in 490 µL of 50 mmol/L of K2HPO4-KH2PO4 buffer (pH 7.4) mixture, which contained 0.5 mg of microsomal protein, 5 µmol of magnesium chloride, 2.5 µmol of β-NADP and 5 µmol of G-6-P. The mixture was preincubated for 5 min at 37 °C in a shaking water bath, and the reaction was initiated by addition of 0.5 IU of G-6-PDH (10 µL) and incubated for 30 min. The reactions were terminated by cooling in icy water.

Chromatographic conditions The HPLC system is Hewlett Packard series 1050, which include a pump (HP 1050), a degasser (HP 1050), an analytical column (HP ODS Hypersil 5 µ, 250 mm x 4 mm, 799260D-584), and a column oven (HP 1050). The detector is an electrochemistry detector (HP 1049A). The mobile phase is acetic acid buffer-acetonitrile (50:50, v/v, pH 4.5). The flow rate is 1.0 mL/min, and the potential is +0.7 V vs Ag/AgCl.

Storage solution preparation Estradiol 9.01 mg, estradiol 6.96 mg, 16α-hydroxyestrone 1.13 mg, 2-hydroxyestradiol 1.10 mg, and stilbestrol 4.92 mg were dissolved into 25 mL methanol respectively. These solutions were stored below zero centigrade. Stilbestrol was used as internal standard.

Sample extraction After cooled the microsome in ice water and added 50 µL of internal standard (0.98 mg/L of stilbestrol in methanol) to it, 2 mL of cool chloroform were added to the mixture. Mixed the microsome in a vortex mixer for 1 min, and centrifuged in 2500 × g for 10 min. The organic layer was separated and dried under high purity N2 flow below 35 °C. After dissolved the residues with 100 µL of mobile phase, 20 µL was applied to the HPLC system.

Validation study Preparing series of standard solution in blank incubation system (without G-6-PDH) to determine the linear range. The minimum detectable concentration is determined at the same time (S/N = 3:1). Three concentration levels of standard were prepared in blank incubation system to study the accuracy and precision of the assay, and to study the stability of the test solution within day and between days.

RESULTS

Pure standards of E2, E1, E3, 2-OHE2, 16α-OHE2, and stilbestrol were added to 500 µL incubate system (without NADPH generate system) to incubate 30 min, and extract with 2 mL of chloroform. The organic phase was dried in N2 flow at 35 °C, after dissolved with 100 µL of mobile phase, it was applied to the system. With a mobile phase of acetic acid buffer-acetonitrile (50:50, v/v, pH 4.5) at flowrate of 1.0 mL/min, and a potential of +0.7 V vs Ag/AgCl, all six composition were well separated, and satisfactorily detected (Fig 1). The retention time of E2, 16α-OHE1, 2-OHE2, E2, E1, and stilbestrol were 3.4, 4.5, 5.7, 9.0, 11.9, and 15.6 min, respectively.

The relationship between the concentration of each estrogen and the peak area ratio to the internal standard (stilbestrol) was linear in the following range (Fig 2): E2: 18.0 - 360.0 µg/L, E1: 11.4 - 227.0 µg/L, E3: 13.9 - 278.0 µg/L, 2-OHE2: 11.0 - 220.0 µg/L, 16α-OHE1: 11.3 - 226.0 µg/L. The minimum detectable amount of E3, 16α-OHE1, 2-OHE2, and E1 were about 100 pg on column (S/N = 3:1).

The average relative recoveries of E3, 16α-OHE1, 2-OHE2, E2, and E3 were 99.46 %, 97.32 %, 83.21 %, 92.04 %, and 94.30 %, respectively (Tab 1). The intra-day and inter-day variations showed in Tab 2.

The blank graph of human liver microsomes and standard graph of all compositions that extract from human liver microsomes showed in Fig 3. The characterized chromatograms of different substrate concentrations
Fig 1. The relationship of signal (area/concentration) and the potential (vs Ag/AgCl). Above +0.7 V vs Ag/AgCl, all the compositions have relatively strong signal, and the signal become stronger far more slowly than that under +0.7 V vs Ag/AgCl. The ideal potential is +0.7 V vs Ag/AgCl.

that incubated in human liver microsome are showed in Fig 3. When substrate concentration is 1 μmol/L, only E2 and E1 can be detected. In 10 μmol/L substrate concentration, E2, E1, and 2-OH E2 can be detected, whereas, in 100 μmol/L, there are more six metabolites can be detectable. In all studied substrate concentration, E1 and 2-OHE2 are the most important metabolites.

Tab 1. Average recoveries of E3, E1, E3, 2-OHE2, and 16α-OHE2 in different concentrations. n = 3. ± s.

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>E3</th>
<th>16α-OHE2</th>
<th>2-OHE2</th>
<th>E2</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recoveries (% , n=3)</td>
<td>35.6±0.17</td>
<td>104.16±0.42</td>
<td>81.83±0.75</td>
<td>92.51±0.43</td>
<td>85.40±0.21</td>
</tr>
<tr>
<td>Concentration (μg/L)</td>
<td>166.8</td>
<td>135.6</td>
<td>122.0</td>
<td>216.3</td>
<td>136.2</td>
</tr>
<tr>
<td>Recoveries (% , n=3)</td>
<td>100.61±0.11</td>
<td>98.69±0.33</td>
<td>85.51±0.58</td>
<td>90.96±0.49</td>
<td>105.32±0.30</td>
</tr>
<tr>
<td>Concentration (μg/L)</td>
<td>278.0</td>
<td>226.0</td>
<td>220.0</td>
<td>360.0</td>
<td>227.0</td>
</tr>
<tr>
<td>Recoveries (% , n=3)</td>
<td>100.33±0.23</td>
<td>89.72±0.39</td>
<td>82.28±0.59</td>
<td>84.65±0.37</td>
<td>91.18±0.29</td>
</tr>
</tbody>
</table>

Tab 2. The intra-day and inter-days variations.

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>E3</th>
<th>16α-OHE2</th>
<th>2-OHE2</th>
<th>E2</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day variation (RSD % , n=5)</td>
<td>4.6</td>
<td>6.5</td>
<td>7.4</td>
<td>5.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Inter-day variation (RSD % , n=8)</td>
<td>9.2</td>
<td>9.7</td>
<td>10.9</td>
<td>7.2</td>
<td>8.1</td>
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</table>

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>E3</th>
<th>16α-OHE2</th>
<th>2-OHE2</th>
<th>E2</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day variation (RSD % , n=5)</td>
<td>2.9</td>
<td>3.2</td>
<td>5.0</td>
<td>2.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Inter-day variation (RSD % , n=8)</td>
<td>5.6</td>
<td>6.6</td>
<td>9.4</td>
<td>4.9</td>
<td>5.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>E3</th>
<th>16α-OHE2</th>
<th>2-OHE2</th>
<th>E2</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day variation (RSD % , n=5)</td>
<td>3.1</td>
<td>2.8</td>
<td>4.3</td>
<td>2.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Inter-day variation (RSD % , n=8)</td>
<td>5.9</td>
<td>4.2</td>
<td>7.4</td>
<td>4.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>
Fig 3. With a mobile phase of acetic acid buffer-acetonitrile (50:50, v/v, pH 4.5) at flowrate of 1.0 mL/min, and a potential of +0.7 V vs Ag/AgCl, all six composition were well separated. Graph A is the blank graph of human liver microsome, and B is the standard graph of $E_2$ (360 µg/L), $E_1$ (227 µg/L), $E_6$ (278 µg/L), 2-OHE$_2$ (55 µg/L), 16α-OHE$_1$ (226 µg/L) and Stibestrol (492 µg/L) extracted from human liver microsome.

Fig 4. The characterized chromatograph of different concentration of $E_2$ incubated in human liver microsomes. When $E_2$ concentration is 1 µmol/L, $E_1$ is the main metabolite. In $E_2$ 10 µmol/L, $E_2$ is still the main metabolite, 2-OHE$_2$ and an unidentified metabolite ($X_1$) are the other two metabolites, while in $E_2$ 100 µmol/L, $E_4$ and 2-OHE$_2$ are the main metabolites, 16α-OHE$_1$ and three unidentified metabolites ($X_1$, $X_3$, $X_5$) have moderate concentration.

Simultaneously, except $E_3$, 16α-OHE$_1$, 2-OHE$_2$ and $E_1$, the other two are unidentified. The minimum detectable
amount of E\textsubscript{2}, 16α-OHE\textsubscript{1}, 2-OHE\textsubscript{2}, E\textsubscript{2}, and E\textsubscript{1} were about 100 pg on column, the method can be modified to detect the E\textsubscript{2}, E\textsubscript{1}, 2-OHE\textsubscript{2}, and 2-OHE\textsubscript{1} level in urine. Usually the excretion amount of 2-OHE\textsubscript{2} (include conjugated) in children, men, perioualation women, pregnant women urine are 20, 1045, 2365, and 5100 μg/L respectively\textsuperscript{19}.

We use the method to study the E\textsubscript{2} metabolism mechanism in human liver microsomes. It is sensitive enough to determine the metabolites of 1 μmol/L of E\textsubscript{2} incubated in 500 μL microsome. Each sample takes only 17 min; the method is more time saving than the previous method. In the following studies we found that the 2-OHE\textsubscript{2} and E\textsubscript{2} are the most two important metabolites in human liver microsomes. E\textsubscript{2} 2-hydroxylation was catalyzed mainly by CYP1A2 and CYP3A4.

2-OHE\textsubscript{2} is very easy to be oxidized when exposure in air, and in high temperature. The incubated microsome should be process as soon as possible and the extracted organic layer should be dry under high purity N\textsubscript{2} flow below 35 °C. The processed sample should be assay within day.

REFERENCES

高效液相色谱-电化学检测器法测定人肝微粒体中
雌二醇的代谢产物

程泽能，黄松林，谭志荣，王 伟，周宏灏
（湖南医科大学基础与临床药理学研究所，长沙
410078，中国）

目的: 建立雌二醇在人肝微粒体中代谢产物的测定

方法: 研究低浓度雌二醇在人肝微粒体中的代谢机
制。方法: 有机溶剂提取、蒸发、重溶的方法处理
样品，高液相色谱法分离组分，电化学检测器测
定含量。结果: 流动相为醋酸缓冲液-乙腈（50:50，
v/v，pH 4.5，电压为 +0.7 V vs Ag/AgCl) 时，在 C18
c柱上分离较好。最低检测量为 100 pg。结论: 本法
可用于测定底物浓度在 1 ～ 100 μmol/L 时，人肝微
粒体中的代谢产物。

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