The development of an ultra performance liquid chromatography-coupled atmospheric pressure chemical ionization mass spectrometry assay for seven adrenal steroids

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Received 12 April 2007
Available online 1 October 2007

Abstract

An ultra performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (UPLC–APCI–MS) method was developed for the separation and quantification of adrenal steroid metabolites from heterologous expression media. Steroids were extracted by liquid–liquid extraction, separated on a Waters UPLC BEH C18 column, ionized by APCI, and detected using a triple quadrupole mass spectrometer in APCI positive mode with single ion monitoring. The incorporation of UPLC enabled the detection of seven structurally closely related steroids at between 5 and 40 ng/ml using run times of 11 min. The adrenal steroidogenic enzyme cytochrome P450 17-hydroxylase/17,20-lyase (CYP17) was expressed in the yeast Pichia pastoris and in nonsteroidogenic COS-1 cells, and used as a model system to evaluate the detection and quantification of adrenal steroid metabolites by UPLC–APCI–MS.

Keywords: Cytochrome P450 17-hydroxylase/17,20-lyase (CYP17); Atmospheric pressure chemical ionization (APCI); Ultra performance liquid chromatography (UPLC)

The mammalian adrenal gland is an important source of steroid hormones. The three end products of adrenal steroidogenesis are the mineralocorticoids, glucocorticoids, and adrenal androgens. The adrenal steroidogenic cytochromes P450 are a unique group of enzymes responsible for the biosynthesis of these hormones that are vital for the control of water and mineral balance, stress management, and reproduction. Within the adrenal steroidogenic pathway, cytochrome P450 17-hydroxylase/17,20-lyase (CYP17) catalyzes two distinctly different reactions, the 17α-hydroxylase and the C17-C20 lyase reaction [1], placing this enzyme at a key branch point in the biosynthesis of aldosterone, cortisol, and the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione (A4). The 17α-hydroxylation of pregnenolone (PREG) and progesterone (PROG) yields 17α-hydroxypregnenolone (17-OHPREG) and 17α-hydroxyprogesterone (17-OHPROG), respectively. The 17,20-lyase reaction catalyzes the cleavage of the C17,20...
bond of 17-OHPREG and 17-OHPROG to yield DHEA and A4, respectively. 3β-Hydroxysteroid dehydrogenase (3βHSD) converts the 3β-hydroxy-Δ5-steroid precursors PREG, 17-OHPREG, and DHEA to the corresponding Δ4 3-ketosteroids PROG, 17-OHPROG, and A4 (Fig. 1). In addition, it is possible for human CYP17, as well as CYP17 of other primate species, to convert PROG to 16α-hydroxyprogesterone (16-OHPROG) [2,3]. These steroids are structurally closely related, and in some instances differ only by the position of a double bond or an OH group, complicating their separation and quantitation in biological extracts.

The detection and quantification of steroid metabolites from various heterologous expression systems expressing steroidogenic cytochromes P450 are commonly performed by thin layer chromatography (TLC) [3,4–7] and high-performance liquid chromatography (HPLC) [2,8,9]. Because a number of the steroids, in particular the Δ5 steroids, have poor UV absorption [10], both of these methods rely on the use of expensive tritiated steroids. Although it is possible to separate either the Δ5 or Δ4 steroid metabolites of CYP17 adequately by TLC, it is impossible to separate a complex mixture containing both the Δ5 and Δ4 steroid metabolites. Even though a better chromatographic separation can be achieved by HPLC, the resolution remains unsatisfactory because metabolites coelute and run times of up to 30 min are required [8].

The detection of adrenal steroids in biological fluids is critical for diagnostic purposes because clinical conditions, such as congenital adrenal hyperplasia and polycystic ovary syndrome [11], are characterized by abnormalities in adrenal steroid production. Gas chromatography–mass spectrometry (GC–MS) and immunoassays are employed regularly for the detection of steroids in serum [12–16]. Although GC–MS methods exhibit good specificity, efficiency is impeded by low throughput and the requirement of large sample volumes [17,18]. Immunoassay analyses, on the other hand, are hampered by cross-reactivity of immunoglobulins and matrix effects [19]. Furthermore, immunoassays often are method specific and cannot be used interchangeably [20–25].

Recently, Nithipatikom and coworkers [26] developed a liquid chromatography–mass spectrometry (LC–MS) assay for the simultaneous detection of several adrenal steroids, demonstrating that LC–MS is a viable alternative to conventional methods. However, this assay made use of conventional HPLC resulting in run times of 50 min.

We have developed a method of LC–MS for the simultaneous detection of seven adrenal steroids obtained in the conversion of PREG and PROG catalyzed by CYP17 expressed in Pichia pastoris, a yeast expression system, and in COS-1 cells, a mammalian expression system. Steroids were extracted by liquid–liquid extraction, separated by ultra performance liquid chromatography (UPLC), ionized by atmospheric pressure chemical ionization (APCI), and detected by a tandem quadrupole mass spectrometer.

Materials and methods

Reagents

PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, A4, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Sigma Chemical (St. Louis, MO, USA). COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Penicillin–streptomycin, trypsin–EDTA, and Dulbecco’s phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal calf serum was purchased from Highveld Biological (Lyndhurst, South Africa). P. pastoris strain GS115 was purchased from Invitrogen (Carlsbad, CA, USA), and all yeast media components were obtained from Difco Laboratories (Detroit, MI, USA). Nucleobond AX plasmid preparation kits were purchased from Macherey–Nagel (Duren, Germany). A bicinchoninic acid (BCA) protein

![Fig. 1. Enzymatic reactions catalyzed by CYP17 and 3βHSD.](image-url)
assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest analytical grade and were purchased from scientific supply houses.

**Standards**

Stock solutions of PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, and A4 (2 mg/ml) were prepared in ethanol. A series of standards (2, 20, 200, and 2000 ng/ml) were prepared in methanol from the stock solutions.

**UPLC–APCI–MS conditions**

A mixture of the Δ5 steroids (PREG, 17-OHPREG and DHEA) and Δ4 steroids (PROG, 16-OHPROG, 17-OHPROG, and A4) was separated by UPLC (ACQUITY UPLC, Waters, Milford, MA, USA) using a Waters UPLC BEH C18 column (2.1 mm, 1.7 μm) at 50 °C. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile). A linear gradient from 85% A to 80% B in 3.5 min, followed by a linear gradient from 80% B to 100% B in 0.1 min and an isocratic elution with solvent B for 1 min, was applied. A linear gradient returned the column to 85% A in 1 min. The total run time per sample was 5 min at a flow rate of 0.4 ml/min. The injection volume of standards and samples was 5 μl.

Samples containing both the Δ5 steroids and Δ4 steroids (PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, and A4) were separated by UPLC (ACQUITY UPLC) using a Waters UPLC BEH C18 column (2.1 x 100 mm, 1.7 μm) at 50 °C. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (3:1 acetonitrile/methanol with 1% isopropanol). The column was eluted isocratically with 56% A and 44% B for 6 min, followed by a linear gradient from 44% B to 80% B in 0.01 min and a subsequent linear gradient from 80% B to 100% B in 2.49 min. A linear gradient returned the column to 56% A and 44% B in 0.5 min. The total run time per sample was 11 min at a flow rate of 0.3 ml/min. The injection volume of standards and samples was 5 μl.

An API Quattro Micro tandem mass spectrometer (Waters) was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters) was used for the APCI interface in positive mode. The corona pin was set to 7 μA, the cone voltage to 30 V, and the APCI probe temperature to 450 °C. All other settings were optimized to obtain the strongest signal possible. Calibration curves were constructed by using weighted (1/x2) linear least squares regression. Data were collected with the MassLynx 4.0 software program.

**Extraction procedure**

Steroids were extracted from the samples (0.5 ml) by liquid–liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 2 min and centrifuged at 500g for 5 min. The water phase was aspirated off, and the dichloromethane phase was transferred to a clean test tube. The samples were dried under a stream of nitrogen. The dried steroids were reconstituted in 100 μl of methanol prior to analysis.

**Validation of UPLC–APCI–MS assay**

A standard curve (10, 20, 40, 100, 200, 400, and 1000 ng/ml) was generated (n = 6). The reproducibility of the assay was determined by replicate analysis (n = 6) of cell culture media (0.5 ml) and yeast culture media (0.5 ml) that had been spiked with each steroid, extracted, and subsequently quantified. Absolute recoveries were calculated at a concentration of 200 ng/ml for all of the steroids, from spiked cell culture media and yeast culture media, by comparing the peak areas of the extracted samples with standard solutions.

**Enzymatic assays in P. pastoris expressing CYP17**

Single colonies of *P. pastoris* strain GS115 (Invitrogen), previously transformed with wild-type human CYP17 [27], were grown in 25 ml BMGY growth media (1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer [pH 6.0], 1.34% yeast nitrogen base without amino acids, 4 x 10^-7% Biotin, and 1% [v/v] glycerol) for 48 h (OD600 = 10.5 x 10^2 cells/ml) in a shaking incubator at 30 °C.

Cell suspension aliquots (8 ml) were harvested by centrifugation at 2000g for 5 min at room temperature, washed with PBS, and resuspended in 2 ml of fresh BMMY induction media (1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer [pH 6.0], 1.34% yeast nitrogen base without amino acids, 4 x 10^-7% Biotin, and 0.5% [v/v] methanol). Cells were preincubated for 15 min at 30 °C in an Erlenmeyer flask (100 ml). PREG and PROG metabolism was assayed after the addition of an equal volume of BMMY media supplemented with either PREG (10 μM) or PROG (10 μM). Aliquots of 50 μl were removed at specific time intervals to assay substrate conversion. Control reactions were preformed using the parent vector strain (GS115/pPIC3.5K), which had not been transformed, following the same protocol.

**Enzyme assays in COS-1 cells expressing CYP17 and 3βHSD**

COS-1 cells were grown at 37 °C and 5% CO2 in DMEM supplemented with 10% fetal calf serum, 1% penicillin–streptomycin, 4 mM l-glutamine, and 25 mM glucose. Cells were plated in 12-well dishes at 1 x 10^5 cells/ml 24 h prior to transfection. Cells were transiently transfected with 0.5 μg DNA using GeneJuice transfection reagent (Novagen, Darmstadt, Germany) and were incubated for 72 h before adding steroid substrate. PREG metabolism was assayed in COS-1 cells cotransfected with Angora goat CYP17 (0.25 μg) and 3βHSD (0.25 μg) plas-
mid constructs. PROG metabolism was assayed in COS-1 cells transfected with either Angora goat, baboon, or human CYP17 constructs (0.5 µg). Control transfection reactions were performed using the mammalian expression vector pCI-neo (Promega, Madison, WI, USA) containing no insert. Substrate conversion was assayed by removing aliquots (0.5 ml) at specific time intervals. On the completion of each experiment, the cells were washed with and collected in 0.1 M phosphate buffer (pH 7.4). The cells were homogenized with a small glass homogenizer, and the protein content of the homogenate was determined by the Pierce BCA method according to the manufacturer’s instructions.

**Results and discussion**

**Separation and characteristics of steroids by UPLC–APCI–MS**

This study aimed to develop a new method to separate and quantify complex mixtures of seven adrenal steroids that form the substrates and metabolites of CYP17. Current methods use conventional HPLC and rely on retention times alone for the identification of the steroids. This complicates the detection and quantification of steroids from complex biological samples and results in run times of up to 30 min [8]. However, coupling HPLC with MS ion extraction analysis allows the detection and quantification of steroids from complex samples as well as the detection and quantification of steroids that were not well resolved by conventional HPLC previously [28]. In addition, incorporating the new technology of UPLC as an analytical tool has the potential to reduce run times significantly.

A UPLC method was initially developed for the separation of the Δ5 steroids (PREG, 17-OHPREG, and DHEA) and Δ4 steroids (PROG, 16-OHPROG, 17-OHPROG, and A4). A cone voltage of 30 V and a capillary voltage of 3.5 kV in MS mode were used for the detection of the Δ5 steroids. PREG and DHEA exhibited a high abundance of protonated molecules due to the loss of water (M – H2O + H) +, whereas 17-OHPREG exhibited high abundance of protonated molecules due to the loss of both a single water molecule (M – H2O + H) + and two water molecules (M – 2H2O + H) + (Fig. 2). The ionization of PREG was further increased by reducing the cone voltage to 15 V. Tandem mass spectrometry (MS/MS) mode resulted in reduced sensitivity due to quantitatively inconsistent daughter ions. Therefore, MS mode was the preferred mode of detection and quantification. Good separation (Fig. 3) was achieved for PREG within 5 min, whereas 17-OHPREG and DHEA eluted within 0.10 min of each other (Table 1). Comparing the spectra of these two steroids revealed that characteristic ion m/z 297 of 17-OHPREG is absent in the spectra for DHEA and similarly that the characteristic ion m/z 271 of DHEA is absent in the spectra for 17-OHPREG (Fig. 2). Therefore, these extracted ions were chosen for quantification.

Using the same system, the Δ4 steroids exhibited mass spectra with a high abundance of protonated molecules ([M + H] +) (Fig. 2). Good separation was achieved for PROG and 16-OHPROG (Fig. 3), whereas 17-OHPROG and A4 had similar retention times (Table 1). The extracted ions m/z 331, unique to 17-OHPREG, and m/z 287, unique to A4, were chosen for quantification.

The separation of a mixture of the Δ5 and Δ4 steroids, using this method, resulted in the coelution of 17-OHPREG, DHEA, 17-OHPROG, and A4 (data not shown). Therefore, the separation of the Δ5 and Δ4 steroids necessitated an alternative UPLC method using a longer column and a different solvent system, resulting in an increased run time of 11 min. This method achieved good resolution for all of the steroids, including 17-OHPREG, DHEA, and 17-OHPROG (Fig. 4). Although the retention time of A4 overlapped negligibly with the retention times of 17-OHPREG and DHEA, the extracted ion m/z 287 of A4 was not detected in the 17-OHPREG and DHEA spectra. Similarly, neither of the extracted ions for 17-OHPREG (m/z 297) or DHEA (m/z 271) was detected in the A4 spectra (Fig. 2). PREG and PROG shared the same retention time using this method. Although these steroids could be separated by increasing the gradient elution time, this would unnecessarily increase the run time because each of these steroids is characterized by a unique extracted ion (PREG, m/z 299; PROG, m/z 315).

**Recovery efficiencies**

The recoveries of steroids were determined by the extraction of standards from both COS-1 cell culture media and yeast cell culture media, followed by UPLC–APCI–MS analyses. The recoveries are shown in Table 2. These data were calculated from the peak areas of the extracted steroids as compared with standards not extracted by media. Steroid recovery from mammalian cell culture media always was higher than that from yeast culture media, with the exception of the recovery of PROG. There are no interfering chromatographic peaks from the mammalian cell culture media (data not shown). Therefore, this effect most likely is due to a matrix effect resulting from the more complex mammalian cell culture media.

The use of an internal standard is not necessary when determining the relative amounts of steroids extracted from heterologous expression media and, therefore, was not used in this study. However, including an internal standard may reduce the variability of the recovery and is recommended, especially when working with low steroid concentrations.

**Standard curves**

Standard curves were generated for each steroid for concentrations ranging from 10 to 1000 ng/ml. The calibration curves were linear over these concentration ranges, with regression correlation coefficients (r²) always greater than 0.99.

The limit of detection (LOD) for each steroid was determined by the lowest concentration at which a signal/noise
A ratio greater than 3 was detected. The limit of quantification (LOQ) was determined by the lowest concentration that was detected with a signal/noise ratio greater than 10 and at which the accuracy was within ±20%. The data for LOD and LOQ are shown in Table 2. These values are relevant for the detection of steroids assayed in the heterologous expression systems used in this study.

**PREG and PROG metabolism of CYP17 expressed in P. pastoris**

Wild-type human CYP17 (GenBank accession number NM000102) was expressed in *P. pastoris*, and the conversion of PROG (10 μM) and PREG (10 μM) was assayed. After 60 min, PROG was metabolized to approximately 63% 17-OHPROG and 37% 16-OHPROG, with no detectable A4, whereas PREG metabolism yielded approximately 76% 17-OHPREG and 14% DHEA (Fig. 5). No PREG or PROG metabolites were detected in *P. pastoris* cells transformed with the pPIC3.5K vector containing no insert. The 16α-hydroxylation of PROG by CYP17 is characteristic of human CYP17, with 16-OHPROG being identified in humans and other primate species [2,3]. Expression of CYP17 in COS1 cells yielded a 16-OHPROG: 17-OHPREG ratio of 1:4 [2].

Fig. 2. APCI–MS mass spectra of steroid standards.
Fig. 3. Selected ion chromatogram of Δ⁵ steroid (A) and Δ⁴ steroid (B) standards separated on a Waters UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) at 50 °C as described in Materials and methods. Total ion current (TIC) of m/z 200 to 400 for mixtures of standards is also shown. Specification of individual ions (m/z) is as in Table 1.
current study, the ratio obtained in the expression of human CYP17 in *P. pastoris* was approximately 1:2 (16-OHPROG:17-OHPROG).

Table 1

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Extracted ion (m/z)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50-mm column</td>
<td>100-mm column</td>
</tr>
<tr>
<td>PREG</td>
<td>299</td>
<td>2.9</td>
</tr>
<tr>
<td>17-OHPREG</td>
<td>297</td>
<td>2.2</td>
</tr>
<tr>
<td>DHEA</td>
<td>271</td>
<td>2.3</td>
</tr>
<tr>
<td>PROG</td>
<td>315</td>
<td>2.9</td>
</tr>
<tr>
<td>16-OHPROG</td>
<td>331</td>
<td>1.9</td>
</tr>
<tr>
<td>17-OHPROG</td>
<td>331</td>
<td>2.3</td>
</tr>
<tr>
<td>A4</td>
<td>287</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**PREG and PROG metabolism of CYP17 expressed in COS-1 cells**

Angora goat CYP17 (GenBank accession number EF524063) and 3βHSD (GenBank accession number EF524065) were transiently cotransfected in nonsteroidogenic mammalian COS-1 cells, and the conversion of PREG (1 μM) was assayed. In the metabolism of PREG, the major metabolites detected were 17-OHPROG (~52%) and A4 (~32%) (Fig. 6). The PROG levels remained low during the entire time course, reaching a maximum of approximately 7% at 3 h, indicating that on formation PROG was metabolized to 17-OHPROG by CYP17. Similarly, 17-OHPREG reached a maximum of

Table 2

<table>
<thead>
<tr>
<th>Steroid</th>
<th>LOD (ng/ml)</th>
<th>LOQ (ng/ml)</th>
<th>Reproducibility a (200 ng/ml) (% RSD)</th>
<th>Recovery b (200 ng/ml) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>COS-1 cells</td>
<td>Yeast</td>
</tr>
<tr>
<td>PREG</td>
<td>20</td>
<td>30</td>
<td>2.6</td>
<td>9.7</td>
</tr>
<tr>
<td>17-OHPREG</td>
<td>20</td>
<td>40</td>
<td>4.8</td>
<td>14.9</td>
</tr>
<tr>
<td>DHEA</td>
<td>40</td>
<td>110</td>
<td>5.0</td>
<td>15.6</td>
</tr>
<tr>
<td>PROG</td>
<td>5</td>
<td>10</td>
<td>17.3</td>
<td>13.6</td>
</tr>
<tr>
<td>16-OHPROG</td>
<td>10</td>
<td>10</td>
<td>12.9</td>
<td>15.8</td>
</tr>
<tr>
<td>17-OHPROG</td>
<td>5</td>
<td>10</td>
<td>14.1</td>
<td>19.4</td>
</tr>
<tr>
<td>A4</td>
<td>10</td>
<td>10</td>
<td>11.2</td>
<td>15.5</td>
</tr>
</tbody>
</table>

a *n* = 6.
b *n* = 3.
approximately 15% at 3 h, demonstrating that on formation 17-OHPREG was metabolized to DHEA by CYP17 and to 17-OHPROG by 3βHSD. Because Angora goat CYP17 is unable to metabolize the conversion of 17-OHPROG to A4 (data not shown), 17-OHPROG is the end product of the Δ4 pathway. No DHEA was detected due to the immediate conversion of DHEA to A4 by 3βHSD.

COS-1 cells expressing Angora goat CYP17 and 3βHSD also produced low, yet detectable, levels of 16-OHPROG (Fig. 6). To date, only CYP17 from primate species has been shown to convert PROG to 16-OHPROG [2,3]. Human CYP17 (GenBank accession number NM000102), baboon CYP17 (GenBank accession number AF297650), and Angora goat CYP17 were subsequently expressed in COS-1 cells and assayed for activity with PROG (1 μM) as substrate. The 17-OHPROG/16-OHPROG ratio was determined after 8 h (Fig. 7). As expected, human CYP17 produced the highest levels of 16-OHPROG, with a 17-OHPROG/16-OHPROG ratio of 2. Baboon CYP17 produced significantly less 16-OHPROG (17-OHPROG/16-OHPROG ratio of 7), whereas Angora goat CYP17 produced significantly less 16-OHPROG than did both primate species, with a 17-OHPROG/16-OHPROG ratio of 22. Our data indicate that CYP17 of other species may well produce small amounts of 16-OHPROG but that the detection methods used previously were unable to detect these low levels. The physiological implications of this finding will be investigated in the future.

Conclusion

We have developed a UPLC–APCI–MS method for the rapid and accurate quantification of seven adrenal steroids that are structurally very similar and thus, difficult to separate effectively. The incorporation of UPLC allows for higher throughput and smaller sample volumes than do current methods, whereas the use of MS offers better selectivity as well as better LODs and LOQs. This method was used successfully for the quantification of both PREG and PROG metabolites formed by CYP17 expressed in the yeast P. pastoris and in nonsteroidogenic COS-1 cells. In addition, 16-OHPROG production by CYP17 was indicated for the first time in a nonprimate species.

Acknowledgments

The authors thank Patricia Storbeck for help with the preparation of this manuscript. This work was financially supported by the South African Mohair Council, National Research Foundation (NRF), University of Stellenbosch, and Wilhelm Frank Bursary Fund.
References


