Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2007 September 1; 856(0): 141–147. doi:10.1016/j.jchromb.2007.05.028.

Development and validation of a high-performance liquid chromatography-mass spectrometric assay for the determination of 17-alpha Hydroxyprogesterone Caproate (17-OHPC) in human plasma

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Abstract

A sensitive and specific method for the determination of 17 α -hydroxyprogesterone caproate (17-OHPC) in human plasma using high performance liquid chromatography and mass spectrometry has been developed and validated. Plasma samples were processed by a solid phase extraction (SPE) procedure using Oasis HLB extraction cartridge prior to chromatography.

Medroxyprogesterone acetate (MPA) was used as the internal standard. Chromatography was performed using Waters C18 Symmetry analytical column, 3.5 μ m, 2.1 $\times$ 10 mm, using a gradient elution with a mobile phase consisting of acetonitrile [A] and 5% acetonitrile in water [B], with 0.1% formic acid being added to both [A] and [B], at a flow rate 0.2 ml/min. The retention times of 17-OHPC and MPA were 8.1 and 5.0 min, respectively, with a total run time of 15 min.

Analysis was performed on Thermo Electron Finnigan TSQ Quantum Ultra mass spectrometer in a selected reaction monitoring (SRM), positive mode using electron spray ionization (ESI) as an interface. Positive ions were measured using extracted ion chromatogram mode. The extracted ions following SRM transitions monitored were m/z 429.2 $\rightarrow$ 313.13 and 429.2 $\rightarrow$ 271.1, for 17-OHPC and m/z 385.1 $\rightarrow$ 276 for MPA. The extraction recoveries at concentrations of 5, 10 and 50 ng/ml were 97.1, 92.6 and 88.7%, respectively. The assay was linear over the range 0.5 - 50 ng/ml for 17-OHPC. The analysis of standard samples for 17-OHPC 0.5, 1, 2.5, 5, 10, 25 and 50 ng/ml

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demonstrated a relative standard deviation of 16.7, 12.4, 13.7, 1.4, 5.2, 3.7 and 5.3%, respectively (n=6). This method is simple, adaptable to routine application, and allows easy and accurate measurement of 17-OHPC in human plasma.

Keywords

17-OHPC; MPA; liquid chromatography-mass spectrometry; Pregnancy

1. Introduction

Preterm delivery is a major determinant of infant mortality in developed countries [1]. Preterm delivery is more common in the United States than in many other developed countries and is the major factor responsible for the relatively high infant mortality in this country [2].

17-alpha-hydroxyprogesterone caproate (17-OHPC) has recently been suggested to be beneficial in preventing preterm labor in women with a prior preterm birth [3,4,5]. 17-OHPC is a synthetic hormone, which is administered intramuscularly once a week at a dose of 250 mg from weeks 16-20 and continued until 37 weeks in women with a history of prior preterm birth. The dose used currently is based on empiric observations and is based on earlier literature in different patient population [6]. There are no pharmacokinetic data of 17-OHPC in pregnant subjects. The only pharmacokinetic study, which measured 17-OHPC drug concentrations in a group of non-pregnant women, used a non-specific immunoassay methodology [7].

A specific and sensitive analytical methodology is necessary for characterizing the pharmacokinetics of 17-OHPC and for determining an optimal dosage regimen of 17-OHPC in pregnant subjects. Previous methods for determination of 17-OHPC were based on immunoassay or HPLC, where the assay was non-specific or the limit of quantification was not low and the assay was not sensitive enough to measure the levels of 17-OHPC in pregnant subjects [8,9,10]. A recent publication used a gas chromatographic method to measure 17-OHPC in equine plasma, however this method requires derivatisation of 17-OHPC and is very time consuming [11]. The objective of this study was to develop a sensitive, specific and reproducible method to estimate the concentration of 17-OHPC in plasma following intramuscular administration of 17-OHPC to pregnant women.

2. Experimental

2.1. Chemicals and solvents

The chemical structures of 17-OHPC and medroxyprogesterone acetate (MPA) are represented in Fig. 1. 17 α -hydroxyprogesterone caproate (17-OHPC) (USP reference standard) was purchased from United States Pharmacopoeia. Medroxy progesterone acetate (MPA) used as an internal standard was purchased from Sigma Chemicals (St. Louis, MO). Oasis ® HLB 1cc (30mg) extraction cartridges were purchased from Waters (Milford, MA). Optima HPLC grade methanol, HPLC grade water and acetonitrile were obtained from

Fischer Scientific (Fair Lawn, NJ, USA). Analytical grade formic acid was obtained from Sigma Chemicals (St. Louis, MO). Human plasma was procured from central blood bank.

2.2. Standards

Primary stock solutions for standards and quality control samples were prepared in methanol (1 mg/ml). Working standards and quality control samples were diluted from primary stock solution with methanol. The working internal standard solution (125 ng/ml) was prepared from the primary stock in methanol. Routine daily calibration curves and controls were prepared by spiking human plasma with working solution. The range of concentration of the spiked plasma standard was from 0.5 - 50 ng/ml and the concentration of quality control samples were 5, 10 and 50 ng/ml. Working and internal standard solutions were stored at 2°C to 8°C, and spiked plasma standard and quality control samples were stored at -20°C.

2.3. Sample preparation

Routine daily calibration curves, controls and the clinical samples were thawed at room temperature. Exactly 400 µl of plasma was diluted with 1 ml of water and passed through Oasis ® HLB 1 cc (30 mg) extraction cartridges, previously conditioned with methanol and water, under vacuum. After washing with 1ml of 50% methanol, 17-OHPC was eluted with 1ml of methanol and the eluent was evaporated to dryness under air. The residue was reconstituted in 100 µl of methanol and 25 µl of IS (125ng/ml), and 10 µl was injected into the HPLC system connected to the mass spectrometer.

2.4. Chromatographic and mass spectrometer conditions

The HPLC unit consisted of a Surveyor MS Pump plus and Micro AS injector (Thermo Electron, Finnigan). Separation was performed at ambient temperature on 3.5 µm, 2.1×150 mm Waters C18 Symmetry analytical column with 2.1×10 mm Waters C18 Symmetry guard column. The mobile phase consisted of 0.1% formic acid in mobile phase A (acetonitrile) and mobile phase B (5% of acetonitrile in water). The total run time was 15 min at a flow rate of 0.2 ml/min with a gradient program starting from 65% phase B, with a linear increase of phase B to 95% over 5 min; following another 5 min the gradient was changed to the initial condition of 65% phase B to achieve the base line. The sample tray temperature was set at 5° C in the Micro AS injector.

A Thermo Electron Finnigan TSQ Quantum Ultra mass spectrometer with electrospray ionization in positive ion mode was used for mass detection. The mass spectrometer was operated in the selected reaction-monitoring (SRM) mode. For 17-OHPC and internal standard, SRM setting used was as follows: spray voltage 3.5 kV; vaporizer temperature 250°C; capillary temperature 300°C; sheath gas pressure 50 psig; auxiliary gas pressure 40 psig; source CID -8 V; argon pressure 20+10 psig; nitrogen pressure 100+20 psig; The following SRM *m/z* transitions were monitored for each analyte: 429.2→313.1 and 429.2→271.1 for 17-OHPC, and 385.1→267.0 for MPA. The LC system and mass spectrometer were controlled by the Xcalibur®, and data were collected with the same software.

2.5. Assay validation

2.5.1. Standard Curve and Linearity—Human plasma spiked with working solutions of standard 17-OHPC in methanol in the concentration range of 0.5 - 50 ng/ml was used for preparing standard curve. Standard curve consisted of 17-OHPC at 0.5, 1, 2.5, 5, 10, 25, and 50 ng/ml. Response in peak area ratio of 17-OHPC to the internal standard (Y) was plotted against 17-OHPC concentration (x). A linear regression with no weighting was used to determine slopes, intercepts, and correlation coefficients. Unknowns were calculated by the equation: $x = (y - \text{intercept}) / \text{slope}$. Coefficient of variation (CV) was examined for each standard concentration. The CV should be less than 15% for each concentration level of all calibration points in the curves, except the lower limits of quantification (LLOQ), where it should not exceed 20% of CV.

2.5.2. Lower Limit of Quantification—LLOQ was determined as the lowest concentration of a given drug giving a response that should be at least 5 times compared to blank response and quantified with both inter assay variation and bias lower than 20%.

2.5.3. Accuracy and precision—Accuracy was measured as the percentage difference from theoretical according to the equation:

$$\text{Bias } (\%) = [(Con_M - Con_T) / (Con_T)] \times 100$$

Where Con_M = measured concentration, Con_T = theoretical concentration.

The precision of the developed method was determined by analysis of six standard samples. Injecting six standard samples at each concentration on the same day assessed intra-day variation of the assay. Inter-day variation was assessed by injecting another six samples of each concentration on three subsequent days. The precision of the method was expressed in %CV.

2.5.4. Recovery—Extraction recoveries from human plasma were determined by comparing the response to extracted plasma samples spiked with known amounts of 17-OHPC (5, 10 or 50 ng/ml, n=4) and fixed concentration of internal standard (25ul of 125 ng/ml) before extraction with the response of extracted blank plasma samples to which analyte has been added at the same nominal concentration just before injection. The extraction recoveries obtained for each concentration should be $100 \pm 20\%$ of actual concentrations.

2.5.5. Ion suppression—Ion suppression was investigated by adding analyte to blank matrix extracts following extraction. The response of the analyte was compared with non-extracted external standard solution (in methanol) at the same nominal concentration. The difference from 100% recovery can be attributed to matrix effect as ion suppression. Three concentrations of 17-OHPC (5, 10 and 50 ng/ml, n=4) and fixed concentration of internal standard (25 μ l of 125 ng /ml) were analyzed. To be acceptable, relative response should be $100 \pm 20\%$ for all QC samples.

2.6. Specificity

The specificity of the method was examined by analyzing different blank human plasma extracted in a same way as sample preparation procedure. Six different source of blank human plasma and ten different plasma samples from pregnant subjects not on 17-OHPC treatment have been tested to document the lack of interference with 17-OHPC or its internal standard, which was ensured at LLOQ.

2.7. Stability

The stability of 17-OHPC in plasma was evaluated with 3 quality control samples (5, 10 and 50 ng/ml, n=4 each) under different conditions *viz* following extraction of plasma kept for 24h at room temperature, following 24h at 5°C post sample extraction and following 3 freeze-thaw cycles. Stability was expressed in percentage of nominal concentration. The values should be $100 \pm 20\%$, as compared to samples extracted and injected immediately.

3. Results

3.1. Mass spectral analysis

When 17-OHPC and MPA were injected directly in the mass spectrometer with a positive ion interface, the protonated molecules $(MH)^+$ of 17-OHPC and MPA were seen in abundance. Both parent ions and product ions were observed to have a mass of m/z 429.2→313.13 and 429.2→271.1 for 17-OHPC, and m/z 385.1→267.0 for MPA. The spray voltage 3.5 kV; vaporizer temperature 250°C; capillary temperature 300°C; sheath gas pressure 50 psig; auxiliary gas pressure 40 psig; source CID -8 V; argon pressure 20+10 psig; nitrogen pressure 100+20 psig, were selected to optimize specificity and selectivity of both parent and product ion detection. The full scan mass spectra (m/z) for 17-OHPC is shown in Fig. 2.

3.2. Separation and relative retention time

The retention times for 17-OHPC and MPA were 7.9 and 4.9 min, respectively. The total run time is 15 min. Blank plasma was tested for endogenous interference. No additional peaks due to endogenous substances were observed that would interfere with the detection of compounds of interest. Typical chromatogram of human blank plasma without internal standard (MPA) and 17-OHPC, plasma spiked with 0.5 ng/ml 17-OHPC, and clinical sample collected following intramuscular administration of 250 mg 17-OHPC is shown in Fig. 3A. Also blank plasma without MPA and 17-OHPC, plasma spiked with 3.125 ng/ml of MPA and clinical sample spiked with MPA is shown in Fig. 3B.

3.3. Linearity

The ratio of mean peak area of 17-OHPC to MPA was linearly related to the concentration of 17-OHPC ($R^2 = 0.9963$) in concentration range of 0.5 – 50 ng/ml. Results of LLOQ (0.5 ng/ml) met the criteria for limit of detection.

3.4. Recovery data

The percent recovery of 17-OHPC was measured by dividing the ratio of concentration levels of extracted samples with that of the unextracted control samples. The extraction recovery and relative response when tested for matrix effect (n=4) for 17-OHPC (5, 10, 50 ng/ml) was 97.1%, 92.6%, 88.7%, and 104.3%, 103.9%, 104.1%, respectively. (Table 2).

3.5. Precision and accuracy

Intra-day and inter-day coefficients of variation for 17-OHPC samples were within the acceptable limits to meet the bioanalytical method guidelines for bioanalytical validation. At concentrations of 0.5, 1, 2.5, 5, 10, 25 and 50 ng/ml the intra-assay precision expressed as CV were 16.7%, 12.4%, 13.7%, 1.4%, 5.2%, 3.7% and 5.3% and inter-assay precision expressed as CV were 14.2%, 12.3%, 13.6%, 13.0%, 7.9%, 10.5% and 4.6% respectively. Data for precision is shown in Table 3. The accuracy of the QC's are presented in Table 1, demonstrating that the measured concentrations are within 15% of the actual concentration.

3.6. Storage stability data

There was no difference in the concentration of 17 OHPC in plasma samples that were thawed and maintained for 24h at RT and those that were kept frozen. There was no loss of 17 OHPC in samples that underwent three freeze-thaw cycles. Repeat injection of extracted samples kept at 5°C for 24 hours resulted in lower estimate of 17-OHPC. (Table 4).

Application to clinical sample analysis—The method developed was applied to a set of samples collected in a clinical study of 17-OHPC in pregnant subjects. An intramuscular dose of 250 mg was administered to volunteers who were undergoing treatment for preterm delivery. Blood samples were collected over a period of 7 days and plasma analyzed using this method. A representative plasma concentration versus time profile in one pregnant volunteer is shown in Fig. 4.

Discussions

17-OHPC appears to provide great promise in the prevention of premature delivery in pregnant women. A lack of pharmacokinetic data in pregnant women has hampered the optimization of the dosing regimen of this agent. There has been no simple, specific, sensitive and reproducible analytical method available to quantify 17-OHPC in human plasma or serum. Consequently, the aim of this study was to develop an LC-MS/MS method for the quantification of 17-OHPC in plasma.

We first established that there is no endogenous interfering substance that is similar to 17-OHPC in blank female human plasma by using HPLC and mass spectrometry. Multiple blank plasma samples were analyzed to assure that there was no endogenous 17-OHPC in female human plasma as well. Using a solid phase extraction more water soluble materials from the plasma were removed first. Some lipid soluble materials and 17-OHPC were then eluted from the solid phase extraction column using methanol. The extraction recovery was in the range of 89 - 97% using the methodology developed. This extraction procedure also

allows for minimal ion suppression (no matrix effect) for 17-OHPC in the mass spectrometry.

17-OHPC was separated from other components in plasma using an analytical column and a gradient profile. The internal standard MPA is structurally related to 17-OHPC and is well separated from 17-OHPC. The gradient profile, while shortening the retention time for 17-OHPC, also allows for future quantification of some of the metabolites of 17-OHPC in a single run.

Preliminary analysis was performed with both +ve and –ve ion modes, but the sensitivity obtained with +ve mode was much better and hence we selected +ve mode for future studies. Electro spray ionization (ESI) gave a higher signal than the atmospheric pressure chemical ionization and was selected for further 17-OHPC analyses.

The method developed was also highly reproducible. The intra-day and inter-day coefficient of variation were within the 15% acceptability limits, except for the lowest quantification value of 0.5 ng/ml (16.7%). The method was also very accurate with a bias of less than 6% at all levels tested.

In pharmacokinetic studies plasma samples are normally stored at –20°C or –80°C until analysis and plasma samples are exposed to various temperatures during the assay procedures. It is important to understand the stability of the analyte at various storage and handling conditions. Stability of 17-OHPC was determined by comparing the results obtained following extraction of the plasma samples immediately after thawing and immediately injecting the extracted samples into HPLC, after extracting the plasma samples kept at room temperature (RT) for 24 hrs following thawing and immediately injecting the extracted sample into HPLC, and extracting the plasma samples after three freeze-thaw cycle and injecting the extract immediately. These steps simulate various conditions that the samples may be subjected prior to analysis. There was no change in the 17-OHPC content independent of the sample processing conditions indicating the stability of 17-OHPC under several conditions tested. We observed a loss of 18% of 17-OHPC when extracted control samples kept at 5°C are reinjected after 24 hrs. However, any error in measuring drug level in biological samples is significantly minimized when control samples and calibration curve samples are injected on the same day. We used the assay developed to analyze the 17-OHPC content in plasma samples obtained in a pharmacokinetics study following multiple intramuscular administration (250 mg) to a pregnant woman. The plasma concentration time profile could be readily measured over the dosing interval. The clinical plasma concentrations of 17-OHPC are very low and require sensitive and reliable analytical methodologies. This method has good sensitivity that can measure the trough levels of 17-OHPC in pregnant women undergoing 17-OHPC treatments by intramuscular injections. The assay sensitivity can further be increased by using a larger volume (1 ml) of plasma sample for extraction and injecting a larger volume into the HPLC.

In summary, a sensitive and selective LC-MS/MS assay procedure using solid phase extraction for the specific and quantitative analysis of 17-OHPC in human plasma was developed and validated. The assay uses MPA as an internal standard; the extraction

procedure uses simple solid phase extraction that allows sufficient sample throughput to be applied to clinical pharmacokinetic studies of 17-OHPC. The assay has been validated, and the results of validation shows the method was reproducible and accurate. The analysis requires only 400 µl of plasma, which has an advantage in pharmacokinetic studies. This LC-MS/MS method has a LLOQ of 0.5 ng/ml, which is suitable for the assessment of 17-OHPC levels in plasma samples from clinical studies.

Acknowledgments

This work is supported in part by NICHD grant HD-047905-2

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Figure 1.
Chemical structures of 17 α -hydroxyprogesterone caproate (bottom, molecular weight : 428.59) and medroxyprogesterone acetate (internal standard, top, molecular weight : 386.53).

Figure 2.
Representative positive ion ESI MS/MS spectrum for 17 α -hydroxyprogesterone caproate.

Figure 3.

Representative SRM chromatograms of 17α-hydroxyprogesterone caproate in human plasma. (A) Blank human plasma without 17α-hydroxyprogesterone caproate (Left), 17α-hydroxyprogesterone caproate spiked 0.5 ng/ml in blank plasma (middle) and Clinical plasma sample following 250 mg intramuscular administration of 17α-hydroxyprogesterone caproate (Right), (B) Blank human plasma without internal standard (Left), Internal standard spiked 3.125 ng/ml in blank plasma (middle) and Internal standard spiked in clinical plasma sample (Right).

Figure 4.

A representative plasma concentration time profile of one pregnant women following intramuscular administration of 250 mg 17 α -hydroxyprogesterone caproate. The patient has received at least 5 weekly injections prior to the first sample time.

Table 1

Accuracy and precision from quality control data for 17-OHPC in human plasma.

Added Concentration (ng/ml)	Mean $\pm$ SD N=6	CV (%)	Bias (%)
5.00	5.24 $\pm$ 0.38	7.31	4.84
10.00	9.43 $\pm$ 0.72	7.62	-5.68
50.00	49.97 $\pm$ 2.90	5.81	-0.07

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Table 2

Extraction and ion suppression recovery data of 17-OHPC in human plasma.

Concentration (ng/ml) (n=4)	Extraction Recovery		Ion Supression Relative Recovery	
	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)
5	97.1 $\pm$ 2.0	2.1	104.3 $\pm$ 9.9	9.5
10	92.6 $\pm$ 5.0	5.3	103.9 $\pm$ 6.6	6.4
50	88.7 $\pm$ 2.1	2.3	104.1 $\pm$ 8.9	8.6
Internal Standard 25 μ l of 125 ng/ml	95.7 $\pm$ 3.2	4.3	79.2 $\pm$ 4.2	5.2

Table 3

Intra-day and inter-day precision from assay calibration data for 17-OHPC in human plasma

Added Concentration (ng/ml)	Intra-Assay Precision (%) N=6			Inter-Assay Precision (%) N=6		
	Mean $\pm$ SD	CV (%)	Bias (%)	Mean $\pm$ SD	CV (%)	Bias (%)
0.50	0.57 $\pm$ 0.10	16.72	15.63	0.47 $\pm$ 0.07	14.26	-6.52
1.00	1.14 $\pm$ 0.14	12.46	14.12	1.08 $\pm$ 0.13	12.33	7.77
2.50	2.62 $\pm$ 0.36	13.79	4.89	2.59 $\pm$ 0.35	13.63	3.63
5.00	4.94 $\pm$ 0.07	1.46	-1.26	5.09 $\pm$ 0.67	13.09	1.87
10.00	10.22 $\pm$ 0.53	5.22	2.23	9.88 $\pm$ 0.79	7.97	-1.18
25.00	24.58 $\pm$ 0.93	3.77	-1.68	24.76 $\pm$ 2.61	10.53	-0.98
50.00	50.13 $\pm$ 2.70	5.38	0.27	50.13 $\pm$ 2.24	4.66	0.27

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Table 4

Stability data for 17-OHPC in human plasma stored at RT for 24 h, extracted plasma samples stored at RT for 24 h, and the effect of freeze-thaw cycle.

Nominal Conc. (ng/ml)	Analysis immediately following thawing (n=4)			Analysis after 24 h at RT following thawing (n=4)			Analysis after 24 h at 5°C post sample preparation (n=4)			Analysis following 3 Freeze-Thaw Cycle (n=4)		
	Mean Observed Value	Relative Recovery (%)	CV (%)	Mean Observed Value	Relative Recovery (%)	CV (%)	Mean Observed Value	Relative Recovery (%)	CV (%)	Mean Observed Value	Relative Recovery (%)	CV (%)
5	5.0	99.7	7.6	4.8	96.4	1.2	4.3	92.7	5.1	4.6	92.0	10.9
10	10.1	101.5	13.9	10.4	104.2	2.4	8.6	84.5	8.0	10.0	99.9	8.1
50	49.1	98.3	9.4	49.7	99.3	6.2	41.0	82.4	8.0	51.1	102.3	4.2