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Purity assignment of 17α-hydroxyprogesterone by mass balance method to establish traceability in measurement

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Abstract: Traceability establishment in chemical measurements is a like a linkage established through an unbroken chain from the measured results to the international system (SI) of units. The primary process for traceability establishment is the purity assignment of a target material to be measured. In this study, we studied the purity assignment of 17α-hydroxyprogesterone (17-OHP). The presence of 17-OHP is indicative of congenital adrenal hyperplasia (CAH) and it builds up due to the deficiency of 21-hydroxylase and 11β-hydroxylase enzyme in the human blood. The purity assignment of 17-OHP was performed by the mass balance method, in which the impurities are categorized into four classes: total related structural impurities, water, residual organic solvents, and nonvolatiles/inorganics. The total related structural impurities were characterized by HPLC-UV; water content was determined by Karl-Fisher coulometer; and the total residual solvents and nonvolatiles/inorganics were determined by TGA. The purity of 17-OHP from a commercial manufacturer was calculated as 993.30 mg/g, and the expanded uncertainty was 0.58 mg/g. The proposed method was validated by uncertainty evaluation and comparing with the actual value of purity.

Key words: 17-OHP, purity, traceability, mass balance method, CAH

1. Introduction

17α-hydroxyprogesterone (17-OHP) is a metabolic precursor of cortisol and a marker for congenital adrenal hyperplasia (CAH).[1-4] Deficiency in several enzymes including 21-hydroxylase, 11-hydroxylase, 3-beta-hydroxy dehydrogenase, and 17-alpha-hydroxylase cause CAH.[5-8] The resulting hormone imbalances (reduced glucocorticoids and mineralocorticoids, and elevated steroid intermediates and androstenedione) can lead to life-threatening, salt-wasting crises in the newborn period, incorrect gender assignment of virilized females, and symptoms of hyperhidrosis in young women.[9-11] Therefore 17-OHP is tested in clinics as part of a neonatal screening test to identify an inherited disorder affecting the adrenal gland.[12-14] Normal and abnormal values of 17-OHP differ for babies born with low birth weight. In general,

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normal results are as follows: babies more than 24 hours old - less than 400 to 600 ng/dL, children before puberty - around 100 ng/dL, and adults - less than 200 ng/dL. A high level of 17-OHP may be due to tumors of the adrenal gland and CAH. In infants with CAH, the 17-OHP level ranges from 2,000 to 40,000 ng/dL. In adults, a level greater than 200 ng/dL may be due to nonclassical adrenal hyperplasia. CAH shows a disease rate of one prevalent per population of 16,000.

In the present research, we have studied the purity assignment of 17-OHP for traceability establishment in CAH diagnostic tests. The structure of 17-OHP is shown in *Fig.* 1. Metrology traceability means the property of a measurement result to be linked to a reference through a documented unbroken chain of calibrations contributing to measurement uncertainty.[15-16] Purity analysis and development of primary methods of measurement play an important role in metrology because they provide the essential first link in traceability chain from the abstract definition of SI Units to its practical use.[17] The primary method of purity analysis is the mass balance method. In the mass balance method, impurities are categorized into four classes: total related structure impurities, water, residual organic solvents, and nonvolatiles/inorganics.[18-24] In the present study, the total related structure impurities were characterized by HPLC-UV, water was determined by a Karl-Fisher coulometer, and the total residual solvents and nonvolatiles/inorganics were determined by TGA. The measured results were validated through uncertainty evaluation and comparison with the provided purity value.

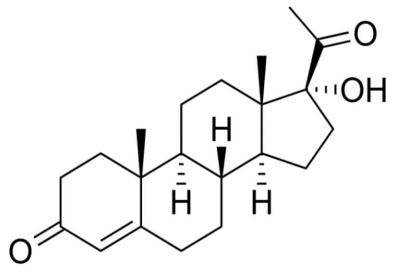

Fig. 1. Structure of 17α-hydroxyprogesterone.

2. Experimental

2.1. Chemicals and reagents

Pure 17-OHP was purchased from Sigma (≥ 95 %, USA). Acetonitrile and methanol were purchased from Fisher Scientific Korea Ltd (HPLC grade, USA), and ethanol was purchased from Merck (ACS reagent, Germany). Water was filtered through a filtration system (Millipore Alpha Q, Germany) and then distilled with a few drops of alkalic potassium permanganate solution. Distilled water was filtered with 0.45 μm PVDF filter (Supelco, USA) for degassing before use.

2.2. Analysis of total related structure impurities

The total related structure impurities were characterized by HPLC with diode array detector (Agilent 1260 series, Germany). Column was BDS Hypersil C_{18} (Thermo, 5 μm, 4 × 250 mm), and the mobile phase was the gradient of water (A) and methanol (B): 0 (50 % B)-5 min (50 % B)-50 min (65 % B)-60 min (65 % B)-65 min (90 % B)-70 min (90 % B)-72 min (50 % B)-82 min (50 % B). The flow rate was 0.5 mL/min, and the injection volume was 1 μL. The detection wavelength of UV was 240 nm, and the sample was prepared with 95 % ethanol at a concentration of 3,000 mg/kg.

2.3. Determination of water content

Water content was determined by 851 Karl-Fisher coulometer with an 885 Compact Oven Sc (Metrohm, Herisau, Switzerland). The oven system was used for water measurement of solids. Oven temperature was set at 170 °C to vaporize water in the sample. The carrier gas was nitrogen (99.9999 %, Rep. of Korea), and was used after drying with a water-removing agent trap. Sample weight was ranged about 0.15 g ~ 0.2 g, and titration extraction time was 600 s. The experiment was started after 15 μg/min drift rate was reached.

2.4. Analysis of nonvolatile/inorganic impurities

Nonvolatile/inorganic impurities were checked by thermogravimetric analyzer (TGA/DSC STARe

System, Mettler Toledo, Switzerland), and measured as a mass residue after oxidative combustion at high temperature. The sample container was an alumina vessel of 70 μL volume. The sample vessel was accurately weighed after six heat treatments under the same experimental conditions. Next, the sample was placed into the sample vessel, and the sample vessel was weighed and loaded into the system; then the experiment was started. Sample amount was about 4 mg. After the experiment was completed, the vessel was cooled, weighed again accurately and compared with the initial weight. The presence or absence of nonvolatile/inorganic impurities was determined from the difference between the two weights. The temperature programing and atmosphere were as follows: 25 ºC (10 min, N_2) – 150 ºC (3 ºC/min, N_2) – 600 ºC (10 ºC/min, Air) – 600 ºC (60 min, Air).

2.5. Analysis of volatile organic compounds

Since 17-OHP is derived from progesterone precursor via enzymes, it is expected that there would be very few residual organic compounds. Therefore, the existence of volatile organic compounds (VOCs) was checked by observing the amount of weight change at temperatures below 80 °C using TGA. During the experiment period, the nitrogen atmosphere was provided to prevent combustion reactions.

2.6. Analysis of volatile organic compounds

The purity of 17-OHP, P, was calculated by Eq. (1).

$$P = [1-(P_W+P_{IN}+P_{VOC})]\cdot P_{RS} \quad (1)$$

where P_W is the mass fraction of water in the sample, P_{IN} is the mass fraction of nonvolatiles/inorganics in the sample, P_{VOC} is the mass fraction of residual organic solvents in the sample, and P_{RS} is the mass fraction of the total related structure organic compounds in the sample. Normally, final purity is reported in units of milligram per gram (mg/g)

2.7. Uncertainty evaluation

Uncertainty evaluation was performed according to Guide to the expression of uncertainty in measurement (GUM).[25-26] The combined standard uncertainty ($u(P)$) of 17-OHP purity is obtained by the quadratic combination of the uncertainties associated with each contributing impurity as in Eq. (2):

$$u(P) = \sqrt{u(P_W)^2+u(P_{IN})^2+u(P_{VOC})^2+u(P_{RS})^2} \quad (2)$$

where $u(P_W)$ is the standard uncertainty of water, $u(P_{IN})$ is the standard uncertainty of nonvolatile/inorganic impurities, $u(P_{VOC})$ is the standard uncertainty of residual organic compounds, and $u(P_{RS})$ is the standard uncertainty of the total related structure impurities.

3. Results and Discussion

3.1. Measurement of the total related structure impurities

In general, the total related structure impurities are characterized by chromatographic analysis using universal detector that responds to every organic material. Methods using GC-FID or HPLC-UV are generally regarded as meeting the requirements for the universal detection of organic analytes. Since 17-OHP has a high melting point, GC-FID was not used. Thus, the total related structure impurities of 17-OHP were characterized by HPLC-UV, because 17-OHP has a chromophore. 17-OHP was dissolved in 95 % ethanol and prepared at a concentration of 3,000 mg/kg to facilitate the detection of impurities. Because the related structure impurities were mostly other steroid hormones or their precursors, they were overlapped with the 17-OHP peak in HPLC analysis. For a complete separation of the related structure impurities, the gradient of the mobile phase was controlled at a very low rate as described in experiments. Although this experiment took a long time, it was possible to achieve a complete separation of the related structure impurities. HPLC chromatograms of ethanol blank and 17-OHP are shown in *Fig.* 2. The ethanol blank peak was excluded from the impurity peaks of 17-OHP sample. The mass fraction of 17-OHP was calculated as a ratio of the peak area of 17-OHP to the sum of peak area of 17-OHP and peak areas of all related structure impurities. The 17-OHP peak area was calculated by subtracting the adjacent peak

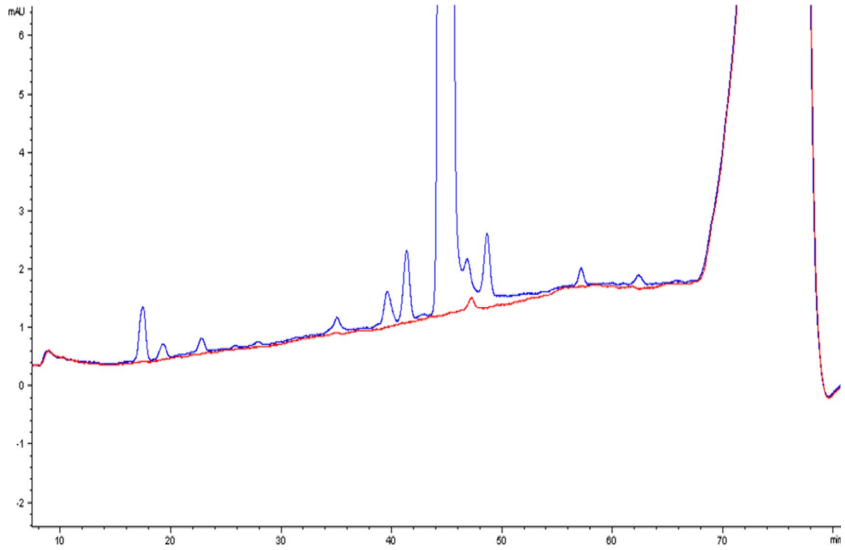

Fig. 2. HPLC/UV chromatograms of ethanol blank and 17-OHP according to gradient elution (red:ethanol blue:17-OHP, A: water, B: methanol): 0 (50 % B)-5 min (50 % B)-50 min (65 % B)-60 min (65 % B)-65 min (90 % B)-70 min (90 % B)-72 min (50 % B)-82 min (50 % B).

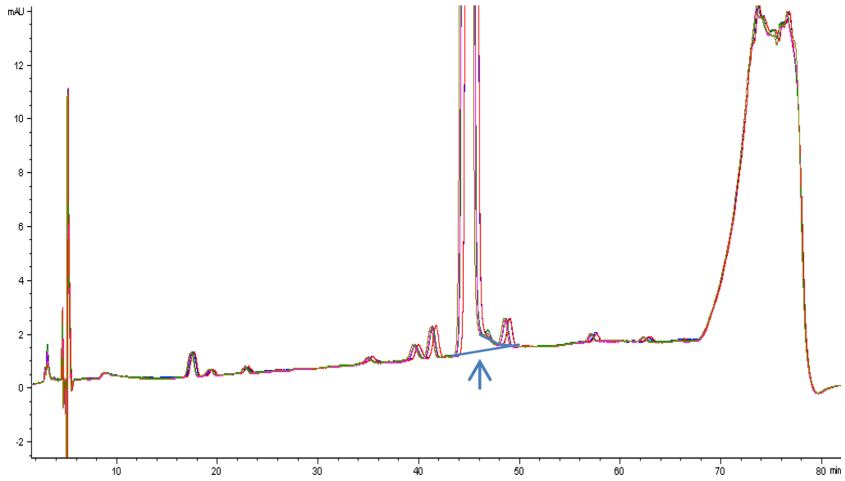

Fig. 3. Repeatability of five times chromatographic analysis and integration process of 17-OHP and adjacent peaks.

areas from the area of the main peak including adjacent peaks as shown in *Fig.* 3. HPLC-UV experiments were repeated five times indepen- dently and showed good repeatability as also shown in *Fig.* 3. The mass fraction of 17-OHP determined by HPLC-UV was 993.91 mg/g (99.39 %), and the standard uncertainty was 0.07 mg/g (0.0067 %), exhibiting a good reproducibility as shown in *Table* 1. Standard uncertainty was obtained by Eq. (3).

$$u(x) = \frac{S(x)}{\sqrt{n}} \quad (3)$$

Where *u(x)* is the standard uncertainty, *s(x)* is the standard deviation, and *n* is the number of measurement.

3.2. Determination of water

The water content of 17-OHP was determined using a Karl-Fisher coulometer. The number of blank

Table 1. Analysis results of the total related structure impurities of 17-OHP by HPLC/UV

Measurement no	Total related structure impurity (mg/g)	Total related structure impurity (%)
1	993.97	99.397
2	993.67	99.367
3	993.88	99.388
4	993.95	99.395
5	994.06	99.406
Average	993.91	99.391
Standard deviation	0.15	0.015
Standarduncertainty	0.07	0.0067

was three, and the number of sample was four. All of them were prepared under the same air condition. The average water value of the three blank was subtracted as the water content of the blank. The measured water contents of 17-OHP was 230.6 μg/g (0.023 %), and standard uncertainty was about 7.9 μg/g (0.001 %) as shown in *Table* 2. The uncertainty evaluation was also calculated by Eq. (3).

3.3. Measurement of nonvolatile/inorganic impurities

A thermo gravimetric analyzer (TGA) was used for the determination of nonvolatile/inorganic impurities. Nonvolatile/inorganic impurities comprise inorganics such as metals and metal oxides, and non-volatile materials. The vessel used in this experiment was an alumina pan; the alumina pan was six times heat treated under the TGA experimental conditions before use. Then, the weight of the alumina pan and sample were measured with external balance. Since temperature equilibrium is important in weighing, an external balance was used for sufficient temperature equilibrium. The weight of the sample was calculated according to the weight difference before and after the loading of the sample. Then, the sample was placed in a TGA autosampler; the temperature was raised from ambient temperature to 150 ºC at a 10 ºC/min rate in nitrogen atmosphere and then to 600 ºC at a 10 ºC/min rate in air atmosphere, and maintained at 600 ºC for 60 min. 17-OHP was combusted with oxygen in air atmosphere above 150 ºC and decomposed into carbon dioxide and water, and showed a rapid weight change. However, at the final temperature of 600 ºC, there was no further weight change, so only the nonvolatile/inorganic materials were considered to remain. After cooling, the pan was weighed again and the amount of nonvolatile/inorganic impurities

Table 2. Measurement results of water contents of 17-OHP determined by KF-coulometer

Measurement no	Blank (μg/g)	Water contents (μg/g)	Water contents (%)
1	115.2	254.2	0.025
2	114.7	222.6	0.022
3	120.3	224.5	0.023
4		221.1	0.022
Average	116.7	230.6	0.023
Standard deviation	3.1	15.8	0.002
Standard uncertainty		7.9	0.001

Table 3. Analysis results of nonvolatile/inorganic impurities by TGA

Crucible (mg)	17-OHP (mg)	Crucible after TGA (mg)	Residues (mg)	Residues (%)	Standard uncertainty (%)
180.0111	4.4595	180.0127	0.0017	0.037	0.0166

Table 4. Uncertainty evaluation in weighing measurement by analytical balance

Readability (mg)	Repeatability (mg)	Uncertainty of weight (mg)	Uncertainty of weight difference (mg)	Standard uncertainty (%)
0.0001	0.0009	0.0005	0.0007	0.0166

was calculated according to the weight difference before and after the TGA experiment. The mass of nonvolatile/inorganic residues was 0.0017 mg (0.037 %) and the standard uncertainty was about 0.017 % as shown in *Table* 3. The standard uncertainty was evaluated from the readability, repeatability of analytical balance as B-type. Assuming a rectangular distribution, the uncertainty was calculated by Eq. (4).

$$u = \frac{a}{\sqrt{3}} \quad (4)$$

Where a is the standard deviation.

Since the inorganic impurities were obtained by measuring the pan weight twice before and after the experiment, the measurement uncertainty was calculated to be twice the weight measurement uncertainty as shown in *Table* 4.

3.4. Measurement of residual organic solvents

In this study, the residual organic solvents were simultaneously determined with nonvolatiles/inorganics using TGA. Because residual organic solvents have a low boiling point, the weight change was checked at below 80 ºC. Nitrogen atmosphere was provided so that no combustion reaction occurred. Since no inflection point indicating a change in weight was observed below 80 ºC, residual solvent impurities were considered to be nonexistent. As a result, the residual organic solvents were calculated as 0 %.

3.5. Calculation of purity

The purity of 17-OHP was calculated using Eq. (1); the uncertainty budget was evaluated by Eq. (2). The assigned value and the expanded uncertainty of 17-OHP are shown in *Table* 5. Because residual organic solvents were not found, only the related structure impurities, water, and inorganic impurities were considered as impurities within 17-OHP. Therefore, the purity of 17-OHP was 993.30 mg/g and the expanded uncertainty was 0.58 mg/g at a 95 % confidence level.

3.6. Method validation

In the purity assignment of 17-OHP, uncertainty was evaluated according to the GUM and calculated including all factors affecting each impurity analysis. The purity of 17-OHP provided by Sigma manufacturer was ≥ 95% without any other information. Although purity has been provided by the manufacturer, accurate purity assignments are required to establish the traceability in measurement. The difference between the purity provided by the manufacturer and the purity assigned in this study was approximately 4.3 %, which directly affects the accurate measurement results. Method validation can also be assessed through the uncertainty evaluation of the measurement results. Another method validation was made through a comparison of the 17-OHP measurement results in serum. The comparison was made for the two levels of 17-OHP in serum and the purity of 17-OHP used in this comparison was prescribed to use the assigned value by each their own laboratories. The final results are shown in *Fig.* 4. Our result (KRISS) showed an equivalent result to those of other laboratories. Although it was not a direct comparison of purity assignment, it is considered to be meaningful because purity assignment processes are included in the measured results. Therefore, the method for the purity assignment of 17-OHP developed in this study was proven to be valid and can be applied for other purpose such as the development of certified

Table 5. Calculation of purity of 17-OHP

	Contents (%)	Standard Uncertainty (%)	DOF	k	Expanded Uncertainty (%)
Water	0.023	0.001	3	3.18	0.0025
Inorganics	0.037	0.0166	1E+99	2	0.0340
Total related organics	99.390	0.0067	4	2.36	0.0158
Mass fraction	99.330	0.018	3	3.18	0.058

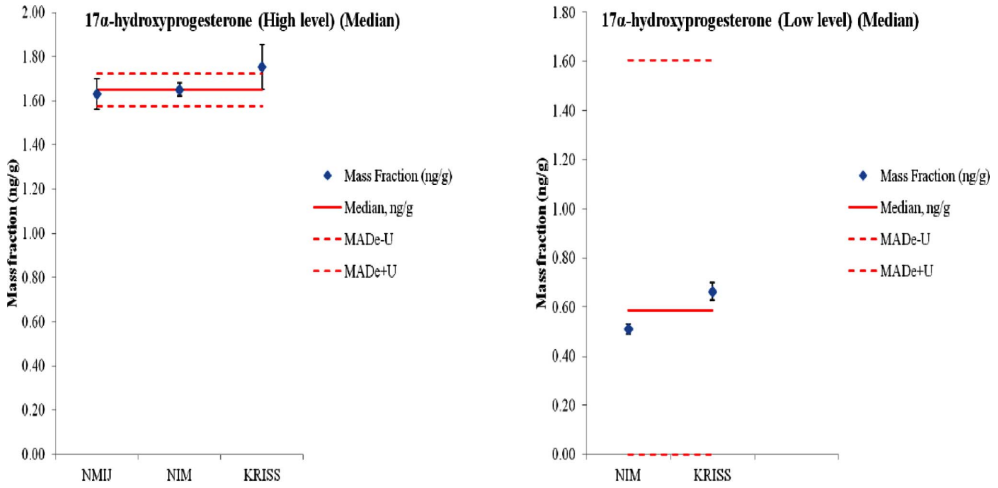

Fig. 4. Comparison results of two levels of 17-OHP in serum: At high level, all results were agreed within uncertainty. At low level, two results were also agreed within uncertainty because uncertainty level was enough large.

reference material (CRM).

4. Conclusions

A method for the purity assignment of 17-OHP was developed according to the mass balance method. Impurities were classified into four classes: related structure compounds, water, nonvolatile/inorganic impurities, and residual solvents. The related structure compounds were characterized by HPLC-UV; water content was determined by Karl-Fisher coulometer, and the nonvolatile/inorganic impurities and the residual organic solvents were determined simultaneously by TGA. The developed method was validated through the uncertainty evaluation and comparison with the supplied purity, and international comparison of 17-OHP in serum. The purity value of 17-OHP from a commercial manufacturer was 993.30 mg/g by this method and the extended uncertainty was 0.58 mg/g.

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