Cold-induced phase separation for the simple and reliable extraction of sex hormones for subsequent LC-MS/MS analysis

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Abstract Sex hormones, including androgens, estrogens, and progestogens, are important biomarkers for various diseases. Quantification of sex hormones is typically conducted by LC-MS/MS. At present, most methods require liquid-liquid extraction or solid phase extraction for sample preparation. However, these pretreatments are prone to compromise LC-MS/MS throughput. To improve on the current standard practices, we investigated cold-induced phase separation for sex hormone extraction. After protein precipitation with acetonitrile and adjusting the solution constitution with water, samples were stored at −30°C for 10 min to generate two distinct phases: an acetonitrile-rich layer on top of a water-rich layer. During this process, the hydrophobic sex hormones spontaneously separate into the upper layer. This simple and reliable cold-induced phase separation-based LC-MS/MS methodology was used here to simultaneously detect estrone, estradiol, estriol, testosterone, androstenedione, dehydroepiandrosterone, progesterone, and 17-hydroxyprogesterone in serum. Validation of this method indicated satisfactory performance, including acceptable linearity, accuracy, precision, and tracability. Compared with the mainstream liquid-liquid extraction-based method, this new method exhibits significant progress in throughput, which shortens the time cost of sample preparation from 90 to 40 min. We propose that this method can be an excellent alternative for sex hormone analysis in routine clinical laboratories.

Supplementary key words cold-induced phase separation • LC-MS/MS • sex hormone • steroid • solid phase extraction • acetonitrile • liquid-liquid extraction • dansyl chloride • derivatization • estradiol

Sex hormones include androgens, estrogens, and progestogens in vivo. In the past decade, these compounds have been utilized for early diagnosis of various diseases, such as infertility, polycystic ovary syndrome, breast cancer, and adrenal tumors (1, 2). Recently, their potential effect for severe acute respiratory syndrome coronavirus 2 treatment has attracted much attention (3). Thereupon, accurate quantification of sex hormones possesses essential value for diverse fields of clinical science.

In modern clinical laboratories, LC-MS/MS has become the preferred platform for analyzing endogenous sex hormones (4). Numerous methodologies have been developed recently (5–16). As excellently summarized in the latest reviews, to achieve profiling of sex hormones, most, if not all, of the published methods introduced liquid-liquid extraction (LLE) or solid phase extraction (SPE) for sample preparation (17, 18). Taking our MS center as a typical example, the methods in use to detect sex hormones in serum samples are both accompanied with LLE (19, 20). The inherent nitrogen-drying process significantly compromises the method throughput. For improvement, we are constantly looking for other advanced extraction strategies.

In 1994, Gu et al. (21) found that the homogenous solution of acetonitrile (ACN)/water was able to spontaneously separate into two phases after storing below −132°C, which finally resulted in an ACN-rich layer on the top (organic phase) and a water-rich layer down below (aqueous phase). During such process, hydrophobic components would distribute into the organic phase, whereas hydrophilic targets would preserve in aqueous phase. In other words, an ACN/water-based “in situ LLE” can be well realized through a simple cooling down process. In the past few years, this mechanism (naming as “cold-induced phase separation (CIPS),” “low-temperature-induced phase separation,” “homogeneous liquid-liquid microextraction,” “low-temperature partition,” and others) has been mainly utilized for drug/pesticide-residue analysis in food/environmental science (22–32). However, its applicability for pretreatment of biotargets remains to be clarified.

In this study, by importing CIPS for the extraction of sex hormones, we put forward a novel and efficient LC-MS/MS method to simultaneously quantify estrogens (estrone [E1], estradiol [E2], and estril [E3]), androgens (testosterone [T], androstenedione [AD], dehydroepiandrosterone [DHEA]), and progestogens (progesterone [P]...
and 17-hydroxyprogesterone (17-OHP) in serum. For extraction, samples are allowed to stand under subzero temperature after protein precipitation by ACN. Along with phase separation, targets are enriched into the upper layer owing to their hydrophobicity. As the components of this layer are ACN and water, it can be transferred for LC-MS/MS analysis directly. To the best of our knowledge, it is the very first time to realize sex hormone profiling without traditional LLE/SPE processes. Under optimized condition, counting derivatization and postcleanup procedure, the whole sample preparation can be completed within 40 min for this CIPS approach, which is much faster than previous methods (up to 90 min, as shown in “Conventional LLE-based method with slight modifications for sex hormone analysis” in the supplemental data section). We expect that the new method can be a promising alternative for daily sex hormone analysis in routine clinical laboratories.

MATERIALS AND METHODS

Chemicals and resources

Standards of E1 (E-075—1 ml), E2 (E-060—1 ml), E3 (E-074—1 ml), DHEA (D-063—1 ml), P (P-069—1 ml), E2-d2 (E4260—5 mg), T-d3 (T-046—1 ml), AD-13C3 (A-084—1 ml), DHEA-d6 (709549—5 mg), and 17-OHP-d8 (H996—1 ml) were all purchased from Sigma-Aldrich (Beijing, China). Standards of T (T102169—5 g) and AD (A10164—1 g) were purchased from Aladdin Industrial Corporation (Shanghai, China). Standards of E1-d4 (E889052—25 mg), E3-d3 (E88962—1 mg), P-d9 (P755902—1 mg), 17-OHP (H952330—5 g), and dansyl chloride (DC) (D17500—5 g) were purchased from Toronto Research Chemicals (Toronto, Canada). Methanol, ACN, ammonium acetate, and formic acid at HPLC grade were purchased from Fisher-Scientific (NJ). The water used throughout the study was purified by a Milli-Q apparatus (Millipore, Bedford, MA).

Traceable materials of SRM-971F, SRM-971M, BCR-576, BCR-577, and BCR-578 were purchased from the National Institute of Standards and Technology (NIST) and the Institute for Reference Materials and Measurements (IRRM). The serum samples used for method comparison were derived from the Department of Clinical Laboratory of Renmin Hospital of Wuhan University (Wuhan, China). These samples were obtained from the excess samples after routine detection services. The whole study was supervised under the Ethics Committee of Renmin Hospital of Wuhan University and abided by the Declaration of Helsinki principles.

Apparatus and parameters

An Ekspekt ultraLC 100- XL system coupled with AB SCIEX 4500 QTRAP mass spectrometer was utilized to perform the detection of sex hormones. For LC separation, formic acid in water (0.2% v/v) with ammonium acetate (10 mmol/l) and formic acid in methanol (0.2% v/v) with ammonium acetate (10 mmol/l) were used as mobile phase A and B, respectively. The gradient was set as 0–0.2 min 60% B, 0.2–3.5 min 60–100% B, 3.5–4.5 min 100% B, 4.5–4.6 min 100–60% B, and 4.6–6 min 60% B. A Kinetex 26 μm C18 100 Å (100 × 3 mm) column was used as chromatographic column. The flow rate and column temperature were set as 0.5 ml/min and 65°C. The injecting volume was 20 μl. To diminish contamination, LC fluid for the first 1 min was discarded by using exchange value. For MS detection, all the targets were analyzed with electrospray ionization source under positive ion mode. Androgens and progestogens were detected in their original forms, whereas estrogens were quantified as DC-modified products (namely as E1-DC, E2-DC, and E3-DC). The multiple reaction monitoring parameters are listed in supplemental Table S1. The ESI parameters were as follow: curtain gas (25.0 psi), collision gas (medium), ionspray voltage (5500.0 V), temperature (500.0°C), ion source gas 1 (30.0 psi), and ion source gas 2 (30.0 psi). Data acquisition and processing were performed using AB SCIEX Analyst 1.6.2 software.

The cooling-down treatment was carried out in refrigerators of Haier-BCD-253WDPDU1 for −10°C storage, Haier-HYCD-282A for −20 or −30°C storage, and Haier-DW-86L728 for −80°C storage.

Calibrators and quality controls

All the standards were dissolved and diluted with ACN to prepare stock solutions (1 μg/ml). Further stepwise dilution was carried out by ACN/water solution (1/1, v/v) to prepare working solutions at proper concentrations. Specifically, a working solution containing mixed internal standards (ISs) was prepared. The concentrations of IS in this working solution were 1 ng/ml for E1-d4, E2-d2, and E3-d3, 10 ng/ml for T-d3 and P-d9, 20 ng/ml for DHEA-d6, 5 ng/ml for AD-13C3, and 17-OHP-d8. The steroid-free serum (lot number: M411D) from Scantibodies Laboratory, Inc (CA) was used as blank matrix. The calibration samples of E2, T, AD, DHEA, P, and 17-OHP were prepared by using 6PLUS® Multilevel Serum Calibrator Set MassChrom® Steroid Panel 2 (Chromsystems; catalog no: 72039; lot number: 4217). And lower limit of quantification (LLOQ) level was derived from mixing level 1 sample of the aforementioned calibrator with blank matrix (one-third, v/v). As a result, the actual calibration ranges were 0.01–5.33 ng/ml for E2, 0.015–11.6 ng/ml for T, 0.05–15.3 ng/ml for AD, 0.25–60.7 ng/ml for DHEA, 0.04–26 ng/ml for P, and 0.025–15 ng/ml for 17-OHP. In addition, the calibration samples of E1 and E3 were prepared by spiking working solutions into blank matrix. The obtained calibration was 0.01, 0.02, 0.05, 0.1, 0.25, 1, and 2 ng/ml for both E1 and E3. The quality controls (QCs) of the four levels (high-, medium-, low-, and LLOQ-QC) were also prepared by spiking working solutions into blank matrix. The detailed information is listed in supplemental Table S2.

Optimization of CIPS condition

We optimized CIPS condition by using blank matrix spiked with E1, E2, E3, T, AD, DHEA, P, and 17-OHP (each 2 ng/ml). This sample (100 μl) was mixed with ACN (200 μl). Then several parameters were investigated for CIPS, including initial ACN-water proportion, freezing temperature, and freezing time.

Experiment 1. Initial ACN-water proportion—different volumes of water (0, 50, 100, 200, and 300 μl) were added to create a gradient of ACN content (67, 57, 50, 40, and 33%). The resulting solution was centrifuged (10,000 g) at 4°C for 2 min and stored at −30°C for 10 min to conduct CIPS.
**Experiment II.** Freezing temperature—after adding water (100 μl), samples were centrifuged (10,000 g) at 4°C for 2 min and stored at −10, −30, or −80°C for 10 min to conduct CIPS.

**Experiment III.** Freezing time—after adding water (100 μl), samples were centrifuged (10,000 g) at 4°C for 2 min and stored at −30, −30, or −80°C for 10 min to conduct CIPS. The upper and lower layers (20 μl) of all the samples were diluted with IS mixture in ACN (each 0.5 ng/ml, 80 μl), sodium bicarbonate solution (100 μl, 0.1 mol/l in water), and DC solution (10 μl, 10 mg/ml in ACN). After incubation (10 min) at 65°C, the mixture was transferred for LC-MS/MS analysis.

**Sample preparation**

Serum samples (100 μl) were combined with IS working solution (10 μl) and ACN (200 μl). After vortexing (1 min), water (100 μl) was added. Then, protein precipitation was conducted by centrifugation (10,000 g) for 2 min at 4°C. And CIPS was performed by storing samples at −30°C for 10 min. The resulting solution was separated into two phases. The upper phase (100 μl) was collected and mixed with sodium bicarbonate solution (100 μl, 0.1 mol/l in water) and DC solution (10 μl, 10 mg/ml in ACN). After incubation (10 min) at 65°C, CIPS was performed again at −30°C for 10 min. The obtained upper phase (50 μl) was pipetted into water (50 μl) for LC-MS/MS analysis. The whole process was summarized in Fig. 1.

**Method validation**

The method was validated according to the guidelines for bioanalytical method validation from Food and Drug Administration and document C62-A from the Clinical and Laboratory Standards Institute. All the determinations were performed by triplicate unless expressly stated.

The calibration was built by the linear least-squares regression model utilizing peak area ratios of targets to their IS versus the nominal spiked concentrations. The linearity was evaluated by the coefficient of determination ($R^2$).

The method sensitivity was reflected by the lowest concentrations of the calibrators (LLOQ level). The carry-over effect was studied by detecting the blank matrixes after three successive analyses of upper limit of calibrators. The residual signal should be no more than 15% of LLOQ. The matrix effect was evaluated by detecting three groups of samples. Group I was ACN-water solution (1/9, v/v) spiked with 0.2, 0.5, 0.2, 1, 1.5, 2, and 1.5 ng/ml for E1, E2, E3, T, AD, DHEA, P, and 17-OHP. Group II was six serum samples from different donors (four males and two females). Group III was serum samples in group II spiked with the same concentration of targets as group I. The difference between group III to group II was compared with the result from group I. The consistency should be within 85–115% for all the six samples. Accuracy was determined as apparent recovery (dividing the measured concentrations to the spiked values) by analyzing all the four levels of QC samples in 1 day ($n=6$, intraday) and in consecutive days ($n=10$, interday). The results should be within 80–120% for LLOQ-QC and 85–115% for others. And imprecision was calculated as coefficient of variation (CV) of those measurements, which should be lower than 20% for LLOQ-QC and 15% for others.

Moreover, the proposed method was certified by using traceable materials. SRM-971F and SRM-971M from NIST were utilized for T and P quantification. And BCR-576, BCR-577, and BCR-578 from IRRM were utilized for E2 detection. The measured results were compared with the certified values. The consistency should be within 85–115%.

**Fig. 1.** Schematic diagram of LLE-based and CIPS-based method for sex hormone analysis in serum.
Clinical application and method comparison

To test the practical applicability, serum samples from early second trimester pregnant women (n = 36) were enrolled for sex hormone quantification. The newly developed method and a previous LLE-based method with slight modifications (as shown in “Conventional LLE-based method with slight modifications for sex hormone analysis” in the supplemental data section) were utilized in parallel. The bias was calculated by dividing the difference between the two methods to the average of their results.

RESULTS

Optimization of CIPS condition

Although CIPS has rarely been used for biomedical fields, there was some experience in chemical engineering and food science. Accordingly, initial ACN-water proportion, freezing temperature, and freezing time were the main parameters influencing CIPS. In result, they were specifically evaluated by using partition coefficient, enrichment factor, and enriching recovery. Partition coefficient was defined as the ratio of target concentration in upper phase to lower phase after CIPS. Enrichment factor was the ratio of target concentration in upper phase after CIPS to the original solution before adding water. And absolute recovery was the ratio of target weight in upper phase after CIPS to the spiked weight.

According to the results from experiment 1, when ACN content was 33%, CIPS can hardly be seen. And along with the decrease of ACN content (67, 57, 50, and 40%), the volume of upper phase reduced gradually (170, 140, 110, and 30 μl). To be noticed, the partition coefficients did not significantly change in these cases for all the targets (CV <12%). This could be ascribed to the fact that the distinction of ACN percentage in upper phase (88%) to lower phase (35%) after CIPS was relatively independent to the ACN-water proportion in initial solution. The average partition coefficients were calculated as 31, 37, 26, 25, 27, 29, 33, and 29 for E1, E2, E3, T, AD, DHEA, P, and 17-OHP, respectively. In practice, higher partition coefficient indicated stronger extracting affinity. As T exhibited the lowest partition coefficient, it was taken as a representative in the following discussion. As shown in Table 1, although enrichment factor reached 6.1 when ACN-water proportion was 40%, the extracting recovery was only 61%. And the volume of upper phase (30 μl) was too small for convenient pickup and not compatible for further derivatization. In contrast, when ACN-water proportion was 50%, obvious enrichment (2.5-folds), excellent absolute recovery (91%) and proper volume of upper phase (110 μl) can be achieved simultaneously. As a result, after precipitating sample (100 μl) with ACN (200 μl), water (100 μl) was introduced to modify the initial ACN-water proportion for the following CIPS process.

According to the results from experiment II, when storage was carried out at −10°C for 10 min, the solution was still homogeneous. Additional tests demonstrated that the steady CIPS can be obtained unless prolonging the incubation up to 60 min at −10°C, which would obviously compromise method throughput. In contrast, when −80°C was utilized for storage, not only CIPS but also solidification of both phases took place at the same time. This phenomenon may be ascribed to that −80°C was already below the melting point of ACN (−45°C). Comparing storage at −30°C could efficiently arouse CIPS without causing solidification, so that it was chosen for further optimization.

According to the results from experiment III, after storing for 5 min at −30°C, CIPS cannot be completely induced. The volume of upper phase was only less than 50 μl in this case. While after storage for 10 min or longer, the volume of upper phase was stable at 110 μl. Moreover, partition coefficients, enrichment factors, and absolute recoveries of all the targets were investigated as the freezing time increased from 10 to 60 min. No remarkable difference was observed (CV <1%). In consequence, storage at −30°C for 10 min was regarded as the optimal condition for CIPS in this study.

Method validation

Linearity and sensitivity. The retention time, linear range, and regression of all the targets are shown in Table 2. The coefficient of determination (R^2) was at least 0.9886, which demonstrated excellent linearity. The sensitivity (as LLOQ) was determined at 0.01 ng/ml for E1, E2, and E3, 0.015 ng/ml for T, 0.025 ng/ml for 17-OHP, 0.05 ng/ml for AD, 0.04 ng/ml for P, and 0.25 ng/ml for DHEA. At this level, the ratio of signal to noise was all higher than 10. The representative LC-MS/MS chromatograms were shown in Fig. 2.

Carry-over and matrix effect. After analyzing three upper limit of calibrators in succession, no residual signals can be detected when a blank sample was injected. Such result indicated negligible carry-over effect of the present method. To investigate matrix effect, spiking experiments were performed in real matrixes (n = 6) rather than blank matrix. Thereby, the potential effect of global interference can be fully investigated. After LC-MS/MS analysis, the consistency of all the targets was 87.1–108.8% from the enrolled six samples.

Accuracy and imprecision. As shown in Table 3, accuracy of all the targets was calculated as 86.5–106.6% at

| TABLE 1. The effect of initial ACN-water proportions on extracting performance of CIPS toward T |
|-----------------------------------------|----------------|----------------|----------------|----------------|----------------|
| Parameters                              | Initial ACN-Water Proportion Before CIPS |
|                                        | 33% | 40% | 50% | 57% | 67% |
| Volume of upper phase/                  | 30/470 | 110/290 | 140/210 | 170/130 |
| lower phase (μl)                        |     |      |     |     |     |
| Partition coefficient                   | 25 | 27 | 24 | 24 |     |
| Enrichment factor                       | 61 | 25 | 29 | 17 |     |
| Absolute recovery                       | 61% | 92% | 98% | 97% |     |
high, medium, and low levels for both intraday and interday. And for LLOQ level, accuracy was also satisfying (84.6–105.9%). Besides, in all cases, imprecision was found lower than 13.2%. Such results clearly demonstrated that CIPS extraction coupling LC-MS/MS analysis can provide robust quantification of sex hormones in wide concentration ranges.

Stability. The stability of sex hormones in serum has been extensively studied (33, 34). Here, we focused on how long the two distinct phases after CIPS can be preserved at room temperature for pipetting. According to the results, after standing for 10 min, it was difficult to find any boundary in the solution. While within 5 min, the phase separation can be well preserved. And the apparent recovery did not obviously change in these samples (98–104% for 0 min, 92–105% for 2 min, and 94–101% for 5 min). However, comparing with 0 and 2 min, the absolute signal intensity for all the targets and their IS has been partly decreased (11–23%)

### Table 2. The calibration curves and sensitivity for LC-MS/MS analysis

| Analytes | Retention Time (min) | Linear Range (ng/ml) | Slope | Intercept | $R^2$ | LLOQ (ng/ml) |
|----------|----------------------|----------------------|-------|-----------|-------|--------------|
| E1       | 3.85                 | 0.01–2               | 9.7844| 0.0057    | 0.9980| 0.01         |
| E2       | 3.95                 | 0.01–5.33            | 9.4645| 0.0015    | 0.9967| 0.01         |
| E3       | 3.10                 | 0.01–2               | 9.4988| 0.0034    | 0.9984| 0.01         |
| T        | 1.41                 | 0.015–11.6           | 10041 | −0.0037   | 0.9886| 0.015        |
| AD       | 1.30                 | 0.05–15.3            | 19466 | 0.0097    | 0.9945| 0.05         |
| DHEA     | 1.57                 | 0.25–60.7            | 0.6581| −0.0578   | 0.9952| 0.25         |
| P        | 2.11                 | 0.04–26              | 0.9810| 0.0113    | 0.9979| 0.04         |
| 17-OHP   | 1.53                 | 0.025–15             | 22331 | −0.0103   | 0.9981| 0.025        |

![Fig. 2. LC-MS/MS analysis of (A) E1 as E1-DC, (B) E2 as E2-DC, (C) E3 as E3-DC, (D) T, (E) AD, (F) DHEA, (G) P, and (H) 17-OHP in spiked blank matrix at LLOQ level.](image-url)
after 5 min storage. In result, although the deviation from losing targets during storage could be normalized by IS, the upper phase after CIPS was suggested to be collected in 2 min at room temperature to prevent potential adverse impact on detection.

Traceability. The detecting results for reference materials are shown in supplemental Table S3. For T and P from SRM-971F and SRM-971M, the bias was −11.4 to 4.1. And for E2 from BCR-576, BCR-577, and BCR-578, the bias was −7.1 to 3.7. The good consistency revealed that the proposed method was reliable for accurate quantification of these targets.

Clinical application and method comparison

In the end, the clinical applicability of the proposed method was investigated by using real samples from pregnant women in the second trimester (n = 36). Sex hormones can be successfully quantified in all these samples. The typical chromatograms from one sample were shown in supplemental Fig. S1. During the detection, two medium-QC samples were also enrolled. They were injected both before and after the real samples. The results exhibited no obvious differences (apparent recovery of 97.4–106.8% for all the targets), which demonstrated that at least three dozens of samples can be run in a batch without compromising method robustness. Moreover, four samples were also detected by a traditional LLE-based method for method comparison. As shown in supplemental Table S4, in all cases, the bias between two methods ranged from −22 to 18.2%. Such results clearly indicated that the much simpler CIPS-based method can achieve comparable analytical performance with the intricate traditional LLE-based method.

DISCUSSION

For quantification of sex hormones in serum, majority of the published methods included LLE for extraction of targets from serum matrix. And derivatization with DC was then frequently conducted for estrogens. After reaction, to eliminate interference (including alkaline catalyst NaHCO3 and byproduct NaCl), a second LLE was further suggested by many researchers (6, 13, 35). Such purification can efficiently prolong the lifetime of LC column and preserve the stability of MS response. As a result, the mainstream strategies for sex hormone detection contained two cycles of LLE (as shown in Fig. 1). This unfavorably compromised the throughput for pretreatment (over 90 min). For improvement, we substituted CIPS for conventional LLE. The schematic diagram was shown in Fig. 1. During the first CIPS, all the hydrophobic sex hormones were spontaneously apportioned into the ACN-rich layer on the top, whereas the endogenous hydrophilic interference was excluded to the water-rich phase at the bottom. And for the second CIPS, targets (androgens and progestogens in their original form and estrogens as DC-derivatized products) were efficiently purified from the exogenous nonvolatile salts derived from derivatization reaction. After inductively coupled plasma–optical emission spectrometry analysis, the residual sodium concentration in the upper phase was less than 2% of the lower phase. In consequence, CIPS realized extraction and purification of sex hormones by simply cooling down the can-containing system for several minutes.

The newly developed CIPS-coupled LC-MS/MS method was carefully validated according to the latest guidelines from the Food and Drug Administration and Clinical and Laboratory Standards Institute. First, the linearity and sensitivity were found comparable to previous methodologies and can well fulfill the requirements of detecting clinical samples with trace levels of sex hormones. Then, profiting from CIPS extraction and IS normalization, carry-over and matrix effect was proved acceptable. Afterward, satisfying accuracy, precision, and traceability were demonstrated by studying QC samples and traceable materials from NIST and IRRM. In the end, the sample collection was suggested to be completed within 2 min at room temperature (24–26°C) after CIPS. To be noticed, this time limit can be obviously prolonged, if the samples were stored at lower temperature after CIPS (e.g., more than 20 min in ice-water bath). However, as one can perform pipetting for more than 12 samples in 2 min in practice (such throughput was enough for our routine applications), liquid transfer was directly conducted at room temperature for convenience in the present work.

### TABLE 3. The method accuracies (as apparent recoveries) and imprecisions (as CV) for detecting sex hormones at four concentration levels

| Targets | High Level | Medium Level | Low Level | LLOQ Level | High Level | Medium Level | Low Level | LLOQ Level |
|---------|------------|--------------|-----------|------------|------------|--------------|-----------|------------|
| 17-OHP  | 103.5 (4.4)| 97.4 (7.1)   | 103.3 (8.0)| 93.7 (9.2)| 101.2 (7.6)| 86.5 (9.2)  | 90.9 (9.1)|           |
| E2      | 102.4 (5.5)| 96.1 (5.8)   | 105.9 (11.8)| 93.1 (5.1)| 99.1 (6.9) | 91.3 (7.8) | 92.2 (10.6)|           |
| E3      | 105.8 (5.7)| 101.4 (5.1)  | 93.7 (6.2) | 99.2 (7.1) | 91.3 (4.3) | 94.3 (7.8) | 93.9 (9.3) |           |
| T       | 93.8 (5.0) | 96.1 (5.8)   | 95.5 (8.9) | 94.7 (5.1) | 105.2 (5.0) | 97.3 (6.9) | 95.0 (8.7) |           |
| AD      | 93.0 (5.9) | 96.1 (4.5)   | 95.5 (8.9) | 94.7 (5.1) | 94.3 (4.5) | 97.3 (6.9) | 95.1 (10.4)|           |
| DHEA    | 99.6 (6.0) | 96.1 (5.8)   | 95.5 (8.9) | 94.7 (5.1) | 93.5 (4.3) | 99.2 (7.1) | 92.2 (10.6)|           |
| P       | 96.6 (3.7) | 103.2 (6.9)  | 93.7 (6.2) | 94.7 (5.1) | 94.3 (4.3) | 99.2 (7.1) | 92.2 (10.6)|           |
| E1      | 103.5 (4.4)| 97.4 (7.1)   | 103.3 (8.0)| 93.7 (9.2)| 101.2 (7.6)| 86.5 (9.2) | 100.9 (9.1)|           |
In addition, as the freezers were also frequently set at −20°C in clinical laboratories, we further tested the applicability of CIPS-based pretreatment for sex hormones at this temperature. After similar optimization and validation processes, we found that (I) CIPS could be well completed after storing the samples for 15 min at −20°C; (II) the partition coefficients, enrichment factors, and absolute recoveries of all the targets were not significantly changed after substituting CIPS at −30°C for 10 min to CIPS at −20°C for 15 min; (III) the matrix effects (89.7–109.4%), apparent recoveries (87.5–108.1%), and intraday, n = 6, including LLOQ level), and imprecisions (6.4–12.1%) were all satisfying when CIPS was taken at −20°C for 15 min; (IV) the stability of the two distinct phases after CIPS at −20°C for 15 min could be well preserved for 2 min under room temperature before sample collection. According to all these results, the CIPS strategy can also be efficiently functioned at −20°C in practice.

In conclusion, the present study developed a novel LC-MS/MS method for sex hormone quantification. Through importing CIPS for extracting targets, traditional LLE/SPE processes were successfully circumvented. The new method can offer comparable detecting performance as traditional LLE/SPE methods whereas exhibits obviously higher throughput. We expect this contribution can further promote the popularization of accurate LC-MS/MS analysis of sex hormones in routine clinical laboratories.

Data availability
All data are contained within the article and supplemental data.

Supplemental data
This article contains supplemental data (6, 19).

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Author contributions
S.-T. W. conceptualization; R. P. methodology; J. L. and J.-R. C. validation; J. L. and S.-L. Y. investigation; Y. L. resources; S.-T. W. writing—original draft; R. P., J. L., S.-L. Y., and S.-T. W. writing—review & editing; S.-T. W. supervision.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations
17-OHP, 17-hydroxyprogesterone; ACN, acetonitrile; AD, androstenedione; CIPS, cold-induced phase separation; DC, dansyl chloride; DHEA, dehydroepiandrosterone; E1, estrone; E2, estradiol; E3, estril; IRMM, Institute for Reference Materials and Measurements; IS, internal standard; LLE, liquid-liquid extraction; LLOQ, lower limit of quantification; NIST, National Institute of Standards and Technology; P, progesterone; SPE, solid phase extraction; T, testosterone.

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