The internal link of serum steroid hormones levels in insomnia, depression and Alzheimer's disease rats: is there an effective way to distinguish among these three diseases based on potential biomarkers?

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Running title: Distinguishing insomnia, depression and AD by steroid hormones.

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Abbreviations:

AD: Alzheimer's disease;

HPA: hypothalamic-pituitary-adrenal

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PLS-DA: partial least square discriminant analysis

VIP: values of variable importance in the projections

LLOQ: the lower limit of quantification

QC: quality control

Keywords: Alzheimer's disease, depression, high performance liquid chromatography-tandem mass spectrometry, insomnia, steroid hormone

Abstract

Insomnia, depression and Alzheimer's disease are all neurodegenerative diseases and are associated with the levels of steroid hormones. To investigate the internal connection and difference of steroid hormones among these three diseases and distinguish them from the perspective of biomarkers, an easy, quick, and efficient HPLC-MS/MS method was established and validated to determine six steroid hormones simultaneously in rat serum. The separation was accomplished on a SHIM-PACK XR-ODS chromatographic column with 0.1% (*v/v*) formic acid and methanol as the mobile phase and the detection was performed with electrospray ionization source in the positive ion mode. Based on the concentrations of steroid hormones, all the groups could be distinguished obviously from each other by using partial least square discriminant analysis. Meanwhile, 11-deoxycortisol, corticosterone, and cortisol

were identified as potential biomarkers and 100% of samples were classified correctly by Bayes discriminant function. These biomarkers were further screened by one-way ANOVA analysis and cortisol was significantly different among all these groups. Bayes discriminant function was also built by cortisol and the classification accuracy was 87.2%. This workflow including determination of steroid hormones and discrimination among three neurological diseases would provide a basis for further clinical studies.

1. Introduction

Insomnia, depression and Alzheimer's disease (AD) are all neurodegenerative diseases and become more and more prevalent around the world, especially among the older adults. The occurrence of these three diseases is closely related. Insomnia is frequently co-morbid with depression and it is also considered as a clinical predictor of subsequent depression [1-2]. Insomniacs reach up to 10 times more likely to suffer from depression than regular sleepers [3-4]. Evidence also shows that people with mood disorders exhibit a higher incidence of sleep disturbance compared with the general population [5]. Depression, which may impair the reliable cognition in the short term, is both a symptom and risk factor of AD [6]. It was reported

that 28.4% of patients with late-life depression showed reliable global cognitive declines, whose risk was 6.4 times higher than that of normal older adults [7]. In addition, AD is typically associated with sleep disorders in older adults. Sleep disorders including insomnia, sleep inadequacy, poor sleep quality, prolonged sleep duration and excessive daytime sleepiness all increase the risk of developing cognitive decline [8-10]. Compared to those without AD or cognitive decline, insomnia is more likely to occur in AD patients [11-12]. Memory impairment is frequently occurred in insomniacs, and the AD patients with insomnia also show a faster progression to dementia [13].

A body of evidence demonstrates that insomnia, depression and AD are all closely related with the levels of steroid hormones [1, 7, 14]. Glucocorticoids, which are released by the regulation of hypothalamic-pituitary-adrenal (HPA) axis, play an essential role in the pathogenesis of insomnia, depression and AD. Excessive glucocorticoids could impair the hippocampal neurogenesis and reduce the volume of hippocampal, and then lead to the impairment of episodic memory performance and psychological resilience [15]. Moreover, the damage of hippocampal diminishes its negative-feedback to HPA axis, which will cause further increase in glucocorticoids secretion. Sex differences are widespread in the incidence of these three neurodegenerative diseases. Reportedly, females were about 1.6 times at higher risk for insomnia than males and were twice as likely to suffer from depression [16]. For AD, it is not only affects males and females disproportionately, but also varies in women's different life

period [17]. One of the most prominent hypotheses for sex differences is the pronounced difference in the levels of gonadal steroid hormones. Contrary to the glucocorticoids, gonadal hormones are always thought to have neuroprotective effect, including neuronal excitability and memory enhancing properties [18], as well as improve the depression symptoms [19-20]. Furthermore, studies have found that gonadal steroid hormones, like pregnenolone and allopregnanolone, are also involved in the generation of slow wave sleep [21].

The diagnostic methods of these diseases are complicated and subjective. Insomnia and depression mainly depend on psychiatric examination of the psychiatrist, while AD is mainly diagnosed by magnetic resonance imaging, positron emission topography and other radiological technologies [22]. Thus, it's meaningful to study the changes of steroid hormones levels in the rats of insomnia, depression and AD, and then distinguish them by biomarkers. Unfortunately, most studies have only investigated the changes of steroid hormones in insomnia, depression and AD, respectively [18-19, 21]. Therefore, monitoring steroid hormones in the serum of rats with these three diseases synchronously is necessary.

The concentrations of endogenous steroid hormones are relatively low [23], so enough sensitivity is required for the analysis method. Traditional immunoassay methods are hard to satisfy the need for their nonspecific antibody interactions, dissatisfactory sensitivity and inconsistent reproducibility, such as enzyme-linked immunosorbent assay [24]. With the advent of MS/MS technology, the MS/MS-based detection methods combined with GC or

HPLC have proved as useful tools for hormone analysis on account of their notably improved specificity, sensitivity and possibility for multi-analyte detection [25-26]. GC-MS usually needs sample derivatization and the process is complicated, so the HPLC-MS method is selected preferentially by virtue of its higher sensitivity and convenient operation.

In our study, the changes of steroid hormones in insomnia, depression and AD were investigated from an overall perspective. Six steroid hormones including cortisol, 11-deoxycortisol, corticosterone, dehydroepiandrosterone, androstenedione and pregnenolone were determined simultaneously in the serum of insomnia, depression and AD rats by an easy, quick and efficient HPLC-MS/MS method. The molecular structures and metabolic pathways of these analytes are listed in Figure 1. Data processing is one of the most important steps in experimental research. Partial least square discriminant analysis (PLS-DA) was applied to classify the data for its reliability and intuition. Partial least squares regression is a statistical method that integrates the basic functions of multiple linear regression analysis, principal component analysis and canonical correlation analysis. In PLS-DA, the contribution of potential biomarkers to the grouping was analyzed by taking the values of variable importance in the projections (VIP) as the indicator and Bayes linear discriminant function was established to validate the potential of biomarkers. Bayes linear discriminant analysis is widely accepted as a good way for the analysis of reliability data, which calculates the between scatter matrix and the within scatter matrix only, then figures out the problem of eigenvalue decomposition easily

with efficient computation. As a result, three model groups and control group all could be distinguished from each other and 11-deoxycortisol, corticosterone, and cortisol were proved to be the biomarkers to distinguish insomnia, depression, AD and healthy rats. The prediction accuracy of classification was 100% by Bayes discriminant analysis. To distinguish these diseases easier, these biomarkers were further screened by one-way ANOVA analysis. After further screening, these three diseases were also could be distinguished from each other only by cortisol. The classification accuracy was 87.2%. It's the first time to distinguish these three diseases based on the levels of steroid hormones, which may be helpful for the preliminary diagnosis and treatment of these diseases.

2. Materials and Methods

2.1 Chemicals and reagents

Androstenedione was purchased from Aladine (Shanghai, China), dehydroepiandrosterone and 17-methyltestosterone (IS) were obtained from Aike (Chengdu, China), corticosterone was purchased from TCI (Shanghai, China), cortisol and pregnenolone were purchased from Selleckchem.com (Texas, USA). 11-Deoxycortisol was purchased from Yuanye (Shanghai, China). The purity of all standard substances was above 98%. HPLC-grade methanol was provided by Fisher Scientific (Fair Lawn, NJ). Distilled water was supplied by Wahaha Co. Ltd (Hangzhou, China). HPLC-grade formic acid and methyl tert-butyl ether were

supplied by Shandong Yuwang Industrial Co. Ltd. (Yucheng, China). $A\beta_{25-35}$, D-galactose, para-chlorophenylalanine were provided by Sigma (St. Louis, MO), chloral hydrate was obtained from Regent Chemical Reagent Co. Ltd (Tianjin, China). Penicillin sodium needle was supplied by Northeast pharmaceutical corporation (Shenyang, China). Sucrose was purchased from Kemiou Chemical Reagent Co. Ltd (Tianjin, China). All other reagents were of analytical grade.

2.2 Animals

Forty-eight male Wistar rats (230 ± 20 g) were supplied by Shenyang Pharmaceutical University Experimental Animal Center (Shenyang, China) with the permission number of SCXK 2014-0001 and raised with accessible water and food in an environment-controlled animal center (temperature 25 ± 2°C, 50 ± 10% relative humidity) under a natural light-dark cycle over seven days. These rats were separated into three model groups and three control groups equally. The animal research was carried out following the Guideline for Animal Experimentation of Shenyang Pharmaceutical University and was agreed by the Animal Ethics Committee of the institution.

2.2.1 Preparation of the insomnia model

Insomnia model was established by classical chemical injection method [27]. The model group rats were intraperitoneal injected para-chlorophenylalanine (350 mg kg^{-1}) once a day for

four days. All the rats were weighted at 9:00 am everyday (Figure S1). The insomnia model was successfully established according to the change of weight and behavior of rats, as well as the brain histopathological examination (Figure S2). Blood samples were acquired by orbital vein 24 h after the last administration and stood in non-heparinized tubes at room temperature for 30 min, then centrifuged at 4000 rpm for 5 min to produce serum samples. All the serum samples were stored at −80°C.

2.2.2 Preparation of the depression model

Chronic unpredictable mild stress combined with solitary kept method was employed to establish the depression model [28-29]. Chronic unpredictable mild stress stimulations consist of tilting the cage for 45^{o} (12 h), fasting (24 h), water deprivation (24 h), nipping the tail (1 min), restricting moving (4 h), shaking the cage (5 min), circadian rhythm conversion (24 h), damp cage (12 h). The rats in chronic unpredictable mild stress group were raised individually while the control rats were paired-raised. The detailed process was listed in the Table S1. Open-field test (Table S2) and novel inhibition of feeding test (Table S3), as well as the brain histopathological examination (Figure S2) were conducted to determine whether the depression model was successfully established or not. Results showed that the model was successfully established. Serum samples were collected and prepared in the same way as the insomnia group.

2.2.3 Preparation of the Alzheimer's disease model

The animal model of AD was established by a widely recognized method throughout the world [30-32]. The rats in model group were intraperitoneal injected D-galactose at a dosage of 50 mg kg^{-1} once a day meanwhile the control group were injected the same amount of 0.9% NaCl and last for six weeks. In the end of the fourth week, the rats in model group were injected into 10 μL of $A\beta_{25-35}$ in the double-test hippocampus (including 10 μg $A\beta_{25-35}$, dissolved by 0.9% sterile NaCl). In the control group, 0.9% sterile NaCl was used instead of $A\beta_{25-35}$. Moris water maze experiment (Figure S3) and brain histopathological examination were conducted and finally validated the model was reasonable. Serum samples were collected and prepared in the same way as the insomnia group.

2.3 Preparation of stock solutions and calibration standards

Stock solutions of analytes were prepared in methanol with a concentration of 1.0 mg mL^{-1}, further dilution was carried out with methanol to acquire a series of working solutions. Cortisol, androstenedione were ranged from 10.00 to 1000 ng mL^{-1}, dehydroepiandrosterone, 11-deoxycortisol were ranged from 100.00 to 10000 ng mL^{-1}, pregnenolone were ranged from 200.00 to 20000 ng mL^{-1}, while corticosterone ranged from 500.00 to 50000 ng mL^{-1}. The IS was prepared by methanol to 0.1 mg mL^{-1}, and then diluted to 10.0 μg mL^{-1}.

One hundred microliter blank serum (mixture of serum from eight healthy rats) was spiked with a range of working standards to prepare the calibration standard samples of analytes and IS. The quality control (QC) samples were prepared in the same way as calibration standard samples and prepared at three concentration level (2.5, 12.5 and 80 ng mL^{-1} for cortisol and androstenedione; 25, 125 and 800 ng mL^{-1} for dehydroepiandrosterone, 11-deoxycortisol and pregnenolone; 125, 625 and 4000 ng mL^{-1} for corticosterone), which were used in validating the analytical method and guarantee the reliability of determination during the analytical run. All stock solutions and calibration standards were stored at 4°C refrigerator.

2.4 Sample preparation

Liquid-liquid extraction technique was applied to extract target analytes in rat serum. One hundred microliter serum sample, 10 μL IS and 10 μL methanol were added to a 5 mL Eppendorf tube and mixed with 300 μL methyl tert-butyl ether, vortex-mixed for 30 s and then centrifuged at 4°C with a rotate speed of 12000 rpm for 5 minutes. The supernatant was quantitatively transferred to another tube, and the bottom liquid phase was extracted by methyl tert-butyl ether in the same way. Merging the organic phase into one Eppendorf tube and evaporated to dryness under a gentle 35°C nitrogen stream. The residue was dissolved with 100 μL original mobile phase, vortex-mixed for 2 min and sonicated for 5 min followed by

centrifugation at 12000 rpm, 4°C for 5 min. Finally, 4 μL of supernatant was injected into HPLC-ESI-MS/MS system for analysis.

2.5 Apparatus and operation conditions

The XR LC-20AD Prominence™ HPLC system (Shimadzu, Japan) equipped with a binary pump, a degasser and an autosampler was applied in the experiment and a SHIM-PACK XR-ODS column (100 × 3.0 mm, 2.2 μm) was used for liquid chromatography separation. The mobile phase was composed of 0.1% formic acid-water (A) and methanol (B) with the elution program (B%) as follows: 0.01-1 min, 10-70%; 1-4 min, 70%; 4-4.01 min, 70-90%; 4.01-5 min, 90%; 5-5.01 min, 90-10%; 5.01-7 min, 10%. All analytes could be effectively separated at 0.4 mL min^{-1} with a sample injection volume of 4 μL.

Mass spectrometry analysis was performed on a 4000 QTRAP™ MS/MS system (AB Sciex, Foster city, CA) with a turbo ion spray source. Analytes were detected via MRM pattern in the positive ion mode. The source temperature was set at 500°C and the ion spray voltage was 4500 V. Curtain gas, gas 1 and gas 2 all adopted nitrogen and set at 20, 40 and 40 psi, respectively. Mass parameters including declustering potential, entrance potential, collision energy and cell exit potential values were optimized for analytes and IS as shown in Table 1.

2.6 Method validation

2.6.1 Specificity

Considering all analytes are endogenous substances, it is difficult to get an authentic blank matrix. The chromatograms of the rat serum without reference solutions and the chromatograms of standard solutions were compared to validate the specificity. A pooled sample of rat serum from control group (n=8) was investigated.

2.6.2 Linearity and the lower limit of quantification

The linearity was generated by plotting the peak area ratio (y) of each analyte subtracted those of blank samples to IS versus nominal concentrations (x) of analytes by $1/x^2$ weighted least square linear regressions. The lower limit of quantification (LLOQ) was considered to be the lowest quantitative, precise and accurate concentration within the calibration levels.

2.6.3 Accuracy and precision

The inter- and intra-day precision as well as accuracy were validated through the LLOQ and three levels of QC samples in six replicates. The concentrations of analytes in the LLOQ and QC samples were calculated by the intraday calibration curves.

2.6.4 Matrix effect

The matrix effect was measured by "IS-normalized matrix factor", which was calculated as the ratio of peak area with matrix (measured by analyzing blank matrix after extraction

spiked with analytes at low and high levels subtracted those of blank samples) to pure solution containing equivalent amount of the analytes and IS at low and high levels.

2.6.5 Stability

Stability tests were conducted by analyzing three replicates of QC samples at low and high levels under these conditions: 4 h at ambient temperature, −80°C for 30 days and three freeze−thaw cycles before preparation; 8 h in autosampler (4°C) after preparation.

2.7 Analysis and data processing

These serum samples were prepared and analyzed according to the above method. In addition, three-level QC samples were prepared to determine whether the data was accepted rejected, QC samples were more than 5% of all samples. The data was reasonably chosen according to Geblas principle and no data was rejected. Then, the data was further processed PLS-DA by the SIMCA-P software package (Version13.0, Umetrics AB, and Ume, Sweden). Potential biomarkers were extracted by VIP>1, which were further confirmed by one-way ANOVA analysis. Differences were regarded as statistically significant at $P<0.05$. Finally, Bayes discriminant analysis was performed on SPSS 19.0 (SPSS Inc, Chicago, IL) to classify the data and validate the predictive accuracy after the biomarkers were found.

3. Results and Discussion

3.1 Optimization of HPLC-MS/MS

The concentrations of steroid hormones in the serum are relatively low, so a sensitive and effective analysis method is needed. Most of endogenous substances are cyclopentane polyhydric phenanthrene derivatized with carbonyl [33] and could be detected in both positive and negative ion modes. Thus, both positive and negative ion modes were studied and the results showed that a higher sensitivity could be achieved in the positive ion mode. Then the mass parameters were further optimized until the product ion of molecular ion could be scanned effectively. Results showed that formic acid could greatly enhance the response of the analytes and the symmetry of the chromatographic peak, so different concentrations of formic acid were investigated. Finally, 0.1% formic acid was able to meet the requirements of separation and response.

3.2 Optimization of sample preparation

An appropriate sample preparation process is essential for the quantitative analysis. As most of steroid hormones are low-weight lipophilic molecular with weak polarity, liquid-liquid extraction and solid phase extraction technique are suitable for sample preparation [34]. Solid phase extraction method is frequently used for its high purification efficiency, but it is expensive and time-consuming. Liquid-liquid extraction technique not only possesses high purification efficiency and simple preparation process, but also has a low

cost. So liquid-liquid extraction was chosen to prepare the samples. Extraction reagents including methyl tert-butyl ether, ethyl acetate, isopropyl and their extraction volume were studied. Finally, triploid methyl tert-butyl ether was proved to be the most suitable method which has the highest recovery rate. Extraction times were also investigated and finally found that the recovery rate was the highest when extracted twice. Thus, these samples were extracted with methyl tert-butyl ether twice. In addition, 50 μL, 100 μL, 200 μL, 400 μL of original mobile phase were investigated to dissolve the residue. The result showed that 100 μL mobile phase could meet the demands of LLOQ and meanwhile the matrix effect was significantly decreased.

3.3 Method validation

The specificity experiment demonstrated that other endogenous substances and metabolites didn't interfere the determination of analytes as listed in Figure S4 and Figure S5. All standard calibration curves were linear and the calibration regression coefficients were ranged from 0.9911 to 0.9997. And the LLOQs of analytes were listed in Table S4. The precision and accuracy data was listed in Table S5, which demonstrated satisfactory results for accuracy and precision. The RSD of IS-normalized matrix effect which evaluated the matrix effect at different concentration levels were less than 15% (Table S6), which proved that the endogenous substances didn't affect the content of all analytes. The stability experiment showed that all analytes were stable in the cases studied within a 15% standard

deviation (Table S6). In total, the specificity, carryover, linearity, LLOQ, precision, accuracy, matrix effect and stability of the method all satisfied the requirements of biological sample analysis.

3.4 Method application

3.4.1 Determination of steroid hormones in insomnia, depression and Alzheimer's disease rats

Three control groups that were parallel to each model group were combined into one group considering that there was no marked difference of steroid levels among these three control groups by one-way ANOVA analysis. As shown in Figure 2, dehydroepiandrosterone and corticosterone were increased in insomnia, depression and AD groups. Meanwhile, the content of androstenedione was decreased. The similar change of these steroid hormones indicated some common pathological mechanism among these three neurodegenerative diseases. In addition, cortisol, 11-deoxycortisol and pregnenolone presented different variation tendency in insomnia, depression and AD. Cortisol was increased in insomnia group and AD group, counter to depression group. 11-Deoxycortisol, the precursor of cortisol, was decreased in depression group and showed no significant difference with insomnia group and AD group. Pregnenolone was increased in insomnia and AD group, but there was no significant difference between depression group and control group. The different changes of

these steroid hormones provided a possibility to distinguish these three diseases based on potential steroid biomarkers.

3.4.2 Distinctions of steroid hormones among insomnia, depression and Alzheimer's disease rats

Four groups including one combined control group and three model groups could be separated obviously by PLS-DA as shown in Figure 3. 11-Deoxycortisol, corticosterone and cortisol were screened out by VIP>1 as shown in Figure S6. Then Bayes discrimination function was established by 11-deoxycortisol, corticosterone and cortisol and 100% of samples were classified correctly. Among the four discriminant functions, the absolute value of the cortisol coefficient was much greater than other coefficients (Table 2), indicating that the change of cortisol had the greatest impact on the discriminant results. To distinguish these diseases easier, one-way ANOVA analysis was applied to further confirm the potential biomarkers. In the end, cortisol exhibited a significant difference among control group and model groups. Thus, Bayes discrimination function was established by cortisol and 87.2% of samples were classified correctly. The Bayes linear discriminant function coefficients of cortisol and constant were listed in Table 3.

As we could see from the data processing results, cortisol played an essential role in the differentiation of these three diseases. In our study, the content of cortisol was significantly

elevated in the serum of insomnia group and AD group. Cortisol, a kind of glucocorticoid released in stressful situations, is also known as the hormone of awakening because the release peak occurs in the morning [23]. High level of cortisol may increase wakefulness in insomniacs and further aggravate insomnia. In AD rats, the stability and functions of hippocampal neurons may be damaged by a large amount of cortisol, and then impaired memory and cognitive function [17]. The release of cortisol was mainly regulated by HPA axis and the increased cortisol indicated the hyperactivity of HPA axis in insomnia and AD rats. Although the content of cortisol increased in both insomnia group and AD group, the variation degree was different in these two groups. The level of cortisol was higher in AD rats compared with insomnia rats. Thus, we can infer that the HPA axis damaged more seriously in AD, which was also consistent to the score plot of PLS-DA. The concentration of cortisol was significantly decreased in depression rats, inconsistent with the insomnia and AD rats, which indicated that the dysfunction of HPA axis in depression rats. In addition, chronic unpredictable stress induced both the dysfunction of HPA axis as indicated by increased corticosterone levels and changes in the immune system and other pathophysiological aspects [35]. Thus, we predicted that the decrease of cortisol and its precursor may be a result of the joint action of HPA axis disorder and other abnormal metabolic pathway [36]. Corticosterone, the main stress hormone in the rodents, was increased in all these three neurodegenerative diseases. Evidence has shown that repeated corticosterone injections

reliably produce depressive-like behavior by impairing gap junctions in the prefrontal cortical and hippocampal astrocytes in rodents and deteriorated the sleep patterns, as well as impair the cognition [37]. The increased corticosterone level in insomnia, depression and AD rats may be caused by the hyperactivity of HPA axis.

4. Conclusion

There is a growing tendency in the occurrence of insomnia, depression and AD [38-40]. Considering that the early symptoms of the three kinds of neurogenic diseases are similar, discriminating and diagnosing them are still difficult. Here, we investigated the changes of the steroid hormones in insomnia, depression and AD rats by an easy, quick and efficient HPLC-MS/MS method. And these diseases were distinguished obviously by potential biomarkers. The study preliminarily revealed the relationship of these three diseases and will provide an effective way to understand the inner mechanisms from the perspective of steroid hormones. The results may greatly help the early detection and effective treatment of these diseases, as well as the development of therapeutic drugs. And by further large-scale study, it is with great possibility to elucidate the potential of the steroid hormones for early diagnosis of insomnia, depression and AD.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure captions

Figure 1. Structures and metabolic pathways of the analytes.

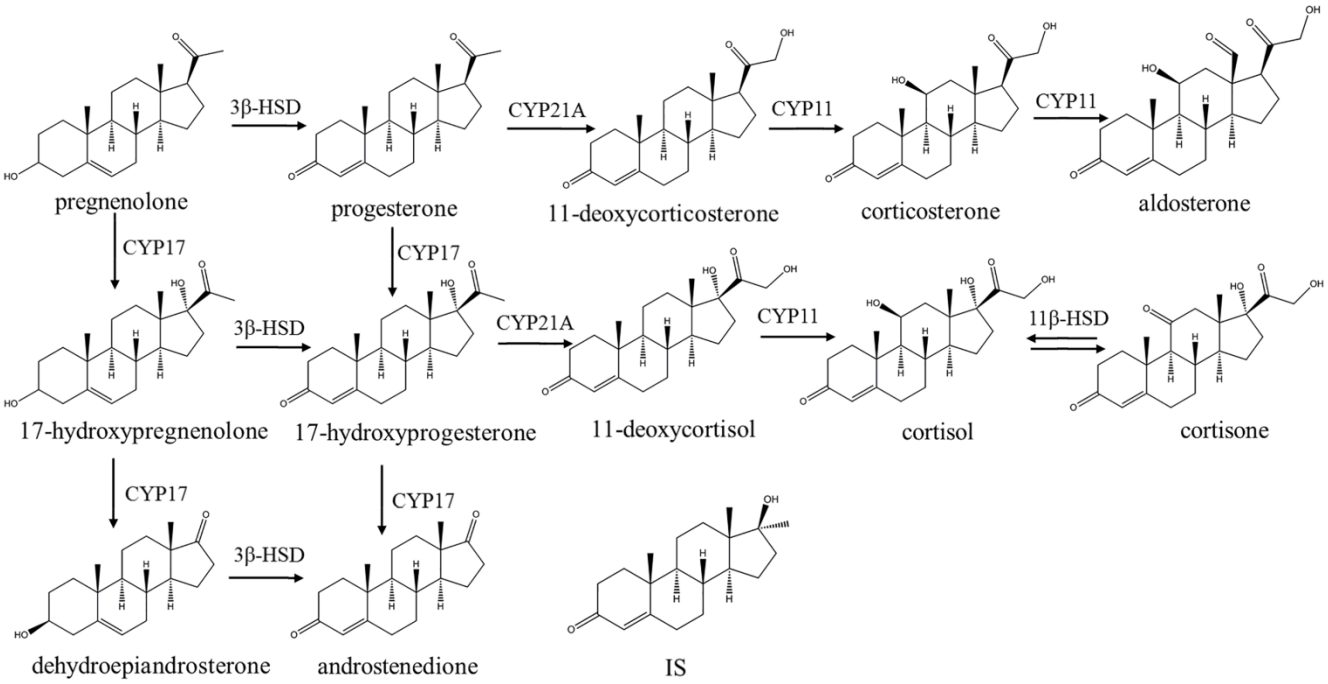

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Figure 2. The concentrations of steroid hormones in rat serum samples.

For statistical significance [a]P< 0.05 compared with control group, [b]P<0.05 compared with insomnia group, [c]P<0.05 compared with depression group.

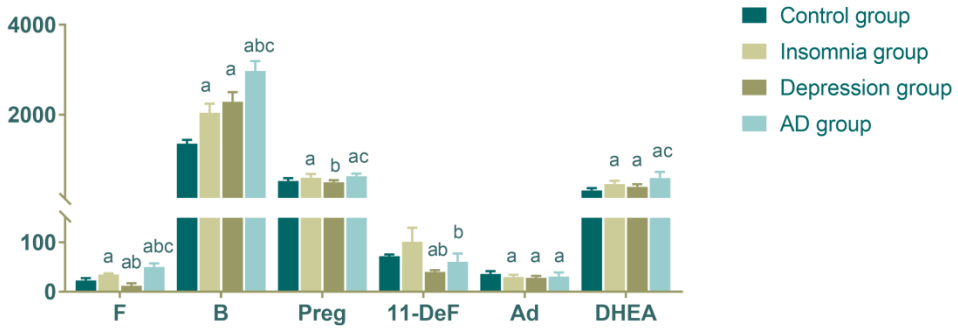

Figure 3. PLS-DA score plot of serum samples from different model groups and control group.

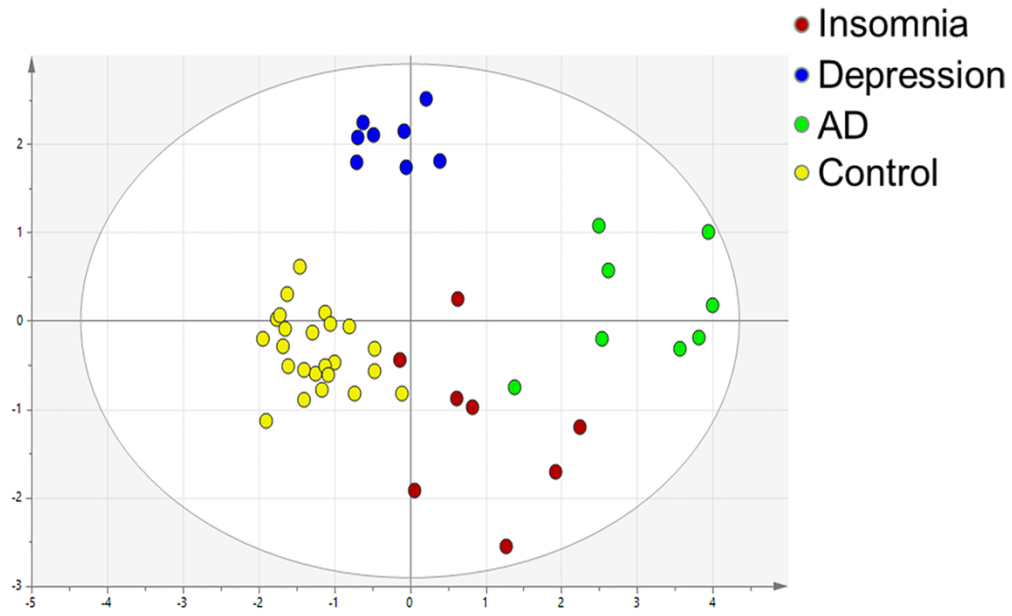

Table 1. Mass spectrometric characteristics of the analytes and IS.

Analytes	Q1mass	Q3mass	DP (V)	EP (V)	CE (V)	CXP (V)
Cortisol	363.1	121.1	110	11	49	16
Dehydroepiandrosterone	337.9	97.2	71	14	27	20
Corticosterone	348.2	313.0	64	4	18	21
Pregnenolone	318.0	255.9	79	5	30	20
11-Deoxycortisol	338.2	97.1	102	14	32	26
Androstenedione	287.2	97.2	90	10	33	6
IS	303.5	108.9	68	8	30	27

declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP).

Table 2. Bayes linear discriminant function coefficients of 11-deoxycortisol, corticosterone, cortisol and constant.

Analytes	Groups			
	Blank	Insomnia	Depression	AD
11-Deoxycortiso	0.248	0.333	-0.048	-0.033
Corticosterone	0.071	0.108	0.122	0.171
Cortisol	1.351	2.049	1.141	2.977
Constant	-73.906	-163.993	-147.407	-335.646

Table 3. Bayes linear discriminant function coefficients of cortisol and constant.

Analytes	Groups			
	Blank	Insomnia	Depression	AD
Cortisol	0.955	1.453	0.514	2.097
Constant	-12.285	-26.212	-4.545	-53.896