Research Paper

Development of a method for quantification of serum dehydroepiandrosterone sulfate by stable isotope-dilution MALDI-MS

Shoujiro Ogawa[1,2], Masahiro Nakamura[1], Mamoru Satoh[3], Takayuki Ishige[4], Fumio Nomura[5], Yuki Sugiura[6], Tatsuya Higashi[1]*

[1] Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278−8510, Japan
[2] Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 1 Sanzo, Gakuen-cho, Fukuyama-shi, Hiroshima 729−0292, Japan
[3] Division of Clinical Mass Spectrometry, Chiba University Hospital, 1−8−1 Inohana, Chuo-ku, Chiba-shi, Chiba 260−8677, Japan
[4] Division of Laboratory Medicine, Chiba University Hospital, 1−8−1 Inohana, Chuo-ku, Chiba-shi, Chiba 260−8677, Japan
[5] Division of Clinical Genetics, Chiba University Hospital, 1−8−1 Inohana, Chuo-ku, Chiba-shi, Chiba 260−8677, Japan
[6] Department of Biochemistry, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160−8582, Japan

Abstract The quantification of dehydroepiandrosterone sulfate (DHEAS) in the human circulation might be of diagnostic help for several diseases, such as diabetes, depression, Alzheimer's disease, osteoporosis and chronic heart failure. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) could be an effective approach to quantify the serum DHEAS due to its high specificity, handleability, rapid analysis and ruggedness. Nonetheless, to the best of our knowledge, no MALDI-MS assay has been reported for the quantification of DHEAS in biological samples. In this study, we developed and validated a method to quantify DHEAS in human serum by MALDI-MS using a stable isotope-dilution technique with $^{2}H_{4}$-DHEAS as an internal standard. This method enabled the reproducible quantification of the serum DHEAS (intra- and inter-assay relative standard deviations, 9.3% or lower) with a small sample volume (20 μL) and effort-saving pretreatment (only deproteinization and crystallization with 9-aminoacridine). The measurable range was 0.250−5.00 μg/mL and the analysis time after the sample preparation was 3 min. The serum DHEAS concentrations measured by the newly-developed MALDI-MS method agreed well with those by liquid chromatography/electrospray ionization-MS/MS; this result demonstrated that the developed MALDI-MS method yielded reliable results.

Key words: matrix-assisted laser desorption/ionization-mass spectrometry, dehydroepiandrosterone sulfate, quantification, serum, stable-isotope dilution

Introduction

Dehydroepiandrosterone (DHEA) is an androgen synthesized by the steroidogenic enzyme P450c17 within the adrenal zona reticularis. More than 99% of DHEA is circulating in the blood as the sulfated conjugate (DHEAS, Fig. 1) and its serum/plasma concentration is reported to be about several hundred nanograms−several micrograms/mL in healthy adult males[1-4]. A number of studies demonstrated that the decline in the circulating DHEAS levels is associated with several diseases, such as diabetes[5,6], depression[7,8], Alzheimer's disease[3], osteoporosis[9] and chronic heart failure[10]. Therefore, the determination of the serum/plasma DHEAS concentration might be of diagnostic help for these diseases. DHEAS has a long half-life (*ca.* 11 h) in blood and no significant diurnal rhythm in its

* **Corresponding author**
Tatsuya Higashi
Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278−8510, Japan
Tel/Fax: +81−4−7121−3660
E-mail: higashi@rs.tus.ac.jp
Received: November 26, 2020. Accepted: February 5, 2021.
Epub March 19, 2021.
DOI: 10.24508/mms.2021.06.003

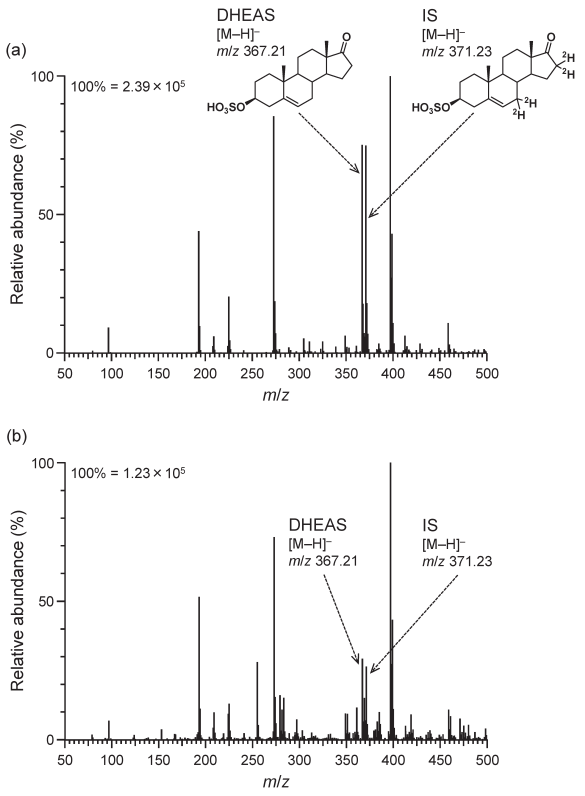

Fig. 1. MALDI-MS spectra of DHEAS and IS. (a) Standard DHEAS and IS (2.5 ng each/spot). (b) Serum sample spiked with IS; the DHEAS concentration of this sample was subsequently determined to be 2.85 μg/mL.

serum/plasma level unlike DHEA[11], which are advantages of DHEAS over DHEA as the diagnostic marker.

DHEAS has conventionally been measured in serum/plasma by immunoassay in clinical practice. However, some of the commercially-available immunoassays for DHEAS have several limitations and drawbacks[12]. Different immunoassay kits sometimes provide different measured values even for the same sample. Poor quality assurance of the standard solutions is considered a major cause of this discrepancy. Furthermore, there is a well-known inaccuracy in the immunoassay due to cross-reaction of antibodies with steroids that are structurally similar to DHEAS. The mass spectrometry (MS)-based method has a high specificity, therefore, it would be a promising alternative to immunoassay for the DHEAS measurement. Many methods using liquid chromatography (LC)/electrospray ionization (ESI)-tandem MS (MS/MS) have been developed for the quantification of DHEAS in serum/plasma[1–4]. However, to the best of our knowledge, there is no matrix-assisted laser desorption/ionization (MALDI)-MS method for this specific purpose though MALDI-MS has a number of attractive properties, such as handleability and rapid analysis due to the absence of LC process, ruggedness (allowance for contamination of ion source), low consumption of organic solvent (environmentally preferred method) as well as high specificity. A major cause for this is the low quantitative capability of MALDI-MS due to the poor reproducibility of the signal intensities. The stable isotope-dilution technique, which is widely used in LC/ESI-MS/MS-based quantitative assays, would be useful to correct the ion abundance fluctuation in MALDI-MS. Recently, two MALDI-MS-based quantitative methods of steroids (including a secosteroid) were reported[13,14], in which the stable isotope-dilution technique worked well for increasing the quantitative accuracy.

Based on this background information, the objective of this study was to develop a simple and rapid method for the quantification of DHEAS in human serum by MALDI-MS. The applicability of this method was evaluated by the analysis of serum samples of healthy subjects and by comparison to the conventional LC/ESI-MS/MS assay.

Experimental

Materials and chemicals

DHEAS and [7,7,16,16−$^2H_4$]-DHEAS (internal standard, IS, Fig. 1) were synthesized in our laboratories and the same as those used in a previous study[15]. Standard solutions of DHEAS (0.500, 1.00, 2.50, 5.00 and 10.0 μg/mL) were prepared in ethanol. The IS was used as the ethanolic solution of 5.00 μg/mL. 9-Aminoacridine (9-AA) was purchased from Sigma-Aldrich Japan (Tokyo, Japan) and used as the matrix. The saturated 9-AA solution (10 mg/mL) was prepared in acetonitrile-water (1 : 1, v/v). All other reagents and solvents were of analytical grade or LC/MS grade.

Serum samples

Serum samples were collected from apparently healthy adult male volunteers (n=21) at the Chiba University Hospital (Chiba, Japan). The experimental procedures were approved by the ethics committee of Chiba University (No. 640). Based on the approved experimental procedures, the serum DHEAS concentrations were measured at Tokyo University of Science. Written forms of informed consent were obtained from all the volunteers. All the serum samples were stored at − 30°C until used.

MALDI-MS

MALDI-MS was performed using a Waters Synapt^{TM} G1 quadrupole time-of-flight (ToF) mass spectrometer (Milford, MA, USA). The ToF mass analyzer was operated in the negative-ion V mode. A Nd:YAG laser (wavelength, 355 nm and energy, 350 arbitrary value) was employed at the firing rate of 200 Hz. The laser was fired for 180 s to obtain a mass spectrum. The intensities of the ions derived from DHEAS (*m*/*z* 367.21) and IS (*m*/*z* 371.23) were measured to quantify DHEAS. Masslynx software (version 4.1, Waters) was used for the system control and data processing.

Pretreatment procedure

The serum sample (20 μL) was added to acetonitrile (200 μL) containing IS (50.0 ng), vortex-mixed for 30 s, then centrifuged at 2000×*g* for 10 min. The supernatant (200 μL) was transferred to another test tube. After the solvent was evaporated, the residue was dissolved in the saturated 9-AA solution (20 μL), 1.0 μL of which was spotted onto the MALDI sample plate and allowed to dry (dried droplet method[16]).

Calibration curve

IS (50.0 ng) and a graduated amount of the standard DHEAS (5.00, 10.0, 25.0, 50.0 or 100 ng, corresponding to 0.250, 0.500, 1.25, 2.50 or 5.00 μg/mL, five points) were dissolved in the saturated 9-AA solution (20 μL), 1.0 μL of which was spotted onto the MALDI sample plate. The peak intensity ratio (DHEAS/IS, *y*) was plotted versus the amount of DHEAS (ng per tube, *x*) with a weighting of 1/*x* to construct the calibration curve.

Assay precision and accuracy

The intra- and inter-assay precisions were assessed by the repeated measurements of three pooled serum samples with different concentrations on one day (n=5) and over three days, respectively. The precision was determined as the relative standard deviation (RSD, %). The acceptance criterion was met if the RSD values were ≤15% according to the guidance from the US Food and Drug Administration[17].

The assay accuracy (%) was calculated by dividing the mean measured concentrations by the theoretical concentrations for the DHEAS-spiked samples. The acceptance criterion was met if the calculated accuracy values were within the range of 85−115%[17].

LC/ESI-MS/MS method for quantification of serum DHEAS

To further evaluate the accuracy of the MALDI-MS assay, the serum DHEAS concentrations measured by the MALDI-MS method were compared with those by the LC/ESI-MS/MS method.

A Waters LC-e2695 chromatograph and a Waters Quattro Premier XE triple quadrupole-mass spectrometer were used. The LC/ESI-MS/MS conditions were as follows: column, J'sphere ODS-H80 (4 μm, 150×2.0 mm i.d., YMC, Kyoto, Japan); column temperature, 40°C; mobile phase, methanol-10 mM ammonium formate (4 : 3, v/v); flow rate, 0.2 mL/min; capillary voltage, −2.8 kV; cone voltage, −50 V; collision energy, −35 eV; source temperature, 120°C; desolvation temperature, 350°C; desolvation gas (N_2) flow rate, 600 L/h; cone gas (N_2) flow rate, 50 L/h; and collision gas (Ar) flow rate, 0.19 mL/min and selected reaction monitoring (SRM) transitions, *m*/*z* 367.2 $[M-H]^- \rightarrow$ 96.8 $[HSO_4]^-$ (DHEAS) and *m*/*z* 371.2 → 96.8 (IS). Masslynx software (version 4.1) was used for the system control and data processing.

The serum sample (20 μL) was pretreated in the same way for the MALDI-MS method. The residue was dissolved in the mobile phase (100 μL), 10 μL of which was subjected to the LC/ESI-MS/MS. DHEAS and IS eluted at 5.9 and 5.8 min, respectively, under the stated conditions and the LC/ESI-MS/MS run time per one sample was 7 min. The calibration samples were prepared by dissolving IS (50.0 ng) and a graduated amount of the standard DHEAS (5.00, 10.0, 25.0, 50.0 or 100 ng, five points) in the mobile phase (100 μL). A portion (10 μL) of these samples was injected into the LC/ESI-MS/MS. The peak area ratio (DHEAS/IS, *y*) was plotted versus the amount of DHEAS (ng per tube, *x*) with a weighting of 1/*x* to construct the calibration curve.

Results and Discussion

Selection of optimum mode for quantification of DHEAS

DHEAS (5.0 ng) was dissolved in the saturated 9-AA solution, and 1/20 of the resulting sample (equivalent to 250 pg of DHEAS) was spotted onto the plate for the MALDI-MS analysis. Because DHEAS has a sulfuric acid group, MALDI-MS was operated in the negative-ion mode, in which the deprotonated molecule ($[M-H]^-$) was detected at *m*/*z* 367.21 with a satisfactory intensity (signal-to-noise

ratio of *ca.* 12). We also tested α-cyano-4-hydroxycinnamic acid, 2,3-dihydroxybenzoic acid and sinapinic acid as the MALDI matrices for the DHEAS quantification. However, no characteristic ion was detected from 250 pg of DHEAS when these matrices were used. Based on these results, we concluded that 9-AA was the suitable matrix to sensitively quantify DHEAS by the MALDI-MS. By the collision-induced dissociation (CID) of $[M-H]^-$, only a sulfuric acid group-derived product ion ($[HSO_4]^-$) was observed at *m*/*z* 96.88. The IS also produced the product ion at *m*/*z* 96.88 from its $[M-H]^-$ (*m*/*z* 371.23) during the MS/MS. The Synapt™ G1 mass spectrometer could not individually detect DHEAS and IS in their mixture by the MS/MS mode because the product ions derived from these compounds were the same. For this reason, in this study, the serum DHEAS was quantified based on the intensity ratio of $[M-H]^-$ of DHEAS and IS in the MS mode (without CID).

Pretreatment of serum sample

Our method required only deproteinization and crystallization with 9-AA for the serum sample prior to the MALDI-MS analysis. The MS spectrum obtained from a serum sample is shown in Fig. 1(b), in which the ions derived from DHEAS (*m*/*z* 367.21) and IS (*m*/*z* 371.23) were readily identifiable. These ions were fully resolved from the interfering ions derived from the 9-AA and serum matrix.

Calibration curve

Although a blank (DHEAS-free) serum is preferable for drawing the calibration curve to correct the matrix effect, a blank serum was not available. For this reason, the calibration curve was constructed using a standard solution as described in the experimental section. The MALDI-MS spectrum of standard DHEAS and IS (2.5 ng each/spot; second highest point of calibration curve) was shown in Fig. 1(a). All the regression lines constructed three times showed a good linearity ($r^2 \geq 0.999$) and the slopes were reproducible [0.0192±0.0002 (mean±SD, *n*=3) and 1.12% (RSD)]. The *y*-intercept (0.0107±0.0037) was also close to zero. The applicability of this calibration curve was verified by the analytical recovery test, which will be described in the next section.

Assay precision and accuracy

As is obvious from Table 1, the proposed MALDI-MS method was highly precise; the intra- (*n*=5) and inter- (*n*=3) assay RSDs did not exceed 9.3% and 3.2%, respectively, for the samples with the different DHEAS concentrations.

Table 2 shows the accuracy (analytical recovery) of the MALDI-MS method. The DHEAS concentrations of the serum samples spiked with the standard DHEAS were determined using the calibration curve described in the preceding section. As shown in Table 2, the accuracy values

Table 1. Assay precision

	Serum A		Serum B		Serum C	
	Measured concentration[a] (μg/mL)	RSD (%)	Measured concentration[a] (μg/mL)	RSD (%)	Measured concentration[a] (μg/mL)	RSD (%)
Intra-assay (*n*=5)	3.56±0.33	9.3	1.91±0.04	2.1	0.85±0.02	2.4
Inter-assay (*n*=3)	3.67±0.08	2.2	1.85±0.06	3.2	0.85±0.01	1.2

[a] Mean±SD.

Table 2. Applicability of calibration curve and assay accuracy

	Serum D		Serum E		Serum F	
	Measured[a] (μg/mL)	Accuracy (%)	Measured[a] (μg/mL)	Accuracy (%)	Measured[a] (μg/mL)	Accuracy (%)
Intact	1.04	—	1.06	—	0.98	—
Spiked (+0.25 μg/mL)	1.36	105.4	1.35	103.1	1.33	108.1
Spiked (+0.50 μg/mL)	1.59	103.2	1.60	102.6	1.59	107.4
Spiked (+1.25 μg/mL)	2.32	101.3	2.29	99.1	2.32	104.0
Spiked (+2.50 μg/mL)	3.57	100.8	3.59	100.8	3.39	97.4

[a] Mean measured concentration.

ranged from 97.4 to 108.1%. This result demonstrated that the calibration curve using the standard solution was applicable for the quantification of the serum DHEAS, and moreover, the proposed MALDI-MS method was sufficiently accurate. This method employed the stable isotope-dilution technique, which worked well in correcting for the influence of the serum matrix.

Reliability of measured values by MALDI-MS

Because no chromatographic separation occurs during the MALDI-MS analysis, the isomeric steroids are hardly discriminated by the MALDI-MS, which is a drawback compared to LC/ESI-MS/MS[13,14]. Testosterone sulfate is the isomer of DHEAS, but it was reported that its serum concentration is too low to be detected by the LC/ESI-MS/MS[18]. Furthermore, we could not find any information in the literature that an isomer other than testosterone sulfate is present in human serum. Therefore, it is a good assumption that the effects of the isomers on the measurement of the serum DHEAS are not usually significant. The serum DHEAS concentrations of apparently healthy male subjects (n=21) were determined to be 1.89 μg/mL (mean) and 0.85−3.56 μg/mL (range) [1.77 μg/mL (median) and 1.56−1.91 μg/mL (interquartile range)] by the MALDI-MS method; these concentrations are in good agreement with the literature values[1−4]. To make extra sure that the developed MALDI-MS method yields reliable measured values, the serum DHEAS concentrations determined by the MALDI-MS were compared to those by the LC/ESI-MS/MS. As shown in Fig. 2, a strong positive relationship was observed between the measured values of the two methods (Pearson's correlation coefficient test, P<0.01). Furthermore, the Wilcoxon signed-rank test revealed that there was no significant difference in the measured values between the two methods (P<0.05). These results demonstrated that the MALDI-MS method provided accurate measured values.

The time required for the MALDI-MS analysis per sample was 3 min by our method and thus, the short analysis time is an advantage of the MALDI-MS versus the conventional LC/ESI-MS/MS (7 min for one sample). Furthermore, MALDI-MS was easier to operate than LC/ESI-MS/MS.

Conclusion

In this study, a stable isotope-dilution MALDI-MS method was developed and validated for the rapid quantification of DHEAS in human serum. This method had a satisfactory precision and provided reliable serum DHEAS concentrations. To the best of our knowledge, this is the first validated and workable quantitative MALDI-MS assay for the serum DHEAS. We believe that the developed MALDI-MS assay would become a method of choice for the DHEAS measurement in clinical laboratories.

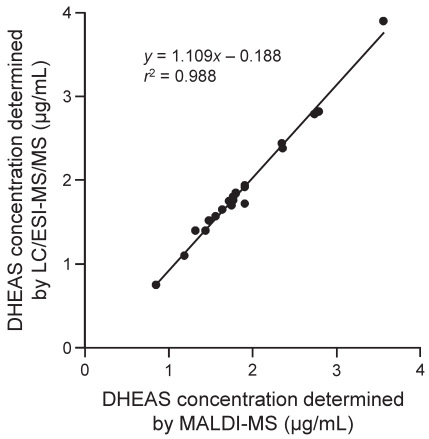

Fig. 2. Scatter diagram comparing the measured DHEAS concentrations by MALDI-MS to those by LC/ESI-MS/MS. Serum samples from apparently healthy male subjects were analyzed (n=21).

Acknowledgment

This study was supported in part by JSPS KAKENHI Grant Number 17K08250 (to T. H.).

Conflicts of Interest

The authors declare no conflicts of interest.

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