Analytical Methods

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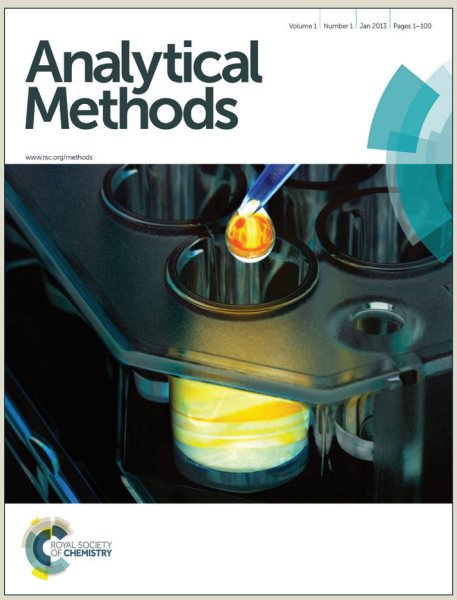

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ARTICLE

Analysis of steroid hormones in human saliva by matrix-assisted laser desorption/ionization mass spectrometry

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This work describes a simple and rapid method for the quantitative analysis of four steroid hormones, including testosterone, progesterone, hydrocortisone and cortisone in human saliva sample by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using nanomaterials as the matrix. The optimal pretreatment processes of saliva samples as well as MALDI conditions for efficient desorption/ionization of these steroid hormones are systematically explored. Parameters, including the concentration and pH level of ammonium citrate buffer, the nature and concentration of MALDI matrix, laser energy, and sample extraction method were systematic investigated in this study. The limits of detection at a signal to noise ratio of 3 for testosterone, progesterone, hydrocortisone and cortisone provided by the proposed approach are 6.0, 6.0, 6.0, and 3.0 μM, respectively, with satisfied quantitative linearity (R^2 > 0.985) and recovery (89.3~111.2%) for the four analytes. Our technique provided advantages of sensitivity, repeatability, speed, and simplicity for the quantitation of the four steroid hormones in saliva samples. This method holds great potential for the high-throughput biological screening and disease detection.

Introduction

Many biological specimens such as blood, urine, saliva, spinal fluid, and feces can be used for the detection of disease. In these samples, collecting saliva is a fast, simple, convenient and non-invasive method[1]. Saliva contains polysaccharides, glycoproteins, and a variety of enzymes as well as steroid hormones. In the human body there are two forms of steroid hormones-the bound form and the free form. The bound form is a combination between steroid hormones and globulin or albumin, and represents about 98% of the total steroid hormones[2]. Due to their large molecular weight, the bound form steroid hormones can't pass through the cell membrane of the blood vessel wall, and its biological activity is very limited. The free form of steroid hormones (about 2-3% of the total steroid hormones) can pass through the cell membrane and penetrate into the blood and saliva, thereby affecting the body's physiological responses[3]. Thus, analyzing the free type steroid hormones in saliva is a convenient way to indicate the health condition of the human body

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful tool for the identification of samples[4]. When traditional organic matrices are used, they crystallize with the analytes and form sweet spots in the MALDI-MS analysis[5]. Sweet spots cause a low accuracy and poor reproducibility[6, 7]. Using nanomaterials as a matrix in MALDI-MS minimizes this problem[8-10] and provides some additional advantages. For example, nanoparticles have a high surface area with good adsorption and uniformity, leading to great sensitivity and reproducibility[11]. Furthermore, nanoparticle reduce the interference in the low molecular region of mass spectra thus it is more suitable used to detect the small molecular.

In this study, we attempted to develop a simple method for detecting steroid hormones in saliva by using nanomaterials as the MALDI-MS matrix. We provided a high-throughput assay, which can rapidly and conveniently determine the concentration of steroid hormones present in saliva.

Experimental

Chemicals and sample handling

Acetic acid, citric acid, cortisone, graphite, hydrocortisone, progesterone, sodium citrate dihydrate, sodium nitrate, sodium tetrachloroaurate (III) dihydrate, testosterone and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), ammonium hydroxide solution (28%), methanol, potassium permanganate and sulfuric acid were acquired from J. T. Baker (Phillipsburg, NJ, USA). Hydrogen peroxide solution (35%) was obtained from Acros Organics (Geel, Belgium). The structures of the four steroid hormones (testosterone, progesterone, cortisone, and hydrocortisone) used in this experiment are shown in Figure S1.

Preparation of the Matrices and the standard solutions

α-CHCA 10 mg/mL was dissolved in MeOH: ACN (v / v, 1:1). Gold nanoparticles (AuNPs) were prepared by citrate reduction of $NaAuCl_4$. The method was described in detail in the previous report[11]. The graphene oxide (GO) was synthesized from graphite powder according to a modified Hummer's method[12]. The stock GO solution at a concentration of 20,000 mg/mL was diluted with deionized water to prepare GO solutions at 2000, 200, 20, 2, 0.2, 0.02 or 0.002 mg/mL. The AuNPs solutions were also diluted with deionized water to different concentrations (7.5~105 nM). The standard solution of four steroid hormones (10^{-4} M) was prepared from mixing their corresponding stock solutions (10 mM) with methanol.

Sampling and pre-treatment of hormones in saliva

A saliva sample was taken from a healthy 22-year-old male. The saliva was collected directly into a 15 mL centrifuge tube, and then stored at -20°C until it was used. Before the analysis, the sample must be thawed to room temperature and then centrifuged at 3000 rpm for 10 min.

The 20 μL of standard steroid hormones solution (1×10^{-2} M) was added into the saliva (1 mL), and then one of the tested organic solvents was added into the solution for extraction of the steroid hormones. The mixture sample was vortexed for 3 min and centrifuged at 3500 rpm for 10 min. The organic phase was then transferred to another vial and dried under nitrogen. The residue was re-dissolved in methanol as the analysis solution. The mixture of the analysis solution (0.75 μL) and gold nanoparticles (0.75 μL) was pipetted onto the layer, allowed to dry in the air and was then measured by MALDI-MS.

MALDI-MS measurements

MS experiments were used in the positive-ion mode on a reflection-type time-of-flight (TOF) mass spectrometer (Microflex, Bruker, Germany) equipped with a 1.96 m flight tube. The samples were irradiated by a nitrogen laser at a wavelength of 337 nm with a frequency of 10 Hz. The available accelerating voltage was 20 kV. To obtain good resolution and signal-to-noise (S/N) ratios, the laser energy was adjusted to a suitable intensity, and each spectrum was generated by an average of 150 laser shots.

Results and discussion

Optimization of MALDI-MS Analysis

In MALDI analysis, the matrix plays a significant role in the signal sensitivity because different matrices have different abilities to transfer the energy of the laser to the analytes. We tried to find the most suitable matrix for the steroid hormones. Different matrices (the AuNPs, the α-CHCA and the GO) were used in the MALDI-MS measurement. By using both AuNPs and GO as matrices, we were able to detect the major ion peaks for the sodium adducts of testosterone, progesterone, cortisone, and hydrocortisone at *m/z* 311.4, 337.4, 383.4, and 385.4, respectively. These signals are assigned to $[M + Na]^+$. However, when α-CHCA was used as the matrix we obtained both $[M + H]^+$ and $[M + Na]^+$ peaks. The major ion peaks for testosterone, progesterone, cortisone, and hydrocortisone were at m/z 289.4, 315.4, 361.4, and 363.4, respectively. These signals are assigned to $[M + H]^+$. The other peaks including *m/z* 329, 353, 354, 360, 382, 383 were from the matrix or the sample plate. Compared to GO and AuNPs, the mass spectrum from α-CHCA had higher MS background. The analyze signals separated into both $[M + H]^+$ and $[M + Na]^+$ caused less sensitivity and quantified difficulty (data not shown). In addition, the sweet spots caused greater variation of quantitation. Thus we believe that α-CHCA is not an appropriate matrix in our system.

We also investigated the ionization efficiency for various concentrations (7.5, 15, 45, 75 and 105 nM) of AuNPs. The MS signal intensities of the four steroid hormones achieve the strongest at 45 nM AuNPs matrix(Figure S2). When the AuNPs concentration is higher than 45 nM, the overcastted laser energy would lead to higher MS background. In addition, the fewer analytes per AuNPs cause the smaller MS signals. On the other hand, when the AuNPs concentration is less than 45 nM, the energy of the transfer laser was too weak to desorb the analytes.

We also varied the concentration of GO from 2000 to 0.002 mg/mL to find the preferable signal intensity of the analyte. The strongest signal intensity was found with 0.2 mg/mL of GO. GO at concentrations greater than 0.2 mg/mL provide lower MS signals than that at 0.2 mg/mL, mainly due to less amounts of analyes per GO. When the concentration of GO is less than 0.2 mg/mL, inefficient energy was absorbed from laser light, leading to poor desorption/ionization efficiencies and thus lower MS signals. The comparison of the mass spectra of steroid hormones obtained using 0.2 mg/mL GO and 45 nM AuNPs as matrixes is shown in Figure 1.

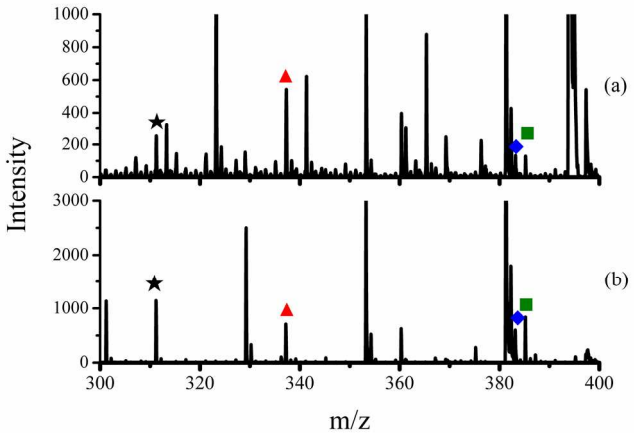

Fig. 1 The mass spectra of the four steroid hormones using various matrix. (a) 0.2 mg/mL GO (b) 45 nM AuNPs. The laser fluence at (a) is 27.57 μJ and (b) is 20.62 μJ. Symbol: (★) $[testosterone+Na]^+$, m/z 311.42; (▲) $[progesterone+Na]^+$, m/z 337.26; (◆) $[cortisone+Na]^+$, m/z 383.44; (■) $[hydrocortisone+Na]^+$, m/z 385.46.

The MS signal intensities of the analytes from using GO as the matrix were lower than those using the AuNPs, mainly because it is relatively poor matrix to absorb energy from laser light and then to transfer its energy to the analyte. With GO, the optimal signal intensities were obtained at 27.57 μJ laser, and the optimal signal intensities from the AuNPs were obtained at 20.62 μJ. The lower laser energy shows that the AuNPs is a better energy transfer matrix than GO in our system. We also obtained a lower noise mass spectrum from the AuNPs system. The AuNPs at the concentration of 45 nM was chosen as the best matrix for this study.

The pH values of the samples influence the MS signal intensity of the steroid hormones. The previous study found that ammonium ions could be used as the proton donors for the ionization of the analytes[13]. We measured the MS intensities of the analytes with the AuNPs matrix in different concentration buffers (0-0.40 mM) at various pH values (4.0-9.5). The strongest analyte signal intensities were detected with the 0.096 mM ammonium citrate buffer at pH of 5.0. (Figure S3, S4)

Validation of Method

To confirm the feasibility of our method, we listed the linear range of the four steroid hormones, as well as R^2, LOD and LOQ. Table 1 shows that the linear ranges of the testosterone, progesterone, and hydrocortisone were all between 20.0-100 μM, while that was 10.0-100.0 μM for cortisone. The correlation coefficients (R^2) for testosterone, progesterone, hydrocortisone and cortisone were 0.992, 0.995, 0.993 and 0.985, respectively. The LOD at S/N = 3 for testosterone, progesterone, hydrocortisone and cortisone was 6, 6, 6, and 3 μM, respectively. The LOQ for testosterone, progesterone, hydrocortisone and cortisone was 20.0, 20.0, 20.0, and 10.0 μM, respectively.

Table 1 Linear ranges, LODs, LOQs, linear equation and R^2 of steroid hormones measured by MALDI-MS.

Analyte	Liner range (mM)	R^2	Linear Equation	LOQ (mM)	LOD (μM)
Progesterone	0.02-0.10	0.9952	Y=2736.74X-217.66	0.02	6
Testosterone	0.02-0.10	0.9921	Y=1620.35X-33.19	0.02	6
Cortisone	0.01-0.10	0.9854	Y=936.47X 164.31	0.01	3
Hydrocortisone	0.02-0.01	0.9934	Y=4426.30X-26.58	0.02	6

Analysis of steroid hormones in the saliva matrix

In addition to hormones, there are many other chemical compounds in saliva. These matrixes may affect the detection of hormones. First, we tried to analyze the steroid hormones without any pre-treatment under optimum conditions. The saliva samples (1 mL) were spiked with the four hormones at 2.0×10^{-4} M and then the spiked samples were measured by MALDI-MS directly. Almost no mass spectrum signals showed up in the direct analysis. Other compounds in the saliva such as salt or other proteins could interferes the desorption/ionization of the hormones. Thus it is important to develop a pretreatment method prior to MALDI-MS measurement of the steroid hormones in saliva. We compared three extraction conditions. Saliva was extracted with diethyl ether (method A), ethyl acetate (method B), and ethyl acetate containing organic acid (method C). When using diethyl ether, the recovery values of the four steroid hormones ranged between 9.34 and 18.68%, while with ethyl acetate, the recovery values ranged between 29.71 and 48.96%. The extraction recovery values were higher using ethyl acetate than using diethyl ether. To increase the recovery percentage we adjusted the pH by adding different concentrations of acetic acid (0-2.0%) into the ethyl acetate. Upon increasing the concentration of acetic acid form 0% to 1%, the recovery values of the steroid hormones increased. As the decreasing of pH value, the dissociation degrees of these steroid hormones decrease (they become more neutral). The neutral molecular could be extracted into organic solvent more efficient. There was no significant improvement in the recovery of the analytes between 1% and 2% acetic acid in ethyl acetate. Therefore we selected ethyl acetate containing 1% acetic acid as the extraction solvent. The recovery values of the four steroid hormones in ethyl acetate containing 1% acetic acid ranged from 89.3 to 111.2 %. Figure 2 displays the recovery values of the four analytes through direct analysis and three other pretreatment methods, showing that ethyl acetate containing 1% acetic acid is the best solvent for the recovery of the four analytes.

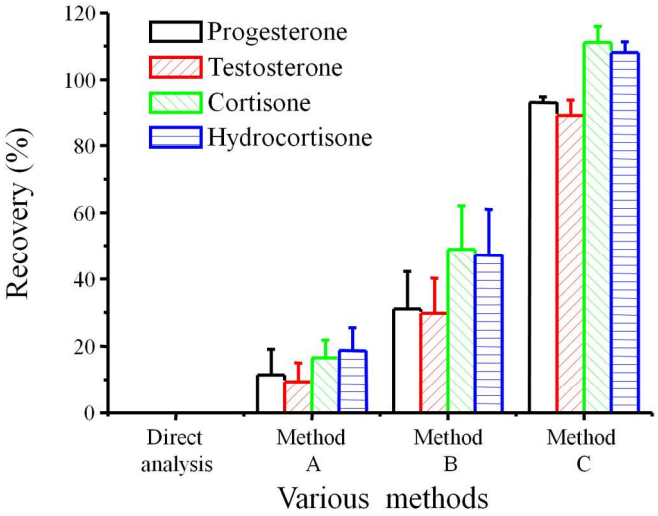

Fig. 2. The recovery of the 4 steroid hormones in saliva was obtained through direct analysis, extracted with diethyl ether (method A), extracted with ethyl acetate (method B), and extracted with ethyl acetate contain 1% acetic acid (method C). (error bar, n=3)

The mass spectrum of the four steroid hormones in the human saliva extracted with ethyl acetate containing 1% acetic acid, was showed in the figure 3. After the suitable pre-treatment and optimum MS conditions, the low noise background and the clear sample peaks were obtained. Also our detection limits were not low enough to detect the real concentration of steroid hormones in saliva[14], our proposed MALDI-MS technique combined with an appropriate pretreatment method proved to be an effective and rapid method for analyzing steroid hormones in saliva samples. We believe this method can be used to measure the real concentration of steroid hormones in saliva after a suitable concentrated process.

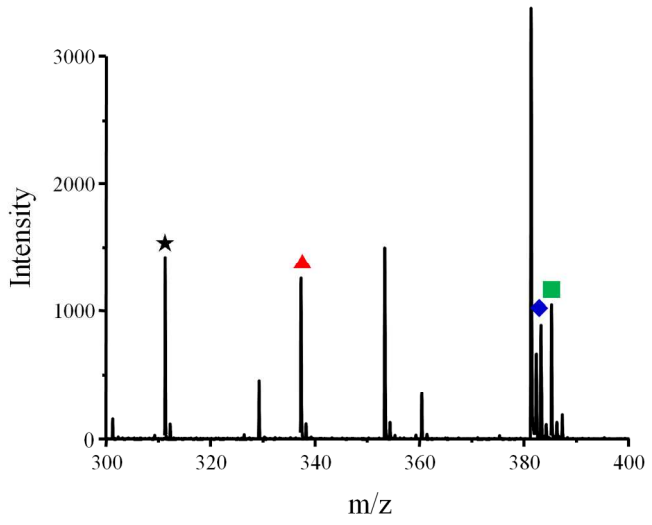

Fig. 3. The mass spectrum of the four steroid hormones in saliva extracted by ethyl acetate containing 1 % acetic acid. Symbol: (★) $[testosterone+Na]^+$, m/z 311.42; (▲) $[progesterone+Na]^+$, m/z 337.26; (◆) $[cortisone+Na]^+$, m/z 383.44; (■) $[hydrocortisone+Na]^+$, m/z 385.46.

Conclusions

In this study, we employed AuNPs as the MALDI-MS matrix to analyze steroid hormones in saliva samples. This approach provided good linearity and enough sensitivity for the analysis of four steroid hormones in saliva. The total analysis time including the sample pretreatment and MS analysis was estimated less than 30 minutes in a 96 wells MALDI sample plate. To the best of our knowledge, this is the first work to measure hormones in saliva by MALDI-MS using nanomaterial as matrix. This technique is a high-throughput assay, which can rapidly screen for steroid hormones in saliva. It has a strong potential to be applied in medical examination.

Acknowledgements

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Notes and references

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