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Full Length Research Paper

Salivary amino acids quantification using RP-HPLC during normal menstrual cycle

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The present study was carried out to detect amino acids profile in women saliva in order to establish the qualitative and quantitative differences that might have potential value in detection of ovulation by noninvasive methods. For the collection of sample, the stages of menstrual cycle were decided by the physical and morphological examination of salivary fern pattern. The saliva from various reproductive phases (prepubertal, preovulatory, ovulatory, postovulatory phases and menopause) was collected and analyzed by reverse phase high performance liquid chromatography (HPLC) after precolumn derivitization of amino acids using O-Pthaldehyde (OPA) by means of RP-HPLC amino acid analyzer. Among the various amino acids identified the compounds such as tryptophan, arginine and phenylalanine were comparatively found to be higher during ovulatory phase when compared to that of other phases. The increase in amino acid concentration during ovulatory phase may be due to the circulation of steroid hormones. Thus, the presence of specific amino acids in ovulatory saliva makes the possibility to develop a biomarker for detection of ovulation by noninvasive methods.

Key words: Ovulation steroid hormones, O-Pthaladehyde, chromatography, amino acids.

INTRODUCTION

A number of techniques such as basal body temperature, ultrasound examination and plasma LH (Lutenising hormone). But these methods are not sufficient to detect the time of ovulation accurately. It has been recently reported that women facial attractiveness increases during follicu-lar phase than the luteal phase (Roberts et al., 2004). The exact time of ovulation is important because it would help to identify the fertile period and thereby give treat-ment in fertile therapy. The prediction of ovulation period still remains a challenge for the investigators.

Human saliva an easily available biological fluid, which shows cyclic variation in its composition during the menstrual cycle (Tandra and Bhattacharaya, 1989). Historically, salivary analyses of female sex hormones were used for fertility monitoring (Read, 1993; Hofman, 2001). How-ever, recent findings indicate that these assays may be useful beyond the study of reproductive concerns.

Estradiol, which was at high levels in women during their reproductive years, may cause increased immuno-reactivity responsible for these phenomena (Markovic, 2001). Normally, the day of ovulation is determined by observing the change in physical characteristics of hu-man cervical mucus (Moghissi et al., 1972; Billings, 1981). In 1969, Cassals reported that the saliva would fern or crystallize during hormonal changes, almost iden-tically to the changes observable in cervical mucus. These changes in cervical mucus have helped to predict when a woman is about to ovulate. Further the salivary ferning showed that the saliva could also help to predict ovulation in some extent (Berardono et al., 1993). Cervical

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mucus is not an easily available body fluid and at the same time needs patient motivation, where as saliva has practical advantages over cervical mucus for the detection of ovulation.

Cyclic changes in various physical properties and biochemical constituents of saliva are known to reflect accurately the hormonal changes associated with a menstrual cycle and may be utilized clinically to determine the time of ovulation. For example, the biochemical substances like salivary amino acids as well as hormones like estrogen and progesterone have been found to fluctuate during the ovulatory period of the menstrual cycle (Landau and Lugibihil, 1967; Lyons et al., 1989). In blood plasma, the large amount of neutral amino acids exhibited maximum reduction and remained low during postovulatory periods (Wall and Truswell, 1991) . To date, analysis of amino acid in blood is considered as a valuable diagnostic tool in cases of suspected inborn errors of amino acid metabolism. The presence of a characteristic pattern of elevated amino acids is very useful in diagnosis of these rare disorders. To overcome this, the preliminary reports prove the changes in the salivary amino acids during reproductive phases of menstrual cycle provide an evidence for detection of ovulation. These applications have led to an increase in the number of salivary amino acid determination and the need for a cost-effective, rapid, reliable and automated method for the prophecy of ovulation through noninvasive methods.

MATERIALS AND METHODS

Collection of samples

Saliva was collected according to the spitting method (Navazesh, 1993; Bosch et al., 1996). The stages of reproductive phases were confirmed through salivary fern pattern (Alagendran et al., 2007) and the sample was processed in preweighed ice-chilled tubes and the collection period was about 10 min. The saliva was collected from 20 different women volunteers during various periods viz, preovulatory, ovulatory, postovulatory phases (aged 22 - 35 years old) and also from prepubertal (7 - 9 years) and menopause stages (aged 45 - 55 years).

The volunteers were instructed to abstain from smoking and drinking 10hrs prior to testing. And also the volunteers were asked for tooth brushing to prevent minimal gingival bleeding.

Total amino acids (acid hydrolysis)

1 ml aliquots of saliva were deproteinized with 50 mg sulfosalicyclic acid (Sigma st, Louis, USA) and centrifuged for 10 mins at 4000 xg at 4°C. The pellet was washed with distilled water. A known weight of the pellet was transferred to a hydrolysis tube. 5 ml of 6 N HCl was added to the pellet and incubated the tube at 110°C for 18 h. Transferred the contents to a china dish after hydrolysis. Acid vapors were completely removed by keeping the china dish over a boiling water bath by repeated evaporation using distilled water. The residue was made up to a known volume and kept in the refrigerator.

Extraction of samples (total free amino acids estimation)

The sample was extracted with 80% ethanol (if necessary the mixture was heated at 70 - 80°C for 30 min in a water bath) and centrifuged at 10,000 xg for 10 min. The clear supernatant was concentrated and used for the assessment of amino acids. Standard amino acids were also run parallel with the unknown samples. Amino acid standard samples were prepared by mixing 95 L of the 250 pmol/ L amino acid standard mixture with 5 L of 10 mM norvaline and analyzed directly by RP-HPLC, within 24 h from preparation. Solutions for linearity study were prepared in duplicate by diluting the 1 nmol/ L amino acid standard solution, and contained 20, 50, 120, 220 or 500 pmol/ L of amino acid standard mixture together with 0.5 mM norvaline (The internal standard l-norvaline was obtained from Sigma-Aldrich). From the standard profile, the amino acid concentration in saliva was quantified (Gnanou et al., 2004) through RP-HPLC. Due to technical constraints, rest of the amino acids in the samples was not quantified. For derivatization 10 µl of supernatant was used.

Instrumentation

The gradient HPLC system was used an LC- 10AT VP (Shimadzu Corporation, Kyoto, Japan) attached with auto injector. Separations were performed at 40°C on a 5 µm Luna C19 column (250 x 4.6 mm (i.d) from Phenomenx (St. Torrance, CA, USA)), protected by a reverse phase guard column (4.0 mm x 3.0 mm (i.d)) from the same supplier. The C-19 column was placed in the column oven (Mayura Analytical Pvt. Ltd, Bangalore, India) and maintained at 40°C. Peak monitoring was performed with a model RF-10Axl fluorescence detector (Shimadzu Corporation, Kyoto, Japan), excitation, wavelength 350 nm and emission cutoff filter at 450 nm. LC workstation CLASS-VP software from the same supplier was used for data processing.

Derivatization procedure

The fully automated precolumn derivitization was performed using SIL- 10AD VP autoinjector ((Shimadzu Corporation, Kyoto, Japan). The auto injector rack contained 2 reagents working OPA reagent and neutralization buffer. The samples 10 µl were loaded on to sample vials and dervitization was started by transferring 90 µl of working OPA reagent. The sample and OPA reagent were assorted by two cycles of aspiration and dispensing. After incubation for 3 min at room temperature. 100 µl of neutralizing buffer was added to the sample OPA reagent mixture, then added 20 µl if the final mixture was injected on to the column. During the chromatography of one sample, the next sample was being derivatized.

HPLC conditions

Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH, while mobile phase B was acetonitrile/methanol/water (45/45/10 v/v/v). The separation was obtained at a flow rate of 2 mL/min with a gradient program that allowed for 1.9 min at 0% B followed by a 16.3-min step that raised eluent B to 53%. Then washed at 100% B and equilibration at 0% B was performed in a total analysis time of 40 min. Fluorometric detection was done using an excitation wavelength of 350 nm and an emission cut off filter of 450 nm. Amino acid concentrations were calculated using the determination and peak areas relative to the area of the internal standard.

Statistical methods

Data are expressed as Mean ± SEM. Those means in the same vertical column that are not marked with the same superscript letters are significantly different at $p<0.05$. The relationship between changes in amino acid concentration was explored by means of