



AUTOMATED DERIVATIZATION WITH O-PHTHALDEHYDE FOR THE ASSESSMENT OF AMINO ACIDS IN WOMEN SALIVA USING REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Aim: To find out the ovulation (fertile) specific marker precursor in menstrual cycle phases of women by amino acid profiling during HPLC. **Objectives:** The current study was carrying out to analysis the feasible changes in salivary amino acids during fertile period. **Methods:** To detect the ovulatory day we used both cervical/saliva mucus ferning test on a microscope slide to form fern like crystals when it dries which shows the rise and fall of estrogenic and crystals shape which is due to the formation of electrolytes such as sodium and potassium during the spike day (14th day) of LH surge. The present study is expected next to perceptive the existence and amount of amino acids in the saliva of human female at the age of varies reproductive state. High Performance Liquid Chromatography (HPLC) analyses of amino acids during the fertile and infertile period in human saliva followed by OPA photodiode array derivatization followed by FD method. **Results:** Glutamic acid, Arginine, aspartic acid and GABA are the amino acids uniquely present during the fertile period which revealed the brain noradrenaline precursor, which may cause instability of diverged effect noradrenaline-mediated central functions in susceptible subjects. Further, the amount of GABA and arginine is significantly than that of other amino acids during ovulatory phase (13th day) which posses electric signal followed by electrolytes like sodium and potassium. **Conclusion:** The method is to assess the clinically important amino acids in saliva may be exceedingly accurate result for fertile period and to avoid the assisted reproductive technology/IVF. Detailed knowledge of the possible variations in human saliva is very important because we can develop a noninvasive problem-solving diagnostic method by assessed the biomarker for amino acids.

KEYWORDS: Amino acids, OPA, estrogen, Saliva, HPLC.

INTRODUCTION

A number of evidence subsists a propos metabolic changes connected with the reproductive phases.^[1,2,3] Increased nutritive rate, body mass index, flow rate, pH and buffer capacity of salivation.^[12,13] and lower plasma amino acid concentrations^[1,5,6] have been reported in women in the menstrual and proliferative phases of the menstrual cycle.^[4] The use of tracer method has formed contradictory outcome concerning protein or amino acid turnover in the different phases of the menstrual cycle. Using glycine with an ammonia end product, Garrel et al^[7] reported that there was no variation in whole body protein turnover in the fed state between the two phases of the menstrual cycle. However, later the same group Lariviere et al^[8] revealed that the leucine turnover during the fasted state in the follicular phase was lower than in

the luteal phase. In addition, it was found that leucine oxidation^[8] and tryptophan catabolism^[9] increased during the luteal phase. Fluctuations in sex hormones provide an appealing explanation for the metabolic differences observed between the two phases of the menstrual cycle. Progesterone and estrogen levels are higher during the luteal compared with the follicular phase. Toth et al^[10] described the positive relationships between estradiol and rate of leucine appearance and between estradiol and leucine oxidation.

Presently, it has been accepted that the IAAO technique is a suitable method to define amino acid requirements in humans.^[11] Human saliva an easily accessible biological fluid, which shows cyclic variation in its composition throughout the menstrual cycle.^[12] Traditionally, salivary

analyses of female sex hormones were used for fertility study.^[13] However, most recent findings indicate that these assays may be useful far-off the study of reproductive apprehension. Sex steroid hormones emerge to take part in a significant role in the physiology of the human oral cavity. Saliva, a heterogeneous fluid comprising proteins, glycoproteins, electrolytes, small organic molecules and compounds transported from the blood, constantly bathes the teeth and oral mucosa. Saliva possesses antimicrobial components and a buffering agent that act to protect and maintain oral tissues. Proteins that are found in saliva, such as lactoferrin, lysozyme, peroxidase, defensins and histatins, can destroy or inhibit the growth of microorganisms in the oral cavity^[12]. There are very few studies on the salivary flow rate, pH, buffer capacity, calcium, protein content and relationship between oxidant-antioxidant defense systems of the saliva and their relations with oral lesions. Changes in amino acid turnover and metabolism that occur during the menstrual phase may affect amino acid requirement; however, this issue has never been investigated. There are some direct methods available in the diagnostic company such as laparoscopy and high-resolution transvaginal ultrasound examination, which are too invasive for this is repetitive or customary in use^[17]. To follow the noninvasive test, Cervical or salivary ferning test is better for the earlier detection of LH spike in menstrual cycle formation of fern like formation during the period of ovulation.^[14,15] Importantly, the identification of estrogen accessibility in these tissues has significant clinical importance and suggests a direct role for estrogen in the physiology of oral mucosa and salivary gland function. Furthermore, no consideration was given to the phases of the menstrual cycle [FIOM, 2002]. The present study is aimed at knowing the existence and mass of amino acids in the saliva of human female during different reproductive phases followed by hormonal mimics such as estrogen and LH spike using HPLC – OPA photodiode array FD method which could be used as the analytical or metabolomic tools of detecting fertility.

MATERIALS AND METHODS

The saliva was collected using Salivette spitting method (Sardeste, Germany) from 50 female volunteers during the age (16-30 years) having normal menstrual cycle.^[18,19,20] The samples were collected during fertile and infertile periods with help of the day of menses. The samples were screened through nylon mesh (16-120 μ m) at the time of collection and were stored at -20°C for further use.

CERVICAL MUCUS FERNING TEST

Taking a drop of sample of cervical mucus, placing it on a microscope slide and allowing it to dry, and see if it forms a distinct 'ferning' pattern. The ferning pattern was believed to be caused by changes in estrogen levels and certain minerals in the cervical mucus at the day of 14th of menstrual periods. However there is also a change in hormones variation and minerals in saliva around the

time of fertile day, which cause the cervical mucus or by saliva to form fern like crystals when it dries. A miniature microscope uses this phenomenon, which is a useful aid for the detection of ovulation.^[16]

AMINO ACIDS PROFILING IN HUMAN SALIVA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY BY OPA DERIVITIZATION METHOD

The separation of compounds is based on the separation coefficients flanked by two immiscible phases, in which the compound distributes by deproteinization of saliva using TCA or Sulphosalicylic acid and OPA derivitization method.^[25]

Amino acid separation

The saliva samples were collected in tubes mixed with 50 mg TCA/sulphosalicylic acid and centrifuged for 6000 rpm for 12 mins at ambient temperature, the supernatant is used for amino acid derivatization. Salivary amino acids concentrations were measured by high performance liquid chromatography (HPLC) with fluorescent detectors (Agilent Technologies, Palo Alto, CA). Deproteinization was performed by adding 100 μ L of 4% sulphosalicylic acid (Pliva – Lachema, Zagreb, Croatia) with homocysteic acid (18.4mg/l) to 100 μ L of saliva. Homocysteic acid (Sigma-Aldrich, Prague, Czech Republic) was as an internal standard. The sample was mixed thoroughly and centrifuged for 5 min at 4000xg.^[26] The chromatographic conditions were maintained and the elution program performed according to the manufacturer's instructions. Amino acids were quantified after calibration with a standard solution and internal standard correction. Samples were analyzed within 1 week of sampling and the results expressed in absolute values 100 μ L).

Instrument and methods

Eluent A: 500 ml 20 mM Sodium acetate + 2 mg EDTA + 0.018% Triethylamine (v/v) adjusted to pH 7.2 with Acetic acid + 0.3% Tetrahydrofuran. Eluent B: 100 ml 20 mM Sodiumacetate adjusted to pH 6.8 with Acetic acid + 200 ml Methanol + 200 ml Acetonitrile.^[27]

Gradient

Flow: 0.45ml/min; Temperature: 40°C; Injection: injection program, including derivatisation steps with OPA. Injected mixture contains 1 μ L AA sample. Column: Each system consisted of a binary pump, a photodiode array detector, a fluorescence detector and an autosampler. A reverse phase Agilent Zorbax Eclipse C18 column AAA (4.6x150 mm, 3.5micron) was used for the chromatographic separation. The linearity and accuracy of the method were assessed using seven different concentrations between 5 and 2500 A mol/l (2.5–1250 A mol/l for Arginine, Glutamic acid, GABA and Aspartic acid) of amino acid standard mixtures, injected in duplicate. Linearity and accuracy were evaluated. To evaluate between-run imprecision, aliquots of the same spiked deproteinized saliva were injected in

different days for a total of 23 analyses over a 2-month period, on two HPLC instruments with both mobile phases. In order to sustain column integrity, a mixture of 90% isopropanol and 10% methanol was run at the end of each set of samples for 30 min and/or every 10–15 injections. This column washing step was essential for both chromatographic separations. 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^[7]. Experiments to establish the recovery for amino acids mixture through different phases including precipitation step was carried out through the procedure and the percent of recovery was assessed. All other solvents were HPLC grade from Fisher Scientific, Pittsburgh, PA. To prevent algae/fungal growth in mobile phase, sodium azide (5 mg/l) was added.^[28]

Gradient Rate

Time (min)	%B
0	0
0	0
1.9	0
18.1	57
18.6	100
22.3	100
23.2	0
26	0

Signal A: $\lambda = 340$ nm bw = 12 nm; reference = 400 nm bw = 20 nm (for OPA amino acids); Signal B: $\lambda = 270$ nm, bw = 18 nm; reference = 330 nm, bw = 10 nm (for FMOc-amino acids); FLD time = 0 min: Ex/Em = 340/450 nm, gain = 10; time = 15 min: Ex/Em = 266/305 nm, gain = 9.

Preparation of Amino acids

The analysis was performed using two identical Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA). Each system consisted of a binary pump, a photodiode array detector, a fluorescence detector and an autosampler. Adding 1 ml of OPA reagent in 200 μ l of amino acids; 0.5 μ l of OPA reagent in 100 μ l of Amino acids in two consecutive ways for detection. 50 μ l of amino acids = 5ml of millipore water (sample use: 40 μ l of amino acids = 200 μ l OPA reagent) (injection; 20 μ l). Concentration settings; 20mins exhausted; Pumping system switch on 1, 2, 3: purging line. (Fixed concentrated Amino acids detection in biological samples). The separated solutes in the solvent stream, emerging from the column (Zorbax Eclipse C18 column 190-800nm) based on their Retention Time are detected by a photodiode array detector and the resulting elution chromatogram is automatically recorded in the form of resultant peaks. Comparing the Retention Time (RT) of the sample with that of the standard Amino acids^[29].

Quantification of Amino acid by Ninhydrin Method

Amino acids were separated by varying temperature, ionic strength and pH according to the instrument manufacturer recommendation. The amino acids eluted were mixed in a high temperature reaction coil with ninhydrin to form colored compounds. The color produced was measured photometrically at 570 nm (amino acids) and 440 nm (imino acids). The run time, injection to injection, for each sample was 130 min^[3].

STATISTICAL ANALYSIS

The results obtained were tabulated and statistically analyzed by independent sample t-test, anova test and Whitney–Mann U-test and software used is SPSS (IBM Corporation, India)

RESULT

The Salivary demographic chart represented as the unstimulated flow rate is more vital than stimulated flow rate because only a small fraction of the day is worn-out eating. The flow rate increases, pH, buffer capacity & concentrations of sodium, potassium rises, while other electrolytes concentration falls during the fertile periods of menstruation.^[21,22,23] In this study, unstimulated saliva was collected during acrophase as salivary flow rate peaks during afternoon time. Salivary electrolytes play a key role in the overall protection of a healthy homeostatic condition in the oral cavity. Since, several factors can influence salivary secretion and composition there is a necessity of precise preanalytical range of salivary electrolytes in mixed dentition during the reproductive phases. Daily samples of saliva were collected from fifty females during a complete menstrual cycle. Findings revealed significant electrolyte changes in the fertile and infertile saliva. Comparison of menstrual and fertile period the concentrations of sodium and potassium confirmed a notable increase in sodium and potassium at midcycle, whereas potassium levels increased significantly ($P < 0.05$). Changes were attributed to a probable hormonal effect on salivary composition (Fig.1).

Fig. 2 shows the separation of amino acid standards with Eluent phase A starting at pH 7.2. Separation of GABA and Arginine can only be achieved with this mobile phase during the fertile phase of menstrual period which possesses a putative chemosignalling, when there is a hormonal change of LH surge at the day of 14th shows 70% of amino acid level of dependency limits shows highly significant at 5%. FMOc was added to OPA in the derivatization mixture to detect and quantitate hydroxyproline and proline using fluorescence detector^[5] (Fig. 1). Hydroxyproline, proline and taurine are separated and quantitated with Eluent phase A (pH 7.2). Cystine was separated with either Eluent phases (A or B), but baseline separation was obtained only with mobile phase A (pH 6.8). This is why we have chosen to quantitate GABA and Arginine using mobile phase A which is highly significant at fertile phase ($P < 0.05$).

Fig. 3 shows the separation of amino acid standards with Eluent phase A and B starting at pH 6.8 in infertile saliva during the menstrual periods. Separation and quantitation of Threonine, Tryptophan, Tyrosine and phenylalanine can only be achieved with this mobile phase. Lysine, Cystine and ornithine can be separated with both pHs (7.2 and 6.8), but since their recovery is affected by FMOC; they are quantitated with Eluent phase B (at pH 6.8) where FMOC is not added.

All other amino acids studied can be separated and quantitated with either Eluent phases A (pH 7.2) or B (pH 6.8). Although most of the amino acids separations are not at baseline, comparison data with (Fig.1 and 2) show that this level of separation is sufficient for accurate quantitation. The method performance for both chromatographic separations is comparable. The use of two mobile phases with different pHs facilitates the identification of rare, but clinically significant amino acids.

Linearity studies were done to evaluate the analytical measurement range of each amino acid. The method was linear within allowable systematic error of 10% up to 2500 A mol/l for all the amino acids studied (1250 μ mol/l for cystine and homocystine; 500 A mol/l for proline and hydroxyproline). The wide analytical measurement range for all the amino acids studied could only be achieved using photodiode array UV detection, with the exception of hydroxyproline and proline. Since these imino acids were detected using fluorescence detector, a lower linearity range was obtained.

The accuracy of the method, evaluated by the highest maximum deviation from the theoretical value of the measured amino acid concentration was 10% and observed for GABA, Arginine, Glutamic acid and Aspartic acid at their maximum level during the fertile phase of menstrual periods were measured as the concentration of 5 μ mol/l. For all other amino acids the accuracy was eluent phase on both fertile and infertile period will be 10% detection limits. The within-run CVs for all amino acids studied were eluent phase of a and b 5% (Fig.1). The between-run coefficient of variation (CV) was found to be a and b 10% for all amino acids studied (**Fig.2 and 3**). A critical step for the reproducibility of the method was the addition of column washes, performed every 10–15 injections, with a mixture of isopropanol/water (90/10) to regenerate the column. The advantages of this method are its ability to separate all amino acids present in saliva in a short time, although two injections per sample are required, and the wide analytic measurement range obtained using a photodiode array detector. The annoyance of this method are the column washes needed to sustain column integrity and the reality that it requires two injections per sample in order to accomplished separation of all amino acids.

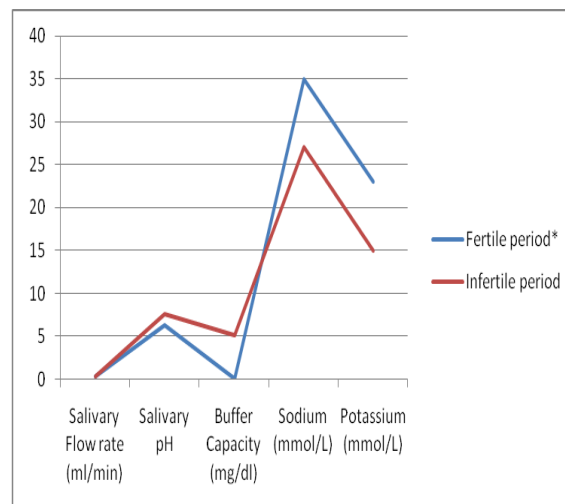


Fig. 1: shows the salivary demographic chart during fertile and infertile period (N=50).

*Salivary physiological variation shows highly significant at fertile phase ($P < 0.05$) followed by One-way Anova using Whitney–Mann U-test.

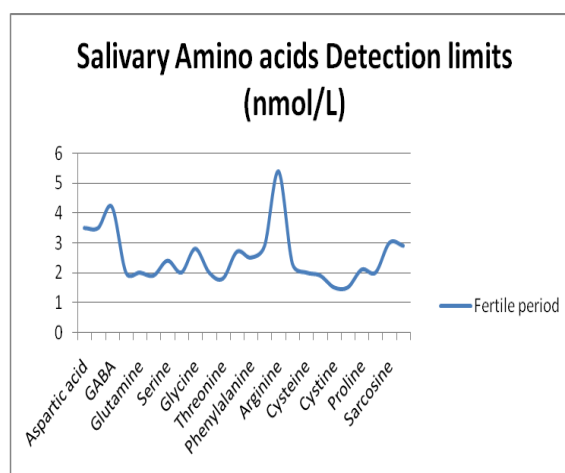


Fig. 2: shows the Salivary amino acid profiling during fertile phase of human menstrual cycle (N=50).

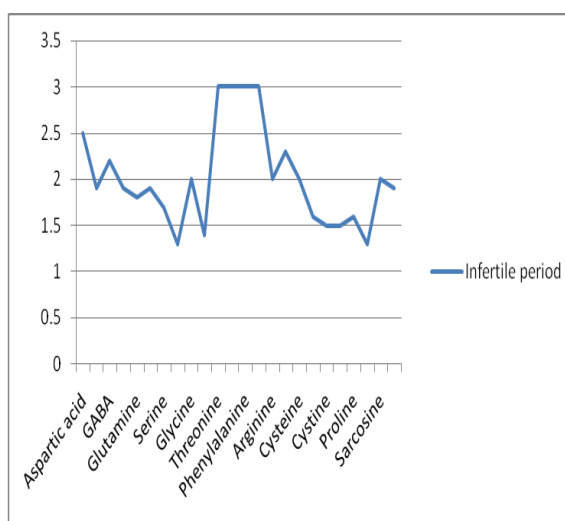


Fig. 3: shows the Salivary amino acid profiling during infertile phase of human menstrual cycle (N=50).

DISCUSSION

Saliva is an attractive body fluid for analyze fast spreading diseases which is simple, safe tearless, non-invasive. Analysis of saliva may provide a cost effective approach for screening of large populations, thus a pre analytical physiologic variations with respect to age and gender is needed to obtain.^[21] Thus the aim of this study was to detect the physiologic levels of different flow rate, pH^[12], buffer capacity^[13], electrolytes and amino acid profiling with the use of HPLC –OPA photodiode array FD methods.^[33,34] The present study was used to demonstrate the salivary factors that may in the potential prove to be useful measures of reproductive phases with the early onset or treatment of IVF/Artificial insemination by using a putative biomarker of detecting the salivary amino acids. In the present study an attempt was made to detect specific amino acids in saliva during fertile and infertile period. So that it can be used as a marker of detecting exact day of fertile period in woman.^[9]

The present investigation revealed that the levels of amino acids in saliva were significantly higher during fertile period than that of infertile cases. Further the increase in amino acids concentration was noted in adolescents rather than elders. These findings suggest that certain amino acids such as Arginine, GABA, glutamic and aspartic acid may be present in higher concentration in saliva especially during the period of fertile day which is fond the day with the analysis of cervical mucus plug and confirmed the day of fertile time followed by cell fragments crystallization due to hormonal and electrolyte fluctuation was arises. Several workers,^[9,10] have reported body fluids amino acid data for normal men and women as subjective by unique diets and modest link was shown between quantitative intake of protein and amino acids excretion^[11,12], from their exertion exposed with the purpose of the amino acid showed higher excretion during pregnancy. Similarly the present results provide novel proof that the amino acid levels are expressly excreted in high concentration during 14th day of menstrual cycle which reveals as fertile period.

The present study shows that the amino acids content in the saliva during the period of menstruation possess a progressive metabolic response that accompanies the alteration from the fluctuations of endocrine signals during the period of ovulation surge^[8]. Endogenous intensity of estradiol, progesterone and other hormones are sporadic during the menstrual cycle (incase PCOs, the amino acids levels will be regress based on the disease factor). In addition to disparity in the core of hormonal prototype between and within women, there are substantial variations in menstrual cycle length^{[12], [13]} have found that the human salivary glands are shown to be the target organs of estrogen action. As noted in the present studies, the amino acids are hypothesize more in high concentration are due to the estrogen action on salivary glands which may have intern increase the

amino acid levels during the fertile day. In the same way significant variation in the salivary components was experimentally observed during the menstrual cycle of the women, where an increase in the activities of salivary components tended to overlap with the increase of estrogen level. Thus the present study provides additional support for flows of salivary components with LH spike physiologically increase as a result of 17 β -estradiol volatility.^[14]

These results are in conformity with previous findings of amino acid analysis clearly indicated that there are three amino acids namely GABA, aspartic acid, glutamic acid and arginine are present in the fertile phase saliva while compare to infertile periods. Among these three amino acids arginine appeared only during ovulatory phase. Such changeability may prove to be clinically imperative.^[26,27,28] While on HPLC analysis GABA, glutamic acid, glutamine and arginine are estrogenic dependent. Among these amino acids, threonine and aromatic amino acids like tryptophan, tyrosine and phenylalanine was reported in the urine of pregnancy women.^[15] Further aspartic acid, glycine, threonine, serine and arginine are more specific and showed the higher concentration in saliva when compared to other amino acids during the period of infertile periods which revealed the brain noradrenaline precursor, which may cause instability of diverged effect noradrenaline-mediated central functions in susceptible subjects.^[29]

Amino acids in human saliva have provided to be a useful biomarker for the detection of fertile period during the menstrual cycle of women.^[16] The remarkable physiological changes put in motion by conception are manifested by several biochemical changes closely connected to amino acid excretion.^[17] The present investigation persuasively influences that the amino acids might be considered as ovulation signifying components. Indeed, precise successful and cost-effective methods are not available to detect the ovulation. The finding of ovulatory correlated components suggests a hopeful impended to expand an easy non-invasive technique to forecast the ovulation very successfully^[18]. Analytical methods for ovulation which allow women to manage the family planning process up to the spot where they necessitate on the way to seek out proficient aid. These agents are suitable for women to execute at domicile and may possibly allow for a better chance of conception.^[32,33,34] Noninvasive profiling test only in human saliva leads to biomarker studies of using amino acids as an indicator for developing a strip which exaggerate the outcome existing in minutes, while other requires obstacle steps and some are excruciating. In fact, the home ovulation detection tests facilitate the people to take an active role in avoiding IVF test and detection of conception rate concomitantly. Advantages of these tests include expediency, decline healthcare expenditure and invitro fertilization.

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