Talanta 119 (2014) 299–305

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Measurement of salivary metabolite biomarkers for early monitoring of oral cancer with ultra performance liquid chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form
23 October 2013
Accepted 1 November 2013
Available online 21 November 2013

Keywords:
Early diagnosis
Saliva
Potential biomarkers
UPLC–ESI–MS
Oral squamous cell carcinoma

ABSTRACT

This study aimed to set-up an ultra performance liquid chromatography–electrospray ionization–mass spectrometry (UPLC–ESI–MS) method for the determination of salivary L-phenylalanine and L-leucine for early diagnosis of oral squamous cell carcinoma (OSCC). In addition, the diagnostic accuracy for both biomarkers was established by using receiver operating characteristic (ROC) analysis. Mean recoveries of L-phenylalanine and L-leucine ranged from 88.9 to 108.6% were obtained. Intra- and inter-day precision for both amino acids was less than 7%, with acceptable accuracy. Linear regression coefficients of both biomarkers were greater than 0.99. The diagnostic accuracy for both biomarkers was established by analyzing 60 samples from apparently healthy individuals and 30 samples from OSCC patients. Both potential biomarkers demonstrated significant differences in concentrations in distinguishing OSCC from control ($P < 0.05$). As a single biomarker, L-leucine might have better predictive power in OSCC with T1–2 (early stage of OSCC including stage I and II), and L-phenylalanine might be used for screening and diagnosis of OSCC with T3–4 (advanced stage of OSCC including stage III and IV). The combination of L-phenylalanine and L-leucine will improve the sensitivity (92.3%) and specificity (91.7%) for early diagnosis of OSCC. The possibility of salivary metabolite biomarkers for OSCC diagnosis is successfully demonstrated in this study. This developed method shows advantages with non-invasive, simple, reliable, and also provides lower detection limits and excellent precision and accuracy. These non-invasive salivary biomarkers may lead to a simple clinical tool for the early diagnosis of OSCC.

1. Introduction

In recent years, there is a growing interest among researchers to use salivary biomarkers in investigation of disease diagnosis, such as lung cancer [1], breast cancer [2], pancreatic cancer [3], oral cancer [4–6], sjögren's syndrome [7], etc. Oral cancer, one of the six most common human cancers, refers to all malignancies arising from the lips, the oral cavity, and pharynx [8,9]. The World Health Organization has reported oral cancer as having one of the highest mortality ratios amongst other malignancies with a death rate of 45% at five years from diagnosis [10]. Approximately 300,000 individuals worldwide are diagnosed with oral cancer annually. More than 90% of oral cancer is squamous cell carcinoma (OSCC). At present, once OSCC detected, it will be at advanced stage, which would generally result in a poor prognosis and a low survival rate. Therefore, early detection of OSCC as well as the screening of high risk populations with precancerous lesions remains to be an urgent need.

Currently, the most definitive method for oral cancer diagnosis and screening is a scalpel biopsy. It is time-consuming and needs extensive experience. In addition, it is also impractical to use imaging techniques for oral cancer screening, since they are expensive and insensitivity for small lesions [11]. Therefore, a number of molecular-based diagnostic markers have been used to detect the presence of OSCC with varying degrees of sensitivity and specificity. Compared with blood samples, using saliva for clinical diagnostics have attracted more and more research scientists and clinical doctors.

Human saliva, a multi-constituent oral fluid, is secreted primarily by three major glands namely parotid gland, submandibular gland and sublingual gland [12,13]. Generally, salivary glands produce about 1–1.5 L of saliva daily. It contains approximately 99% water with minerals, nucleic acids, electrolytes, mucus and proteins [14]. It is one of the most complex, versatile, and important body fluids, supplying a large range of physiological needs. Therefore, saliva is also called the “mirror of the body” or

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http://dx.doi.org/10.1016/j.talanta.2013.11.008

"a window on health status". Non-invasive collection is one of the great advantages of saliva as a diagnostic medium, especially when repeated samples must be taken for particular examinations. Additionally, it has the advantages of easy to store and inexpensive compared to blood sample collection [15].

In order to investigate biomarkers in saliva, recently developed technologies such as proteomics [16], transcriptomics [17] and metabonomics [18] have been explored. Metabolomics is the systematic study of small-molecular-weight substances in cells, tissues or whole organisms as influenced by multiple factors [19,20]. The major analytical techniques that are employed for metabonomics investigations are based on nuclear magnetic resonance spectroscopy [21,22] and mass spectrometry (MS) [23,24]. Sugimoto et al. use CE–TOF–MS to analyze saliva samples collected from both healthy and oral cancer persons, and more salivary metabolite marker candidates ($P<0.05$ Steel–Dwass test) of oral cancer have been found [25]. HPLC–MS analysis was also performed to discriminate individuals with OSCC and oral leukoplakia (OLK) from healthy control and a total of 14 OSCC-related and 11 OLK-related metabolites were discovered [26]. In addition, a panel of three salivary metabolites including lactic acid, phenylalanine and valine were selected in combination yielded sensitivity of 94.6% and specificity of 84.4% in distinguishing OSCC from OLK by Wei et al. [27]. ^{1}H NMR-based metabonomic technique was used by Zhou et al. to differentiate the OSCC patients from the OLK patients and the controls by using PLS-DA analysis [28]. According to the research of Sugimoto, *P*-value of L-phenylalanine and L-leucine are <0.005. L-phenylalanine is an essential amino acid and is a precursor of the neurotransmitters called catecholamines, which are adrenalin-like substances. L-leucine is one of branched chain amino acids, which is critical to human life and is particularly involved in stress, energy and muscle metabolism.

In this study, we aimed to develop and validate an assay for the simultaneous analysis of salivary L-phenylalanine and L-leucine using UPLC–ESI–MS with an intention to build criteria for diagnostics of OSCC. L-phenylalanine and L-leucine in combination yielded satisfactory accuracy for early OSCC prediction. Therefore, accurate detection of both of salivary biomarkers has important clinical value in early diagnosis of OSCC.

2. Materials and methods

2.1. Materials

Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (USA). Distilled water was purified "in-house" using an ULUPURE system (Chengdu Ultrapure Technology Co., Ltd, Chengdu, China). Trifluoroacetic acid (HPLC grade) and ammonium formate were purchased from Adamas (Switzerland). Phenylisothiocyanate (PITC) was purchased from Alfa Aesar (Tianjin, China). Formic acid, triethylamine and *n*-hexane (Ke Long Chemical Reagent Factory, Chengdu, China) were used in this work. The stock standard solutions of L-phenylalanine and L-leucine (Sangon Biotech Co., Ltd, Shanghai, China) at a concentration of 1 mg/mL were prepared by dissolving L-phenylalanine and L-leucine in water with 0.1% formic acid, respectively. These solutions were stored at 4 °C and diluted to the required concentrations with water containing 0.1% formic acid prior to use.

2.2. Saliva specimens

Saliva samples were collected from a group of 30 OSCC patients (25 men and 5 women, 7 of stage I, 6 of stage II, 2 of stage III and 15 of stage IV), whose mean age was 62 years. The OSCC patients were all recruited from the West China Hospital of Stomatology, West China School of Stomatology, Sichuan University. There was no history of receiving medication and none had been treated with chemotherapy and radiotherapy. Diagnosis for all OSCC cases was based on clinical and histopathologic criteria. In addition, we obtained control samples from a group of 60 healthy individuals, cancer-free individuals with 35 males and 25 females. The Ethical Committee of the West China Hospital of Stomatology, Sichuan University, approved the protocol and all of the subjects signed an Ethical Committee consent form agreeing to serve as saliva donors for the experiments.

2.3. Saliva collection and preparation

A well-defined and standardized protocol was used for collection, storage, and processing of all the saliva samples. All the donors were asked to be on an empty stomach, without eating, drinking, smoking, or using oral hygiene products for at least 1 h prior to sample collection, and then rinse their mouth thoroughly with water. All samples were kept on ice during the process. Roughly 3 mL of clear unstimulated whole saliva was obtained. The samples, once collected, were centrifuged at 2500 rpm for 15 min at 4 °C. Thereafter, the supernatant was removed and a further centrifugation at 12,000 rpm for 20 min at 4 °C was performed to remove insoluble materials, cell debris, and food remnants. Finally, the samples were divided into 400 μL and frozen at −40 °C until the laboratory analyses.

The samples were thawed at room temperature first. To precipitate proteins, acetonitrile (800 μL) was added to the 400 μL saliva sample and the mixture was shaken for 60 s vigorously, and then was allowed to stand for 10 min. The obtained samples were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant (1 mL) were used for amino acid derivation.

2.4. Amino acid derivation

Amount of 500 μL triethylamine (1 mol/L) and 500 μL PITC (0.1 mol/L) were added into the supernatant, and reacted for 1 h at room temperature after shaking 60 s vigorously. Then, 1.5 mL *n*-hexane was used as extractant for three times to remove unreacted PITC. After extraction, 250 μL 1.0 mol/L trifluoroacetic acid was added in the solution, and refluxed at 70 °C for 30 min. The final product (PTH-phe and PTH-leu) was centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was filtered through a syringe filter (0.22 μm) for UPLC–TOF/MS analysis.

2.5. UPLC–ESI–MS

The UPLC–ESI–MS system consisted of a Waters ACQUITY™ Ultra Performance Liquid Chromatography system and a Micromass LCT Premier™ orthogonal accelerated time of flight mass spectrometer (Waters, Milford, USA). An ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm, Waters, Milford, USA) was used as the analytical column. The column was maintained at 45 °C. The flow rate of the mobile phase was 0.2 mL/min. Gradient elution was performed with the following solvent system: (A) 0.1% formic acid—water with 1 mM ammonium formate, (B) Acetonitrile; 5% A for 1.0 min, 5%∼10% A in 2.0 min, 10%∼25% A in 3.0 min, 25%∼50% A in 6.0 min, 50%∼90% A in 8.0 min, 90%∼5% A in 10.0 min and then holding at 5% A for 2 min. TOF/MS was operated with an ESI source with positive ion and W-geometry mode. The ionization parameters are listed as follow: capillary voltage, 3.0 kV; cone voltage, 100 V. "Aperture 1" voltage was set to 0 V. The flow rates of cone gas and desolvation gas were 40 and 700 L/h, respectively. The source temperature and desolvation temperature were 110 and 350 °C, respectively.

In the present study, the TOF mass spectrometer fitted with a lock spray enabled Z-spray ion source was used to acquire intact mass date. Lock mass correction was performed using a leucine-enkephalin (Sigma-Aldrich, L9133, lot 095K5109, Steinheim, Germany, $[LE+H]^+$, m/z, 556.2771). The solution of LE was infused through the reference probe at the flow rate of 0.03 mL/min with the help of a second LC pump (Waters). Exact mass data were acquired over a 100–800 m/z range.

2.6. Date and statistical analysis

All saliva samples were divided into three groups: healthy control as group 1 (HC); early stage of OSCC including stage I and II as group 2 (T1–2); advanced stage of OSCC including stage III and IV as group 3 (T3–4). The differences of both saliva biomarkers concentrations in three groups were investigated with the non-parametric Mann–Whitney U test. Then receiver operating characteristic (ROC) curves also were used to evaluate the diagnostic effectiveness of both potential biomarkers and to find an optimal cut-off point based on the maximum corresponding sensitivity and specificity. L-Phenylalanine and L-leucine in combination for early diagnosis OSCC were investigated using logistic regression (LR) mode. The ROC curve was constructed and area under curve (AUC) was computed, either from single biomarker concentrations or for multimarker panels. All statistical analyses were operated using SPSS 16.0, and results were considered significant at a 2-tailed P value of <0.05.

3. Results

3.1. Method performance

The UPLC system provided a rapid, effective, and convenient analytical method for the detection of amine acid in saliva. The UPLC conditions, such as flow rate of mobile phase, temperature of column, gradient, have been optimized. Based on the optimum conditions, PTH-phe and PTH-leu were fully separated chromatographically. The TIC chromatogram of PTH-phe and PTH-leu were shown in Fig. 1A. The reproducibility of the data was also studied and the retention time variability was determined. The retention time was 6.61 min (RSD%=0.18, $n=11$) and 6.77 min (RSD%=0.11, $n=11$) for PTH-phe and PTH-leu, respectively.

For MS operation, ESI positive ion mode was evaluated for determination of PTH-phe and PTH-leu. MS parameters such as capillary voltage, cone voltage, flow rates of cone gas and desolvation gas, source temperature and desolvation temperature, were determined by flow injection analysis of derivatized amino acid standard solution using the inbuilt syringe pump. PTH-phe and PTH-leu gave m/z 283.0898 ($[M+H]^+$) and 249.1061 ($[M+H]^+$) as the base ions, respectively (Fig. 1B and C). The difference of m/z between measured and theoretical value of both analytes is lower than 0.8 mDa because of excellent accuracy and precision of TOF/MS [29].

3.2. Chemical compositions

Chemical compositions were calculated from the acquired MS data using the Masslynx 4.1 analysis software. The spectra routinely collected with 12,000 mass resolution, ~0.6 mDa precision and ~10 ppm tolerances is adequate to generate an initial list of possible chemical compositions. Taking PTH-phe as an example (Fig. 1D), element limits were set to C, H, N, O, and S. The three restrictions used were (a) the maximum number of double bond equivalent was set to 12, (b) the mass tolerance was 2 mDa, and (c) the maximum number of nitrogen atoms in the chemical formula was set to 3. Using these restrictions, the maximum number of chemical formulas for each compound detected was 6. Among these formulas, the selection of the best match was strongly dependent on its i-FIT value, which is an index of the deviations observed from the predicted masses and intensities of monoisotopic peaks corresponding to a given chemical formula. The final searched formula with i-FIT value was 0.5 and 0.1 for PTH-phe and PTH-leu, respectively.

3.3. Linearity and limit of quantitation

In order to evaluate the linearity, L-phenylalanine and L-leucine were spiked to saliva sample before derivation at the concentrations of 0, 50, 100, 200, 400, 600, 800, and 1000 ng/mL. The calibration curve was constructed by subtraction of endogenous L-phenylalanine and L-leucine concentration in non-added saliva from added-saliva samples respectively. The assay was found to be linear up to 1000 ng/mL for both biomarkers. The linear equations of calibration curves of L-phenylalanine and L-leucine in human saliva was $y=0.01742x+0.32336$ with $r^2=0.995$, and $y=0.03735x-0.581$ with $r^2=0.994$, respectively (y, peak area; x, concentration (ng/mL) of L-phenylalanine or L-leucine; r, correlation coefficient). Under our UPLC-MS conditions, the detection limits ($S/N=3$) of L-phenylalanine and L-leucine were 3.9 and 6.8 ng/mL, respectively. This is generally better than other methods, such as GC-MS [30], optical biosensor [31], HPLC-MS/MS [32]. The limit of quantification (LOQ) was defined as the concentration when the S/N ratio is 10. Therefore, LOQ was 13.0 ng/mL for L-phenylalanine and 22.7 ng/mL for L-leucine.

3.4. Recovery

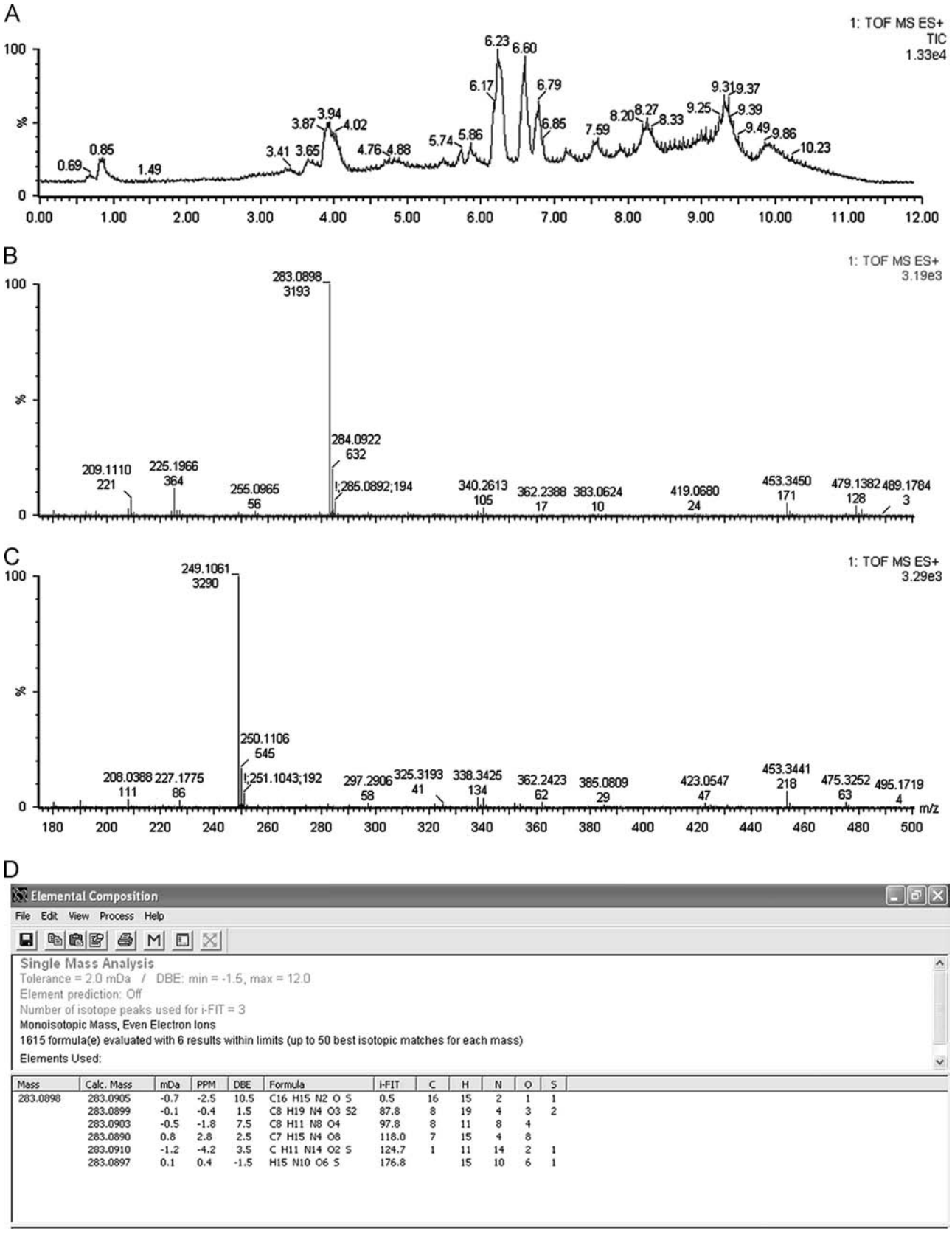
In order to confirm the validity of this method, the recovery of L-phenylalanine and L-leucine were investigated. Recovery of both biomarkers was found to be within acceptable limits. Three saliva samples were spiked known amounts of L-phenylalanine and L-leucine with concentrations of 50, 200 and 800 ng/mL. The recovery was performed by comparing the concentrations calculated based on calibration curve with standard concentrations. For L-phenylalanine, the mean recoveries for each increasing spike concentration were 100.8, 100.3 and 96.9%, with a range of 92.6–109.3%. Mean recoveries for L-leucine were 108.6, 103.0 and 88.9% with increasing spike concentrations, with a range of 87.6–109.4%.

3.5. Precision and accuracy

Precision including intra- and inter-day precision were determined by calculating relative standard deviations (RSD) using in-house spiked samples at three different concentrations (50, 400 and 1000 ng/mL). For intra-day precisions, samples were run 6 times in a single batch. For inter-day precisions, samples were run once a day for six days. The intra- and inter-day precisions were summarized in Table 1. Intra-day precisions range from 3.0 to 4.3% ($n=6$) for L-phenylalanine and 2.9 to 5.6% ($n=6$) for L-leucine. Inter-day precisions of both amino acids were lower than 6.0% ($n=6$), which revealed that the method presented good precisions for the analysis of L-phenylalanine and L-leucine in saliva.

3.6. Sample stability

The stability of samples was assessed in two ways: one is the derivatized saliva placed onto the auto-sampler board for a prolonged period; and the other is the untreated saliva stored in the freezer. As shown in Table 2, for the derivatized saliva on board stability, samples were determined after 3 days, 5 days, 1 week,



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O	S
283.0898	283.0905	-0.7	-2.5	10.5	C16 H15 N2 O S	0.5	16	15	2	1	1
	283.0899	-0.1	-0.4	1.5	C8 H19 N4 O3 S2	87.8	8	19	4	3	2
	283.0903	-0.5	-1.8	7.5	C8 H11 N8 O4	97.8	8	11	8	4	
	283.0890	0.8	2.8	2.5	C7 H15 N4 O8	118.0	7	15	4	8	
	283.0910	-1.2	-4.2	3.5	C H11 N14 O2 S	124.7	1	11	14	2	1
	283.0897	0.1	0.4	-1.5	H15 N10 O6 S	176.8		15	10	6	1

Fig. 1. TIC chromatogram of PTH-phe and PTH-leu (A), mass spectra obtained from PTH-phe (B), PTH-leu (C), and chemical composition of PTH-phe (D).

and 2 weeks. The changes in response were compared with the initial signal were within $\pm 10\%$ for all samples, and therefore, those variations were considered acceptable [33].

For the stability of the samples held in the freezer, saliva samples from three different subjects were divided into aliquots and stored at $-35\ ^{\circ}C$. Samples were thawed after 1 week, 2 weeks,

3 weeks, 1 month, and 3 months' storage. The obtained results were compared with the initial values determined when the samples were fresh. The changes of both L-phenylalanine and L-leucine were within ± 10%. These results suggest that L-phenylalanine and L-leucine are stable in human saliva for at least 3 months when stored at −35 °C.

Table 1
The intra- and inter-day precisions of L-phenylalanine and L-leucine.

Metabolite	Spiked (ng/mL)	Intra-day R.S.D. (%)[a]	Inter-day R.S.D. (%)[a]
Phe	50	4.32	5.28
	400	3.01	6.42
	1000	3.40	3.51
Leu	50	4.59	5.86
	400	2.94	5.25
	1000	3.36	4.80

[a] $n=6$.

Table 2
Sample stability characteristics for saliva.

Metabolite	Derivatized saliva storage stability (R.S.D.%)			Untreated saliva storage stability (R.S.D.%)		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Phe	9.2	8.6	9.6	7.9	6.5	5.0
Leu	8.7	7.8	8.4	8.6	6.9	9.1

3.7. Human saliva analysis

The saliva samples collected from 60 healthy individuals and 30 OSCC patients have been systematically investigated. As shown in Fig. 2, box plots of two metabolite biomarkers in distinguishing OSCC T1–2, and T3–4 from control were provided. From the result we can find that L-phenylalanine and L-leucine content in the early stage of OSCC decreased as compared to control. The average concentration of L-phenylalanine and L-leucine in saliva is 4100 and 2300 ng/mL of healthy people, 2500 and 600 ng/mL in OSCC patient with T1–2, and 1900 and 500 ng/mL in OSCC patient with T3–4. Average concentrations for OSCC patients with T1–2 compared with healthy individuals were 1.6 times lower for L-phenylalanine ($P=0.028$) and 3.8 times lower for L-leucine ($P=0.001$).

According to the nonparametric Mann–Whitney *U* test, the *P*-value in HC *vs* T1–2 was 0.028 for L-phenylalanine and 0.001 for L-leucine. In HC *vs* T3–4 mode, the *P*-values of both biomarkers were <0.05. Both potential biomarkers showed a notable difference between OSCC patients and healthy individuals. In addition, there were no significant differences in the distribution of smoking (Phe: $P=0.830$; Leu: $P=0.788$), alcohol consumption (Phe: $P=0.747$; Leu: $P=0.125$), and sex (Phe: $P=0.628$; Leu: $P=0.595$) between OSCC patients and the cancer-free individuals.

The ROC curve is a graphical display of the false-positive rate and the true-positive rate from multiple classification rules. As shown in Fig. 3, the ROC curves were created to demonstrate the early predictive power of L-phenylalanine and L-leucine for OSCC. The detailed sensitivity and specificity of two salivary biomarkers

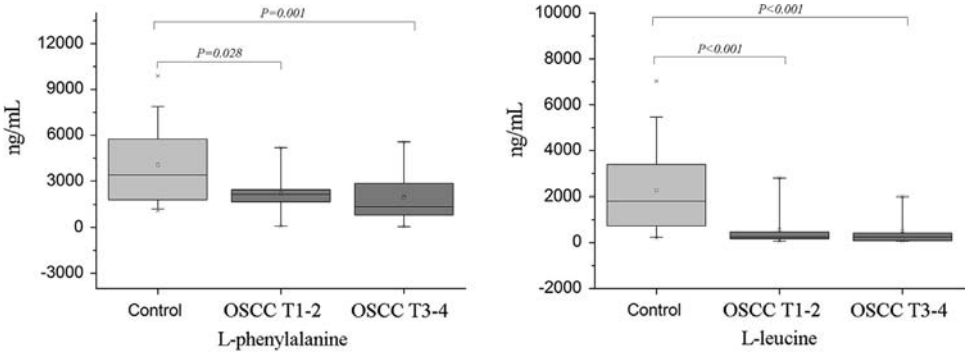


Fig. 2. The box plots of both biomarkers in distinguishing OSCC with T1–2 and T3–4 from healthy control. Horizontal lines represent from bottom to top: the minimum, 25th, 50th, 75th percentiles and the maximum. The *P* values of the comparisons of OSCC with control were marked.

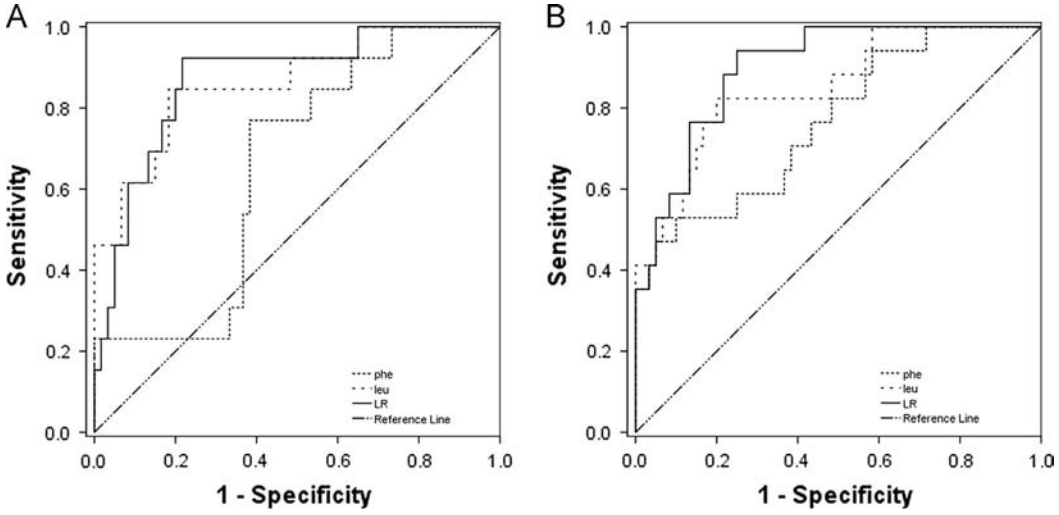


Fig. 3. ROC analysis for two biomarkers and LR mode in diagnosis OSCC. (A) HC *vs* T1–2 mode. (B) HC *vs* T3–4 mode.

Table 3
ROC curve analysis of salivary biomarkers.

Metabolite	AUCs	95% Confidence interval	Sensitivity (%)	Specificity (%)	Std. error[a]
	HC *vs* T1-2				
Phe	0.695	0.560–0.830	84.6	61.7	0.069
Leu	0.863	0.747–0.979	84.6	81.7	0.059
LR model	0.871	0.767–0.974	92.3	81.7	0.053
	HC *vs* T3–4				
Phe	0.767	0.637–0.896	47.1	95.0	0.066
Leu	0.852	0.748–0.956	82.4	80.0	0.053
LR model	0.899	0.827–0.971	94.1	75.0	0.037

[a] Under the nonparametric assumption.

for OSCC T1–2 and T3–4 prediction are listed in Table 3. L-Leucine as a single biomarker in saliva gives a sensitivity of 84.6% and a specificity of 81.7% (AUC=0.863) to predict OSCC with T1–2. In addition, L-phenylalanine give a sensitivity of 84.1% and a specificity of 95.0% (AUC=0.767) to predict OSCC with T3–4. AUC value is known to be a useful measure of overall predictor quality, with a value of 100 for a perfect predictor and 50 for a random predictor. Compared with AUC values in HC *vs* T1–2 and HC *vs* T3–4, we can find that as a single salivary biomarker, L-leucine might have better predictive power in OSCC with T1–2, and L-phenylalanine might be used for screening and diagnosis of OSCC with T3–4.

In order to demonstrate the utility of L-phenylalanine and L-leucine in combination for diagnosis OSCC, a conventional logistic regression model was built using the binary outcome of the healthy individuals and OSCC patients as dependent variables and validated by using leave-one-out cross validation. The ROC curve was also plotted for LR model of HC *vs* T1–2 and HC *vs* T3–4. The AUC was 0.871 (sensitivity 92.3%; specificity 91.7%; 95% confidence interval, 0.767–0.974; $P<0.0001$) for HC *vs* T1–2. These results illustrated that the combination of L-phenylalanine and L-leucine will improve the diagnostic accuracy for OSCC.

4. Discussion

OSCC cells are immersed in the salivary milieu, therefore, analysis of the salivary metabolites to find potential biomarkers for OSCC is of great value. Saliva testing, a non-invasive alternative to serum testing, is rapidly advancing. Additionally, it has a simple collection method, is easy to store, and is inexpensive. Our method exhibited comparative advantages of less analysis time, higher sensitivity, and lower LOD. UPLC play a key role in performance enhancement. The reasons include the sub-2 μm particles, the mobile phases at high linear velocities, and the instrument operated at higher pressure than that used in HPLC, dramatic increase in resolution, sensitivity, and the fast speed of analysis.

L-Phenylalanine is an essential amino acid and the precursor for tyrosine. Like tyrosine, it is the precursor of catecholamines in the body (tyramine, dopamine, epinephrine and norepinephrine). The decreased L-phenylalanine level in OSCC saliva appears to be the result of enhanced energy metabolism or upregulation of the appropriate biosynthetic pathways, and required cell proliferation in oral cancer tissues. But the result differs from other serum and tissue studier, which observed an increased level of L-phenylalanine [34]. It is propose because of the different metabolite rates in the different biological compartments [27]. L-Leucine as a branched chain amino acids is critical to human life and is solely to fats metabolism. It stimulates protein synthesis, increase reutilization of amino acids in many organs and reduce protein breakdown. Substantial amounts of L-leucine are generated by protein breakdown. The intermediates of L-leucine become involved in the tricarboxylic acid (TCA) cycle when there is a shortage in energy supply. In OSCC, L-leucine content in saliva decreased as compared to the healthy people, probably because the increased metabolic utilization by the TCA cycle in oral cancer cells [35]. Additional, it may also be associated with cancer cachexia and enhanced protein synthesis in excessive proliferation of cancer cells.

5. Conclusions

This is the first report for systematic investigation of salivary metabolites (L-phenylalanine and L-leucine) as potential biomarkers for OSCC diagnosis with UPLC–ESI-MS. This assay shows advantages with non-invasive, simple, and reliable. A total run time need only about 10 min. The analysis was rapid, robust and specific permitting a more accurate vision of potential biomarkers for both amino acids present in the saliva. The present method also provides lower detection limits and excellent precision and accuracy.

Both biomarkers demonstrated significant differences ($P<0.05$) in concentrations between OSCC and control. Compared to the healthy people, their contents were lower in the OSCC patients. As a single biomarker, L-leucine might have better predictive power in OSCC with T1–2, and L-phenylalanine might be used for screening and diagnosis of OSCC with T3–4. L-phenylalanine and L-leucine in combination will improve the sensitivity and specificity (sensitivity: 92.3%; specificity: 91.7%; AUC=0.871; $P<0.0001$) for diagnosis OSCC in the early stage because OSCC is a complex disease resulting from an interdependent series of genetic alterations rather than a single decisive event. The discovery of these targets may lead to a simple clinical tool for the noninvasive early diagnosis of OSCC.

Acknowledgement

The authors are grateful to the financial support from National Recruitment Program of Global Experts (NRPGE), the Hundred Talents Program of Sichuan Province (HTPSP), and the Startup Funding of Sichuan University for setting up the Research Center of Analytical Instrumentation.

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