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RESEARCH ARTICLE

Estimation of lead in biological samples of oral cancer patients chewing smokeless tobacco products by ionic liquid-based microextraction in a single syringe system

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Abstract Several studies have reported that the chewing habit of smokeless tobacco (SLT) has been associated with oral cancer. The aim of the present study was to evaluate the trace levels of lead (Pb) in biological samples (blood, scalp hair) of oral cancer patients and referents of the same age group (range 30–60 years). As the concentrations of Pb are very low in biological samples, so a simple and efficient ionic liquid-based microextraction in a single syringe system has been developed, as a prior step to determination by flame atomic absorption spectrometry. In this procedure, the hydrophobic chelates of Pb with ammonium pyrrolidinedithiocarbamate (APDC) were extracted into fine droplets of 1-butyl-3-methylimidazolium hexafluorophosphate [C_4MIM][PF_6] within a syringe while using Triton X-114 as a dispersant. Factors influencing the microextraction efficiency and determination, such as pH of the sample, volume of [C_4MIM][PF_6] and Triton X-114, ligand concentration, and incubation time, were studied. To validate the proposed method, certified reference materials were analyzed and the results of Pb^{2+} were in good agreement with certified values. At optimum experimental values of significant variables, detection limit and enhancement factor were found to be 0.412 μg/L and 80, respectively. The coexisting ions showed no obvious negative outcome on Pb preconcentration. The proposed method was applied satisfactorily for the preconcentration of Pb^{2+} in acid-digested SLT and biological samples of the study population. It was observed that oral cancer patients who consumed different SLT products have 2–3-fold higher levels of Pb in scalp hair and blood samples as compared to healthy referents ($p<0.001$). While 31.4–50.8 % higher levels of Pb were observed in referents chewing different SLT products as compared to nonconsumers ($p<0.01$).

Keywords Lead · Oral cancer · Smokeless tobacco products · Blood · Scalp hair · Ionic liquid-based microextraction

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Introduction

Oral cancer is the sixth most common cancer in the world and is a common malignancy among peoples who have consumed different tobacco products (Khandekar et al. 2006; Oji and Chukwuneke 2007; Kazi et al. 2010a). The annual estimated incidence is ~300,000 new cases (Khandekar et al. 2006); two thirds of these cases are occurring in developing countries (Ferlay et al. 2004). Oral cancer is characterized by a high rate of morbidity and mortality (Boyle and Ferlay 2005). Both tobacco smoking (cigarettes, cigars, and pipes) and chewing smokeless tobacco (SLT) products with and without other ingredients have been shown to increase the risk of developing oral cancer (Accortt et al. 2005; Kazi et al. 2010b; Rodu and Cole 2002). The International Agency for Research on Cancer recently reported that there is "sufficient evidence" that the use of SLT is carcinogenic to humans (Cogliano et al. 2004). Cancers caused by SLT products may often begin as leukoplakia or erythroplakia, which generally has a higher chance to becoming cancerous over time (Bouquot 1991).

Tobacco plant (*Nicotiana tabacum*) is well known for its capacity to concentrate toxic elements from its growing environment (Golia et al. 2007). Tobacco is known to contain numerous classes of carcinogenic substances such as tobacco-specific nitrosamines, which are often regarded as a major factor in smokeless tobacco-related carcinogenesis. The combined exposure to nitrosamines and other classes of organic and inorganic substances, including toxic metals, enhances the carcinogenetic effects (Stepanov and Hecht 2005). Lead (Pb) is known to be a toxic metal that accumulates in the human body throughout the lifetime (Godwin 2001). Typical symptoms of Pb poisoning are abdominal pain, anemia, headaches, chronic nephritis of the kidney, brain damage, and central nervous system disorders (Jaffe et al. 2001).

Flame atomic absorption spectrometry (FAAS) has been widely used for the determination of trace metal ions because of the relatively simple and inexpensive equipment (Citak and Tuzen 2010; Ghaedi et al. 2010). However, direct determination of metal ions at trace levels by FAAS is limited, not only due to insufficient sensitivity but also due to the matrix interference. Under these circumstances, to determine trace levels of Pb, a separation and enrichment step prior to the determinations is beneficial. Several methods have been proposed for the separation and preconcentration of trace levels of Pb including liquid–liquid extraction (LLE) (Comitre and Reis 2005), solid-phase extraction (Ghaedi et al. 2013; Gundogdu et al. 2009), cloud point extraction (Arain et al. 2013; Citak and Tuzen 2010), and liquid–liquid microextraction (LLME) (Shah et al. 2012). The use of classical extraction method requires large amounts of high purity solvents, which may also result in environmental and safety problems, due to high volatilization. Ionic liquids (ILs) consist of large organic cations like quaternary ammonium, imidazolium, or pyridinium ions combined with anions of smaller size and more symmetrical shape such as Cl^-, Br^-, I^-, $AlCl_4^-$, BF_4^-, PF_6^-, $ROSO_3^-$, NTf_2^- (bis(trifluoromethylsulfonyl) imide), triflate (trifluoromethanesulfonate), and others (Abdolmohammad-Zadeh and Sadeghi 2009). Many of the possible combinations of these ions exist as liquids at room temperature and some of them have turned out to be stable at temperatures up to 500 K (Heintz 2005). ILs are low melting point ionic compounds having unique physicochemical properties, such as broad liquid ranges, negligible vapor pressures, good thermal stabilities, nonflammability, and good extractabilities for various organic compounds and metal ions as neutral or charged complexes, as well as tunable viscosity and miscibility with water and organic solvents, which make them very attractive in separation processes (Heintz 2005; Liu et al. 2009; Marsh et al. 2004). ILs have the capability to form a wider range of intermolecular interactions than typical volatile organic solvents (Łuczak et al. 2008). Several studies have been reported in which ILs have successfully been utilized for extraction of metal ions as chelates (Haixia et al. 2007; Martinis et al. 2008). A number of new procedures with different extraction performances have been developed based on classical LLME (Baghdadi and Shemirani 2008; Martinis and Wuilloud 2010). Recently, chemists have started finding different ways to miniaturize the classical dispersive microextraction techniques (Naeemullah et al. 2013; Arain et al. 2015).

The objective of study is to present an efficient extraction method to avoid the centrifugation step, thermal dispersion, and usage of hazardous organic solvents. This novel approach uses two syringes: one 20 mL plastic syringe as extraction unit and another 1 or 3 mL syringe for the recovery of extractant. The proposed procedure was termed as ionic liquid-based microextraction in a single syringe system (SS-ILμE). All the variables, pH, incubation time, concentration of Triton X-114 and ammonium pyrrolidinedithiocarbamate (APDC), as well as the volume of the sample and IL, affecting the proposed procedure, have been studied and optimized. After optimization, the proposed method has been applied successfully to real samples.

Materials and methods

Chemicals and reagents

Ultrapure water obtained from ELGA LabWater system (Bucks, UK) was used throughout the work. Concentrated nitric acid (65 %) and hydrogen peroxide (30 %) were obtained from Merck (Darmstadt, Germany). All chemicals and reagents were of analytical grade. 1-Butyl-3-methylimidazolium hexafluorophosphate [C_4MIM][PF_6] and the nonionic surfactant, Triton X-114, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonionic surfactant

dilute solutions (0.025–0.2 % *v*/*v*) were prepared by dissolving an appropriate amount of Triton X-114 in 100 mL of distilled water; 0.1 mol/L of acetate buffer was used to control the pH of the solutions. The pH of the samples was adjusted to the desired pH (3–8) by the addition of 0.1 M HNO_3/NaOH solution in acetate buffer. The certified standard solutions of Pb (1000 mg/L) and APDC were obtained from Fluka Kamica (Bush, Switzerland). HNO_3 (0.2 mol/L) was used for the dilution of stock standard solution to make working standards. For the accuracy of methodology, certified reference materials (CRM) of human hair BCR 397 (Brussels, Belgium), Clincheck® control-lyophilized human whole blood (Recipe, Munich, Germany), and Virginia tobacco leaves (ICHTJ-cta-VTL-2) (Vienna, Austria) were used.

Instrumentation

A Perkin-Elmer Model AAnalyst 700 (Norwalk, CT, USA) FAAS was used. A single element hollow cathode lamp was operated at 7.5 mA at a spectral bandwidth of 0.7 nm. The analytical wavelength was set at 283.3 nm. Overall analysis was carried out with an air/acetylene flame, using 10 cm long slot-burner head. Peak heights were recorded as signals. A pH meter (Ecoscan Ion 6, Malaysia) was employed for pH adjustments. A PEL domestic microwave oven (Osaka, Japan), programmable for time and microwave power from 100 to 900 W, was used for digestion of samples.

Study population

A survey was carried out about SLT products (gutkha, mainpuri, and snuff) and the chewing and inhaling habits of both genders, age ranged 30–60 years, residing in the different cities of Sindh, Pakistan. The data of hospital-based case–control study for oral cancer patients were collected from the Nuclear Institute of Medicine and Radiotherapy (NIMRA) Jamshoro and Larkana Institute of Nuclear Medicine and Radiotherapy (LINAR), during the years 2011–2013, by collecting files and extracting important information. During a 1-year study period (2010), the information department of both hospitals recorded >5200 cases of cancers of all types, and oral cancer comprised 3.6 % of the total. Oral cancer patients were divided into subgroups according to different locations of oral cancer: lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (throat). Oral cancer patients and referents were further grouped according to their SLT chewing habits: non-SLT (NU), gutkha (GU), snuff (SU), and mainpuri users (MPU). Complete demographic information is listed in Table 1.

Physical examinations were performed to measure participant's weight, height, blood pressure, and biochemical data. The biochemical tests of patients and referents were performed to estimate hemoglobin, red blood cells, packed cell volume, mean corpuscular hemoglobin concentration, mean corpuscular volume, and transferrin iron-binding capacity in the blood. The biochemical results and histological

Table 1 Characteristics of study subjects (30–60) age groups

Referents/oral cancer patients		Non-SLT consumer	Gutkha users	Snuff users	Mainpuri users
Male					
Referents		192	158	135	209
Oral cancer patients	Lips	31	54	47	55
	Tongue	23	51	29	34
	Cheeks	18	37	26	32
	Floor of the mouth	21	27	24	38
	Hard and soft palate	20	34	21	27
	Sinuses	22	26	25	31
	Pharynx (throat)	18	23	30	26
	Total	153	252	202	243
Female					
Referents		136	120	107	98
Oral cancer patients	Lips	23	31	28	24
	Tongue	18	31	23	27
	Cheeks	20	15	22	19
	Floor of the mouth	14	19	15	26
	Hard and soft palate	18	19	25	23
	Sinuses	17	28	15	20
	Pharynx (throat)	19	15	19	26
	Total	129	158	147	165

information are not reported in the present study. The criteria for the selection of patients were biopsy-proven oral squamous cell carcinoma prior to any treatment and patients were not taking any mineral supplements during the last 3 months. The selection criteria for the 1155 referent subjects were the same age group, socioeconomic status, dietary habits, and not taking any mineral supplement. Prior to the collection of biological samples, the referent subjects also underwent a standard routine medical examination. This study was approved by the Ethics Committee of Sindh University, working under the auspices of the Higher Education Commission of Pakistan.

Sampling of SLT products

Different brands of SLT products (n=46) of snuff (dry and moist), gutkha, and mainpuri were purchased from local markets of the different cities of Pakistan as per their availability over a 3-year period (March 2011–January 2014). The samples were packed in their original packing and placed in prewashed dried plastic bags separately and stored at 4 °C, until tested. Five composite samples of each brand of snuff, gutkha, and mainpuri were prepared by homogenizing the mixture after removing the wrappers. Care was taken to avoid any source of contamination, and this preparation was carried out in a clean environment. All samples were dried at 80 °C. The dried samples were ground with agate mortar and pestle, sieved through nylon sieves with mesh sizes of 125 μm, and then stored in the labeled sample bottles.

Biological samples

Venous blood samples (5 mL) were collected by 7 mm heparinized lithium Vacutainer® tubes (Becton Dickinson). About 2 mL of venous blood samples were stored at −20 °C until metal analysis. The scalp hair samples were taken from the nape of the neck. The hair was tied together from 1 cm of the scalp with Teflon thread, cut with a stainless steel scissor, and pretreated as reported in our earlier work (Panhwar et al. 2013). Hair samples were put into separate plastic envelopes for each participant, tightly sealed, and attached with the identification number of the participant and questionnaire.

Microwave-assisted acid digestion (MAD)

Six replicate samples of each CRM (0.5 mL Clincheck® control-lyophilized human whole blood, 0.2 g of Virginia tobacco leaves and BCR 397 human hair) and duplicate samples of different types of SLT products (0.2 g), whole blood (0.5 mL), and scalp hair (0.2 g) were taken separately in polytetrafluoroethylene (PTFE) flasks (25 mL in capacity), which were added with 3 mL of a freshly prepared mixture of concentrated HNO_3–H_2O_2 (2:1, v/v) and kept at room temperature for 10 min. Then, the flasks were placed in a covered PTFE container and heated at 80 % of total power (900 W) for 3–4 min. The digested samples were diluted up to 10 mL with 0.1 mol/L concentrated HNO_3. A blank extraction (without sample) was carried out through the complete procedure.

Ionic liquid-based microextraction in a single syringe system

To develop the SS-ILμE system, a 20-mL plastic syringe was used as an extraction unit and another 1 mL plastic syringe was used for the injection of the ligand. Replicate 10 mL of standard solution (10–80 μg/L) whose pH was adjusted in the range of 3–8 with appropriate volume of buffer was sucked into the syringe and added with 1 mL of 0.1–0.5 % (w/v) APDC. Then, 500 μL of 0.025–0.20 % (v/v) Triton X-114 was added by an adjustable micropipette, and a cloudy solution was immediately formed. Then, 50–200 μL of IL as extractant $[C_4MIM][PF_6]$ was added and slightly agitated to disperse the IL. Afterward, the piston of the syringe was slowly moved up and down to allow the recovery of IL from syringe walls. After phase separation, the aqueous phase was drawn out and the IL phase with analyte was easily recovered from the syringe. To the IL phase, 0.2 mL of acidic ethanol was added to reduce its viscosity. Samples of 100 μL were injected into FAAS through a self-made injection system of teflon funnel and Eppendorf pipette, which was connected with the capillary tubing of a nebulizer (Fig. 1).

Statistical analysis

All statistical analyses were performed using the computer program, Excel X State (Microsoft Corp., Redmond, WA, USA) and Minitab 13.2 (Minitab Inc., State College, PA, USA). Data from triplicate samples of each composite sample were expressed as means±SD. Student's t test was used to assess the significant difference of Pb in certified and experimentally found values. ANOVA was used to assess the significance of differences between the concentrations of Pb observed in the biological samples of patients and referent subjects, calculated by the unpaired two-sample t test. A $p<0.05$ was considered as significant difference.

Results

To achieve a high recovery and enrichment factor, the influence of different parameters, such as pH, concentration of APDC, incubation time, amounts of IL, and disperser solvent, for Pb determination in biological samples by SS-ILμE was investigated and optimized.

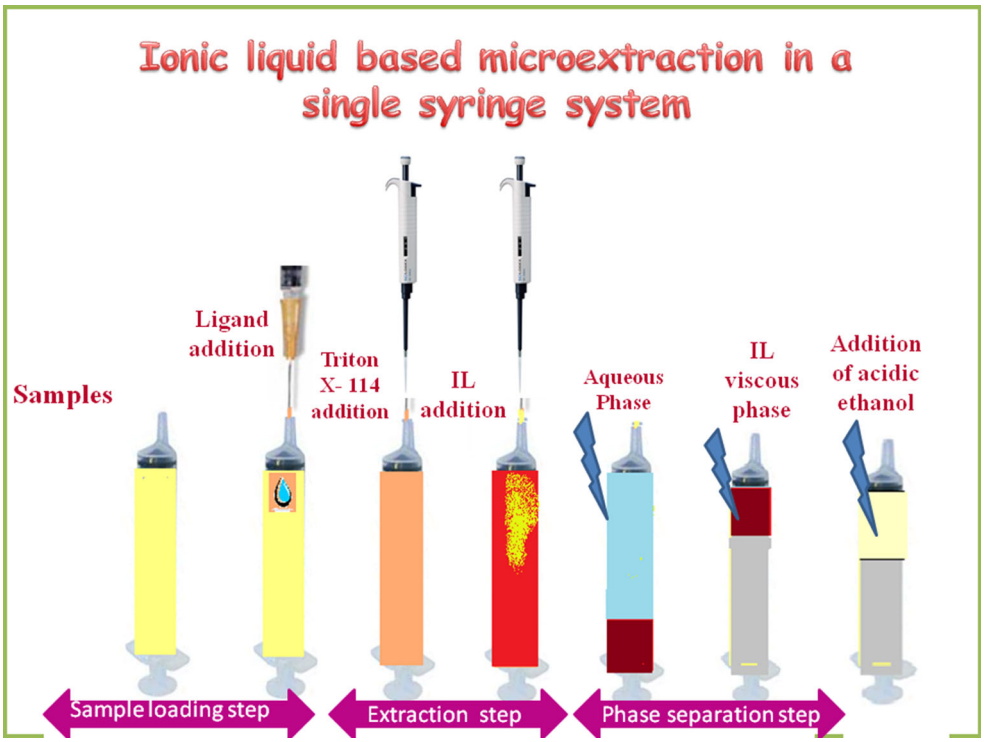

Fig. 1 Ionic liquid-based microextraction in a single syringe system

Optimization of factors affects the SS-ILμE

The effect of pH on microextraction of Pb was investigated by using six replicate standard solutions of the analyte (10 μg/L) in the pH range of 3–8, while other parameters were at their optimum levels. Each operational desired pH value was obtained by the addition of 0.1 mol/L of HNO_3/NaOH, in the presence of acetate/borate buffer. The maximum extraction efficiency was obtained at pH range 5.0–6.0. For subsequent work, pH 6.0 was selected. The concentration of APDC was used in range of 0.1 to 0.5 % (*w*/*v*). The optimum recovery of Pb is enhanced up to 0.35 % (*w*/*v*), and the APDC concentration is further increased and caused no changes in the signals. Hence, 0.35 % (*w*/*v*) of the APDC was selected for the microextraction of Pb.

The nonionic surfactant was chosen as a dispersive solvent. The solubility of "hydrophobic" IL in nonionic surfactant occurs due to the existence of significant interactions (H-bonding, dipole-induced dipole) among $[C_4MIM][PF_6]$ and Triton X-114 (Zhao et al. 2008). The effect of Triton X-114 volume on Pb recovery was examined in the range of 0.025–0.20 % (*v*/*v*). The maximum recovery was obtained at 0.075 % of Triton X-114 and remained constant up to 0.125 %. It indicated that a further increase in Triton X-114 concentration results in a decrease of recovery. While at lower concentration, recovery of analyte was low because there were few molecules of Triton X-114 to disperse IL. So Triton X-114 of 0.075 % (*v*/*v*) was selected for subsequent study. The variations in recoveries against IL volume were studied in the range of 50–200 μL. The result shows that IL quantitatively extracts Pb when its volume was 100 μL. No significant changes in recoveries were observed at higher IL volume. So, 100 μL of IL was used for subsequent experimental work.

The incubation time for Pb recovery was studied in the range of 1–5 min. The optimum recovery was obtained between 2 and 3 min, so 3 min of incubation time was chosen as the optimum incubation time. In fact, when the extraction mixture (dispersant + extractant) was dispersed into the sample solution, a cloud of tiny droplets of extractant was observed, which increased the contact surface area between the two phases. Consequently, equilibrium was achieved quite quickly which is one of the main advantages of our proposed procedure. For interference study, different amounts of coexisting ions were added to standard solutions of 10 μg/L of Pb and the procedure was followed. Table 2 indicates the quantitative recovery of Pb in the presence of understudied interfering ions which proved the applicability of the proposed procedure for Pb determination in real samples.

Analytical figures of merit

In order to assess the performance of the proposed method, the following three main parameters were employed: extraction recovery (ER), enhancement factor (EF), and consumptive

Table 2 Influences of some foreign ions on the % recoveries of Pb (10 μg/L) determined by applying the SS-ILμE method

Ions	Concentration (mg/L)	Pb
Na^+	4000	98.3±1.12
K^+	3000	96.6±2.41
Ca^{2+}, Mg^{2+}	4000	97.9±2.34
Cl^-	2000	99.1±2.51
F^-	1000	97.9±3.43
NO_3^-	3000	99.2±1.45
HCO_3^-	1000	98.4±1.90
Al^{3+}	500	97.5±3. 12
Fe^{3+}	50	96.3±2.23
Ni^{2+}, Cd^{2+}, Zn^{2+}	50	97.2±2.45
Cr^{3+}	100	97.1±3.21

index (CIn). The ER was defined as the percentage of total analyte which was extracted into the IL phase:

$$\mathrm{ER} = \frac{m_{\mathrm{IL\ phase}}}{m_{\mathrm{aq}}} = \frac{C_{\mathrm{IL\ phase}} \times V_{\mathrm{IL\ phase}}}{m_{\mathrm{aq}} \times V_{\mathrm{aq}}} \times 100$$

In the above equation, $m_{\mathrm{IL\ phase}}$ and m_{aq} are the analyte masses in the final IL phase and the initial concentration in the sample solution, respectively. The $C_{\mathrm{IL\ phase}}$ and C_{aq} are the analyte concentrations in the IL phase and in the aqueous phase, respectively. The $V_{\mathrm{IL\ phase}}$ and V_{aq} are the concerned volumes of the phases (Cruz-Vera et al. 2009). The calibration graph for the preconcentration of Pb was linear with correlation coefficients >0.995, at the range of 10–80 μg/L. Relative standard deviation (RSD) of a minimum of six independent analyses of CRMs after SS-ILμE of Pb was <7 %. The limit of detection (LOD), calculated as the ratio of three times the standard deviation of ten blank readings to the slope of the calibration curve, was 0.412 μg/L. EF is the ratio of calibration curve slopes before and after the application of the SS-ILμE procedure and was found to be 80. Another term, CIn, is defined as:

$$\mathrm{CIn} = {}^{V_s}/_{\mathrm{EF}}$$

where V_s is the sample volume (in milliliters) (Cruz-Vera et al. 2009; Fang et al. 1997). The CIn obtained for the proposed method for enrichment of Pb was 0.126. Thus, CIn reflects the efficiency of sample utilization, and it is a useful tool for selecting a preconcentration method when sample amount is limited, such as in the case of body fluid analysis (Fang et al. 1997). The validity and efficiency of the analytical method were checked with certified values of human hair CRM 397, Clincheck® control-lyophilized human whole blood, and Virginia tobacco leaves (ICHTJ-cta-VTL-2) (Table 3). Paired *t* test was applied to compare the results obtained by SS-ILμE and showed that the $t_{\mathrm{experimental}}$ value is lower than t_{critical} (2.75) at a confidence interval of 95 % (p=0.05), which indicated a nonsignificant difference in the obtained and certified value of Pb in CRM (Table 3). The high sensitivity and low detection limits of the present SS-ILμE method suggest that it is efficient and sensitive for the determination of very low concentrations of Pb in biological samples. A comparative study was carried out to evaluate the efficiency of SS-ILμE with other reported extraction and preconcentration methods for Pb determination, as shown in Table 4. The LOD and EF of the present study were comparatively better that those of previously reported works (Alonso et al. 2006; Arain et al. 2013; Bai et al. 2010; Gama et al. 2006; Matoso et al. 2003; Minami et al. 2005; Naseri et al. 2008; Shah et al. 2012). Therefore, the proposed method could be of key interest especially for routine analytical laboratories.

Applications

Determination of Pb in different SLT products

The pH of different types of SLT (gutkha, snuff, and mainpuri) products was highly basic, in the range of 8.2–8.9, which favors the formation of tobacco-specific amines, thus making the product potentially toxic (IARC 2007).

Multiple samples of different brands of each SLT product were analyzed, and the mean concentrations along with the standard deviation for five composite samples of each brand are provided in Table 5. The Pb level in the different brands of gutkha (G) and mainpuri (MP) were observed in the range of 1.85–5.64 and 7.44–11.9 μg/g, respectively. In brown and green moist (BM and GM) and dry brown and black snuff (DB and DBK), the concentration of Pb was found in the range of 8.46–15.7, 7.10–10.1, 4.38–6.12, and 6.05–8.64 μg/g, respectively. The concentration of Pb in different SLT products (gutkha, mainpuri, and snuff) were found in increasing order as BM>MP>GM>DBK>DB>G.

The Pb concentration in biological samples of referents and oral cancer patients

The mean concentrations with standard deviations of Pb in biological samples of referents and patients are shown in Table 6. The resulted data indicated that the concentration of Pb was significantly higher in scalp hair and blood samples of cancer patients (lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx) than those of referents (p<0.001). The Pb in scalp hair samples of male referents, NU, GU, SU, and MPU, were found in the range of 4.49–5.12, 5.70–6.21, 6.10–6.95, and 7.02–8.03 μg/g, respectively, while the range of Pb levels in scalp hair samples of male oral cancer (lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx) patients were 10.5–19.7, 11.8–

Table 3 Determination of Pb in certified sample of human hair, blood, and Virginia tobacco leaf by MAD and SS-ILμE

Elements	MAD $\bar{x} \pm s$ [a]	% recovery[b]	SS-ILμE $\bar{x} \pm s$	% recovery	Paired t test[c] $t_{experimental}$	Certified values
Certified sample of human hair (μg/g)						
Pb	32.3±1.9 (5.88)[d]	(97.8)	32.6±1.2 (3.68)	(98.8)	0.501	33±1.2
Certified sample of whole blood (μg/L)						
Pb	103±8.9 (8.64)	(98.1)	104±7.1 (6.82)	(99.0)	0.236	105±24
Virginia tobacco leaf (μg/g)						
Pb	21.4±1.7 (7.94)	(96.8)	21.9±1.0 (4.60)	(98.6)	0.287	22.1±1.2

$t_{critical}$ at 95 % confidence limit=2.57

[a] Average value±confidence interval (p=0.05)

[b] % recovery=[experimental value]/[certified value]×100

[c] Paired t test between certified values vs found values, degree of freedom (n−1)=5

[d] Values in parenthesis are RSD

22.9, 11.3–19.8, 12.5–22.6, 12.9–24.6, 12.2–19.2, and 13.2–25.4 μg/g, respectively (p<0.001) (Table 5). The same trend was observed in females. The Pb concentrations in blood samples of male referents, NU, GU, SU, and MPU, were observed in the range of 113–122, 149–161, 158–170, and 72–185 μg/L (Table 6), whereas the levels of Pb in blood samples of male with different oral cancers (lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx) were found in the range of 246–302, 215–352, 221–318, 229–322, 252–362, 232–329, and 242–369 μg/L, respectively, which were significantly higher as compared to those values obtained for referents (p<0.001) (Table 6). The same trend was observed in females.

The unpaired Student's t test between cancerous patients and referents for different degrees of freedom was calculated at different probabilities. Our calculated $t_{experimental}$ exceeds that of $t_{critical}$ values at 95 % confidence intervals, which indicated the significant differences between the data of Pb in biological samples of oral cancer patients than referents (p<0.001).

Discussion

A survey in Karachi indicates that 36 % of males and 44 % females chew different SLT products (Bhurgri et al. 2000). Population-based surveys from India, Pakistan, and Nepal over the past couple of decades have reported a prevalence of use of SLT products between 20 and 40 % among adolescents and adults (Gupta and Ray 2003; Qidwai et al. 2002). In Pakistan, a recent study among adolescents and adults of Karachi reported that 40 % of the population was using at least one chewable product of smokeless tobacco on a daily basis (Mazahir et al. 2006). In Pakistan and

Table 4 Comparison of SS-ILμE with other reported preconcentration techniques for Pb determination

Technique	Instrumentation	EF	LOD (μg/L)	Reference
CPE	GFAAS	40	1.12	Arain et al. (2013)
SPE	FAAS	30	6.1	Matoso et al. (2003)
LPME	FAAS	40	9.5	Bai et al. (2010)
SPE	FAAS	37	3.75	Gama et al. (2006)
IL-DLLME	FAAS	273	0.6	Yousefi and Shemirani (2010)
DLLME	GFAAS	150	0.02	Naseri et al. (2008)
Coprecipitation	GFAAS	20	0.5	Minami et al. (2005)
Online SPE	GFAAS	20.5	0.012	Alonso et al. (2006)
VLLME	FAAS	54.2	0.307	Shah et al. (2012)
SS-ILμE	FAAS	80	0.412	Present study

CPE cloud point extraction, SPE solid-phase extraction, *LPME* liquid-phase microextraction, *DLLME* dispersive liquid–liquid microextraction, *VLLME* vortex liquid–liquid microextraction, *GFAAS* graphite furnace atomic absorption spectrometry

Table 5 Lead concentration in different SLT products (μg/g)

Mainpuri (MP)	$\bar{x} \pm ts/\sqrt{n}$ [a]	Gutkha (G)	$\bar{x} \pm ts/\sqrt{n}$ [a]	Moist snuff	$\bar{x} \pm ts/\sqrt{n}$ [a]	Dry snuff	$\bar{x} \pm ts/\sqrt{n}$ [a]
MP1	8.61±0.37	G1	4.57±0.19	BM1	12.6±0.26	DB1	5.97±0.11
MP2	8.19±0.38	G2	3.77±0.25	BM2	15.2±0.48	DB2	4.72±0.24
MP3	8.20±0.45	G3	3.32±0.15	BM3	14.8±0.42	DB3	5.73±0.41
MP4	11.1±0.51	G4	3.79±0.23	BM4	12.1±0.38	DB4	5.91±0.12
MP5	8.18±0.19	G5	5.27±0.31	BM5	14.5±0.77	DBK5	6.24±0.23
MP6	8.69±0.32	G6	3.08±0.20	BM6	10.9±0.46	DBK6	7.37±0.16
MP7	8.34±0.47	G7	3.67±0.14	BM7	9.33±0.62	DBK7	8.42±0.12
MP8	9.41±0.43	G8	4.82±0.25	GM8	9.89±0.30	DBK8	7.21±0.25
MP9	8.87±0.52	G9	2.07±0.17	GM9	7.72±0.52	DBK9	8.23±0.13
MP10	7.96±0.34	G10	3.05±0.28	GM10	8.55±0.79	–	
MP11	7.70±0.35	G11	4.15±0.28	GM11	7.46±0.17		
MP12	8.78±0.45			GM12	8.46±0.68		
				GM13	7.89±0.75		
				GM14	8.89±0.92		

BM brown moist, *GM* green moist, *DB* dry brown, *DBK* dry black

[a] Average value±confidence interval (p=0.05)

South Asian subcontinent, the popular chewing products are betel quid, betel nut, gutkha, and snuff (Johnson 2001). Oral cancer is the second most common malignancy in Pakistan. It was observed that oral cancer is now common in a younger population. According to the World Health Organization (WHO), the use of SLT products is a culturally acceptable habit in Asian countries including Pakistan (Bhurgri et al. 2006).

Table 6 Lead concentrations in scalp hair and blood samples of referents and oral cancer patients

Biological samples	Types of patients	Referents	Different types of oral cancer patients						
			Lips	Tongue	Cheeks	Floor of the mouth	Hard and soft palate	Sinuses	Pharynx (throat)
Male									
Scalp hair (μg/g)	NU	4.82±0.60	10.5±1.21	11.8±0.92	11.3±1.05	12.5±1.42	12.9±1.08	12.2±1.52	13.2±0.95
	GU	5.95±0.54	17.8±1.35	19.8±1.77	17.5±1.64	18.1±1.55	19.5±1.81	17.1±1.43	20.4±1.08
	SU	6.52±0.79	19.3±1.98	20.9±1.73	18.7±1.21	19.1±1.81	20.4±1.97	18.9±1.99	21.8±2.01
	MPU	7.51±0.98	19.7±1.79	22.9±1.11	19.9±1.67	22.6±1.17	24.6±1.88	19.2±1.97	25.4±1.44
Blood (μg/L)	NU	118±9.82	246±17.5	215±14.2	221±14.1	229±17.5	252±16.5	232±16.1	242±17.2
	GU	155±11.6	285±18.2	311±15.8	291±16.4	298±19.8	323±18.5	301±16.1	341±19.7
	SU	163±14.5	292±17.2	343±14.9	305±18.4	311±14.5	352±14.3	322±13.6	357±19.2
	MPU	178±12.9	302±13.8	352±18.7	318±15.7	322±13.2	362±20.9	329±18.9	369±21.8
Female									
Scalp hair	NU	3.35±0.36	8.38±0.85	9.48±0.98	9.78±1.99	10.5±1.05	8.21±0.60	9.29±1.02	10.9±1.95
	GU	5.08±0.67	14.2±1.53	18.7±1.92	16.9±2.35	17.5±1.94	18.5±2.05	15.6±2.18	19.6±2.72
	SU	5.99±0.79	15.2±2.38	19.8±2.05	17.9±1.81	19.1±1.76	19.9±1.85	16.9±2.54	20.7±2.62
	MPU	6.92±0.95	17.8±2.05	21.2±2.74	19.6±1.62	20.1±1.98	23.9±1.74	17.3±1.42	24.9±1.98
Blood	NU	101±7.18	223±16.2	209±15.1	219±16.5	215±18.1	232±17.8	230±17.7	240±18.1
	GU	148±10.9	276±17.1	303±13.4	287±15.4	287±16.7	319±15.6	297±17.2	332±14.3
	SU	159±11.2	286±14.9	337±17.6	299±17.3	309±15.3	342±16.9	316±15.8	342±16.8
	MPU	171±15.5	298±19.1	340±14.4	309±14.5	312±14.8	354±17.1	322±15.2	354±19.8

NU non-SLT users, *GU* gutkha users, *SU* snuff users, *MPU* mainpuri users

With regard to the habit of chewing different SLT products (gutkha and mainpuri), the referents and patients gave information that they use 2–10 packets (2–5 g) per day, kept it in the mouth for 30 min to 1 h, chewed, and mostly swallowed, and only 5 to 10 % claimed that they spitted it out, while patients suffering from different oral cancers informed that mostly at night, they kept SLT in their mouth and sleep without using any mouthwash. Therefore, it appears that more amount of SLT contents is absorbed by the buccal mucosa or posterior region of the mouth. Patients who consumed different types of snuff placed 2–3 g between the gingival and buccal mucosa, and this habit is repeated 8–15 times a day depending upon individual mood. It was observed that cancer patients, who had consumed SLT products, were not aware of the symptoms till the severity developed.

This case–control study provides data to evaluate the possible association between Pb exposure via consumption of different types of SLT products and its altered levels in the blood and scalp hair of different oral cancer patients and referents of both genders. It was observed that the Pb level varies in biological samples of referents and patients, according to the types of SLT products consumed, but the difference was not significant ($p>0.05$).

The World Health Organization/Joint Expert Committee on Food Additives (WHO-JECFA) as well as the Food and Agriculture Organization/World Health Organization (FAO/WHO) has established provisional maximum tolerable daily intake (PMTDI) of Pb to be 3.6 μg/kg bodyweight/day (Baars et al. 2001). The intake of Pb via consumption of 10 g of gutkha, mainpuri, and snuff product was found in the range of 19.1–55.7, 74.3–113, and 43.1–157 μg/day/person, respectively, contributing 8.9–73 % of the PMTDI for Pb in adults (60 kg).

The present analysis, based on the dataset available on different types of oral cancer in the population of both genders, confirms that chewing SLT products could be major risk factors for oral disease. The resulted data indicated that significantly higher levels of Pb were observed in blood samples of referents GU (23.4 %), SU (35.3 %), and MPU (55.8 %) than those values obtained for referents who did not consume any SLT product ($p<0.01$), while 31.4–50.8 % higher levels of Pb were observed in scalp hair samples of referents chewing different SLT products (GU, SU, and MPU) as compared to referents who did not consume any SLT products. The significantly higher levels of Pb were observed in tongue, pharynx, and hard and soft palate cancer patients as compared to referents ($p<0.001$), shown in Table 6.

Many epidemiological studies have reported that oral cancer is strongly associated with tobacco and alcohol drinking (Kazi et al. 2010a; Kingsley et al. 2008). The International Agency for Research on Cancer (IARC) classified inorganic Pb as a class 2A carcinogen in 2006 (Gwini et al. 2012). Higher Pb contents were linked to cancers of the gastrointestinal, brain, breast, lung, and bladder and leukemia (Alatise and Schrauzer 2010; Gwini et al. 2012; Pasha et al. 2010; Van Wijngaarden and Dosemeci 2006). There are some lines of evidence that suggest that Pb increases the susceptibility to cancer. Pb may exert diverse toxic effects on cells, disrupting the ability of cells to develop appropriate and precise responses to genotoxic agents. It may also interfere with the ability of DNA to repair itself. By binding with histones, Pb may decrease the protection, which these proteins give to DNA, so it has direct exposure to DNA as damaging agents (Quintanilla-Vega et al. 2000; Silbergeld et al. 2000). It is reported in the literature that individuals with high levels of Pb had increased cancer-caused mortality (Lustberg and Silbergeld 2002). Substantial experimental evidence implicates oxidative stress via oxidation–reduction-inactive metal pathways for Pb, resulting in increased reactive oxygen species that lead to depletion of nitric oxide and create secondary upregulation of endothelial nitric oxide synthase (Prozialeck et al. 2008; Vaziri and Khan 2007). High exposure to Pb changes the intracellular calcium homeostasis (Silbergeld 2003). It was reported in a study that Pb induced tumors due to enhancement of cellular proliferation (Calabrese and Baldwin 1992).

The reported data of Pb in scalp hair samples of healthy human subjects were in the range of 0.22–7.26 and 4.8–5.7 μg/g, respectively (Nowak and Chmielnicka 2000; Rodushkin and Axelsson 2000). It was reported in the literature that the reference value of Pb in the blood of Nigerian referents is in the range of 18–85 μg/L (Alatise and Schrauzer 2010). On the basis of different epidemiological studies, it has been recommended that levels of blood Pb should be kept below 100 μg/L (Menke et al. 2006; Yakub and Iqbal 2010).

It was also observed in the present study that socioeconomic factors also play a role in higher mortality rates in oral cancer patients, such as poor nutrition, irregular screening, late diagnosis, and unequal access to health care due to poverty. On the other side, the cost of treatment for different types of cancer is very high, which is commonly not affordable. The local hygiene center facilities are poor in the country and there are no routine monitoring and screening carried out for those people living in small towns.

Conclusion

An efficient experimental setup for ionic liquid-based microextraction in a single syringe system is presented to determine trace levels of Pb in SLT products and biological samples. In the proposed method, the use of toxic organic extractants and dispersant solvents (i.e., chloroform, carbon tetrachloride, methanol, acetone, etc.) has been replaced with green alternative solvents such as IL and Triton X-114. Our proposed technique requires only a conventional plastic

syringe as extraction unit avoiding the centrifugation step to reduce the extraction time. The present study evidenced marked significant divergences of Pb in the blood samples of oral cancer patients in comparison with the referents who consumed or not any SLT products ($p<0.001$). The imbalance in Pb level in oral cancer patients could be due to change of cellular metabolism in the cancer process. Since the role of Pb in the mechanism of oral cancer development is still unclear, further detailed and comprehensive investigations are necessary.

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