Trends in cotinine level in urine samples of smokeless tobacco chewers from Belagavi region

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Abstract

Background: Tobacco consumption is a major reason for morbidity and mortality in India and more than 20% of worldwide tobacco related mortality occurs in India. Various analytical methods are used to check the levels of different biomarkers hereto and HPLC method has proved to be of wide acceptance for such studies.

Aim: The present study was aimed to evaluate the cotinine level in urine samples of smokeless tobacco chewers using RP-HPLC-PDA and GC-MS.

Methods: Out of 600 urine samples, 300 of smokeless tobacco chewers (STC) and 300 from non-tobacco chewers (NTC) were analyzed. The cotinine level in urine was analyzed by RP-HPLC-PDA and confirmed using GC-MS.

Results: The cotinine concentration in urine samples of STC and NTC were found in the range of 100.84 to 5810 ng/mL urine sample and 10.01 to 59.02 ng/mL respectively.

Conclusion: Determination of cotinine level in STC and NTC along with its further confirmation using GC-MS method served an efficient analytical tool for such studies.

Keywords: Cotinine, Urine sample, RP-HPLC-PDA, GC-MS, Smokeless tobacco

Introduction

The use of tobacco dates back as early as 5000-3000 BC, when tobacco plants were first cultivated in South America[1]. Initially, tobacco was regarded as a medicinal plant used for medical purposes such as a painkiller for earaches and toothaches[2]. However, later studies clearly established the deleterious effects of tobacco smoking on health. Tobacco consumption is a major reason for morbidity and mortality in India[3]. It is estimated that, approximately 5 million deaths occur due to tobacco consumption annually and it is expected to reach 10 million by 2025. Today more than 20% of worldwide tobacco related mortality occurs in India[4,5].

The tobacco consumption is identified by two main methods: smoked tobacco products and smokeless tobacco products. The later being the cheapest and widely circulated habit in Indian community. Smokeless tobacco may include different forms of non conventional Manufactured Smokeless Tobacco Product (MSTP) *viz. Ghutka*, *Khaini*, *Zarda*, *Mawa* etc.[6,7] or conventional tobacco *pan masala*, tobacco with lime and more commonly tobacco with '*pan*' (betel leaf) and betel quid[8]. MSTP due to its easy affordability, lesser cost and misconceptions is important contributory factors for increased smokeless tobacco consumption[9]. In some parts of India like Bihar and Maharashtra, use of smokeless tobacco is more common than smoking. In one report, the age-adjusted relative risk of mortality for users of smokeless tobacco like that of smokers is elevated compared to that of non-tobacco users[3]. The major health consequences associated with the use of smokeless tobacco in South Asia caused cancers of several sites (e.g. the upper respiratory and digestive tracts), and poor reproductive outcomes. Some study exposed that the impact of smokeless tobacco on blood pressure and cardiac disease[10].

Nicotine is the common major alkaloid found in all of the above products. It was first reported from

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tobacco plant (*Nicotiana tabacum* L.), hence was named after its genus. The alkaloid constitutes of 0.3 to 5 % of tobacco plant on dry weight basis. It is very toxic with a LD-50 of 40-60 mg in humans[8]. About 70-80% of nicotine metabolizes rapidly and extensively to form its lactam derivative, cotinine. It is a biomarker for intake of nicotine[11]. Due to the long half-life of cotinine, it has been used as a biomarker for tobacco smokers and chewers. Urinary cotinine is recommended as the quantitative parameter to access intake of nicotine[12,13]. Several analytical techniques have been reported for determination of cotinine and nitrosamines. They include gas chromatography (GC)[14] enzyme linked immunosorbent assay[15,16] liquid chromatography-mass spectrometry (LC-MS)[17], and high-performance liquid chromatography (HPLC) with UV detection[12,18].

The present work describes a RP-HPLC-PDA method for evaluation of cotinine in urine samples of smokeless tobacco chewers. The study also attempts to confirm its presence in urine samples using GC-MS.

Materials and methods

Reagents and standards: HPLC grade methanol acetonitrile and distilled water and analytical-reagent grade dichloromethane, diethyl ether was obtained from Qualigens, Mumbai, India. Reference standard cotinine ≥98%, (*S*)-1-methyl-5-(3-Pyridyl)-2-pyrrolidinone was procured from Sigma-Aldrich, Mumbai, India (Lot No. #092M4002V).

Subjects and sample collection: Six hundred samples were collected from Belagavi Institute of Medical Sciences (BIMS), Belagavi, Karnataka, India. Out of these samples, three hundred were collected from smokeless tobacco chewers and three hundred from non-tobacco chewers. Ethical clearance 25/IEC/BIMS/2013-13 and KIMS/IEC/2/2013 was obtained prior for the proposed study from the respective Institution and Informed consent was taken from the subjects included in the study. Urine samples were taken from day one of the conducting study.

Urine samples: Fasting urine samples were preferred from the subjects. The collected samples were transferred immediately to the laboratory at BIMS, Belagavi and frozen at –20 °C until analysis.

Extraction procedure of urine sample for HPLC analysis: Extraction of cotinine from urine sample was achieved using three important steps. **Step 1:** A 0.5 mL aliquot of individual urine sample was placed into a micro centrifuge tube with 100 μL 5.0 M NaOH, then vortex mixed for 1 min; **Step 2:** One milliliter

dichloromethane– diethyl ether (1:1 v/v) was added to the resulting solution mixture and vortex mixed again for 2 min. The mixture was centrifuged at 3,500 rpm for 3 min and the resulted organic layer formed was transferred to a new glass tube. **Step 3:** To this 20 μL of 0.25 M HCl was added and the organic phase was then evaporated under a stream of nitrogen at 45°C until dryness. Further it was dissolved in 100 μL of mobile phase consisting of a mixture of 0.272 g of KH_2PO_4, 0.184 g of sodium 1-heptane sulfonate, 820 mL of water, and 180 mL of methanol[19]. A 20 μL of extract from urine sample was injected into the HPLC system.

Preparation of standard: Standard cotinine (10 mg) was accurately weighed and dissolved in 10 mL of methanol. Working standard solutions from a range of 10, 25, 50, 125, 250, 500, 1000, 2000, 4000, 10000 ng/mL were prepared using the stock solution (1.0 mg/mL).

Reverse Phase - High - Performance Liquid Chromatography - Photo Diode Array (RP-HPLC-PDA) analysis: RP-HPLC apparatus consisted of a Shimadzu system (Model no. LC-20AD) equipped with SPD-M20A Photo Diode Array (PDA) detector. The chromatographic separations were performed on a Reverse Phase (RP) C18 column (Synergi™, 150 mm × 4.6 mm i.d., particle size 4 μm) manufactured by Phenomenex, USA. The column temperature was kept constant at 25 ± 2°C. Separations were performed in isocratic mode using a mobile phase consisting of a mixture of 30 mM citrate, 0.272 g of 30 mM KH_2PO_4, 1mM of heptane-1-sulphonic acid, sodium salt, and 50 mL of HPLC grade ACN. The pH of the mobile phase was maintained at 2.6. The flow rate used was 0.3 mL/min, and the wavelength was fixed at 262 nm.

GC-MS analysis: The analysis of the cotinine in urine sample by gas chromatography-mass spectrometry was carried out on Thermo Scientific Trace Ultra gas chromatograph interfaced with Thermo Scientific ITQ 1100 mass spectrometer. A column fitted with BP-1 (30 m × 0.25 mm i.d., 0.25-μm film thickness) was used and the oven temperature was programmed between 60-220°C at 3°C/min. using helium as a carrier gas at 1.0 mL/min. The injector temperature was 250°C and injection volume was 0.1 μL of 1% solution in methanol (split ratio 1:50). The mass spectra were taken at 70 eV with a mass scan range of 40-450 amu. The mass spectrometric parameters were those reported earlier[20,21].

Identification of cotinine was done on the basis of MS library search NIST 08 MS Library (Version 2.0 f; Thermo Fisher Scientific Austria) and WILEY MS 9th Edition

(Thermo Fisher Scientific Austria), and co-injection of authenticated samples of cotinine purchased from Sigma-Aldrich, India (≥98%purity).

Statistics: All statistical analyses have been performed by using means of different biochemical parameters using Microsoft Excel 2007 Software for Windows. Simple linear and logarithmic correlation analysis was used to indicate a measure of the correlation and the strength of the relationship.

Results

The cotinine concentration was analyzed by using the HPLC-DA methodology, statistical calculation was done by Microsoft Excel 2007 Software for Windows. The estimated mean Retention time and mean cotinine maximum and minimum values were expressed from STC & NTC urine samples. P-value showed extremely significant relationship with STC & NTC cotinine concentrations. (Table.1)

Cotinine was quantitatively estimated using RP-HPLC method and the results were expressed in ng/mL urine sample. Clear and sharp peaks for standards (Figure 1a) and urine samples (Figure 1b) were obtained during the analysis. Calibration curves were constructed correlating the detector signals with the corresponding cotinine concentrations (ng/mL). A 10 point calibration curve was obtained using different concentrations of cotinine in triplicates. Linearity for the results was carried out using the least-square method, revealing good linear fit in the concentration range. The attributes and results for RP-HPLC analysis of standard cotinine such as concentration range, linearity equation, and coefficient of regression, limit of detection and limit of quantification are presented in Table 2. The high R^2 value was indicative for the good linearity, and the low values of standard deviations of the intercept and slope were indicative for the significant.

Table 1. Retention time (RT in min) along with cotinine content (μg/mL) determined in urine samples using RP-HPLC analysis.

	STC*	NTC**
RT mean value	9.49 ±1.55	9.39 ±0.269
Min. Value	100.84	10.01
Max. Value	5810.7	59.02
Cotinine mean concentration (ng/mL)	1563.34 ±1199.4	23.48 ±11.08
P. value	<0.001***	

*STC= Smokeless tobacco chewer,

NTC=Non-Tobacco chewer,

***Comparison of STC & NTC showed extremely significant p value

Table 2. RP-HPLC analysis attributes and results obtained for cotinine

Parameters	Cotinine
Concentration range (ng/mL)	10 - 10000
Linearity equations	y = 118.8761x - 7453.8158
Coefficient of Regression (R²)	0.9952
LOD (ng/mL)	0.081 ± 0.004
LOQ (ng/mL)	0.245 ± 0.012

Validity of the calibration points were used for constructing calibration curve. Precision was
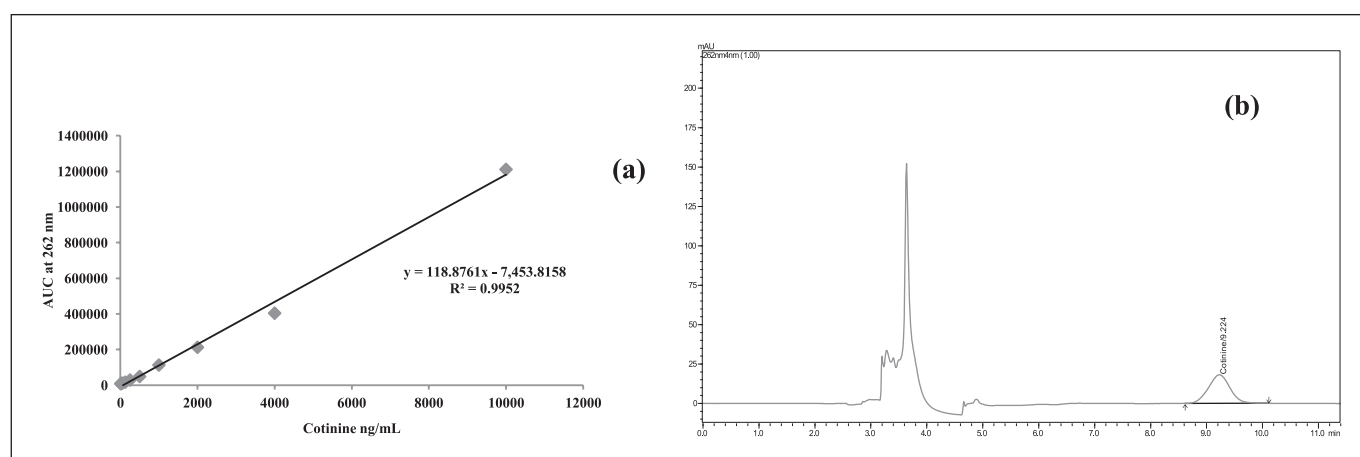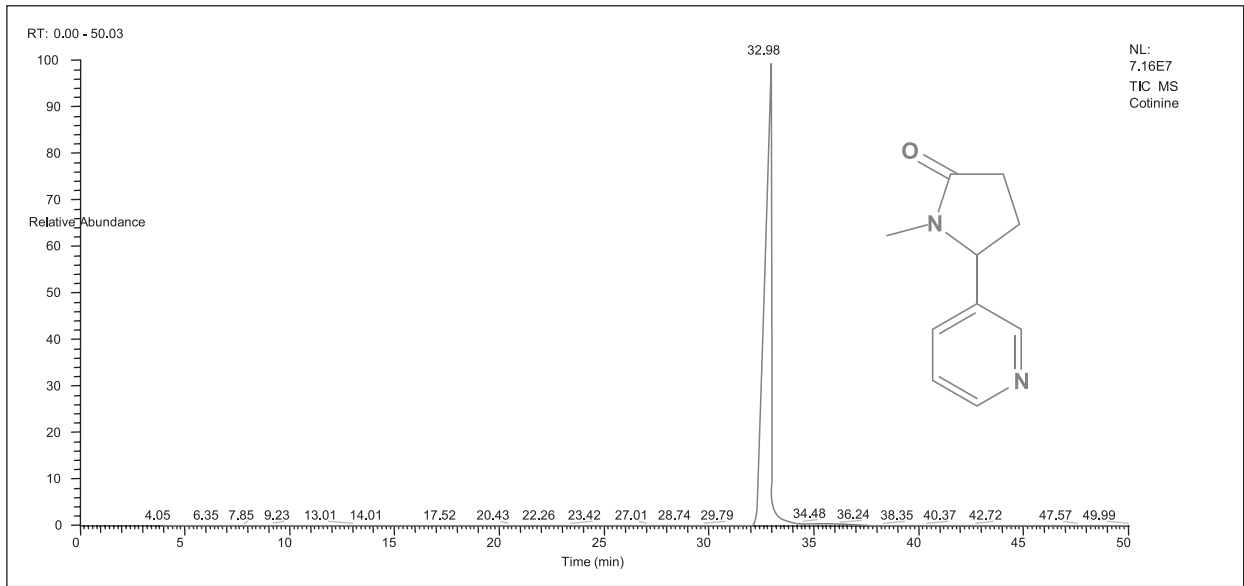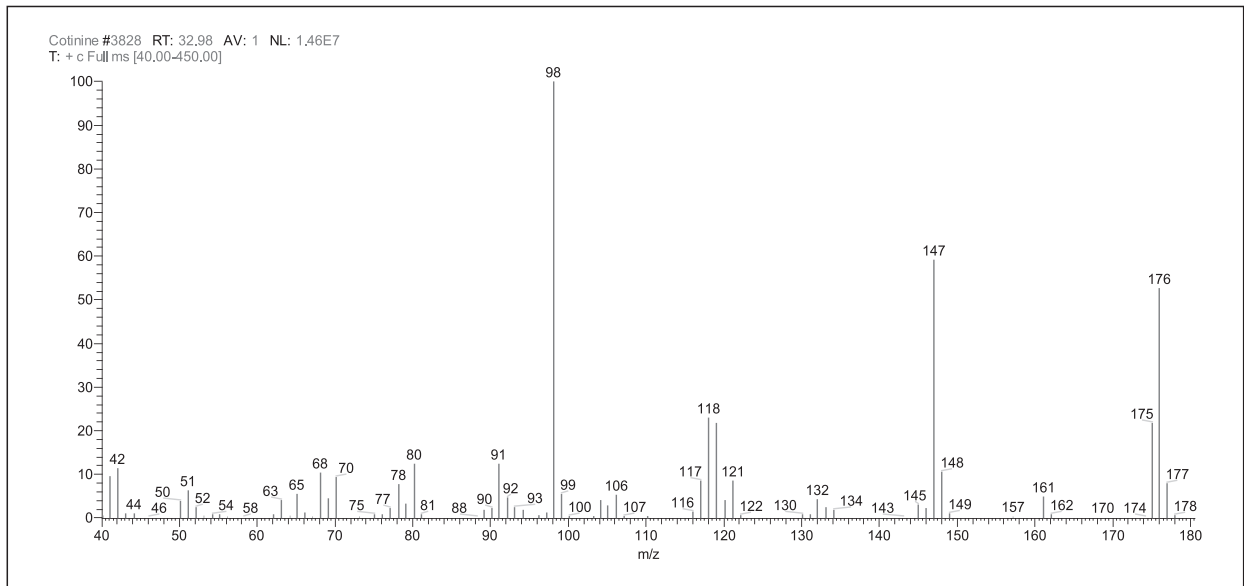

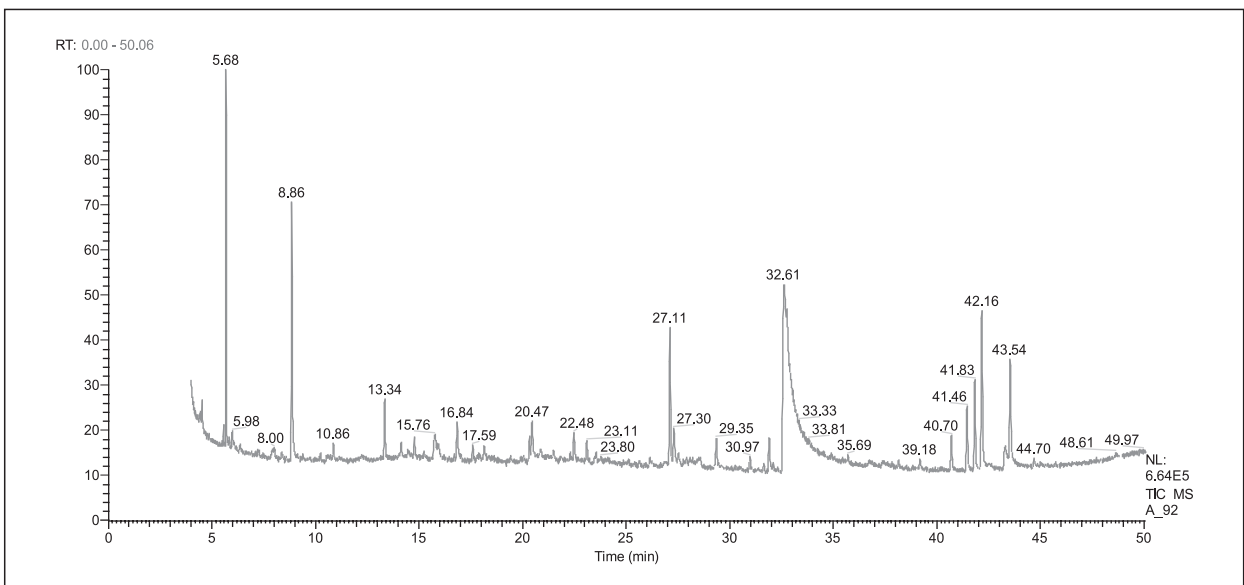**Figure 1. RP-HPLC profiles of - a: Calibration curve; b: Standard cotinine (2000 ng/mL)**
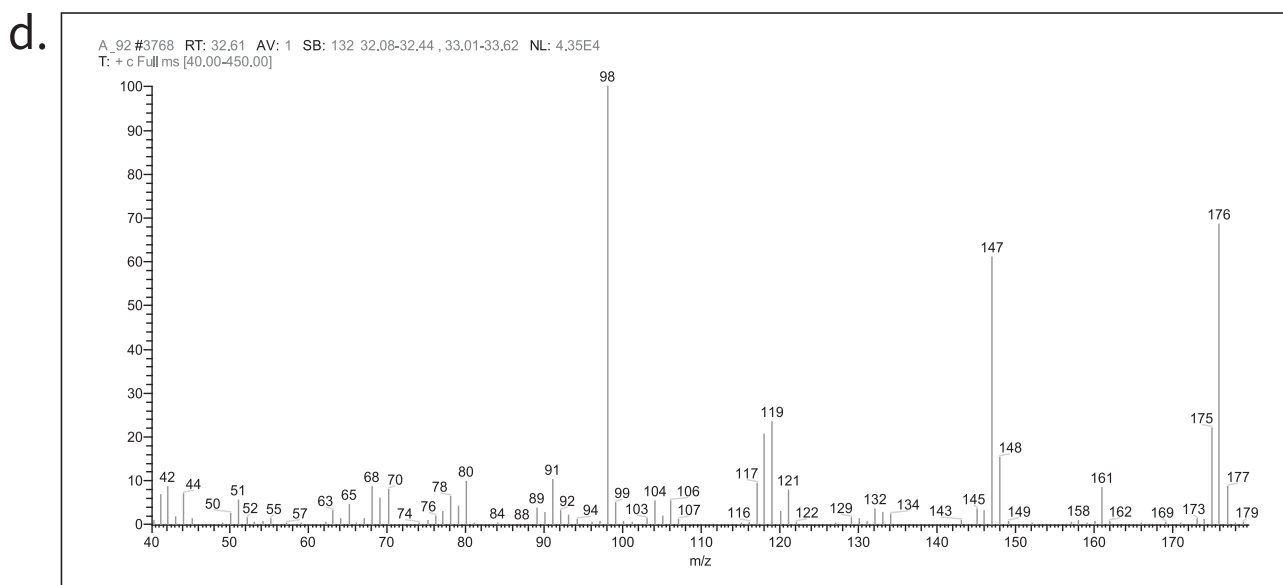
a.

b.

c.

d.

Figure 2 a: GC-TIC chromatogram of standard cotinine; b: Mass spectra and structure of cotinine; c: GC-TIC chromatogram urine sample C15; d: Mass spectra and structure of urine sample C15

calculated by injecting 3 consecutive cotinine standard at three varying concentrations (500, 2000, and 4000 ng/mL). The relative standard deviation (RSD) of the signal values did not exceed 4.072%. Inter-day precision (reproducibility) was determined by measuring inter-day variation for triplicate determination of cotinine at the same concentrations. The lower RSD values (≤5.167%) indicated the acceptable reproducibility of the method. Tailing factor (T) for cotinine at 500 ng/mL concentration, was found to be 1.104, with good peak symmetry. The theoretical plate number (N) was found to be 1937.276 for the column used in the study (150 mm × 4.6 mm i.d., particle size 4 μm), demonstrating acceptable column efficiency.

Profiles with retention time of 9.334 ± 0.220 minutes for standards and samples were obtained. Same chromatographic system was used for estimating cotinine from samples. Auto generated chromatograms of samples were obtained as final output of the study. In present study, cotinine values in smokeless tobacco consumers (STC) ranged from 100.84 to 5810 ng/mL urine sample and 10.01 to 59.02 ng/mL urine sample in non tobacco consumers (NTC). The detection of the cotinine in the urine samples of NTC could be due to the contribution of dietary cotinine from different food sources[22] or passive smoking[23]. The GC-TIC analysis of standard cotinine and its mass spectra is represented in Fig. 2a and b. Presence of cotinine in urine sample by GC-MS is showed in Fig. 2c and d. The cotinine values obtained from RP-HPLC-PDA analysis were directly related to their smokeless tobacco chewing habits.

Discussion

In India, tobacco is consumed both in smoking and non-smoking forms. Smoking forms include cigarette, bidi, hukka and chutta. Tobacco chewing is the main non-smoking form of tobacco use. A number of biochemical markers like thiocynate, nicotine, cotinine and carbon monoxide in the expired air and carboxyhaemoglobin in blood have been used to validate claims of non-smoking. Among these cotinine is possibly the best marker for situations where accuracy is paramount. Cotinine is a major metabolite of nicotine but its level in the blood is not a good marker of nicotine content of blood. In contrast, urinary excretion of cotinine is a good marker as it is less influenced by the flow of urine and pH[23].

Urinary cotinine has been recommended as a quantitative measure of nicotine intake. Several analytical techniques for the determination of urinary cotinine are described in the literature. These include gas chromatography (GC), enzyme linked immunosorbent assay, liquid chromatography (LC)–mass spectrometry (MS)–MS, and high-performance liquid chromatography (HPLC) with UV detection[24]. The modified methods used in the study are applicable and reliable for the determination of cotinine in urine using HPLC and GC–MS. This method has good results regarding (Limit of detection) LOD, (Limit of Quantification) LOQ, correlation coefficient, %R.E (Absolute Mean bias)., %RSD (Relative Standard Deviation). Similar methodology and results were quoted in other reported article[26, 27, 28, 29, 30,31].

According to the results obtained by our study, the comparison table no.3 shows the sample volume considered was similar to that of other studies i.e. 0.1mL and the Limit Of Quantification (LOQ) standardized, was 0.24 ng/mL, which correlates with Andrew N. Hoofnagle and et al study (2006). The R2 value for our study showed similar positive correlated values with other studies. The run time for the analysis of urinary cotinine in the present study was 9.49 minutes, which was similar to the previous study (7.5,14,8.31,16,6.5 minutes) The concentrations of cotinine in urine showed higher levels in tobacco Chewers around 100.84 – 5810 ng/mL and in non tobacco chewers 10.01- 59.02 ng/mL respectively. The average calculated for the STC and NTC were 1563.34 and 23.48 ng/mL, Moreover same cotinine concentrations were also in observed in other studies[26, 27, 28, 29, 30,31]. The variation in range of cotinine depends on the tobacco chewer's dietary intake of nicotine, cotinine excretion, metabolic activity, passive smoking and environmental smoke[23].

Table 3. Comparison with Other Methods Using HPLC-PDA and GC-MS

Parameters	Present study	Moyer et al[30]	Hoofnagle et al[27]	Xu Xu[28]	Massadeh[26]	Yen-Hsia Wen[29]
Biological fluids	Human Urine	Human Urine	Human Urine	Human Urine	Human Urine	Human Urine
Sample volume (mL)	0.1	0.1	0.1	0.1	0.5	0.1
LOQ (ng/mL) cotinine/urine	0.24	0.1	0.25	0.1	0.4	0.1
R2	0.9952	0.999	0.9971	0.9983	0.9986	0.999
Run time (min)	9.49	7.5	14	8.31	16	6.5
Cotinine range ng/mL	10.01-5810	73–6680	5.5-4,920	0.2–4000	36–3946	551-2631

These biomarkers have also been used in epidemiological studies, to assess the effects of tobacco use on human health. While studies on nicotine and cotinine levels in cigarette smokers as well as those for passive smoking in other ethnic groups are well documented, information on tobacco used in non smoking forms (tobacco chewing) is lacking[23]. In the present study, we estimated the amount of cotinine excretion in urine in a group of healthy individuals from Belagavi region who were users of tobacco in different forms and also in nonsmokers and compared the values amongst tobacco users showed Significant p value ($p < 0.001$) which is consistent with the reported urinary levels of cotinine levels are being found in previously reported articles[23, 31]. The mean values of the determined cotinine metabolite concentration for tobacco chewer are consistent with reported concentrations in smokers' urine (Adnan M. Massadeh et al[26]).

Finally, we must note that further evaluation of this method with urine samples relevant to active and passive tobacco chewing will be helpful, our results indicate that cotinine may be useful biomarker for identifying and validating the smokeless tobacco status in Indian population.

Conclusion: Determination of cotinine concentration levels from urine sample of STC and NTC by HPLC-DA and conformation of cotinine metabolite by Gas Chromatography-Mass spectrometry (GC-MS) serves as useful marker to determine the effects of different forms of tobacco consumption. Thus, we conclude that, our paper describes a simple, sensitive, and rapid HPLC method for cotinine determination in urine may be useful to assess the effect of different types of tobacco use in our population.

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