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Determination of nicotine and cotinine in tobacco harvesters' urine by solid-phase extraction and liquid chromatography

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Abstract

A solid-phase extraction method using Drug Test-1 column containing chemically modified silica as a solid support for sample clean up and reversed phase ion-paired high-pressure liquid chromatography method have been developed for the simultaneous determination of nicotine and its metabolite cotinine from the urine samples. Mobile phase was consisted of acetate buffer (containing 0.03 M sodium acetate and 0.1 M acetic acid) pH 3.1 and acetonitrile (78:22% (v/v)) containing 0.02 M sodium octanosulfonate as an ion pair agent. pH of the mobile phase was adjusted to 3.6 with triethylamine for better resolution and to prevent peak tailing. The linearity was obtained in the range of 0.5–10 μg/ml concentrations of nicotine and cotinine standards. The correlation coefficients were 0.998 for cotinine and 0.999 for nicotine. The recoveries were obtained in the range of 79–97% with average value of 85% for nicotine and in the range of 82–98% with average value of 88% for cotinine. The limit of detection was 2 ng/ml for cotinine and 5 ng/ml for nicotine with 2 ml urine for extraction, calculated by taking signal to noise ratio 10:3. The intra-day co-efficient of variation (CV) were <4 and 7% and inter-day CV were <9 and 7% for nicotine and cotinine, respectively. The method was applied to the urine samples of tobacco harvesters, who suffer from green tobacco sickness (GTS) to check the absorption of nicotine through dermal route during the various processes of tobacco cultivation due to its good reproducibility and sensitivity.

Keywords: Nicotine; Cotinine

1. Introduction

Several biological markers have been proposed for the assessment of direct or passive exposure to tobacco or tobacco smoke. Measurements of nicotine and cotinine in biological fluids have become an important component of behavioral studies on the role of nicotine in smoking [1,2]. These biochemical markers have been used to estimate active smoking behavior, to validate abstinence smoking, to evaluate exposure to environmental tobacco smoke [3] and to monitor tobacco withdrawal by substitution treatment [4,5]. Number of analytical methods have been developed during the last few years for determination of these biochemical markers of active and passive smoking from urine, saliva and plasma. Majority investigators preferred urine as a sample instead of plasma and saliva, because urine sample is much easier to obtain and concentrations of nicotine and cotinine in urine are higher than in plasma and saliva [6,7]. Moreover, cotinine is more stable than its parent component, nicotine. Matsushita et al. [8] reported that urinary cotinine elevation after active smoking lasted for 60 h. Many investigators have reported Green Tobacco Sickness (GTS), an occupational illness in the field workers during various processes of tobacco cultivation. This has been attributed to the acute nicotine poisoning resulting from the absorption through the dermal route. They observed the elevated levels of nicotine and its major metabolite cotinine in the urine of the tobacco harvesters [9–11]. Number of methods has been developed for the determination of nicotine and cotinine in urine, but currently most often used analytical methods are gas chromatography [12] and HPLC [13–23]. Some investigators have established reversed phase ion-paired liquid chromatography for the analysis of basic analytes, alkaloids in body fluids [24–26] and nicotine in commercial tobacco products [27].

Pretreatment of samples prior to any instrumental analysis is very important. It is necessary for selective characterization or confirmation of the analytes. Solid-phase extraction (SPE) technique has become the most powerful technique

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currently available and it offers a faster, high quality and cost effective sample preparation technology [3,15,27–32].

In this paper, we have described solid-phase extraction technique using Drug Test-1 column for the clean up of urine sample and a simple and sensitive reversed phase ion-paired liquid chromatographic method using PDA detector. Drug Test-1 is an indigenous SPE silica column. It bears multiple properties such as cation exchange, hydrophobic and polar activities. It selectively retains and elutes the analytes by mixed mode interaction mechanism. Due to the multiple interaction properties nicotine and cotinine are retained onto the column almost quantitatively, there is no loss during rinse steps, and it requires very small amount of elution solvent and also we can work with as low as 2 ml of sample. This method is applied to determine the concentrations of nicotine and cotinine simultaneously in the urine samples of tobacco harvesters.

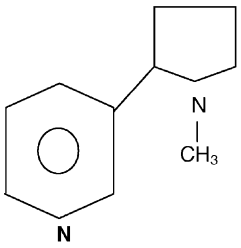
2. Experimental

2.1. Reagents and standards

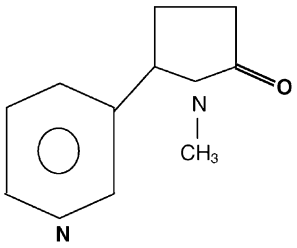
Acetonitrile (HPLC grade from E. Merck, India), methanol, glacial acetic acid, dichloromethane (DCM), isopropanol (IPA), ammonia (NH_3; HPLC grade) and sodium acetate (Excelar grade) from Qualigenes Fine Chemicals (India) and HPLC water (Thomas Baker, India) were used through out the study. Nicotine and cotinine drug standards in methanol were obtained from Sigma (USA). 2-Phenylimidazole, sodium octanesulfonate and triethylamine (TEA) were procured from Aldrich (USA). All liquids used for experiments were filtered through 0.22 μm membrane filters from Millipore (USA).

Stock solutions of nicotine and cotinine were prepared at a concentration of 100 μg/ml in methanol and 2-phenylimidazole as internal standard (IS) at a concentration of 10 μg/ml in methanol. These stock solutions were stored at −20 °C until used in assay.

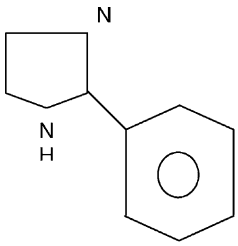
The chemical structures of nicotine, cotinine and 2-phenylimidazole are given below.



Nicotine



Cotinine



2-phenylimidazole

The buffer for the mobile phase was an aqueous acetate buffer containing 0.03 M sodium acetate and 0.1 M glacial acetic acid at pH 3.1.

Drug Test-1 columns from Analchem Ltd., Allahabad, India, were used for the extraction of urine samples. This column contains chemically modified silica as a solid support. Phase mass/column volume are 130 mg/ml and 3 ml, respectively. This is a mix mode sorbent column and the nature of this column is a combination of hydrophobic, hydrophilic and cation exchange.

EnvirEx column is a chemically modified silica gel having C18 group on the surface was used for the preparation of blank urine. Its heavily loaded C18 surface suited for purification.

2.2. Instrumentation

Chromatographic analysis was performed on a Shimadzu, Japan LC-10AVP System, consisting of binary gradient pumps, a Rheodyne manual injector with 20 μl loop, thermostated column oven and PDA detector.

The stationary phase was a Shimpack C18 ODS stainless steel column (25 cm × 4.6 mm i.d., 5 μm particle size). The system was monitored by class VP software (version 6.12 SP4).

2.3. Sample collection

Urine samples were collected from tobacco harvesters after the completion of work shift and preserved at −40 °C till used for assay.

2.4. Preparation of blank matrix

Twenty milliliter of urine sample from non-smoker, non-exposed subject cleaned up by passing through EnvirEx Column, preconditioned with 20 ml methanol and 20 ml HPLC water. This urine was used as a blank matrix for all validation parameters.

2.5. Extraction of samples

The Drug Test-1 column were connected to a Vac elute—a vacuum manifold (Varian, USA) conditioned with 2 ml of methanol followed by 2 ml phosphate buffer pH 6.0. A mixture of 2 ml urine sample of tobacco harvesters and 1 ml of phosphate buffer spiked with 0.1 ml of 0.5 μg/ml

2-phenylimidazole (IS) was passed through the conditioned Drug Test-1 column at a slow flow rate (1 ml/min) by applying vacuum. The column was rinsed with 2 ml HPLC water and dried under full vacuum for 3–5 min. The analytes were eluted slowly with 2 ml of DCM–IPA–NH_3 mixture (96:2:2% (v/v)) in labeled collection tubes kept in a rack in Vac elute. The organic phase was dried under the slow stream of N_2 at 40 °C and reconstituted in 100 μl of mobile phase.

2.6. Assay condition

Mobile phase consisted of acetate buffer (0.03 M sodium acetate and 0.1 M glacial acetic acid) and acetonitrile (78:22% (v/v)), containing 0.02 M sodium octanesulfonate as an ion pair agent. The pH of the mobile phase was adjusted to 3.6 with triethylamine to prevent the co-elution of caffeine with cotinine and to minimize the problem of peak tailing. The mobile phase was degassed by filtration under reduced pressure with glass filter assembly using HAWP filter (0.22 μm) from Millipore followed by ultrasonication in transonic digital ultrasonic cleaning bath (ELMA, Germany) for 15 min.

The PDA detector was monitored at 259 nm wavelength and column was kept at 40 °C in column oven. The flow rate was 1.00 ml/min.

2.7. Linearity

Calibration curves were prepared by processing various concentrations, i.e. 0.5, 1, 1.5, 2.0, 2.5, 5.0 and 10 μg/ml of working standard of nicotine and cotinine prepared by diluting stock standards in mobile phase. An amount of 0.1 ml of each concentration was spiked in mixture of 2 ml blank urine and 1 ml phosphate buffer (pH 6.0) and processed it through conditioned Drug Test-1 column. Eluent was concentrated under nitrogen stream. Twenty microliter from each concentration was injected into HPLC. The peak areas of nicotine and cotinine were plotted versus the original concentrations and evaluated by linear least square regression analysis.

2.8. Recovery and reproducibility

The recovery of nicotine and cotinine was measured under the extraction conditions described above. The recovery was calculated by comparing the peak area ratio of the spiked standards with those of standards injected directly without extraction.

Reproducibility of the method was checked by intra- and inter-day variations. Three different concentrations 2.5, 5.0 and 10.0 μg/ml were processed and checked the intra-day variations by injecting five times the same concentrations on the same day, while inter-day variations were checked by running the three standards everyday for 5 days.

2.9. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection and limit of quantification for nicotine and cotinine were calculated by the VP Software programme provided by Shimadzu. The LOD and LOQ values were calculated by considering signal to noise ratio 3.3 and 10, respectively.

3. Results and discussion

Fig. 1 shows the chromatograms of extracts of urine blank, blank urine spiked with nicotine, cotinine and 2-phenylimidazole. The total run time was 18 min with cotinine eluting at 4.9 min, nicotine at 9.1 min and 2-phenylimidazole at 16.9 min. There is no peak in the extract of non-exposed nonsmokers' urine at the time of elution of cotinine and nicotine.

3.1. Linearity

Calibration curve was prepared by processing mixture of blank urine and PO_4 buffer spiked with various concentrations of nicotine and cotinine standards over the range of 0.5–10 μg/ml. The calibration curves were linear over the range of 0.5–10 μg/ml.

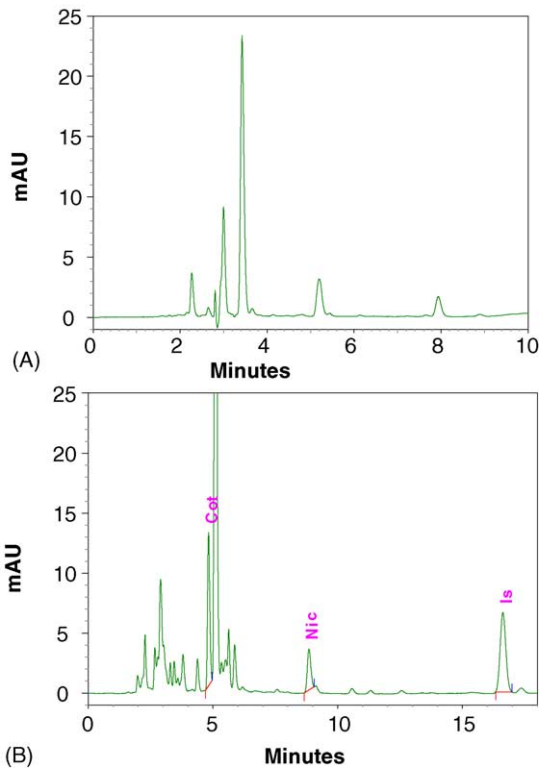


Fig. 1. Chromatograms of extract of: (A) blank urine and PO_4 buffer; (B) mixture of blank urine and PO_4 buffer spiked with 2.5 μg/ml cotinine, 2.5 μg/ml nicotine and 0.5 μg/ml of 2-phenylimidazole.

Table 1
Calibration data of nicotine and cotinine

Compound	Concentrations (μg/ml)	No. of experiments	Correlation coefficient (*r*)
Nicotine	0.5–10	5	0.999
Cotinine	0.5–10	5	0.998

The correlation coefficient (*r*) for cotinine was 0.998 and for nicotine it was 0.999. The results are given in Table 1.

3.2. Recovery and reproducibility

The recovery of nicotine and cotinine was measured under the extraction conditions described above. The recovery for the nicotine was in the range of 79–97% with the average value of 85% and it was in the range of 82–98% with the average value of 88% for cotinine. The reproducibility of the method was demonstrated by repeated injections of urine spiked with nicotine and cotinine standards. Five daily injections over a 5-day period gave intra-day coefficient variation (CV) below 7% for cotinine and below 4% for nicotine while inter-day CV was below 7% for cotinine and below 9% for nicotine (Tables 2 and 3).

3.3. Limit of detection and limit of quantification

The LOD values were 2 and 5 ng/ml and LOQ values 6 and 17 ng/ml for cotinine and nicotine, respectively, as calculated by signal to noise ratio with the help of VP software programme provided by Shimadzu Ltd., Japan.

Table 4
Concentrations of nicotine and cotinine in the urine samples of tobacco harvesters

Subjects with smoking habits	Concentrations of nicotine (mean ± S.E.M., μg/ml)	Concentrations of cotinine (mean ± S.E.M., μg/ml)
Non smokers (10)	1.60 ± 0.59	3.36 ± 0.79
Smokers (10)	5.40 ± 1.71	10.28 ± 3.25
Chewers (10)	2.22 ± 0.48	5.87 ± 0.66
Snuff users (10)	2.12 ± 0.47	3.87 ± 0.73

Figures in parentheses indicate number of subjects.

3.4. Application of method

This method of solid-phase extraction and reversed phase ion-paired liquid chromatography was applied to determine the concentrations of nicotine and its major metabolite cotinine in the urine samples of the tobacco harvesters.

Many investigators reported that the absorption of nicotine takes place through dermal route among tobacco harvesters as a consequence of occupational contact with tobacco leaves [33–36]. The levels of nicotine and cotinine in their urine samples collected after the work shift were reported in Table 4. The workers were classified according to their smoking habits. Majority of the women workers were nonsmokers and occasional snuff users. The concentrations of nicotine and cotinine were found higher among smokers followed by chewers; snuff users and nonsmokers tobacco harvesters. Some investigators have reported

Table 2
Recovery and reproducibility of nicotine and cotinine from spiked sample (intra-day variations)

Compound	Spiked concentrations (μg/ml)	Actual recovered (mean ± S.D., μg/ml)	Recovery (mean ± S.D., %)	Intra-day CV (%)
Nicotine (5)	2.5	1.94 ± 0.07	77.60 ± 2.61	3.4
	5.0	3.99 ± 0.14	79.80 ± 2.86	3.6
	10.0	8.50 ± 0.31	85.00 ± 3.08	3.6
Cotinine (5)	2.5	2.34 ± 0.05	93.60 ± 2.19	2.4
	5.0	4.14 ± 0.05	82.80 ± 1.10	1.3
	10.0	8.92 ± 0.56	89.20 ± 3.08	6.3

Figures in parentheses indicate number of experiments.

Table 3
Recovery and reproducibility of nicotine and cotinine from spiked sample (inter-day variations)

Compound	Spiked concentrations (μg/ml)	Actual recovered (mean ± S.D., μg/ml)	Recovery (mean ± S.D., %)	Inter-day CV (%)
Nicotine (5)	2.5	2.10 ± 0.17	84.12 ± 6.93	8.1
	5.0	4.21 ± 0.34	84.24 ± 6.70	8.1
	10.0	8.78 ± 0.56	87.80 ± 5.60	6.4
Cotinine (5)	2.5	2.19 ± 0.13	87.52 ± 5.05	5.9
	5.0	4.40 ± 0.22	88.04 ± 4.42	5.0
	10.0	8.91 ± 0.62	89.12 ± 6.18	6.9

Figures in parentheses indicate number of experiments.

the levels of nicotine and cotinine in the urine of exposed workers exceed those of novice smokers, who had smoked three cigarettes in succession [9,37]. In this study, urine samples were selected because the concentrations of nicotine and cotinine in urine are 10–100-fold greater than the concentrations in plasma and saliva [7,39]. According to Jarvis et al. [7] and Keller-Stanislawski-Kellerm et al. [39], after environmental exposure, the average nicotine and cotinine levels in saliva, plasma and urine of nonsmokers varies from 0.5 to 4.0 μg/ml, whereas the average amount of nicotine in the serum of cigarette smokers ranges from 15 to 40 μg/ml and lies between 500 and 2000 μg/ml in saliva and urine. Cotinine concentration varies from 150 to 350 μg/ml in plasma, from 150–400 μg/ml in saliva and can go up to 2000 μg/ml in urine. D'Alessandro et al. [38] from Italy reported that tobacco harvesters absorbed approximately 0.8 mg of nicotine daily. Our results also showed 1.6–5.4 μg/ml of nicotine and 3.36–10.28 μg/ml of cotinine in the urine samples of tobacco harvesters. The approximate half-life of nicotine is 2–2.2 h and cotinine has approximate 10–20 h. However, after dermal application of nicotine the apparent half-life may double to 4–5 h [39].

The present method of solid-phase extraction using Drug Test-1 column offers a simplified, faster, high quality and cost effective sample preparation procedure. The concept of mixed mode consists in using combined mode of sorption usually reversed phase and cation exchange. The mechanism of isolation is both low energy (reversed phase) and high energy (ion exchange). One mechanism retains the solutes and interferences but only the analyte is retained by the second mechanism and interferences may be eluted from the SPE sorbent. One of the major applications of mixed mode SPE is in the isolation of drugs and basic metabolites, from urine and blood samples [40]. It also improves the reproducibility of analysis.

The reversed phase ion-paired liquid chromatography method improves procedure for the simultaneous determination of nicotine and cotinine levels from the urine samples of tobacco harvesters with suitable resolution, sensitivity, recovery and reproducibility. The high concentration of acetic acid (0.1 M) in the acetate buffer improved the separation of nicotine and cotinine. The addition of 0.02 M sodium octanesulfonte as an ion pair agent to the mobile phase has increased the reproducibility of the separation and improved the resolution of cotinine from the caffeine. Addition of 0.01 M TEA in mobile phase increased the sharpness of the peak and minimized the problem of peak tailing [24,40].

Our results show that solid-phase extraction with Drug Test-1 column offers quick, simple and cost effective extraction technique and reversed phase ion-paired liquid chromatography permits simultaneous determination of nicotine and cotinine from the urine samples of tobacco harvesters with suitable resolution, sensitivity, recovery and reproducibility.

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