Evaluation of Dried Urine Spot Method to Screen Cotinine among Tobacco Dependents: An Exploratory Study

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ABSTRACT

Background and Objectives: Assessment of cotinine, a metabolite of nicotine in body fluids, is an important approach for validating the self-report among tobacco users. Adaptation of assays on dried urine spots (DUSs) has advantages of ease of collection, transportation, minimal invasiveness, and requirement of small volume. The aim of the present study was to develop an efficient method for testing cotinine in DUSs and evaluating its clinical applicability. Methods: This involved optimization of conditions for detection, recovery, and stability of cotinine from dried urine, spotted on filter paper. Enzyme-linked immunosorbent assay was used for screening, whereas confirmation was done by gas chromatography. For clinical applicability, urine samples of tobacco users were tested. Results and Interpretation: Water was found to be a suitable extracting solvent as compared to carbonate-bicarbonate buffer (pH 9.2) and saline. Screening was achieved by two punches taken from a 20 µl (diameter 1.3 cm) spotted urine samples, and confirmation was achieved by five complete circles each of 20 µl sample volume. The recovery was found to be 97% in water. Limit of detection for the method was found to be 100 ng/ml. No signs of significant degradation were found under all storage conditions. All the urine samples of tobacco users were found to be positive by a conventional method as well as DUSs, and the method proved to be efficient. Conclusions: DUS samples are a useful alternative for biological monitoring of recent nicotine use, especially in developing countries where sample logistics could be an important concern.

Key words: Cotinine, dried urine spot, enzyme-linked immunosorbent assay, gas–liquid chromatography, nicotine, urine

INTRODUCTION

Tobacco dependence is a global pandemic and a major cause of morbidity and preventable mortality.\(^1\) Prevalence of tobacco use is higher in low- and middle-income group of countries. In India, the overall prevalence of tobacco use among adults is 35%, of whom 21% use smokeless tobacco, 9% smoking, and 5% use a combination of both.\(^2\)

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A recent nationwide study on tobacco-associated deaths in India reported smoking to cause 1 in 5 deaths in men (aged 30–69 years) and estimated to cause 1 million deaths annually.\[13] Smoking is reported to be higher and heavier in drug using population\[4] including drug abuse treatment settings.\[14,15] Therefore, it is very important to develop interventions that can reduce and prevent tobacco use.

Nicotine, a natural alkaloid 3-[(1-methyl-2-pyrrolidinyl) pyridine present in tobacco leaves, is considered to be the major psychoactive and dependence-producing substance in tobacco products.\[7] Like other drugs of abuse, chronic consumption of nicotine has been shown to produce both tolerance and dependence in humans.\[8–10]

National Drug Dependence Treatment Centre (NDDTC) of All India Institute of Medical Sciences (AIIMS), New Delhi, has two community clinics and various programs running all over the country. Screening of drugs of abuse of the patients belonging to remote areas is to be considered the exercise fraught with many logistic problems. Furthermore, to minimize variations, it is always advisable for analysis to be carried out in one centralized laboratory. Transportation to a distant laboratory involves problems such as the requirement of trained staff, sample spillage, breakage, cross-contamination, and shipment in cold, adds to the cost. A suitable transport system that circumvents these problems is, therefore, a prerequisite for analysis at a centralized laboratory.\[11]

Dried stains spotted on filter paper have several advantages over liquid samples for screening/diagnosis. This is ideally suited to countries like India which have limited health budgets and where sampling and handling theoretically vary widely, making storage and transportation of samples difficult. Urine stains (spotted filter paper) proved to be a viable means for storing samples for drug screening. There is a paucity of literature regarding the usage of urine spotted dried samples for screening drug of abuse in drug addicts from field settings. Till date, only a few reports have been published for drug abuse testing in urine spotted onto filter paper.\[12,13] In general, drug abuse testing is a two-stage process, i.e., primary screening followed by confirmation. Thus, there is a need to carry out such studies in this regard. In view, it was considered worthwhile to undertake the current study. The aim of the present study was to develop a simple, efficient, and low-cost method for testing cotinine in dried urine spots (DUSs) and evaluating its clinical applicability.

**MATERIALS AND METHODS**

**Chemicals**

All the reagents used were of analytical grade and were obtained from Merck except cotinine standard which was obtained from Sigma-Aldrich. Whatman Filter paper 903 was obtained from GE Healthcare India.

**Apparatus**

Primary screening was performed by enzyme-linked immunosorbent assay (ELISA) technique (Tecan GENios ELISA reader, Austria GmbH, Austria) using Magellan software. Further confirmation was done on gas chromatograph model 7890A, Agilent India Pvt. Ltd., USA, equipped with 7683B series Autosampler; split/splitless inlet, fused silica capillary column coated with HP-5 cross-linked 5% diphenyl and 95% dimethylpolysiloxane (30 m × 0.320 i.d., 0.25 μm film thickness); and nitrogen phosphorus detector (NPD) with electronic pneumatic control. System control, data acquisition, and analysis were performed with gas chromatography (GC) ChemStation G2075BA software.

**Urine samples**

Urine samples were collected from fifty tobacco-dependent participants seeking treatment at NDDTC, AIIMS, New Delhi, India. Drug-free urine samples were obtained from laboratory staff volunteers.

**Experimental design**

DUSs were prepared from supplemented urine spotted onto the Whatman filter paper 903 by pipette in 20 μl aliquots. To establish an efficient method for the extraction of cotinine from filter paper, three different solvents (deionized water, carbonate-bicarbonate buffer, and saline) were compared concerning their extraction efficiency. Final analyses for urinary cotinine were performed on DUSs and urine sample of nicotine users using ELISA technique and GC technique.\[16]

The study was carried out in accordance with the Declaration of Helsinki and written informed consent was taken from all the patients. The study protocol was approved by the Institutional Ethics Committee of AIIMS, India. The study was completed in 1-year duration (June 2013–June 2014).

**Standardization of conditions for extraction and analysis of cotinine from dried urine spotted onto Whatman filter paper (903)**

The following steps were involved as under:

**Preparation of spiked standards**

Urine samples with zero baseline drug level were spiked with various concentrations (1, 10, 50, and 100 μg/ml) with cotinine standard. Stock standard of cotinine was prepared at the strength of 100 μg/ml and was serially diluted to 50, 10, and 1 μg/ml.

**Spotting of standard**

The spiked urine standards of cotinine were spotted (20 μl each) onto a marked circle (diameter 1.3 cm)
of filter paper (Whatman 903) kept on a nonabsorbent surface. These standards were air dried overnight at room temperature.

**Optimal elution and recovery of the drugs from filter paper**

Optimal conditions were worked out to check the maximum recovery of drug from filter disc using different eluting solvents, temperature, and time conditions. Briefly, dried filter papers with spiked cotinine standards were punched manually using a manual puncher of diameter 3.2 mm. Punches were obtained in various numbers and were preceded for cotinine elution in three different solvents (deionized water, saline, and sodium carbonate-bicarbonate buffer, pH 9.2). Elutes were tested for the presence of cotinine using COT One-step Cotinine Test Device (Cutoff 200 ng/ml, Confirm Biosciences). The detection of cotinine was further checked with ELISA kits (Calbiotech Pvt. Ltd., USA) onto ELISA reader. This technique is based on the principle of competitive binding immunoassay. Assay was carried out as directed by the manufacturers. Urinary cotinine concentrations corresponding to the optic densities which were calculated using standard curve, and the results were expressed in units of nanogram per milliliter. The sensitivity limit of cotinine assay was 1 ng/ml. After the optimization of the number of punches, selection of the suitable eluting solvent with different volumes was worked out. Thereafter, the extraction was carried out at different time intervals in a water bath shaker at 37°C. Moreover, the standard curve was prepared using quantitative ELISA technique, from direct as well as filter paper extracted drugs to observe the recovery at various conditions. Thus, analysis of the elute was done by ELISA (Quantitative) for the extraction of spiked urinary cotinine standard spotted on filter paper and recovery was calculated, based on a comparison between the standard and the extracted cotinine from filter paper. Limit of detection (LOD) and intra- and intra-assay precision (% coefficient of variation [CV]) were calculated. The within-day precision was done on three replicates of each concentration, whereas between-day precision was carried out for 3 consecutive days 3 times of the each concentration. The within-day and between-day precisions were expressed in relative standard deviation (CV).

**Screening and confirmation of clinical samples**

To investigate the feasibility of using filter paper in a clinical setting for drug screening, fifty urine samples (30 ml of each) were collected from patients coming to the outpatient department of NDDTC, AIIMS. Comparison of urinary cotinine concentrations was done in urine samples collected by conventional method and urine extracted from filter paper. Collection was done after obtaining the informed consent from the patients as per the inclusion and exclusion criteria. Patients fulfilling the International Classification of Diseases-10 criteria for harmful use or dependence for nicotine and last nicotine use within the past 48 h with no known major physical/psychiatric comorbidity were included in the study. They were all male patients, aged between 18 and 60 years. Patients who were currently abstinent from nicotine (past 48 h) based on self-report and unwilling to participate were excluded from the study.

The optimized conditions were further used for the clinical validity of primary screening of patients’ urine samples vis-à-vis urine spotted filter paper samples in the following exercises. All the samples were subjected to ELISA for primary screening that involves both direct urine screening and extracted urine samples spotted onto filter paper. The qualitative results were given as the presence or absence of cotinine as positive and negative based on the ELISA screening.

The samples came positive during primary screening were further subjected to the confirmation using GC-NPD using a modified method of Verebey et al. Briefly, the pH of 1 ml filter paper elute was adjusted to 11–12 with 10 N NaOH and then extracted with chloroform and isopropanol (3:1) once. Organic layer (2.5 ml) was separated and dried under the stream of nitrogen. The dried residue was reconstituted with 100 µl of methanol and 2 µl was injected to GC. The parameters used for the GC analyses were as follows:

- **Carrier gas**: Nitrogen at a flow rate of 10 ml/min
- **Air flow** at a flow rate of 60 ml/min and hydrogen flow at the flow rate of 3 ml/min
- **Injection**: Split (10:1), 1 µl
- **Inlet temperature**: 250°C
- **Oven ramps**: 150°C for 5 min; 150°C–200°C at 5°C/min; 200°C for 5 min
- **Detector temperature**: 300°C
- **Run time**: 20 min.

**Stability of urinary cotinine dried on filter paper**

Urine-spiked standards of different concentrations were stored at three different temperatures, i.e., room temperature (25°C–30°C), 4°C, and −20°C. The estimation was done using ELISA at different points of interval, i.e., within 24 h after collection, at the end of every week for first 4 weeks and then every other week for the next 4 weeks at all three temperature conditions. Simultaneously, the collected urine samples from nicotine users were also spotted on filter paper and effect of storage was evaluated in aforesaid storage conditions. All the samples were analyzed in duplicates. All the DUSs were kept with a desiccant in a polyethylene zip-lock bag to avoid any contact with environmental humidity.
RESULTS

Optimization of the extraction of analytes from dried urine spots

Extraction of the analytes from the spots was tested using three different solvents (deionized water, saline, and sodium carbonate-bicarbonate buffer, pH 9.2). It was determined that cotinine could be recovered from urine drug stains and quantitated by ELISA and gas–liquid chromatography (GLC).

The ideal matrix used for elution was Whatman 903 filter paper with a maximum of 20 µL of urine. Drug recovery was optimum with the use of deionized water (97%) as an elution solvent [Figure 1]. The details of the solvent with appropriate volumes used for elution are discussed in Table 1.

Among the three solvents used, deionized water appeared to be the best solvent for elution of cotinine from filter paper as it did not interfere while performing ELISA. Final conditions for cotinine elution using filter paper in the current experiment are shown in Table 2. LOD for the method was found to be 100 ng/ml. Results of inter- and intra-day precision exercise are shown in Table 3.

Results of the final phase of the study where a comparison of the drug detection in urine samples collected by conventional method vis-à-vis using filter paper in clinical settings was done for cotinine

Urine samples were collected from nicotine-dependent participants seeking treatment at NDDTC, AIIMS. All the participants (n = 50) were males with the mean age of 34.51 ± 8.3 years. All the urine samples were taken from smokers; however, many of them were chewing tobacco (40.8%) along with smoking cigarettes/bidi. Table 4 shows the demographic profile of the included participants.

After screening with ELISA, confirmation of cotinine in the urine samples was done with GLC equipped with nitrogen phosphorous detector. Figure 2a and b shows the representative GLC chromatograms of the cotinine-spiked urine standard by a conventional method and cotinine-spiked urine standard by filter paper method, with a retention time of 10.49 and 10.48 min, respectively. Figure 3a and b shows the GLC chromatogram of a patient’s urine sample using the conventional method and the filter paper method with retention time at 10.44 and 10.44 min, respectively.

Table 1: Eluents and percentage recovery for extraction of cotinine from filter paper

| Eluents                     | Volume of buffer (µl) | Percentage recovery obtained using ELISA |
|-----------------------------|-----------------------|----------------------------------------|
| Deionized water             | 250                   | 52.0                                   |
|                             | 500                   | 97                                     |
|                             | 1000                  | 55                                     |
| Sodium carbonate-bicarbonate buffer (pH 9.2) | 500 | >100 (blank was run and found to be interfering while performing ELISA) |
| Saline                      | 500                   | 85 (blank was run and found to be interfering while performing ELISA) |

Table 2: Optimized conditions for elution of cotinine from urine spotted onto filter paper (primary screening)

| Variables                        | Optimal conditions                  |
|----------------------------------|-------------------------------------|
| Number of punches                | 2                                   |
| Buffer used                      | De-ionized water                    |
| Volume of buffer used            | 500 µl                              |
| Time taken for proper elution (incubation) | 24 h in a water bath-shaker assembly kept at 37°C |

Table 3: Within-day (intra-day) precision and between-day (inter-day) precision

| Amount (µg/ml) | CV % (intra-day) | CV % (inter-day) |
|----------------|------------------|------------------|
| 1              | 9.62             | 10.72            |
| 10             | 11.02            | 9.78             |
| 100            | 9.75             | 8.75             |

Table 4: Demographic profile of the participants (n=50)

| Variable                | Frequency (%) |
|-------------------------|---------------|
| Marital status          |               |
| Married                 | 28.5          |
| Unmarried               | 71.5          |
| Education               |               |
| Higher degree           | 16.3          |
| Illiterate              | 12.3          |
| Literate                | 71.4          |
| Employment status       |               |
| Unemployed              | 8.2           |
| Business/self-employed | 42.8          |
| Employed                | 48.9          |
| Monthly income          |               |
| High                    | 12.3          |
| Medium                  | 38.8          |
| Low                     | 48.9          |

Figure 1: Standard curve for quantitative enzyme-linked immunosorbent assay technique from direct as well as extracted filter
The results showed 100% concordance between filter paper-extracted urine specimens and direct urine specimens in patients’ sample.

**Stability of urinary cotinine dried on filter paper**

In the current study, cotinine did not show signs of significant degradation in spiked standards or patient’s urine samples under all tested storage conditions. Figure 4 shows the results of the short-term (up to 4 weeks) and long-term stability (up to 3 months) of cotinine in different patient’s samples.

**DISCUSSION**

A screening method to detect cotinine in small volumes of urine spotted onto filter paper has been developed. Although the technique is mainly applied for blood analysis, other biological fluids can be considered in a similar manner. In case of drug abuse testing, urine is the preferred specimen. Regarding urine specimens, the methodology is referred to as DUSs. The DUS technique provides a suitable procedure for the storage and analysis of samples in clinical toxicology because they are easy to handle, to transport, and to store in the laboratory, even in the absence of refrigeration, which can be a problem in some countries. It permits the analysis of a small volume of sample. The DUS technique is, therefore, recommended as an additional procedure to be used in conjunction with conventional methods for preserving unstable drugs from decomposition and for avoiding potential errors in interpretation of analytical results resulting from the addition or absence of preservatives. The DUS assay has potential as a precise and inexpensive option for the determination of cotinine in small urine samples. In particular, the DUS sampling can be considered a good candidate to be applied, in the near future, for testing the recent use of nicotine. This study was an attempt to explore the analysis of cotinine using microsampling method. As per our experience, in de-addiction clinics, recent use of drug by the patient is the main concern, i.e., within 24–48 h after the last intake. Moreover, this is the first report for the detection of urinary cotinine in DUSs. There were certain limitations of the study such as cutoff and LOD for cotinine were higher, and further research with larger sample size is needed to differentiate between active, passive, and currently abstinent smokers. Further work is currently in progress to evaluate storage and stability of urine specimens on filter paper for detection of cotinine among tobacco users.

**CONCLUSIONS**

The finding of the study will help to improve the clinical service available at various community settings of nicotine-dependent participants and also
in tobacco control and cessation programs. As a simple microextraction method, collecting urine samples of participants on filter paper and analyzing the dried spots for the presence of nicotine appears promising.

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Conflicts of interest
There are no conflicts of interest.

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