Dried urine spots for detection of benzodiazepines

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Abstract:
BACKGROUND AND AIM: Benzodiazepines (BZD) are widely prescribed to substance users. However, the nonmedical use of prescription BZD often leads to abuse and dependence. Therefore, it is important to detect BZD among substance users seeking treatment. The aim of the present study was to develop an efficient method for testing BZD on dried urine spot (DUS) and evaluating its clinical applicability.

METHODS: This involved optimization of conditions for the detection, recovery, and stability of BZD from dried urine, spotted on filter paper. Enzyme linked immuno-sorbent assay was used for screening whereas confirmation was done by gas chromatography. For clinical applicability, urine samples of BZD users were tested.

RESULTS: The recovery was found to be 99.7% in de-ionized water from 20 μl spotted urine samples. Limit of detection, inter-day and intra-day CV were found to be 100 ng/ml, 4.22% and 3.83%, respectively. BZD were found stable in DUS for 3 weeks at room temperature, and for 3 months at 4°C and −20°C. All the urine samples of benzodiazepine users were found positive by conventional method as well as the DUS method.

CONCLUSION: DUS method proved to be efficient for BZD testing with advantages of ease of collection, transportation, minimal invasiveness and small sample volume. It offers a useful alternative for BZD testing especially in developing countries where logistics of sample collection and transportation could be an important concern.

Keywords: Benzodiazepines, dried urine spots, drug abuse testing, oxazepam

Introduction

Benzodiazepines (BZD) are central nervous system depressants and are widely prescribed for the treatment of anxiety, seizures, sleep disorders, and drug withdrawals symptoms among dependent substance users. When used for the treatment of alcohol withdrawal, they are proven to reduce withdrawal severity and the incidence of seizures and delirium tremens. BZD use is considered to be relatively safe.[] Apart from their therapeutic benefits, BZD are also commonly used for nontherapeutic purpose. Although a small minority of patients with prescribed BZD increases their dosages excessively, the recreational use of BZD is a growing concern. The issue of abuse of BZD is especially relevant among opioid-dependent patients because these individuals are at increased risk of using BZD as an anxiety-coping strategy during withdrawal[5] or to enhance the intoxication associated with opioids.[6] Thus, screening of BZD in biological specimens among substance users is an important area of research and clinical practice.

In the human body, BZD are rapidly metabolized and excreted in the form of glucuronides. Acid hydrolysis followed by heating at 90°C cleaves the BZD ring structure to form benzophenones, which is then detected in human urine using sensitive
techniques. For instance, hydrolyzing Diazepam produces 2-amino-5-chlorobenzophenone (ACB) and 2-methylamino-5-chlorobenzophenone (MACB), while hydrolyzing nordiazepam, chlordiazepoxide and oxazepam produces ACB only. For a urine sample to be declared as positive for diazepam both ACB and MACB should be present.\[5\]

The screening for potent compounds remains an analytical challenge for clinical toxicological laboratories. Drug abuse screening is an important standard of care in the addiction treatment setting. It offers an unbiased, reproducible, and accurate method to monitor patients and provide objective support for clinical observations. However, such laboratories are resource-intensive and hence available in only specialized settings. Screening for drugs of abuse in patients belonging to remote areas is considered to be fraught with many logistic problems. In addition, to minimize variations, it is always advisable for the analysis to be carried out in one centralized laboratory. Transportation to a distant laboratory often involves challenges such as the requirement of trained staff, sample spillage, breakage, cross-contamination, and shipment in cold; all these challenges add to the cost. A suitable transport system to address these problems is a prerequisite for analysis at a centralized laboratory.\[6\]

Dried stains spotted on filter paper have several advantages over liquid samples for screening/diagnosis.\[7\] The technique of drying biological fluid on filter papers has been widely applied in blood analysis in forensic laboratories, but in the case of drug abuse screening, urine is the specimen of choice. Dried urine spots (DUS) are ideally suited to countries like India with limited health facilities and large remote areas, making the storage and transportation of samples more difficult. The main advantage offered by the DUS method is the requirement of a small volume of the samples. The DUS assay has the potential as a precise and inexpensive option for the determination of BZD in small urine samples. This technique comes across as a suitable procedure for the storage and analysis of samples in clinical toxicology laboratories and addiction treatment settings because DUS are easy to handle, transport, and store in the laboratory, even in the absence of refrigeration, which can be a concern in some settings.

Considering the above-mentioned merits, DUS could be a feasible technique as an alternative approach for biological monitoring of the prescribed and nonprescribed use of BZD. There is a dearth of published research regarding usage of urine spotted dried samples for screening drug of abuse in patients with substance use disorders from field settings, though reviews of dried blood spot (DBS) are available.\[8-12\] To the best of our knowledge, no study has reported the use of DUS for detection of BZD.

The aim of the present study was to develop a simple, efficient and low-cost method for the analysis of BZD in DUS and evaluating its clinical applicability.

Materials and Methods

Chemicals

All the reagents used were of analytical grade and were obtained from Merck, USA. Oxazepam standard was extracted from tablet Anxozap15 (Sun Pharmaceuticals Industries, India). 2-ACB was obtained from SRL (Sico Research Labs, India) and 5-chloro-2-methylaminobenzophenone was a gift from Forensic and Toxicology Laboratory, Institute of Legal Medicine (Padova, Italy). Benzophenones were also prepared from the parent BZD by acid hydrolysis for 1 h followed by extraction into diethyl ether.\[13\] Whatman Filter paper 903 was obtained from GE Health Care India.

Instrumentation and gas chromatography conditions

The screening was performed by enzyme linked immunosorbent assay (ELISA) technique (Tecan GENios ELISA reader, Austria GmbH, Austria) using Magellan software.

Further confirmation of the samples was done using Gas chromatograph (model 7890A, Agilent India Pvt. Ltd., USA). The gas chromatography (GC) was equipped with 7893B series Auto Sampler; (split/splitless inlet, fused silica capillary column coated with HP-5 cross-linked 5% diphenyl and 95% dimethylpolysiloxane (30 m x 0.320 i. d., 0.25 μm film thickness). The temperature gradient used was 1 min at 200°C, 5°C/min from 200°C to 250°C, 5 min at 250°C. Nitrogen was used as a carrier gas at a flow rate of 10 ml/min. The injector temperature was 280°C. The injection volume was 2 μl for each GC run, and the split ratio was kept 1:10. The nitrogen phosphorus detector (NPD) was used with electronic pneumatic control at 300°C. System control, data acquisition, and analysis were performed with GC Chem Station G2075BA software.

Sample collection

The study was conducted at a tertiary care addiction treatment center of north India. Male patients fulfilling ICD-10 criteria for harmful use or dependence for BZD with recent drug use and willing to participate were included in the study. Patients who were currently abstinent from BZD (past 48 h) based on self-report and unwilling to participate were excluded from the study. After inclusion, 30 ml of urine samples were collected from subjects using BZD (prescribed or non-prescribed). Drug-free urine samples as controls were obtained from laboratory staff volunteers and were screened by urine cassette test for opiates, BZD cannabis and nicotine use.
Informed consent was obtained from all participants who fulfilled the inclusion criteria. The study was carried out in accordance with Declaration of Helsinki, and the study protocol was approved by the Institutional ethics committee. The study was completed in 1 year duration (August 2012–2013).

**Dried urine spots preparation**
Control urine samples with zero baseline drug level were spiked with various concentrations (1 mg/ml, 500 μg/ml, and 100 μg/ml) with oxazepam standard. The spiked urine standards and patient samples (20 μL) were spotted on to filter paper. During spotting, the filter paper was kept on a non-absorbent surface. The spotted samples were allowed to dry overnight at ambient temperature.

**Extraction procedure**
Optimal conditions were worked out to check the maximum recovery using three different eluting solvents at different temperature and time conditions. DUS with spiked oxazepam standards were punched manually using a manual puncher of diameter 3.2 mm. Three different solvents, i.e., deionized water, sodium carbonate-bicarbonate buffer (pH 9.2), and phosphate buffer (pH 11) were tried for BZD extraction from DUS. Elutes were tested for the presence of BZD using Instant-View® Benzodiazepine urine drug test cassettes (Alpha Scientific, Ltd. USA) with a cutoff 300 ng/ml. The detection of oxazepam was further checked with ELISA kits (Calbiotech Pvt. Ltd., USA) onto ELISA reader as per the manufacturer’s guidelines. After the optimization of the number of punches, selection of the suitable eluting solvent with different volume was worked out. Thereafter, the extraction was carried out at different time intervals in a water bath-shaker at 37°C followed by confirmation by GC-NPD.[14]

**Method validation**
Percentage recovery was calculated based on comparison between the standard and the extracted oxazepam from DUS. Following formula was used to calculate the recovery:

$$% \text{ Recovery} = \frac{X_e}{X_s} \times 100$$

Where, $X_e$ = Mean conc. obtained from extracted standard using DUS method, and $X_s$ = Mean conc. obtained from the direct standard.

For inter-day precision, the above procedure was carried out in triplicate for each concentration for three consecutive days using ELISA whereas for intra-day precision, the said procedure was repeated three times in triplicate within the same day. Both are expressed as standard deviation and coefficient of variation (%CV).

**Screening and confirmation of clinical samples**
Feasibility of using filter paper to screen drug use in a clinical setting was checked by collecting urine samples from 50 patients who were using BZD. The results of urinary BZD as measured from direct urine samples were compared with DUS extracted samples. In brief, the samples were subjected to ELISA for primary screening of BZD that involved both direct urine screening and filter paper extracted urine samples. The qualitative results were given as positive or negative based on the cut-off (100 ng/ml) of ELISA kit being used. The samples reported positive in primary screening were further subjected to confirmation using GC-NPD.

**Stability**
The stability of BZD onto DUS was investigated using urine spiked standards stored in sealed plastic bags to protect them from contamination and humidity. These sealed plastic bags were stored at three different temperatures (–20°C, 4°C and room temperature [21°–28°C]). Estimation was carried out at different points of time, i.e., within 24 h after collection and then at the end of the 1st, 2nd, 3rd, 4th, 6th, 8th, 10th, and 12th week (up to 3 months). The urine samples collected from BZD users were also evaluated in the above mentioned storage conditions.

**Results**

**Optimization of extraction of benzodiazepines from dried urine spots**
The extraction of BZD was carried out using three extracting solvents for different time period. The recovery of BZD was found to be maximum (99.71%) using four punches (3.2 mm each) in 1000 μL of de-ionized water at 37°C in a water bath-shaker for 24 h [Figure 1].

Limit of detection (LOD) for the method was found to be 100 ng/ml. The inter-day CV and intra-day CV were found to be 4.22% and 3.83%, respectively.

**Clinical validation**
Urine samples were collected from 50 patients reporting BZD use as per the inclusion criteria. All the patients were males with the mean age of 35.63 ± 9.67 years. Majority of patients were married (74%) and employed (86%). Among 50 patients, 24 (48%) had been educated until primary, whereas 26% were graduates.

The urine samples were screened for the presence or absence of BZD using ELISA technique. All the 50 samples were found to be positive for BZD in direct urine as well as filter paper spotted urine samples.

After screening with ELISA a subsample (20 samples) was taken for further confirmation by GLC. In the current study, all the patients were on diazepam. Thus, the confirmation of benzodiazepine was done in the form of benzophenones (ACB and MACB) which is a routine procedure of our laboratory.[14-17]. Representative GLC chromatograms of the ACB and MACB spiked control urine with a retention time of 6.1 and 6.7 min, respectively, is presented in Figure 2a and b, respectively. Figure 3 shows the GLC chromatogram of a patient’s urine sample by filter paper method with retention time of 6.1 and 6.7 min for ACB and MACB, respectively.

The results showed 100% concordance between filter paper extracted urine specimens and direct urine specimens in patients’ sample using GC.

**Stability**

BZD was found to be stable in urine samples spotted on filter paper stored for a period of 3 weeks at room temperatures, while the samples stored at −20°C and 4°C were found to be stable even at the end of 3 months [Figure 4].

**Discussion**

Despite the fact BZD are invaluable in the treatment of anxiety disorders, they also have the potential for abuse and dependence. The use of DUS in addiction treatment settings for patients represents an inexpensive, rapid, precise, and simple method, with a satisfactory low detection limit for urine of only 20 μL per spot. Through the study, a validated method that can be used for the qualitative analysis of BZD in small volumes of urine contained in DUS has been developed. The extraction, identification, and screening of BZD in DUS were found comparable to that in a conventional sampling method.

In terms of transportation and storage, DUS is much more space saving and can easily be transported by mail. In the past two decades, DUS method has gained popularity in drug testing.[15-19] However, as per our
knowledge, this is the first study to report the use of DUS for screening BZD. Importantly, the findings of the previous work on the stability of BZD metabolites in DBS complements our findings of stability.[20] The study suggests that the DUS can improve BZD stability even when stored at room temperature (up to 3 weeks) but was found more stable at 4°C or −20°C (up to 3 months). Keeping this in view, it is advisable to store DUS at lower temperatures for long-term stability of BZD.

GC-NPD was the method of choice for detecting BZD in dried urine, but if compared with GC–mass spectrometry the LOD was considerably lower (i.e., lower amounts of urine can be used for analysis to reach a lower limit of the same order of magnitude). It has been shown that filter paper stabilizes the analyte thus increasing the chance of getting a positive result even with low concentrations and/or a considerable period after sample collection.

A higher cutoff for BZD was among the limitations of the study. In addition, this methodology was developed for the qualitative screening of BZD; future studies with quantitative estimation are required. The finding of the study will help to improve the clinical service available at various community settings for patients with prescribed or non-prescribed use of BZD.

Conclusion

The DUS technique is, therefore, recommended as an alternative 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 environmental influences. As a simple microextraction method, collecting urine samples of subjects on filter paper and analyzing the dried spots for the presence of BZD appears promising. In particular, the DUS sampling can be considered a good candidate to be applied, in the near future, for testing BZD among substance users.

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Conflicts of interest

There are no conflicts of interest.

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