Development of an Aseptic Urine Collection Kit and its Evaluation in Preventing the Bacterial Decomposition of Nitrazepam

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A novel aseptic urine collection kit has been devised, and its effectiveness in collecting and storing bacteria-free urine specimens was evaluated. The kit, which is based on filtration sterilization, was proven effective for these purposes. For the evaluation of its efficiency, urine samples spiked with nitrazepam at 0.5 μg/ml were treated with the kit and monitored for 6 months at 4°C and 25°C. An automated column-switching liquid chromatography-mass spectrometry (LC-MS) procedure was utilized for the direct determination of the analyte in urine. In severely-contaminated urine with bacteria, there were significant losses of nitrazepam at 25°C. However, such degradation was successfully suppressed by the use of the kit even at a storage temperature of 25°C, while requiring no chemical additives.

Key words —— aseptic urine collection kit, sterilization, bacterial decomposition, column-switching liquid chromatography-mass spectrometry, nitrazepam, urine

INTRODUCTION

Urine analysis for determining drug intake is an indispensable part of criminal investigation. Recent improvement in analytical techniques not only enables the detection of trace-level drugs in biological specimens, but it also has reduced specimen requirements.[1–6] Consequently, the specimen remaining should carefully be stored for a reexamination, often after a long period of time. Thus, there is a critical concern that the drug concentration which was first detectable may fall below the limit of detection, when it is reexamined in response to the defendant's claim. However, urine specimens submitted to laboratories have some complicated features, as follows: specimens are more or less contaminated with bacteria, even at the time of urination (with naturally-occurring and/or infectious bacteria); unlike taking blood specimens, their aseptic collection into a test tube is nearly impossible; and specimens can be exposed to ambient temperature when they are collected and transported. Moreover, remarkable bacterial contamination is often observed in postmortem urine specimens.[7,8] Thus, precautions should be taken against potential decomposition of drugs during the storage, which may lead to disagreements between the results of the primary and subsequent examinations due to various reasons, such as the effects of bacteria propagation. This paper reports the development of a novel aseptic urine collection kit and the evaluation of its effectiveness in collecting bacteria-free urine samples. The kit was devised for these purposes by taking the advantage of filtration sterilization, and its effectiveness was double-checked by monitoring changes in pH and the concentration of nitrazepam (NZ), which is among the most unstable drugs for bacteria, in the urine samples. For efficiently analyzing a large number of samples, an automated column-switching liquid chromatography-mass spectrometry (LC-MS) procedure was first established for the direct determination of NZ in urine, based on our previous studies.[9–11]

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MATERIALS AND METHODS

Chemicals and Materials —— NZ was purchased from Sigma (St. Louis, MO, U.S.A.). Stock stan-

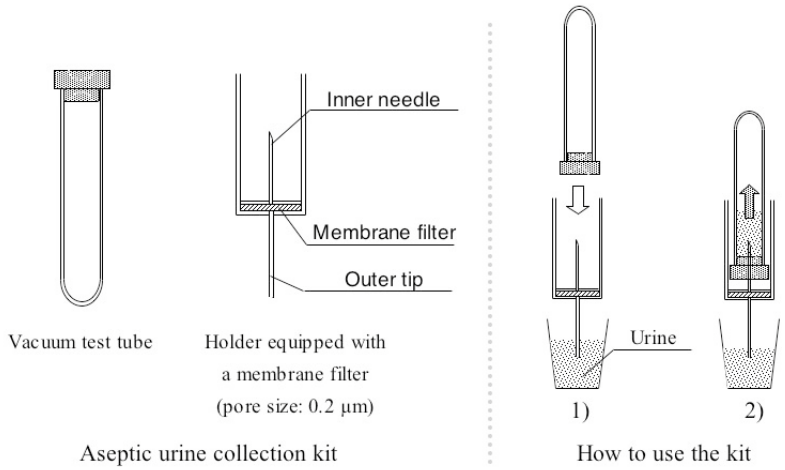

Fig. 1. Illustration of the Aseptic Urine Collection Kit (2-ml Vacuum Test Tube; Membrane Filter, Pore Size, 0.2 μm; Diameter, 17.5 mm) and How to Use the Kit

1) The outer tip of the holder is immersed into the urine collected in a cup. 2) As the tube is set up on the inner needle of the holder, the bacteria-free urine is withdrawn into the tube.

dard solution of NZ (100 μg/ml) was prepared in distilled water, and used for preparing spiked urine samples. Dibenzylamine (DBA), used as an internal standard (I.S.), and sodium azide (NaN_3) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Acetonitrile (AN) and formic acid (FA) were of HPLC grade and other chemicals used were of analytical grade. The aseptic urine collection kit tested was manufactured by Kunimune Co., LTD. (Osaka, Japan).

Drug-free urine specimens were collected from 24 random volunteers (12 males and 12 females, aged 18 to 58 years). Spiked urine samples were prepared by adding the stock standard solution of NZ at 0.5 μg/ml. To compare the efficiencies between filtration sterilization using the aseptic urine collection kit and chemical preservative, the following methods were applied before storage: addition of NaN_3 (0.5 wt%) as a preservative or use of filtration sterilization with the aseptic urine collection kit. The samples with either or without any preservative treatment were stored up to 6 months in the dark at 4°C and 25°C.

Bacterial Contamination Levels of Urine Samples —— In order to investigate the bacterial contamination levels in random urine specimens, the urine specimens collected from 24 volunteers were examined using the Luria-Bertani (LB) media, according to the bacteriological examination method prescribed in Japan's Standard Methods of Analysis in Health Science.[12] The urine specimen that was found to be among the least contaminated with bacteria (from a 27 year-old male; No. 21 in Table 1; number of bacterial cells, 1.9×10^3 counts by the Standard Methods; see below in Results) was used as the Slightly-contaminated Sample in the experiments. The urine specimen that was found to be among the most severely contaminated (from a 29 year-old female; No. 20 in Table 1; number of bacterial cells, 1.0×10^7 counts) was used as the Severely-contaminated Sample.

Aseptic Collection of Urine Specimens —— Figure 1 illustrates the aseptic urine collection kit devised and tested in this study. The kit consists of a vacuum test tube (polyethylene terephthalate ; 2-ml capacity) and a holder equipped with a membrane filter (polysulfone; pore-size, 0.2 μm; diameter, 17.5 mm). These were packed in a plastic wrapper and thoroughly sterilized by gamma-ray irradiation.

Sample Pretreatment for LC-MS Determination —— I.S. solution (10 μg/ml DBA, 30 μl) was added to urine samples (600 μl), and these were filtrated through 0.45-μm membrane filters and 15-μl aliquots were automatically injected into the instrument.

Instruments and Conditions —— LC-MS was performed on a Shimadzu LCMS-QP2010 HPLC-quadrupole mass spectrometer equipped with a six-port column-switching valve, and an electrospray ionization (ESI) interface. The on-line extraction column employed was a Shodex MS-pak PK-2A (*N*-vinylacetamide-containing copolymer gel, 10 × 2.0 mm i.d.; Showa Denko, Tokyo, Japan). The mobile phase used for introducing samples to the

Table 1. Number of Bacterial Cells Obtained from the Bacteriological Examination of 24 Random Urine Specimens, before and after Filtration Sterilization

No.	Number of bacterial cells		No.	Number of bacterial cells	
	before	after		before	after
1	3.9×10^5	n.d.	13	1.0×10^7	n.d.
2	1.0×10^7	n.d.	14	5.0×10^3	n.d.
3	1.0×10^7	n.d.	15	1.1×10^4	n.d.
4	1.0×10^7	n.d.	16	4.0×10^3	n.d.
5	1.0×10^6	n.d.	17	7.0×10^5	n.d.
6	1.0×10^7	n.d.	18	6.0×10^3	n.d.
7	1.5×10^5	n.d.	19	1.0×10^7	n.d.
8	6.0×10^3	n.d.	20	1.0×10^7	n.d.
9	1.0×10^7	n.d.	21	1.9×10^3	n.d.
10	1.0×10^6	n.d.	22	1.0×10^6	n.d.
11	1.3×10^4	n.d.	23	4.0×10^3	n.d.
12	1.0×10^6	n.d.	24	3.3×10^6	n.d.

n.d.: Not detected.

trap column, and washing out urinary matrices was 5 mM ammonium acetate (0.5 ml/min). The separation column was an L-column ODS semi-micro column (150 × 1.5 mm i.d.; Chemicals Evaluation and Research Institute, Tokyo, Japan). The trapped analyte and I.S. were eluted and chromatographed by gradient elution with mobile phases A (10 mM FA-AN; 95 : 5, v/v) and B (10 mM FA-AN; 70 : 30, v/v) at a flow rate of 0.2 ml/min (0–4 min, B 5%; 4–14 min, B 5–100%; 14–20 min, B 100%; 20–21 min, B 100–5%; 21–30 min, B 5%). Both LC separation and on-line extraction were carried out at 30°C, and the entire flow of the eluate was introduced to the ESI interface. ESI-MS was performed in the positive mode. Quantitative analyses were carried out in duplicate in the selected ion monitoring mode, while confirmation was done in the full-scan mode. The operating parameters were as follows: nebulizer nitrogen gas flow-rate, 1.5 l/min; curved desolvation line (CDL) voltage, 25V; CDL temperature, 250°C; Q-array Bias, 15 V.

RESULTS

Bacterial Contamination Levels of Random Urine Specimens and the Efficiency of the Aseptic Urine Collection Kit

The bacterial contamination levels in 24 random urine specimens were examined using the LB media, according to Japan's Standard Methods of Analysis in Health Science.[12] The results are summarized in Table 1, together with the results after sterilization using the aseptic urine collection kits. There was a significant variation in number of bacterial cells among the unprocessed specimens, but no bacterium was detected in any of the samples after filtration sterilization. To confirm the effectiveness and reliability of the kits, the Severely-contaminated Sample (one of the most contaminated urine specimens, No. 20 in Table 1) was divided into ten 2-ml-portions, and each was examined after sterilization using the kits. No bacterium was detected from any of the samples. In addition, there were no losses of NZ, the analyte to be tested caused by filtration using the kits. Thus, the satisfactory effectiveness and reliability of the kit were confirmed.

Effect of Bacteria on pH Changes

When the Severely-contaminated Sample was stored at 25°C without any preservative measures, its pH (initially *ca.* 7) gradually increased and exceeded 9 after a storage period of 150 days. A slighter pH increase (*ca.* 1) was observed for the Slightly-contaminated Sample under the same conditions. There were no pH changes for either of the samples, either with filtration sterilization or the addition of NaN_3.

Validation of Analytical Methodology

In this study, NZ in urine was automatically extracted and determined by utilizing the optimized column-switching on-line extraction LC-MS system. An *N*-vinylacetamide-containing, hydrophilic polymer on-line extraction column was employed, based on our previous studies.[10,11] A combination of an

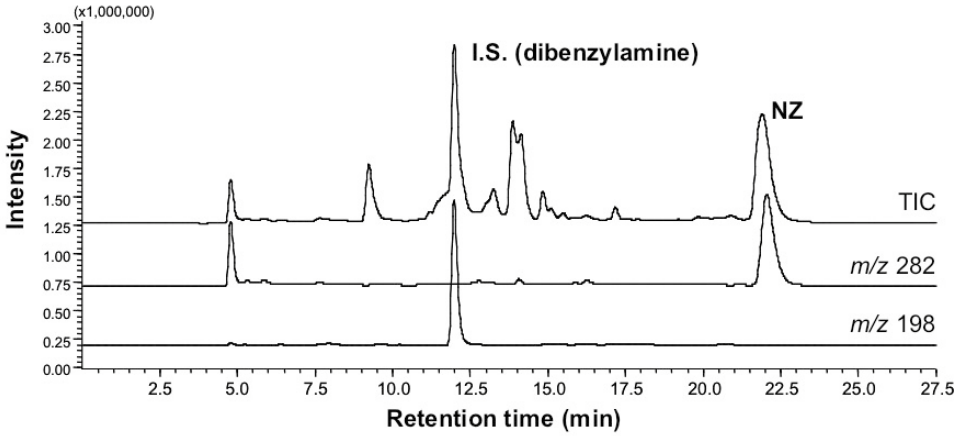

Fig. 2. Total Ion Chromatogram and Mass Chromatograms Obtained in the Full-Scan Mode from Severely-Contaminated Sample before Storage

The concentrations of NZ and I.S. (dibenzylamine) were 0.5 μg/ml each.

Table 2. Validation Data of the Established LC-MS Procedure for NZ in Urine

Recovery[a)]	103%
Accuracy[a)]	0.51 μg/ml
Precision[a)]	3.5%
Calibration Curve	
Linearity range	0.01–1.0 μg/ml
Coefficient of correlation	0.999
Lower limit of detection	0.005 μg/ml

a) Evaluated by analyzing a urine sample spiked with NZ at 0.5 μg/ml ($n = 3$).

ODS semi-micro column and a gradient mobile phase containing FA and AN was employed to achieve the best MS response of the analyte (see Experimental). Figure 2 shows the total ion chromatogram and the mass chromatograms obtained from the Severely-contaminated Sample spiked with NZ and I.S. (0.5 μg/ml each), before storage. As shown in Fig. 2, no interference by urinary components was observed. Table 2 shows the validation data of the present method evaluated by replicate measurements (n = 3) of urine spiked with NZ at 0.5 μg/ml. As shown in Table 2, satisfactory results were obtained. Thus, the optimized LC-MS methodology was proven effective for the use in present study.

Long-Term Storage of Urine Samples Containing NZ

Figure 3 shows changes in the concentration of NZ spiked into the Slightly-contaminated and the Severely-contaminated Samples under various storage conditions, with or without preservative measures. NZ was completely disappeared within two weeks in the Severely-contaminated Sample when stored at 25°C without any preservative measures. An approximate fivefold slower decrease of NZ was observed in the Slightly-contaminated Sample under the same conditions. However, such decreases were completely inhibited by either the use of the aseptic collection kit or the addition of NaN_3. Thus, the effectiveness of the kit in collecting and storing urine specimens containing such an unstable drug was demonstrated.

DISCUSSION

Evaluation on the Aseptic Urine Collection

Urine specimens submitted to laboratories are normally contaminated with bacteria. Such contamination occurs for endogenous and exogenous reasons: endogenous contamination includes bacteria infections and contamination in the genitals, while exogenous contamination occurs in collecting specimens via the sampling vessels. Also, propagation of bacteria during sample transport and storage may remarkably aggravate the degree of bacteria contamination. Such bacterial contamination and propagation must be particularly severe in postmortem specimens.[7,8)] It is well known that such saprogenic bacteria transform certain amino acids and urea in urine into putrefactive amines and ammonium, respectively.[13)] Such basic compounds increase the pH of urine samples, which may lead to the loss of un-

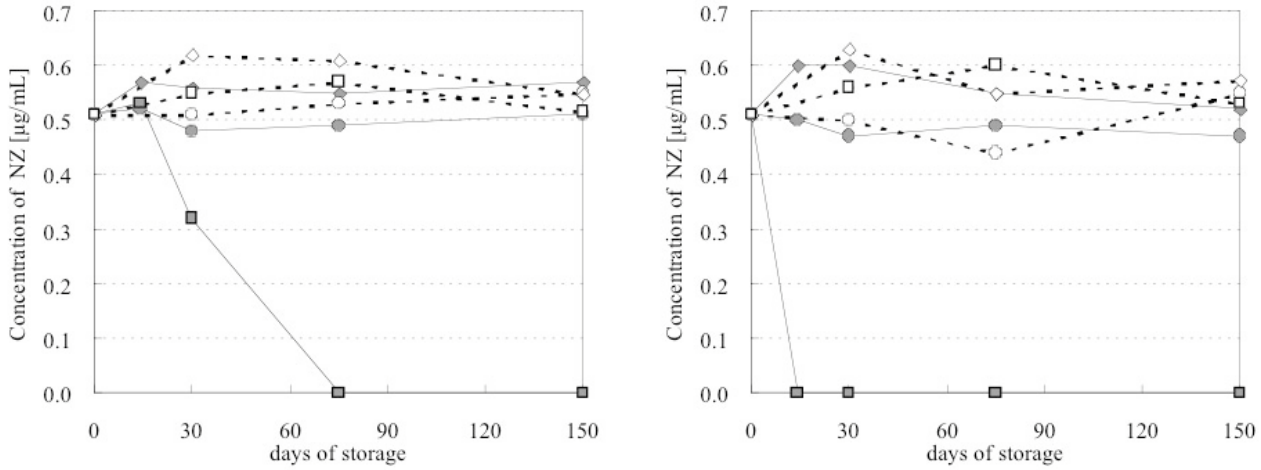

Fig. 3. Time-Course Changes in the Concentration of NZ in the Slightly-Contaminated (No. 21 in Table 1) (A) and Severely-Contaminated (No. 20 in Table 1) (B) Urine Samples, under Different Sampling and Storage Conditions

These plots were the averages of duplicate measurements of each sample. ●: with filtration, at 25°C; ◆: with NaN_3, at 25°C; ■: without any preservative measures, at 25°C; ○: with filtration, at 4°C; ◇: with NaN_3, at 4°C; and □: without any preservative measures, at 4°C.

stable drugs. Thus, proper preservative treatments against bacteria are preferable for the storage of urine specimens. In the development of the aseptic urine collection kit, various pore-sizes were tested for the membrane filter. Because filters with a pore-size lesser than 0.2 μm did not allow smooth filtration of urine samples, the pore-size was set at 0.2 μm.

As demonstrated in the preceding evaluation, the aseptic collection kit completely removes bacteria from urine because the pore-size of the filter is 0.2 μm in diameter and bacteria are typically larger than 1 μm in diameter. (see Fig. 1 in Experimental) Consequently, the use of the kit can inhibit secondary pH increase by bacteria propagation, which may cause the denaturation of drugs.

Effectiveness of the Kit in Preventing the Decomposition of NZ

NZ was unstable when the sample was contaminated with bacteria and the storage conditions allowed their propagation. We detected 7-aminonitrazepam in all the samples in which losses of NZ were detected. It is well established that nitrobenzodiazepienes, such as NZ, are readily converted to the corresponding 7-amino-metabolites by bacteria.[14–16] Most of bacteria contain an oxygen-sensitive enzyme capable of reducing nitroaromatic compounds into the corresponding amines, and such bacteria in the gastrointestinal tract is the most important site of the reduction of nitrobenzene in humans.[17,18] Robertson and Drummer demonstrated that NZ was completely converted to 7-aminonitrazepam within 240 min at 22°C in the post-mortem blood specimen from a cadaver 48 hr after death, where various kinds of enteric bacteria were detected.[7]

In this study, the concentration of NZ in the Severely-contaminated Sample reached undetectable level within 15 days, when stored at 25°C without any preservative measures. In contrast, there were no losses of NZ when stored at 4°C, regardless of the bacterial contamination levels. Also, Robertson and Drummer reported that NZ was stable up to 24 months when samples were stored at 4°C, where bacterial activity was almost suppressed.[7] Thus, an appropriate preservative treatment for removing bacteria or inhibiting their propagation, such as the addition of NaN_3 or filtration sterilization, should be performed immediately after sample collection when it is to be examined for such nitorobenzodiazepines. As mentioned above, the use of the aseptic urine collection kit is effective in correcting and storing bacteria-free urine specimens, and thus, successfully suppressed the conversion of NZ, which is among the most labile in bacteria propagation. In addition, the kit is quite user-friendly because it does not require tedious sample handling. Also, it allows the storage of intact urine samples because it requires no additives, such as NaN_3, which itself is one of the most important analytes of forensic interest and it can react with drugs in the sample due to its strong reactivity.

In conclusion, unlike collecting blood samples from humans, the aseptic collection of urine is nearly

impossible, and urine specimens submitted to laboratories are normally contaminated with bacteria. The newly developed kit removes bacteria from such urine specimens. In order to evaluate its efficiency, urine samples spiked with NZ, which is among the most unstable in biological matrices contaminated with bacteria, were treated with the kit. Degradation of NZ was successfully suppressed by the use of the kit, while requiring no chemical additives. Thus, the use of the aseptic urine collection kit is recommended not only for forensic urine analysis but also for "postal urinalysis" because such specimens are normally contaminated with bacteria and can be exposed to ambient temperatures when they are collected and transported.

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