Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International 174 (2008) 189–196
Forensic Science International
www.elsevier.com/locate/forsciint

Long-term stability of various drugs and metabolites in urine, and preventive measures against their decomposition with special attention to filtration sterilization

Kei Zaitsu *, Akihiro Miki, Munehiro Katagi, Hitoshi Tsuchihashi

Received 22 January 2007; received in revised form 17 April 2007; accepted 19 April 2007
Available online 6 June 2007

Abstract

The long-term stability of drugs and metabolites of forensic interest in urine, and preventive measures against their decomposition have been investigated, with special attention to filtration sterilization. An aseptic urine collection kit, which was recently developed based on filtration sterilization, was utilized for the aseptic collection and storage of urine samples. For evaluating preservation measures, methamphetamine (MA), amphetamine (AP), nitrazepam (NZ), estazolam (EZ), 7-aminoflunitrazepam (7AF), cocaine (COC), and 6-acetylmorphine (6AM) were spiked into urine at 500 ng/mL each, and were monitored for 6 months at 25, 4, and −20 °C, after the addition of NaN_3 and/or filtration sterilization using the aseptic collection kit. In severely contaminated urine with bacteria, there were significant losses of 7AF and NZ, and slight decomposition of MA and AP at 25 °C. However, such degradation was successfully suppressed by the use of the kit, though the use of the kit and NaN_3 were preferred for 7AF. The kit was also effective in preventing the hydrolyses of COC and 6AM, while it was suggested that the common preservative NaN_3 can accelerate the hydrolysis of such ester-type drugs and metabolites.

Keywords: Aseptic urine collection kit; Sterilization; Bacterial decomposition; Long-term stability; Column-switching LC–MS

1. Introduction

Urine analysis for determining drug use is an indispensable part of drug enforcement procedures in many countries. Recent technical improvement in analytical toxicology not only enables us to detect trace-level drugs and metabolites in biological specimens, but it also has reduced specimen requirements [1,2]. Consequently, the specimen remaining should carefully be stored for a reexamination, often after a long period of time. Also, in forensic and workplace urine examinations, ''Sample B'', the reserve sample, must be stored properly. It is also common to set a certain cutoff value for each drug in such analyses, and cutoffs are generally set at a relatively low concentration, often near the limit of detection. Thus, there is a critical concern that the drug concentration which first exceeded the cutoff may fall below the cutoff level, for some reasons, when it is reexamined in response to the defendant's claim. The detection of trace metabolites, in addition to that of the parent drug (and for unstable drugs, the detection of trace parent drug, in addition to that of its metabolites) is also preferred, or even mandatory, for reporting indisputable proof of drug use [3,4]. However, urine specimens submitted to forensic laboratories have some complicated features, as follows: (1) it is usually not known what drugs at what concentrations may be present; (2) specimens are more or less contaminated with bacteria, even at the time of urination (with naturally occurring and/or infectious bacteria); (3) unlike taking blood specimens, their aseptic collection into a test tube is nearly impossible; and (4) specimens can be exposed to ambient temperature when they are collected and transported (e.g., from a distant police station in summer). Moreover, remarkable bacteria contamination is often observed in postmortem urine specimens [5,6]. Thus, precautions should be taken against potential decomposition of drugs during storage, which may lead to disagreements between the results of the primary and subsequent examinations due to various reasons, including the direct and secondary effects of bacteria

* Corresponding author at: Forensic Science Laboratory, Osaka Prefectural Police Headquarters, 1-3-18 Hommachi, Chuo-ku, Osaka 541-0053, Japan. Tel.: +81 6 6268 1234; fax: +81 6 6271 8066.
E-mail address: k_zaitsu_fsl_opp@ybb.ne.jp (K. Zaitsu).

0379-0738/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.forsciint.2007.04.224

propagation. Although there have been several studies about such effects of bacteria [3,5–7], there is a pressing need for the comprehensive investigation that covers various types of drugs and metabolites in urine, and preservative measures against their decomposition.

In this paper, various types of drugs and metabolites, which are often detected in forensic laboratories, were used as analytes, as follows: methamphetamine (MA), amphetamine (AP), cocaine (COC), 6-acetylmorphine (6AM), estazolam (EZ), nitrazepam (NZ), and 7-aminoflunitrazepam (7AF). The above were selected for the following reasons: (1) MA, COC, and EZ can be detected in urine in their unchanged forms; (2) AP, 6AM, and 7AF are metabolites of grate forensic importance, and their detection is indispensable for the proof of intake of their parent drugs; (3) NZ, which is readily decomposed by bacteria, can be used as an indicator of bacteria propagation; and (4) COC and 6AM are common ester-type drug and metabolite, which are readily hydrolyzed. Although conjugated metabolites are generally significant, we excluded conjugates in this research. This was because of technical difficulties in the preparation of the authentic standards, in the accurate quantitation of both the conjugated and free-form metabolites, and in the interpretation of their quantitation results. We primarily investigated the long-term stability of these drugs/metabolites in urine at low concentrations, with special attention to the efficiency of a newly invented aseptic urine collection kit. The kit has been devised to aseptically collect urine samples based on filtration sterilization [8]. To accurately and efficiently analyze a large number of samples containing unstable drugs/metabolites toward hydrolysis, an automated column-switching liquid chromatography–mass spectrometry (LC–MS) procedure was established and utilized for the simultaneous determination of these analytes in urine, based on our previous studies [9–11].

2. Experimental

2.1. Chemicals and materials

d-MA hydrochloride was purchased from Dainippon Pharmaceutical Industries (Osaka, Japan). *dl*-AP sulfate, COC hydrochloride and EZ were purchased from Takeda Pharmaceutical Industries (Osaka, Japan). NZ and 7AF were purchased from Sigma (St. Louis, MO). 6AM was prepared from morphine by the method of Fehn and Megges [12]. Stock standard solutions of these analytes (100 μg/mL each) were prepared in distilled water or methanol, and used for preparing spiked urine samples. Dibenzylamine, used as an internal standard (IS), and sodium azide (NaN_3) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Acetonitrile and formic acid were of HPLC grade and other chemicals used were of analytical grade. The aseptic urine collection kits were manufactured and provided by Kunimune Co. Ltd. (Osaka, Japan). Silanized-glass Target I-D™ Vials (National Scientific Company, Rockwood, TN, U.S.A.) were used for sample preparation.

Drug-free urine specimens were collected from 24 random volunteers (12 males and 12 females, aged 18–58 years). The urine specimen that was found to be among the least contaminated with bacteria (from a 27-year-old male; number of bacterial cells, 1.9×10^3 by the Standard Methods of Analysis in Health Science [13], established and authorized by the Pharmaceutical Society of Japan) was used as the slightly contaminated urine in the experiments. The urine specimen that was found to be among the most severely contaminated (from a 29-year-old female; number of bacterial cells, 1.0×10^7) was used as the severely contaminated urine.

Spiked urine samples were prepared by adding the stock standard solutions to these samples at 500 ng/mL each. To compare several preservation techniques, the following methods were applied before storage: addition of NaN_3 (0.5 wt%) as a preservative and/or use of filtration sterilization with the aseptic urine collection kit. The samples with either or both, or without any preservative treatment were stored up to 6 months in dark at 25, 4, and −20 °C (only for EZ, NZ, and 7AF).

2.2. Aseptic collection of urine specimens

Fig. 1 illustrates the aseptic urine collection kit tested in this study. The kit consists of a vacuum test tube (polyethylene terephthalate; 2-ml capacity) and a holder (polyethylene terephthalate) 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.

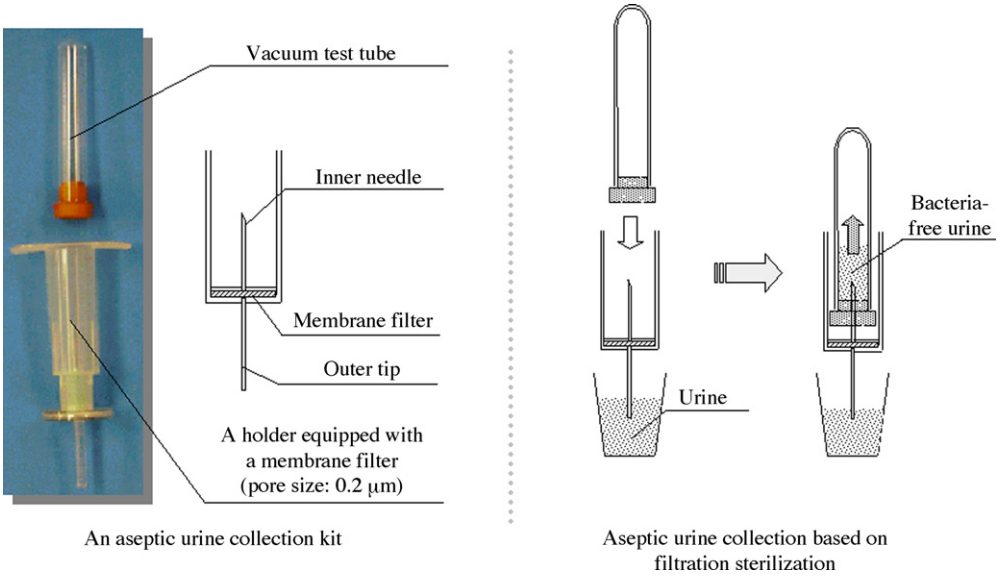

Fig. 1. The aseptic urine collection kit used in this study.

2.3. Sample pretreatment for LC–MS determination

IS 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 LC–MS instrument.

2.4. 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 mm × 2.0 mm i.d.; Showa Denko, Tokyo, Japan). The mobile phase used for introducing samples to the 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 (pore-size, 120 Å; 5-μm particles; 150 mm × 1.5 mm i.d.; Chemicals Evaluation and Research Institute, Tokyo, Japan). The trapped analytes were eluted and chromatographed by gradient elution with mobile phases A (10 mM formic acid–acetonitrile; 95:5, v/v) and B (10 mM formic acid–acetonitrile; 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 (SIM) 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, 25 V; CDL temperature, 250 °C; Q-array Bias, 20 V.

3. Results

3.1. Establishment of analytical methodology

In this study, an automated on-line extraction LC–MS procedure was established and utilized to accurately analyze a large number of urine samples containing unstable drugs and metabolites. An *N*-vinylacetamide-containing, hydrophilic polymer on-line extraction column was employed for extracting the seven analytes with a wide range of polarity difference, based on our previous studies [9–11]. To pursue the best LC separations and MS responses of such analytes, a strong cation exchanger (SCX) column (CAPCELL PAK SCX, 150 mm × 1.5 mm i.d.; Shiseido, Tokyo, Japan), a gel filtration chromatography (GFC) column (Shodex MS-pak GF-310 2D, 150 mm × 2.0 mm i.d.; Showa Denko, Tokyo, Japan), and the ODS column described in the experimental section were compared, with the optimization of mobile phase for each column. The GFC column provided blunt peaks with poor separations and insufficient MS responses, despite trials with various gradient mobile phase systems. The SCX column gave excellent MS responses with an optimized mobile phase containing 65% acetonitrile and adjusted to pH 4.0, but resulted in ordinary peak separations; NZ and EZ coeluted despite careful optimization of mobile phase. The ODS column only provided satisfactory peak resolutions when the formic acid-based gradient mobile phase described in the experimental section was used, instead of an ordinary ammonium acetate-based gradient mobile phase. Fig. 2 shows the extracted mass chromatograms obtained from a urine sample spiked with the seven drugs/metabolites at 500 ng/mL each, by using the optimized method, in the SIM mode. No chromatographic or spectral interference with urinary components was observed for each analyte. The optimized methodology was validated for the seven analytes in urine at 500 ng/mL each. The validation data are summarized in Table 1. No interference was observed even in determining closely eluting NZ and EZ, owing to differences in their mass spectra. Also, no degradation of analytes during analytical procedure were observed even for COC and 6AM which are unstable toward hydrolysis. The lower limits of detection (LODs) were 5–10 ng/mL in the SIM mode. Thus, the established methodology was proven effective for the present study.

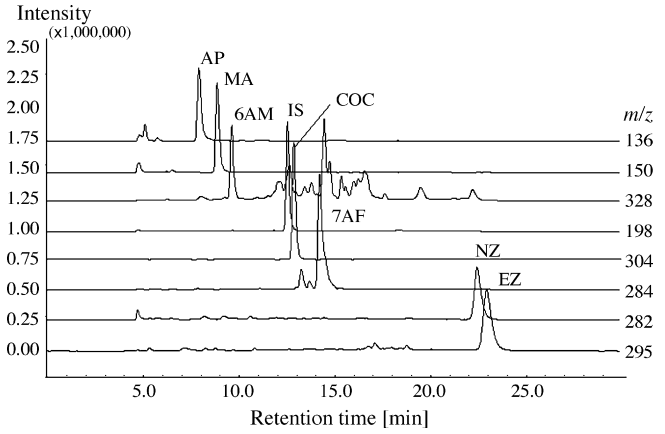

Fig. 2. Mass chromatograms obtained by the automated on-line extraction LC–MS of seven drugs and metabolites in urine at 500 ng/mL each.

Table 1
Validation data of the method for the determination of target analytes ($n = 3$)

	Recovery (%)	Accuracy and precision (mean[a] ± S.D.%)	Linearity[b]	LOD (ng/mL)
Methamphetamine (MA)	97	0.50 ± 3.3	0.999	5
Amphetamine (AP)	97	0.51 ± 3.6	0.998	10
Nitrazepam (NZ)	101	0.55 ± 2.1	0.998	5
Estazolam (EZ)	95	0.50 ± 3.9	0.999	5
7-Aminoflunitrazepam (7AF)	102	0.52 ± 2.5	0.999	5
Cocaine (COC)	96	0.51 ± 3.6	0.999	5
6-Acetylmorphine (6AM)	101	0.50 ± 1.5	0.999	5

[a] Evaluated by analyzing urine samples spiked at 0.5 μg/mL each.
[b] Evaluated range: 50–1000 ng/mL.

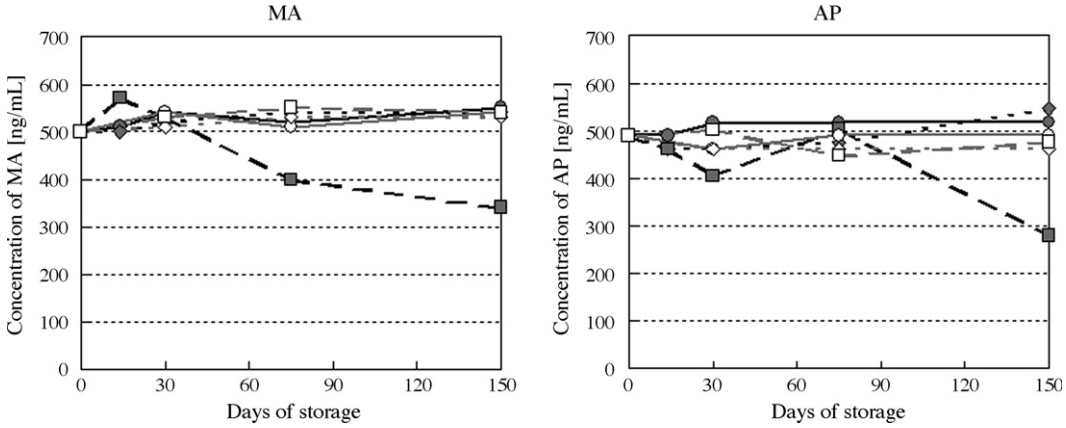

Fig. 3. Time-course changes in the concentrations of MA and AP in the severely contaminated urine under various storage conditions ((●) with filtration, at 25 °C; (◆) with NaN_3, at 25 °C; (■) without any preservative measures, at 25 °C; (○) with filtration, at 4 °C; (◇) with NaN_3, at 4 °C; (□) without any preservative measures, at 4 °C).

3.2. Stability of MA and AP

Fig. 3 shows time-course changes in the concentrations of MA and AP in the severely contaminated urine under various storage conditions, with or without preservative measures. For the slightly contaminated urine, there were no noticeable changes of AP and MA over 150 days, even when stored at 25 °C, without any preservative measures (data not shown in Fig. 3). Decreases of MA and AP by 32 and 44%, respectively, were observed in the severely contaminated urine after a storage period of 150 days under the same conditions. However, the losses of both analytes were completely prevented by either the use of the aseptic collection kit or the addition of NaN_3.

The same kind of experiment was also carried out for an additional five random urine specimens. Of these actual specimens, in urine from a 28-year-old female, the concentrations of MA and AP decreased by 60 and 23%, respectively, after a 150-day storage at 25 °C, though no changes were noticed in the other four. However, there were no decreases of both drugs in this female's urine after filtration sterilization. Thus, filtration sterilization using the kit, as well as the addition of NaN_3, was found to completely prevent the decomposition of MA and AP over 150 days, even at room temperature.

The losses of MA observed in the present study, however, suggested additional experiments to check whether MA in urine can be converted into AP during the storage, as follows: a total of five relatively severely contaminated drug-free urine samples were spiked with MA at 500 ng/mL, and were then stored at 25 °C for 30 days without any preservative measures. Although decreases of MA by up to 24% were observed after 30 days, no traces of AP formation were noticed (LOD of AP being 10 ng/mL in the SIM mode) in all of the samples tested.

3.3. Stability of NZ and EZ

Fig. 4 shows changes in the concentrations of NZ and EZ in the severely contaminated urine under various storage conditions, with or without preservative measures. No changes were noticed over 150 days at −20 °C (data not shown in Fig. 4). There were no noticeable losses of EZ over 150 days at 25 °C, even in the severely contaminated urine, without any preservative measures. Unlike EZ, all of the NZ disappeared

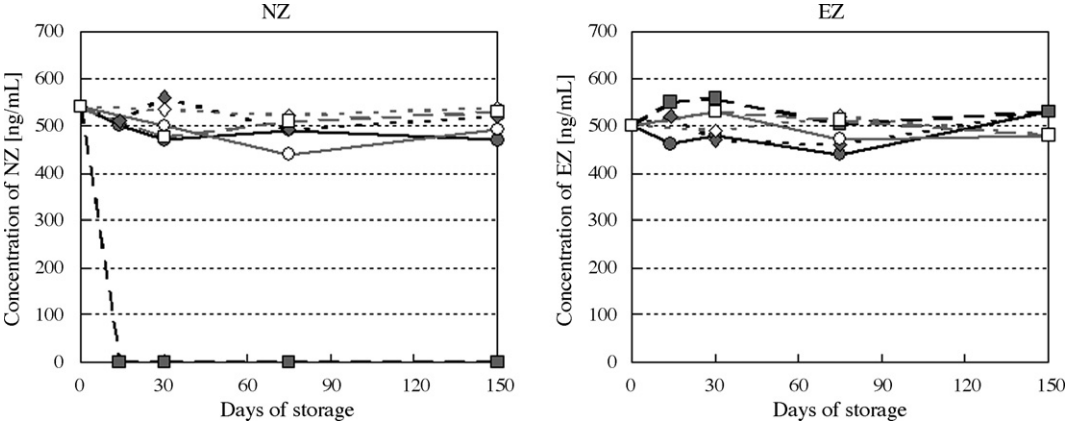

Fig. 4. Time-course changes in the concentrations of NZ and EZ in the severely contaminated urine under various storage conditions ((●) with filtration, at 25 °C; (◆) with NaN_3, at 25 °C; (■) without any preservative measures, at 25 °C; (○) with filtration, at 4 °C; (◇) with NaN_3, at 4 °C; (□) without any preservative measures, at 4 °C).

within 14 days in the severely contaminated urine when stored at 25 °C without any preservative measures. An approximate five-fold slower decrease of NZ was observed in the slightly contaminated urine under the same conditions (data not shown in Fig. 4). However, such decreases of NZ were completely inhibited by either the use of the aseptic collection kit or the addition of NaN_3.

3.4. Stability of 7AF

Fig. 5 shows changes in the concentration of 7AF in the severely contaminated urine under various storage conditions, with or without preservative measures. No changes were noticed over 150 days at −20 °C (data not shown in Fig. 5). There were only slight differences in the loss of 7AF between the severely and slightly contaminated urine samples. Also, filtration sterilization provided poor effect in preventing the loss of 7AF. Although the addition of NaN_3 alone provided moderate effect, a combination of NaN_3 with filtration sterilization was found to be most effective, and 7AF was fairly stable over 75 days even at room temperature.

It was noticeable that 7AF concentration seemed to reach a plateau at 30 days. This implied its possible decrease due to its surface adsorption onto the sample vessel during storage because no noticeable loss due to adsorption onto the filter was observed in a preliminary experiment. To investigate such adsorption, a series of bacteria-free aqueous solutions of 7AF (250–2000 ng/mL, with stepwise dilutions) were processed and stored for an equilibrium period of 30 days, using the kit. The results are shown in Fig. 6. The losses were estimated at an equivalent of approximately 100 ng/mL at each concentration. Because the losses seemed not to be dependent on the concentration, the surface adsorption of 7AF onto the vessels was estimated at an equivalent of 100 ng/mL or less. However, as shown in Fig. 5, the decreases of 7AF observed in the storage

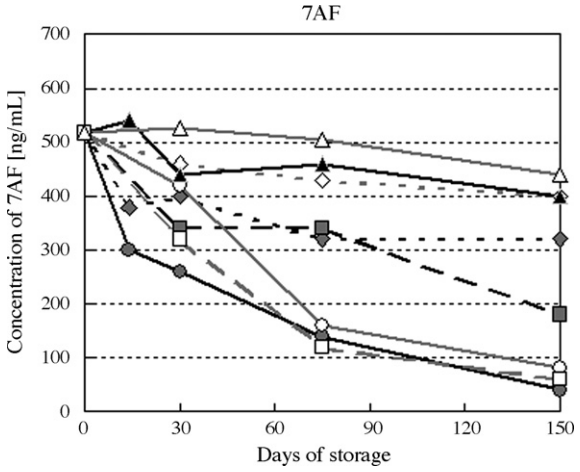

Fig. 5. Time-course changes in the concentration of 7AF in the severely contaminated urine under various storage conditions ((●) with filtration, at 25 °C; (◆) with NaN_3, at 25 °C; (▲) with filtration and NaN_3, at 25 °C; (■) without any preservative measures, at 25 °C; (○) with filtration, at 4 °C; (◇) with NaN_3, at 4 °C; (△) with filtration and NaN_3, at 4 °C; (□) without any preservative measures, at 4 °C).

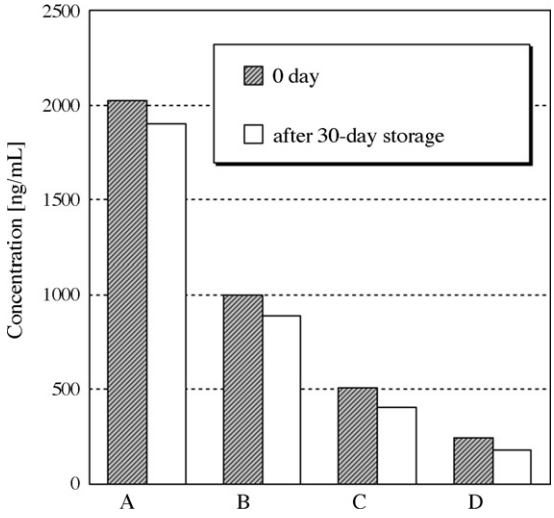

Fig. 6. Changes in the concentration of bacteria-free 7AF diluted standard solutions at various concentrations (250–2000 ng/mL): estimation of the surface adsorption of 7AF onto the sample vessel.

experiments were far more than 100 ng/mL in some cases. This indicates that the surface adsorption of 7AF was not the main cause of its losses in such cases.

3.5. Stability of COC and 6AM

Fig. 7 shows changes in the concentrations of COC and 6AM in the severely contaminated urine under various storage conditions, with or without preservative measures. There were no significant losses of both COC and 6AM over 150 days for all the samples stored at 4 °C. For the severely contaminated urine, both compounds were completely lost within 30 days when they were stored at 25 °C without any preservative treatments. Both compounds were also lost even in the slightly contaminated urine after 75 days under the same conditions (data not shown in Fig. 7). Decreases of COC and 6AM were completely inhibited by the use of the aseptic urine collection kit, even when samples were stored at 25 °C. However, without the use of the kit, the addition of NaN_3 resulted in the complete loss of COC after 75 days, and a nearly 75% loss of 6AM after a 150 days, when stored at 25 °C.

4. Discussion

4.1. Efficiency of the aseptic urine collection kit

Urine specimens submitted to forensic 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 and pipettes. Also, propagation of bacteria during sample transport and storage may remarkably aggravate the degree of bacteria contamination. Bacterial contamination and propagation should be particularly severe in postmortem specimens [5,6]. It is well known that such

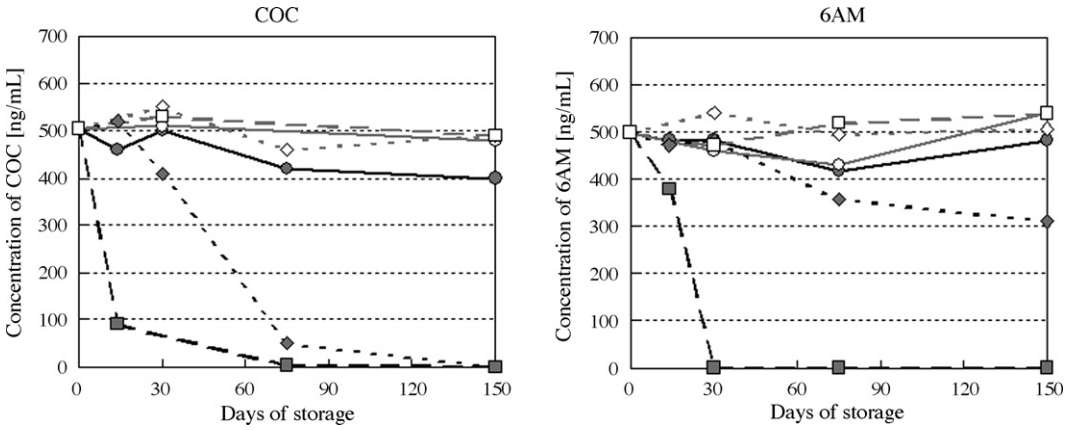

Fig. 7. Time-course changes in the concentration of COC and 6AM in the severely contaminated urine under various storage conditions ((●) with filtration, at 25 °C; (◆) with NaN_3, at 25 °C; (■) without any preservative measures, at 25 °C; (○) with filtration, at 4 °C; (◇) with NaN_3, at 4 °C; (□) without any preservative measures, at 4 °C).

saprogenic bacteria transform certain amino acids and urea in urine into putrefactive amines and ammonium, respectively [14]. Such basic compounds increase the pH of urine specimens, which may lead to the conversion of ionized amine-type drugs into the free base forms. Thus, proper preservative treatments against bacteria are preferable for the storage of urine specimens.

In our previous study [8], the bacterial contamination levels (i.e., numbers of bacterial cells) in random urine specimens submitted to our laboratory ($n = 24$) were examined using the LB (Luria-Bertani) media [13]. The effectiveness of the aseptic urine collection kit in removing bacteria and keeping bacteria-free samples was also evaluated. Although there was a significant variation in numbers of bacterial cells among the unprocessed specimens, no bacteria was detected in any of the samples after filtration sterilization using the kit. This was because the pore-size of the filter is 0.2 μm in diameter and bacteria are typically larger than 1 μm in diameter, and, thus, bacteria are completely excluded with the filter. The use of the kit was also found to be effective in inhibiting secondary pH increase by bacteria propagation, which may cause the denaturation of drugs [15]. In this study, although slight losses of 7AF probably due to its adsorption onto the sample vessels were observed, no losses relating to the use of the kit (e.g., adsorption onto the filter and sample vessel) were noticed for the other drugs and metabolites tested here.

4.2. Stability of MA and AP

There were only slight decreases of MA and AP even in severely contaminated samples, when stored at room temperature without any preservative measures. Clauwaert et al. [16] reported similar results for the stability of 3,4-methylenedioxy derivatives of MA and AP (i.e., MDMA and MDA). These drugs in urine were reported to be stable for 21 weeks, even when stored at room temperature without any preservative treatments. Thus, amphetamines in urine seem to be relatively stable against bacteria propagation.

In forensic urine analysis for MA, the detection of its relevant metabolite AP, in addition to the parent drug MA, is mandatory for the proof of MA intake [3,4]. MA is readily *N*-demethylated into AP enzymatically in the body [17]. However, no data have been reported about the transformation of MA into AP in biological specimens during sample storage. Thus, the losses of MA observed in this study suggested the additional experiments to check whether MA can be changed into AP. As a result, no traces of AP generation were noticed in the SIM mode (the limit of detection for all of the samples tested. This indicates that the secondary amine moiety of MA is rather stable against its reductive decomposition. Another supplementary experiment was conducted to examine whether the losses of MA and AP occurred due to the adsorption of free-base MA and AP onto the inside of the test tube, at an alkalized pH. As a result, no such adsorption was noticed. In bacterial-reductive reactions of nitrogen-containing biological substances, the cleaving of carbon–carbon bonds normally occur to form putrefaction amines [14]. Thus, the losses of MA and AP observed are most probably due to the breaking of a carbon–carbon bond caused by bacteria, though no specific degradation products were determined in the present study.

4.3. Stability of NZ and EZ

Contrary to the high stability of EZ, NZ was unstable when the sample was contaminated with bacteria and the storage conditions allowed bacteria propagation. It is well established that nitrobenzodiazepienes, such as NZ, are readily converted to the corresponding 7-amino-metabolites by bacteria [18–20]. Most of bacteria contain an oxygen-sensitive enzyme capable of reducing nitroaromatic compounds into the corresponding amine, and such bacteria in the gastrointestinal tract is the most important site of the reduction of nitrobenzene in humans [6,21,22]. In the present study, we detected 7-aminonitrazepam in all the samples in which losses of NZ were detected. Robertson and Drummer [5] reported that NZ was stable up to 24 months when samples were stored at 4 °C, where bacterial activity must be suppressed. Thus, an appropriate preservative treatment that removes bacteria or inhibits bacteria 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 benzodiazepines and their metabolites.

4.4. Stability of 7AF

The stability profile of 7AF was rather complex. There were only slight differences in the loss of 7AF between the severely and slightly contaminated urine samples, as well as between samples with and without filtration sterilization. Also, slight differences were observed between the storage temperatures of 25 and 4 °C. The use of NaN_3 alone was also found to be insufficient. However, because a combination of the use of NaN_3 and filtration sterilization was most effective, the main reason for the loss of 7AF was probably due to its chemically unstable properties, even though bacteria propagation probably accelerates its spontaneous conversion.

Robertson and Drummer [18] reported similar results for the decrease of 7AF in blood specimens. They observed that the concentration of 7AF in whole blood declined by ~10% during 28-day storages at 4, 22 and 37 °C, where sodium fluoride was used as a preservative. They also observed a significant loss of 7AF (ca. 10% within 2 days) in a bacteria-contaminated blood sample, when stored at 22 °C. Although there are differences between the natures of urine and blood, it is certain that 7AF is unstable in biological specimens, particularly when samples were severely contaminated with bacteria. Curiously, Robertson and Drummer [18] also reported obvious losses of 7AF in blood at −20 °C, but no such losses were observed at −20 °C in the urine samples tested in the present study. In conclusion, a combination of the use of NaN_3 and filtration sterilization is recommendable for the storage of urine specimens containing 7-amino-metabolites of benzodiazepines.

4.5. Stability of COC and 6AM

COC and 6AM are readily hydrolyzed enzymatically and/or spontaneously; the former into benzoylecgonine and methylecgonine, and the latter into morphine [3]. The spontaneous hydrolysis is pH-dependent, occurring more rapidly under alkaline conditions [15]. As described earlier, the hydrolysis rates of this drug and metabolite in the severely contaminated urine was several times faster than those in the slightly contaminated urine. This was most probably because of the spontaneous hydrolysis under elevated pH conditions due to bacteria propagation. As expected, the use of the aseptic collection kit completely inhibited the decreases of both compounds, even for the severely contaminated urine, stored at 25 °C. Interestingly, however, slight decreases were still noticed for both compounds even when NaN_3 was added, and the sample was stored at room temperature. Very similar decomposition rates were also observed in severely and slightly contaminated urine samples when these were stored after the addition of NaN_3 (0.5 wt%) and filtration sterilization. These results suggested that NaN_3 accelerated the decomposition of these drugs. The azide anion functions as a strong nucleophile, which attacks the carbonyl carbon and probably accelerates the hydrolysis of such an ester bond in a slightly alkaline aqueous medium, as illustrated in Fig. 8. Fuller and Anderson [23] reported that chemical preservation with 0.1 wt% NaN_3 had no observable effect on the stability of 6AM for 12 weeks at 20 °C. Although there are slight differences between our results and theirs, they would be probably due to the differences in the concentration of NaN_3 and storage period. In this study, there was no noticeable difference in 6AM concentration after 90 days between those with and without NaN_3, but the difference became apparent after a 150-day storage. Although no azide adducts of such compounds were detected in our additional analysis, the use of NaN_3 can affect the long-term stability of drugs and metabolites containing an ester moiety. Thus, the use of the aseptic collection kit, instead of chemical preservation with NaN_3, is recommendable for urine samples that are to be analysed for determining such analytes.

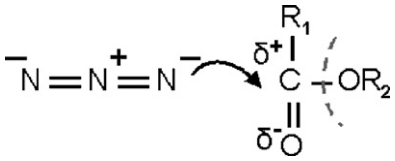

Fig. 8. Possible mechanism of the hydrolysis of ester-type drugs catalyzed by azide anion.

5. Conclusion

Among the various types of urinary drugs and metabolites of forensic interest, those with an aromatic nitro moiety, an ester moiety, and amino-benzodiazepine metabolites were found to be readily decomposed, especially the samples were severely contaminated with bacteria. Unlike collecting blood samples from humans, however, the aseptic collection of urine specimens is nearly impossible, and, therefore, urine specimens submitted to forensic laboratories are normally contaminated with bacteria. The aseptic collection and storage of urine specimens has become possible by the use of the recently developed collection kit. The filtration sterilization using the kit was found to be effective in preventing the decomposition of various kinds of unstable drugs and metabolites. Its additional advantages over chemical preservatives include its user-friendly operativity and its requiring no additive. Thus, filtration sterilization using the kit is recommended in collecting urine specimens because forensic specimens should be analyzed for ‘‘unknowns’’ though these are often exposed to ambient temperatures when they are collected and transported.

References

[1] M. Nishikawa, H. Tsuchihashi, Applications of LC/MS in forensic chemistry, J. Toxicol. Toxin Rev. 17 (1998) 13–26.
[2] K. Kudo, H. Tsuchihashi, N. Ikeda, Meeting challenges in forensic toxicology in Japan by liquid chromatography/mass spectrometry, Anal. Chim. Acta 492 (2003) 83–104.
[3] O. Suzuki, K. Watanabe, Drugs and Poisons in Humans. A Handbook of Practical Analysis, Springer, 2005.
[4] Pharmaceutical Society of Japan, Standard methods of Chemical Analysis in Poisoning, Tokyo Kagaku Dozin, Tokyo, 2006.
[5] M.D. Robertson, O.H. Drummer, Postmortem drug metabolism by bacteria, J. Forensic Sci. 40 (1995) 382–386.

[6] M.D. Robertson, O.H. Drummer, Postmortem distribution and redistribution of nitrobenzodiazepines in man, J. Forensic Sci. 43 (1998) 9–13.
[7] B. Levine, R.V. Blanke, J.C. Valentour, Postmortem stability of benzodiazepines in blood and tissues, J. Forensic Sci. 28 (1983) 102–115.
[8] K. Zaitsu, A. Miki, M. Katagi, N. Kunimune, H. Tsuchihashi, Development of an aseptic urine collection kit and its evaluation in preventing the bacterial decomposition of nitrazepam, J. Health Sci. 52 (2006) 724–729.
[9] M. Katagi, M. Nishikawa, M. Tatsuno, A. Miki, H. Tsuchihashi, Column-switching high-performance liquid chromatography/electrospray ionization mass spectrometry for the simultaneous microanalysis of herion metabolites in human urine, J. Chromatogr. B 751 (2001) 177–185.
[10] A. Miki, M. Tatsuno, M. Katagi, M. Nishikawa, H. Tsuchihashi, Simultaneous determination of eleven benzodiazepine hypnotics and eleven relevant metabolites in urine by column-switching liquid chromatography–mass spectrometry, J. Anal. Toxicol. 26 (2002) 87–93.
[11] A. Miki, M. Katagi, H. Tsuchihashi, Determination of methamphetamine and its metabolites incorporated in hair by column-switching liquid chromatography–mass spectrometry, J. Anal. Toxicol. 27 (2003) 95–102.
[12] J. Fehn, G. Megges, Detection of O6-monoacetylmorphine in urine samples by GC–MS as evidence for heroin use, J. Anal. Toxicol. 9 (1985) 134–138.
[13] Pharmaceutical Society of Japan, Standard Methods of Analysis for Hygienic Chemists, Kinbara Publisher, Tokyo, 2000.
[14] M.W. Miller, The Pfizer Handbook of Microbial Metabolites, McGraw-Hill, New York, NY, 1961.
[15] D.S. Isenschmid, B.S. Levine, Y.H. Caplan, A comprehensive study of the stability of cocaine and its metabolites, J. Anal. Toxicol. 13 (1989) 250–256.
[16] K.M. Clauwaert, J.F. Van Bocxlaer, A.P. De Leenheer, Stability study of the desighner drugs ''MDA, MDMA and MDEA'' in water, serum, whole blood, and urine under various storage temperatures, Forensic Sci. Int. 124 (2001) 36–42.
[17] T. Baba, H. Yamada, K. Oguri, H. Yoshimura, Participation of cytochrome P-450 isozymes in *N*-demethylation, *N*-hydroxylation and aromatic hydroxylation of methamphetamine, Xenobiotica 18 (5) (1988) 475–484.
[18] M.D. Robertson, O.H. Drummer, Stability of nitrobenzodiazepines in postmortem blood, J. Forensic Sci. 43 (1998) 5–8.
[19] H. Kelly, A. Huggett, S. Dawling, Liquid-chromatographic measurement of nitrazepam in plasma, Clin. Chem. 28 (1982) 1478–1481.
[20] H.M. Stevens, The stability of some drugs and poisons in putrefying human liver tissues, J. Forensic Sci. Soc. 24 (1984) 577–589.
[21] J. Rieder, G. Wendt, Pharmacokinetics and metabolism of the hyponotic nitrazepam, in: S. Garattini, E. Mussini, L.O. Randall (Eds.), The Benzodiazepines, Raven Press, New York, 1973.
[22] F. Rafii, W. Franklin, R.H. Heflich, C.E. Cerniglia, Reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract, Appl. Environ. Microbiol. 57 (1991) 962–968.
[23] D.C. Fuller, W.H. Anderson, A simplified procedure for the determination of free codeine, free morphine, and 6-acetylmorphine in urine, J. Anal. Toxicol. 16 (1992) 315–318.