

TECHNICAL NOTE

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Stability study of opioids and benzodiazepines in urine samples by liquid chromatography tandem mass spectrometry

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Abstract

Background: The stability of opioids and benzodiazepines was studied in patient urine samples stored at different temperatures over 30 days.

Methods: Samples were prepared by solid phase extraction and quantified by liquid chromatography coupled to a tandem triple quadrupole mass spectrometer.

Results: Sample storage conditions affected the stability of all drugs investigated. The concentrations of hydromorphone and oxycodone in urine samples from patients who had taken hydrocodone, morphine, and/or oxycodone increased significantly over 30 days of storage.

Conclusions: These data suggest that results from long-term-stored urine samples with opioids and benzodiazepines should be cautiously interpreted. This study indicates that the biotransformation of parent drugs to metabolites still occurs during sample storage at ambient temperatures.

Keywords: Benzodiazepine; Opioid; Opiate; Solid phase extraction; Triple quadrupole mass spectrometry; Liquid chromatography

Introduction

In the USA, more attention has been placed during the past decade on effective pain management due to its contribution to the healing process and the improvement of the quality of patients' lives. However, according to the US Centers for Disease Control and Prevention, pain medication prescription overdoses have increased rapidly and are now at epidemic levels (Centers for Disease Control and Prevention 2012). Recent studies have shown that physicians, internists, and family practitioners are more likely than ever before to be supervising care of patients with chronic pain who regularly have other comorbidities and may be taking medications that could affect the results of urine drug test (Couto et al. 2009). Couto et al. found in their recent article that three quarters of patients from nearly one million patient test

samples were not taking their medications according to their physician's prescription. Altogether, 38% of patients did not have a detectable level of their prescribed medication, while 27% had higher drug levels and 15% had lower drug levels than expected (Couto et al. 2009).

A review on the topic of drug instability by Peters indicates that storage conditions are important factors for data interpretation. Of particular note, compounds that are structurally labile are most susceptible to degradation (Peters 2007). Benzodiazepines and opioids are among the most commonly prescribed drugs in pain management therapy. Benzodiazepines are prescribed particularly in anxiety-induced depression cases, stress, panic, sleep disorders, and seizures, while opioids have long been used to treat acute pain such as postoperative pain and to alleviate the severe and disabling pain of terminal conditions such as cancer. However, these substances are regularly present in emergency intoxication episodes and drugs-of-abuse testing. They are also regularly responsible for

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overdose in suicide attempts (Lader 1999; Drummer 2004). Consequently, clinical and forensic laboratories must be able to accurately screen, confirm, and quantify benzodiazepines and opioids along with their metabolites in biological samples.

The knowledge on drug concentration changes in the sample and on the time of its storage period is crucial particularly if there is a considerable time interval between sample collection and toxicological analysis (Skopp 2004). Samples are usually kept at undefined storage conditions during transportation to the laboratory, and toxicology laboratories are obliged to keep biological samples for a certain period in order to allow reanalysis if it is required by the practitioner. Hence, the instability of the drugs (or benzodiazepines and opiates in this case) in different biological samples (or urine in this case) is a problem of data interpretation in analytical toxicology. Biological samples for toxicological analysis frequently remain at room temperature for some hours during transport, reception, and registration. Refrigeration of samples at 4°C is common following drug screening and analysis confirmation. For forensic purposes, samples may be frozen for long time periods due to legal constraints and subsequent retest. Some factors such as storage time and temperature can influence drug stability. It is one of the most important issues challenging the interpretation of postmortem toxicology results (Drummer 2007). A number of studies in the literature are focused on the effects of sample storage temperature and the formation of metabolites (El Mahjoub and Staub 2000; Skopp et al. 2001a; Skopp et al. 2001b; Klingmann et al. 2001; Clauwaert et al. 2001), but these mainly report data from blood and/or plasma samples.

Due to the high number of intoxications per year with drugs and pharmaceuticals, fast drug screening methods are necessary in clinical and forensic intoxication cases. Traditional methods usually include immunoassay testing and gas chromatography/mass spectrometry (GC/MS) for confirmation. There are intrinsic strengths and weaknesses associated with these methods. Immunoassays are only available for a small number of substance classes, and it suffers from lack of specificity and sensitivity (Hammett-Stabler and Cotten 2012). GC/MS requires a rigorous sample preparation often involving derivatization of certain compounds and relatively long chromatographic runs (Anizan et al. 2012). Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been increasingly used for confirmation of drugs of abuse in various matrices (de Castro et al. 2009a; de Castro et al. 2009b; Cheng et al. 2006; Mueller et al. 2005; de Jager and Bailey 2011). Multiplex triple quadrupole mass spectrometry has enabled

simultaneous detection of a drug and its fragment ions in one single run.

The aim of the present study was to evaluate the stability of opioids and benzodiazepines in patient urine samples after different storage periods and temperatures. A triple quadrupole mass spectrometer was used to monitor the stability of six opioids and five benzodiazepines in urine samples from four patients with chronic pain, who have been under physicians' supervision. Patient I was prescribed morphine and oxycodone (Oxy Ir®), while patient II was prescribed oxymorphone (Opana®) and oxycodone. Patient III was prescribed hydrocodone and acetaminophen (Lortab®), oxycodone and acetaminophen (Percocet®), carisoprodol (Soma®), and diazepam (Valium®), and patient IV was prescribed oxycodone and acetaminophen (Endocet®), carisoprodol (Soma®), and alprazolam (Xanax®). The stability of parent compounds and corresponding metabolites was evaluated in urine samples stored at laboratory temperature or in the refrigerator over 30 days.

Materials and methods

Materials and reagents

Certified reference standards 6-acetylmorphine, morphine, buprenorphine, hydromorphone, norbuprenorphine, hydrocodone, oxycodone, oxymorphone, alpha hydroxy alprazolam, alprazolam, nordiazepam, oxazepam, and temazepam were purchased from Cerilliant (Round Rock, TX, USA). For quality control samples, hydromorphone, oxycodone, oxymorphone, alprazolam, oxazepam, and temazepam were purchased from Restek (Bellefonte, PA, USA) while buprenorphine, alpha hydroxy alprazolam, and nordiazepam were purchased from Grace (Deerfield, IL, USA). Norbuprenorphine, 6-acetylmorphine, and alpha hydroxy alprazolam quality control samples were different lot numbers from Cerilliant. Deuterated internal standards oxycodone-d3, buprenorphine-d4, hydromorphone-d3, norbuprenorphine-d3, oxymorphone-d3, alpha hydroxy alprazolam-d5, alprazolam-d5, oxazepam-d5, and temazepam-d5 were purchased from Cerilliant. Each standard compound (1 mg/mL) and internal standard (0.1 mg/mL) in methanol was prepared in 30% methanol as working solutions. Acetonitrile, methanol, and water were LC-MS grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). Reagent-grade formic acid, phosphate buffer, and β -glucuronidase enzyme were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate was purchased from Hampton Research (Aliso Viejo, CA, USA). The 96-deep-well plates for solid phase extraction (SPE; GV-65C 96-deep-well) and sample collection were purchased from Scienceware (Wayne, NJ, USA), while the Agilent 96-well

plate (Agilent Technologies, Santa Clara, CA, USA) was used on the LC-MS autosampler.

Sample collection and storage

Urine samples were obtained from four patients (I to IV) with prescribed medications containing opioids and/or benzodiazepines. Samples were received in our laboratory within 24 h after collection and the analyses were performed immediately. For compound stability investigation, urine samples were separated into two aliquots, in polypropylene tubes, and stored at room temperature (about 20°C) and at 4°C for 30 days. Urine samples were prepared by SPE and analyzed by liquid chromatography tandem mass spectrometry.

Sample preparation and SPE extraction

Samples were prepared using a Tecan Genesis RSP 100 Liquid Handler (Tecan Group Ltd., Männedorf, Switzerland). For the standard calibration curve, 100 µL of a 100-µg/mL stock solution (final concentration 5 µg/mL) of internal standards was combined to 30% methanol in water including the reference standard. The final volume of each calibration standard level (1 to 6) was 2 mL. For patient urine samples, 150 µL of urine samples, 38 µL of internal standard mixed (250 ng/mL final concentrations), 300 µL of 0.1 M phosphate buffer pH 6.8, 130 µL of β-glucuronidase enzyme, and 108 µL of water were added in a 96-deep-well plate. An incubation at 47°C for 2 h on a shaker followed. Following the enzymatic digestion, 24 µL of 10% ammonium hydroxide was added to each well and the plate was left at room temperature for 20 min.

For SPE, the 96-deep-well SPE plate on a vacuum manifold was preconditioned with methanol and 250 mM phosphate buffer pH 9. Samples (500 µL) were then added, washed twice with 200 µL of 250 mM phosphate buffer pH 9, and eluted with three additions of 400 µL methanol. The eluate (1,200 µL) was evaporated under nitrogen using a 96-well-plate evaporator system. Samples were then reconstituted in 500 µL of 30% methanol in water, incubated on a shaker for 10 min, and centrifuged at 3,000 rpm for 10 min. The supernatant (250 µL) was then transferred to the Agilent 96-well plate for LC-MS analysis.

LC-MS analysis

LC-MS analyses were performed on an Agilent 1290 Infinity LC coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization source. Chromatographic separation was carried out in a Poroshell 120SB-C18 column (2.1 × 100 mm, 2.7 µm; Agilent Technologies, Santa Clara, CA, USA) with 99.9%

water, 0.05% formic acid, and 0.05% ammonium formate as mobile phase A, and 99.95% acetonitrile and 0.05% formic acid as mobile phase B. The autosampler and the column temperature were maintained at 6°C and 50°C respectively. Gradient elution with mobile phase A and mobile phase B at a flow rate of 0.7 mL/min was as follows: 5% to 50% B over 3 min, 50% to 95% B over 0.5 min, isocratic at 95% B for 0.6 min, 5% to 95% B over 0.1 min, and isocratic at 5% B over 0.7 min. Prior to the next injection, the column was re-equilibrated at 5% B over 1 min. A divert valve switched the LC flow initially to waste for 0.3 min, subsequently to the mass spectrometer for 4.5 min, and then back to the waste for the remaining chromatographic run.

Mass spectral data were obtained in positive electrospray mode, with the following parameters: nitrogen gas temperature, 350°C; gas flow rate, 12 L/min; nebulizer pressure, 45 psi; and capillary voltage, 4,000 V. Ion transitions for each analyte were optimized by flow injection analysis of individual compounds (0.1 µg/mL in methanol) using the Agilent MassHunter Optimizer software. Table 1 shows the transitions, the internal standard used for each compound, the optimized fragmentor voltages for the parent ion, as well as the collision energy for fragmentation of the product ions. Detection and quantitation were performed by dynamic multiple reaction monitoring (MRM) of at least two transitions for each analyte and one transition for each internal standard. The LC-MS analysis data were processed using Agilent MassHunter Workstation.

Validation

Selectivity, sensitivity, linearity, intra-assay imprecision and analytical recovery, extraction efficiency, matrix effect, process efficiency, hydrolysis, and drug carry over were evaluated. Potential endogenous interferences were determined by the analysis of blank urine samples from laboratory volunteers. Sensitivity of the method was examined by establishing the limit of quantitation (LOQ), the half limit of quantitation (1/2 LOQ), and the introduction of the initial calibration verification standards (ICV) in the calibration curve standards. The accuracy of each compound at each calibration level was between 80% and 120%. Linearity was determined using quadratic regression with two replicates for each level. Six-level calibration curves were used with the dilution pattern 1:2:2:2:2:4 for all compounds.

Extraction efficiency, matrix effect, and process efficiency were calculated at three QC concentrations. A set of samples containing the same amount of internal standard and blank urine were spiked with different amounts of analytes corresponding to levels 1, 3, and 5 of the calibration curve. The matrix effect was assessed by comparing the peak areas of the analyte standards in

Table 1 Multiple reaction monitoring (MRM) transitions with optimized fragmentation voltage and collision energy

Compound	RT (min)	MW	ISTD	MRM transition (m/z)	Fragmentor (V)	CE (eV)
Opiates						
6-Acetylmorphine	1.73	327.2	Oxycodone-d3	<i>328.2 > 165.1</i>	173	48
				328.2 > 211.1	173	24
Buprenorphine	3.27	467.3	Buprenorphine-d4	<i>468.3 > 55.1</i>	208	44
				468.3 > 396.2	208	40
Hydromorphone	0.92	285.2	Hydromorphone-d3	<i>286.2 > 185.1</i>	158	28
				286.2 > 128.1	158	64
Norbuprenorphine	2.70	413.3	Norbuprenorphine-d3	<i>414.3 > 152.1</i>	235	100
				414.3 > 165.1	235	104
Oxycodone	1.69	315.2	Oxycodone-d3	<i>316.2 > 241.1</i>	178	28
				316.2 > 256.1	178	24
Oxymorphone	0.75	301.1	Oxymorphone-d3	<i>302.1 > 284.2</i>	179	16
				302.1 > 227.1	179	28
Morphine	0.7	285.2	Morphine-d3	<i>286.2 > 165.1</i>	161	48
				286.2 > 157.1	161	44
Hydrocodone	1.74	299.2	Hydrocodone-d3	<i>300.2 > 199.1</i>	167	28
				300.2 > 171.1	167	40
Benzodiazepines						
Alpha hydroxy alprazolam	3.35	324.1	Alpha hydroxy alprazolam-d5	<i>325.1 > 216.1</i>	105	40
				325.1 > 279.1	105	40
Alprazolam	3.69	308.1	Alprazolam-d5	<i>309.1 > 281.1</i>	99	28
				309.1 > 205.1	99	44
Nordiazepam	3.72	270.1	Temazepam-d5	<i>271.1 > 140.1</i>	161	24
				271.1 > 208.1	161	28
Oxazepam	3.38	286.1	Oxazepam-d5	<i>287.1 > 241.1</i>	167	16
				287.1 > 269.1	167	8
Temazepam	3.73	300.1	Temazepam-d5	<i>301.1 > 255.1</i>	198	20
				301.1 > 177.1	198	40

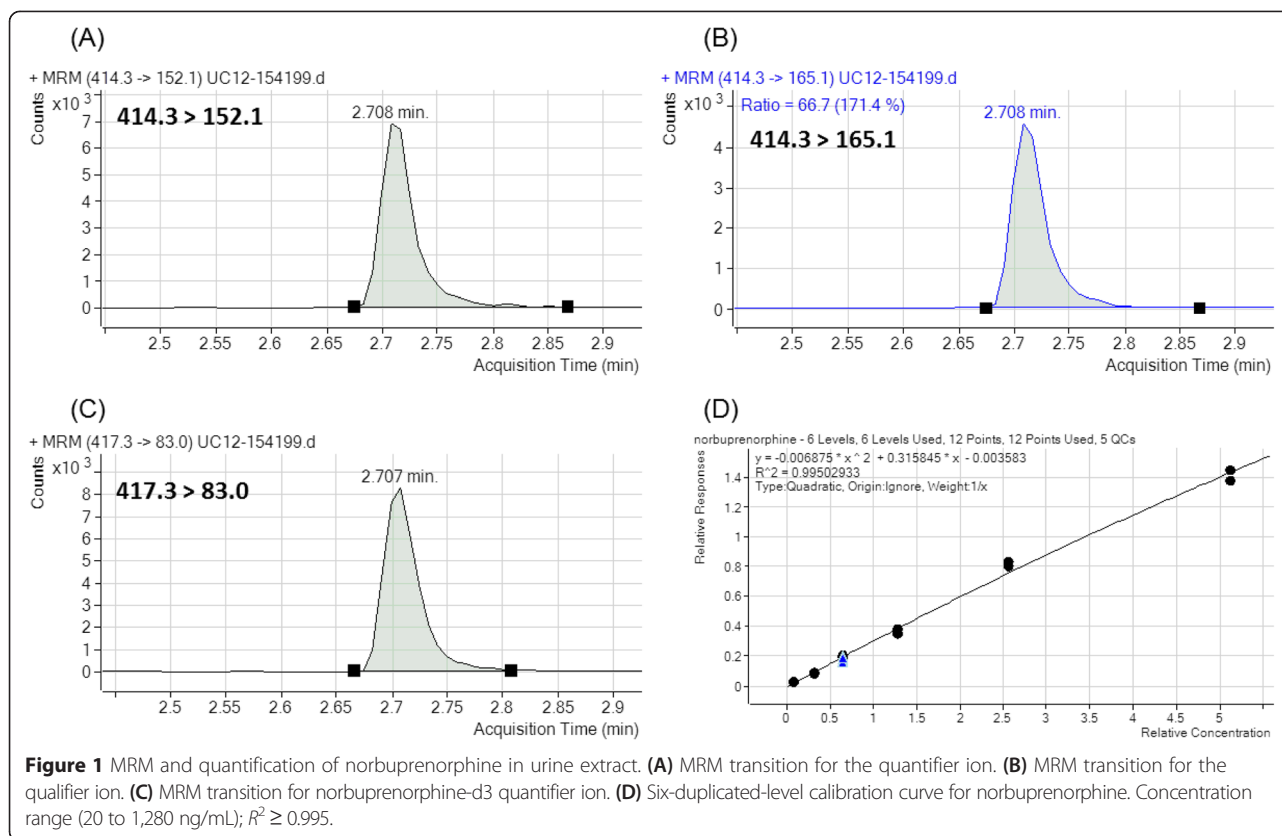
Italicized transitions used for quantification. RT, retention time; MW, molecular weight; ISTD, internal standard; CE, collision energy.

matrix-free solvent with standards spiked into blank urine samples after extraction. The recovery and ion suppression or enhancement associated with the blank urine was evaluated by dividing the concentration of standards from matrix-free with standards in blank urine multiplied by 100.

Results and discussion

The combination of SPE followed by LC-MS analysis is routinely used for metabolomics (Cheng and Mok 2004; Lu et al. 2008; Zhou et al. 2012). A similar procedure has been used to successfully quantify opioids and benzodiazepines from patient urine samples, using a triple quadrupole mass spectrometer. Triple quadrupole tandem mass spectrometry provides sensitive quantitation by acquiring the signal

corresponding to the highest response product ion (quantifier) from the fragmentation of the compound parent ion or precursor ion. The transition between the parent ion and the product ion, known as MRM, is subsequently used to acquire an additional signal corresponding to the next highest product ion (qualifier) (Table 1) (Yost and Enke 1979; Yao et al. 2010). This information may be considered as adequate for confirmation and quantitation of analyte, particularly if the ratio of signal between the two product ions is consistent between the calibration standards and the unknown samples. Dynamic MRM enables MRM acquisitions of compounds during a retention time window corresponding to the elution time of each compound, thus increasing the minimum dwell time (Liu et al. 2011).



Method validation

Using six calibration levels, linear calibration curves were obtained for all analytes with correlation (R^2) equal to or greater than 0.99 (Figure 1). The accuracy of all analytes at all calibration levels was within 20% of the nominal value. The method was also evaluated for the potential presence of ion suppression or enhancements due to matrix effects. The matrix effect was found to be less than $\pm 20\%$ of the nominal values for all compounds. This value is within the acceptable range, as it has been found to vary between 4.5% and 39.5% in umbilical cord samples (de Castro et al. 2009a) and $\leq 25\%$ in urine samples (Cheng et al. 2006; de Jager and Bailey 2011).

Compound stability

Urine samples from four patients under physicians' supervision for pain management were analyzed over 30 days of storage. Table 2 shows patient medical prescriptions, the expected compounds in the urine analyses, the detected compounds and metabolites, as well the initial and final concentrations of the detected analytes after 30 days of storage. Analytes with initial concentrations higher than the higher level of quantitation (HLOQ) were not selected for stability study. Successive quantitation of drugs from patient urine samples was

made in duplicate after 3, 8, 15, and 30 days of storage at room temperature and at 4°C. The percentage of the original amount of drug or metabolites remaining in patient urine samples was calculated at day 30. After 30 days of investigation, no significant difference was detected on the stability of opioids and benzodiazepines in urine samples stored at 4°C and at room temperature (Figure 2). The coefficient of variation of the remaining drug stored at 4°C and at room temperature was 5% or less. A comparison between the recoveries of blood buprenorphine and morphine kept at 4°C and at 25°C has previously been investigated (Hadidi and Oliver 1998). Morphine in blood was found to be reasonably stable regardless of the storage time and temperatures with a recovery ranging from 85% to 94% and from 76% to 80% after 6 and 12 months of storage respectively. Blood buprenorphine was found to be reasonably stable at the storage temperatures with a recovery ranged from 77% to 101% and from 70% to 99% after 6 and 12 months respectively (Hadidi and Oliver 1998). Anagnostis et al. (2011) have recently investigated the stability of buprenorphine in solution stored at room temperature (20°C to 25°C) and in the refrigerator (2.2°C to 7.8°C). Their findings revealed that more than 95% of buprenorphine in ethanolic solution is recovered after 30 days regardless of storage temperatures.

Table 2 Concentration of opioids and benzodiazepines in patient urine samples

Patient	Prescriptions	Expected compounds	Detected compounds and metabolites	LLOQ (ng/mL)	HLOQ (ng/mL)	Concentrations (ng/mL)		Change ^c
						Initial ^a	Final ^b	
I	OxyIR [®]	Morphine, oxycodone	6-Acetylmorphine	3	192	17	9	-
			Hydromorphone	100	6,400	639	900	+
			Morphine	100	6,400	(91,475)	(146,125)	
			Oxycodone	50	3,200	(8,525)	(12,346)	
			Oxymorphone	100	6,400	1,432	2,426	+
			Nordiazepam	80	5,120	4,147	4,092	-
			Oxazepam	80	5,120	4,830	4,684	-
II	Opana [®] , oxycodone	Oxymorphone, oxycodone	Buprenorphine	10	640	23	24	+
			Norbuprenorphine	20	1,280	94	86	-
			Oxycodone	50	3,200	(13,076)	(23,402)	
			Oxymorphone	100	6,400	(10,109)	(15,006)	
			Alpha hydroxyl alprazolam	80	5,120	620	528	-
			Alprazolam	80	5,120	163	154	-
			III	Lortab [®] , Percocet [®] , Soma [®] , Valium [®]	Hydrocodone, acetaminophen, oxycodone, carisoprodol, diazepam	Hydrocodone	100	6,400
Hydromorphone	100	6,400				207	259	+
Oxycodone	50	3,200				3,197	2,941	-
Oxymorphone	100	6,400				365	774	+
Alpha hydroxyl alprazolam	80	5,120				133	57	-
Nordiazepam	80	5,120				1,733	1,533	-
Oxazepam	80	5,120				(6,604)	(5,646)	
IV	Endocet [®] , Soma [®] , Xanax [®]	Oxycodone, acetaminophen, carisoprodo, alprazolam	Temazepam	80	5,120	3,891	2,953	-
			Oxycodone	50	3,200	1,413	1,182	-
			Oxymorphone	100	6,400	491	803	+
			Alpha hydroxyl alprazolam	80	5,120	253	161	-
			Alprazolam	80	5,120	1,003	890	-
			Meprobamate	100	6,400	(14,016)	(13,838)	

LLOQ, lower level of quantification (level 1); HLOQ, higher level of quantification (level 6). ^aInitial concentration of the detected compounds; ^bconcentration of the detected compounds after 30 days of storage at 4°C; ^cchange in the compound concentration after storage: increased (+), decreased (-).

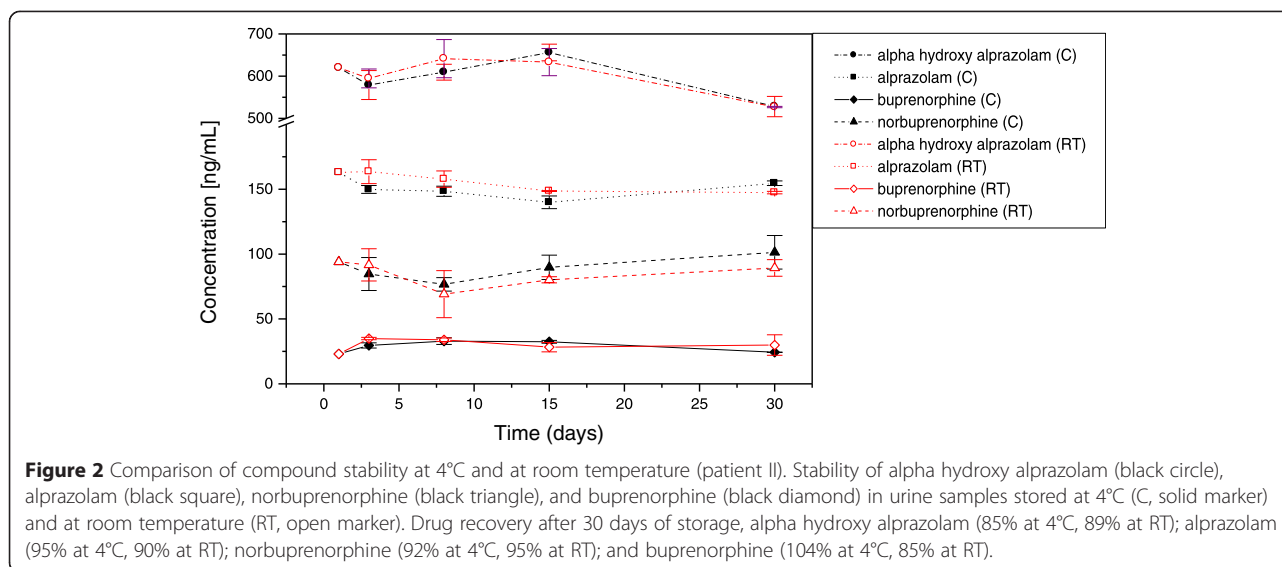
Stability of opioids

No significant difference was detected on the stability of opioids in urine samples stored at 4°C and at room temperature. The coefficient of variation of the remaining drug stored at 4°C and at room temperature was 5% or less for all compounds. Buprenorphine, norbuprenorphine, hydrocodone, and oxycodone showed the best stability with 104%, 92%, 80%, and 84% respectively of the concentration at day 1 after 30 days of storage. 6-Acetylmorphine showed the least stability with 52%, while the concentration of hydromorphone and oxymorphone increased drastically with 125% to 141% and 164% to 212% respectively (Figure 3).

6-Acetylmorphine, a biologically specific indicator marker of heroin, has a high occurrence in oral fluid from

users of this drug, but due to its short half-life, its detection in urine is limited (Allen 2011). Less than 50% of this metabolite remained in the patient urine sample after 3 days (Figure 3), and its concentration remained nearly constant for the rest of the storage duration. After ingestion of heroin, it breaks down in minutes to 6-acetylmorphine and then to morphine. This drug is not medically prescribed in the USA, and it has been given schedule 1 status by the US Drug Enforcement Administration. This drug has been detected in patient I's urine sample with prescribed medications containing morphine and oxycodone.

Buprenorphine and its metabolite norbuprenorphine were both detected in patient II. The concentration of the parent compound decreased over 8 days of storage, while the concentration of its metabolite increased



(Figure 3). The stability of buprenorphine and norbuprenorphine has recently been investigated in whole blood samples from suspected drug users (Selden et al. 2011). This investigation revealed that buprenorphine and norbuprenorphine showed some degradation when stored at 4°C for 3 weeks, and in many cases, the concentrations of buprenorphine were very low compared to norbuprenorphine. These findings support the evidence that buprenorphine may be converted to norbuprenorphine in whole blood sample at 4°C, but this conversion in urine sample has not previously been demonstrated. In the present study, the increase in the concentration of norbuprenorphine is probably due to degradation of buprenorphine.

Hydromorphone and oxymorphone were detected in patients I, III, and IV who were prescribed medications containing morphine and/or oxycodone. Evidence for morphine metabolism to hydromorphone in pain patients chronically treated with morphine has extensively been confirmed (Cone et al. 2006; Hughes et al. 2012). Likewise, the metabolism of oxycodone to oxymorphone in pain patients has also been demonstrated (Yee et al. 2012). However, the occurrence of this transformation in stored urine samples has not been previously demonstrated. After ingestion, morphine is metabolized in the liver into morphine-3-glucuronide and morphine-6-glucuronide (Kilpatrick and Smith 2005). It may also be metabolized into normorphine, codeine, and hydromorphone, but the metabolism rate is determined by gender, age, diet, genetic makeup, disease state, and the use of other medications. In humans, oxycodone is primarily metabolized in the liver to α and β oxycodol and oxymorphone (Moore et al. 2003). Some patients are fast metabolizers of oxycodone, resulting in reduced analgesic effect but increased adverse effects, while others

are slow metabolizers resulting in increased toxicity without improved analgesia (Gasche et al. 2004). The concentrations of the metabolites hydromorphone and oxymorphone in urine samples after 30 days of storage were very high compared to the initial concentration at day 1 and independently to patients. This trend once more supports the evidence that parent compounds may be converted into corresponding metabolites in urine samples stored at 4°C. The metabolite concentration levels depend on the concentration of the parent compounds. These two metabolites can still be analyzed in patient urine samples stored at 4°C for 30 days, when patients have been prescribed medications containing the main compounds.

Stability of benzodiazepines

Unlike the variation in the concentrations of opioids investigated, the concentrations of all benzodiazepines decreased over 30 days of storage at 4°C. Alprazolam, nordiazepam, and oxazepam showed the best stability with 95%, 90% to 98%, and 97% of the original concentration at day 1 respectively, while alpha hydroxy alprazolam (43% to 85%) and temazepam (68% to 78%) showed more degradation (Figure 3).

In humans, alprazolam is extensively metabolized to alpha hydroxy alprazolam and 4-hydroxy alprazolam, whose plasma concentrations relative to unchanged alprazolam concentration are less than 4% (Smith et al. 1984). The stability of alprazolam is attributed to the presence of the triazole ring in its structure which makes the compound more resistant to hydrolysis. In a recent study on the stability of benzodiazepines in postmortem samples, estazolam whose structure is similar to alprazolam was the most stable in unpreserved blood samples at 4°C (Melo et al. 2012). The coefficient of variation

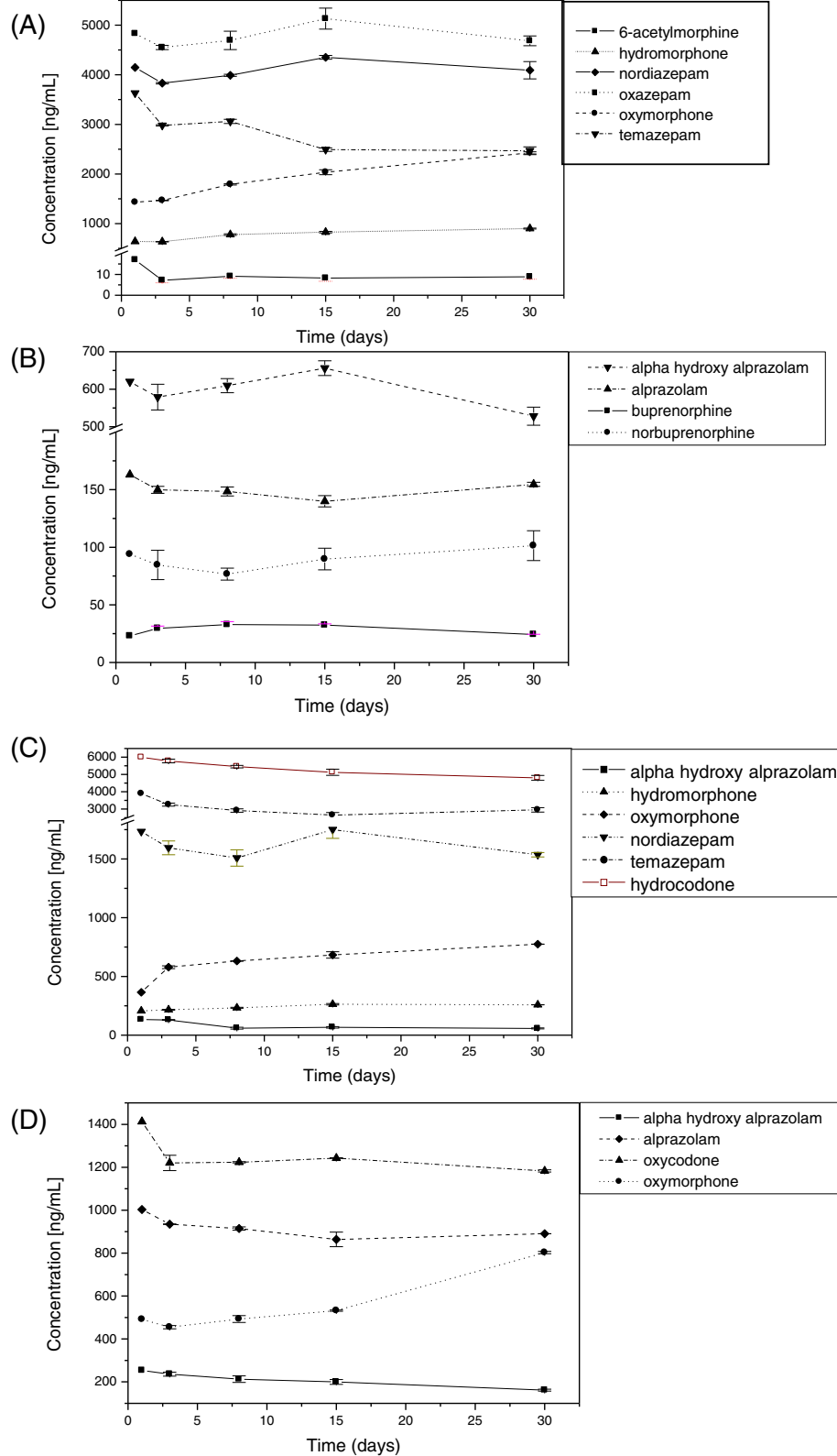


Figure 3 Change in urine opioid and benzodiazepine recovery with time. Change in detected compounds from patient I (A), patient II (B), patient III (C), and patient IV (D).

ranged from 1.4% to 6% over 4 weeks of storage. Alprazolam and alpha hydroxy alprazolam were detected in patients II and IV, but only patient IV has been prescribed medications containing alprazolam.

The main active metabolite of diazepam is nordiazepam and the minor active metabolites are oxazepam and temazepam (Riss et al. 2008). The half-life of diazepam is about 1 to 3 days and 2 to 7 days for nordiazepam. The elimination half-life of diazepam and nordiazepam increases considerably in the elderly, which may result in the prolonged action and accumulation of the drug during repeated administration (Vozeh 1981). Nordiazepam, oxazepam, and temazepam were detected in patients I and III, but only patient III was under prescription of medications containing diazepam, the parent compound. Nordiazepam and oxazepam were the most stable compared to temazepam. Atanasov et al. recently investigated the stability of diazepam in blood samples at different storage conditions (Atanasov et al. 2012). The authors showed that the concentration of diazepam decreased by 30% after storage for 4 weeks at 4°C. However, diazepam and chlordiazepoxide are benzodiazepines with long-acting active metabolites like nordiazepam. We could also observe an increase in the concentration of nordiazepam (patients I and III) after 2 weeks of storage at 4°C, thus suggesting diazepam degradation into nordiazepam (Figure 3). The data obtained from this study suggested that opioid and benzodiazepine degradation with consequent metabolites may occur by chemical breakdown during storage. In postmortem samples, it has been suggested that decomposition may occur due to microorganisms' activity (Melo et al. 2012; Levine et al. 1983).

Conclusions

This work implies that some patients do not take their medications according to their physician's prescription or take illicit drugs in addition to their prescriptions. When interpreting toxicological results related to possible opioid or benzodiazepine exposure, it is important to consider that the presence of their metabolites in urine samples can be due to patient biotransformation of the drug (in vivo), but also due to their decomposition during storage (in vitro). Opioid and benzodiazepine concentrations in urine samples can change due to degradation during sample storage. This study revealed that opioid and benzodiazepine degradation increased with storage time. The change in concentration of metabolites after sample storage suggests that the biotransformation of parent drugs to metabolites still occurs during storage at positive temperatures. Quantitative results from long-term-stored urine samples with opioids or benzodiazepines should be interpreted cautiously. Ongoing monitoring of these patients will help the laboratory and clinician draw conclusions on patient adherence to their

prescription regimen. In efforts to enable accurate interpretation of the toxicological results, it is important to know the stability of the drugs at several storage conditions and in different matrices. This study demonstrates the need for more detailed studies of in vitro changes of analytes in biological samples and in urine samples particularly. By considering other work related to this topic, and by conducting more research aimed at particular storage conditions, degradation models may be applied.

Findings

The purpose of this study was to investigate the stability of opioid and benzodiazepine drugs and metabolites in stored urine specimens. This is an area of interest for clinical and forensic toxicology applications. The data was collected under validated methodologies with quality control samples run with each batch. The results demonstrated that sample collection and storage conditions are important considerations in the context of drug testing.

Competing interests

Drs. Brent Dixon and Flaubert Mbuenkui are scientists employed by Physicians Choice Laboratory Services. Joseph Wiegel is an owner of Physicians Choice Laboratory Services.

Authors' contributions

RBD made substantial contributions to the study design, interpretation of data, and manuscript revision. FM made substantial contributions to the study design, analysis and interpretation of data, and manuscript preparation. JWW made substantial contributions to the interpretation of data, manuscript revision, and final approval. All authors read and approved the final manuscript.

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