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Concerns in the development of an assay for determination of a highly conjugated adsorption-prone compound in human urine

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Abstract

Concerns in pre-analytical handling of urine samples are discussed using a new KDR kinase inhibitor, 3-[5-(4-methanesulfonyl-piperazin-1-ylmethyl)-1*H*-indol-2-yl]-1*H*-quinolin-2-one (compound A), as an example of a case where high light sensitivity and low analyte recovery (high affinity for container surface) were found. The absence of these problems in plasma samples may be a result of the plasma protein content. Low recovery of the analyte from urine can be remedied by either changing the container or by using additives, such as bovine serum albumin (BSA) or non-ionic surfactant Tween-20. In the case of compound A, changing containers (polypropylene versus glass vial) or addition of BSA did bring analyte recovery up to 80%. However, the addition of 0.2% Tween-20 into urine quality controls (QCs) gave more than 95% analyte recovery, indicating effective reduction of analyte loss to the surface of containers. The urine assay using mixed-mode SPE and LC–MS/MS was not affected significantly by introducing Tween-20 into the samples. The mean SPE extraction recovery was 68.4% and matrix suppression of ionization on MS was less than 8% at all analyte concentrations. The linear range of the calibration curve was 0.5–400 ng/mL on PE Sciex API 3000 LC–MS/MS system. The assay intraday accuracy and precision were 92.1–104.8% and <4.2% (%CV), respectively. Urine QC samples, containing 0.2% Tween-20, gave excellent recovery after three cycles of freeze and thaw. Since analyte loss to its urine container surface is not unique to compound A (M. Schwartz, W. Kline, B. Matuszewski, Anal. Chim. Acta 352 (1997) 299–307; A.L. Fisher, E. DePuy, T. Shih, R. Stearns, Y. Lee, K. Gottesdiener, S. Flattery, M. De Smet, B. Keymeulen, D.G. Musson, J. Pharm. Biomed. Anal. 26 (2001) 739–752), we suggest an evaluation of the potential problem in the early stages of urine assay development to ensure reliable quantitation of analytes. The addition of Tween-20 can serve as a useful analytical tool to other analytes with similar situations.
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Keywords: Urine assay; Low QC recovery from urine; Tween-20; Solid-phase extraction and LC–MS/MS

1. Introduction

Urinary excretion is an essential issue in determination of renal clearance of investigational drugs. As a consequence, developing a reliable urine assay is important to support phase I clinical trails for drug development. The major difference between urine and plasma in terms of their compositions is that the latter contains 6–8% proteins while the former does not [1]. Lack of proteins in urine sometimes makes the pre-analytical handling of urine samples a challenge, especially for the light sensitive and adsorption-prone compounds. A number of cases demonstrating low recovery of analytes from urine samples has been reported previously [2–5]. Although the addition of control human plasma or bovine serum albumin into human urine solved the problem [2,3], their effect may vary significantly depending on the protein-binding properties of different analytes. Polyoxyethylene sorbitan detergents, such as Tween-20 and Tween-40, were used to effectively improve the recovery of proteins and antigens in urine and urinary tissue by reducing nonspecific binding of protein in ELISA assay and membrane filtration assay [4,5]. However, the effectiveness of these surfactants for small molecular drugs and their interference on solid-phase extraction and MS/MS detection are still unclear.

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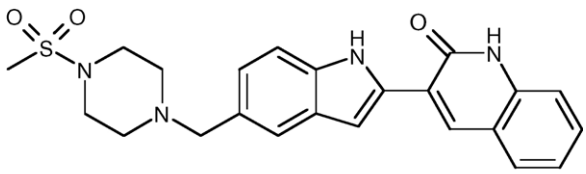
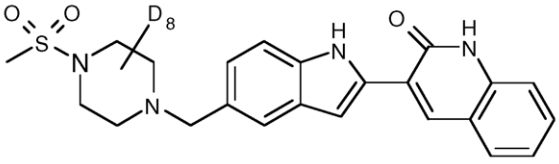


Compound A



I.S.

Fig. 1. Chemical structures of compounds A and the internal standard (I.S.).

Compound A, 3-[5-(4-methanesulfonyl-piperazin-1-ylmethyl)-1*H*-indol-2-yl]-1*H*-quinolin-2-one (Fig. 1), is a drug candidate that is currently under clinical investigation for the treatment of cancer. This compound is light sensitive and adsorption prone to the surface of containers, with a molecular weight of less than 500 Da. It is a highly protein bound drug with 2.8–3.7% unbound in human plasma at 2 and 10 μM concentrations. In rats, unchanged drug excreted in urine and bile accounted for about one-third of the dose, and *N*-dealkylation followed by glucuronidation was the major route of metabolism. Development of a urine assay for human samples was required before starting the first clinical study. In this report, the behavior of compound A in human urine samples is presented as an example of a case where special pre-analytical handling was addressed, and the effectiveness of additives on improving analyte recovery was evaluated. Furthermore, a urine assay using solid-phase extraction (SPE) coupled with LC–MS/MS for the determination of compound A was developed, and the validation results suggested that the addition of Tween-20 effectively improved recovery of compound A from urine samples without significant interference on SPE extraction and LC–MS/MS analysis.

2. Experimental

2.1. Materials and solutions

Compound A, and deuterium labeled internal standard (I.S.) (Fig. 1) were synthesized at the Merck Research Laboratories, Merck & Co. (West Point, PA). Human urine was collected from healthy males from the laboratory staff. Human control plasma (sodium heparin as anticoagulant) was purchased from Biological Specialty Co. (Colmar, PA, USA). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA) and surfact-amps 20 (active 10% Tween-20) was purchased from Pierce (Rockford, IL, USA). HPLC grade acetonitrile and ammonium acetate, optima grade methanol, laboratory grade formic acid (90%) and ACS grade acetic acid and ammonium hydroxide (29.7%) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Water was purified by a Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA). Oasis® MCX (mixed-mode cation exchange) 96-well SPE plate (10 mg/well) was purchased from Waters Corporation.

2.2. Equipment

A Packard MultiPROBE II automated liquid handling system (Meriden, CT, USA) and TomTec Quadra 96 workstation (Model 320, Hamden, CT, USA) were used to perform sample transfer and automated solid-phase extraction, respectively. Light sensitivity test under 254 nm UV light was conducted with a Rayonet Photochemical Reactor from the Southern New England Ultraviolet Company (Branford, CT, USA). LC–MS/MS was performed on a Perkin Elmer Series 200 LC micro pump (Ontario, Canada) and Leap Technology HTS PAL System (96-Well Plate Autosampler, Carrboro, NC, USA), coupled to a Sciex API 3000 triple-quadrupole mass spectrometer with a Sciex Turbo Ion Spray Interface (Sciex, Toronto, Canada). The data were collected and processed through Analyst 1.1 or Analyst 1.2 software (Sciex, Toronto, Canada).

2.3. Urine standards and quality controls (QCs)

Stock and working stock solutions of compound A and I.S. were prepared in acetonitrile and 0.1% formic acid in the ratio of 50:50 (v/v). The calibration curves consisting of at least seven concentrations of compound A in a dynamic range of 0.5–400 ng/mL were prepared on a daily basis. To test the effectiveness of additives in preventing the adsorption of compound A, QC samples were prepared in human urine with or without additives, BSA or Tween-20. To test the effect of different containers, both plasma and urine QCs were transferred into either polypropylene (Sarstedt, Germany) or glass (Fisher Scientific, PA, USA) or siliconized glass vials (treated with trimethylsilanol, PPD Pharmaceutical Inc.), and stored at −70 °C freezer. The QC recovery was calculated by comparing mean measured concentration with nominal concentration of compound A to reflect the analyte loss in the different containers and in the absence or presence of additives.

2.4. Urine sample preparation and SPE extraction

Compound A was extracted from urine in the presence of 0.2% Tween-20. Tween-20 was added to the clinical samples during urine collection and to control urine during standard curve and QC preparation. After thawing and centrifuging at 3000 rpm for 5 min, 400 μL of control urine or QC samples were mixed with 40 μL of working standard or solvent

(for QCs), 40 μL of 100 ng/mL of I.S. and 520 μL of 0.1 M acetic acid, handled by a MultiPROBE II. The acidified sample (900 μL) was then loaded on Oasis® MCX SPE plate under applied vacuum. The sample wells were washed with 800 μL of 0.1 M acetic acid followed by 800 μL of acetonitrile, and centrifuged at 1500 rpm for 10 min. The analytes were eluted with 0.5 mL of acetonitrile/29.7% ammonium hydroxide (95/5, v/v). The collected extract was dried under nitrogen at 35 °C, and the residues were reconstituted into 150 μL of acetonitrile/0.1% formic acid (50/50, v/v). All procedures were conducted under yellow light except where indicated.

2.5. Chromatographic conditions and MS/MS detection

The HPLC separation was performed on an Aquasil C18, 50 mm × 2.1 mm (3 μm) column from Keystone Scientific (Bellefonte, PA, USA), with a mobile phase of acetonitrile/ammonium acetate buffer (5 mM, pH 5.0) at a ratio of 60/40 (v/v) at the flow rate of 0.25 mL/min. The injection volume was 10 μL and the autosampler temperature was set at 5 °C.

MS/MS detection was performed on a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray interface in a positive ion mode. The ion transitions (precursor ion → product ion), *m/z* 437 → 273 for compound A and *m/z* 445 → 273 for I.S., were selected for multiple reaction monitoring (MRM). A high voltage of 4.5 kV was applied to the sprayer. The turbo gas temperature was 450 °C, and the auxiliary-gas flow was set at 8 L/min. The nitrogen flow rates of nebulizing gas, collision gas, curtain gas were set at 12, 8 (CGT = 2.0×10^{15} molecules per cm^2) and 8 L/min, respectively. The optimized declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and focusing potential (FP) were 27, 15, 19 and 150 V for compound A, and 35, 17, 22 and 240 V for I.S., respectively. The optimized entrance potential (EP) were 10 V for all compounds. The dwell times were 350 and 400 ms for compound A and I.S., respectively. Calibration curve was obtained by weighed ($1/x \times x$) least squares linear regression on the peak area ratio of analyte to I.S. versus the nominal concentration (x) of analyte.

2.6. Method validation for urine assay

The urine method was validated in the presence of Tween-20 (0.2% in control urine and QCs). The selectivity of the assay was confirmed by processing control urine from six different healthy volunteers. Intraday precision and accuracy were determined by analyzing five sets of spiked standard samples in five lots of control Tween-20 urine. The final concentrations on the standard curves were 0.5, 2, 10, 20, 100, 300 and 400 ng/mL. QC samples containing 0.2% Tween-20 were analyzed after first freezing and thawing, and the calculated concentrations were considered as the initial values. Freeze–thaw stability was evaluated using QC samples that went through three cycles of freezing and thawing, with at least one-day storage at −70 °C between each thaw.

SPE extraction efficiency was calculated by comparing peak areas of five replicates of Tween-20 urine standards with peak areas of standards that were post-spiked into the drug-free urine SPE extract at the concentrations of 0.5, 20 and 200 ng/mL of compound A. It is worth to clarify that SPE extraction recovery is different from the QC recovery mentioned in Section 2.3. The former reflects analyte loss during SPE extraction, while the latter reflects analyte loss prior to sample analysis, including sample collection and storage.

The matrix effect was evaluated by comparing the absolute peak areas of urine standards that was post-spiked into the drug-free urine SPE extract with those of the neat standards prepared in reconstitution solvent at the concentrations of 0.5, 20 and 200 ng/mL ($n = 5$ at each concentration) of compound A.

2.7. Plasma assay

To compare pre-analytical handling of urine sample versus plasma sample, plasma QCs were evaluated using the method reported recently [6]. The plasma assay procedures were similar to the urine assay's in terms of preparation of standards, QCs and clinical samples, SPE extraction, chromatographic conditions and MS/MS detection, except for the following differences: (1) no Tween-20 was added to standards, QCs and clinical samples; and (2) SPE eluting solvent was 0.5 mL of methanol/29.7% ammonium hydroxide (95/5, v/v). The assay was validated with a calibration range of 0.05–400 ng/mL and QCs of 0.15, 10 and 200 ng/mL.

3. Results and discussion

3.1. Comparison of pre-analytical handling of urine samples versus plasma samples and the effect of additives on recovery of compound A from human urine

Compound A is a highly conjugated molecule, which can absorb energy from light and is subject to oxidation. In order to provide a guideline for sample handling during clinical trials, its light sensitivity was evaluated with plasma and urine QC samples. Three replicates of samples at each of the three concentrations were kept under yellow light, regular laboratory white light and 254 nm UV light, respectively, at room temperature for 4 h. The results indicated that compound A was more stable in plasma than in urine (Table 1), which may be the result of protection provided by plasma proteins. Thus, urine samples required more strict light protection procedures during clinical sample collection and analysis.

Following the development and validation of the plasma assay [6] for compound A, an attempt to directly apply the method to urine was not initially successful because of low recovery (about 60%) observed for compound A from urine QC samples (Table 2). The loss of compound A was most likely

Table 1
Light sensitivity of compound A in plasma and Tween-20 urine quality control samples

Sample matrix	Nominal concentration (ng/mL)	Accuracy (%)[a] [%CV] ($n = 3$)		
		Yellow[b]	White[c]	UV[d]
Plasma	0.15	105.0 [2.3]	105.8 [4.5]	89.0 [3.9]
	0.30	102.5 [8.0]	105.3 [1.7]	86.3 [2.0]
	10.0	94.0 [8.6]	100.6 [0.5]	83.7 [1.6]
	200.0	101.2 [0.8]	98.8 [0.7]	83.6 [2.1]
Urine	1.5	94.9 [7.0]	86.1 [6.8]	50.0 [5.6]
	20	99.1 [2.0]	95.9 [2.5]	3.8 [105]
	200	97.7 [0.4]	91.3 [2.7]	30.6 [10.5]

[a] Expressed as [(mean measured concentration/initial concentration) × 100]%.
[b] QC samples placed under yellow light at room temperature for 4 h.
[c] QC samples placed under regular white light at room temperature for 4 h.
[d] QC samples placed under UV light (254 nm) at room temperature for 4 h.

Table 2
Comparison of recovery of compound A from plasma and urine (no Tween-20) quality control samples in different containers

Sample matrix	Nominal concentration (ng/mL)	QC recovery (%)[a] [%CV]		
		PP[b]	Glass	Sil. glass[c]
Plasma	0.15	102.5 [2.1]	106.9 [13.2]	–[d]
	0.30	106.3 [1.9]	106.9 [0.6]	–[d]
	10.0	109.0 [4.7]	111.7 [5.8]	–[d]
	200.0	106.9 [5.0]	109.5 [6.7]	–[d]
Urine	2	60.6 [5.5]	79.8 [9.4]	84.3 [3.1]
	20	63.8 [5.3]	80.9 [3.7	80.9 [1.7]

[a] Expressed as [(mean measured concentration/nominal concentration) × 100]%. All data were average of three measurements, except five replicates for plasma in polypropylene tubes.
[b] QC stored in polypropylene tubes.
[c] QC stored in siliconized glass tubes.
[d] Not determined.

due to the adsorption of analyte to the surface of containers. The reason for good plasma recovery (Table 2) may be due to plasma proteins that keep the analyte away from container surface through stronger interactions, such as hydrogen bonding and hydrophobic attractions, i.e., protein binding. Since blood samples are usually collected in heparin containing glass tubes, the recovery from plasma in glass container was also tested. No analyte loss was observed in the glass container (Table 2), and this suggested the plasma collection procedure is suitable for compound A. In contrast, special pre-analytical handling for urine samples has to be considered.

Three methods were evaluated based upon their effects on analyte recovery from urine. First, glass containers (with or without treatment of trimethylsilanol) were tested in an attempt to minimize interaction of the analyte to their container surface. The result showed that changing the urine container from polypropylene to glass can increase recovery from 60% to about 80% (Table 2), but not to the expected ∼100%. Introducing proteins such as plasma and BSA has been reported in the literature to successfully remedy the low-recovery problem in urine [2,3]. However, for compound A, the improvement of urine recovery by the addition of BSA up to 1.5% was not satisfactory (Table 3). Finally, the addition of Tween-20 was considered and evaluated. It has been reported that non-ionic surfactants, such as Tween-series, can reduce nonspecific protein binding in assays such as ELISA and membrane filtration assay [4,5]. These detergents, which bind to the proteins in their monomeric form, do not usually denature proteins and do not

Table 3
Effect of bovine serum albumin (BSA) and Tween-20 on recovery of compound A from human urine

Additive	Concentration of additive in urine (%)	QC recovery (%)[a] [%CV] ($n = 3$)	
		2 ng/mL of A	20 ng/mL of A
BSA	0	60.6 [5.5]	63.8 [5.3]
	0.5	83.0 [1.2]	81.2 [0.8]
	0.75	81.5 [5.9]	81.4 [0.9]
	1.0	84.5 [9.4]	80.6 [0.5]
	1.5	78.6 [8.6]	79.7 [1.9]
Tween-20	0	60.6 [5.5]	63.8 [5.3]
	0.1	92.4 [1.0]	87.3 [9.9]
	0.2	97.9 [0.8]	95.2 [0.3]
	0.5	97.8 [4.4]	94.8 [1.0]
	0.75	96.1 [1.8]	94.8 [1.4]

[a] Expressed as [(mean measured concentration/nominal concentration) × 100]%.

Table 4
Precision and accuracy of Tween-20 urine quality control (QC) samples of compound A

Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Accuracy[a] (%)	Precision[b] (%CV)
Tween-20 + A[c] ($n = 5$)			
1.50	1.51[d]	100.8	3.6
20.0	18.06[d]	90.3	4.0
200.0	178.8[d]	89.4	2.8
A + Tween-20[e] ($n = 3$)			
1.5	1.54	102.7	5.8
20.0	18.17	90.8	4.4
200.0	183.3	91.7	8.8

[a] Expressed as [(mean measured concentration/nominal concentration) × 100]%.
[b] Expressed as coefficient of variation (%CV) based on peak area ratios.
[c] The QCs prepared by spiking compound A into Tween-20 containing control urine.
[d] Considered as the initial concentration of urine QCs.
[e] The QCs prepared by spiking compound A into control urine, mixing, and then adding Tween-20.

disturb the specific protein–protein interaction [7]. Applying these characteristics to our study, Tween-20 was expected to reduce nonspecific binding between the small molecular analyte and container surface without disturbing the interaction between analyte and SPE sorbent during sample extraction. Our results showed that addition of Tween-20 did significantly increase analyte recovery in urine. Among the tested Tween-20 concentrations in urine (0.1, 0.2, 0.5 and 0.75%), 0.2% gave the best result—more than 95% of compound A was recovered from urine at 2 and 20 ng/mL of analyte levels with limited variation (Table 3). In order to figure out the timing effect of Tween-20 addition, the recoveries of compound A from urine were compared between two situations: one is addition of Tween-20 to control urine followed by spiking of compound A (the situation used for preparation of standard and QC samples); the other is addition of Tween-20 to the urine that contains compound A (the situation similar to urine sample collection at the clinical site). The result showed that there was no significant difference between two procedures (Table 4) and suggested that Tween-20 can be added to the urine anytime before the sample is transferred

Table 6
Intraday precision and accuracy for the determination of compound A in five lots of control human Tween-20 urine

Nominal concentration (ng/mL)	Mean concentration (ng/mL) ($n = 5$)	Accuracy[a] (%)	Precision[b] (%CV)
0.5	0.511	102.2	4.2
2.0	1.84	92.1	2.3
10.0	9.68	96.8	2.1
20.0	19.7	98.6	2.1
100.0	105	104.8	1.2
300.0	305	101.7	1.3
400.0	415	103.9	1.6
Linear regression	$R = 0.9987$; slope = 0.15; intercept = −0.0237		

[a] Expressed as [(mean measured concentration/nominal concentration) × 100]% ($n = 5$).
[b] Expressed as coefficient of variation (%CV) based on peak area ratios ($n = 5$).

to other containers. Based on this result, we recommended that during sample collection, urine specimen should be weighed by subtracting the weight of the empty bottle from that of the urine containing bottle. The appropriate amount of Tween-20 calculated based on the weight of the specimen in grams (0.2% Tween-20 in the final solution) should be added and recorded. An aliquot of the Tween-20 treated specimen can then be transferred to a pre-labeled polypropylene centrifuge tube for storage and shipment.

3.2. Effect of Tween-20 on SPE extraction and MS/MS detection, and validation of the urine assay

Based on the above results, 0.2% Tween-20 was added during urine sample handling. Since the assay utilized mixed-mode Oasis MCX 96-well extraction plate in conjunction with LC–MS/MS, further experiments were conducted to determine whether Tween-20 interferes with solid-phase extraction and MS/MS detection, and consequently affects the assay accuracy and precision.

SPE extraction recovery and the matrix effect in the presence of Tween-20 were evaluated. Five replicates of Tween-20 urine QC were used at each of the nominal concentrations (0.5, 20 and 200 ng/mL) of compound A, and generated 15

Table 5
Extraction recovery and matrix effect of compound A and IS in human Tween-20 urine

Nominal concentration (ng/mL)	Mean peak area ($n = 5$) (pre-spiked)[a]	Mean peak area ($n = 5$) (post-spiked)[b]	Mean peak area ($n = 5$) (neat)[c]	Extraction recovery[d] (%)	Matrix effect[e] (%)
Compound A					
0.5	4114	5128	5568	89.1	92.1
20	198400	322200	345400	68.4	93.3
200	2282000	3396000	3542000	74.7	95.9
IS ($n = 15$)					
10	67753	110400	119933	68.2	92.1

[a] Pre-spiked is the standard spiked into urine before extraction.
[b] Post-spiked is the standard spiked in the extract of control urine.
[c] Neat is the standard in reconstitution solvent without Tween-20.
[d] Calculated as [(mean pre-spiked peak area/mean post-spiked peak area) × 100]%.
[e] Calculated as [(mean post-spiked peak area/mean neat peak area) × 100]%.

replicates for I.S. at the working concentration of 10 ng/mL. The mean extraction recovery was higher than 68.2%, and matrix suppression of ionization was less than 8% for both compound A and I.S. at all tested concentrations (Table 5). Compared to 80% extraction recovery and about 10% matrix effect in the plasma assay (no Tween-20) [6], the effect of Tween-20 in urine assay is negligible. It is worth mentioning that introducing Tween-20 into the mass spectrometer can cause ion suppression, more significantly to the analyte at low concentrations (as observed with neat standards that contained 0.2% Tween-20 and were directly injected into the LC–MS/MS). The mixed-mode SPE used in this assay allowed efficient washout of the non-ionic surfactant Tween-20, and resulted in a negligible matrix effect.

The urine assay was validated in the concentration range of 0.5–400 ng/mL using 0.4 mL of Tween-20 urine. The mean accuracy of five replicates intra-day determination was between 92.1% and 104.8%. The precision, as measured by coefficient of variation (%CV), was less than 4.2% for each concentration on the calibration curve (Table 6). The selectivity of the urine assay was assessed in six different lots of human control urine containing 0.2% Tween-20. No interfering peak was observed in the retention time window of the analyte and internal standard under the assay conditions. Representative extracted ion chromatograms of a control Tween-20 urine single blank and a Tween-20 urine containing 0.5 ng/mL of compound A (LLOQ) with 10 ng/mL of I.S. are shown in Fig. 2 (panels A and B).

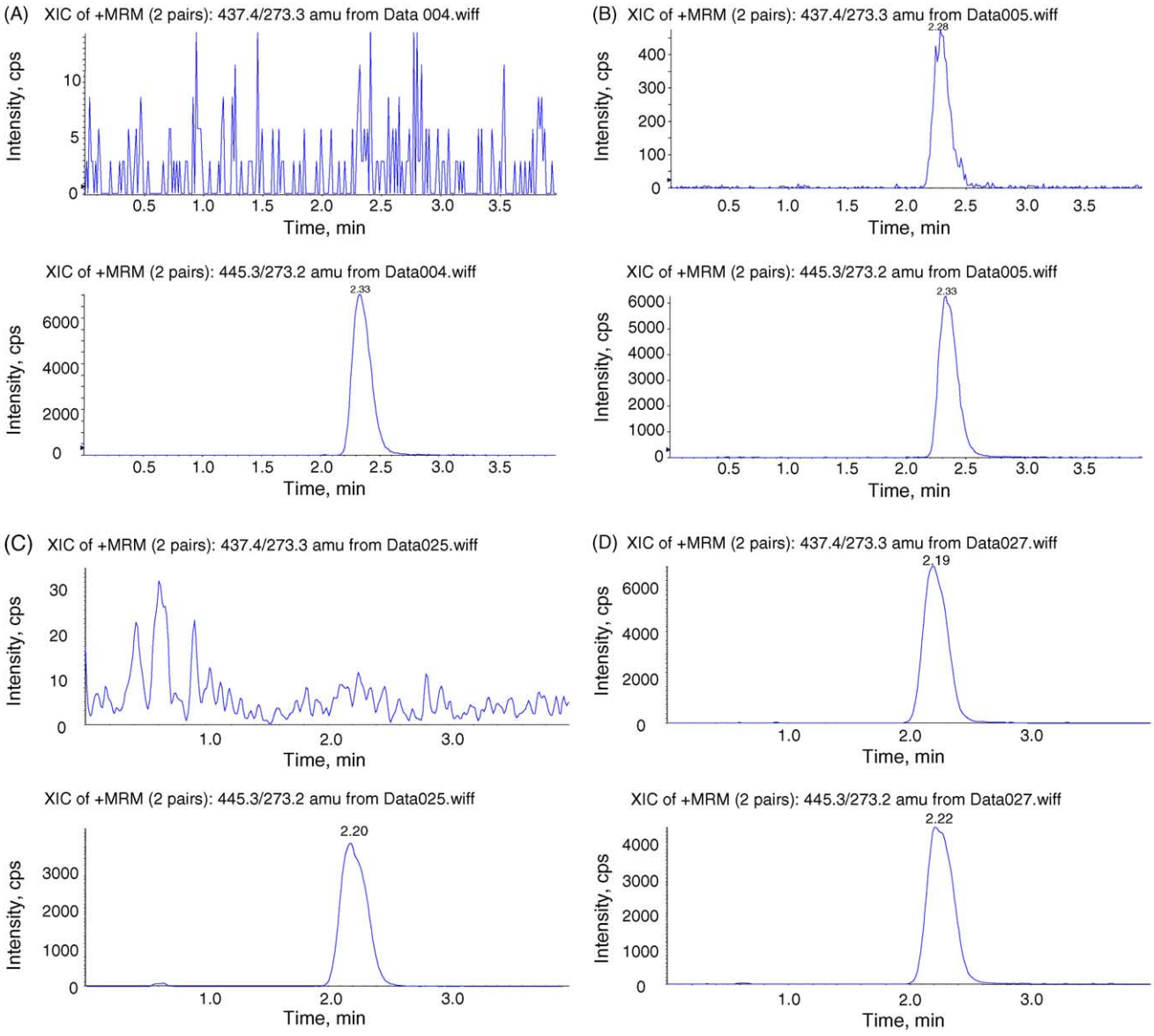


Fig. 2. Representative extracted ion chromatograms (XIC) of (A) single blank, control human Tween-20 urine fortified with 10 ng/mL I.S.; (B) lower limit of quantification (LLOQ), 0.5 ng/mL of compound A with 10 ng/mL I.S. in urine standard. (C) Predose urine sample from Subject #002; (D) 4–8 h post-dose urine sample from subject #002 following a 12-mg single oral dose of compound A. (In all panels, upper: compound A; lower: I.S.)

Table 7
Stability of QC samples of compound A in human Tween-20 urine

Nominal concentration (ng/mL)	Percentage of initial concentration[a]		
	3*F*/*T*[b] (*n* = 3) [%CV][c]	20 days/−70 °C (*n* = 3) [%CV][c]	3 months/−70 °C (*n* = 2) [%CV][c]
1.50	100.0 [2.9]	100.7 [4.7]	111.9 [1.7]
20.0	99.6 [2.1]	99.5 [4.7]	110.5 [3.5]
200.0	100.5 [6.4]	101.6 [7.9]	107.8 [3.7]

[a] Expressed as [(mean measured concentration/initial mean concentration in Table 4) × 100]%.
[b] QC samples after three cycles of freeze and thaw.
[c] Coefficient of variation.

QC samples in Tween-20 urine were prepared and assayed at concentrations of 1.5 (three times of LLOQ for compound A), 20 and 200 ng/mL of compound A. The precision and accuracy ($n = 5$ at each concentration) are given in Table 4, and the measured mean concentrations were used as initial values to evaluate the stability of the analytes. The stability test indicated that compound A is stable in Tween-20 urine after three cycles of freezing and thawing, and stable at −70 °C for at least 3 months (Table 7).

3.3. Application to clinical studies

The described pre-analytical handling procedures and analytical method have been successfully applied to the determination of urine concentrations of compound A in support of pharmacokinetic analysis in phase I clinical studies. Representative chromatograms of human clinical urine samples obtained from a dosed subject are shown in Fig. 2 (panels C and D).

3.4. Pros and cons of using different additives in urine assay

Low urine QC recovery (due to sample loss in a container) has been observed in many other cases in our laboratory during method development of urine assay to support clinical studies. The most commonly used approach to solve this problem for small-molecule drug candidates, so far, is to add BSA to urine samples. The work described here provides a useful alternative tool which uses non-ionic surfactant Tween-20 as an effective additive to prevent the sample loss during sample collection and storage.

As always, every technology has its pros and cons. BSA, as part of plasma protein, generally works well for the urine assay that is modified from the validated plasma assay, regardless whether the sample preparation is liquid-liquid extraction, solid-phase extraction or on-line extraction. However, for some drug candidates such as compound A, addition of BSA may not be adequate to bring the QC recovery up to 100% (Table 3). Besides, sometimes, BSA itself such as its availability in European clinical sites, its cost (especially when more than 1% of BSA is required) and the difficulty to prepare and transfer because of its high viscosity can be a concern during the clinical studies. In contrast, addition of a small amount of Tween-20 can effectively prevent analyte adsorption to the surface of the container, and it is cost effective and easy to handle at the clinical site. In the urine assay using mixed-mode SPE, the MS/MS detection was not affected by introducing Tween-20 into the urine samples. However, special attention should be made to the chosen extraction method because Tween-20 may cause MS ion suppression at low drug concentration if Tween-20 were not removed from the matrix during sample preparation.

The extraction technique used in this paper is ideal because the mixed-mode (ion exchange and reverse phase) SPE allowed washout of any non-ionic components (such as Tween-20) and retention of the ionic analytes. Along this line, Tween-20 is recommended for the basic/or acidic drug candidates that can be potentially extracted using ion (cation/or anion) exchange techniques, such as Oasis MCX or MAX (mixed mode of reversed phase and anion exchange) SPE products. Since most drug candidates are weak bases or weak acids, and low urine QC recovery is fairly common, the findings described here will serve as a valuable tool for the bioanalyst to develop reliable urine assays in support of clinical studies in the future.

4. Conclusion

Concerns in pre-analytical handling of urine samples were discussed using compound A as an example of a case where light sensitivity and low QC recovery were found. A low-recovery problem can be remedied by either changing containers or by using additives, such as BSA or non-ionic surfactant Tween-20. For compound A, addition of Tween-20 in urine was the most effective approach in preventing analyte adsorption to the surface of the container. The urine assay combining mixed-mode SPE and LC–MS/MS was not affected by introducing Tween-20 into the samples. Since low recovery of drug from urine is not unique to compound A [2,3], to ensure reliable quantitation of analytes in urine, evaluation of the potential problem in the early stages of assay development is recommended. And, the addition of Tween-20 can be extended to other small molecular drug candidates with similar situations.

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