Journal of Chromatography B, 878 (2010) 583–589

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantitative determination of BAF312, a S1P-R modulator, in human urine by LC–MS/MS: Prevention and recovery of lost analyte due to container surface adsorption

Wenkui Li*, Suyi Luo, Harold T. Smith, Francis L.S. Tse

Department of Drug Metabolism and Pharmacokinetics, Novartis Institutes for Biomedical Research, One Health Plaza, East Hanover, NJ 07936, USA

ARTICLE INFO

Article history:
Received 19 August 2009
Accepted 21 December 2009
Available online 4 January 2010

Keywords:
BAF312
Urine assay
Tween-80
CHAPS
Bovine Serum Albumin (BSA)
β-cyclodextrin
LC–MS/MS
Container Surface Absorption

ABSTRACT

Analyte loss due to non-specific binding, especially container surface adsorption, is not uncommon in the quantitative analysis of urine samples. In developing a sensitive LC–MS/MS method for the determination of a drug candidate, BAF312, in human urine, a simple procedure was outlined for identification, confirmation and prevention of analyte non-specific binding to a container surface and to recover the 'non-specific loss' of an analyte, if no transfer has occurred to the original urine samples. Non-specific binding or container surface adsorption can be quickly identified by using freshly spiked urine calibration standards and pre-pooled QC samples during a LC–MS/MS feasibility run. The resulting low recovery of an analyte in urine samples can be prevented through the use of additives, such as the non-ionic surfactant Tween-80, CHAPS and others, to the container prior to urine sample collection. If the urine samples have not been transferred from the bulk container, the 'non-specific binding' of an analyte to the container surface can be reversed by the addition of a specified amount of CHAPS, Tween-80 or bovine serum albumin, followed by appropriate mixing. Among the above agents, Tween-80 is the most cost-effective. β-cyclodextrin may be suitable in stabilizing the analyte of interest in urine via pre-treating the matrix with the agent. However, post-addition of β-cyclodextrin to untreated urine samples does not recover the 'lost' analyte due to non-specific binding or container surface adsorption. In the case of BAF312, a dynamic range of 0.0200–20.0 ng/ml in human urine was validated with an overall accuracy and precision for QC sample results ranging from −3.2 to 5.1% (bias) and 3.9 to 10.2% (CV), respectively. Pre- and post-addition of 0.5% (v/v) Tween-80 to the container provided excellent overall analyte recovery and minimal MS signal suppression when a liquid–liquid extraction in combination with an isocratic LC separation was employed. The compound was stable in 0.5% Tween-80 treated human urine QC samples for at least 24 h at room temperature, after three freeze/thaw cycles with storage at ≤−60 °C and for at least 3 months when stored at ≤−60 °C. The current work could serve as a simple example in trouble shooting non-specific binding or container surface adsorption in quantitative analysis of urine samples.

1. Introduction

Non-specific binding or container surface adsorption of drug candidates in urine samples has drawn increasing attention in pharmaceutical bioanalysis [1–6]. Failure to promptly and adequately address this issue would result in underestimated urine drug concentrations and lead to inaccurate pharmacokinetic assessment of an investigational drug.

Urine does not normally contain protein and lipids that are present at approximately 8% in whole blood, plasma or serum [7]. This lack of protein and lipids in urine samples can be associated with the issue of non-specific binding or container surface adsorption of drug molecules, especially those lipophilic and highly protein bound, in quantitative analysis of urine samples. The non-specific binding or container surface adsorption is often evidenced by the unusually low extraction recovery of the analytes of interest and/or non-linearity of the calibration curves or highly variable QC sample results [1–6,8–11]. Unfortunately, the issue has been often overlooked during the early stages of bioanalytical method development, especially when assay sensitivity is not an issue and/or both the calibration standards and QC samples are prepared in the same fashion, i.e. freshly spiked, daily prepared or pre-pooled. In the latter cases, the unexpected low recovery of analyte would often be interpreted as a result of matrix effect or signal suppression, but not non-specific binding or container surface adsorption because the problem, if any, may be masked by a similar degree of LC–MS/MS signal loss of the analyte from the injections of both the calibration standards and QC samples that are prepared in the

* Corresponding author. Tel.: +1 862 778 4255; fax: +1 973 781 7579.
E-mail address: wenkui.li@novartis.com (W. Li).

1570-0232/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2009.12.031

same fashion. In some cases, it might not be until after many failed feasibility runs could the issue of non-specific binding or container surface adsorption be realized. Thus, a simple approach is needed to quickly identify and confirm and effectively prevent the loss of analytes due to non-specific binding or container surface adsorption.

BAF312 (structure not shown) is a S1P-R modulator currently under clinical investigation for the treatment of multiple sclerosis. This compound has a molecular weight of less than 550 Da and is highly protein bound (>99.9%) with pK_a values of 2.69 and 8.81 and log *P* value of 6.90. A human urine assay was needed to support several clinical studies. During urine assay development, an issue of very low extraction recovery was observed in the first feasibility run by using freshly spiked calibration standards and pooled QC samples. Systematic assay trouble shooting led to the confirmation of non-specific binding or container surface adsorption and the associated modification of urine sample collection procedure. Although non-specific binding or container surface adsorption and the corresponding preventive approaches have been reported in some previous publications [1–6], it has been unclear from these articles how the issues could be quickly identified and confirmed during assay development and how the 'lost' analytes in the original bulk container could be cost-effectively recovered if no sample transfer occurs. In this paper for the development and validation of a rugged LC–MS/MS method for quantitative analysis of BAF312 in human urine samples, we recommended steps for quickly identifying and confirming, and cost-effectively preventing analyte non-specific binding or container surface adsorption, as well as recovering, in some cases, the 'non-specific loss' of an analyte in quantitative urine sample analysis.

2. Experimental

2.1. Materials and solutions

BAF312 (structure not shown) and the deuterium labeled internal standard ([M + 11]BAF312, structure not shown) were synthesized at Novartis Pharma AG (Basel, Switzerland). The compound has a molecular weight of less than 550 Da and is highly protein bound (>99.9%) with pK_a values of 2.69 and 8.81 and log *P* value of 6.90. Control human urine was collected internally from healthy donors. Human plasma (sodium EDTA as an anticoagulant) was purchased from Bioreclamation (Hicksville and/or Westbury, NY, USA). Bovine serum albumin (BSA), CHAPS [3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CAS No. 75621-03-3)], β-cyclodextrin, and Tween-80 were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol and acetonitrile, laboratory grade methyl *tert*-butyl ether (MTBE), formic acid (85%), acetic acid, trifluoroacetic acid, hydrochloric acid and ammonium hydroxide (~29%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified by an ELGA ultrapure water system (ELGA, Oxford, UK). Polypropylene sample tubes/vials were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Chromatography

An integrated Shimadzu liquid chromatography system consisting of a model SCL-10Avp controller, a multi-channel mobile phase degasser (DGU-20A), a column heater (CTO-10A), two LC-20AD pumps (Shimadzu, Columbia, MD, USA) and an ACE 5 C_{18} (50 mm × 4.6 mm, 5 μm particle size, MAC-MOD Analytical, Chadds Ford, PA, USA) HPLC column was used for the chromatographic separation of BAF312 and the internal standard from the matrix components. The autosampler was an HTS-PAL from Leap Technologies (Carrboro, NC, USA). The Guard column (C_{18}, 2.0 mm × 3.0 mm, 0.5 μm pore size) was obtained from Analytical Sales & Services (Pompton Plains, NJ, USA). Mobile phase A was water (containing 0.5% formic acid) and mobile phase B was acetonitrile (containing 0.5% formic acid). Isocratic elution using 70% mobile phase B was employed from 0 to 2.0 min at a flow rate of 1.0 ml/min, followed by a column wash-out using 95% mobile phase B at a flow rate of 1.5 ml/min. Column re-equilibration was achieved with 70% mobile phase B at a flow rate of 1.0 ml/min. The injection cycle time was approximately 3.5 min.

2.3. MS/MS detection

A Sciex API4000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Concord, Ontario, Canada) with a TurboIonSpray® (TIS) interface was operated in the positive ionization mode for the multiple reaction monitoring (MRM) LC–MS/MS analyses. The mass spectrometric conditions were optimized for both the analyte and the internal standard by infusing a 10 ng/ml standard working solution in 50% acetonitrile (containing 0.5% formic acid) at 10 μl/min using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA) directly connected to a "Tee", where the analyte and the internal standard were mixed with a mobile phase mixture (70% B) before entering the mass spectrometer. The optimized instrumental conditions were as follows: TIS source temperature of 550 °C; TIS voltage was 3500 V; curtain gas set at 30 units; nebulizing gas (GS1) set at 35 units; TIS (GS2) gas set at 75 units; CAD gas set at 8 units; collision energy (CE) was 27 eV for the analyte and 29 eV for the internal standard. The following precursor → product ion transitions were used for multiple reaction monitoring (MRM): analyte, *m*/*z* 517.2 → 416.3; ISTD, *m*/*z* 528.5 → 427.3, with a dwell time of 300 ms for each analyte and internal standard mass transition. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.7 Da) for both the first quadrupole and the third quadrupole.

2.4. Data analysis

Data was processed using Watson LIMS version 7.2.0.01 (Thermo Fisher Scientific, Philadelphia, PA, USA.). The calibration curves (analyte peak area/ISTD peak area *versus* analyte concentration) were constructed using the least squares linear regression model $y = ax + b$ with a weighting factor of $1/x^2$. Acceptance criteria were established to be >0.99 for the calibration curve coefficient of determination (r^2) with the observed QC concentrations within ±15% Bias of the nominal concentration (accuracy) and ≤15% CV (precision) for the intra-day and inter-day assay at all levels, except at the LLOQ, where the intra-day and inter-day assay accuracy and precision limits were within ±20% Bias and ≤20% CV.

2.5. Urine calibration standards (Cs) and quality controls (QCs)

Two primary BAF312 stock solutions were prepared in acetonitrile, each at a concentration of 1.0 mg/ml in 20-ml vials. The stock solutions were stored at <−60 °C. For validation purposes, the stock solutions from the two weighings must have LC–MS/MS responses within 5% of each other. The stock solution was serially diluted with 50% methanol to prepare the standard working solutions at the desired concentrations. An internal standard working solution containing 2.00 ng/ml of [M + 11]BAF312 was prepared from the ISTD stock solution using 50% methanol.

Prior to assay validation and sample analysis, Tween-80 was mixed with blank human urine to achieve a final concentration of 0.5% (v/v) to prevent analyte surface adsorption. The calibration standards were freshly prepared by spiking 100 μl of the appropriate standard working solution into 0.100 ml of human urine

pre-treated as noted. Eight non-zero calibration standards were prepared at concentrations of 0.0200, 0.0500, 0.250, 0.500, 2.50, 5.00, 16.0 and 20.0 ng/ml. Each calibration standard concentration was assayed in duplicate in each assay run. Blank and zero (blank + ISTD) samples were also assayed, but not included in the calibration regression.

Pooled quality control (QC) samples were prepared by spiking appropriate amounts of the standard working solutions, at less than 2% of the final volume, into pools of the pre-treated blank human urine. Five QC sample concentrations at 0.0200, 0.0600, 0.400, 1.00 and 15.0 ng/ml were prepared and employed in assay validation. All QCs were transferred to 2-ml polypropylene vials and stored at ≤−60 °C.

2.6. Urine sample preparation

A 100 μl aliquot of the appropriate standard working solution was added to the wells designated for calibration standards in a 2-ml 96-well plate, followed by the addition of 100 μl of blank urine to each well for the standards. To the wells designated for control blanks and zero samples, a 100 μl aliquot of blank urine was added. A 100 μl aliquot of each QC concentration or study sample was added to the appropriate wells of the plate, followed by the addition of 100 μl of 50/50 (v/v) methanol/water to the control blanks, zero samples, QCs and study samples followed by brief vortex-mixing. A 25.0 μl aliquot of the ISTD working solution ([M + 11] BAF312, 2.00 ng/ml in 50% aqueous methanol, v/v) was added to all wells except for the control blanks, to which a 25.0 μl aliquot of 50% aqueous methanol (v/v) was added. A 600 μl aliquot of methyl *tert*-butyl ether (MTBE) was added to each well and the plate was covered and vortex-mixed for approximately 15 minutes on a pulse vortex-mixer at a motor speed setting of approximately 50. The plate was centrifuged at approximately 3500 rpm (∼2000 × *g*) for 10 min at ∼10 °C, the resulting supernatant transferred to a clean 1-ml 96-well plate using a TomTec system and then evaporated to dryness under a stream of nitrogen at 45 °C. The sample residues were reconstituted with 125 μl of reconstitution solution (50% aqueous methanol), followed by vortex-mixing and centrifugation at 3500 rpm (∼2000 × *g*) for 5 min at ∼25 °C. A 20 μl aliquot of the reconstituted extract was injected onto the LC–MS/MS system.

2.7. Urine assay method validation

Three validation batches were used to assess the precision and accuracy of the method. Each batch was processed on a separate day and had two replicates of each calibration standard and six replicates of each QC sample concentration (0.0200, 0.0600, 0.400, 1.00 and 15.0 ng/ml). The QC samples and other test samples were interspersed between the two replicates of calibration standards. A blank sample was always placed immediately after the upper limit of quantification (ULOQ) standard to evaluate the carry-over of the LC–MS/MS system.

The matrix effect of the method was determined by comparing the LC–MS/MS response of extracted Tween-80 (0.5%, v/v) treated blank urine samples fortified post-extraction with neat solutions of the analyte at low (0.0200 ng/ml), medium (0.250 ng/ml) and high (20.0 ng/ml) concentrations with the response obtained from neat solutions of the analyte at the same concentrations. Recovery was determined by comparing the LC–MS/MS response of extracted QC samples at concentrations of 0.0200, 0.250 and 20.0 ng/ml with the response obtained from extracted Tween-80 (0.5%, v/v) treated blank samples fortified post-extraction with the same analyte concentrations.

For the short-term stability assessment, QC samples at 0.0600, 0.400 and 15.0 ng/ml were subjected to three cycles of freeze–thaw (freeze-thaw stability) or storage on the laboratory bench at room temperature for approximately 24 h (bench-top stability) and processed together with calibration standards and regular QC samples. As part of the stability assessment, one batch of extracted samples was stored in the auto-sampler for approximately 5 days before re-injection onto the LC–MS/MS system to determine the storage and re-injection reproducibility of the processed samples.

Table 1
Result summary of the QC samples prepared without any pre-treatment ∼1 h prior to assay using freshly prepared calibration standards in the initial assay run.

QC Nominal concentration (ng/ml)	Initial urine assay ($N = 6$)		
	Mean (ng/ml)	Mean bias (%)	Mean CV (%)
0.0200	0.00553	−72.4	11.2
0.0600	0.0108	−82.0	5.9
0.400	0.0760	−81.0	1.3
1.00	0.214	−78.6	2.2
15.0	2.91	−80.6	2.0

3. Results and discussion

3.1. Method development

Following the development and validation of the plasma sample assay method (protein precipitation followed by transfer of supernatant, drying and reconstitution prior to LC–MS/MS analysis) for BAF312 and the implementation of the method in support of the first-in-human study, an initial attempt to directly apply the same methodology to urine sample analysis was not successful. Failure of this initial approach was evidenced by the overall low recovery (18.0–27.6%) of urine QC pools (Table 1) prepared ∼1.5 h prior to the analytical run that used freshly spiked calibration standards. The accuracy and precision results (not shown) for the calibration standards, however, were excellent with a coefficient of determination (r^2) better than 0.999. This observation triggered quick investigations, which led to identification and confirmation of non-specific binding or container surface adsorption of the compound in urine and the associated assay method modification.

Quick test by adjusting the pH value of the urine matrix using formic acid, acetic acid, trifluoroacetic acid (TFA), hydrochloric acid and ammonium hydroxide (up to 5% each, v/v) prior to preparation of pooled QC samples was not successful either (data not shown), suggesting that the low recovery of the analyte was independent of pH in the matrix. In another quick attempt, β-cyclodextrin, a cyclic oligosaccharide, was added to the blank matrix prior to preparation of pooled QCs. Given the unique nature of its structure, β-cyclodextrin has a hydrophobic core and hydrophilic exterior in aqueous phase and could form host-guest complexes with hydrophobic molecules via hydrophobic interactions, hydrogen bonding and/or electrostatic interactions [12–13]. The addition of β-cyclodextrin to the aqueous matrices has been reported to create and maintain stable homogeneous distributions for the hydrophobic compounds in the aqueous phase, resulting in improved assay accuracy and precision [14–15]. In this attempt, β-cyclodextrin was added to blank human urine at a final concentration of 0.2% (w/v) prior to preparation of the QC sample pools. As seen in Fig. 1, there was a significant increase in recovery up to 80% across the three concentration levels tested. However, post-addition of β-cyclodextrin (0.2%, w/v) to the QC samples that were prepared without pre-treatment and stored at ≤−60 °C for ∼24 h did not reverse the low analyte recovery. Similar recoveries were observed before and after the addition of β-cyclodextrin to the untreated QC sample pools (Fig. 1). This suggested that the analyte loss might not have occurred in the aqueous phase. Furthermore, the loss of the analyte was not related to instability in matrix as analyte stability

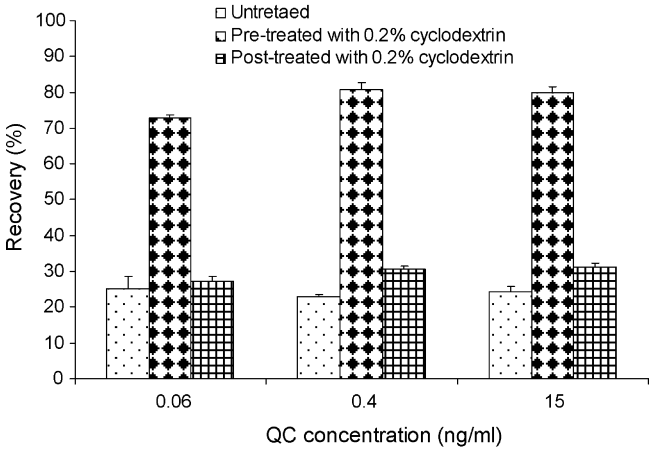

Fig. 1. Extraction recoveries (%) of human urine QC samples with addition of 0.2% of β-cyclodextrin (w/v) prior to or after preparation.

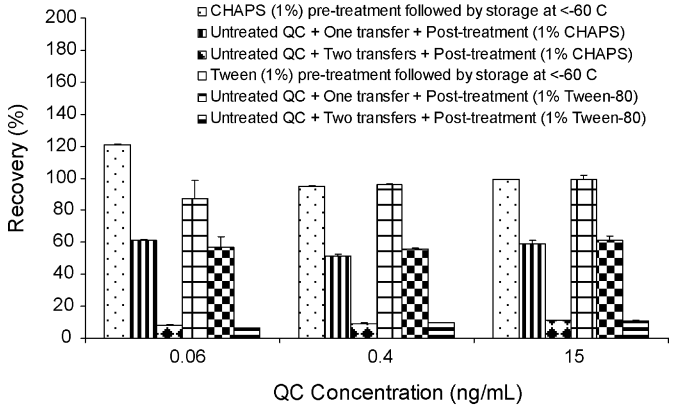

Fig. 2. Extraction recoveries (%) of the human urine QC samples that were prepared via pre-addition of 1% of CHAPS (w/v) or Tween-80 (v/v) and the untreated human urine QC samples with post-addition of CHAPS (1%, w/v) or Tween-80 (v/v) after one or two steps of transfer from one container to the another.

for up to 3 freeze/thaw cycles, up to 24 h at room temperature and up to 9 months in human plasma stored at ≤−60 °C had been confirmed. In addition, the standard working solution (in 50% aqueous methanol, v/v) was also found to be stable for up to 7 months when stored at ≤−15 °C, even at the 0.0200 ng/ml concentration level. The exact cause of analyte loss remained to be investigated.

The urine and plasma assay methods both employed freshly spiked calibration curves and these curves had similar calibration slopes and intercepts (details not shown). One significant difference between the plasma and urine assays is that proteins and lipids are abundant (~8%) in plasma, but normally absent in urine [7]. Since BAF312 is approximately 99.9% protein bound in human plasma, the presence of proteins and lipids likely help to stabilize BAF312 in the aqueous (plasma) phase. Based on this hypothesis, a test was conducted by preparing a very high concentration BAF312 human plasma QC sample, and then spiking this sample into untreated blank human urine to achieve the target analyte concentrations in human urine (containing less than 5% of human plasma, v/v). These urine QC samples were analyzed along with a set of freshly spiked calibration standards. As anticipated, the overall recovery of the QC samples at all concentration levels ranged from 86.3 to 112% (Figure not shown) with the higher recovery observed at the lower concentration QCs. These results confirmed that a small amount of plasma protein in the urine matrix help, via protein binding, retain the analyte in solution.

Taking these results into consideration, the loss of BAF312 in the untreated urine QC samples was attributed to apparent non-specific binding of the analyte to the surface of the sample tube. For confirmation and establishing a mechanism to address the problem, several surfactants including the well known CHAPS [6] and Tween-80 were evaluated. In these tests, blank human urine was pre-treated with CHAPS or Tween-80 at a final concentration of 1%. This treated blank matrix was used to prepare QC sample pools at concentrations of 0.0600, 0.400 and 15.0 ng/ml. For comparison purposes, QC samples at the same concentrations were prepared in 2-ml polypropylene vials using untreated blank human urine. Thirty minutes after preparation, the contents of the untreated QC sample vials were transferred into new sample vials and then followed by the addition of CHAPS or Tween-80 at a final concentration of 1% (w/v for CHAPS and v/v for Tween-80) to prevent further loss of the analyte. After vortex-mixing for approximately 5 min, both the pre-treated and untreated/post-transfer treated samples were analyzed along with a freshly spiked calibration curve. The results from these evaluations are summarized in Fig. 2. Pre-treatment of the blank human urine with either CHAPS or Tween-80 prevented analyte loss due to container surface adsorption as shown by approximately 100% recovery for all QC concentration levels. Not surprisingly, within a very short period of time following preparation of the untreated QC samples, approximately 40% of the analyte was adsorbed to the container surface. The incorporation of one additional transfer step resulted in almost 80% adsorption of the analyte from the aqueous phase to the container surface. Thus, the loss of analyte due to container surface adsorption was confirmed. On the other hand, the above assessment further demonstrated the necessity of using freshly spiked calibration standards and pre-pooled untreated QC samples in early stage of a urine assay method development in order to quickly identify the issue of non-specific binding or container surface adsorption of analyte of interest. Ideally, the pre-pooled urine QC samples should be left on bench for a minimum of one hour after preparation, during which non-specific binding or container adsorption, if any, should occur after equilibration of the matrix. Although it is compound dependent, non-specific binding of an analyte to container surface may occur very quickly in the untreated urine samples [10]. Thus, preparation of calibration standards on daily basis [3–5] may not be appropriate if they are left on the bench for various periods of time after preparation before the feasibility run using untreated pooled QC samples.

From these results, it is apparent that any untreated human urine samples transferred from one or more containers (i.e. from bulk container to sample vial/tubes) during sample collection would not be viable and should be discarded. However, samples that were not subjected to any transfers and were still in their original bulk containers might be viable in that the 'lost or adsorbed' analyte concentrations could be recovered through the post-collection addition of CHAPS [6], Tween-80 or bovine serum albumin (BSA). The results shown in Fig. 3 indicate that the post-addition of up to 2% CHAPS (w/v) and 5% BSA (w/v) to the bulk urine sample containers could retrieve the 'adsorbed' analyte concentrations. Addition of Tween-80 at a final concentration of 0.5% (v/v) is not only equally effective at recovering the adsorbed compound but also much less expensive and easier to handle in the large clinical study setting compared to CHAPS [6] or BSA. Thus, Tween-80 was selected for further assay optimization and validation. However, introduction of Tween-80 into the mass spectrometer may be a source of ion suppression, especially for the detection of analyte at very low concentrations, if the sample matrix is directly injected onto the LC–MS/MS system. Nonetheless, the current liquid–liquid extraction in combination of isocratic LC separation resulted in negligible matrix effect (figure not shown) for the detection of analyte in MS due to Tween-80.

Table 2
Precision and accuracy of quality control samples.

	0.0200 (ng/ml)	0.0600 (ng/ml)	0.400 (ng/ml)	1.00 (ng/ml)	15.0 (ng/ml)	40.0 (ng/ml) (×10)	40.0 (ng/ml) (×5)	400 (ng/ml) (×25)
Day-1	0.0188	0.0614	0.382	0.970	15.2			
	0.0179	0.0671	0.406	0.997	15.1			
	0.0188	0.0626	0.404	1.02	15.5			
	0.0212	0.0594	0.391	0.996	16.0			
	0.0215	0.0641	0.396	0.969	15.6			
	0.0195	0.0585	0.406	1.01	14.9			
Intra-run Mean	0.0196	0.0622	0.398	0.994	15.4			
Intra-run SD	0.00144	0.00316	0.00971	0.0207	0.397			
Intra-run % CV	7.3	5.1	2.4	2.1	2.6			
Intra-run % Bias	−2	3.7	−0.5	−0.6	2.7			
n	6	6	6	6	6			
Day-2	0.0160	0.0511	0.401	0.980	15.5	40.7	40.7	374
	0.0180	0.0611	0.412	1.00	15.9	42.3	41.7	376
	0.0183	0.0559	0.405	1.02	16.1	42.6	42.1	390
	0.0142	0.0539	0.421	1.02	16.0	42.4	41.4	382
	0.0205	0.0565	0.409	1.04	16.1	41.9	41.7	373
	0.0197	0.0539	0.408	1.03	16.3	40.4	41.7	386
Intra-run Mean	0.0178	0.0554	0.409	1.02	16.0	41.7	41.6	380
Intra-run SD	0.00234	0.00337	0.00683	0.0217	0.271	0.937	0.472	6.94
Intra-run % CV	13.1	6.1	1.7	2.1	1.7	2.2	1.1	1.8
Intra-run % Bias	−11	−7.7	2.3	2	6.7	4.3	4.0	−5.0
n	6	6	6	6	6	6	6	6
Day-3	0.0209	0.0680	0.356	0.948	14.2			
	0.0213	0.0667	0.424	1.09	15.7			
	0.0200	0.0639	0.407	0.975	15.7			
	0.0200	0.0681	0.421	1.07	16.2			
	0.0219	0.0673	0.462	1.05	17.1			
	0.0200	0.0679	0.435	1.07	16.6			
Intra-run Mean	0.0207	0.067	0.418	1.03	15.9			
Intra-run SD	0.000813	0.0016	0.0353	0.0581	0.999			
Intra-run % CV	3.9	2.4	8.4	5.6	6.3			
Intra-run % Bias	3.5	11.7	4.5	3	6			
n	6	6	6	6	6			
Overall mean (ng/ml)	0.0194	0.0615	0.408	1.01	15.8	41.7	41.6	380
Inter-run SD	0.00198	0.00556	0.0219	0.0393	0.662	0.937	0.472	6.94
Inter-run % CV	10.2	9.0	5.4	3.9	4.2	2.2	1.1	1.8
Inter-run % Bias	−3.2	2.5	2.0	1.4	5.1	4.3	4.0	−5.0
n	18	18	18	18	18	6	6	6

3.2. Validation of the final method procedure

3.2.1. Specificity, selectivity and sensitivity

Under the LC–MS/MS conditions described in the Experimental Section, BAF312 was well separated from interferences in blank matrix. Analysis of six lots of the Tween-80 (0.5%, v/v) treated blank urine did not show any co-eluting endogenous peaks with the analyte and internal standard in the LC–MS/MS chromatograms (Figure not shown).

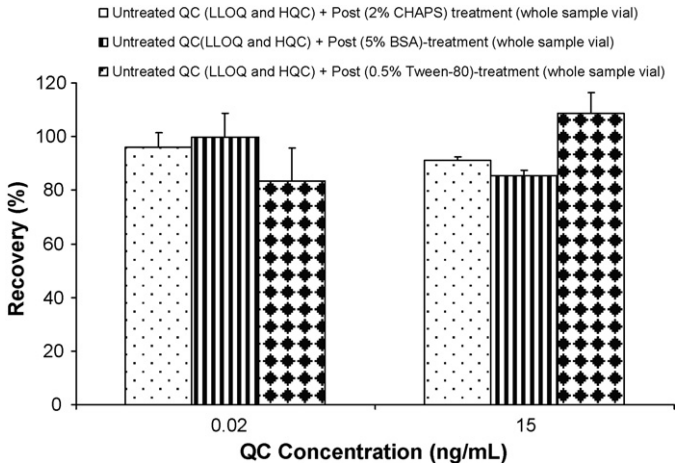

Fig. 3. Extraction recoveries (%) of the untreated and un-transferred human urine QC samples with post-addition of CHAPS (2%), bovine serum albumin (5%) or Tween-80 (0.5%).

The current human urine assay has an analyte LLOQ of 0.0200 ng/ml using a 0.100 ml urine volume. Reliable precision (CV 10.2%) and accuracy (bias −3.2%) was obtained from the analysis of six replicates of LLOQ samples (Table 2) along with two sets of calibration standards and six replicates of QCs at low (0.0600 ng/ml), medium (0.400 and 1.00 ng/ml) and high (15.0 ng/ml) concentration levels in each of three validation runs. A representative LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 4. During assay validation, a column wash was required to consistently achieve the desired sensitivity and reproducibility. The column was washed using mobile phase B at a higher flow rate (1.5 ml/min) for at least 0.5 min prior to column re-equilibration with the starting mobile phase. In order to extend column life, the autosampler needle height must be adjusted to avoid injection of sample particulates.

3.2.2. Matrix effect and recovery

Since Tween-80 was used in the blank urine for QC sample preparation and in the urine sample collection process, possible matrix effects had to be assessed during the validation. The matrix effect was estimated by spiking neat solutions of the analyte (0.0200, 0.250 and 20.0 ng/ml, $N = 4$) into the extracted Tween-80

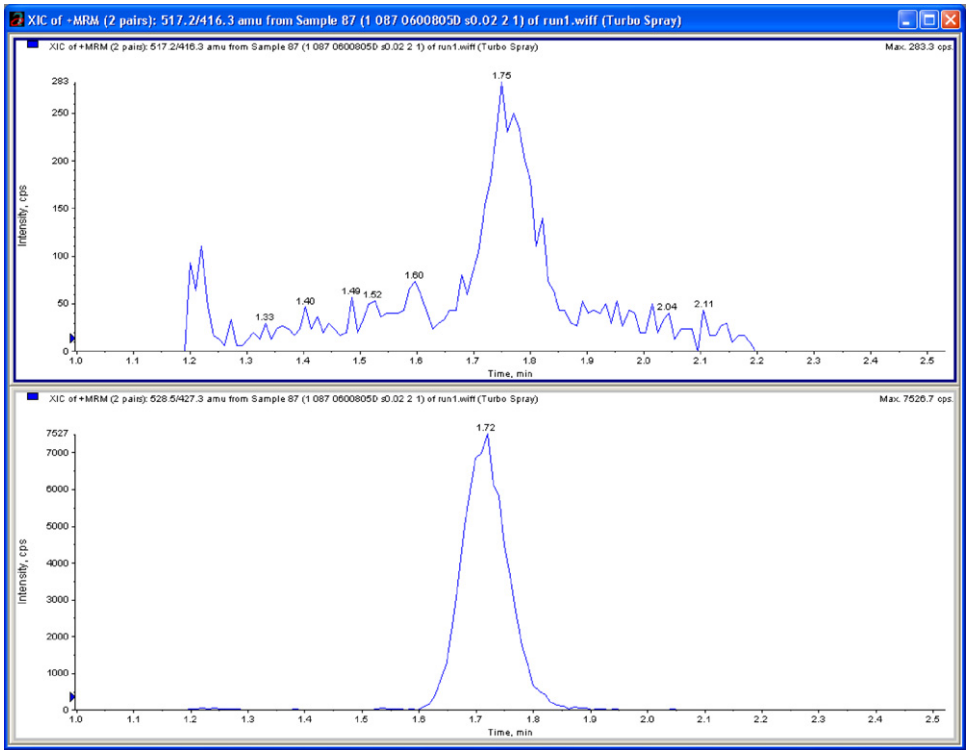

Fig. 4. A representative LC–MS/MS chromatogram of a human urine LLOQ sample (0.0200 ng/ml) with responses of BAF312 and the internal standard shown in top panel and bottom panel, respectively.

(0.5%, v/v) treated blank urine samples and comparing the mean analyte peak areas obtained from the above post-spiked samples with those from the corresponding neat solutions. The overall matrix effect ranged from 8.5 to 19.6% across the three concentration levels.

The recovery was assessed by comparing the mean analyte peak areas from the extracted QC samples at concentrations of 0.0200, 0.250 and 20.0 ng/ml with those from the extracted Tween-80 (0.5%, v/v) treated blank urine samples, to which the analyte was post-spiked at the same concentrations as above. The overall recovery was estimated at 67.5–80.3% across the three concentration levels.

3.2.3. Precision and accuracy

The accuracy and precision of the method were determined by analyzing six replicates of each QC sample concentration level (0.0600, 0.400, 1.00 and 15.0 ng/ml) along with two sets of each calibration standard (0.0200–20.0 ng/ml) for the analyte in each of the three validation runs. The accuracy of the method was obtained by calculating the bias(%) and the precision by calculating CV(%). Table 2 summarizes the accuracy and precision of the three validation runs for the analyte in human urine with the bias(%) ranging from −7.7 to 11.7% and the CV(%) ranging from 1.7 to 8.4% over the concentration range evaluated.

3.2.4. Dilution integrity

The dilution integrity was assessed using 5-, 10- and 25-fold dilutions with Tween-80 (0.5%, v/v) treated blank urine, respectively, of the 40, 40 and 400 ng/ml QC samples, prior to extraction. Six replicates of each dilution were assayed along with a calibration curve and regular QCs in a validation run. As shown in Table 2, the precision and accuracy of the dilution QC sample results ranged from 1.1 to 2.2% (CV) and from −5.0 to 4.3% (bias), respectively, demonstrating that samples with higher concentrations can be analyzed to obtain acceptable data after dilution with blank urine.

3.2.5. Stability

The stability of the analyte in reconstituted samples stored in the autosampler at 2–8 °C was assessed for 5 days after the initial analytical run. The analyte was stable as demonstrated by the acceptable bias (%) that ranged from −1.0 to 5.0% for the 5 concentrations (0.0200–15.0 ng/ml) tested. The bench-top stability of analyte in human urine was evaluated at ambient temperature (~22 °C) over a 24-h period using QC samples at 0.0600, 0.400 and 15.0 ng/ml. The measured concentration of analyte in these QC samples sitting at room temperature for 24 h was compared to the nominal values with bias (%) ranging from −1.0 to 3.3%, indicating that the analyte was stable for at least 24 h in human urine when stored at ambient temperature. The freeze–thaw stability of QC samples at 0.0600, 0.400 and 15.0 ng/ml concentration levels experiencing three cycles of freeze–thaw were analyzed together with one set of calibration standards and regular QC samples. The bias(%) for these results ranged from −3.7 to 3.3%. The long term stability of the analyte in QC samples stored at ≤−60 °C immediately following preparation and analyzed along with calibration standards and regular QCs showed that the analyte is stable in urine for up to three months when stored at ≤−60 °C.

3.2.6. Incurred sample reanalysis

The validity of the current method was further demonstrated via incurred sample reanalysis (ISR), for which 24 incurred samples (containing ~0.5% Tween-80, v/v) from at least three subjects were re-analyzed in single repeat. The results were summarized in Table 3 with the difference between the repeated and the initial data all within ±30%.

4. Application to clinical studies

The sample collection procedure and analytical method described have been successfully applied to the determination of urine BAF312 concentrations in pharmacokinetic samples obtained

Table 3
Summary of incurred sample reanalysis (ISR).

No.	First determination (ng/ml)	Repeat determination (ng/ml)	Normalized difference (%)	No.	First determination (ng/ml)	Repeat determination (ng/ml)	Normalized difference (%)
1	0.236	0.242	2.5	13	0.0381	0.0376	−1.3
2	0.0798	0.0999	25.2	14	0.126	0.143	13.5
3	0.0470	0.0550	17.0	15	0.145	0.158	9.0
4	0.0444	0.0436	−1.8	16	0.136	0.149	9.6
5	0.116	0.119	2.6	17	0.0854	0.0869	1.8
6	0.0943	0.104	10.3	18	0.0426	0.0486	14.1
7	0.0792	0.0814	2.8	19	0.452	0.445	−1.5
8	0.177	0.185	4.5	20	0.277	0.283	2.2
9	0.0629	0.0635	1.0	21	0.244	0.263	7.8
10	0.152	0.146	−3.9	22	0.144	0.153	6.3
11	0.0826	0.0789	−4.5	23	0.201	0.200	−0.5
12	0.0962	0.104	8.1	24	0.110	0.110	0.0

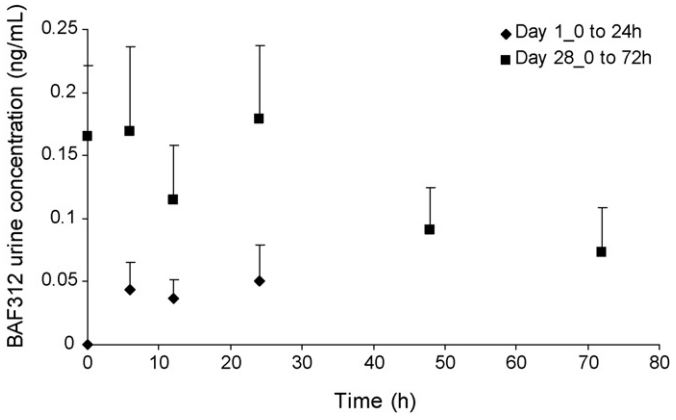

Fig. 5. A representative BAF312 urine sample concentration versus time interval (0–6, 6–12 and 12–24 h for Day 1 and 0–6, 6–12, 12–24, 24–48 and 48–72 h for Day 28) profile obtained from healthy volunteers orally administrated BAF312 at 20 mg/day for 4 weeks.

in a phase I clinical study. A representative urine concentration versus time interval profile for one study cohort (20 mg daily, $N = 9$) is shown in Fig. 5. The overall analyte urine concentration on study day 28 is higher than on study day 1.

5. Conclusion

A validated LC–MS/MS method was developed and validated for quantitative analysis of BAF312 in human urine in support of clinical studies. To rapidly identify the cause of low urine sample analyte recovery due to non-specific binding or container surface adsorption, it is highly recommended to employ freshly spiked calibration standards and pre-pooled QC samples in the early stage of urine assay method development. The pre-pooled QC samples might be prepared on the day of analysis, but they should be left on bench for a minimum of 1 h prior to extraction and analysis using freshly spiked calibration standards. The 'lost or adsorbed' analyte in untreated QC and/or incurred samples due to non-specific binding or container surface adsorption may be recovered through post-addition of simple reagents such as BSA, Tween-80 and CHAPS. However, this approach is only effective if the sample has not been transferred from its original container. β-cyclodextrin may be suitable in stabilizing the analyte of interest in urine samples via pre-treating the matrix with the agent. However, post-addition of β-cyclodextrin to untreated QC and/or incurred urine samples does not reverse the 'lost' analyte due to non-specific binding or container surface Adsorption. For the above reasons, a validated procedure should be in place prior to urine sample collection if container surface adsorption is identified and confirmed. In the case of BAF312, addition of Tween-80 (0.5%, v/v) to blank urine or to the collection containers was the most cost-effective method in eliminating analyte container surface adsorption. The employed liquid–liquid extraction in combination with isocratic LC separation of sample extract eliminated the potential suppression effect during the LC–MS/MS analysis. The validated assay method is rugged as demonstrated by excellent intra- and inter-day precision and accuracy of QC sample results during validation and very good incurred sample reanalysis (ISR) results during study sample analysis. The current practice can be extended to quantitative analysis of other small molecule drug candidates in urine.

References

[1] M. Schwartz, W. Kline, B. Matuszewski, Anal. Chim. Acta 352 (1997) 299.
[2] 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.
[3] Y. Xu, L. Du, M.J. Rose, I. Fu, E.J. Woolf, D.G. Musson, J. Chromatogr. B 818 (2005) 241.
[4] M. Groff, K. Riffela, H. Songa, M.-W. Lo, J. Chromatogr. B 842 (2006) 122.
[5] C. Chen, L. Bajpai, N. Mollova, K. Leung, J. Chromatogr. B 877 (2009) 943.
[6] B.T. Hoffman, S. Bhadresa, L. Zhao, G. Hade, E. Moore, S. Nowak, S. Heit, D. Mulvana, M.Q. Huang, N. Weng, S. Unger, 54th ASMS Conference on Mass Spectrometry, Seattle, WA, USA (2006).
[7] C. Lentner (Ed.), Geigy Scientific Tables (8th, Revised and enlarged edition), CIBA-GEIGY, West Caldwell, New Jersey, 1981, p. 53 (volume 1) and p. 65 (volume 3).
[8] E. Vittinghus, Scand. J. Clin. Lab. Invest. 50 (1990) 843.
[9] A.A.J. van Landeghem, Y.T.J. Somers-Pijnenburg, W.J.H.M. Somers, C. Stokwielder, W. de Bruyn, G.B. van den Berg, J. Inher. Metab. Dis. 22 (1999) 293.
[10] J.A. Blanc, V.A. Manneh, R. Ernst, D.E. Berger, S.A. de Keczer, C. Chase, J.M. Centofanti, A.J. DeLizza, Clin. Chem. 39 (1993) 1705.
[11] J.J. palmgren, J. Monkkonen, T. Korjamo, A. Hassinen, S. Auriola, Eur. J. Pharm. Biopharm. 64 (2006) 369.
[12] M.V. Pekharsky, Y. Inoue, Chem. Rev. 98 (1998) 1875.
[13] M.E. Davis, M.E. Brewster, Nat. Rev. 3 (2004) 1023.
[14] W. Zhou, T. Steege, R. Tollefson, A. LaFayette, S. Zhou, N. Weng, J. Seroogy, AAPS J. 6 (S1) (2004) 1322.
[15] L. Sun, J.A. Stenken, J. Chromatogr. A 1161 (2007) 261.