Consideration Of Adsorption Issues During Sample Handling In Urine Bioanalysis

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Introduction

Quantitation of analytes in the matrix urine can be challenging from a bioanalytical standpoint. Beside the large variability of this matrix between individuals, for example the large pH-range in contrast to blood (pH(urine)=4-8 vs pH(blood)=7.35-7.45), the virtual lack of proteins and lipids, both important mediators of solubility, induce non-specific binding on a variety of surfaces. The standard approach to tackle adsorption at the bioanalytical site by switching to inert container surfaces like glass is often not possible since the urine collection containers used at the clinical sites are usually limited to polypropylene or high-density polyethylene. Thus, alternative solutions like adding anti-adsorption reagents (detergents, proteins, etc.) have to be applied. Importantly, in many cases this has to be implemented already at the clinical sites. The following case studies demonstrate the detection and resolution of adsorption issues and how this led to adjusted clinical protocols for sample collection.

Case Study 1

This case study demonstrates in detail the identification of strong adsorption effects for a proprietary compound (Compound A, MW=710.8 g/mol) in human urine, the inhibition of this adsorption by addition of the anti-adsorptive reagent Triton X-100 and thus the requirement to adjust the clinical protocol for sample collection.

The developed method for quantification of Compound A was based on an already established method in human plasma. Both methods for the determination of the compound, in plasma or in urine, were based on analogous protein precipitation/sample dilution procedures. In plasma, Compound A did not exhibit any significant adsorptive characteristics. However, in initial precision and accuracy runs in urine, quality control (QC) samples showed reduced accuracies (Table 1).

The reductions were independent of freezing the QC aliquots and reanalyzing them, decreasing the likelihood of solubility problems of Compound A in urine. Since the quality control samples were generated in bulk and sub-aliquotted, contrary to the standard samples, adsorption was suspected as main reason for the reduced accuracies.

Spiked standard Condition	S1 fresh	S2 fresh	S3 fresh	S4 fresh	S5 fresh	S6 fresh	S7 fresh	S8 fresh
Nominal Conc. [ng/mL]	1.00	2.00	8.00	25.0	80.0	250	950	1000
Calculated Conc. [ng/mL]	1.03	1.87	8.07	25.1	80.6	254	972	976
Accuracy [%]	103	93.5	101	100	101	101	102	97.6
CV [%]	8.04	0.698	1.63	8.56	2.61	2.69	3.49	6.85
n	2	2	2	2	2	2	2	2

Spiked QC Condition	QC1 fresh	QC2 fresh	QC3 fresh	QC4 fresh	QC1 frozen	QC2 frozen	QC3 frozen	QC4 frozen
Nominal Conc. [ng/mL]	1.00	3.00	30.0	800	1.00	3.00	30.0	800
Calculated Conc. [ng/mL]	0.705	1.76	16.5	555	0.620	1.76	19.7	562
Accuracy [%]	70.5	58.8	55.0	69.3	62.0	58.8	65.7	70.2
CV [%]	11.2	8.43	7.40	2.25	7.67	1.89	4.54	2.08
n	5	5	5	5	6	6	6	6

Table 1: Initial precision and accuracy data of standards and QC samples.

Adsorption in urine was checked in the 15mL tubes used for sample shipping, which were also made of polypropylene like the 2mL tubes used for the standard and QC samples. Samples at QC Low concentration were exposed consecutively to 5 different tubes (see Figure 1). For each step the QC solution was incubated for 10 min, vortexed, poured into a new tube and vortexed again.

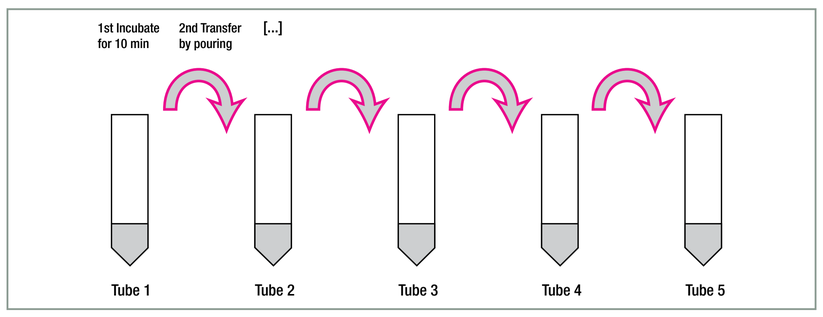

Figure 1: Schematic of sequential sample transfer assay.

This procedure resulted in a reduction of the analyte concentration by 32% (Table 2, Column 1 & 2). Another indication for adsorption was the fact that generating QC Low samples in a smaller volume, but in the same tubes, i.e. with a larger ratio of tube surface to analyte amount, also reduced the recovery (Table 2, Column 1 & 3).

Tube# Vol(Solution)/Vol(Tube)	Tube 1 5ml / 15ml	Tube 5 N/A	Tube 1 1ml / 15ml
Mean peak area (Analyte)	2998	1998	2489
Mean peak area (IS)	39559	38990	39491
Mean peak ratio	0.0758	0.0512	0.0630
Recovery [%]	100	67.6	83.1

Table 2: Test for adsorption in 15mL tubes with sequential sample transfer.

To counteract adsorption, various anti-adsorption reagents were tested for efficacy (Table 3). All tested approaches, by adding protein, organic solvent, acid or detergent, increased recovery. The group of detergents (CHAPS, Tween-20 and Triton X-100) showed the strongest inhibition of adsorption. Both Tween-20 and Triton X-100 gave similar recovery values, but the addition of Tween-20 resulted in a decrease of signal intensity of both analyte and internal standard (IS). In addition, adding Tween-20 in retrospect reversed the adsorption only partially, in contrast to Triton X-100, which did not induce a significant difference between before or after spiking. Thus, Triton X-100 was chosen as the anti-adsorption reagent for further tests.

Urine supplement Reagent addition	H2O before spiking	1% BSA before spiking	10% DMSO before spiking	10% MeOH before spiking	100mM HCl before spiking
Mean peak area (Analyte)	2519	3313	3435	3445	3844
Mean peak area (IS)	41000	39244	40374	40929	40575
Mean peak ratio	0.0614	0.0844	0.0851	0.0842	0.0947
Recovery [%]	60.6	83.3	83.9	83.0	93.4

Urine supplement Reagent addition	0.2% CHAPS before spiking	0.2% Tween-20 before spiking	0.2% Tween-20 after spiking	0.2% Triton X-100 before spiking	0.2% Triton X-100 after spiking
Mean peak area (Analyte)	2737	3722	3655	4122	4062
Mean peak area (IS)	28597	36799	38117	41365	40628
Mean peak ratio	0.0951	0.101	0.0959	0.0996	0.100
Recovery [%]	93.8	100	94.5	98.2	98.7

Table 3: Inhibition of adsorption with various anti-adsorption reagents.

Successful resolution of the adsorption issues was verified by repeating the sequential sample transfer assay (Table 4, Figure 1). No Triton X-100, 0.02% or 0.2% Triton X-100 were added to QC Low and these solutions were transferred between 5 tubes. Untreated urine again confirmed strong adsorption. Addition of 0.02% Triton X-100 reduced adsorption, and 0.2% Triton X-100 eliminated it. This showed that 0.2% Triton X-100, but not 0.02%, was sufficient to avoid adsorption in this assay.

Urine supplement Tube#	H2O Tube 1	H2O Tube 5	0.02% Triton X-100 Tube 1	0.02% Triton X-100 Tube 5	0.2% Triton X-100 Tube 1	0.2% Triton X-100 Tube 5
Mean peak area (Analyte)	1230	668	1269	1124	1376	1445
Mean peak area (IS)	24640	24182	21974	22834	22105	23511
Mean peak ratio	0.0499	0.0277	0.0578	0.0493	0.0622	0.0614
Recovery [%]	80.2	44.4	92.8	79.2	100	98.7

Table 4 Inhibition of adsorption in 15mL tubes with Triton X-100.

Most importantly, due to the observed extent of adsorption, it was investigated if adsorption was also a problem in the primary 3L urine collection containers and if addition of 0.2% Triton X-100 was necessary. To test this, small and large volumes of urine were spiked in the urine containers as QC Low and incubated for 24 hours at room temperature to mimic sample collection procedures (see Figure 2). After this incubation period an aliquot was taken for analysis. The containers were supplemented with 0.2% Triton X-100, in 3 consecutive cycles five times inverted over head and incubated for 10 minutes. Finally, another aliquot was taken. Similar to the 15mL tubes, the extent of adsorption was strong and volume-dependent and the addition of 0.2% Triton X-100 reversed this adsorption (Table 5). The addition of Triton X-100 to urine in the urine collection containers was therefore required prior further sample transfer into the 15ml tubes.

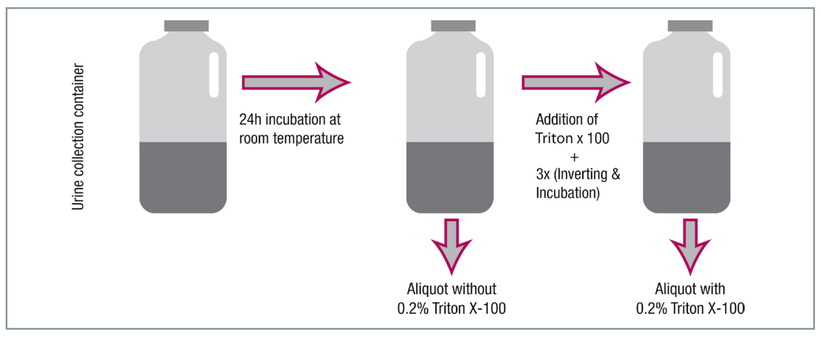

Figure 2: Schematic of adsorption test in urine collection containers.

Sample Addition of 0.2% Triton X-100	400mL in 3L container no	400mL in 3L container yes	2000mL in 3L container no	2000mL in 3L container yes
Nominal Conc. [ng/mL]	3.00	3.00	3.00	3.00
Calculated Conc. [ng/mL]	2.05	3.30	2.81	3.05
Accuracy [%]	68.4	110	93.7	102
CV [%]	6.47	0.591	1.76	2.01
n	4	4	4	4

Table 5: Redissolving adsorbed compound in urine collection containers with Triton X-100.

Case Study 2

This case study demonstrates the identification of adsorption of a proprietary compound (Compound B, MW=365.4 g/mol) in human urine and the necessity to adjust the clinical protocol for sample collection. This compound exhibited adsorptive characteristics in pure solutions, but not in human plasma. An extraction procedure for human urine based on sample dilution was established. Adsorption in polypropylene tubes was suspected. Thus, adsorption of Compound B in the 15mL tubes used for sample shipping was quantified in the sequential sample transfer assay and the inhibition of this potential adsorption with 0.2% Triton X-100 was tested (Table 6, Figure 1). As expected, without addition of detergent, the recovery in the fifth tube was significantly reduced. In contrast, by addition of Triton X-100, nearly no difference was detected after repetitive transfer. Therefore, it was demonstrated that addition of 0.2% Triton X-100 to urine solved the adsorption problems in this assay.

Urine supplement Tube#	H2O Tube 1	H2O Tube 5	0.2% Triton X-100 Tube 1	0.2% Triton X-100 Tube 5
Mean peak area (Analyte)	8666	5793	8648	8342
Mean peak area (IS)	9053	8599	8224	8157
Mean peak ratio	0.956	0.673	1.05	1.02
Recovery [%]	90.8	63.9	100	97.3

Table 6: Test for adsorption in 15mL tubes with sequential sample transfer assay.

Inhibition of adsorption in primary urine collection containers was also tested by adding 0.2% Triton X-100 retrospectively, similar to the experiment for Compound A depicted in Figure 2. The same maximum volume but a smaller minimum volume was used since the urine collection periods ranged from 6 hours to 24 hours contrary to the ones for the determination of Compound A which were always 24 hours (Table 7). The extent of adsorption without Triton X-100 was smaller than for Compound A, but it was similarly volume-dependent. Importantly, the retrospective addition of 0.2% Triton X-100 to the urine resolubilized Compound B sufficiently. Thus, also for Compound B an early introduction of Triton X-100 into the bioanalytical procedure was required and solved the occurring adsorption problems.

Sample Addition of 0.2% Triton X-100	100mL in 3L container no	100mL in 3L container yes	2000mL in 3L container no	2000mL in 3L container yes
Nominal Conc. [ng/mL]	3.00	3.00	3.00	3.00
Calculated Conc. [ng/mL]	2.51	2.87	2.78	2.89
Accuracy [%]	83.8	95.5	92.8	96.3
CV [%]	4.42	3.77	5.24	6.26
n	4	4	4	4

Table 7 Redissolving adsorbed compound in urine collection containers with Triton X-100.

Case Study 3

This case study demonstrates the identification of a small adsorption effect of a third proprietary compound (Compound C, MW=443.5 g/mol) which did not result in an adjusted clinical protocol for sample collection. The method was based on the determination of Compound C in human plasma using a sample dilution approach. The compound exhibited adsorptive characteristics in pure solutions, but not in human plasma. However, the preparation of urine QC samples resulted in incorrect accuracies (data not shown). Adsorption issues in polypropylene were suspected as the reason for this. This was tested using the sequential sample transfer assay. Indeed, analyte loss was detected in 2mL PP-tubes used for aliquotting the QC samples (Table 8). However, in the 15mL tubes used for shipping the urine aliquots, the relative loss was considerably smaller than for Compound A or B. Compound C-levels were reduced by around 13%, in contrast to the reduction of Compound A and B by 32% and 30%, respectively (Table 2, 6 and 8). Thus, it was decided not to change the clinical sampling protocol since only a small impact of adsorption was anticipated.

Tube-types Tube#	2ml PP-tubes Tube 1	2ml PP-tubes Tube 5	15ml PP-tubes Tube 1	15ml PP-tubes Tube 5	2.5ml Glass-tubes Tube 1	2.5ml Glass-tubes Tube 5
Mean peak area (Analyte)	14569	12118	14510	12425	14894	14448
Mean peak area (IS)	21789	22384	22175	21772	21338	21535
Mean peak ratio	0.669	0.541	0.654	0.571	0.698	0.671
Recovery [%]	100	81.0	100	87.3	100	96.1

Table 8: Adsorption in different types of tubes.

The problem of adsorption during aliquotting in 2mL PP-tubes was resolved by switching to glass-tubes since this already reduced adsorption to a minimum (Table 8). Further addition of detergents was not necessary as this increased the recovery only slightly (Table 9)

Tube-types Urine supplement	2.5ml Glass-tubes -	2.5ml Glass-tubes 0.2% Tween-20	2.5ml Glass-tubes 0.2% Triton X-100
Mean peak area (Analyte)	14001	15362	15311
Mean peak area (IS)	19097	19963	19851
Mean peak ratio	0.733	0.770	0.771
Recovery [%]	95.1	99.8	100

Table 9: Inhibition of adsorption with anti-adsorption reagents Tween-20 and Triton X-100.

Effect On Clinical Sampling Protocol

In the first two case studies it was demonstrated that significant adsorption already takes place in the primary urine collection containers. Without additional action at the clinical sites the accuracy of the measurements would have been already compromised, in a volume-dependent manner, with the first transfer from the primary urine collection containers to the 15mL shipping tubes. Therefore, modification of the proposed clinical protocol to incorporate the addition of Triton X-100 and resolubilization of compounds was required (Figure 3). First, the amount of urine collected was accurately measured. The easiest precise approach was by comparing the weight of the empty container with the filled container and converting weight to volume using the estimated density of ρ=1g/L. Secondly, commercially available 10% Triton X-100 solution was used to ease preparation at the clinical sites, i.e. to avoid generation of additional dilutions, and to facilitate pipetting. Thirdly, the retrospective addition of Triton X-100 was used to allow a precise final concentration of Triton X-100 in urine. Fourthly, an easy, clearly defined, non-work intensive and fast resolubilization protocol was required. This resulted in the adjusted clinical sampling protocol depicted in Figure 3.

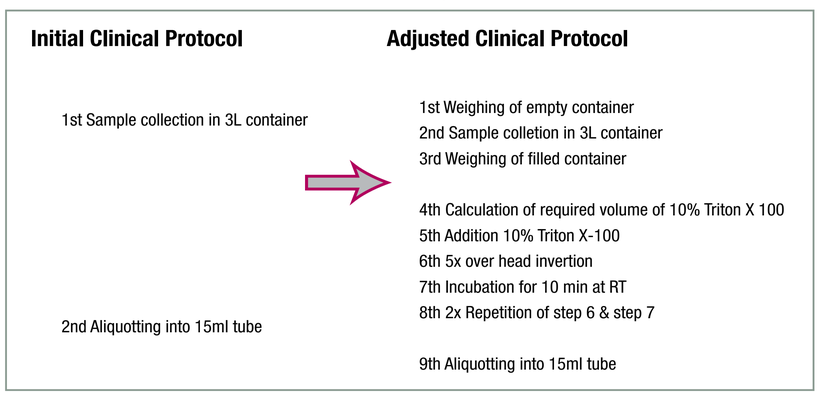

Figure 3: Overview of clinical protocols.

Conclusion

Non-specific binding of compounds to urine containers, small or large, can significantly impact the accuracy of quantification methods. Taking care of this issue should not start at the bioanalytical site, but has to be dealt with already at the clinical sites. This results in non-bioanalysts participating in an important part of the bioanalytical pipeline, and thereby significantly impacting the quality of the analysis. Thus, it is crucial to develop and implement clinical protocols which minimize the likelyhood of introducing additional errors. These adjusted clinical protocols have to be easy, fast, well defined and should require only minimal preparation.

Another important consequence of such adjusted clinical protocols is the requirement to finalize them prior to the first sample collection. Indeed, even more time is required as such changes of protocol need to be communicated to all clinical sites, and these sites also need time for preparation, e.g. for ordering the required reagents and materials. In summary, the accurate determination of compounds in urine may frequently require support at the clinical sites, and the bioanalytical community should endeavor to make the tasks as simple as possible.

Poster presentation at EBF 7th Open Meeting, November 19-21 2014

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