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Overcoming non-specific adsorption issues for AZD9164 in human urine samples: Consideration of bioanalytical and metabolite identification procedures

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ABSTRACT

A key challenge in the development of robust bioanalytical methods, for the determination of drug analyte in human urine samples, is the elimination of potential analyte losses as a result of non-specific adsorption to container surfaces in which the samples are collected, stored or processed. A common approach to address adsorption issues is to treat the urine samples with additives that serve to increase analyte solubility and/or minimise interaction with the container surfaces. A series of adsorption experiments were performed on human urine samples containing an adsorption-prone in-house development compound (AZD9164). A roller-mixing methodology was employed to maximise sample interaction with container surfaces and quantification of analyte was performed by LC–MS/MS following minimal sample preparation. In the absence of any urine additive, adsorptive losses averaged 35% but were highly variable between different lots of urine. In the presence of a range of additives, including the surfactants Tween 80, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) and sodium dodecylbenzenesulphonate (SDBS), analyte adsorption was shown to be eliminated. Of particular academic interest was the finding that adsorptive losses could also be reduced upon the addition of phospholipid. The presence of additive generally had no marked impact on the analyte MS response but the use of an isotopically labelled internal standard satisfactorily compensated for instances in which ion suppression was observed, e.g. in the presence of Tween 80. Since metabolite profiling/identification investigations are often performed on urine samples originating from early clinical pharmacology studies, the elution of selected additives was also monitored by MS. CHAPS, dimethylacetamide (DMA) and HP-β-cyclodextrin eluted as single chromatographic peaks in, or just after, the column void volume whilst polymeric Tween 80, and to a lesser extent SDBS, eluted over a wide retention time window. The potential of the latter surfactants to obscure the detection of unknown metabolites is significant and therefore their use in urine samples, upon which metabolite investigations are to be performed, is not recommended. Upon consideration of other factors such as additive cost and toxicity, CHAPS was selected for use in development of the validated assay.

1. Introduction

The development of bioanalytical methods for the determination of drug analytes in human urine samples is often only an after-thought to the development of the corresponding plasma method. However, the development of a successful urine assay has distinct challenges to those faced with plasma [1]. Chief amongst these challenges is the issue of non-specific adsorption to container surfaces. Urine is an aqueous medium and, unlike other matrices such as plasma or blood, is lacking in any significant amount of protein capable of binding drug analytes and retaining them in solution. Therefore, urine samples are particularly susceptible to analyte losses on the surfaces of containers in which they are collected, stored or processed.

A number of methodologies have been adopted in order to overcome adsorptive losses. A common approach [2–5] focuses on replicating the plasma environment by adding a sufficient quantity of bovine serum albumin (BSA) to the urine samples. The resultant samples from the clinic may then be analysed using a simple protein precipitation procedure or, alternatively, the same methodology employed in the validated plasma method. However, this approach is not always successful and more recently, the use of surfactants such as Tween 20 [6,7], Tween 80 [8], CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate] [8,9] and SDBS (sodium dodecylbenzenesulphonate) [8], have been successfully employed in urine methods. The effectiveness of such approaches is evidenced in the method validation accuracy and precision results, for the determination of drug analyte in

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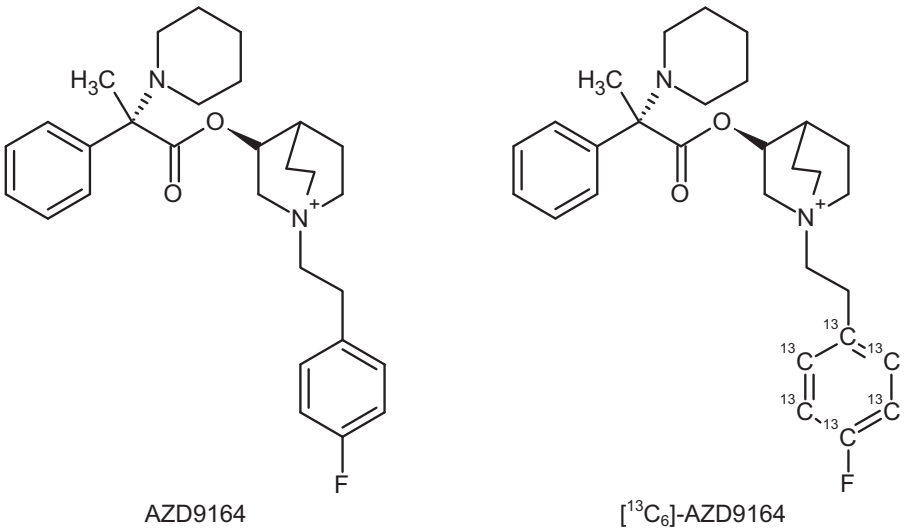

Fig. 1. Chemical structures of AZD9164 and [13C6]-AZD9164.

quality control samples, and also from incurred sample reproducibility (ISR) data [9].

Unfortunately, the use of surfactants (and other additives) in urine samples presents the bioanalyst with a different challenge. Relatively high concentrations of surfactant in the sample introduces a further, and not insignificant, interference in an already complex matrix. For analysis methods employing liquid chromatography coupled with tandem mass spectrometric detection (LC–MS/MS), this interference may manifest itself as suppression (or enhancement) of the analyte response [10]. For metabolite profiling/identification investigations, which are often performed on samples originating from early clinical pharmacology studies, this interference may also impede the detection and identification of unknown metabolites. Moreover, in such metabolite investigations (including quantitative bioanalytical assays for parent and metabolites), removal of the surfactant through selective sample processing [e.g. solid phase extraction (SPE)] may be precluded. Therefore, it is important that the surfactant (or other additive) is characterised appopriately for the anticipated analyses to be performed on the urine samples; selection of a suitable surfactant may then obviate the need for any sample preparation (other than centrifugation).

AZD9164 (Fig. 1), an in-house development compound, is a quaternary amine with considerable lipophilic character. The analyte is prone to non-specific adsorption in human urine samples as evidenced by the non-linear calibration curves observed in initial method development studies. The aim of the work presented in this paper is to assess the applicability of a number of surfactants (and other additives) for their use in developing a simple and fit-for-purpose method for determining AZD9164 in urine human samples from clinical studies. Firstly, a simple methodology is outlined to assess the degree of adsorptive losses of analyte from human urine to the surfaces of polypropylene containers; this material is routinely used in the clinic for the purposes of collecting, storing and transporting urine samples. Secondly, the effectiveness of a variety of surfactants, and other additives, in preventing losses of analyte from human urine is reported. Thirdly, the potential impact of the additive with regard to mass spectrometric determination/identification of parent analyte and metabolites is evaluated. Lastly, factors relating to the practical use of the additives in the laboratory (method validation) and the clinic (safety, ease-of-use) are discussed.

2. Experimental

2.1. Materials and solutions

AZD9164 and the carbon-13 labelled internal standard ([$^{13}C_6$]-AZD9164, Fig. 1) were synthesised at AstraZeneca R&D Charnwood (Loughborough, UK). Control human urine was collected from healthy volunteers at AstraZeneca R&D Charnwood. CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate], CHAPSO [3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate], Tween 80, acetonitrile (Far UV grade), ammonium acetate (HPLC grade) and formic acid (AR grade) were purchased from Thermo Fisher Scientific (Loughborough, Leics., UK). SDS (sodium dodecyl sulphate), SDBS (sodium dodecylbenzenesulphonate), 2-hydroxypropyl-β-cyclodextrin, methanol, DMA (dimethylacetamide), L-α-phosphatidylcholine [16:0 (∼33%), 18:0 (∼13%), 18:1 (∼31%), 18:2 (∼15%)], L-α-lysophosphatidylcholine [16:0 (∼69%), 18:0 (∼27%), 18:1 (∼3%)], were purchased from Sigma–Aldrich (Gillingham, Dorset, UK). Water was purified by a Milli-Q Gradient A10 purification unit (Millipore, Watford, Herts., UK). Polypropylene tubes were obtained from Thermo Fisher Scientific, and polypropylene 96-well plates (1 mL) with pre-split sealing mats were obtained from Crawford Scientific (Strathaven, UK). Glass vials were purchased from Perkin Elmer (Pangbourne, Berks., UK).

2.2. Instrumentation

LC–MS/MS analysis was performed on an Agilent 1100 series HPLC system (G1322A degasser, G1312A binary pump, G1367A well-plate sampler, G1316A column compartment; Agilent Technologies, Stockport, Cheshire, UK) coupled to an AB Sciex API 365 mass spectrometer (AB Sciex, Concord, Ontario, Canada), fitted with a TurboIonspray® (TIS) interface. Data were collected and processed using Analyst v1.4.1 (AB Sciex).

2.3. Chromatographic conditions and MS/MS detection

HPLC separations were performed on a Phenomenex Luna C18, 2 mm × 50 mm (3 μm) column (Phenomenex, Macclesfield, Cheshire, UK) held at 40 °C. Mobile phase A was water, containing 25 mmol/L ammonium acetate, and mobile phase B was acetonitrile. An isocratic elution consisting of 55% mobile phase B was

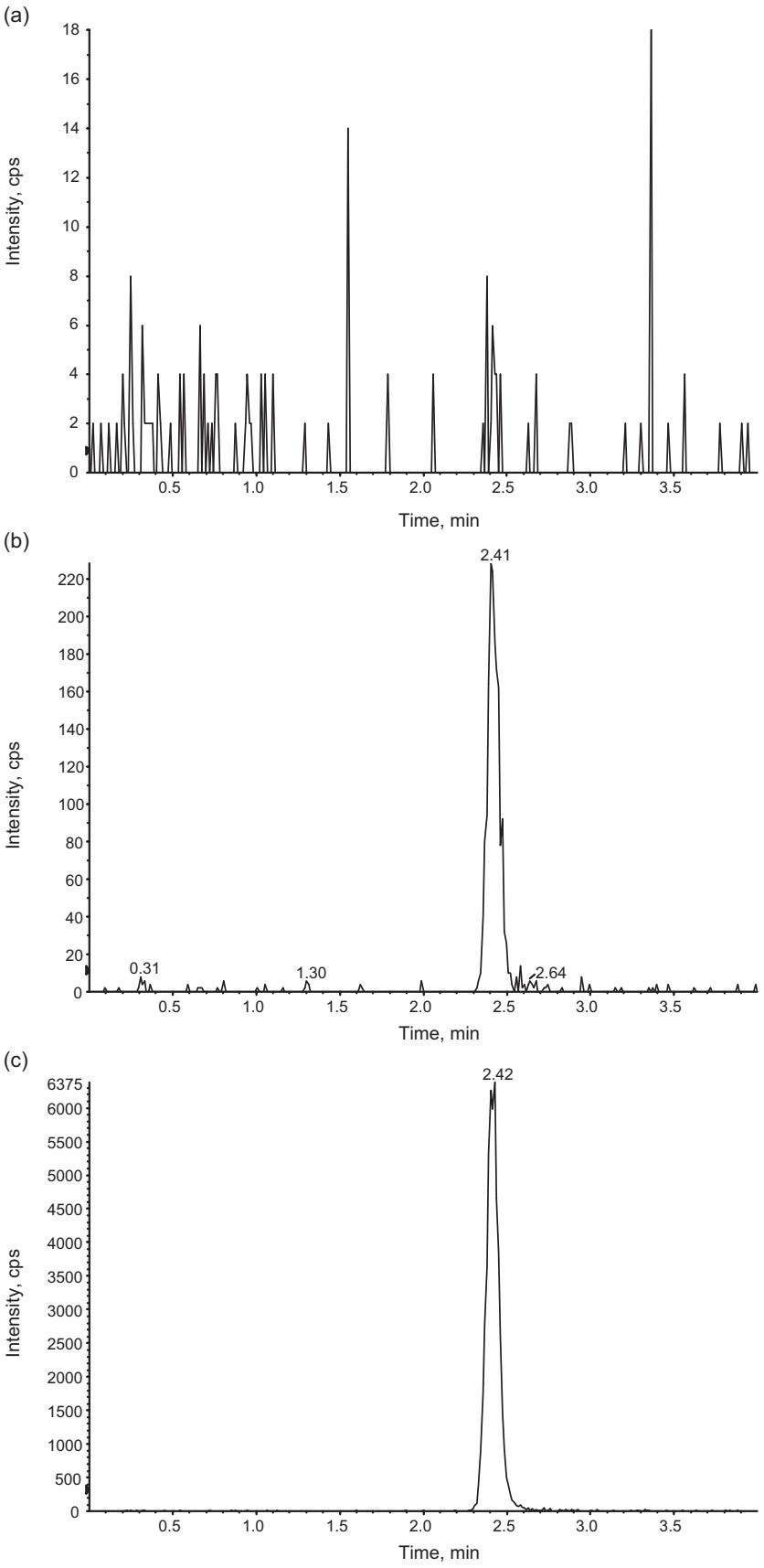

Fig. 2. Representative LC–MS/MS chromatograms of AZD9164 in (a) blank urine and (b) LLOQ calibration sample (5.0 nmol/L), together with a representative chromatogram of its isotopically labelled internal standard (c).

employed at a flow rate of 400 μL/min. The injection volume was 1 μL and the injector programme included a needle wash with 50% (v/v) aqueous methanol, containing 0.1% (v/v) formic acid, to minimise analyte carry-over. Samples were maintained at 15 °C in the autosampler tray.

Quantitation of AZD9164 was achieved by MS/MS detection in positive ion mode, through multiple reaction monitoring (MRM) of *m*/*z* transitions 465.1/250.2 and 471.0/256.2 for AZD9164 and its internal standard (IS), respectively. Unit mass resolution was employed for the first and third quadrupole and the dwell time was 500 and 200 ms for AZD9164 and IS, respectively. The analysis run-time was 4 min. The optimised instrument conditions for analyte and IS were as follows: TIS source temperature of 450 °C; ionspray voltage (IS) of 4500 V; nebuliser gas of 14; curtain gas of 15; CAD gas of 6; collision energy of 35 eV; declustering potential (DP) of 21 V; focussing potential (FP) of 130 V; entrance potential (EP) of 10 V; collision exit potential (CXP) of 10 V. Calibration curves were constructed by plotting the peak area ratio of analyte:IS versus the nominal concentration of analyte and applying a linear regression with a $1/x^2$ weighting.

Chromatographic profiling of surfactants/additives was also achieved by MS detection. Representative masses in single ion monitoring (SIM) or MRM, under positive or negative ionisation, and optimised instrument conditions were determined by infusing solutions of each compound in mobile phase (typically 10–30 μmol/L; approximately 1 mmol/L for polymeric HP-β-cyclodextrin). Unit mass resolution was employed for the first and third quadrupole and the dwell time for each MRM transition was typically 200 ms. The analysis run-time ranged between 4 and 30 min. The typical optimised instrument conditions were as follows: TIS source temperature of 450 °C; ionspray voltage (IS) of (±) 4500 V; nebuliser gas of 14; curtain gas of 15; CAD gas of 6. Additional information regarding SIM/MRM experiments is given in Table 3.

2.4. Preparation of standard solutions

Primary stock solutions of compound AZD9164 and IS (each at 1 mmol/L) were prepared in methanol in glass vials. Subsequently, eight working stocks containing AZD9164 were prepared in methanol at concentrations of 0.25, 0.50, 1.0, 2.5, 5.0, 25, 45 and 50 μmol/L by appropriate dilution of the 1 mmol/L primary stock. Additionally, a working solution of IS was prepared in acetonitrile at 0.20 μmol/L by appropriate dilution of the primary stock. All primary and working stocks were stored in a refrigerator at 10 °C or below.

2.5. Preparation of urine samples and adsorption experiment

For each batch, on the day of analysis, a fresh set of calibration samples, containing AZD9164 at 5.0, 10, 20, 50, 100, 500, 900 and 1000 nmol/L, were prepared by mixing 10 μL of the working stock solutions detailed with 490 μL of blank human urine in 1.5 mL polypropylene centrifuge tubes. The samples were processed by adding 500 μL of the working IS solution (acetonitrile) into the *same* tubes and thoroughly vortex mixed. The samples were centrifuged and 200 μL aliquots transferred to a 96-well plate for analysis.

Quality control samples (20 and 900 nmol/L) were prepared for each batch by spiking 20 μL of the appropriate working stock with 980 μL of blank human urine in 25 mL polypropylene tubes. Six replicates were prepared at both concentrations. Similarly, test samples were prepared using blank human urine (from the same batch as used to prepare QC samples) that had been pre-treated with the test surfactant/additive (solid/liquid added directly to approximately 8 mL of urine – see Table 2 for details regarding the concentrations employed). Test samples were prepared in triplicate at both concentrations.

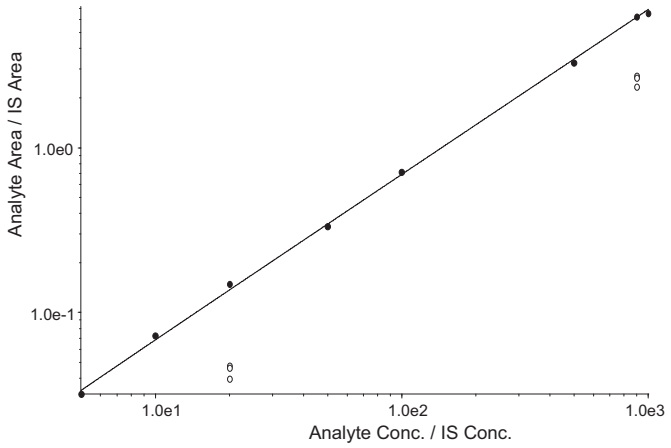

Fig. 3. Representative calibration curve (●) for urine samples containing AZD9164, between 5.0 and 1000 nmol/L, overlayed with QC samples (○), at 20 and 900 nmol/L, subjected to roller-mixing adsorption experiment.

The QC and test sample tubes were placed on a roller-mixer for approximately 30 min at room temperature. At the end of this period, 500 μL aliquots from each of the test samples and from 3 replicates of the QC samples (at both concentrations) were transferred to 1.5 mL polypropylene centrifuge tubes and processed as per the calibration samples [the addition of 500 μL of the working IS solution (acetonitrile), thoroughly vortex-mixing, centrifuging and transferring 200 μL aliquots to a 96-well plate for analysis]. These quality control samples are referred to as "negative" QCs. The remaining 3 replicates of the QC samples (at both concentrations) were processed by adding 1 mL of working IS solution into the *same* tubes and thoroughly vortex mixing. Following centrifugation, 200 μL aliquots were transferred to the 96-well plate for analysis. These quality control samples are referred to as "positive" QCs.

3. Results and discussion

3.1. Methodology to assess adsorptive losses

A common approach to assess the degree of adsorption, and that analyte losses are not due to instability, is to perform serial transfers of urine QC samples from tube to tube [1]. The approach taken here was to prepare small volume urine QC samples (1 mL) in large volume polypropylene tubes (25 mL), and roller-mix for 30 min, in order to maximise the container surface area in contact with the sample and encourage adsorption to occur. Aliquots from the samples were subsequently transferred into acetonitrile to prevent any further adsorptive losses and analysed by LC–MS/MS against a set of calibration samples that had been prepared directly in 1:1 (v/v) urine:acetonitrile (final composition in QC samples). Representative chromatograms of AZD9164 and IS from a calibration sample at the lower limit of quantification (LLOQ, 5.0 nmol/L) and a matrix blank are presented in Fig. 2. The results demonstrated an acceptable calibration curve but determined concentrations in the QC samples, at 20 and 900 nmol/L, were approximately 60% lower than expected (Fig. 3).

Having demonstrated its applicability, the methodology was employed in a series of experiments to assess the adsorptive losses from urine in the presence of test additives. Batches contained calibration samples and "negative" QC samples (as described in Section 2.5) prepared in untreated urine, and test QC samples prepared in urine pre-treated with the test additive. Additionally, "positive" QC

Table 1
Summary of mean accuracies (%, and standard deviation, $n = 3$) obtained for quality control samples in untreated urine.

Batch no.	Nominal concentration of AZD9164			
	20 nmol/L		900 nmol/L	
	Positive QC	Negative QC	Positive QC	Negative QC
1	122 (2.1)	39 (2.4)	115 (4.0)	53 (2.2)
2	111 (4.4)	39 (1.2)	107 (6.7)	50 (3.3)
3	99 (5.1)	34 (1.9)	90 (0.9)	44 (3.7)
4	101 (3.1)	74 (6.6)	96 (1.4)	67 (2.6)
5	106 (5.8)	72 (3.5)	99 (2.8)	71 (1.0)
6	112 (5.7)	82 (2.6)	105 (3.1)	82 (1.4)
7	85 (2.0)	51 (1.7)	90 (0.8)	55 (1.8)
8	115 (5.9)	90 (2.9)	101 (5.3)	83 (0.8)
9	89 (6.1)	82 (2.1)	91 (2.4)	84 (1.5)
10	84 (3.0)	49 (3.0)	94 (1.7)	60 (3.2)
11	96 (3.6)	63 (3.7)	102 (3.7)	78 (2.6)
12	90 (6.2)	46 (4.5)	88 (3.6)	53 (0.4)
13	99 (5.0)	47 (2.1)	98 (2.4)	58 (1.7)
14	130 (5.0)	98 (9.4)	112 (5.1)	85 (3.8)
15	87 (1.8)	53 (4.7)	95 (0.5)	60 (0.7)
16	93 (0.9)	75 (5.8)	96.1 (2.7)	82 (3.2)
17	95 (1.4)	51 (2.9)	99 (1.6)	61 (0.9)
18	95 (2.2)	78 (5.5)	95 (2.5)	79 (1.6)
Overall	101	62	99	67

samples were included to provide confidence in the assay. These samples were subjected to roller-mixing but were subsequently processed by the addition of acetonitrile directly into the 25 mL tube to reverse any adsorption that may have occurred. The mean accuracies (and precision) obtained for "positive" and "negative" QC samples from all batches are presented in Table 1. As can be seen, the "positive" QC results demonstrate acceptable assay performance and confirm that analyte instability is not a concern over the time-frame of the experiment. The overall mean accuracy observed in "negative" QC samples was comparable for the two concentrations tested (20 and 900 nmol/L) and indicated average adsorptive losses of approximately 35%. Although a proportional loss of analyte from samples differing in initial concentration by 45-fold is not entirely expected, and is not consistent with saturation of binding sites [1], other researchers have reported similar findings [9]. Within a given experiment, the same lot of control urine was employed in all samples but different lots of urine were required between experiments. This is reflected in the minimal variability of adsorptive losses observed within an experiment in comparison to the large variability between experiments and further highlights the challenge of non-specific adsorption.

3.2. Assessment of adsorptive losses in the presence of additives

The urine additives investigated included simple organic solvent [acetonitrile (MeCN)], aprotic solvent [dimethylacetamide (DMA)], polymeric non-ionic surfactant (Tween 80), zwitterionic surfactant (CHAPS and CHAPSO), ionic surfactant (SDS and SDBS), complexation agent (HP-β-cyclodextrin) and phospholipids [L-α-phosphatidylcholine (PC) and L-α-lysophosphatidylcholine (lysoPC)]. The latter additives are technically zwitterionic surfactants consisting of a negatively charged phosphate group (in the standard pH range), a positively charged quaternary amine in the choline group and one (lysoPC) or two (PC) hydrophobic hydrocarbon chains. The concentrations of AZD9164 determined in urine samples, in the presence of each additive, are presented as accuracies with respect to the "positive" QC sample results and as recoveries with respect to the "negative" QC sample results (see Table 2). Generally, the effectiveness of each additive in preventing adsorptive losses can be ranked by comparing the accuracy values. However, as discussed in Section 3.1, since there is known variability in the extent of adsorption between different lots of urine (and experiments), consideration of the extent of recovery with respect to the "negative" QC samples is required. For example, simple addition of 5% (v/v) acetonitrile resulted in QC accuracies of 84% and 89% at 20 and 900 nmol/L, respectively. However, the corresponding accuracies for the "negative" QC samples (measure of adsorption in the absence of any additive) in the same batch were 81% and 85% and therefore the actual extent of analyte recovery afforded by the presence of 5% (v/v) acetonitrile is low (17% and 29%, respectively). Additives effective in minimising adsorptive losses should demonstrate accuracies and recoveries approaching 100%. Batches in which minimal adsorption was observed in the "negative" QC samples were repeated.

All test additives were found to reduce adsorptive losses and, for a given additive, the analyte losses were proportional at concentrations of 20 and 900 nmol/L. From the range of additives (and concentrations) evaluated, we found that DMA, Tween 80, CHAPS, CHAPSO, SDBS and lysoPC were all capable of affording (near-)complete recovery of AZD9164. The effectiveness of Tween 80, DMA and CHAPS has also been demonstrated when each was present at concentrations ranging 100, 10 and 5-fold, respectively. Since urine collection volumes will vary considerably within the

Table 2
Summary of mean accuracies* ($n = 3$) and recoveries** for test samples in human urine containing various additives.

Additive	Concentration	Nominal concentration of AZD9164			
		20 nmol/L		900 nmol/L	
		Accuracy (%)	Recovery (%)	Accuracy (%)	Recovery (%)
Acetonitrile	5.00% (v/v)	84	17	89	29
DMA	5.00% (v/v)	99	98	101	101
	0.50% (v/v)	98	95	95	85
Tween 80	2.00% (v/v)	97	89	99	95
	0.20% (v/v)	104	108	104	111
	0.02% (v/v)	98	89	98	88
CHAPS	3 mmol/L	98	97	99	98
	0.6 mmol/L	96	82	101	102
CHAPSO	3 mmol/L	99	98	92	85
SDS	6 mmol/L	94	78	94	80
SDBS	4 mmol/L	102	106	103	111
HP-β-cyclodextrin	3 mmol/L	92	80	94	84
	0.6 mmol/L	59	22	69	25
LysoPC	3 mmol/L	95	90	96	88

* Accuracy $= \frac{(\text{mean conc in test QC})}{(\text{mean conc in post QC})} \times 100\%$.

** Recovery $= \frac{(\text{mean conc in test QC}) - (\text{mean conc in neg QC})}{(\text{mean conc in pos QC}) - (\text{mean conc in neg QC})} \times 100\%$.

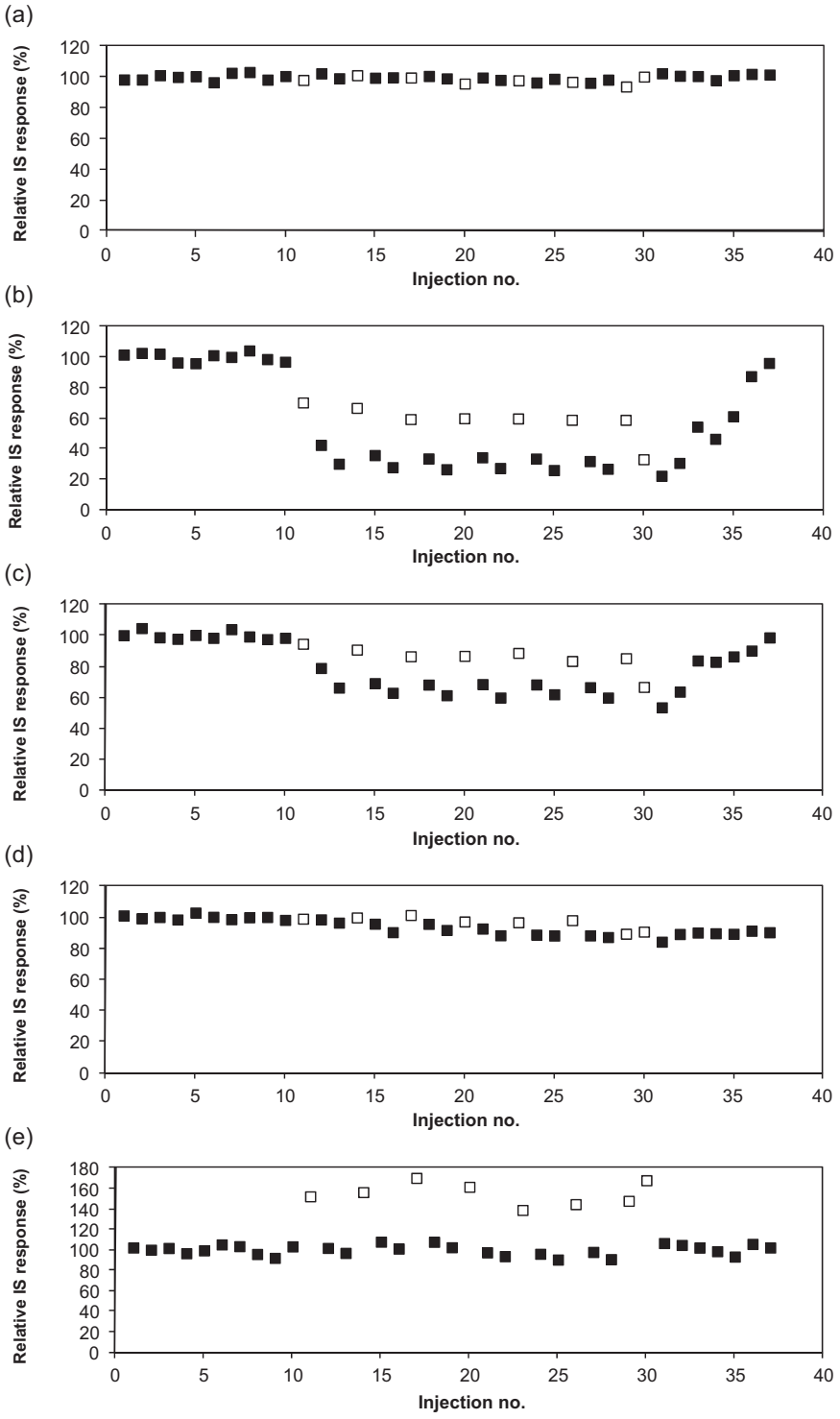

Fig. 4. Plots of internal standard relative peak area response against injection number in LC–MS/MS analysis batches containing urine samples with (□) and without (■) the presence of test additive; (a) 3 mmol/L CHAPS, (b) 2.00% (v/v) Tween 80, (c) 0.20% (v/v) Tween 80, (d) 0.02% (v/v) Tween 80, and (e) 3 mmol/L LysoPC.

clinic, and practicalities dictate that each sample must be treated to a standard protocol, it is important to verify that the chosen additive is effective over a concentration range. HP-β-cyclodextrin, in the concentrations evaluated here, did not afford complete recovery. However, since adsorptive losses were reduced, and analyte solubility is known to increase proportionally with the concentration of cyclodextrin [11,12], its applicability should not be discarded for other analytes. The result obtained for lysoPC is of particular interest since a whole host of procedures and technologies (see for example Ref. [13]) have been developed to remove or avoid endogenous phospholipid species in the analysis of plasma samples. Here, we demonstrate a practical example of where the presence of phospholipids is advantageous; logistically, the cost of lysoPC prohibits its use as a routine urine additive. We have also observed in other bioanalytical methods that the absence (or reduced presence) of phospholipid species in plasma sample extracts results in lower analyte recoveries following dry-down and reconstitution (data not shown). These observations should not be surprising upon

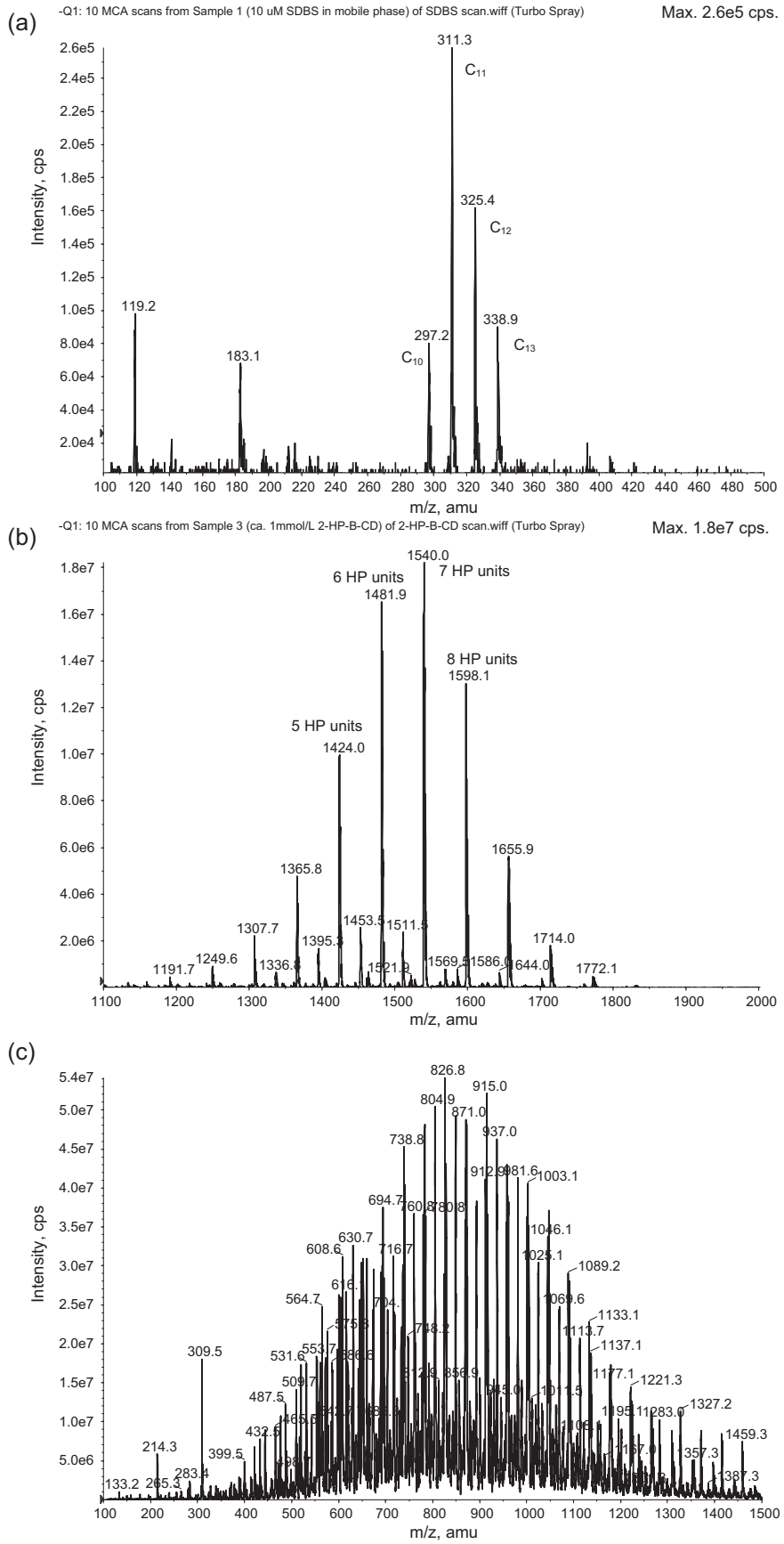

Fig. 5. MS spectra obtained upon infusion of solutions of (a) SDBS, (b) HP-β-cyclodextrin and (c) Tween 80 in mobile phase.

consideration of their amphipathic structure and lecithin (PC), for example, is a well-known emulsifier in food products, has become increasingly important as a formulation excipient in oral drug delivery [14] and has been demonstrated to have a significant impact on drug solubility in human intestinal fluids [15]. In the experiments performed here, PC did not demonstrate any appreciable reduction in adsorptive losses (data not shown) but this is believed to be due to difficulties in solubilising the compound directly in urine.

3.3. Impact of additive on quantitative bioanalysis

Attention was next focussed on evaluating the impact, if any, from the presence of additive on the LC–MS/MS method employed. Peak area responses of the isotopically label IS, obtained within each adsorption test batch, were inspected for any signs of enhancement or suppression for treated urine samples compared to untreated samples. Of the additives affording (near-)complete recovery of AZD9164, DMA, CHAPS, CHAPSO and SDBS had no observable effect on MS sensitivity. However, the presence of Tween 80 or lysoPC in urine had a marked impact on peak area, suppressing MS sensitivity in the case of the former and enhancing sensitivity in the case of the latter (see Fig. 4). Significant signal suppression, due to Tween 80, was also observed to carry through into subsequent injections of untreated urine samples indicating that the elution of this surfactant is quite prolonged. The suppression/enhancement effect on analyte MS sensitivity, in the presence of Tween and phospholipids, is well documented [10,13,16] but it is interesting to note that signal suppression could almost be eliminated by reducing the concentration of Tween 80 down to 0.02% (v/v). It should also be noted that these ionisation effects did not impact on the quantitation of AZD9164 due to use of the isotopically labelled IS.

A further potential issue that could complicate quantification of analyte, and manifest itself as poor recovery in the presence of the test additive, is the formation of analyte–additive clusters/complexes. This is particularly relevant in the use of HP-β-cyclodextrin since the formation of a guest–host complex is the basis upon which this agent is able to solubilise drug analytes. However, the results obtained within the investigation suggest that any clusters/complexes present in the urine test samples (containing analyte and additive) are disrupted sufficiently upon the addition of MeCN and/or injection onto the LC system to prevent underestimation of analyte. A single peak was present in the LC–MS/MS chromatograms of AZD9164, and the retention time and peak symmetry remained consistent irrespective of the presence or the absence of additive in the sample. Similarly, clusters with IS would also be expected to form upon its introduction to additive-spiked samples and yet there was no observable effect on either the IS chromatography or the peak response (excepting the instances of ion-suppression/enhancement noted above).

Therefore, from the perspective of developing and validating a urine bioanalytical method, with minimal sample manipulation, DMA, CHAPS, CHAPSO and SDBS are all potential additives that minimise non-specific adsorptive losses of AZD9164 and have no significant impact on its MS response. Tween 80, in moderate concentrations, may also be an appropriate additive for use in assays that are internally standardised with isotopically labelled material.

3.4. Impact of additive on qualitative/quantitative metabolite investigations

In order to evaluate any possible impact that the presence of additive may have on MS detection with reference to metabolite quantification and/or identification, it is necessary to understand the additive chemically and chromatographically. For this part of the investigation, only the most appropriate of closely related additives were selected. For example, CHAPS and CHAPSO were comparable in their ability to overcome adsorptive losses but the former is a markedly less expensive reagent. Conversely, SDS was simply unable to overcome adsorptive losses to the same extent as SDBS.

Initially, solutions of DMA, CHAPS, SDBS, Tween 80, lysoPC and HP-β-cyclodextrin (all in mobile phase) were infused into the mass spectrometer and a Q1 scan performed in positive and negative ionisation modes, as appropriate, to confirm the parent ions. As expected, the mass spectra obtained for DMA and CHAPS, under positive ionisation, showed protonated molecular ions ($[M+H]^+$) at m/z 88.0 and 615.5, respectively. Similarly, the mass spectrum for lysoPC exhibited molecular ions ($[M^+]$) at m/z 496.2 and 524.3, corresponding to the C16:0 and C18:0 species, respectively, along with characteristic fragments at m/z 184.1 and 103.8 [16]. The mass spectra acquired for SDBS, HP-β-cyclodextrin, and Tween 80 were more complex as shown in Fig. 5. Commercial SDBS actually consists of 4 homologues, decyl-, undecyl-, dodecyl- and tri-decylbenzenesulphonates (C_{10}, C_{11}, C_{12} and C_{13}), giving rise to the observed molecular ions ($[M-H]^-$) at m/z of 297.2, 311.3, 325.4 and 338.9, respectively. The spread of masses observed in the mass spectra of HP-β-cyclodextrin and Tween 80 reflect their polymeric nature. HP-β-cyclodextrin demonstrated numerous molecular ions ($[M-H]^-$) spanning an m/z range from 1191.7 to 1772.1, and increasing in multiples of 58 a.m.u. The observed spectrum is attributable to a distribution of β-cyclodextrin species (expected $[M-H]^-$ m/z of 1134) modified with between 1 and 11 hydroxypropyl units; the most abundant cyclodextrin species observed, with an m/z of 1540.0, contains 7 such units. In the mass spectrum of Tween 80, the expected parent molecular ion ($[M+H]^+$) at m/z 1309.2 (for the mono-oleate ester of sorbitan polyethoxylate) was observed but only as a minor component amongst a distribution of fragment molecular ions, principally differing by 44 a.m.u. and further complicated by adducts with sodium. Such distributions are characteristic of polyoxyethylene surfactants like Tween 80 (see also Refs. [17,18]).

Subsequently, representative single ion monitoring (SIM) or multiple reaction monitoring (MRM) experiments were generated for each additive, as summarised in Table 3, and injections of urine samples, with and without additive, were made under the same chromatographic conditions employed for the analysis of AZD9164. As shown in Fig. 6, peaks were observed in all experiments performed on treated urine samples. Corresponding experiments performed on untreated urine did not exhibit these peaks (data not shown) providing further confidence, together with the inherent specificity of each experiment, that the peaks observed in treated urine samples derive from the test additive. The additives DMA and CHAPS have been shown, from the infusion experiments, to exist as single chemical species and these were found to elute as single chromatographic peaks in, or just after, the void volume of the system; an approximate void time of 0.27 min would be expected for the column and flow rate employed. Similarly, despite a distribution of HP-β-cyclodextrin species, the most abundant forms monitored (containing 5, 6, 7 and 8 HP units) all eluted in the void volume; others [11] have reported comparable chromatographic findings for unmodified β-cyclodextrin. The chromatogram obtained for SDBS showed considerably more than 4 chromatographic peaks as would be expected for the 4 homologues. This is believed to be due to the existence and separation of phenyl isomers of each homologue, with retention times spanning from approximately 0.80 to 2.33 min. Similarly, isomers of the C16:0 and C18:0 forms of lysoPC were chromatographically separated with peaks evident at 2.00, 2.30, 4.45 and 5.13 min. For Tween 80, elution of each monomer resulted in exceptionally broad peaks across the majority of the chromatogram and out to approximately 26 min.

Table 3
Summary of MS experiments employed to monitor chromatography of selected additives.

Additive	MS experiment	Polarity	*m/z* value(s)	DP (V)	FP (V)	CE (eV)
DMA	MRM	Pos	88.0 → 45.9	20	230	5
Tween 80	SIM	Pos	1309.7	100	250	N/A
CHAPS	MRM	Pos	615.5 → 448.3	35	225	46
SDBS	MRM	Neg	297.2 → 183.0 311.3 → 183.0 325.4 → 183.0 338.9 → 183.0	−30	−180	−50
HP-β-cyclodextrin	SIM	Neg	1424.0 1481.9 1540.0 1598.1	−95	−285	N/A
LysoPC	MRM	Pos	496.2 → 184.0 524.3 → 184.0	50	160	35

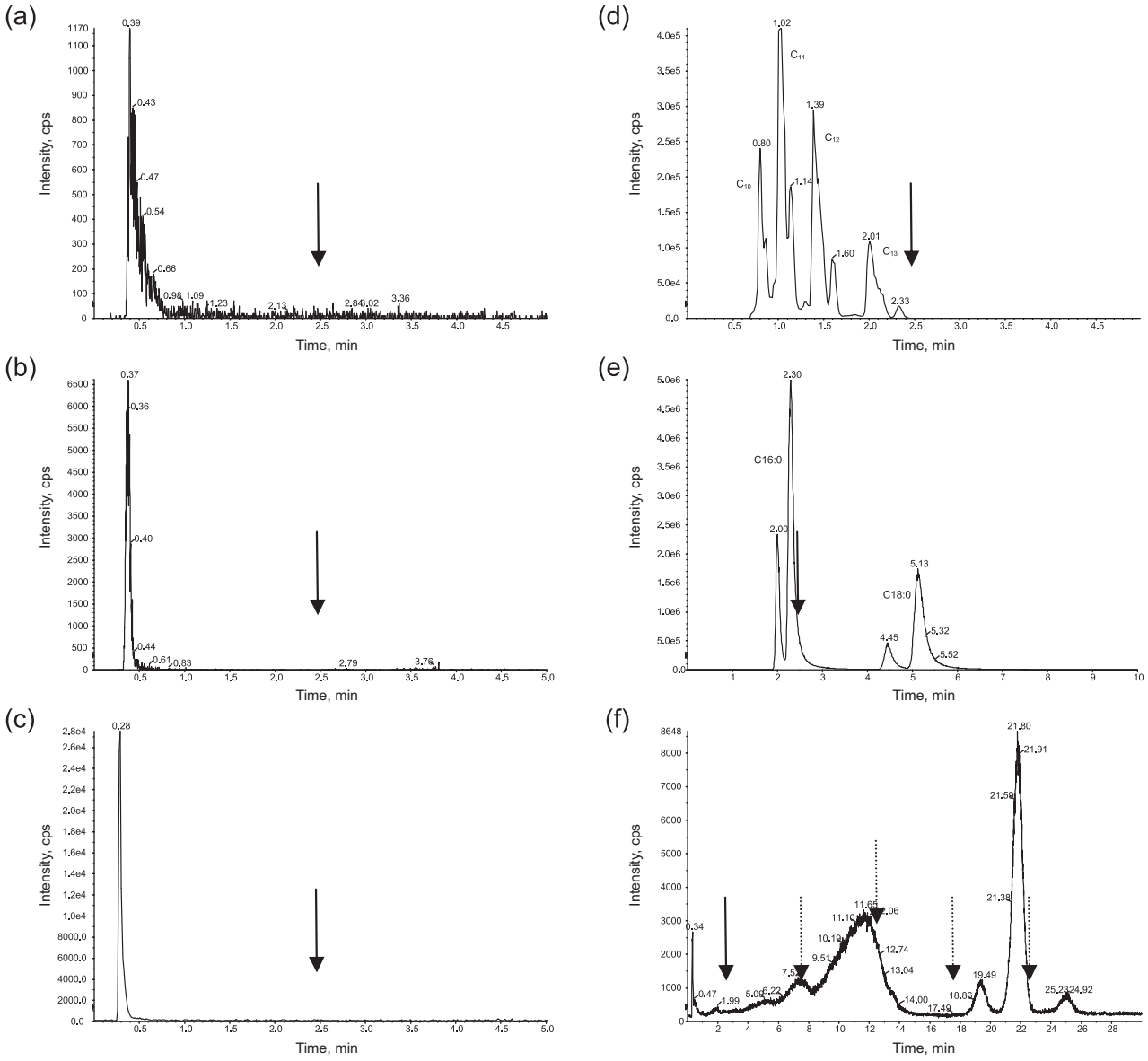

Fig. 6. LC–MS chromatograms obtained, employing the representative SIM or MRM experiments given in Table 3, for urine samples containing (a) 5% (v/v) DMA, (b) 3 mmol/L CHAPS, (c) 3 mmol/L HP-β-cyclodextrin, (d) 4 mmol/L SDBS, (e) 3 mmol/L LysoPC, and (f) 2% (v/v) Tween 80. The retention of AZD9164 is indicated (↓, dotted lines denote expected retention of AZD9164 from subsequent injections).

The elution of additive correlates entirely with the observed effects on analyte ion suppression/enhancement. Additives found to have no impact on analyte MS response have all been shown to be chromatographically separated from the analyte (and IS), albeit marginally in the case of SDBS. However, the surfactants lysoPC and Tween 80, which co-eluted with analyte, resulted in modified MS response and for Tween 80 the effect on ionisation was evident over several injections as the polymer continued to elute.

Moreover, the results clearly show that the use of polymeric surfactants such as Tween 80 would be unsuitable in urine samples upon which metabolite identification investigations are to be performed. Although metabolite analyses will generally employ a gradient elution, rather than the isocratic conditions used in the work presented here, the wide elution profile observed for Tween 80, coupled with the detection of numerous fragment ion masses, is likely to impede the detection and identification of metabolites and cause extensive ion suppression issues. Similarly, although to a lesser extent, SDBS would not appear to be an ideal choice due to the introduction of a series of homologues and positional isomers. The additives CHAPS and DMA however, have both been shown to elute early as single chemical species. As a consequence, they are less likely to obscure the MS detection of unknown metabolites and are appropriate additives for such investigations. Likewise, the co-elution of HP-β-cyclodextrin species, as a single chromatographic peak in the column void volume, indicates the suitability of this additive also.

4. Other considerations

In the investigations performed, a range of surfactants (and other additives) have been evaluated in their potential to overcome the non-specific adsorption of AZD9164 and to interfere with subsequent quantitative and qualitative bioanalytical assays. However, there are other factors that need to be considered in order to select the most suitable additive to take forward into the development of a validated analytical method. For instance, the additive must be appropriate for use in a clinical environment with regard to its toxicity and ease-of-use. In this respect, the commonly used excipients Tween 80 and HP-β-cyclodextrin are ideal, whereas CHAPS, and in particular DMA, carry greater concerns. The cost associated with using each additive is also a significant consideration and especially so if the additive is present (as would be recommended) in containers during the collection of urine; in a typical early clinical pharmacology study (e.g. single ascending dose), the total volume of urine could easily be 200–300 L.

Ultimately, the surfactant CHAPS was selected for use in development of the validated analytical method for AZD9164. Although it carries greater toxicity concerns than some of the other surfactants, its cost is not prohibitive and the investigations performed clearly demonstrated its potential in overcoming adsorptive losses of the analyte with minimal risk of interfering with the assay. It is important that the subsequent method development includes further experiments to demonstrate that adsorptive losses are also overcome under the conditions of urine sample collection (over 24 h at room temperature), storage (at −20 °C for several weeks) and manipulation (following freeze–thaw cycles); it would also be desirable to demonstrate that adsorptive losses could be overcome irrespective of whether the additive is spiked pre- or post-collection.

5. Conclusions

The applicability of a range of surfactants, and other additives, to overcome non-specific analyte adsorption issues in human urine samples has been assessed. Simple adsorption experiments, designed to maximise sample interaction with container surfaces, were performed on urine quality control samples containing an adsorption-prone in-house development compound (AZD9164). From the perspective of developing and validating a urine bioanalytical method, with minimal sample manipulation, DMA, CHAPS, CHAPSO and SDBS were all found to be suitable additives that minimised non-specific adsorptive losses of AZD9164 and had no significant impact on the analyte MS response. Tween 80 was also found to overcome adsorptive losses and, despite its presence suppressing the analyte MS response, in moderate concentrations [~0.02% (v/v)] would also be an appropriate additive for use in assays that are internally standardised with isotopically labelled material. Of particular academic interest was the finding that adsorptive losses could also be reduced upon the addition of phospholipid (lysoPC).

For additives used in urine samples upon which metabolite investigations are to be performed, it is important to also confirm that the presence of additive will not impede the detection and/or quantification of unknown metabolites. Monitoring each additive by LC–MS(/MS) demonstrated that the polymeric surfactant Tween 80 (and to a lesser extent SDBS) eluted over a wide retention time window. Conversely, CHAPS and DMA (and HP-β-cyclodextrin, despite not fully eliminating adsorptive losses) eluted as single chromatographic peaks in, or just after, the column void volume and therefore their presence in urine should not unduly impact on metabolite investigations. Upon consideration of other factors such as additive cost and toxicity, CHAPS was selected for use in development of the validated assay.

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