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Evaluation of LC–MS for the analysis of cleaning verification samples

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Abstract

The cleaning verification of pharmaceutical manufacturing equipment prior to further use is a cGMP requirement. Typically, relevant data are generated by HPLC with UV detection using methods individually developed and validated for each product. This work describes the use of HPLC with mass spectrometry to analyse cleaning verification samples, a novel means of utilising this analytical technology. The initial aim was to produce a single, generic method capable of quantifying a broad range of pharmaceuticals. Ultimately, however, a more effective strategy, in terms of efficiency and reliability, proved to be application of a well-defined approach to the rapid generation of compound specific methods. Results of studies to optimise the sample preparation for a basic compound in drug development (compound 1), together with experimental results for two further compounds are presented. These demonstrated that the combination of a well defined approach to chromatographic method development and mass spectrometric detection provided methodology with advantages in terms of sensitivity. Additionally, and by virtue of its potential for general applicability, the approach proposed has the potential to improve the overall efficiency with which methods for cleaning verification samples can be developed and applied.

Keywords: Cleaning verification; Liquid chromatography–mass spectrometry; Stable isotope labelled internal standards

1. Introduction

LC–MS is widely used in the pharmaceutical industry for applications such as the identification of potential drug candidates in pharmacological screens, the identification of impurities and degradation products obtained during clinical development and the quantification of drugs in biological media, both in vitro and in vivo [1] In the latter case, the technique's key advantages of improved sensitivity of detection and selectivity with consequent reduction in analysis times have led to it becoming widely adopted as the quantitative technique of choice [1]. For those laboratories not concerned with bioanalysis but with establishing the overall quality of active pharmaceutical ingredients (APIs) and their formulated products LC–MS equipment is, today, essential but is most often associated with qualitative rather than quantitative applications. However, given the technique's advantages, it seems likely that LC–MS has similar, as yet unrealised, potential in respect of quantitative work in these laboratories. Accordingly, in this work, the suitability of LC–MS for the quantification of API residues during the cleaning verification of pharmaceutical manufacturing equipment has been briefly assessed using three compounds under development in our laboratory.

After the manufacture of a pharmaceutical formulation has been completed it is a cGMP requirement that the equipment be cleaned prior to being used for the manufacture of a different product [2]. Various analytical methods have been used to verify the success of cleaning operations; including HPLC–UV, which is the most commonly applied [3,4], ion mobility spectrometry (IMS) [5] total organic carbon (TOC) [6] and HPLC with evaporative light scattering detection (ELSD) [7]. Both IMS and TOC have the advantage of speed with respect to HPLC–UV methods but the latter would not be specific for the compound of interest and the former is not generally available at pharmaceutical manufacturing facilities. Similarly, although it allows for the sensitive detection of compounds, including those with a poor chromophore, ELSD has not found general applicability in this area. Recently the reduction in LC–MS equipment prices and the increasing number of applications, have led to much greater access to this type of equipment within facilities where cleaning

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verification is routinely performed. Because of this and the techniques's potential advantages, including improved sensitivity, an investigation into the feasibility of using LC–MS for the analysis of cleaning verification samples was considered appropriate.

Following equipment cleaning, different procedures may be used to confirm the operation's success. These fall into two categories, rinse and direct surface sampling. Rinse samples are obtained by passing a volume of solvent (generally aqueous) through or over the cleaned equipment, which is then analysed for the compound of interest. By contrast direct surface sampling involves the use of swabs (for small surface areas) and wipes (for larger surface areas) which are moistened with the solvent of choice and rubbed over the surface to be monitored. This technique is generally preferred as it allows the use of a relatively small volume of organic solvent, which not only results in greater removal of compounds from the equipment surface, but also avoids excessive dilution of these species prior to analysis [2]. For this reason, within the general aim of testing the feasibility of using LC–MS for the analysis of cleaning verification samples, the scope of the experimentation was limited to the validation of direct surface sampling methodology and the use of the most common surface type, stainless steel.

Validation data required to support the determination of trace API levels during cleaning verification can be viewed as somewhat intermediate in nature between those that would be required to support quantification of an impurity in an API and those required for a limit test [8]. Because of this only validation data sufficient to show the approach to be feasible were produced recognising that, were the proposed approach to be adopted routinely, additional validation data may be necessary. Consistent with the above, methods were developed and applied to a piperidinyl derivative (compound 1), a substituted isoquiniline (compound 2) and a modified pyridazino species (compound 3) which were under development for differing pharmaceutical applications.

2. Experimental

2.1. Reagents and chemicals

Compounds 1, 2 and 3 and their stable isotope labelled (SIL) versions (which all contained >99% labelled compound), lactose monohydrate, povidone, magnesium stearate and sodium starch glycolate were all supplied by sanofi-aventis. HPLC grade acetonitrile was purchased form Fisher Scientific Ltd. (Loughborough, Leicestershire, UK). HPLC grade ethanol and ammonium formate were purchased from BDH Laboratory Supplies (Poole, Dorset, UK). Purified water was produced in-house by use of an Elga Maxima system (Elga LabWater, High Wycombe, UK). Formic acid was purchased from Sigma–Aldrich Co. Ltd. (Poole, Dorset, UK).

2.2. Equipment

HPLC–MS was performed using a Micromass ZMD single quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) coupled to an Agilent 1100 series HPLC system (Agilent Technologies UK Ltd., Stockport, UK). Solvent optimisation was performed using DryLab® chromatography optimisation software, Version 2.05 (LC Resources, Walnut Creek, CA, USA). Wipes used were Kimtex® Lite reference 7271 purchased from Kimberly-Clark Ltd. (Kent, UK) cut to 18 cm × 19 cm.

2.3. Chromatographic and mass spectrometric conditions

2.3.1. Single method for all compounds

For this methodology the HPLC column was a Waters XTerra™ C8 (3.5 μm particle size, 21 mm × 3 mm) purchased from the Waters Corporation (Watford, Hertfordshire, UK). The mobile phase consisted of acetonitrile–water, containing 20 mM formic acid (90:10 v/v) deliver at a flow rate of 2 ml min^{-1} and split 20:1 in favour of waste prior to the mass spectrometer. The column was held at 40 °C and 50 μl of each solution was injected.

The mass spectrometer was operated in electrospray mode with positive ionisation. The cone voltage was set to 30 V, the capillary voltage to 3.5 kV, the desolvation gas flow to 400 l h^{-1}, the source block temperature to 120 °C, and the desolvation temperature to 300 °C. The dwell time was 0.1 s. Each compound and its associated internal standard was monitored using SIM of the most abundant ion which, in each case, was the $[M + H]^+$ ion.

2.3.2. Well defined approach

Methods were developed for all three compounds. In each case two gradient analyses were carried out and DryLab® software used to predict the solvent ratio which would give a retention time of approximately 2 min. This allowed the analyte to be resolved from any interference at the solvent front.

For compounds 1 and 3 the HPLC column used was a Waters XTerra™ MS C8 (3.5 μm particle size, 50 mm × 4.6 mm) whereas for compound 2 a Waters XTerra™ RP C18 (3.5 μm particle size 100 mm × 4.6 mm) was used. Both columns were purchased from the Waters Corporation (Watford, Hertfordshire, UK).

For compounds 1 and 3 a mobile phase of acetonitrile–water, containing 20 mM formic acid (35:65, v/v) and (20:80, v/v) respectively was used at a flow rate of 2 ml min^{-1}. For compound 2 a mobile phase of 20 mM ammonium formate (pH 8)–acetonitrile (60:40, v/v) at a flow rate of 2 ml min^{-1} was used. All flow rates were split 20:1 in favour of waste prior to entering the mass spectrometer. The column was held at 40 °C in all cases. An injection volume of 100 μl was used for the extraction optimisation experiments and 50 μl for all other work.

The mass spectrometer conditions were optimised for each compound. In all cases the instrument was operated in electrospray mode with positive ion detection and a nitrogen gas flow of 400 L h^{-1}. The cone voltage was 30 V for compound 1 and 35 V for compounds 2 and 3; the capillary voltage was 3.5 kV for compounds 1 and 2 and 2.25 kV for compound 3. The source block temperature was 120 °C for compounds 1 and 3, and 150 °C for compound 2, the desolvation temperature was 300 °C for compounds 1 and 2 and 350 °C for compound 3, the dwell time was 0.1 s in all cases. Each compound and its asso-

ciated internal standard, was monitored using SIM of the most abundant ion, which, in each case, was the $[M + H]^+$ ion (Table 3) and smoothed prior to integration.

2.4. Sample preparation and analysis

2.4.1. Extraction optimisation

For the extraction optimisation experiment samples were prepared by impregnating a wipe with 7 ml of ethanol, then placing it in a 100 ml conical flask, 1.0 ml of a 40 μg ml^{-1} compound 1 solution in ethanol was added directly onto the wipe, the flask stopper replaced and the sample allowed to stand for at least 1 h to simulate the time to take and receive wipe samples were they taken from actual manufacturing equipment. Then, either 7, 42 or 67 ml of mobile phase was added to the flask to give final volumes of 15, 50 or 75 ml respectively. The initial samples were 'swirled' and an aliquot diluted (1:1, v/v) with the relevant SIL Internal Standard (1.0 μg ml^{-1} in mobile phase) prior to analysis. Further samples, prepared in this manner, were shaken on a mechanical shaker (approximately 200 shakes per min) and a sample taken after 5, 10, 20, or 30 min, separate samples being used for each timepoint. Sample concentrations were determined by reference to a calibration line constructed from standards containing 0.125, 0.25, 0.5, 1.0 and 1.5 μg ml^{-1} of the respective analyte and 0.5 μg ml^{-1} of the SIL internal standard in mobile phase.

2.4.2. Linearity of response

For each compound the linearity of response was assessed by injecting standards prepared in mobile phase containing either 0.04, 0.1, 0.2, 0.4, 0.6 and 0.8 μg ml^{-1} of the relevant compound and 0.4 μg ml^{-1} of the relevant SIL internal standard.

2.4.3. Accuracy

Samples to test the recovery of each compound from stainless steel at a single level, representative of those that could be obtained during cleaning verification work were prepared as follows. Duplicate 1.0 ml aliquots of a 40 μg ml^{-1} solution of each compound in either ethanol (compounds 1 and 3) or ethanol–water (50:50, v/v, compound 2) were spread evenly over separate 400 cm^2 stainless steel surfaces and allowed to dry Wipes were impregnated with either 8 ml of ethanol (compounds 1 and 3) or 8 ml of ethanol–water (50:50, v/v, compound 2) and the plate wiped as shown in Fig. 1. The wipes were placed in 100 ml conical flasks, 42 ml of mobile phase added, and the samples shaken on a mechanical shaker for 10 min (approximately 200 shakes per min). Finally, each extracted sample solution was diluted (1:1, v/v) with the relevant SIL internal standard (0.8 μg ml^{-1}) in mobile phase to give solutions containing, nominally, 0.4 μg ml^{-1} of the relevant compound and 0.4 μg ml^{-1} of the relevant SIL internal standard.

Fig. 1. Method used to wipe stainless steel plate. The area was wiped from top to bottom, the wipe folded and the surface wiped from left to right.

Lower limit of quantification samples, containing 0.01 μg ml^{-1} of the relevant drug and 0.4 μg ml^{-1} of the relevant SIL internal standard in mobile phase were prepared for each compound and analysed with the recovery samples. This analyte concentration, representing 1 μg wipe^{-1} prior to extraction as described above.

In order to confirm that any sample recoveries found to be significantly below 100%, such as those obtained for compounds 1 and 2, were not due to poor extraction from the wipes used, the following samples of each were also prepared and analysed. In each case a wipe was impregnated with 7 ml of the relevant wiping solvent and the wipe placed in a 100 ml conical flask. About 1.0 ml of the relevant 40 μg ml^{-1} solution was added directly onto the wipe and the wipe taken through the extraction process outlined above, including the final dilution with SIL internal standard.

In all cases sample concentrations were determined by reference to a calibration line constructed from standards containing 0.04, 0.2, 0.4 and 0.6 μg ml^{-1} of the respective analyte and 0.4 μg ml^{-1} of the SIL internal standard in mobile phase.

2.4.4. Effect of excipients

In order to assess the effect of excipients, a slurry containing 3.6 mg ml^{-1} lactose monohydrate, 0.1 mg ml^{-1} povidone, 0.04 mg ml^{-1} magnesium stearate and 0.2 mg ml^{-1} sodium starch glycolate in ethanol was prepared and mixed well. About 1.0 ml was spread evenly over a 400 cm^2 stainless steel surface and allowed to dry. An amount of 1.0 ml of a 40 μg ml^{-1} or 4 μg ml^{-1} compound 1 solution in ethanol was then spread evenly on top of the dried excipient mix, or directly onto the stainless steel surface. Each wipe was impregnated with 8 ml of ethanol and used to wipe the stainless steel as shown in Fig. 1. The wipe was placed in a 100 ml conical flask, 42 ml of mobile phase added and the flasks shaken by mechanical shaker (approximately 200 shakes per min) for 10 min. The sample was filtered and a final (1:1, v/v) dilution made with a 0.8 μg ml^{-1} SIL internal standard solution in mobile phase.

3. Results and discussion

In attempting to maximise the efficiency of LC–MS based methodology for quantifying API residues on manufacturing equipment several approaches to sample introduction were tried. It was initially envisaged that a generic and very rapid means of analysing a variety of compounds would result if no chromatographic column were used and the sample solution directly infused into the spectrometer. Unfortunately, this approach led to band broadening as the sample aliquot travelled between the LC injector and the MS inlet and, consequently, each flow injection analysis cycle would have been relatively slow (approximately 2 min). This was rectified by introduction into the flow path of a short (21 mm × 3 mm) HPLC column which, in conjunction with a mobile phase containing a high percentage of

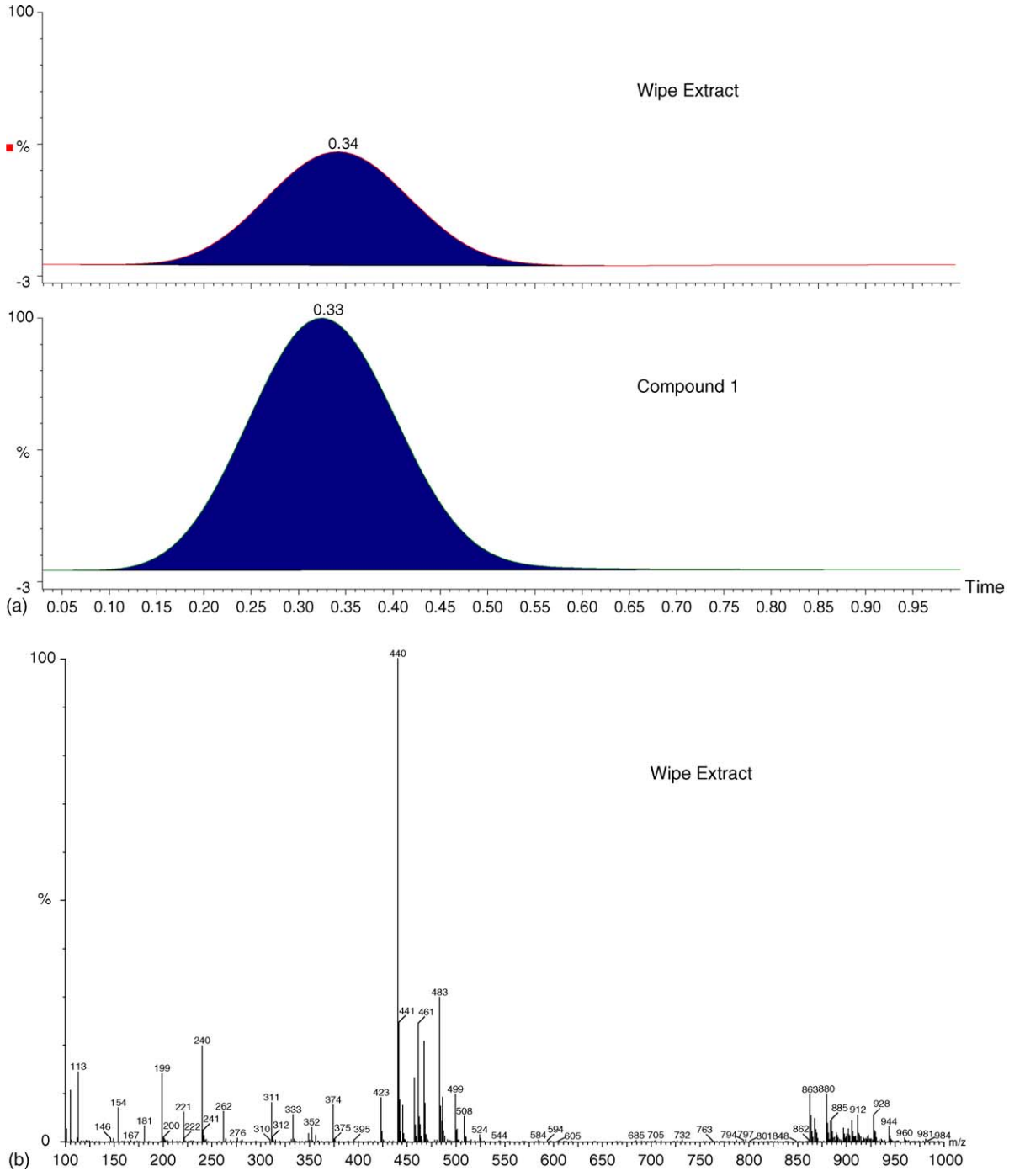

Fig. 2. Comparison of (a) MS response of a wipe extract at the $[M+H]^+$ for compound 1 together with the MS response at the same $[M+H]^+$ for a 0.4 μg ml^{-1} solution of compound 1 injected under the same conditions and (b) a full scan mass spectrum of the peak observed for the wipe extract showing the multiple, potentially, interfering signals.

organic modifier, allowed sample bands to be 'focused' prior to entering the spectrometer, without significantly retaining the compounds of interest or compromising the generic character of the approach. In terms of peak shape and speed of analysis (analysis time of approximately 1 min per injection, Fig. 2) this means of sample introduction appeared promising. However, as attempts were made to validate the approach it was discovered that unidentified polymeric species, extracted from the wipes used coeluted with all of the compounds of interest, which in turn were at or close to the solvent front. Since this significant interference (Fig. 2), could not be overcome using a single quadrupole detector the approach was abandoned in favour of a well defined method development approach designed to ensure chromatographic separation of the interfering species and the compounds to be quantified whilst keeping injection cycle times relatively short.

3.1. Well defined approach to method development

The use of a well defined approach to enable the rapid development of LC–MS conditions for a compound has been described previously [9]. In short this requires that the analyst make use of only a limited number of LC column types and mobile phase components in order to rapidly develop suitable, relatively fast, LC–MS compatible chromatographic conditions. In this work the approach was refined slightly in that ammonium formate buffer, rather than formic acid was used in some of the mobile phases, since this yielded improved peak shapes and allowed for the use of a relatively high pH buffer in one instance (compound 2). The previously selected XTerra class of HPLC columns [9] was retained as the stationary phase of choice since it is known to be suitable over a wide pH range and consistent, therefore, with a general methodology designed to be applied to a range of analytes. Using this approach chromatographic conditions for each compound were developed in a matter of hours and resulted in analysis times of approximately 4 min from injection to the end of data collection (Fig. 3). Following brief investigations into sample extraction conditions and the potential effect of commonly used pharmaceutical excipients, methods for each compound were validated.

3.2. Extraction optimisation and detection sensitivity

For drug development candidates with a high UV absorbance, sensitivity of detection, even at the levels requiring detection and quantification during cleaning verification studies, may not be an issue. For those that do not possess a high UV absorbance, additional measures such as the use of reduced extraction volumes, or even a pre-concentration step, are necessary to maintain an appropriate detection sensitivity [10]. Sensitivity issues can be further exacerbated for potent compounds as limits of quantification required for cleaning verification methodology are generally determined by the acceptance limits for the equipment concerned which, in turn, may be related to the potency of the compound [11] and reduced accordingly.

It was reasoned that the sensitivity generally afforded by MS detection could not only obviate the need for time consuming sample pre-treatment for those compounds with a poor or moderate UV absorbance but would also allow use of standard extraction volumes which may be in excess of those that would otherwise be employed for an HPLC–UV method without fear of compromising sensitivity. This would avoid concerns about potentially poor sample extraction reproducibility due to insufficient exposure of the whole wipe to the solvent when reduced volumes were employed to improve sensitivity. To determine a suitable extraction volume for general use with LC–MS based methodology for the analysis of wipe samples only compound 1 was used as described in Section 2.4.1. This was considered justified as poor extraction of compounds due to lack of solubility in the extraction solvent would be unlikely given the low levels involved and because using the approach described in this work, the extraction solvent (mobile phase) will have a relatively high level of organic modifier consistent with the development of relatively rapid chromatography. The results, which are given in Table 1, show that, for the 15 ml extraction volume, the extraction reproducibility was relatively poor until samples had been shaken for 20 min. Conversely the 75 ml extraction volume resulted in relatively reproducible extraction throughout the experiment. Ultimately, the use of a 50 ml extraction volume in a 100 ml stoppered conical flask and 10 min of shaking at approximately 200 shakes per min were selected as the optimal conditions for further use. It was felt that these conditions represented the best compromise between reproducibility of extraction, quantity of extraction solvent used and sample preparation time. Using these extraction conditions, and for all of the compounds tested, 1 $\mu g\,wipe^{-1}$ (nominally 0.01 $\mu g\,ml^{-1}$ after extraction with 50 ml of extraction solvent and dilution with internal standard) could be readily quantified using LC–MS (Table 3). Taking this to be a level below which sample quantification is not generally required and bearing in mind that the final dilution with an internal standard would not be necessary, cleaning verification samples for compounds 2 and 3 could be analysed by HPLC–UV although they would be at or close to their respective limits of quantification using the extraction condition advocated. Compound 1, however, provides an example of an API under development which has a relatively poor UV absorbance which, although suitable for other standard HPLC–UV applications such as potency and impurity determinations, would require a modified sample extraction/pre-treatment if HPLC–UV were to be used to quantify cleaning verification samples. From the chromatograms given in Fig. 3, and allowing for the absence of an internal standard dilution, it can be seen that for HPLC–UV samples concentrations below that representing approximately 5 $\mu g\,wipe^{-1}$ (approximately 0.1 $\mu g\,ml^{-1}$ following extraction) could not be quantified. By comparison the LC–MS methodology would allow facile quantification at a concentration representing 0.5 $\mu g\,wipe^{-1}$ (approximately 0.005 $\mu g\,ml^{-1}$ following extraction and dilution with internal standard), an improvement in sensitivity of at least 10 times. In making this comparison it should be noted that, for this sensitivity gain to be routinely realised, it is important that the SIL internal standard be free from significant contamination with the unlabelled molecule since, otherwise, interference will result and limits of quantification increased proportionately.

Table 1
Extraction optimisation analysis

Time (min)	% Recovered		
	15 ml extraction solvent	50 ml extraction solvent	75 ml extraction solvent
Initial	57.8, 96.0	88.8, 107.3	102.4, 101.6
5	57.7, 86.7	106.7, 103.4	103.9, 104.3
10	84.9, 70.8	104.3, 103.4	111.1, 103.1
20	96.7, 105.9	115.9, 109.8	110.9, 104.1
30	100.6, 106.1	105.7, 103.1	102.8, 102.9

3.3. Effect of excipients

As well as the API, pharmaceutical formulations contain a variety of excipients which may be present in samples taken for

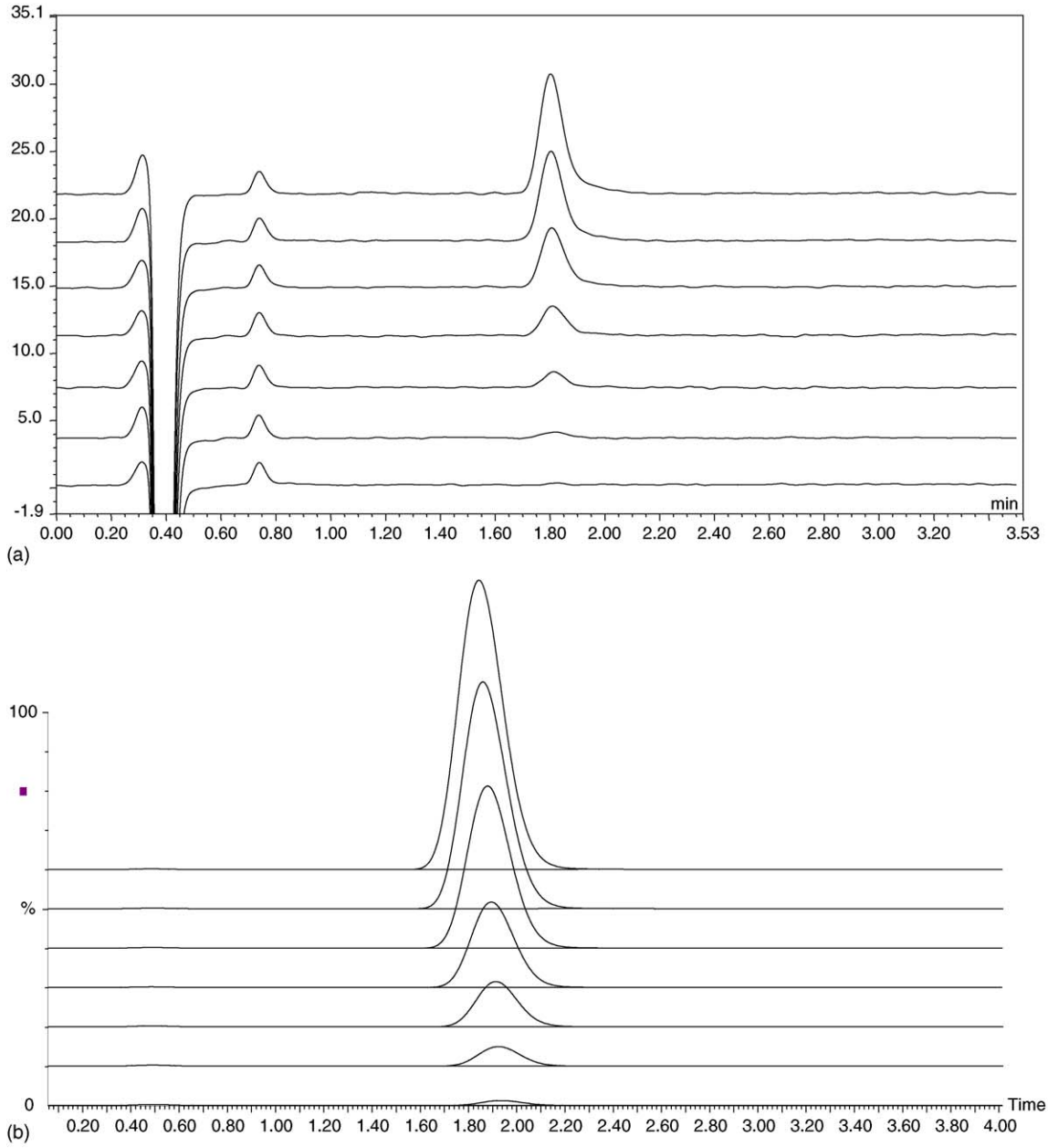

Fig. 3. Comparison of (a) UV and (b) MS responses for compound 1. In each case the chromatograms are, in ascending order, 0.01, 0.04, 0.1, 0.2, 0.4, 0.6 and 0.8 $\mu g\,ml^{-1}$.

cleaning verification. For this reason the effect of several common, solid dose form excipients on the proposed methodology were also investigated at two API levels, that used for the other experimentation, 40 $\mu g\,ml^{-1}$, and one tenth of this. Again, consistent with only establishing the feasibility of using LC–MS for the analysis of cleaning verification samples, the effect of excipients was only assessed using compound 1 as described in Section 2.4.4. The excipient residue was well in excess of that likely to be encountered during a 'real' analysis, as it was visible on the stainless steel. If this were the case during an actual cleaning operation, the equipment would be re-cleaned prior to submitting samples for analysis. However, for both API levels tested the data given in Table 2 demonstrate that the presence of excipients did not significantly effect the results obtained. Based on these data excipients were not used during experiments to validate the recovery of either compounds 1, 2 or 3 from stainless steel.

Table 2
Effect of excipients

	Sample	
	Excipients present	No excipients present
Mean % recovered 40 $\mu g\,wipe^{-1}$ sample	74.0	70.8
Mean % recovered 4 $\mu g\,wipe^{-1}$ sample	67.8	71.5

Table 3
Validation data

Parameter	Compound 1	Compound 2	Compound 3
$[M+H]^+$ (analyte)	621	383	382
$[M+H]^+$ (internal standard)	627	386	392
Specificity	Free from interference[a]	Free from interference[a]	Free from interference[a]
Accuracy[b]	70.8% (103.9%)	51.3% (97.1%)	91.2% (100.8%)
Precision of injection (RSD) (repeatability of calibration standards)	1.1%	1.1%	1.8%
Limit of quantitation (LC–MS)[c]	0.005 μg ml^{-1} 0.5 μg wipe^{-1}	0.004 μg ml^{-1} 0.4 μg wipe^{-1}	0.005 μg ml^{-1} 0.5 μg wipe^{-1}
Limit of quantitation (LC–UV)[c]	0.1 μg ml^{-1} 10 μg wipe^{-1}	0.015 μg ml^{-1} 1.5 μg wipe^{-1}	0.02 μg ml^{-1} 2 μg wipe^{-1}
Linearity[d]			
Coefficient of correlation	>0.999	>0.999	>0.999
Slope	0.00568	0.01278	0.00766
y-Intercept[e]	0.022 (3.7%)	<−0.001 (−0.4%)	<−0.001 (−0.06%)

[a] No interference from the wipe extract. Small peak, well below the limit of quantification, detected in all cases due to trace levels of unlabelled material in the SIL internal standard.
[b] Mean of duplicate results at, nominally, 40 μg wipe^{-1} following recovery of samples present on stainless steel surfaces at 0.1 μg cm^{-2}. Figures in parenthesis refer to samples taken through the procedure but not exposed to stainless steel.
[c] Taken to be the level equating to a signal to noise ratio of 10. The μg wipe^{-1} figures assume the extraction conditions described in Section 2.4.3, including the final dilution with internal standard.
[d] Six levels from 0.04 to 0.8 μg ml^{-1}.
[e] Figures in parenthesis as a percentage of the relevant 0.4 μg ml^{-1} response.

3.4. Method validation

Using LC operating parameters and the extraction conditions determined during the preceding experimentation, methods for each compound were briefly validated.

The data generated are summarised in Table 3. In all cases a precision of injection and linearity of response over the range of interest, consistent with the potential use of the methods for analysis of cleaning verification samples, were obtained. In terms of mean accuracy compounds 1 and 2 gave figures below 90%. Although these levels are acceptable for this type of analysis [12,13] in that they can be corrected for by application of an appropriate factor to the results from unknown samples, it suggests that some of each compound is not fully removed from the stainless steel surfaces used. The fact that these losses were not due to poor extraction from the wipe used or some other problem with the sample preparation process was confirmed by analysis of the samples which had not been exposed to stainless steel and which gave recoveries close to 100% for all three compounds.

Limited additional experimentation was performed for compound 2, a substituted isoquinoline, in an attempt to improve its apparent poor recovery from stainless steel since it was thought that the use of an aqueous/organic rather than wholly organic wipe solvent may have contributed to this effect but these were unsuccessful. The solvents considered acceptable for wiping pharmaceutical manufacturing equipment are limited and their selection, together with other factors may influence sample recovery from stainless steel or other surfaces [14,15]. However, given that this work was concerned principally with the means of determining the level of API residues after sampling and not improving the means by which such samples can be obtained, this particular aspect of the cleaning verification process was not explored further.

4. Conclusions

On the basis of evidence from the compounds studied, it appears that the use of LC–MS for the quantification of API residues in cleaning verification samples is feasible. Its principal advantage in this regard is that it allows the facile detection of compounds with only moderate or poor UV absorbance, a factor which can hamper their determination by the most widely used technique, HPLC–UV. This advantage when combined with a well defined approach to method development and standard means of sample extraction, another potential benefit of improved sensitivity, have the potential to improve the efficiency with which methodologies for cleaning verification campaigns can be developed and applied.

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