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October 2018
Volume 31 Number s10
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Advances in Pharmaceutical Analysis

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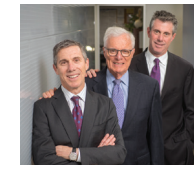

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LC GC europe
solutions for separation scientists

Published by
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Advances in Pharmaceutical Analysis

Adrian Clarke[1] and Davy Guillarme[2], [1]Novartis Technical R&D, Basel, Switzerland, [2]University of Geneva, University of Lausanne, Geneva, Switzerland

An introduction from the guest editors of this special supplement from *LCGC Europe* focusing on recent trends in pharmaceutical analysis.

The pharmaceutical industry is one of the most regulated industries worldwide because the drugs produced have to be safe and effective. To comply with the regulations and guidance of drug production and control, a wide range of analytical techniques have to be used. In this supplement from *LCGC Europe*, leading industrialists share their expertise in some of the most commonly used and promising separation-based techniques. Among them, gas chromatography (GC), two-dimensional liquid chromatography (2D-LC), supercritical fluid chromatography (SFC), and the hyphenation of chromatography with mass spectrometry (MS) can be cited. In addition, an interesting paper dealing with the use of LC–UV and LC–MS based process analytical technology (PAT) in the pharmaceutical industry is also featured.

The first article by Wenya Zhu and colleagues (from Novartis, China and Switzerland) describes a new way of using regular GC methods used for decades to determine residual solvents in pharmaceutical substances. In this work, the authors propose a LEAN approach where multiple solvents can be simultaneously determined based on predetermined relative response factors (RRF) against an internal standard with only one injection of sample solution. This allows laboratory efficiency and instrument utilization to be significantly improved (by about 60% compared to the conventional external standard-based methods).

Mass spectrometry has gained impetus in recent years and considering its potential, there is no doubt that it will be more and more widely implemented in more pharmaceutical laboratories in the coming years. Tony Bristow and Andrew Ray (AstraZeneca, UK) discuss the recent advances in MS hyphenated to chromatography and its application in pharmaceutical analysis. This includes the use of compact, easy-to-use mass spectrometers for simple applications based on well-established chromatography workflows; developments in SFC–MS; the increasing use of high resolution MS in combination with LC and GC; ion mobility-based techniques as orthogonal separation techniques; and the increasing need for MS for more demanding applications such as novel (larger) molecules and complex drug delivery systems.

Liquid chromatography is by far the most widely used separation method in the pharmaceutical R&D and quality assurance (QA) and QC laboratories. 2D-LC is a powerful approach offering novel solutions to problems ranging from complex samples requiring excessively large peak capacity to simple, yet difficult to resolve compounds. In his contribution, C.J. Venkatramani (Genentech, USA) illustrates the potential of 2D-LC in the modern-day pharmaceutical industry to address real problems covering a wide range of applications from coelution, to peak purity assessment, to simultaneous achiral-chiral analysis, to genotoxic impurities, and more.

Doug Richardson (Merck, USA) and Todd Maloney (Eli Lilly, USA) highlight the practical applications of online chromatography (also known as PAT) in pharmaceutical and biopharmaceutical process development and manufacturing. The increasing importance of PAT (using chromatographic approaches and hyphenated techniques) in pharmaceutical industry is emphasized and the current status and recent developments are illustrated through various industrial applications, including control in regulated continuous manufacturing.

Despite the fact that SFC was first introduced in 1962 by Klesper, it is still considered as a niche technique, mostly used for chiral separations and preparative scale applications in the pharmaceutical industry. However, the technique has recently seen a real metamorphosis and renewed interest for analytical achiral applications in the pharmaceutical analysis community. The contribution prepared by Claudio Brunelli (Pfizer, UK) describes the key achievements and strengths of modern SFC in the pharmaceutical industry. A particular focus is dedicated to the implementation of SFC in regulated quality control (QC) laboratories, including the state of the modern instrumentation.

As guest editors of this special issue, we would like to warmly acknowledge all authors for their excellent job, and we hope that these contributions will be of interest to the *LCGC Europe* readers.

Davy Guillarme

Adrian Clarke

A LEAN Approach for the Determination of Residual Solvents Using Headspace Gas Chromatography with Relative Response Factors

Wenya Zhu[1], Guoyi Liang[1], Wenlong Qiu[1], Adrian Clarke[2], Christoph Kolarik[2], and Sebastiano Mozzo[1], [1]Chemical & Analytical Development, Suzhou Novartis Pharma Technology Company Limited, Changshu, Jiangsu, China, [2]Chemical & Analytical Development, Novartis Pharma AG, Basel, Switzerland

This study demonstrates a new LEAN approach and method, where 25 solvents can be simultaneously determined based on predetermined relative response factors (RRFs) against an internal standard (decane) with only one injection of sample solution. The RRF average value of each solvent was determined by comparison of slope against internal standard from linearity experiments in the range of 10–200% and the response factor against internal standard at the ICH Q3C limit based on nominal sample concentration. Validation was performed successfully covering specificity, linearity, sensitivity, precision, accuracy, stability, and robustness. Laboratory efficiency and instrument utilization rates were significantly improved, leading to time and cost savings of more than 60% compared to a conventional external standard headspace gas chromatography (HS-GC) method since this method was implemented in 2014. It has been demonstrated that this generic RRF-based HS-GC method is a LEAN approach for residual solvents testing in pharmaceutical substances, from process controls to quality control (QC) analysis.

Organic solvents are routinely used in the synthesis and manufacture of drug substances, excipients, and drug products. They can also be formed as by-products or side reactions during the manufacturing process. Their use or presence is therefore an inherent part of the pharmaceutical manufacturing process. However, as residual solvents do not provide any therapeutic benefits, can potentially impact the physicochemical properties of pharmaceutical substances, and can even pose a potential safety risk to the patient, they should be controlled as much as possible to meet ingredient and product specifications, good manufacturing practices, or other quality-based requirements (for example, quality guidelines ICH Q3C (R6) [1] and *USP* <467> Residual Solvents [2]), which regulate the solvent's classification and control limits based on the toxicity.

Specific methods (nuclear magnetic resonance [NMR], gas chromatography [GC]) and nonspecific methods (loss on drying [LOD] and thermal gravimetric analysis [TGA]) are often used to determine the solvent residues based on the intended use in the pharmaceutical industry. Among all the methods, headspace (HS)-GC–flame ionization detection (FID) has been the most popular instrumental setup in the pharmaceutical industry because of its high sensitivity and low matrix effects. Generic and fast methods were developed and published from various suppliers and laboratories, which readily facilitated the determination of residual solvents in pharmaceutical substances (2–8).

In the traditional approach, residual solvents are determined by external standard methods using reference standard(s) containing the solvents of interest. This approach is selective and accurate; however, as the value stream map of traditional residual solvents determination using external standards shows (Figure 1), it requires frequent sample and standard preparation. For example, when considering one sample with three interested solvents, the net working time is about 90 min (run and data analysis excluded). Therefore, it is not the preferred solution for the early phase R&D laboratory because of the dynamic needs and short time demands.

Scientists from AstraZeneca R&D developed an intelligent GC–FID (direct injection) relative response factor (RRF) method where predetermined RRFs were used to quantify the solvents of interest (3). It significantly improved the laboratory efficiency; however, the application was not further disclosed if it was routinely used in a regulated environment (for example, GMP) and there is the potential risk of carryover and matrix effects for sample

Figure 1: Value stream map of traditional residual solvents determination. Areas highlighted in red are identified as wasteful activities; areas highlighted in orange are identified as business value activities; areas highlighted in green are identified as value adding activities.

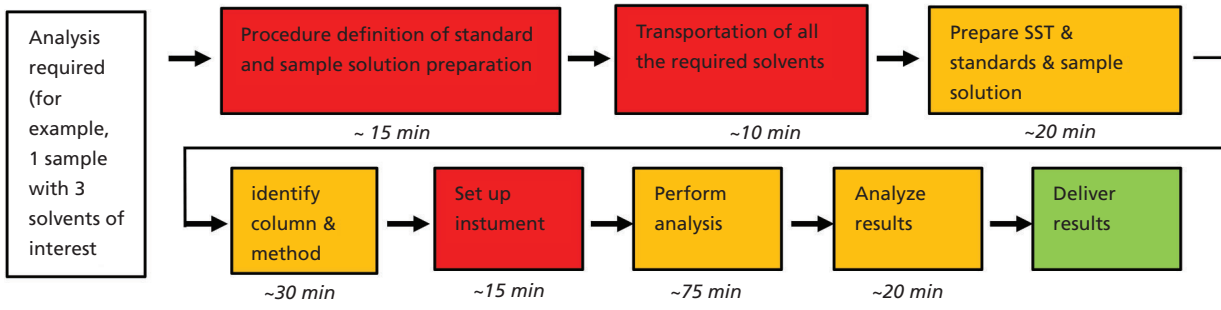
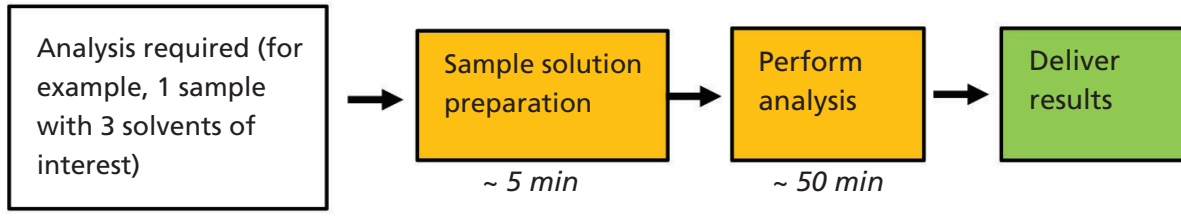


Figure 2: Value stream map of residual solvents determination with HS-GC RRF method. Areas highlighted in orange are identified as business value activities; areas highlighted in green are identified as value adding activities.



analysis, and also lower column robustness and fouling because direct injection was used for this method.

Headspace GC is considered a more suitable technology for the determination of residual solvents in nonvolatile materials, such as drug substances and drug products because only volatile components are introduced into the GC system, potential contamination to the system can be reduced, and therefore robustness can be enhanced (4).

A new generic HS-GC RRF method was developed and further validated under GMP in our R&D laboratories. The process flow is shown in Figure 2. One sample with three interested solvents was again considered; the net working time is only about 5 min (run time and data analysis excluded), which meant that more than 75% of the sample and standard preparation time was saved compared with the traditional method. The study results demonstrated that the method is a fast and economical approach for residual solvents testing from in-process monitoring and controls to end product quality checks. The method is designed to be suitable for the determination of residues in a range of common processing solvents, including newer processing solvents used in "green chemistry" initiatives.

Experimental

Reagents and Chemicals: The solvents were analytical grade or above and purchased from commercial resources.

Instrumentation: An Agilent 7890A GC equipped with a flame ionization detector and a G1888 Head Space sampler was used. GC-HS system control, data acquisition, and processing were all accomplished by Chromeleon software (Thermo Fisher Scientific). The GC column employed was a 30 m × 0.32 mm,1.8-µm Agilent J&W DB-624 (6% cyanopropylphenyl and 94% dimethyl polysiloxane) fused silica capillary column. Headspace sample vials of 20-mL size were utilized with a Teflon-lined septum and an aluminium crimp cap (Agilent).

Final Method Operating Conditions:

GC Conditions:

Inlet temperature: 200 °C; injector mode: split; split ratio: 20:1; carrier gas: helium (or hydrogen). If H_2 is used as carrier gas, the retention times are slightly shifted forwards in comparison to He; flow: 2.0 mL/min; mode: constant flow; vial pressurize: 14.0 psi; oven temperature program: 50 °C hold for 3 min, ramped to 80 °C with 5 °C/min, and finalize to 230 °C with rate 30 °C/min, hold for 2 min; detector: FID; detector temperature: 300 °C; H_2 flow: 40 mL/min; air flow: 400 mL/min; make up flow: 30 mL/min.

Headspace Parameters:

Oven temperature: 120 °C; loop temperature: 130 °C; transfer line temperature: 135 °C; vial equilibration time: 10 min; mixing speed: low; pressurization time: 0.5 min; loop fill time: 0.25 min; loop equilibration time: 0.1 min; injection time: 1.0 min; cycle time: 23 min; headspace vial: 20 mL.

Solution Preparations: Solution for GC analysis, 1 mL of solution in 20 mL headspace vial.

Internal Standard Solution:
The internal standard solution (decane in N-Methyl-2-pyrrolidone [NMP]) was accurately prepared at about 0.05 mg/mL by diluting decane with NMP.
Sample Solutions:
Approximately 50 mg of sample was dissolved by adding 1 mL of internal standard solution into a 20-mL headspace vial, sealed with the cap and ready for injection.
Reference Solution (= Specificity Solution):
Each solvent was accurately prepared at the level equivalent to the appropriate ICH Q3C limit by dilution with internal standard solution based on nominal sample concentration 50 mg/mL. Taking ethanol as an example, where the ICH Q3C limit is 5000 ppm, the appropriate concentration for ethanol to be prepared is 0.25 mg/mL (= 5000 ppm*50 mg/mL/10^6). For methylisobutyl ketone (MIBK), the draft limit of 2250 ppm from the ICH residual solvents Q3C(R6) guideline, step 2 (for consultation and discussion) was used to define the limit. The finalized revision (ICH Q3C(R6) Current Step 4 version dated 20 October 2016) moved MIBK from Class III to Class II solvent with a limit of 4500 ppm (PDE: 45 mg/day) (1).
Reporting Limit (RL) Solution (= 10% of Reference Solution):
The reporting limit solution was prepared by 10-fold dilution of the reference solution with internal standard solution.
System Suitability Test (SST) Solution:
A mixed SST solution containing reporting level of methanol, 2-butanone, ethyl acetate, toluene, decane, and 1, 2-dimethoxyethane at 20% of reference solution was prepared.
Determination of Relative Response Factor (RRF) and Calculation:
A mixture of 25 reference solvents and decane was prepared for RRF determination in NMP. The concentration of each solvent was calculated according to the individual concentration limit in ICH Q3C (step 2) based on a nominal sample concentration of 50 mg/mL. There were two approaches used for the RRF determination and average value was used for calculation. First, RRF1 determination, the RRF of each solvent to decane (internal standard) was determined by comparison of slope from linearity experiments, with solvents in the range of 10% to 200% of the ICH Q3C limit (for example, 0.025 mg/mL to 0.5 mg/mL for ethanol) and decane at 0.01% to 0.2% (m/m) (equation 1, RRF1). Second, RRF2 determination, the relative response factor was determined by comparison of response factor of each solvent to decane from six injections of reference solution at a single concentration (equations 2 and 3), average RRF (equation 4) was calculated for residual solvents determination (equation 6).

$$RRF1 = S_{solvent}/S_{IS} \quad [1]$$

$$RF_{solvent} = A_{solvent}/C_{solvent} \quad [2]$$

$$RRF2 = RF_{solvent}/RF_{IS} \quad [3]$$

Where, $S_{solvent}$ = slope of regression equation from validation for each solvent; S_{IS} = slope of regression equation from validation for decane; $A_{solvent}$ = average peak area of solvent from GC chromatogram (n = 6), pA; $C_{solvent}$ = concentration of solvent, mg/mL; $RF_{solvent}$ = response factor of solvent, pA/(mg/mL); RF_{IS} = response factor of internal standard (decane), pA/(mg/mL).

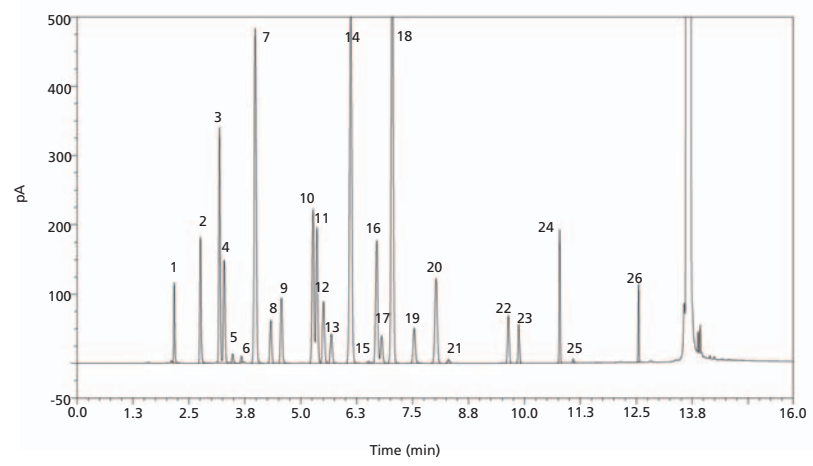
$$RRF = (RRF1+RRF2)/2 \quad [4]$$

$$\text{Concordance (RRF agreement): } = RRF2/RRF1*100\% \quad [5]$$

$$C_{solvent}(ppm) = (As \times C_{decane})/(A_{decane} \times Cs \times RRF) \times 10^6 \quad [6]$$

Where, $C_{solvent}$ = amount of solvent in sample, ppm; A_s = peak area of solvent in sample solution, pA; C_s = concentration of sample solution, mg/mL; A_{decane} = peak area in sample solution, pA; C_{decane} = concentration of decane in solvent, mg/mL; C_s = sample concentration, mg/mL.

Figure 3: Typical chromatogram of specificity solution. 1: methanol; 2: ethanol; 3: acetone; 4: 2-propanol; 5: acetonitrile; 6: dichloromethane; 7: TBME; 8: *n*-hexane; 9: *n*-propanol; 10: 2-butanone; 11: ethyl acetate; 12: 2-butanol; 13: tetrahydrofuran; 14: cyclohexane; 15: 1,2-dimethoxyethane; 16: isopropyl acetate; 17: 2-Me-THF; 18: *n*-heptane; 19: *n*-butanol; 20: methyl cyclohexane; 21: 1,4-dioxane; 22: MIBK; 23: toluene; 24: *n*-butyl acetate; 25: DMF; 26: decane (internal standard).



Results and Discussion

Method Development and Proof of Concept: The idea was to develop a fast method to address business needs focused on speed and efficiency. RRF-based approaches fit well for this purpose: 1) predetermine the relative response factor of each solvent to an internal standard; 2) dissolve sample in internal standard solution; 3) perform GC analysis (≥ 1 injection of sample) and results calculation based on RRF against the internal standard. With this concept, the method was developed.
Internal Standard Selection:
In principle, a good internal standard

Table 1: RRF determination of 25 solvents against decane from the linearity and individual reference standard experiments

Solvent	RRF 1 (Linearity, n = 7)	RRF 2 (Reference Solution, n = 6)	Average RRF Value	Concordance (RRF2/RRF1, %)
Methanol	0.4507	0.4406	0.446	98%
Ethanol	0.5364	0.5302	0.533	99%
Acetone	1.1231	1.1011	1.112	98%
2-Propanol	0.5647	0.5579	0.561	99%
Acetonitrile	0.5597	0.5679	0.564	101%
Dichloromethane	0.2517	0.2319	0.242	92%
TBME	2.3473	2.2507	2.299	96%
n-Hexane	4.3908	3.9873	4.189	91%
n-Propanol	0.4257	0.4182	0.422	98%
2-Butanone	0.9132	0.9042	0.909	99%
Ethyl acetate	0.7969	0.7939	0.795	100%
2-Butanol	0.4276	0.4310	0.429	101%
Tetrahydrofuran	1.2599	1.2463	1.253	99%
Cyclohexane	3.3819	3.2600	3.321	96%
1,2-Dimethoxyethane	0.5786	0.5610	0.570	97%
Isopropyl acetate	0.8503	0.8446	0.847	99%
2-Me-THF	1.2855	1.2707	1.278	99%
n-Heptane	3.4479	3.3357	3.392	97%
n-Butanol	0.3049	0.3020	0.303	99%
Methyl cyclohexane	2.7213	2.6523	2.687	97%
1,4-Dioxane	0.3449	0.3342	0.340	97%
MIBK	0.6168	0.6120	0.614	99%
Toluene	0.9956	0.9851	0.990	99%
n-Butyl acetate	0.4613	0.4603	0.461	100%
DMF	0.1077	0.1036	0.106	96%
Decane	1.0000	1.0000	1.000	100%

RRF1 was determined from comparison of slope between individual solvent and decane from linearity (n = 7) in the range of 10% to 200% of the ICH Q3C limit based on normal sample concentration 50 mg/mL. RRF2 was determined from comparison of response factor between individual solvent and decane at the concentration of ICH Q3C limit (100% level) based on normal sample concentration 50 mg/mL, refer to "Solution Preparations".

for HS-GC measurement should have the following properties: good response and peak shape, no significant carryover, stable and chemically inert, pure, relatively inexpensive, commercially available, minimal chance to overlap with the target analytes, and should also not be potentially present in the samples of interest. Decane and toluene were assessed during method development and finally toluene was ruled out because it was often used in process development and manufacturing processes and could be potentially present in the samples of interest. Decane was selected as it meets all these criteria and elutes later in the chromatogram (Figure 3). It is well separated from all the solvents of interest, is not commonly used as a processing or manufacturing solvent, and perfectly fits as an internal standard for the purpose of residual solvents determination.

As decane is a high boiling solvent, the potential carryover was examined by repeated injections of the decane internal standard solution (0.05 mg/mL decane in NMP) and blank solvent (NMP). The results show that no significant residue was found in the blank solution following the repeated injection of internal standard solutions and this confirmed decane as a good option as internal standard. Furthermore, this low carryover was later demonstrated and confirmed during routine sample testing.

Diluent and Solvents Selection: A good diluent (sample solvent) for residual solvents determination is expected to have the following properties: good solubility for the samples, pure, well separated with the solvents of interest, stable and chemically inert and not susceptible to degradation under the operating conditions, relatively inexpensive, and readily commercially available. Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and NMP were

Table 2: Method validation results for resolution, reporting limit, linearity, and precision

Solvent	Resolution	Reporting Limit			Linearity		Precision	
	Rs	Level (ppm)	*S/N* Ratio	r	*y*-Intercept (%)	Residual Standard Deviation (%)	RSD% at Reference Solution Level	RSD% at Reporting Limit Level
Methanol	12.3	300	617	1.0000	-0.59	0.31	1.0	0.90
Ethanol	7.6	500	1047	1.0000	0.20	0.51	1.1	1.34
Acetone	1.8	500	1910	1.0000	-0.65	0.52	0.97	0.75
2-Propanol	3.0	500	923	0.9998	-0.96	1.4	1.2	1.1
Acetonitrile	3.2	41	106	0.9999	4.8	0.87	0.73	3.5
Dichloromethane	4.3	60	52	0.9980	-8.5	4.4	0.88	3.6
TBME	4.4	500	2848	0.9999	-0.89	0.75	1.19	1.5
n-Hexane	3.3	29	340	0.9994	-3.7	2.5	1.99	2.8
n-Propanol	9.3	500	600	1.0000	0.66	0.70	1.5	2.2
2-Butanone	1.1	500	1346	1.0000	0.47	0.51	1.0	0.62
Ethyl acetate	1.9	500	1160	0.9999	1.2	0.71	0.95	0.59
2-Butanol	2.1	500	589	0.9998	2.1	1.4	1.6	2.5
Tetrahydrofuran	5.0	72	253	1.0000	1.2	0.63	1.2	1.5
Cyclohexane	4.6	388	3005	0.9999	-0.60	0.73	1.2	1.4
1,2-Dimethoxyethane	2.1	10	15	0.9999	-1.3	0.92	2.4	6.0
Isopropyl acetate	1.3	500	1086	1.0000	0.70	0.55	1.0	0.60
2-Me-THF	2.7	72	240	1.0000	0.34	0.35	1.0	0.57
n-Heptane	5.6	500	4375	1.0000	-0.05	0.53	1.1	1.4
n-Butanol	5.1	500	372	0.9999	0.27	0.76	1.9	2.8
Methyl cyclohexane	3.0	118	722	1.0000	0.21	0.27	1.0	0.91
1,4-Dioxane	16.6	38	15	0.9999	1.3	0.71	1.2	1.4
MIBK	3.8	500	225	0.9999	0.76	0.76	1.4	1.2
Toluene	18.3	89	172	1.0000	0.81	0.63	1.3	1.5
n-Butyl acetate	7.4	500	604	0.9999	1.1	0.97	1.5	1.1
DMF	40.2	88	24	0.9994	0.03	2.5	2.8	9.7
Decane	12.3	100	357	0.9998	2.3	1.3	1.4	1.7

Reporting level (ppm) based on nominal sample concentration (50 mg/mL)

investigated as potential diluents. It was found that all three diluents could provide similar solubility, but NMP could accommodate more solvents for analysis because it elutes after DMF and DMSO in the developed GC conditions. In addition, given the fact that impurities in DMF and DMSO were interfering with decane (internal standard), NMP exhibited a "cleaner" chromatogram and was finally selected as the diluent. The most commonly used solvents in process development were identified prior to developing the analytical method. In total, 25 solvents (classes 2, 3, and some unclassified solvents) were in scope. Since class 1 solvents are highly carcinogenic or toxic, they are generally avoided in pharmaceutical manufacturing and are therefore not in the scope of the method requirements.

Headspace and GC Operating Conditions: Selection and Development:

GC method operating conditions were developed based on the well-established and available internal generic method and key parameters, for example, the oven temperature gradient was adjusted by evaluating the resolution of specificity solution, sensitivity of RL solution, and total analysis time. As shown in Figure 3, all the solvents were well separated within 16 min, the final optimized conditions are shown above.

The headspace conditions were developed by considering the following points: ensure elution of DMF, which is the highest boiling solvent of interest; have a good response for all the solvents of interest; and avoid potential carryover during analysis. The finalized conditions are shown in the experimental section. The selected HS-GC operating parameters are within the typical GC parameters for a generic separation of residual solvents reported in previous publications (5).

Determination of RRF Value and Proof of Concept:

After the method operating conditions were established, the RRF was

determined by two approaches (for calculation formula refer to *Determination of Relative Response Factor [RRF] and Calculation)* and the average value was used as a working RRF in the final method. The concordance between the RRF values determined by the two approaches was acceptable for all solvents of interest. The concordance is generally higher for class 3 solvents where precision is better owing to the higher concentration ranges used for the linearity and reference solution. Conversely, the RRF concordance is generally lower when the precision is lower, for example, for non-class 3 solvents where concentration levels and reporting levels are lower and solvents such as DMF, DCM show the lowest detector response. This relationship of RRF concordance to precision is also supported by the results shown in Table 1.

Since the RRF is the key to method accuracy, the RRF value was investigated in a different laboratory on a second instrument (same brand and model) during method development, validation, and transfer, and consistency between each analysis was shown.

With the established method and RRF value, several laboratory samples with known content of solvent residue calculated by external standard methods were also analyzed and consistent results were obtained. Therefore the concept and suitability of the RRF-based method was proven.

System Suitability Test:

To ensure the delivery of accurate, reliable, and consistent results, the suitability of the HS-GC system should be checked periodically, or even in each analysis sequence, for example, for a GMP analysis. Considering the ultimate goal of GMP application, the worst-case scenario was taken to assess the system suitability. A mixture of solvents including methanol, 2-butanone, ethyl acetate, toluene, and decane at reporting limit levels and 1, 2-dimethoxyethane at double the reporting limit (named SST solution) was proposed to check the system performance. The selected solvents were located at early, middle, and late positions (time windows) in the chromatogram and are good indicators to confirm the method specificity and retention times. 2-butanone and ethyl acetate represent the worst separation and most critical resolution (resolution 1.1, criteria >1.0) and 1, 2-dimethoxyethane (signal-to-noise ratio [*S/N*] 15, criteria >10) shows the lowest response and is an effective measure of sensitivity.

To confirm the absence of any potential interference from the sample, the variation between the decane peak area in the internal standard in blank solution (NMP) and sample solution was also proposed as a system suitability test. The acceptance criteria was proposed as variation < 10%, considering the variability from sample preparation and instrument capability.

Method Validation: The method was validated according to the ICH Q2 (R1) guideline by evaluating the specificity, reporting limit (sensitivity), precision, linearity, accuracy, stability, and robustness.

Specificity:

The specificity of this method was evaluated by injecting a solution containing all the solvents of interest at the ICH Q3C limit based on nominal sample concentration 50 mg/mL (named reference solution). As shown in Figure 3, all the solvents of interest were sufficiently separated from each other, and their identity was confirmed by injection of individual solutions. The developed method was found to be sufficiently selective.

Reporting Limit and Limit of Quantification (LOQ):

Limit of quantification (LOQ) is defined as the lowest amount of analyte in the sample that can be quantitatively determined. The reporting limit is equal or greater than LOQ and was confirmed by the *S/N* of each solvent in RL solution (10% concentration of reference solution). The results are shown in Table 2, and all *S/N* ratios of each solvent at the RL level are ≥ 10. The reporting limit of all solvents was defined at 10% of the individual concentration limit based on a nominal sample concentration 50 mg/mL.

Linearity:

The linearity of peak response versus concentration was studied from 10% to 200% of reference solution at seven concentration levels (10%, 30%, 60%, 80%, 100%, 120% ,and 200%). The linearity of decane (internal standard) was studied from 0.01% to 0.2% (m/m). A simple linear regression analysis by the least squares was applied for each solvent. The value of correlation coefficient (r), *y*-intercept (in % relative to the calculated response at reference solution), and residual standard deviation (RSD, relative to the concentration of each solvent in reference solution) were evaluated and criteria were set as $r \geq 0.98$, *y*-intercept ≤25%, and RSD ≤ 10%, respectively. As shown in Table 2, all the linearity parameters meet the requirements and good linearity for each solvent was demonstrated. Slope (a) of the regression line was used for RRF1 calculation.

Precision:

Method precision was assessed by the RSD of the peak area on six injections of reference solution (at relevant ICHQ3C limits) and reporting limit solution (approximately 10% of relevant ICHQ3C limits). Six replicate measurements were performed and RSD% of the area was calculated. For reference solution and RL solution, RSD of the peak area for each solvent was in the range of 0.7–2.8% for the reference solution and 0.6–9.7% at the reporting limit solution (Table 2). The precision is the lowest for non-class 3 solvents, where levels and reporting levels are lower, in particular for those that show the lowest detector response (for example, DMF, 1,2-Dimethoxyethane, Dichloromethane). The resolution was calculated against the closest peak.

Solution Stability

In this method, methanol was the first peak eluted, 2-butanone and ethyl acetate was the worst case for resolution, and 1, 2-dimethoxyethane presented the lowest *S/N* ratio. A mixture solution of these six solvents (SST solution) was prepared for system check and its stability was estimated (mixture at reference level) together with internal standard solution in well-sealed glass both at room temperature and in the refrigerator. The concentration of the solvent was checked by a freshly prepared solution employed

as the external standard.
The difference between the concentrations of the stored and fresh solution was calculated for review and assessment of the stability. The internal standard solution and SST mixture solution were stable for at least 200 days both at room temperature and under 2–8 °C in the refrigerator.
Accuracy:
The accuracy of the RRF method was demonstrated by testing samples where the solvent content is known by determination of external standard methods. In total, 20 laboratory samples containing different residual solvents in the validated linearity range were analyzed and the variation (relative difference) for all the tested solvents was below 10% with no matrix effects observed. The SST check is also evidence of accuracy in a sense since the recovery of internal standard (peak area of decane between blank and sample) can be obtained during carryover check.
Robustness:
The robustness of the method was evaluated by the experiment design with five centre changes of initial column temperature, ramp rate, flow rate, injection time, and equilibration time. The RRF value, resolution, and peak asymmetry were evaluated. The results showed that the method is sufficiently robust and there is no significant variation in the results in the operating ranges assessed.

In addition, the influence of the carrier gas and makeup gas type was evaluated in different laboratories using identical equipment and method conditions during method development. Hydrogen (H_2), helium (He), and nitrogen (N_2) were combined as carrier gas and makeup gas in three groups. The concordance of RRF value was checked, and deviation was within 10%. As demonstrated from the experiment, the influence of carrier gas and makeup gas was very limited on the RRF value and an easy method transfer between laboratories using different carrier gases could be foreseen.

Application and Advantages: The developed method was firstly applied in a non-GMP laboratory for process supporting and finally implemented in a GMP laboratory for in-process monitoring and controls for QC analyses after method validation. In 2017, about 400 samples were analyzed in a non-GMP laboratory using this method with dedicated HS-GC equipment (same brand and model). Based on a 60-min saving for one sample (as compared in Figure 1 and Figure 2), about 50 working days were saved last year. In addition, with the HS-GC RRF method, only 5% of the sample diluent (NMP) was consumed compared with the previous year using traditional external standard method. This method is available for simultaneous determination of 25 residual solvents using just one sample injection, which means it is also a good choice to identify unexpected peaks and determine the content without extra operation.

Conclusions

A simple, efficient, and robust method based on HS-GC for the simultaneous determination of 25 residual solvents has been developed and implemented in routine laboratories. It is sufficiently specific, linear, accurate, precise, sensitive, robust, and suitable as a quick and economic approach for the determination of residual solvents in a range of pharmaceutical substances, from process controls to product quality checks.

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Extractables and Leachables Analysis of IV Bag Systems Based on Thermal Extraction of Materials and Stir-Bar Sorptive Extraction of Aqueous Solutions Using GC–MS and GC–TOF-MS

GERSTEL

Solutions in IV bags are passed directly into the veins of the patient in significant amounts making extractables and leachables studies of IV bags especially critical. In this work, IV bag components were analyzed for extractables using direct thermal desorption/ thermal extraction combined with gas chromatography–mass spectrometry (GC–MS). The results were compared to those obtained for leachables by stir-bar sorptive extraction (SBSE) of an aqueous simulant stored in the exact same type of IV bag. A high resolution GC–time of flight (TOF) mass spectrometer was used to verify or disprove tentative identifications. SBSE is a solvent-free technique commonly used to extract and concentrate analytes from aqueous samples: Analytes are absorbed in the PDMS phase, desorbed by thermal desorption, concentrated in a cryogenic trap, and finally transferred quantitatively to the GC column. Significant concentration factors can be achieved using SBSE–TD–GC–MS, combining high recoveries with very large sample volumes. This means that SBSE can provide extraordinarily low limits of detection when analyzing aqueous samples.

Materials and Instrumentation:

Empty and sterile, 250 mL capacity IV bags were used for analysis. The bags were made from polypropylene, but, as indicated on the outer (secondary) packaging: "some product components contain DEHP-plasticized PVC". Analysis of IV bag components was performed on a 7890B GC coupled with a 5977A MSD (Agilent Technologies), equipped with a PTV Inlet (CIS 4), Thermal Desorption Unit (TDU), and MultiPurpose Sampler (MPS) (all from GERSTEL).

Direct Thermal Desorption (Extraction) of IV Bag Components:

Sample Preparation:

Small sample pieces (between 3 and 15 mg) were taken from the IV bag at the positions displayed in Figure 1. The samples were placed in individual, preconditioned TDU tubes for subsequent thermal extraction at 80, 140, and 200 °C.

Stir-Bar Sorptive Extraction (SBSE) of an Aqueous Simulant:

Sample Preparation:

A 250 mL volume of deionized water was filled into an empty IV bag and stored for 48 h at 40 °C to enable compounds to leach into the aqueous simulant. A 10 mL aliquot of the water was transferred to a vial, a Twister stir bar was added, and the sample extracted for 60 min. The stir bar was removed and placed in a sealed conditioned thermal desorption tube for analysis.

Results and Discussion

Direct Thermal Desorption (Extraction) of IV Bag Components: As a result of the large amount of DEHP in the IV tubing material, the thermal extraction temperature was limited to 140 °C with a 1:30 split. The resulting chromatogram is shown in Figure 2 and the shown annotated peaks are identified in Table 1.

The presence of cyclohexanone and 2-ethylhexanol in the chromatogram seems very plausible: The IV tubing is made of PVC containing large amounts of DEHP plasticizer. Cyclohexanone is a solvent often used in PVC production and 2-ethyl hexanol could be residual reagent from the DEHP production or a degradation product.

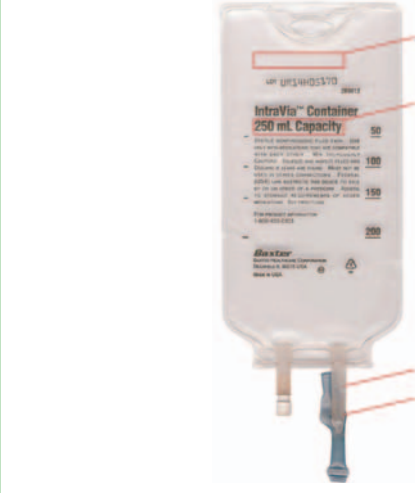

Figure 1: IV bag sampling spots: IV bag sample (A), IV bag sample with imprint (B), tubing (C), and valve (D).

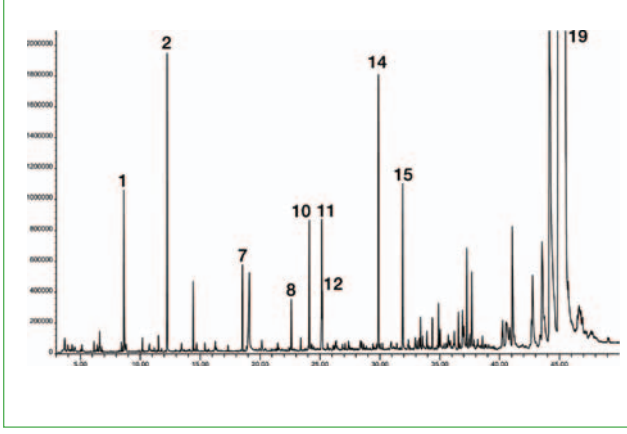

Figure 2: Thermal extraction of 3.7 mg IV tubing (PVC) at 140 °C, split 1:30. Compounds are listed in Table 1.

Table 1: Compounds identified following direct thermal desorption of IV bag samples and stir-bar sorptive extraction of an aqueous simulant placed in an IV bag listed along with the IV bag component source and possible origin.

No.	Compound	Main Source
1	Cyclohexanone	IV tubing
2	2-Ethyl hexanol	plastic valve
3	Nonanal	
4	Nonanol	
5	2-tert-Butyl-1,4-benzoquinone	IV bag
6	Benzothiazole	Vulcanization agent
7	1,3-di-tert-Butyl benzene	IV bag
8	Diphenyl ether	
9	Butylated Hydroxyanisole BHA	IV bag
10	2,6-di-tert-Butyl-p-benzoquinone	IV bag
11	Butylated Hydroxytoluene BHT	plastic valve
12	2,4-di-tert-Butyl phenol	IV bag
13	Diethyl phthalate	plastic valve
14	2-Ethylhexyl benzoate	plastic valve, IV tubing
15	2-Ethylhexyl salicylate	
16	3,5-di-tert-butyl-4-hydroxybenzaldehyde	IV bag
17	Isobutyl phthalate	IV bag
18	7,9-di-tert-butyl-1-oxaspiro-[4.5] deca-6,9-diene-2,8-dione	IV bag
19	DEHP	plastic valve, IV tubing

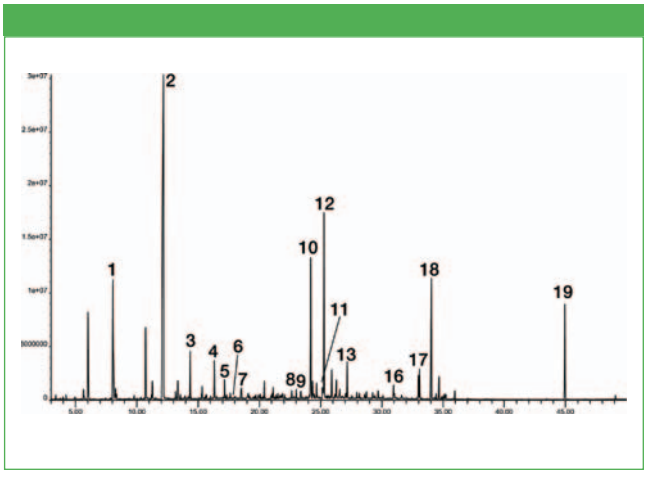

Figure 3: Stir-bar sorptive extraction of a 10 mL aliquot of a 250 mL deionized water sample stored for 48 h at 40 °C in an IV bag, splitless sample introduction. For compound list, see Table 1.

Stir-Bar Sorptive Extraction (SBSE) of an Aqueous Simulant: A GERSTEL Twister was added to a 10 mL aliquot of the water sample from the leachables simulation experiment described previously. In parallel, a Twister extraction of a 10 mL blank water sample was performed followed by thermal desorption–GC–MS. The resulting chromatogram of the blank showed some siloxane background from the Twister PDMS coating, but no significant traces of other organic compounds.

Using SBSE, a couple of compounds could be identified that were also found in the previously mentioned thermal extraction experiments performed on the packaging material. Additional compounds were detected that had not been detected as extractables, possibly as a result of their presence at very low concentrations. Some compounds, among them benzothiazole (confirmed) and N,N-dibutyl formamide (not confirmed), were verified using TOF-MS. The compounds are listed in Table 1.

Conclusions

Thermal desorption of packaging components followed by Twister analysis of aqueous simulants provides a simple, efficient, and highly sensitive means of creating a comprehensive target list for leachables experiments including semivolatile organic compounds (SVOCs). Packaging component sources of extractable compounds were identified. The usefulness of accurate mass GC–QTOF-MS to confirm or exclude the identity of compounds in extractables data was demonstrated. The data presented here were generated for demonstration of concept. In order to comply with FDA guidelines, additional replicate measurements need to be performed as well as quantitative or semi-quantitative estimates of the extractables.

Reference

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Recent Advances in Hyphenated Chromatography and Mass Spectrometry Techniques and Their Impact on Late-Stage Pharmaceutical Development

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This article reviews the changing role of mass spectrometry (MS) hyphenated to reversed-phase liquid chromatography (LC) and alternative separation techniques in late-stage pharmaceutical development. The impact of the changing portfolios within the pharmaceutical industry is discussed as the industry moves from a traditional small-molecule model to a more diverse portfolio. A new generation of high-resolution mass spectrometers and ion mobility mass spectrometers operating as orthogonal separation techniques has greatly increased the ability to resolve impurities and increase the level of knowledge gained from a single experiment. The continued impact and innovation of gas chromatography–mass spectrometry (GC–MS) in late-stage development is also discussed.

The introduction of small, compact mass spectrometers has widened the potential uses for this technique (1) These mass spectrometers may be considered as cheaper options for open access systems, and are used as supplementary and complementary detectors to UV for peak tracking and forced degradation studies, or as quantitative detectors for potentially mutagenic impurities, or for analytes without chromophores. The use of mass spectrometry (MS) to confirm the identity of an impurity during (accelerated) stability analysis and route development activities gives the analyst greater confidence in the data and potentially highlights issues earlier than when using UV detection alone (for example, for the identification of coeluting peaks). The smaller size of these systems makes it much easier to take the mass spectrometer to the sample, for example, for on-line reaction monitoring (2); this has enabled self-optimizing routines to be used where the mass spectrometer is identifying when optimum conditions are reached (2,3)

Recent years have seen an increase in the use of different separation techniques, moving from traditional reversed-phase high performance liquid chromatography (HPLC) and gas chromatography (GC) to ultrahigh-pressure liquid chromatography (UHPLC) with shorter run times, hydrophillic interaction liquid chromatography (HILIC), supercritical fluid chromatography (SFC), and ion chromatography (IC). These can be a challenge to the mass spectrometer as a result of the need for faster scan speeds or issues with interfacing. In SFC–MS, the pressure reduces as the eluent leaves the column, the CO_2 can potentially boil off, and analytes can potentially precipitate. To overcome these challenges, the eluent flow can be split before the back-pressure regulator, or the eluent can be mixed with a solvent miscible with CO_2. The use of a back-pressure regulator alone can compromise the chromatographic integrity (4). SFC–MS has been shown to be applicable to a wide range of pharmaceutical compounds (5), including analysis from dosage forms (6), for chiral analysis (7), and preparative chromatography (8). SFC–MS has also been operated as an open access system in support of an academic MS facility (9). Capillary electrophoresis (CE)–MS has also been shown to have advantages in some instances (10).

The range and capability of mass analyzers available has continued to evolve. An increased number of these systems are capable of high mass resolution; as resolution increases, the mass accuracy and specificity increases such that it becomes easier to make structural assignments. The high resolution also offers an alternative to more traditional MS/MS experiments for quantitative analysis, where the specificity is gained by removing nominally isobaric impurities through mass resolution rather than the formation of different fragment ions (11). The robustness of modern analyzers and their ease of use has to some extent moved the operation of these instruments from MS specialists into the hands of analytical scientists.

The potential for application of ion mobility-mass spectrometry (IM-MS) within the pharmaceutical industry was first demonstrated by Eckers and co-workers in 2007 (12). The use of collisional cross-section (CCS) as an additional characteristic of an impurity,

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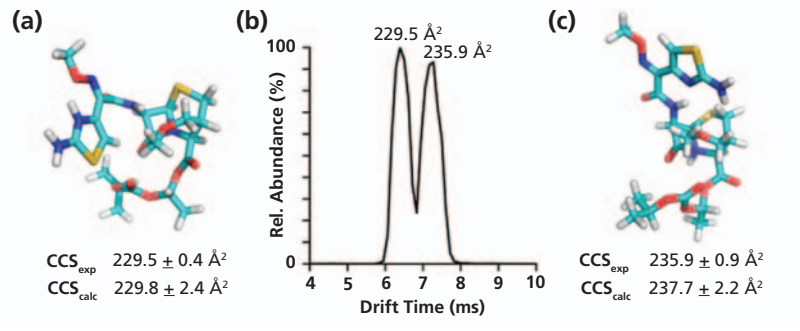
Acetylation of 2,6-Dimethylaniline during the synthesis of Lidocaine. (Impurity N-(2,6-Dimethylphenyl) acetamide, MM0102.08).

LGC

Figure 1: (a) Conformation of cefpodoxime proxetil, obtained through molecular modelling, which had a theoretical CCS value 0.65%, different to that of lower experimental CCS value; (b) bimodal arrival time distribution of cefpodoxime proxetil annotated with the experimental CCS values; (c) conformation of cefpodoxime proxetil, obtained through molecular modelling, which had a theoretical CCS value 0.97%, different to that of the higher experiment CCS value. Adapted with permission from Hines *et al.*, *Anal. Chem.* **89**, 9023 (2017). © 2017 American Chemical Society.



in addition to its retention and molecular weight, has significant potential as a tool within the pharmaceutical industry (13). The peer-reviewed literature contains abundant examples from academic research groups of the application of many different types of ion mobility interfaced to MS for pharmaceutical analysis. The potential impact of the technology is illustrated by the 2018 review by Iain Campuzano and Jennifer Lippens (14), which discusses innovations in ion mobility technology and how they have been applied within research in the pharmaceutical industry. The review outlines the theory of different ion mobility technologies and describes applications to small molecules, metabolites, lipids, peptides, proteomics, proteins, and antibody–drug conjugates (ADCs). The authors note and reflect that ion mobility has seen broad acceptance and adoption within the academic community. However, within the pharmaceutical industry, it is still seen as a niche and specialist technique, which is reflected in its uptake and the resulting limited examples of applications originating from industrial research within the peer-reviewed literature.

An area of particular interest in the pharmaceutical industry is enantiomeric analysis of small molecules and this has been explored by IMS-MS. A recent example is the publication by Donald and co-workers, where differential ion mobility spectrometry (DMS) MS was explored for the rapid and quantitative chiral recognition of small molecules (tryptophan and phenylalanine) using a chiral selector (N-tert-butoxycarbonyl-O-benzyl-L-serine [BBS]) that formed proton bound diastereomeric complex ions (15). The formation of gas-phase charge isomers (protomers) has been shown by Sobott and co-workers to be an additional complication during ion mobility analysis because multiple peaks are observed for the same molecule (16); this has also been observed by Hines *et al.* (13) .

The biggest challenge to the analytical chemist or MS specialist working in late-stage pharmaceutical development is the now immense diversity of molecular entities that are being developed as drug molecules, with a notable shift towards larger molecules (17); these may be peptides, oligonucleotides, or drug delivery systems such as ADCs. This shift can require adoption of new techniques or a retraining in old techniques that have to some degree fallen out of favour (CE and size-exclusion chromatography [SEC], for example). These molecules provide challenges, especially around the identification and quantification of impurities. For example, CE–MS has shown some complementarity with LC–MS for the analysis of peptides through orthogonal separation (18).

Oligonucleotides present a particular challenge as a result of the large number of chiral isomers. The complex structure and multistep synthesis and purification lead to a broad range of impurities such as N-1 and N+1 shortmers and longmers where the impurities have either one less or one more nucleotide (and the similarity between the main component and the impurities). The separation of these molecules are typically based around ion-pair chromatography (19,20), but the presence of coeluting impurities means that MS is used to quantify the purity of the main peak. The importance of therapeutic oligonucleotides is clearly reflected in their increasing prevalence within the peer-reviewed literature. The potential impact of oligonucleotides was illustrated in the 2011 review paper by Niessen and van Dongen, which discussed bioanalytical LC–MS of therapeutic oligonucleotides (21). This review recognized the increasing importance of LC–MS to characterize the parent oligonucleotide and its metabolities in biological fluids. The extensive review covers many of the key aspects of LC–MS of oligonucleotides, including chromatographic retention, ionization efficiency, ion-pair chromatography, pH, organic modifiers, the distribution of multiple charges, and fragmentation efficiency. Bartlett and co-workers have been notably active and this is reflected in two recent publications. A review published in 2018 focuses on the application of chromatographic techniques (including ion-pair reversed phase–HPLC–MS) for the determination of a broad range of oligonucleotide impurities and degradation products (22). The review also describes in detail the vast range of impurities and their synthetic origin. The importance of the characterization of the impurities and understanding their origin in the context of both process optimization and design of commercial synthetic processes is highlighted. In addition to this thorough review, Bartlett *et al.* have also recently described the application of IP–reversed-phase LC–MS/MS for the in-depth characterization of the degradation products formed from four different antisense oligonucleotides under stressed conditions (different pH values and temperatures) (23). There have been a number of recent examples of research in the area of oligonucleotide characterization originating directly for the pharmaceutical industry. Smith and Beck at GlaxoSmithKline described the application of LC–MS and ^{31}P NMR to quantify a low-level coeluting impurity in a modified oligonucleotide (24), and Breda and co-workers at Aptuit have published a validated (10–10000 ng/mL) bioanalytical ion pair LC–MS/

MS assay for the quantification of a 13-mer oligonucleotide in rat plasma to support a four-week toxicology study (25). Though less prevalent within drug project portfolios, therapeutic peptides are of increasing interest within analytical science. This has been reflected in the growing market for counterfeit biopharmaceuticals and the impact on analytical science has been investigated by Vanhee *et al.* (26). Their 2015 paper discusses the analysis of illegal peptide biopharmaceuticals frequently encountered by controlling agencies. It describes the development of a general screening method employing LC–MS/MS for both the identification and quantitation of illegal injectable peptide preparations that covers a range of therapies including oncology. The method was selective for the characterization of 25 different peptides (based on MS/MS fragmentation), and also validated for quantitation according to ISO-17025.

Many peptide separations can require buffers, salts, or additives that render them incompatible with MS. Hao Luo and colleagues at Merck have sort to overcome this challenge by developing two-dimensional (2D)-LC as an on-line desalting tool to allow peptide identification directly from these MS-unfriendly HPLC methods (27). Their method employs a heart-cutting 2D-LC system coupled to a quadrupole time-of-flight (QTOF)-MS. Fractions separated in the first dimension using an MS-incompatible mobile phase are transferred to the second dimension, where fast desalting with an MS-compatible phase allows subsequent MS characterization of impurities. In a novel method, Gammelgaard *et al.* have investigated the use of selenium as an elemental label for the quantification of the cell-penetrating 16 amino acid peptide penetratin (28). Using the labelling method in combination with flow injection combined with inductively coupled plasma–mass spectrometry (ICP-MS) (for total Se), LC–ICP-MS (for quantitative peptide uptake), and liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) (for the characterization of degradation products) provided detailed information of the peptide cellular uptake.

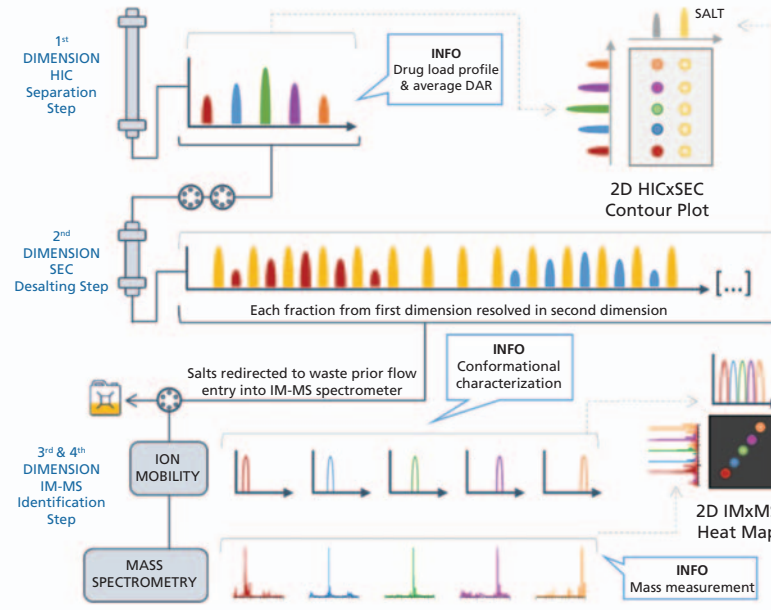
Another class of compound that is becoming increasingly prevalent is the ADC. The challenges involved in the mass spectrometric analysis of these compounds have been investigated by Friese and co-workers (29). For characterization of ADCs, Cianferani and colleagues have described a proof of concept study on the application of an on-line four-dimensional hydrophobic interaction chromatography (HIC)×SEC×ion mobility-mass spectrometry (IM-MS) methodology (Figure 2). The approach allows several critical quality attributes required for process and formulation development, lot characterization, and stability testing to be monitored in a single analysis (30).

Polymeric materials have long played an important role in the pharmaceutical industry, for example as excipients in oral solid-dose drug product formulations. Fiebig *et al.* from Boehringer Ingelheim have taken a novel approach to characterizing the regularly used formulation constituents, polyethylene glycol 400 and polysorbate 80. Their publication describes the application of travelling wave ion mobility spectrometry (TW-IMS) quadrupole time-of-flight high resolution mass spectrometer (QTOF-HRMS) and the use of both the collision cross-section and accurate mass for this characterization challenge (31). The methodology was applied to *in vivo* metabolite studies allowing rapid identification of the formulation constituents.

More recently polymeric materials are being developed as nanocarriers for targeted drug delivery in biomedicine. Examples include nanoparticles that encapsulate an active pharmaceutical ingredient (API) and dendrimer drug conjugates, where a number of API molecules are attached to the surface of a hyperbranched polymer (32). As a result of their relatively recent emergence and novelty, reports on the characterization of dendrimers is limited, however poly(amidoamine) (PAMAM) dendrimers have found some focus, notably by Fernandez-Alba and colleagues in 2013 (33,34). The group have described the application LC–ESI-MS and LC–ESI-MS/MS (using both QTOF and hybrid quadrupole–linear ion trap) to the characterization (accurate mass MS/MS) and quantitation (SRM) of PAMAM dendrimers (generations G0 to G3) in simple aqueous media and more biorelevant urine. The quantitative method was validated and shown to have sensitivity in the micromolar range.

Finally, we should not lose sight that GC–MS remains an essential tool within the pharmaceutical industry for many qualitative and quantitative applications. Continued innovation in GC–MS technology has been demonstrated by the introduction of a number of high-resolution GC–MS systems (35). The authors of this article have themselves demonstrated the capability of GC coupled to an orbital

Figure 2: Flow chart of the analysis from brentuximab vedotin. Adapted with permission from Ehkirch *et al.*, *Anal. Chem.* **90**, 1578 (2018) © 2018 American Chemical Society.



mass spectrometer for structural characterization to deliver process development and understanding (36). Accurate mass GC–electron ionization (EI)-MS and GC–chemical ionization (CI)-MS data were used to characterize key impurities of a synthetic building block for an important drug substance that was under development. Such characterization and impurity tracking of small synthetic building blocks is an essential aspect of process development and design for long-term product quality and patient safety. The quantitative potential of GC with orbital trap MS was also evaluated.

GC–MS plays an important role in the characterization and quantitation of extractables and leachables that may result from devices used within the pharmaceutical industry. GC coupled with HRMS has proved particularly effective in extractable and leachable analysis (37,38).

A recent example of this is the report by Lacorte *et al*. who have assessed the migration of plasticizers from poly(vinyl chloride) and infusion bags both qualitatively and quantitatively using selective extraction and GC–MS (39). PVC is widely used in the pharmaceutical industry for the manufacture of a wide range of medical devices, including tubes, probes, bags, and primary packaging. Therefore, the characterization of the migration potential of plastic additives (for example, phthalates, various phenols, and benzophenone) is of great importance in the context of patient safety and adherence to international regulations.

Summary

The use of mass spectrometry in all areas of the pharmaceutical industry has increased markedly over the last ten years as instruments become smaller and cheaper, or smaller and with increased resolution. The changes in the project portfolios across the pharmaceutical industry with novel (larger) molecules and complex drug delivery devices means that there are many challenges where mass spectrometry will be the analytical technology of choice. However, there is also a requirement to shift to differing separation techniques in front of the mass spectrometer or for ion mobility mass spectrometry, after the ionization has occurred. It is clear that mass spectrometry coupled to a wide range of separation technologies continues to play an essential role throughout the pharmaceutical industry, from discovery to development, to supporting a long-term supply of essential medicines to patients. The continuing evolution of MS technologies will only further strengthen the future impact and importance of MS in the pharmaceutical industry. LC–MS is still a predominant technique and its impact will not only continue, but will be enhanced over the coming years

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Ready-to-Use Impurity Mixture Solutions for Efficient QC Analysis—Paracetamol as a Case Study

Merck

Impurities can significantly impact the safety of drug products. Accurate detection and control of impurities in drug substances and drug products is an important element of quality by design, ICH, and GMP requirements. We offer more than a dozen ready to use impurity mixes for some of the most common APIs.

Introduction

During manufacture of a drug material impurities can arise from many different sources including drug substance residual impurities, degradation, extractables, and leachables, as well as impurities present in or derived from excipients. Accurate impurity identification and quantitation requires highly characterized reference materials.

Typically, monographs written by pharmacopeias require impurities for a given drug substance to be analytically verified by a chromatographic method by comparison against known reference standards. This usually requires a time-consuming process of preparing multiple samples, which can potentially introduce increased uncertainty and error in the analysis.

Solution-based impurity mixtures for several active pharmaceutical ingredients (APIs) have been developed as certified reference materials (CRMs) to be used as reference standards in order to reduce the potential for sample preparation errors and improve accuracy and efficiency in analytical verification of impurities. These CRMs have been manufactured according to ISO Guide 34 and can include several impurities found in specified monograph test methods.

Acetaminophen, or paracetamol as it is known internationally, belongs to a class of drugs called analgesics (pain relievers) and antipyretics (fever reducers). Since its approval in 1951, paracetamol has become the most common drug ingredient in the USA, and is widely used throughout the world.

The paracetamol multicomponent solution reference standard was designed to include paracetamol (API) and its seven related compounds (RC): 4-acetoxyacetanilde (RC-A), N-(4-hydroxyphenyl) propanamide (RC-B), 2- acetamidophenol (RC-C), acetanilide (RC-D), 4-nitrophenol (RC-F), 4-chloroacetanilide(RC-J), and 4-aminophenol.

Experimental Conditions

Column:	25 cm × 4.6 mm, 5-µm (59354-U) Discovery C8
Instrument:	Dionex Ultimate 3000
Mobile phase:	[A] methanol, water, glacial acetic acid (50:950:1); [B] Methanol, water, glacial acetic acid (500:500:1)
Gradient:	20 to 100% B in 10 min; held at 100% B for 1 min
Flow rate:	0.9 mL/min
Pressure:	1880 psi (130 bar)
Column temp.:	40 °C
Detector:	UV at 254 nm
Injection:	5 µL
Sample:	25 mg/mL in methanol
Standards:	20 µg/mL in 100:30, methanol–acetonitrile

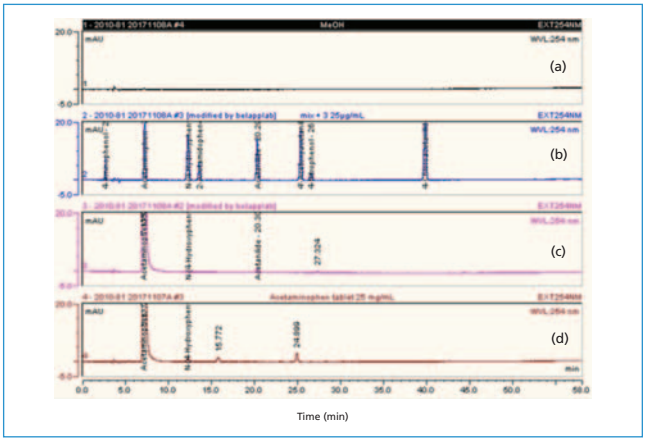

Figure 1: Chromatograms showing (a) injections of the blank, (b) the certified reference material, and (c) and (d) the commercially available paracetamol tablets under the conditions found above.

Results

Figure 1 shows the chromatograms for the screening of impurities in commercially available paracetamol tablets (Figure 1[c] and 1[d]) using a retention time comparison to the available paracetamol solution mixture CRMs. Using this solution mixture can help quickly identify the impurities present in the drug materials and allow for greater efficiency in quantifying each that are observed.

Conclusions

The use of a ready to use CRM solution standard containing a mixture of impurities can help improve efficiencies in the analysis of pharmaceutical impurities. This formulation allows for fewer chances of sample handling error and can also lead to quickly identifying specific impurities that may be found in drug materials. Several monograph impurities were identified in the commercially available paracetamol tablets by simple retention time comparison against the certified reference material solution standard.

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What Can Two-Dimensional Liquid Chromatography Offer the Pharmaceutical Industry?

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The evolution of two-dimensional liquid chromatography (2D-LC) instruments along with improved software capabilities has transferred 2D-LC from the hands of experienced researchers to functioning analytical laboratories in the pharmaceutical industry. 2D-LC offers chromatographers novel solutions to problems ranging from analyzing complex samples requiring excessively large peak capacities to separating simple compounds that are difficult to resolve. Recent developments in 2D-LC and 2D-LC–MS have demonstrated the potential of this technique in practice and 2D-LC is set to become an essential tool in the pharmaceutical sector to address problems ranging from coelution, peak purity assessment, simultaneous achiral-chiral analysis, genotoxic impurities, and more.

The pharmaceutical industry is one of the most regulated industries because the pharmaceutical products have to be safe and efficacious. From a chemistry, manufacturing and controls (CMC) perspective, the level of actual and potential impurities and degradation products in active pharmaceutical ingredients (APIs) and drug products has to comply with International Conference on Harmonization (ICH) guidelines to ensure quality and safety. According to ICH guidelines, the reporting, identification, and qualification thresholds for the impurities in APIs are 0.05%, 0.10%. and 0.15%, respectively, assuming a maximum daily dose of less than 2 g of drug substance (1). In the case of a chiral drug substance developed as a single enantiomer, the enatiomeric purity has to be controlled because there have been cases of severe adversity resulting from inadequate control of undesired enantiomers (2–3). Additionally, genotoxic impurities constitute another class of impurities that have to be limited to low parts per million (ppm) based on daily dosing and duration of exposure, making it extremely challenging to analyze (4).

High performance liquid chromatography (HPLC) with a diode array detector (DAD) and mass spectrometry (MS) has been the technique of choice to assess the chemical purity of drug substances and drug products. DAD relies on comparing the UV profile of the main component at multiple time points (front, apex, and tail of peak) to assess peak purity and cannot discern small spectral differences between structurally similar compounds that are often comparable, especially ones coeluting in proximity to the main component. Similarly, MS cannot differentiate or detect coeluting isomers (isobaric) and neutral compounds, as a result of spectral similarity and poor ionization, respectively. In addition, ion suppression of minor components in the presence of major components can significantly limit MS detection capabilities even if the spectra are distinct. Recently, the advantages and disadvantages of assessing potential coelution (peak purity) in LC using chromatographic data system software, and the use of multivariate curve resolution and two-dimensional liquid chromatography (2D-LC) were published in a series of three articles in *LCGC* (5–7).

In general, developing chromatographic methods of the desired selectivity and sensitivity to resolve structurally similar impurities such as regioisomers, des-halogenated and nitro-substituted APIs, and degradation products from the API can be challenging because they often elute close to the main component. Additionally, the ageing of the chromatographic columns, the long-term impact of modifiers, and minor changes to column chemistry by vendors could impact column selectivity. This could sometimes result in the resolution of previously coeluting impurities during the long-term stability of the drug substance or drug product. This could have a significant bearing on the clinical programme with new unqualified impurities or degradants in the drug substance or drug product. Similarly, assessing chiral purity of the API with multiple chiral centres can be a daunting task. Considering these challenges, it is surprising that the pharmaceutical industry is just beginning to adopt and embrace 2D-LC–MS. In the past few years, several applications of 2D-LC in pharmaceutical analysis have been reported and are beginning to change the landscape (8–21). Dwight Stoll *et al.* captured the recent advances in 2D-LC for pharmaceutical and biopharmaceutical analysis in *LCGC North America* (22). The following

article presents several applications of 2D-LC and 2D-LC–MS in the pharmaceutical industry.

Results and Discussion

Application 1: Simultaneous Achiral-Chiral Analysis by 2D-LC–MS: Developing a chiral HPLC method of the desired selectivity and sensitivity for an API with multiple chiral centres can be challenging because the number of potential stereoisomers increases by 2^n, where n is the number of chiral centres. In addition, the chiral HPLC method should have selectivity for the desired enantiomer and other stereoisomers from process-related impurities such as a des-halogenated API or a nitro-substituted API. 2D-LC can offer a simple, yet effective, solution for these challenging problems. Some of the earliest adoption of 2D-LC in the pharmaceutical industry has been for the analysis of chiral pharmaceuticals (17,23–24).

Results of the simultaneous achiral-chiral analysis of an API with three chiral centres are presented in Figure 1. A 2D-LC–MS method was developed to overcome the shortcomings of one-dimensional (1D) chromatography. The primary achiral column resolves the API (and its enantiomer) from potential diastereomers and process-related

Figure 1: Result of simultaneous achiral-chiral analysis of drug substance using 2D-LC is presented. The primary achiral column resolves the API (RRR and SSS) from diastereomers and process-related impurities while the chiral secondary column resolves the enantiomers. The level of undesired enantiomer is about 0.1% relative to the main component. Adapted with permission from reference 19. The rectangular box around the main component highlights the single heart-cutting of primary column eluent to secondary column.

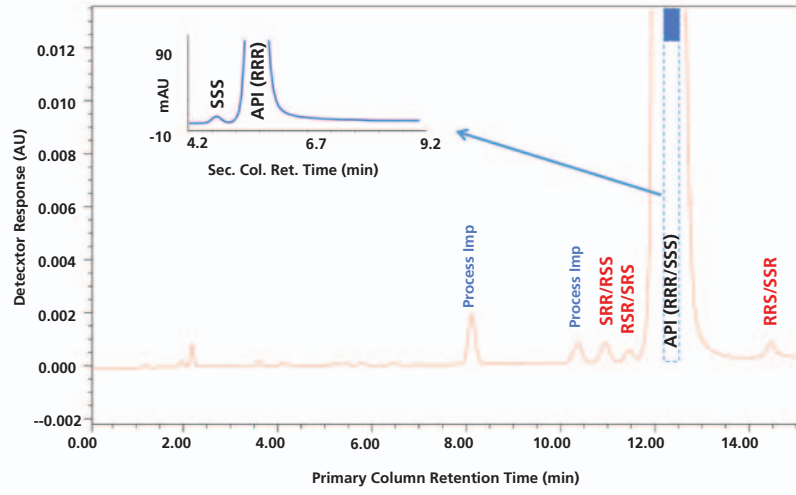
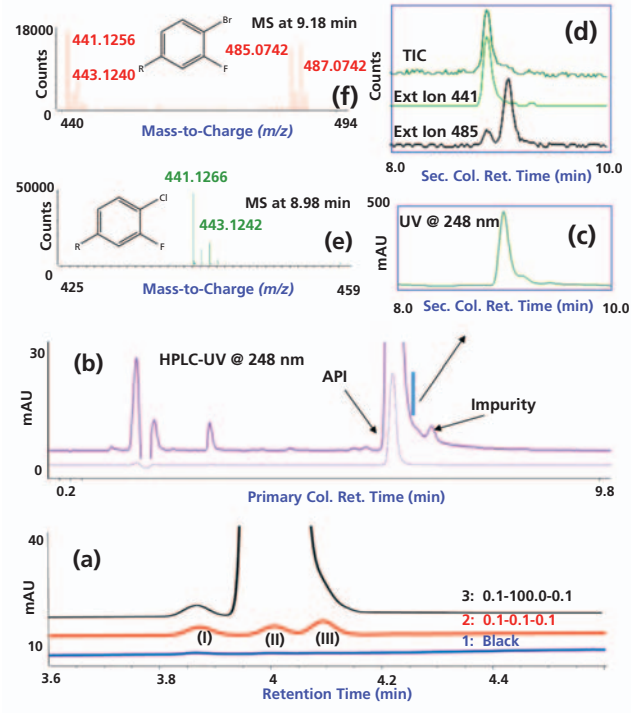


Figure 2: Application of 2D-LC using the same stationary phase in both dimensions to address dynamic range issues of conventional HPLC. (a): Impact of sample component concentration on chromatographic resolution. (b): ^{1}D separation of drug substance showing potential coelution in the tail of the main component. (c): ^{2}D separation of single heart-cut with UV detection. (d): ^{2}D separation of single heart-cut with MS (SIM) detection. (e): Mass spectrum of drug substance resolved in secondary dimension. (f): Mass spectrum of impurity, a bromo-substituted API in the secondary dimension. Adapted with permission from reference 19.



impurities, whereas the secondary chiral column resolves the desired enantiomer (RRR) from undesired enantiomer (SSS). A single heart-cut of the primary column eluent highlighted in Figure 1 (dashed box) was transferred to the chiral secondary column to resolve the enantiomers. By presenting a simple mixture to the secondary chiral column, components of interest were baseline resolved. Attaining similar separation (resolution of potential stereoisomers and process-related impurities from the desired API) would be impractical by one-dimensional chiral chromatography. Using 2D-LC, the enantiomer excess (ee) was determined with ease: 99.8%. Since enantiomers perfectly coelute (are unresolved) on the achiral column, a single heart-cut around the peak apex is adequate to determine the %ee. We demonstrated the comparability and appropriateness of single heart-cutting 2D-LC to selective comprehensive 2D-LC and conventional HPLC in our earlier work on simultaneous, sequential quantitative achiral-chiral analysis (17). For projects in early stages of development with multiple chiral centres, we have been using 2D-LC–MS to assess enantiomer purity until a conventional HPLC method(s) of desired selectivity is developed. We have also successfully coupled reversed-phase HPLC with normal-phase supercritical fluid chromatography (SFC) (2D-LC–SFC) to enable simultaneous achiral-chiral analysis of compounds with multiple chiral centres, extending the capability of multidimensional separation as the majority of chiral compounds are amenable to normal phase chiral chromatography (18).

Recently, we encountered a scenario where differences in cell-killing assay were observed between GMP and GLP tox lots of linker drug intermediates (LDIs) impacting the regulatory filing. We used 2D-LC–MS to determine the enantiomer purity of GMP and GLP tox lots, a potential cause for the anomaly in cell-killing assay. Unlike the example shown in Figure 1, where the four-diastereomer pairs are baseline resolved in an achiral column, in the case of an LDI with three chiral centres, only two of the four-diastereomer pairs would be resolved in the achiral column, as a result of structural similarities. Similarly, the chiral chromatography lacked selectivity and specificity to resolve stereoisomers and process-related impurities thereby limiting its applicability. Using achiral-chiral 2D-LC–MS, two of the four diastereomers were resolved in the primary dimension and the primary column peaks were sampled into a chiral HPLC column for further separation. The secondary chiral HPLC column resolved each peak into four individual stereoisomers (results not presented). In this example, although the individual dimensions had limitations, by effectively coupling the two dimensions, chiral purity of LDI was determined. The 2D-LC–MS results along with other test results demonstrated the comparability of the two lots (GMP and GLP) to enable successful regulatory filing.

Application 2: Addressing the Dynamic Range Issue with 2D-LC: Realizing complementary or orthogonal separation in reversed-phase 2D-LC–MS requires columns of different selectivity and different operating conditions. In reversed-phase LC, finding complementary columns of different selectivity, especially for structurally similar compounds, can be challenging because hydrophobic interaction usually dominates the separation and differences in column chemistries are usually inadequate to resolve structurally similar compounds. There are examples where the same column chemistry could be used in both dimensions to resolve the coeluting components, although the separation is not orthogonal (19). Figure 2(a) shows overlay plots of diluent blank and three sample components. When the relative levels of the sample

components are comparable, they are baseline resolved. However, as the concentration of component #2 is increased by a 1000-fold relative to other components, the resolution between component #2 and component #3 is lost. This is a common problem encountered in the pharmaceutical industry where the relative level of main component to potential impurities differs by several orders of magnitude, which obscures the detection of minor components. Given the structural similarity, the peak-purity tools commonly used in HPLC with a diode array detector or a mass spectrometer are often inadequate to discern residual coeluting impurities. However, because the relative level of the coeluting impurities and the main component at the peripheral of the abundant peak are comparable, re-injecting fractions of the abundant peak (front and or tail) could resolve these coeluting components. The dynamic range issue commonly encountered in the pharmaceutical industry can be readily addressed using 2D-LC, a simple yet powerful application with significant bearing.

A real-world application involving same column chemistry using 2D-LC is shown in Figure 2. The 1D separation of the API on a 15 cm × 4.6 mm, 3-µm cyano column at 248 nm is shown in Figure 2(b). Careful assessment of the chromatogram shows a shoulder peak in between the API and the impurity peak in the tail of the main component. A single fraction of the primary column eluent (100 µL) corresponding to the elution of the potential impurity was transferred to a 5 cm × 3.0 mm, 1.8-µm cyano ^{2}D column. The 2D chromatogram of the transferred fractions (UV and MS) shows partial separation of the API and the impurity as the relative level of the two components is comparable (~10:1) as demonstrated in Figures 2(c) and 2(d). This is more obvious in the extracted ion chromatogram shown in Figure 2(d). Based on MS data (time-of-flight [TOF]), the peak eluting at retention time 8.98 min corresponds to the API with M+H ion of 441.12 [Figure 2(e)], whereas the MS of the peak eluting at 9.18 min is a bromo-substituted API (Figure 2[f]). The bromo- and

Figure 3: Result of selective comprehensive 2D-LC analysis of linker drug intermediate (LDIs) used in ADCs. (a): ^{1}D separation of blank and sample lots shows lot-to-lot variability. The rectangular box around the main component highlights the high-resolution sampling of primary column eluent to the secondary column. (b): Full scale ^{2}D separation of LDI (main component). (c): Expanded plot of ^{2}D separation of LDI (main component). (d–f): Contour plots of three lots of LDI showing resolution of multiple components in the secondary dimension. Adapted with permission from reference 20.

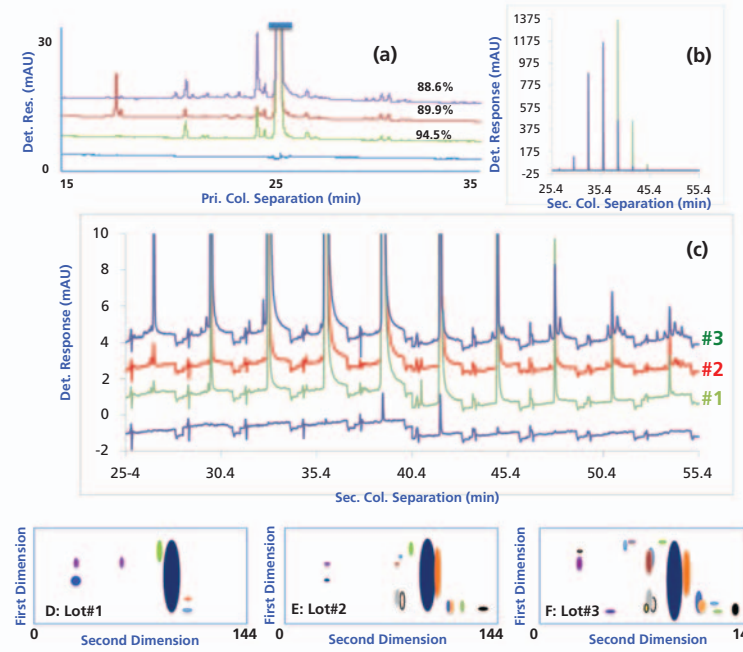


Figure 4: Reproducibility of selective comprehensive 2D-LC analysis of aged LDIs used in ADCs. (a): Expanded ^{2}D separation of blank and triplicate preparations of aged sample. (b): Full scale ^{2}D separation of LDI (main component). (c–e): ^{2}D separation of triplicate preparations of aged LDI demonstrating the reproducibility of 2D-LC. Adapted with permission from reference 20.

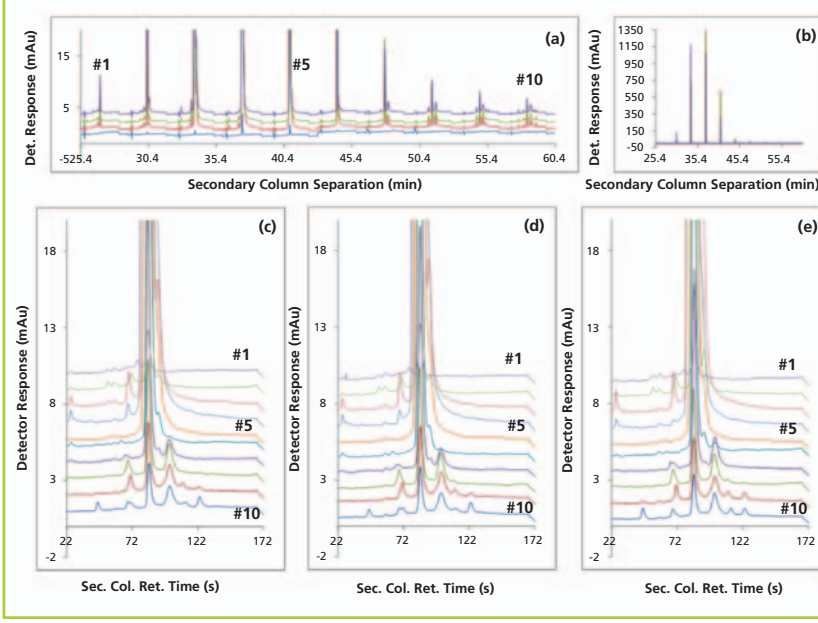
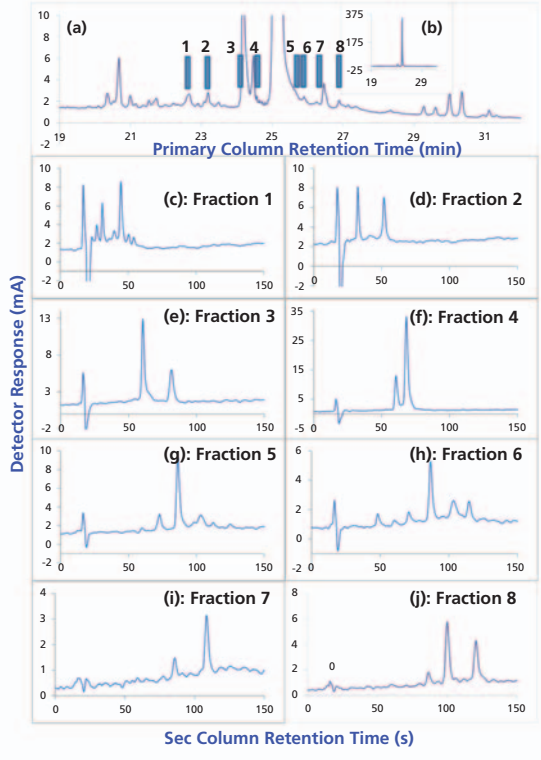


Figure 5: Results of multiple heart-cutting 2D-LC analysis of complex LDIs used in ADCs. (a): Expanded ^{1}D separation of sample showing locations of multiple heart-cutting. (b): Full scale ^{1}D separation of LDI. (c–j): ^{2}D separation of transferred fractions demonstrating sample complexity and resolving power of 2D-LC. Adapted with permission from reference 20.



chloro-substituted API was inferred by comparing the relative intensities of M+1 and M+3 ion. For the API (Figure 2[e]), the intensity of M+3 ion is about one third of M+1 ion, thus confirming the presence of a single chloro-substituent in the API, whereas for the bromo-substituted API (Impurity, Figure 2[f]), the relative intensity of the M+1 and M+3 ions are comparable, suggesting the presence of the bromo-substituent. The bromo-impurity originates from the regulatory starting material (RSM, 5-chloro, 2,4-difluorobenzoic acid) and if its level is not controlled, it undergoes similar chemistry as the RSM and is difficult to purge in the downstream manufacturing process. Controlling the level of bromo-impurity in the RSM is critical in limiting the formation of bromo-substituted API impurity in the drug substance. Upfront monitoring of the RSM by 2D-LC for structurally similar impurities, including isomers using similar columns, is a prudent approach in limiting the formation of difficult to purge impurities downstream. The above example is a simple, yet effective, application of 2D-LC–MS to resolve and identify potential coeluting impurities in the RSM and API. This strategy could also be used to assess the stability-indicating method for potential coeluting impurities, either in the front or tail of API peaks as some of these impurities could potentially grow during stability (degradation products) and show up during long-term stability with the ageing or modification of chromatographic columns or as a result of small changes to column chemistry deemed trivial by vendors.

Application 3: High-Resolution Analysis of Linker Drug Intermediates (LDIs) Used in Antibody-Drug Conjugates (ADCs) Using 2D-LC–MS: The following example demonstrates the application of selective comprehensive 2D-LC–MS analysis of extremely complex LDIs used in the synthesis of ADCs, selective modern chemotherapeutics often used along with radiotherapy and chemotherapy treatments. The unique selectivity of the antibody is exploited to deliver the drug (toxin) to the targeted cells that upon internalization results in the drug release (toxin) directly into the cytoplasm, killing the cancerous cells.

Synthesis of linker drugs, a key intermediate in ADCs, is extremely challenging because it is a multistep process involving more than 20 chemical transformations. The relatively high-molecular-weight of LDIs (over one kDa), the high reactivity, and chemical instability make it challenging, if not impossible, to analyze the structurally similar compounds coeluting with the main compound. Furthermore, significant differences in the concentration ranges of potential impurities relative to the LDI (~ two or more orders of magnitude) aggravates the issue. Upfront control of impurities in LDIs is extremely critical because many of these impurities could potentially conjugate with the antibody in the downstream process and could be difficult to purge and analyze.

Developing a chromatographic method for the LDI was challenging because excessive tailing at low pH and chemical instability at high pH limited the practical pH range of the method from 4.5 to 8.0. A gradient HPLC method was developed on a 15 cm × 3.0 mm, 2.7-µm superficially porous C3 column using 5-mM phosphate buffer at pH 7.0 and acetonitrile. Compared to C8 and C18 stationary phases, a short chain C3 column offered the desired selectivity and retention for hydrophobic LDI, enabling their elution with relatively low organic content. A 2D-LC method was developed using a C3 column in the primary and a C18 column in the secondary dimension. Among various columns assessed in the second dimension, the C18 column offered good peak shape and resolution. The 2D-LC system was operated in high-resolution sampling (HRS) mode enabling complete transfer of the main component to the complementary secondary column.

A 2D-LC separation of three lots of LDIs is shown in Figure 3. The overlay plots of the blank and three

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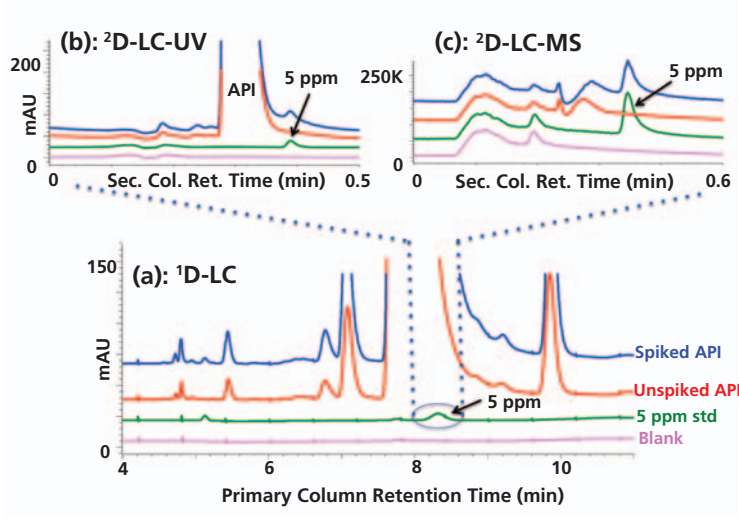
Figure 6: Result of single heart-cut 2D-LC analysis of residual genotoxic impurity (GTI) coeluting with the drug substance. (a): Expanded ^{1}D separation of blank, 5 ppm standard of ethyl besylate, unspiked and spiked drug substance sample showing coelution of GTI with the active. (b): Expanded ^{2}D separation with UV detection demonstrating the power of 2D-LC to resolve residual, coeluting GTI in the complementary secondary column. (c): Expanded ^{2}D separation with MS (SIM) detection demonstrating the specificity and sensitivity of MS detection in the secondary dimension. Adapted with permission from reference 21.



sample lots in the primary dimension (Figure 3[a]) show lot-to-lot variability, with the purity of the main component ranging from 88.6% to 94.5% (20). The complexity of LDIs is obvious from these chromatograms with several sample components either coeluting or being partially resolved from the main component. Changing the column selectivity or its operation did not improve the overall separation. The fractions of primary column eluent transferred to the secondary column using HRS is highlighted in Figure 3(a) with a blue rectangle. The full-scale and expanded 2D separations are shown in Figures 3(b) and 3(c), respectively. Although the fractions were analyzed in reverse order of parking, the results are presented in the order sampled to enable better visualization and interpretation of the results. The overlay plots of the three development lots show lot-to-lot variability, with over 15 coeluting peaks resolved from the main component in sample lot #3. Based on ^{2}D peak shapes, some of the resolved components are probably multiple components substantiating the analytical challenges and sample complexity. The blank chromatogram shows carryover of the main component in some of the fractions from previous injections. As expected, the resolving power of 2D-LC is much pronounced in the contour plots of the sample shown in Figure 3(d) to 3(f) (bottom), where each spot represents a sample component.

With the exception of an impurity in sample lot #3, the levels of other impurities were below 0.05%. Effective peak focusing and high-speed separation in the secondary dimension resulted in sharper peaks in the secondary dimension, resulting in lower detection limits.

The secondary dimension purity of the main component ranged from 99.57% to 99.93%, resulting in an overall purity of 88.25% to 94.47% for the LDI. The overall purity of the main component is determined from the product of primary and secondary dimension peak purity.

Using 2D-LC–MS, we were able to assess lot-to-lot variability of development lots with good precision and detection limits well below 0.01%. These levels are well below the limits mandated by ICH guidelines. This application clearly demonstrates the power of 2D-LC in detailed characterization of structurally similar, coeluting impurities in LDI that is impractical by conventional 1D-LC.

Repeatability of 2D-LC Separation of Linker Drug Intermediate Used in ADCs: Repeatability of 2D-LC is often questioned because of the complexity in the design and operation of the 2D-LC system with multiple valves including the parking deck(s). To demonstrate the repeatability of 2D-LC, an aged sample of LDI was prepared in triplicate and analyzed. The results of this study are presented in Figure 4. The expanded and full-scale ^{2}D separations are shown in Figures 4(a) and 4(b), respectively. Stack plots of replicate sample analysis are shown in Figures 4(c), 4(d), and 4(e). From these chromatograms, it is obvious that the 2D-LC separation of LDIs is reproducible with the multiple valves in sync between runs. The %RSD for the main component from triplicate injections of the sample was less than 0.1%, which is comparable to conventional LC separation.

Additionally, the absolute difference in the percentage peak areas of impurities less than 0.1% was within +/-0.01 and for impurities greater than 0.1% was within +/-0.02, demonstrating the reproducibility of 2D-LC for the quantitative analysis of complex linker drug intermediates. Effective peak focusing resulted in enhanced sensitivity in the secondary dimension (20).

Assessing Sample Complexity Using 2D-LC–MS: The resolving power of 2D-LC–MS in the analysis of complex LDIs is highlighted in Figure 5, where multiple heart-cutting from the primary column was analyzed using a complementary stationary phase in the secondary dimension. What appears to be partially resolved components in the primary dimension is resolved into multiple components in the secondary dimension. This is most obvious in fractions 1 and 6. Most of these impurities are well below 0.05%, the reporting level mandated by the health authorities. Resolving these many components using 1D-LC would be impossible, which demonstrates the power and practicality of 2D-LC (20).

Application 4: Ultra-Trace Analysis of Genotoxic Impurities by 2D-LC–MS: Genotoxic impurities (GTIs) are an important class of compounds, which when present in drug substances could significantly impact patient safety and health by binding to the DNA or protein, causing gene mutation. The levels of these impurities need to be controlled to low ppm as mandated by ICH M7 guidance based on duration of exposure and daily dosing of drug product (4).

Given the significant differences in the volatility, reactivity, and polarity of these compounds, the analysis of GTIs is extremely challenging. This is further compounded by relatively high concentrations of the sample (mg/mL). Compared to conventional impurities that are often limited to a few tenths of a percentage in a drug substance, the GTIs have to be limited to low ppm, therefore requiring techniques of high selectivity and specificity like LC or gas chromatography (GC) coupled to high-end mass spectrometry.

Application of 2D-LC–MS in the analysis of ethyl besylate, a potential GTI, is shown in Figure 6. Ethyl besylate coelutes with the API in the primary column (Figure 6[a]) but is resolved in the complementary secondary column. The results of LC–UV and LC–MS detections are shown in Figures 6(b) and 6(c). Compared to UV, MS with selective ion monitoring (SIM) offers both sensitivity and specificity for residual GTIs, eliminating the impact of the API peak present at much higher concentrations (21).

Conclusions

Several applications of 2D-LC–MS in "real-world" pharmaceuticals have been demonstrated, ranging from simultaneous achiral-chiral analysis of an API with multiple chiral centres, to addressing dynamic range issues commonly encountered in API analysis, to assessing peak purity of complex LDIs with several coeluting impurities in the midst of the main component, to residual GTI analysis (ppm). These examples highlight the challenges commonly encountered in the pharmaceutical industry along with simple, yet novel, solutions provided by 2D-LC. The key attributes of 2D-LC separation, such as linearity, precision, accuracy, detection, and quantitation limits, are comparable to conventional HPLC. This is critical for the transitioning of 2D-LC from R&D to a GMP environment. The misnomer that the application of 2D-LC is limited to complex samples has also been addressed. In reality, 2D-LC can also provide novel solutions to simple but challenging problems that are difficult to address by conventional HPLC. The advent of active solvent modulation should enable easier coupling of "difficult-to-couple" separation mechanisms as a result of solvent mismatch between the two dimensions. For example, reversed phase and hydrophilic interaction chromatography (HILIC), or size-exclusion (SEC) and reversed phase chromatography. Additionally, with 2D-LC it is practical to use an MS-incompatible mobile phase in the primary dimension. In conclusion, the author believes it is a matter of "when" and not "will" 2D-LC and 2D-LC–MS dominate contemporary chromatography to provide novel solutions to the increaasing challenges and needs of the pharmaceutical and other industries.

Acknowledgements

The author would like to thank M. Al-Sayah, S.R. Huang, Ila Patel, Jacob Kay, and Larry Wigman for their contributions, and Genentech Inc., for its continued support and opportunity

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C.J. Venkatramani is a senior scientist at Genentech USA and has more than 15 years experience in the pharmaceutical industry. He was a key member of the Genentech technical team instrumental in the successful launch of Genentech's first small molecule, Erivedge, leading from development to commercial. Erivedge is currently approved in several countries around the world for the treatment of advanced basal cell carcinoma (BCC). His area of interest includes 2D-LC, 2D-LC-SFC, and ultratrace analysis of geneotoxic impurities. Over the years, he has successfully used multidimensional chromatography to address challenging problems encountered in the pharmaceutical industry.

Regulatory Compliance and Data Integrity — Who, What, Why, and How

Gesa J. Schad, Shimadzu Europa GmbH

A current topic related to analytical data is the lack of integrity following modification or replacement. Whether intentional or accidental, such problems are often the result of incorrect operating procedures. Accordingly, the question of how to ensure data integrity has become a pressing issue for analytical laboratories. In addition to the sophisticated software security functions included nowadays in computerized systems, manufacturers aim to improve and simplify data handling while helping to ensure data reliability by preventing undetected data manipulation.

Who Defines and Controls Compliance with Data Integrity?

Regulatory authorities such as the World Health Organization (WHO), the International Conference on Harmonization (ICH), United States Food and Drug Administration (USFDA), or the European Medicines Agency (EMEA), to name just a few, act as safeguards to ensure the safety, efficacy, and quality of drugs available to the public. International organizations establish the rules related to product registration, manufacturing, distribution, price control, marketing, research and development, and intellectual property protection, while responsibility to ensure quality and safety lies with the national regulatory authorities. As a control mechanism, routine inspections are carried out by the different organizations to ensure that regulations are followed, and drug products are produced with consumer safety in mind. The Pharmaceutical Inspection Co-operation Scheme (PIC/S) is meant as an instrument to improve cooperation in the field of good manufacturing practices between regulatory authorities and the pharmaceutical industry.

What are "GMP" and the Importance of 21 CFR Part 11?

Good manufacturing practices or GMP implies a set of standard guidelines fixed by the regulatory bodies to ensure proper design, monitoring, and control of manufacturing processes and facilities. The identity, strength, quality, and purity of drug products is regulated to prevent contamination, mix-ups, deviation, failure, and errors, and to guarantee the safety and efficacy of pharmaceuticals. While generally referred to as guidelines, these are in fact laws and it is mandatory for pharmaceutical companies to abide by them. Omission to follow these laws is a criminal offence, as it would put consumers at risk.

GMP guidelines are designed to be flexible, and to allow companies to use modern technologies and innovative approaches to achieve higher quality through continuous improvement. Therefore, cGMP meaning "current" good manufacturing practice requires the use of up-to-date technologies and systems to comply with the regulations. It describes the minimum standards to be met or exceeded. One of the most talked about guidelines in recent years is certainly Part 11 of Title 21 of the FDA's Code of Federal Regulations (CFR), also known as 21 CFR Part 11 or simply Part 11, which deals with "Electronic Records: Electronic Signatures". It requires the implementation of control measures, including audits, system validations, audit trails, electronic signatures, and documentation for software and computerized systems involved in any stage of the manufacturing of pharmaceutical products, to ensure data integrity.

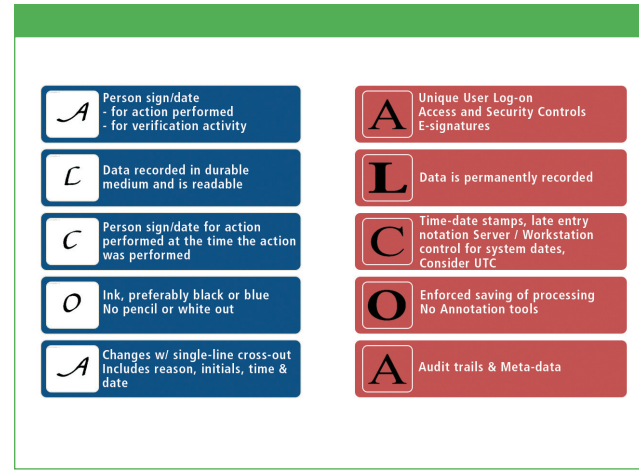

Figure 1: ALCOA for paper (blue) or electronic (red) records (Adapted from D. Stokes, Principal Consultant, Convalido Consulting Ltd, UK).

What are "Data Integrity" and "ALCOA+"?

Data integrity is defined as the extent to which all data are complete, consistent, and accurate throughout the "data life cycle". This encompasses all phases in the life of the data from initial generation and recording through processing (including transformation or migration), use, data retention, archive, retrieval, and destruction. Data must be unequivocally attributable to the operators. All data need to be protected and managed to be tamper-proof throughout the data life cycle. To ensure the reliability of data acquired, operating procedures should be followed and computerized systems need to protect data integrity.

The **"ALCOA+"** principle is meant to ensure that data is trustworthy. The acronym stands for:

- **A**ttributable: Who did it, when did they do it, why did they do it?
- **L**egible: Can it be understood, is it permanent?
- **C**ontemporaneous: Was it captured or recorded as it happened?
- **O**riginal: Is it original or a verified copy?
- **A**ccurate: Are there no undocumented errors or changes?
- **PLUS:** Are the data **complete**, including metadata, audit trails? Are all data and time stamps **consistent**? Is it recorded

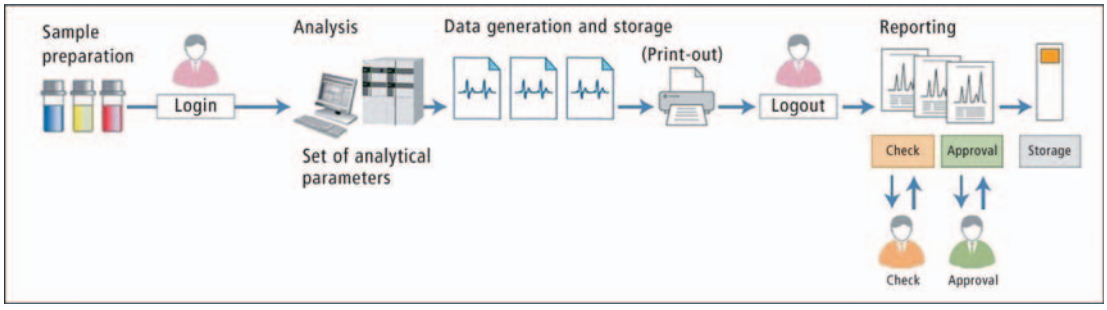

Figure 2: Schematic workflow for acquisition and processing of data using the example of an HPLC analysis.

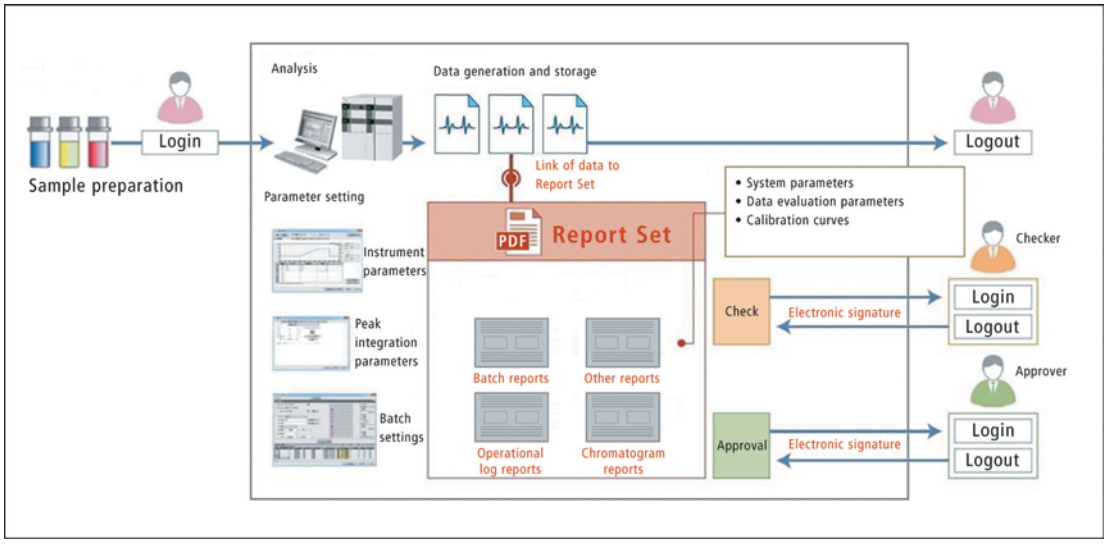

Figure 3: Compliant data integrity using an advanced reporting feature.

on controlled, **enduring** media (hard copy or electronic)? Is it **available** to authorized users throughout the life cycle?

This principle can be applied to the entire life cycle of data in a computerized system, as well as for paper records (1).

The Need for Data Integrity

The increase in digital evaluation and approval of measured data in recent years has made data integrity a key quality topic. Electronic data processing enables data to be modified intentionally or unintentionally, for example, changes to evaluations or substitution of datasets. When these data are used, for example, for the release of medicinal products or active substances, such manipulation could lead to serious consequences for the health of the patient. Regulatory auditors therefore pay close attention to data integrity measures during audits.

Controls by the US FDA (Food and Drug Administration) have revealed several cases where companies could not demonstrate compliance regarding data integrity, prompting the FDA to publish a guideline in April 2016. “Data Integrity and Compliance with cGMP” (1) emphasizes that the FDA regards data integrity as a crucial factor. As a result, numerous “warning letters” were published in recent years pointing out the most common issues as well as the principles of electronic record-keeping according to Guideline 21 CFR Part 11. In some severe cases, contravention led to import bans.

As a result of the many incidents of nonconformity, FDA auditors now apply a “guilty until proven innocent” approach during their inspections, and non-compliance with the regulations is assumed. End-to-end proof of the integrity of measured data has therefore become essential in a controlled environment (2).

Although traditionally associated with the pharmaceutical industry, recent incidents of food fraud, missing forensic samples,

or environmental cover-ups have brought up the subject of data integrity across many industries in Europe.

With the current speed of technological advances, it is difficult to keep abreast of how software-controlled automated analytical equipment can help simplify the path to compliance.

Suppliers of chromatography data systems, that is, software for acquisition, processing, administration, and storage of data arising from analytical measurements, have also adapted to these changing circumstances by implementing dedicated functions for data integrity to support companies operating in a controlled environment with regard to data integrity and FDA compliance.

Pitfalls Associated with Data Integrity

To illustrate the necessary functions and precautions during day-to-day analytical work, possible pitfalls associated with data integrity are highlighted using a high performance liquid chromatography (HPLC) analysis as an example. In a purely paper-based laboratory, the workflow follows the scheme shown in Figure 2.

The user logs in on the PC, records data, processes, and prints it. The printed chromatograms are checked, approved, and then archived in a filing system. This procedure seems acceptable, provided that the corresponding security settings such as access data, settings for the audit trail, and user privileges for deleting data have been set appropriately.

However, in paper-based documentation, only printed chromatograms are generally evaluated. Instrument settings, data processing, sample table, or other parameters are usually not considered, even though it is also necessary to obtain a reliable evaluation of all these factors (3). The printout does not show the exact structure of the total dataset at the time it was printed. Regardless of how secure an electronic data processing system or software is, it always depends on the entries made by the user. Even with the strictest security measures in place, it is very difficult to prevent inadmissible actions.

Clear Visualization of Every Manual Manipulation

Unequivocal proof that no inadmissible action occurred during acquisition or evaluation of the data that could falsify the analytical result can only be established if all operations during data processing are easy to see. This can be achieved by gathering all human-mediated operations, such as parameter settings or data evaluation, and making them readily recognizable as manual steps. By rendering all manipulation obvious, any inadmissible processes such as modification, deletion, or substitution of data can be recognized straight away.

Software features such as the so-called “Report Set” function from Shimadzu create a set of pdf reports automatically, where batch analysis, operational log, and chromatograms are converted into a single document. This Report Set also includes information on manual and automated actions so that any data manipulation leads to documented deviation from a defined procedure that is immediately recognizable (Figure 3).

While still widely used there are some problems associated with a paper-based workflow, such as the time and effort required for printing, checking and archiving of data, storage space issues, and the vulnerability of paper records to destruction or replacement without trace. These can be overcome by use of a computerized system offering the advantage of electronic signatures, thereby obviating the need to print and store paper reports.

Electronic signatures are used for reviewing and releasing reports, for which the original data are consulted at the same time. Overseeing a full project is therefore easier and more reliable than checking through a big pile of printouts.

Conclusion

For users operating in a controlled environment, data management and data integrity is necessary to comply fully with official regulations. Modern data systems aim to support users by providing sophisticated software features to simplify the workflow while ensuring data reliability.

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Practical Applications of Online Liquid Chromatography in Pharmaceutical and Biopharmaceutical Process Development and Manufacturing

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Recent advances in continuous pharmaceutical and biopharmaceutical manufacturing have renewed interest in acquiring process analytical data in real-time. For biologics and small-molecule pharmaceuticals, spectroscopic techniques such as infrared (IR), Raman, and UV–vis have historically been used to monitor reaction kinetics, nutrients and impurity profiling, and reaction progress. While these technologies are beneficial for monitoring specific changes or trends in continuous processes, they do not offer the mass sensitivity, specificity, and selectivity that liquid chromatography (LC) provides. This article describes practical examples of implementing online LC for in-process monitoring of biologics and small molecules with a common goal: the implementation of rapid process analytics for pharmaceutical and biopharmaceutical process development and manufacturing.

Advances in continuous manufacturing for pharmaceutical and biopharmaceutical processes have renewed interest in acquiring analytical data in real-time. Many of the advances in continuous manufacturing have been in response to the Food and Drug Administration (FDA) Pharmaceutical Quality for the 21st Century Initiative to promote modernization of pharmaceutical manufacturing, including elements of quality by design (QbD) and process analytical technology to enable new manufacturing technologies (1–2). The pharmaceutical industry has responded to this initiative by developing continuous manufacturing platforms designed to be more efficient and flexible, and to demonstrate improved process understanding and control. A key element of continuous manufacturing is the ability to monitor a process at a specific location, at any time, at a frequency required to demonstrate the process is in a state of control and the product is of consistent quality. Analytical instruments designed to collect process data and information in real-time have historically been classified as process analytical technology (PAT). PAT has since expanded to include the systematic design, analysis, and control of a manufacturing process (3–4).

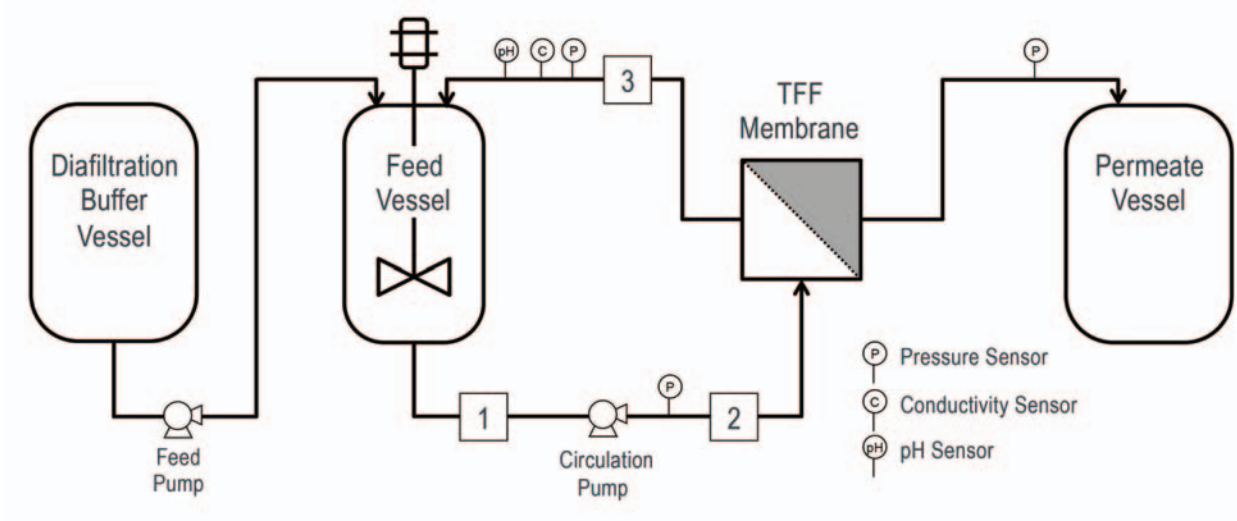
Early applications of PAT for pharmaceutical analysis involved spectroscopic probes (focused-beam reflectance measurement [FBRM], infrared [IR], near infrared [NIR], Raman, UV) to monitor crystallizations, blend uniformity, and reaction kinetics. While these analytical technologies have been effective for monitoring specific quality attributes in pharmaceutical processes, they do not offer the mass sensitivity, specificity, and selectivity of liquid chromatography (LC). Online LC for process monitoring has been demonstrated in the pharmaceutical industry previously, but the instruments were often customized designs built out of necessity to support a specific commercial process (5–9). While implementation of these customized online LC systems was successful, integration into the process was time-consuming, costly, and the instruments were not flexible or portable to support other applications. Recently, online LC instruments have been introduced that provide an integrated and more robust approach to performing online LC (10–13).

In this article, several applications of using online LC to monitor pharmaceutical and biopharmaceutical processes are presented. The applications represent approaches used at two different end-user companies to illustrate the value of online LC as a real-time process monitoring tool.

Sampling Interfaces for Online LC

The sampling interface for any analytical instrument to a process is a critical component for enabling real-time analysis. The “Hippocratic Oath” for sampling any process is “Thou shall not harm the process”. Sampling from a process cannot perturb the process; the volume of sample removed cannot compromise the process to the point that it significantly alters the volume, temperature, reaction kinetics, or composition of the process. This practice can be especially challenging when considering viscous solutions, biphasic mixtures, slurries, suspensions, or other nonhomogeneous mixtures. The ability to pull a representative

Figure 1: Schematic for an ultrafiltration (UF) and diafiltration (DF) unit operation along with sampling locations for online UHP-SEC. Locations include (1) Pre- circulation pump, (2) Pre-membrane TFF feed, (3) TFF retentate. Courtesy of Dr. Mark Brower Ph.D. Merck & Co., Inc., Kenilworth, New Jersey, USA.



sample from the process reproducibly and robustly is critical for successful integration of online PAT. In general, it is undesirable to introduce any material back into a process after a sample is taken because this could increase the risk of contamination for biopharmaceutical processes. For pharmaceutical processes, chemical compatibility, permeability of tubing for air, moisture, or light-sensitive processes, and extractables or leachables should be considered. For biopharmaceutical processes, upstream sampling must also take into account good aseptic practice to ensure contamination is not introduced into the process.

Experimental

The online-LC system used for experiments 1 and 2 was a Patrol UPLC Process Analysis system (Waters) with PDA detection (Waters). Empower 3 software (Waters) was used for all experiments. For experiments 1 and 2 PEEK tubing (Idex) was connected with plastic 3-way tee valve and PEEK to swagelok fitting and ferrule. Figure 3 provides a schematic of the UF/DF setup with sampling locations numbered.

A 4.6 mm × 150 mm, 1.7-µm BEH 200 SEC (Waters) column was used for experiments 1 and 2 with the PDA detector monitoring wavelengths 214 nm and 280 nm. Mobile phase composition included water, 100mM phosphate and 100mM sodium chloride pH 7.0. Method run times were 4.5 min (Experiment 1) and 3.0 min (Experiment 2). Calibration curves using various dilutions of stock mAb were used for experiment 1 and 2.

Experiment 3 used the same on-line LC system connected to an Acquity QDa mass spectrometer (Waters) in place of the PDA detector. PEEK (Idex) was connected from this system to the permeate sampling location. A 2.1 mm × 100 mm, 1.8-µm HSS T3 (Waters) column was used for all experiments. A 2.1 mm × 50 mm, 1.8-µm BEH C4 300 A (Waters) column was used as guard for all experiments to remove hydrophobic species from the cell culture permeate. A column temperature of 40 °C and mobile phase compositions of 10-mM ammonium formate pH 3.7 (MP A) and acetonitrile with 0.1% formic acid (MP B).

Online Ultrahigh-Performance Size-Exclusion Chromatography (UHP-SEC) for Downstream Process Understanding:

The ability to connect process analytical technology, such as online LC, in the downstream biologics purification unit operations, enables advanced process understanding and attribute control during development and manufacturing. This article describes the use of online ultrahigh-performance size-exclusion chromatography (UHP-SEC) to monitor excipients, concentration, and aggregation in the downstream UF/DF unit operation of a monoclonal antibody to support high concentration formulation studies. The initial experiment used a single online LC system sampling from one process location before the UF/DF recirculation pump. The final experiment used two online LC systems sampling in parallel from pre- and post-TFF membrane positions, pre-membrane TFF feed and TFF retentate, respectively.

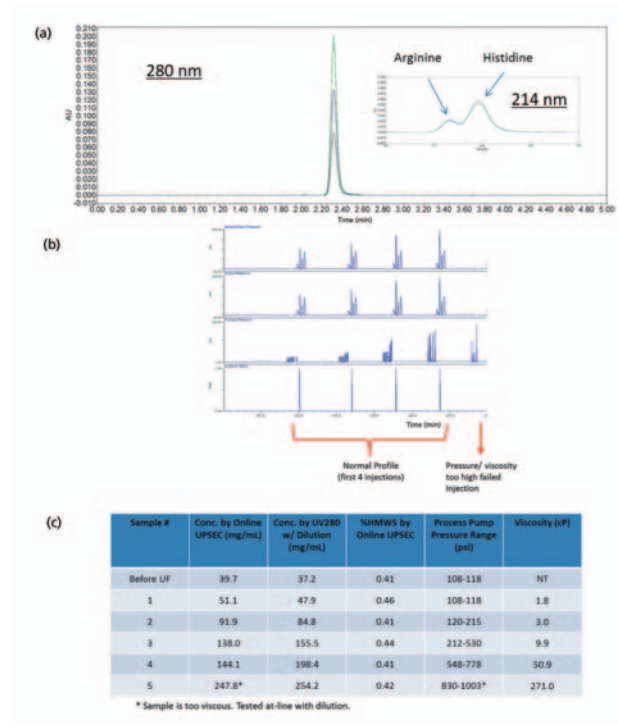
Results and Discussion

Online UHP-SEC was successfully applied for online monitoring of arginine diafiltration, monomer concentration, and aggregation area percentage during ultrafiltration experiments. As described above, Experiment 1 focused on sampling pre-circulation pump (Figure 1, position 1) and on monitoring the diafiltration of arginine and monomer concentration. The online UHP-SEC profile and online concentrations for mAb 2 versus. offline UV (at 280 nm) showed good correlation for each diavolume (DV) tested. Retention times of both arginine and histidine were confirmed with standards. Following the 5 DV buffer exchange the arginine peak area plateaued, indicating the arginine diafiltration was complete. The original experiment called for 8 DV; however, the online LC results allowed for stopping the diafiltration after 6 DV, saving valuable time and more importantly buffer in the experiment. Following successful diafiltration

of arginine, product concentration was increased through ultrafiltration. Figure 2(a) shows UHP-SEC overlays of successful injections with an inlay of the stable excipient peaks. During the fifth injection, the process sampling module used exceeded its maximum pressure of 1000 psi, resulting in system failure. Figure 2(b) shows the process sampling module pressure profile for all injections. Figure 2(c) provides a summary of these results for each online UF sample. The concentration results for each sample correlate well with differences attributed to manual dilution of high viscosity samples. Aggregation results were consistent throughout the experiment demonstrating that the UF/DF process at laboratory-scale did not impact product quality. Viscosity data ranged from 1.8 to >270.0 cP resulting in the increased pressure for the PSM. This online monitoring approach with UHP-SEC was successfully applied to pilot-scale UF/DF experiments.

Experiment 2 for UF/DF focused on sampling from positions pre- and post-TFF membrane (Figure 1 positions #2 and #3). Two online LC systems were connected to the process in parallel, with the goal of monitoring sheer-induced aggregation from the UF/DF pump or TFF membrane. Figure 3(a) shows product concentration for mAb2 pre-membrane TFF feed and TFF retentate. Retentate data are plotted against offline concentration measurements, with UV at 280 nm. The product concentration difference of approximately 5mg/mL pre- and post-membrane was an interesting observation not commonly measured during process development but easily automated with online LC. Figure 3(b) shows pre- and post-TFF membrane UF concentration and aggregation data. Good correlation is observed between online and offline concentration results for the pre-TFF membrane samples. Aggregation was not observed in any samples until the concentration increased past 40 mg/mL. Above this concentration low levels of aggregation were observed in online samples only with slightly higher aggregate amounts post-TFF membrane. During online UHP-SEC, the time from dilution to analysis was only a few seconds while offline SEC results were achieved within 30 min of sampling and dilution. Transient aggregation is suspected and

Figure 2: (a) Overlay of four online UHP-SEC profiles from ultrafiltration samples with inlay of stable arginine and histidine concentrations. (b) Process sampling module pressure profile highlighting four successful injections until pressure failure at fifth injection as a result of high concentration and viscosity. (c) Online UHP-SEC ultrafiltration sample concentration, purity, and viscosity results with PSM pressure ranges.



Sample #	Conc. by Online UPSEC (mg/mL)	Conc. by UV280 w/ Dilution (mg/mL)	%HMWS by Online UPSEC	Process Pump Pressure Range (psi)	Viscosity (cP)
Before UF	39.7	37.2	0.41	108-118	NT
1	51.1	47.9	0.46	108-118	1.8
2	91.9	84.8	0.41	120-215	3.0
3	138.0	155.5	0.44	212-530	9.9
4	144.1	198.4	0.41	548-778	50.9
5	247.8*	254.2	0.42	830-1003*	271.0

* Sample is too viscous. Tested at-line with dilution.

Figure 3: (a) Product concentration for mAb2 pre-membrane TFF feed and TFF retentate during DF. Retentate data are plotted versus offline concentration measurements with UV280, (b) shows pre-membrane TFF feed and TFF retentate UF concentration and aggregation data.

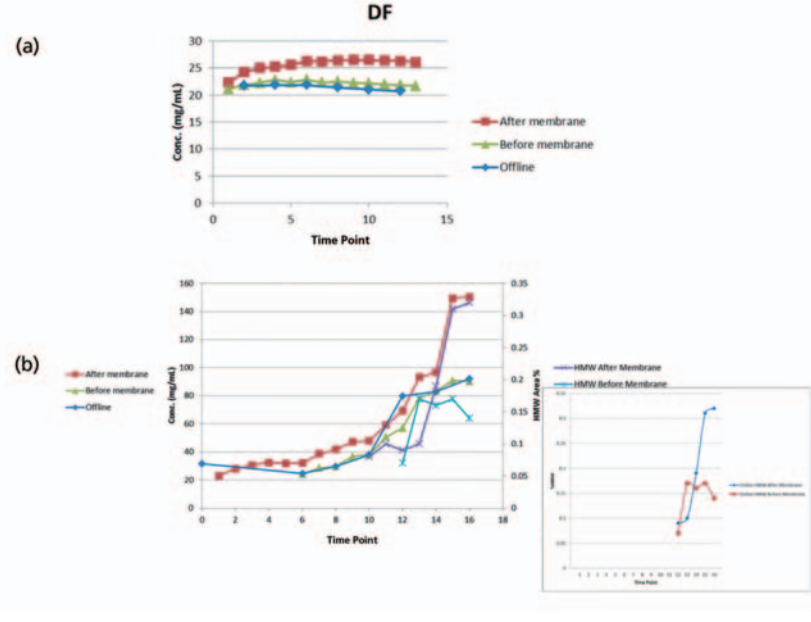
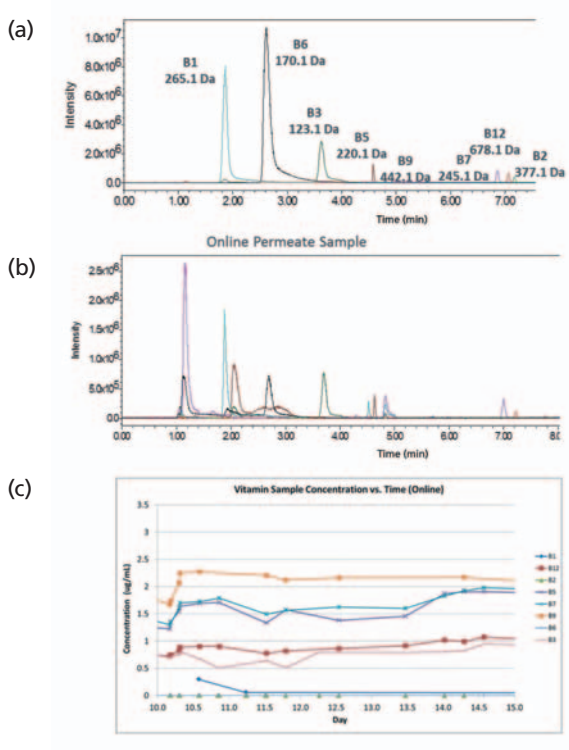


Figure 4: (a) SIR overlay of vitamin B standards with mass in daltons at 1 µg/mL following 10× dilution with an online LC system. (b) SIR overlay of vitamins from online permeate samples for retention time comparison. (c) Online concentrations of water-soluble B vitamins over a 5-day period using SIR.



additional process characterization studies are ongoing for molecules with higher levels of aggregation. Online UHP-SEC provided a powerful PAT tool to support process development and understanding for UF/DF. The ability of UHP-SEC to provide concentration, excipient, and purity information in a single analytical run is a vital method within the bioprocess analytical toolkit.

Online LC–MS for Vitamin Monitoring of Permeate

Media development for cell culture is a critical area of research for bioprocess development. Chemically-defined media are comprised of hundreds of components, with many important for optimal cell growth and productivity for maximizing product titer and quality. Common nutrient components include amino acids, vitamins, inorganic salts, and trace metals. The complexity of chemically-defined media is the ultimate analytical challenge and is complicated further in spent cell culture. This article introduces online LC–MS for monitoring water-soluble B vitamins in permeate from a continuous biologics manufacturing process.

Results and Discussion

This work demonstrates initial success for quantitative online monitoring of eight water-soluble vitamins from permeate samples of a continuous biologics manufacturing process at laboratory-scale. Figure 4(a) shows the SIR overlay for the eight B vitamin standards at 1 µg/mL following 10× dilution. Figure 4(b) shows SIR overlays from an online permeate sample. Additional peaks were observed from the permeate matrix highlighting the need to use both retention time and SIR mass for the vitamin quantitation in the complex permeate matrix. The method was applied to monitor vitamin levels in permeate samples online over a 14-day period; days 10–15 are highlighted in Figure 6(c). Vitamins B1 and B6 did not have good linear regression and required quadratic fitting for quantitation. Vitamin B2 was not detected while Vitamin B6 was observed but below the limit of quantitation (LOQ) for the method (B6 LOQ = 1 ng/mL), indicating that these components were consumed in the culture and may be limiting metabolism. Method development is still underway for alternative guard column options and to improve the dynamic range of the assay. Additional effort will focus on other nutrients to support both feedback control of limiting components and spectroscopy model validation efforts.

Monitoring Continuous Pharmaceutical Processes with Online Liquid Chromatography

In the absence of in-process testing of isolated solids in continuous pharmaceutical processes, online LC is a critical PAT tool for enabling process understanding and control in small molecule continuous manufacturing. Online LC has been deployed across numerous continuous processes at Eli Lilly and Company, measuring reaction completion, monitoring assay and impurity profiles of synthetic processes, and chiral purity across a variety of continuous manufacturing platforms. Sampling of the continuous manufacturing processes was performed with this system or by an in-house designed dilution cart (14) connected to the system. The following examples highlight several pharmaceutical applications of online LC for monitoring continuous processes.

Continuous Monitoring of an Iridium-Catalyzed Reductive Amination Reaction with Online LC

Measuring quality attributes during a continuous process is critical during startup transitions and steady state operation of the process. During the development and scale-up of a continuous iridium catalyzed high-pressure reductive amination process, online LC was implemented to monitor conversion and track a key impurity throughout development and a 24-day GMP campaign that produced two metric tons of the penultimate intermediate for the process (15). Online LC analyzed the product solution exiting the 360 L pipes in series reactor every 30 min for the duration of the campaign. Product solution was collected in tanks that filled over a 24-h period, enabling a composite sample to be analyzed offline for approval to forward process material. Online sampling, quenching, and dilution of the process samples was performed using a dilution cart and delivered to the instrument used for online LC analysis. Figure 5(a) shows the online and offline LC results throughout

the duration of the campaign. While the online LC data were not used to make forward processing decisions for this process, the area percent of the product, residual starting material, and key process impurity were trended throughout the campaign providing invaluable information on the health of the process. The X for each data series in Figure 5(a) represents the offline LC analytical result generated from the product solution can collected every 24 h, demonstrating excellent agreement between the online and offline results. The strong agreement between these data led to a higher confidence in the use of online LC as a PAT tool for monitoring continuous pharmaceutical processes.

An additional concern with using online LC for this process was the duration of processing required to meet the projected material delivery for this product, potentially requiring the reductive amination reaction and online LC to operate continuously for up to six months. Continuous operation of an LC system for six months is not routine practice and generated a significant amount of concern in terms of instrument and method robustness, in addition to routine monitoring of mobile phase, diluent, and solvent waste. In order to determine if online LC could support a continuous process for six months, an experiment was designed to assess instrument robustness over an extended period. A development batch of product solution was prepared and used to emulate the process solution, and a sample set method was configured where the online LC system would sample, quench, dilute, and analyze the product solution every 30 min. Figure 5(b) shows the data collected over a six month time period using an online LC system, with the red lines labelled A–E indicating five shutdowns of the online LC that occurred over the six-month experiment. Shutdown A was investigated after an alarm was generated on the binary solvent pump. A failure of the check valve on channel B of the pump was quickly diagnosed and repaired. Shutdowns B–E were all operator error-induced shutdowns from the instrument depleting the mobile phase before it was replenished resulting in a low pressure alarm. After refilling the mobile phase and priming the pump, restart of the pump

Figure 5: (a) Online and offline LC data over 24 days of continuous processing. (b) Online LC robustness study over six months of continuous instrument operation.

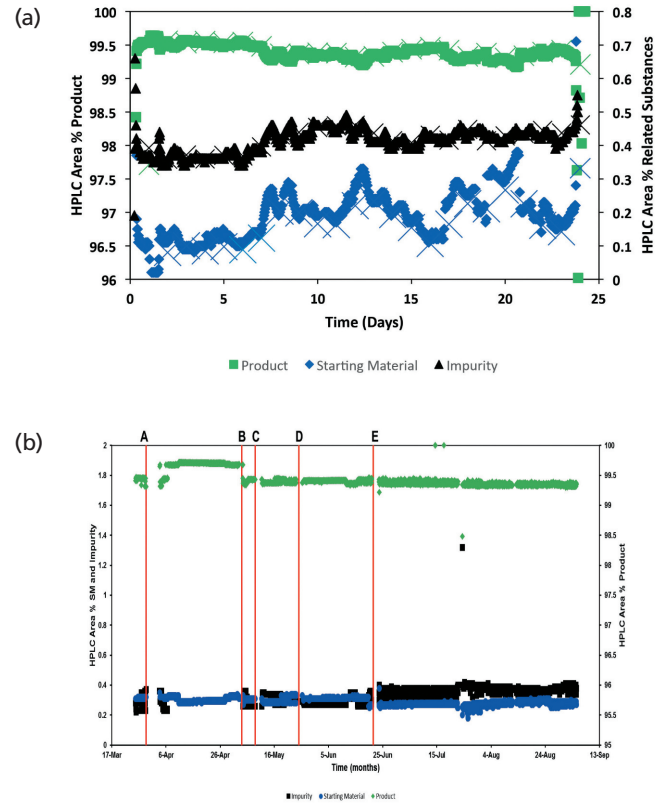


Figure 6: (a) Online LC data for area % product, impurities vs. time for step one. (b) Online LC data for area % product, impurities vs. time for step two. (c) Online LC data for area % product, impurities vs. time for step three. Adapted with permission from reference 16.

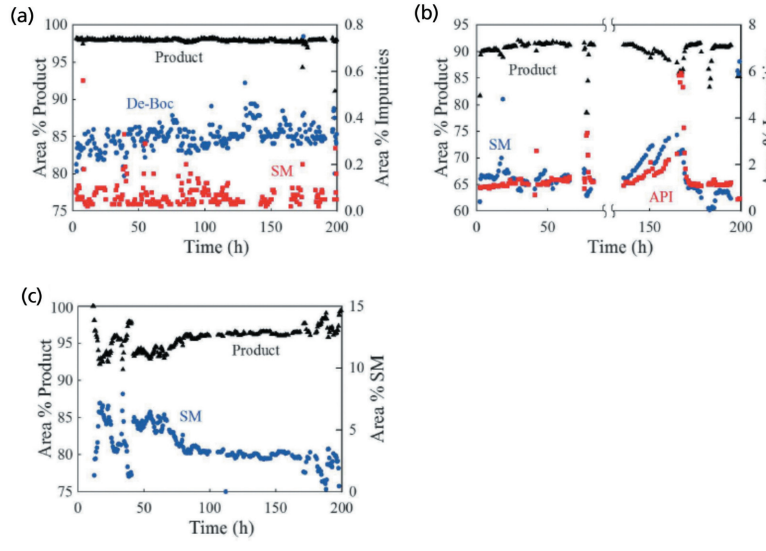
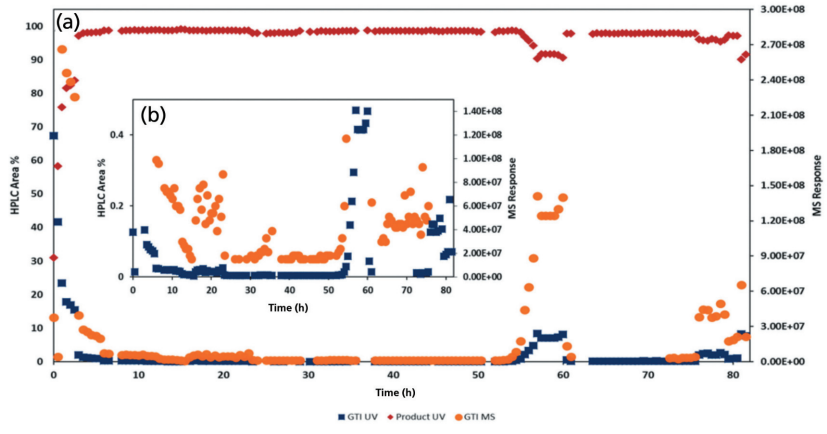


Figure 7: (a) Online LC–MS data over 80 h of continuous processing and (b) enlarged view of UV and MS response.



generated a high-pressure alarm for shutdowns B–E, requiring replacement of the LC column. While a true system suitability was not run on a regular basis for this experiment, the results support continuous operation of online LC for extended periods, and for this experiment operator error played a greater role than instrument failure in instrument downtime.

Online LC Monitoring for a GMP Multi-Step Continuous Flow Process

Online LC was used to monitor the product solution of three steps of a multi-step continuous flow process (16). All three steps used plug flow reactors (PFRs) with step 1 running a condensation reaction, step 2 a nucleophilic aromatic substitution (S_NAR) reaction, and step 3 a deprotection reaction. The product solution for each step was monitored by online HPLC over 200 h of continuous operation. Online sampling, quenching, and dilution of the process samples for each step was performed using a dilution cart and delivered to the online LC system for online LC analysis. Online LC, in conjunction with refractive index sensors and parametric controls including, pressure, temperature, and mass flow rates, enabled minor adjustments to the process to maintain a state of control throughout production. Online HPLC data for the step 1 condensation reaction in Figure 6(a) shows the consistent product quality achieved as the process remained in a state of control throughout production. The start-up of the step 2 S_NAR reaction went smoothly, but halfway through the process the online HPLC had a pump failure, taking the instrument offline (Figure 6[b]). After the instrument was back online, the online LC system immediately detected a disturbance in the step 2 process. The product solution exiting the step 2 PFR showed elevated levels of starting material and active pharmaceutical ingredients (API) (~150 h mark), triggering a pause in processing and an offline analytical investigation. The offline investigation indicated an evaporative loss of base in the step 2 feed stream, which had occurred over time, resulting in a pH imbalance in the step 2 S_NAR reaction and corresponding increase in impurities shown in Figure 6(b). In order to remove the elevated impurity step 2 product from the process, the step 3 deprotection reaction was started to empty the step 2 product solution surge tank prior to restarting the step 2 S_NAR reaction. After the restart of the SNAR reaction, the step 2 product solution returned to the same quality as before the process disturbance, indicating the process was again in a state of control. Figure 6(c) shows the performance of the step 3 deprotection reaction with trending of the online LC data indicating the process was in a state of control. While online LC data were not used for forward processing decisions in this work, the detection of the disturbance in step 2 is an example of detecting special cause variation by online LC, and ultimately it minimized the amount of step 2 product at risk by detecting the process disturbance.

Online LC–MS for Trace Impurity Monitoring

A critical attribute of any chromatographic method is the ability to detect trace-level impurities for a given sample. The ability to perform trace analysis by online LC–MS was explored for a multi-step continuous process where intermediates and their related substances were identified *in silico* as potential genotoxic impurities (GTI). The four-step continuous process included a Suzuki-Miyaura cross-coupling (step 1) (17), hydrogenolysis reaction (step 2), amide bond formation (step 3), and thermal deprotection (step 4) to form the API (18). An online LC system equipped with a mass spectrometer was used to monitor the product solution exiting the step 3 PFR. Online sampling, quenching, and dilution of the process samples was performed using a dilution cart and delivered for online LC–MS analysis. Step 3 was identified as a key control point for this process since the stoichiometric ratio of the feed solutions entering the reactor are key to reducing excess substrate and in-process impurity formation. One of the starting material feeds for step 3 is the step 2 aniline product solution, a known GTI. While the LC–UV method was capable of detecting this impurity to an appropriate alert level, we were interested in evaluating the capabilities of MS as a qualitative online detector for detecting trace-level impurities. Figure 7(a) highlights the online LC–MS data obtained during the 80-h development campaign. Online LC–UV and LC–MS data monitored the step 2 product for the duration of the campaign. Elevated levels of the step 2 product detected by online LC–MS were attributed to plugs in one of the step 3 reaction feeds, resulting in a change in the stoichiometry and an increase in residual step 2 product. Furthermore, the data in Figure 7(b) illustrate that the MS system detected residual step 2 product at several time-points where the online LC–UV did not detect a peak, providing an additional level of sensitivity for online analysis. Continued reaction of the substrates in the product surge tank combined with dispersion and downstream rejection efficiency in the step 3 and 4 crystallizations provided adequate control of residual step 2 product. Samples from each step 3 product can were analyzed offline using a quantitative LC–MS method.

Table 1: LC–MS results comparing offline and online MS instruments

Product Can	Offline MS Result (ppm)	Online MS Result (ppm)
1	318	264
2	137	118
4	107	89
5	111	90
6	167	136
7	124	104
8	186	172

* Product can 3 was not tested.

The same samples were also analyzed offline (at-line) using an online LC system connected to a mass spectrometer to compare the data sets from the quantitative and qualitative LC–MS methods, respectively. Table 1 shows consistent results for residual step 2 product in the tested product cans, demonstrating that online LC–MS is a viable PAT tool for monitoring trace-level impurities in a continuous process when compared with offline analysis.

Significant growth has occurred for the use of online LC–UV and LC–MS as a PAT tool in pharmaceutical and biopharmaceutical development and manufacturing (5–13). The selectivity and specificity provided by online LC enable the monitoring of product quality during production, leading to advances in process understanding. As the pharmaceutical and biopharmaceutical industries transition towards continuous manufacturing, implementation of tools, such as online LC, will be a core component to the overall analytical control strategy helping to streamline drug disposition. Continued technological advances are still needed to support online LC implementation within pharmaceutical manufacturing. These technology needs include advanced sampling interface systems, robust clarification platforms, "plug-and-play" software interfaces, and development of an online 2D-LC system to enable multidimensional separations within the PAT toolkit. In addition, advances in analytical methodology, including column and stationary phase technologies to provide the platform for rapid, robust PAT-focused chromatography assays, will enable a transition from process monitoring with online LC to advanced process control.

Acknowledgements

Merck & Co., Inc., Kenilworth, NJ, USA, Acknowledgements: The author would like to thank the research labs of Merck & Co., Inc., Kenilworth, New Jersey, USA, and specifically the bioprocess development and process analytical technology departments for support and investment in online LC. Special thanks to past and present colleagues from both biologics process development and analytical directly involved in the case studies presented above: Zhi Chen, Xiaodun (Susan) Mou, Jun Heo, Allison Chinsee, Mark Brower, Bill Napoli, Nuno Pinto, David Pollard, Shenjiang Yu, Daisy Richardson, Patricia Rowicki, InKwan Han, Jennifer Pollard, Collette Cuttler, William Rayfield, Nihal Tugcu, and Gregg Nyberg. A special thanks to Bhumit Patel for his technical input and critical review of this manuscript.

Eli Lilly and Company Acknowledgements: The author would like to thank the Small Molecule Design and Development organization and Lilly Manufacturing in Kinsale, Ireland, for their continued support of online LC. Special thanks to the SMDD management team of Bret Huff, Paul Collins, and Derek Berglund for their vision, support, and investment in online LC. The case studies presented in this article involved contributions from numerous Lilly scientists, with significant contributions from Gordon Lambertus, Bradley Campbell, Michael Miller, Mindy Forst, Martin Johnson, Brandon Reizman, Justin Burt, Molly Hess, Wei-Ming Sun, Luke Webster, Jennifer Groh, Richard Spencer, Scott May, Kevin Cole, Tim Braden, Joel Calvin, Tim White, Ian Sherlock, Paul Desmond, Ken Desmond, and Noel Sheehan.

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Supercritical Fluid Chromatography in the Pharmaceutical Industry: **Implementation in Development and Quality Control**

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Supercritical fluid chromatography (SFC) is a well-established analytical technique used in the pharmaceutical industry for decades. However, it is still considered a new technique in some areas, for example, implementing the technique for purity profiling in late-stage development and production. In pharmaceutical analytical departments, SFC serves a wide variety of purposes, including compound purification, purity profile, and chiral analysis. Depending on the phase of drug development, the analytical performance requirements, such as speed of analysis, efficiency, and sensitivity, may vary considerably. The end goal is to provide robust, reliable and transferable analytical SFC methods. The challenges for future development and widespread implementation of SFC and the implementation of SFC in quality control (QC) laboratories using modern instrumentation are also discussed.

Product development life cycle in the pharmaceutical industry can be divided into three distinct phases: discovery, development, and registration or manufacturing. At each stage, supercritical fluid chromatography (SFC) has been implemented for several important applications.

Each of these phases has different requirements for performance attributes of separation techniques used to support product development. In the discovery phase, where very large numbers of compounds are screened against multiple targets, the emphasis is on selectivity and speed of analysis. For the next phase, where the stability of the active ingredient and formulation of the product are established, the emphasis shifts more towards high resolution and high sensitivity. This stage involves development of synthetic route and formulation development. The emphasis on high resolution and sensitivity is a result of the need to be able to separate a large number of components and quantification at low levels, for example, 0.05% by area. Finally, during preparation for registration application and transfer to manufacturing facilities, the key attribute of an analytical method is its focus on robustness, reliability, ease of operation, and transferability of the method. In the case of less popular analytical technology, such as SFC, the availability of instrumentation and relevant skill sets in quality control (QC) laboratories become additional requirements.

The intrinsic nature of supercritical CO_2, with its low viscosity, high density, and relatively low toxicity (compared to other supercritical substances), makes it an ideal mobile phase for certain types of chromatographic applications. Amongst these, it is known to be very efficient for fast separations as well as isolation and purification of compounds. Both of these applications are well established in the drug discovery phase (1,2). For example, the use of carbon dioxide-based mobile phases for preparative chromatography allows clean and rapid recovery of the purified compounds, with dramatic savings of organic solvent, energy, time, and overall cost (3). Another attractive feature of SFC is a higher success rate in chiral method development when compared to other chromatographic modes (normal-phase or reversed-phase LC) (4,5). This makes it the technique of choice for many chiral separations (6,7).

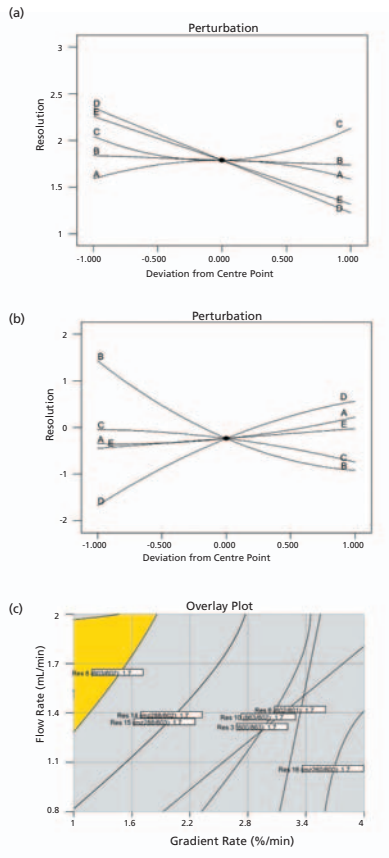
Despite the introduction of commercial instruments in the 1980s, in the development and subsequent phases of drug development, SFC is still regarded primarily as a research tool rather than a routine technology. Consequently, SFC methods are rarely implemented throughout later stages of development and are, equally, rarely used in registration applications.

Important advances in understanding and controlling SFC separations for purity profiling have been made in the past 15 years: the high efficiency and the modelling of selectivity were fundamental in determining the potential and limitation for using SFC for purity profiling (8–14).

The relatively slow penetration of SFC into the manufacturing QC area was mainly a result of real, or sometimes perceived, negative user experiences with former instrument reliability, complex technology transfers, and lack of method robustness.

In this article, we assess the transferability of an SFC method across modern SFC instruments from three different vendors with the aim of assessing some of the performance

Figure 1: Relationship between resolution and instrument settings: factor dependence of resolution of critical pair 11 (a) and of critical pair 8 (b). (c) The optimal robustness separation conditions are set within the area delimited in yellow.



attributes relevant to registration application activities and transfer to manufacturing environments, rather than comparing the performance of the instruments.

Experimental

Instruments and Methods: System 1 was equipped with an autosampler with a 10-µL loop and a diode array detector (DAD) with a 10-mm high pressure flow cell.

System 2 was equipped with an autosampler with a 100-µL loop and a DAD with a 3-mm high pressure flow cell.

System 3 was equipped with an autosampler with a 5-µL loop and a DAD with a 10-mm high pressure flow cell.

Chiral Method 1: The column used was a 150 mm × 3.0 mm, 3-µm Chiralcel IC-3 (Chiral Technologies Europe) (column 1). HPLC-grade methanol with 10 mM ammonium formate (Fisher) was used as organic modifier, 1.9 mL/min flow rate. Gradient programme: 5% (hold 0.5 min) to 20% in 30 min, then to 45% in 35 min. Outlet pressure was set at 105 bar, temperature was 50 °C. Sample was prepared at 1.0 mg/mL in methanol. Detection was set at 275 nm with compensation reference from 310 nm to 410 nm on instrument system 1.

Chiral Method 2: The column used was a 450 mm × 4.6 mm, 3-µm Chiralcel IC-3 (Chiral Technologies Europe) (column 2), organic modifier was HPLC-grade methanol, 3.0 mL/min. Gradient programme: 2% (hold 1.0 min) to 34% in 32 min. Outlet pressure was set at 120 bar, temperature was 25 °C. Sample was prepared at 2.0 mg/mL in dichloromethane. Detection was set at 232 nm, with reference from 440 nm to 540 nm (injection volume was 10 µL on system 1, 30 µL on system 2 to compensate for a shorter DAD flow cell, 5 µL on system 3 in full loop).

Discussion

For SFC to be considered a technique to support registration applications and be transferred to a manufacturing environment, it must satisfy certain attributes that fall into four broad categories:

- Applicability
- Robustness
- Validation
- Transferability.

Applicability: During method development, it must be demonstrated that SFC offers clear advantages over more established techniques, such as LC. This could be, for example, better resolution, better peak shape, and better compatibility with sample components (stability). The field of SFC applications is expanding and it covers a wide range of chemical structures with different polarities and applies to structurally similar compounds, such as stereo- and positional isomers (15–19). In drug development laboratories, SFC has become a technique of choice for chiral separations, when orthogonal selectivity is required, but also for the separation of compounds that are unstable or insoluble in aqueous solutions (20–22).

For example, a normal-phase LC method successfully implemented in a QC laboratory was used for the chiral analysis of an intermediate in the synthesis of a new product. This method, however, exhibited some challenges, such as very long equilibration time and the use of the mobile phase containing *n*-hexane (safety implications) and HPLC-grade ethanol (cost, a controlled solvent, and not always readily available). A newly developed chiral SFC method (chiral method 2) offered several advantages: first, a straight linear gradient with pure methanol as mobile phase modifier; second, instrument and chromatographic separation setup was much simplified, resulting in easy operation; and, finally, improved resolution of all potentially interfering compounds maximized method robustness. An added advantage was the compatibility with mass spectrometry (MS), which allowed simple peak identification.

Robustness: During robustness testing, the ability to separate and quantify required components must be shown and should remain unaffected by changes in operating parameters. This must also be true for chromatographic consumables, such as batch-to-batch column variability.

In order to achieve maximal method robustness, the development had to be approached in a systematic manner as detailed below:

Column and Mobile Phase Choice: The most suitable column should be chosen in a systematic approach, usually achieved through screening a small number of columns with different selectivities. This must be performed together with mobile phase selection because the latter also has a significant impact on resolution. In this case column 1 was selected as the chiral selector because it provided optimal peak symmetry and higher resolution with methanol as organic modifier. When it comes to mobile phase selection, simple composition is preferred where possible. Ammonium formate was used as an additive to methanol. Ammonium formate could be used instead of a isopropylamine and trifluoroacetic acid mixture that is often used during column screening.

Separation: For separation development, a sample mixture that contains all relevant components

Table 1: Values of *s/n*, % RSD of retention times (t_R), and % RSD or area of a reporting limit solution (0.05% of nominal concentration)

	System 1			System 2*			System 3**		
	S/N	t_R RSD%	Area RSD%	*S/N*	t_R RSD%	Area RSD%	*S/N*	t_R RSD%	Area RSD%
Intermediate	29.5	0.03	2.26	14.8	0.09	2.23	10.4	0.09	4.87
Enantiomer	18.7	0.05	1.22	8.3	0.04	1.97	5.0	0.13	4.62

*: The injection volume was 30 µL, to compensate for the 3 mm DAD compared to other instruments
**: The sample loop of system 3 was 5 µL, which means that the column loading was half compared to systems 1 and 2 (10 µL as per method).

that may interfere with the analytes of interest must be used if available. A multifactorial design of experiments (DOE) with three levels of response surface (26 runs plus 3 centre points) was chosen to model the retention and separation of each component (retention time and resolution). Modelling the relationship of retention and resolution from DOE experiments (Figures 1[a] and 1[b]) allows selection of optimal operating parameters to maximize method robustness (Figure 1[c]).

Detection: In order to achieve optimal sensitivity and reduce baseline noise, UV detection settings must be optimized together with consideration for sample loading and its impact on peak shape and resolution.

Figure 2 shows the relative chromatogram obtained (Chiral Method 1).

Validation: Any new technology used to support registration applications must meet established validation criteria, for example, ICH Q2 R1. There are numerous literature reports describing that SFC under optimal operating conditions is perfectly capable of achieving this requirement (23,24).

Transferability: Before using an analytical application in a manufacturing environment, it must be transferred to a receiving laboratory. This is typically performed under a standard analytical transfer protocol. In the case of SFC, suitable instrumentation and appropriate skills for operation must be available at the receiving laboratory.

A recent study tested the performance of a method for the impurity control of salbutamol in 19 different laboratories. The method was run on a single technology platform and the statistical analysis of the quantitative results reported reproducibility equivalent to LC methods (25).

To demonstrate the suitability of modern SFC instruments, the chiral method described previously (see "Applicability" section), which was developed on System 1, was used. Validation criteria, which are often affected by instrumentation, such as specificity, sensitivity, and precision, were used to demonstrate performance and suitability of all of these systems.

Specificity: It was demonstrated that the specificity of the chiral SFC method described in "Applicability" section (Chiral Method 2) was preserved when this method was replicated on three different columns and three different instrument platforms (Figure 3). We believe this was a direct consequence of systematic method development.

Figure 3 shows the overlaid chromatograms of the chiral analysis of the racemic mixture and a laboratory sample analyzed on (a) System 1 on column 2a, (b) on System 2 on column 2b, and (c) on System 3 on column 2c.

Sensitivity: The reporting limit (set at 0.05% of the nominal concentration) needs to be higher than the limit of quantification (LOQ), which is satisfied with a signal-to-noise (*S/N*) ratio >10 or with a RSD <10% on at least six injections.

In Table 1, we report the average *S/N* values and the RSD% on area on six repeated injections, for both intermediate (main) and enantiomer peaks. System 1 showed higher values of *S/N* values compared to other systems for this analysis (The method was first developed on System 1). Lower *S/N* values were obtained on System 2, however, RSD% on area were comparable and were satisfactory with suitability requirement (<10%). Also, on System 3 lower *S/N* values were obtained compared to System 1: it must be noted, though, that the sample loop on System 3 was 5 µL, which means that the column loading was half compared to System 1 and System 2 (10 µL as per method). Also on System 3 RSD% on area were lower than 10% and therefore satisfied the suitability requirement (<10%). The data demonstrate that all instruments are capable of performing analysis compatible with GMP requirements.

Precision: Precision is one of the system suitability tests necessary for GMP applications and is therefore one of the most important parameters to evaluate modern SFC instruments.

The reported values of RSD% for retention time and area on the analysis of the racemic mixture are provided in Table 2. Figure 4 shows the overlay of ten repeated injections of a laboratory sample on three different vendors' instruments: (a) System 1, (b) System 2, and (c) System 3.

The data show comparable values on all instruments, with RSD% of area around 0.5% for both intermediate and undesired enantiomers (NB: for comparison only, Table 2 provides additional data relative to process-related impurities [PRIs] present in the sample. These were not quantified with the described method, but on a dedicated reversed-phase LC method).

Because of difference in performance, primarily sensitivity, it is not guaranteed that a method that is developed on one system can be successfully transferred to a system from a different vendor, or with a different configuration or setup. It is recommended that development and transfer are planned and performed on the same technology platform. Otherwise, it is highly recommended to introduce the necessary adaptation to the method (for example, a higher sample concentration) to ensure successful transfer on a

different instrument at the receiving laboratory. The knowledge of the instrument vendor or configuration at the receiving end is essential. The necessary modification to the method should be already accounted for during the development phase. This approach is no different than for any other LC method.

Implementation New Analytical Technology in a Manufacturing Environment: Equipping a QC laboratory with a "new" technology has financial implications: not only the initial capital expenditure, but also long-term service, maintenance, and running costs need to be budgeted. These costs need to be offset against the number of samples that will be run by SFC throughout the year. Any instrument fault may generate a downtime that could represent a bottleneck when release of product is due and may result in a delay. This creates much more of an impact when there is only one instrument available. The availability of multiple systems may not be justified or financially viable for a relatively low utilization of SFC, compared to other technologies.

However, the return on investment resides with a faster sample turnaround as a result of increased simplicity and speed of the method, and lower instrument downtime as a result of a more robust application.

The implementation of SFC into a laboratory needs to take into consideration the laboratory infrastructure. The delivery of CO_2 needs to be risk assessed and this should include evaluation of the supplier, the storage and handling of the cylinders and pump (for external supplies), and the location of the instrument to ensure adequate venting (fume hood or air circulation).

Figure 2: Chromatogram describing the separation of the undesired enantiomer from the API and all other interfering impurities including diastereomers.

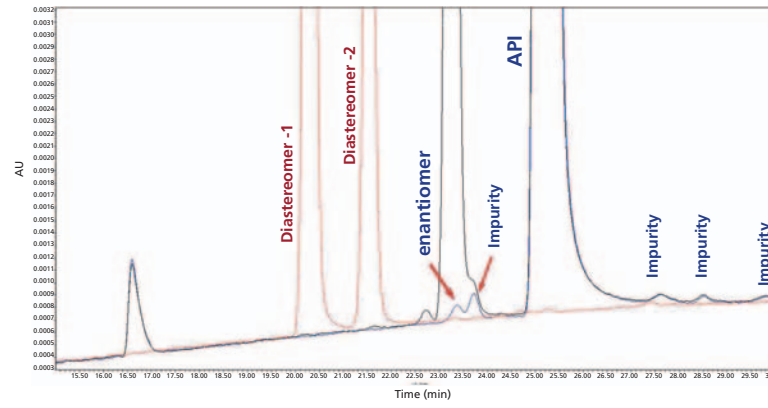


Figure 3: Overlaid chromatograms of the chiral analysis on a) System 1 on column 2a (black: racemic mixture, blue: laboratory sample), b) System 2 on column 2b (red: racemic mixture, blue: laboratory sample), and c) System 3 on column 2c (red: racemic mixture, black: laboratory sample).

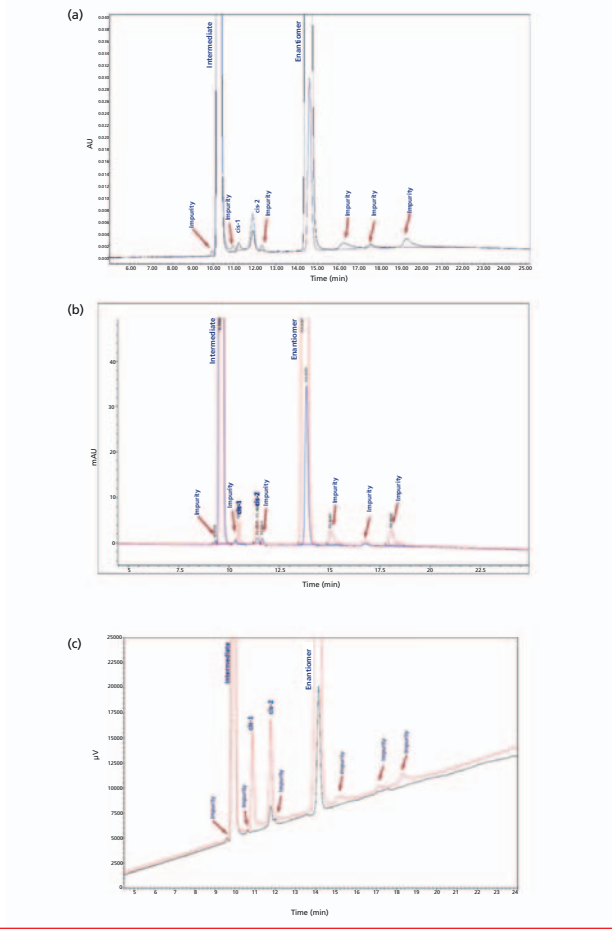


Table 2: % RSD of retention times (t_R) and % RSD of area of a system suitability mix (racemic mixture)

	S/N	%RSD on t_R	%RSD on Area
System 1	Intermediate	0.1	0.46
	cis-isomer 1	0.09	1.21
	cis-isomer 2	0.08	0.7
	Enantiomer	0.04	0.45
	Imp4	0.09	2.51
	Imp5	0.05	2.54
	Imp6	0.06	2.65
System 2	Intermediate	0.06	0.52
	cis-isomer 1	0.06	0.73
	cis-isomer 2	0.06	0.69
	Enantiomer	0.08	0.46
	Imp4	0.06	2.44
	Imp5	0.09	1.93
	Imp6	0.05	2.02
System 3	Intermediate	0.09	0.52
	Enantiomer	0.04	0.51

Staff training is probably one of the most important aspects that needs to be taken into account when equipping a laboratory with SFC. Adequate training is provided by most vendors and analysts are quickly enabled to use the instruments and run a method as received. Most modern systems have automated start-ups and are controlled by software that analysts may already be familiar with if they are used to running LC.

Troubleshooting may be more complicated at first because of unfamiliarity with the technique, but this will become simpler with time and greater experience with the technique. Support from vendors and subject matter experts is essential to facilitate the initial implementation and longer term running of the technique. Effective training and support should mean that SFC will become no different to LC in its longer term routine application.

Conclusions

Modern SFC technology is suitable for QC analysis in regulated environments. Systematic science-based method development is essential to ensure that SFC methods successfully pass validation and transfer to QC laboratories. In particular, SFC should be selected for the right application where it provides a higher degree of speed, robustness, and simplicity over alternative or more established techniques.

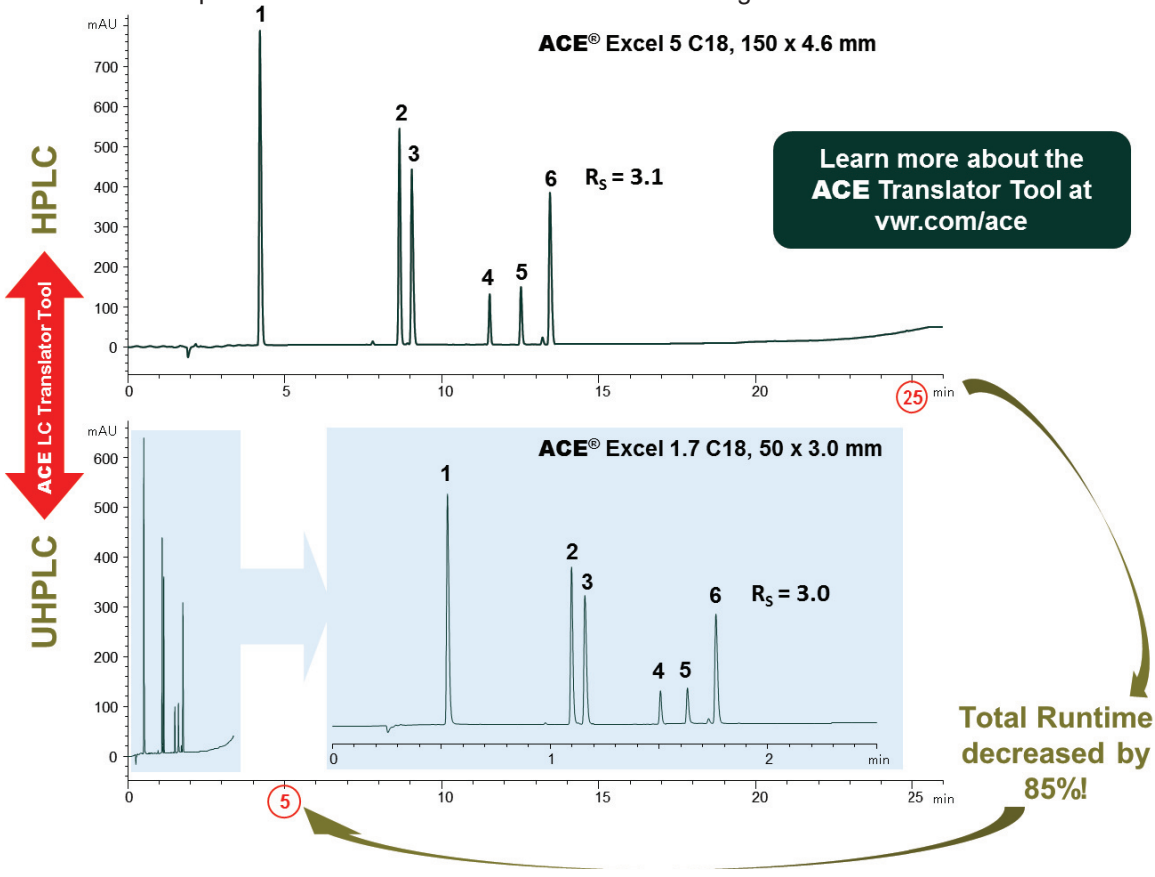
Method transfer among different instrument platforms carries risks (as with any technology). It is advisable to know the differences in the instruments (for example, sensitivity, instrument dispersion, as well as operating parameters and limits typical of each system, such as temperature range, flow, and pressure limits) and to anticipate these differences during development to forecast the necessary adaptations, such as injection volume, and sample concentration.

The value and the challenge in the implementation of SFC in a QC laboratory go beyond the performance of a method or an instrument and associated financial commitment, the investment has to include adequate and appropriate staff training.

The development of an SFC method and its transfer to QC laboratories

Practical Ultra-High Performance Liquid Chromatography (UHPLC)

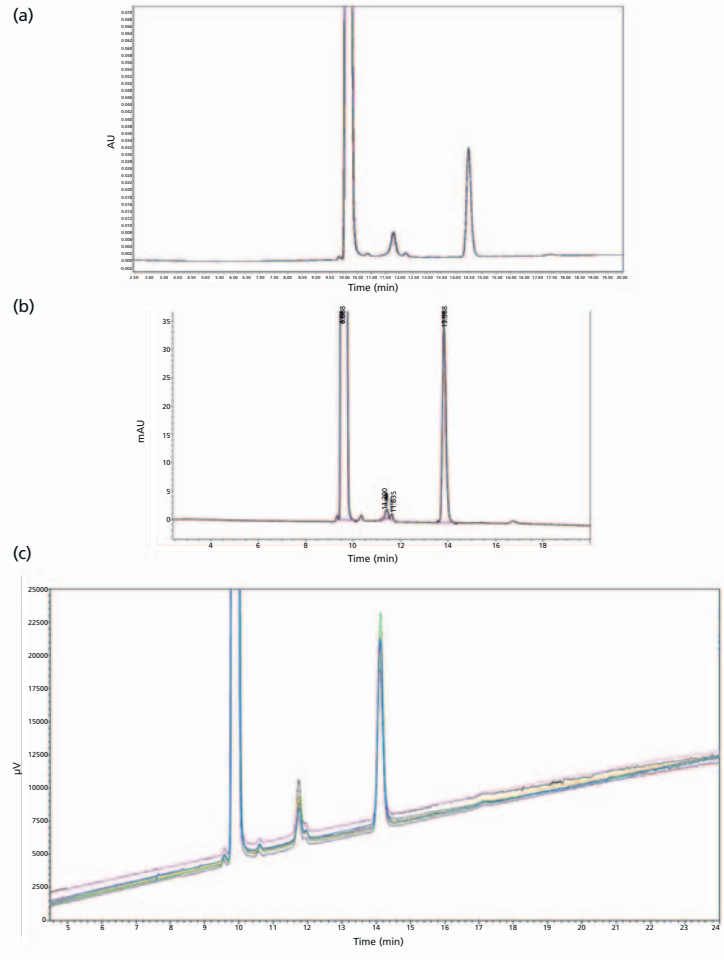
The potential to increase chromatographic efficiency and resolution along with significant savings in solvent cost and analysis time have driven the uptake of UHPLC to many application areas. This discussion outlines how instrument and column technologies continually evolve to meet the requirements of UHPLC, providing new options for chromatographers. Example data are provided to show the high speed and high resolution options of UHPLC. Advanced topics such as HPLC to UHPLC translations using free downloadable tools are also covered.



Top: separation of six peptides on an ACE Excel 5 C18 HPLC column installed on an HPLC instrument. **Bottom**: the same separation translated to an ACE Excel 1.7 C18 UHPLC column installed on a VWR Hitachi ChromasterUltra Rs UHPLC system.

The method translation was achieved using the ACE LC Translator Tool. Mobile phase: A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN. Temperature: 60 °C. Detection: UV, 220 nm. Sample: 1. Gly-Tyr, 2. Tyr-Tyr-Tyr, 3. Val-Tyr-Val, 4. Oxytocin, 5. Angiotensin II, 6. Leu-enkephalin.

Figure 4: Overlay of 10 repeated chromatograms of the chiral analysis of the laboratory sample analysed on a) System 1 on column 2a, b) System 2 on column 2b, and c) on System 3 on column 2c.



is justified when it responds to the demands of robustness, for example, in the case of chiral analysis and the separation of water-insoluble or unstable solutes.

Initially, achiral SFC analyses may be less common in QC laboratories, with LC remaining the preferred technique. SFC has a higher chance of offering a robust method when it is a simpler approach to solving an analytical problem, whether through simpler mobile phase preparation, or rapid separation without the need for complex mobile phase gradients and lengthy system setup.

For good reasons, more and more contract research organizations (CROs) are equipping their analytical laboratories with SFC instruments and investing in staff training to enable it to be run on a routine basis to satisfy the increasing demand from pharmaceutical research and development. A closer interaction with CROs equipped for SFC analysis represents an opportunity for pharmaceutical industry, provided the necessary qualification and data integrity requirements are maintained.

Acknowledgements

We thank David Mason and Concept Life Sciences for the collaboration with executing part of the Transferability experiments.

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