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The use of partially porous particle columns for the routine, generic analysis of biological samples for pharmacokinetic studies in drug discovery by reversed-phase ultra-high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Recent years have seen the introduction of new high performance liquid chromatography (HPLC) instruments and columns that are capable of achieving high resolution, high speed liquid chromatographic separations at back pressures up to 1000 bar, so-called ultra-high performance liquid chromatography (UHPLC). Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) is gaining widespread use for this purpose, and for this approach to be successful a generically applicable, robust column is required. Here, data are presented showing the robustness of a partially porous 2.7 μm diameter particle material in this application and the accuracy and precision of an assay for a typical pharmaceutical in plasma. This stationary phase material is evaluated for performance and compared with other materials frequently used for similar analyses using a test mix currently used routinely in our laboratories to assess the performance of UHPLC–MS/MS systems. The partially porous material demonstrates similar resolving power to sub-2 μm materials under the ballistic gradient chromatography conditions employed and exhibits excellent resilience over the analysis of thousands of protein precipitated plasma extracts. It is suggested that this stationary phase material can be an invaluable tool in generic, high throughput assays for pharmaceutical bioanalysts.

1. Introduction

Modern drug discovery and development is an extremely costly time and resource consuming endeavour and is subject to external pressures to limit the cost of new medicines. There are also challenges in regard to maximising the exclusive patented lifetime of new medicines. It is therefore essential that compounds unlikely to reach market are eliminated as early as possible. This demands the ability to be able to make informed decisions regarding compounds in accordance with tight deadlines. A contributory part of the required information is a knowledge of the drug metabolism and pharmacokinetic properties of potential drug candidates with the implication that fast generic methods for pharmaceutical bioanalysis are essential.

Recent years have seen the introduction of a new generation of high performance liquid chromatography (HPLC) instruments that surpass the pressure limitation of conventional equipment (400 bar) and are capable of achieving liquid chromatographic separations at back pressures up to ~1000 bar. These separations have been termed ultra-high performance liquid chromatography (UHPLC). This technology has enabled the use of small particle size, ≤2 μm, stationary phase materials to obtain very high resolution separations or, in conjunction with high linear mobile phase flow, very fast separations.

The first of the UHPLC instruments, the Waters Acquity™, was introduced in 2004 together with Waters Acquity™ 1.7 μm particle size columns and, coupled to tandem mass spectrometry (UHPLC–MS/MS), applied to bioanalysis in the field of drug metabolism [1]. Subsequently other equipment manufacturers have introduced instruments with similar performance capabilities and many companies have introduced approximately 2 μm particle size stationary phases. In the 4 years since its advent, the use of UHPLC has risen spectacularly and UHPLC has been extensively applied in drug metabolite identification [2–6], metabonomics [7–11] and quantitative pharmaceutical bioanalysis, e.g. [12–27], together with applications outside of the pharmaceutical industry, for example in food analysis [28–29] and forensics [30–31].

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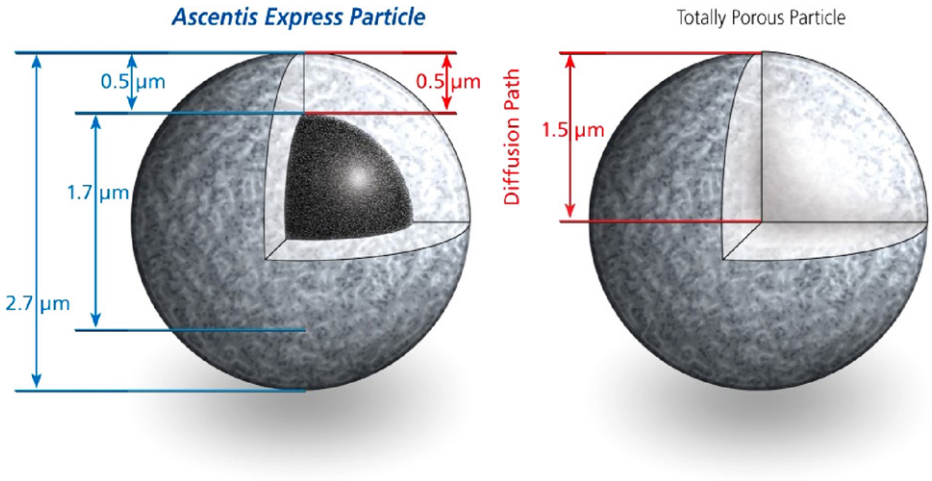


Fig. 1. Structure of Ascentis Express™ Fused-Core™ particle compared to conventional porous silica particle (graphic provided by Sigma–Aldrich Co. Ltd., Poole, Dorset, UK).

In a drug discovery bioanalysis laboratory a huge number of samples derived from studies with a high number of widely differing compounds require analysis within short timelines; though often there will be not a lot of samples per compound. As a consequence it is fair to say that the emphasis in the typical discovery drug metabolism and pharmacokinetics (DMPK) laboratory is on speed rather than maximum resolution, and reliable bioanalysis protocols are essential. The lack of time available for custom method development means that generic methodologies have to be established and as these methods have to retain and elute a wide diversity of analyte chemistries which exhibit a range of lipophilicities, gradient LC procedures are required. These gradient LC protocols can retain strongly hydrophilic compounds under initial conditions whilst still developing the eluotropic strength to elute strongly lipophilic compounds. Such analysis protocols can be defined readily with UHPLC equipment and columns and proffer the high sample throughput demanded.

It should be noted though, that operating very fast, so-called ballistic gradients negates part of the extremely high resolving power and peak capacity that can be obtained from the smallest <2 μm particle stationary phase materials [32] and in the authors' experience these materials do not always offer the best solution for high throughput UHPLC bioanalyses, as under these conditions the steepness of the gradient rather than the particle diameter predominates in determining the resolution. One alternative stationary phase that has recently been introduced and merits investigation in these very fast gradient UHPLC bioanalyses is the partially porous material (referred to as fused-core particles) and marketed as Halo™ (MAC-MOD Analytical Inc. and Hichrom Ltd.) or Ascentis Express™ (Sigma–Aldrich Co.). This material comprises 2.7 μm diameter particles consisting of a 0.5 μm radius "shell" of porous stationary phase surrounding a 1.7 μm non-porous core (Fig. 1). These particles exhibit reduced diffusional mass transfer compared to wholly porous particles and enable the use of higher mobile phase flows without concurrent loss of efficiency and have been shown to demonstrate similar peak capacities to several sub-2 μm porous particulate materials in fast gradient analyses, and furthermore at significantly lower backpressures [33]. Spherical geometry dictates that the porous shell comprises ~80% of the total particle volume and thus the loading capacity of this material is not greatly reduced compared to totally porous particles, particularly as the deep interiors of porous particles probably have limited interaction with analytes under the kinetics of these high linear flow velocity separations. This material has recently been successfully applied to the analysis of a pharmaceutical in mouse plasma [34].

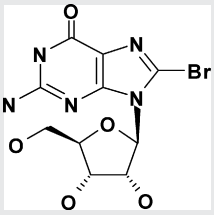
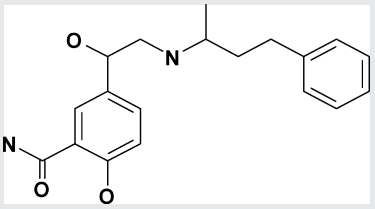
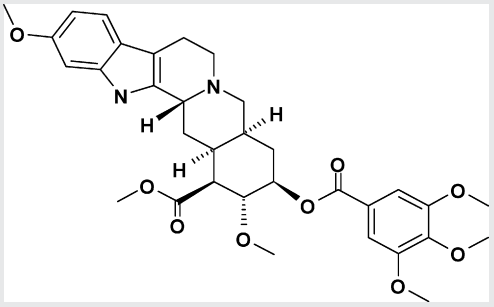
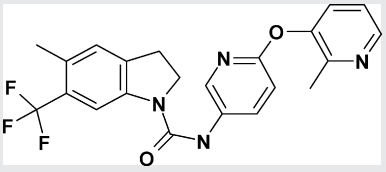
In this report the potential of this fused-core material for use as the routine column for generic, fast gradient UHPLC–MS/MS pharmaceutical bioanalysis is evaluated. Particular attention is paid to column robustness over the course of 1000 s of injections of protein precipitated plasma extracts in terms of both peak shape and retention using a test mix currently used routinely in our laboratories to assess the performance of UHPLC–MS/MS systems. The retention stability, accuracy and precision of a quantitative assay of a proprietary GlaxoSmithKline compound based on this stationary phase are also presented.

2. Materials and methods

2.1. Reagents

HPLC grade water and acetonitrile, and 99% pure formic acid were supplied by Fisher Scientific UK (Loughborough, Leics., UK). A performance test mix comprising bromo-guanosine, reserpine (both obtained from Sigma–Aldrich, Poole, Dorset, UK), labetalol and a GlaxoSmithKline compound SB243213A (both supplied by Medicinal Chemistry, GlaxoSmithKline, Stevenage, Herts., UK) was used to demonstrate column performance. The structures, molecular weights and calculated log *P* values are shown in Table 1. These "druglike" test mix components were carefully chosen from other test mixes found in literatures [35,36] (Labetalol and Reserpine), and from in-house experience (bromo-guanosine and SB243213A) to cover a range of analytical-related physicochemical properties of most pharmaceutical compounds, such us hydrophobicity and molecular weight. Test mix concentrations were optimised to obtain comparable MRM responses in positive and negative modes for all four compounds and within the detection linearity range for each compound when using an API4000 mass spectrometer fitted with either turbo-ionspray or atmospheric pressure chemical ionisation probes. The concentrations of the components were bromo-guanosine 1000 ng/mL, labetalol 125 ng/mL, reserpine 350 ng/mL and SB243213A 25 ng/mL prepared in 0.1% aqueous formic acid. A basic proprietary GlaxoSmithKline compound (GSK A) with a calculated log *P* of 1.2 with an adenine core structure and proprietary functionality was used in the assay reproducibility and precision measurements. The assay used as internal standard a more lipophilic analogue of GSK A (GSK B) with a calculated log *P* of 2.9. The full struc-

Table 1
Test mix components.

Compound name	Molecular formula	MWt (molecule)	Structure	*c* Log *P*
Brguanosine	C10H12BrN5O5	361.36		−2.3
Labetalol	C19H24N2O3	328.2		2.5
Reserpine	C33H40N2O9	608.69		3.9
SB243213A[a]	C22H19F3N4O2	428.42		5.3

[a] GSK compound structure previously published [37].

ture of these two compounds cannot be disclosed for commercial reasons.

2.2. Instrumentation

All UHPLC–MS analyses were conducted using a Jasco X-LC gradient solvent delivery system (Jasco UK, Great Dunmow, Essex, UK), a CTC Analytics HTS PAL autosampler (Presearch, Basingstoke, Hampshire, UK), fitted with a Cheminert 15,000 p.s.i. injection valve, 25 μL injection syringe and a 20 μL loop, a Model 7990 column oven (Jones Chromatography, Hengoed, Glamorgan, UK) and an Applied Biosystems API4000 mass spectrometer (Applied Biosystems, Warrington, Cheshire, UK). Plasma sample extraction was conducted on a Tecan Genesis RSP150 robotic sample processor (Tecan UK, Theale, Berkshire, UK) by solvent addition and filtration through Whatman Unifilter protein precipitation 96-well plates (Whatman, Maidstone, Kent, UK). Plasma extracts were dried down using a Porvair Sciences sample concentrator (Porvair Sciences, Leatherhead, Surrey, UK).

2.3. Chromatographic conditions

Analyses were conducted by gradient UHPLC on a 50 mm × 2.1 mm 2.7 μm particle size Supelco Ascentis Express C18 column (Sigma–Aldrich, Poole, Dorset, UK). Other columns used for comparison purposes included Luna HST C18(2) 2.5 μm and Luna PFP 3 μm (Phenomenex, Macclesfield, Cheshire, UK) and Acquity BEH C18 1.7 μm (Waters, Elstree, Herts., UK). Mobile phase A was 0.1% (aq) formic acid and mobile phase B was 0.1% formic acid in acetonitrile. A linear gradient ran from 95% A to 95% B in 1 min before returning to 95% A at 1.05 min and re-equilibrating for a further 0.25 min. The injection to injection sample cycle time was ∼1.9 min. The flow rate was 1.1 mL/min. Unless specified otherwise, UHPLC analyses were conducted with the column oven kept at 40 °C.

2.4. Mass spectrometry

Detection was by MS–MS multiple reaction monitoring (MRM) operating in positive ion turbo-ionspray mode. The temperature, collision gas, curtain gas and ionspray voltage were 600 °C, 3, 20 and 5500 V, respectively. Nitrogen was used as the collision and curtain gas. The MS–MS reactions monitored were *m*/*z* 362.2 → 230.0, 329.4 → 162.3, 609.4 → 195.3 and 429.3 → 228.3 for bromo-guanosine, labetalol, reserpine and SB243213A, respectively, with a dwell time of 50 ms. The MS–MS reactions monitored for GSK A and its internal standard were *m*/*z* 321.4 → 223.1 and 408.1 → 98.1, also with dwell times of 50 ms. Data were acquired using Analyst 1.4.2 software and processed using Analyst 1.4.1 software (Applied Biosystems).

2.5. Plasma sample extraction procedure

25 μL aliquots of spiked plasma calibration standards over the range 0.5–5000 ng/mL (prepared by serial dilution on the Tecan Genesis RSP 150) and spiked plasma validation samples of GSK A were extracted by protein precipitation with 150 μL of acetonitrile containing GSK B as internal standard, and filtered through a 96-well filter plate. The Tecan robot first aspirated 150 μL of acetonitrile containing internal standard, followed by a small air gap and then the 25 μL plasma sample prior to co-dispensing these to the 96-well filter plate. Following addition of all the samples the filter plate was left to equilibrate for 2 min prior to the application of vacuum and collection of filtrates into a clean 96-well plate. Filtrates were evaporated to dryness under nitrogen at 40 °C and reconstituted in 200 μL of 10:90 acetonitrile:water. The injection volume was 18 μL, which was chosen to allow for an accurate 3× reduced volume injection (6 μL) for a preliminary assessment of the comparability of any matrix effects in both sample and calibrant extracts, so as to ensure that any ion suppression or enhancement was compensated for within the assay. This test is routinely applied in the authors' laboratories.

2.6. Column robustness testing

Six complete calibration sample sets of 16 (14 calibration standard concentrations plus 2 wash samples) for GSK A in plasma were prepared per 96-well plate according to the above procedure, and the whole plate of samples was injected six times. This entire procedure was conducted on multiple occasions, with the column performance monitored periodically by interspersing these batches of injections of protein precipitated plasma extracts with injections of the bromo-guanosine, labetalol, reserpine and SB243213A test mix and assessment of the resulting retention times and peak shapes for these analytes.

2.7. Intra-assay accuracy and precision of the generic UHPLC–MS/MS analysis procedure

The intra-assay and precision of the methodology was assessed by designating one set of calibration samples (processed as described above) as standards and interpolating the concentration data for the next six consecutive sets of calibration standards. The precision was determined as the coefficient of variation for the mean interpolated concentration and the assay bias determined as the difference between the mean interpolated concentration and the nominal concentration, with both results expressed as percentages.

2.8. Carryover

The carryover present in the assay was assessed by comparison of the apparent concentration values interpolated from the calibration line for a blank plasma extract injected immediately following a 5000 ng/mL calibration standard.

3. Results and discussion

3.1. Column robustness

Fig. 2 shows the UHPLC–MS/MS chromatograms obtained for injections of the bromo-guanosine, labetalol, reserpine and SB243213A test mix interspersed between the UHPLC–MS/MS analysis of many hundreds of protein precipitated plasma sample extracts. As can be seen there is some slight reduction in the retention time of the labetalol and reserpine peaks over the initial ~650 injections, presumably due to irreversible adsorption of components in the plasma sample extracts to the stationary phase surface. However, it was observed that this retention shift was gradual rather than sudden and would have been unlikely to have caused difficulty in the quantitative analysis of either component for either a small batch of samples or for a batch of several hundred samples. Indeed, injections 1658 (c) and 3668 (d) demonstrate virtually identical retention and furthermore, the resolution between peaks (iii) and (iv) is appreciably greater than observed when the column was new, injection 9 (a). It is also clear that the injection of approaching 4000 protein precipitated plasma extracts has not had a deleterious effect on the observed peak shapes with peak widths being not significantly wider at injection 3688 ($w_{1/2}$ for labetalol ~0.9 s) (d) than at injection 9 (a) ($w_{1/2}$ for labetalol ~0.8 s).

Another indicator of the excellent robustness of this column to these typical bioanalytical samples is that the injection of approaching 4000 plasma extracts has led to only a minor increase in the system backpressure, from 434 to 458 bar, and given that the column has a manufacturer's specification of a 600 bar pressure limit, indicates that the column could probably have continued to successfully achieve these analyses for many thousands more extracted plasma samples. Fig. 3 shows UHPLC–MS/MS chromatograms for the bromo-guanosine, labetalol, reserpine, SB243213A test mix on several possible alternative columns for these analyses. Within this laboratory it should be noted, disappointing peak shapes have sometimes been observed with Zorbax Eclipse XDB-C18 Rapid Resolution HT 1.8 μm columns; and also significant inter-column variation, from excellent to poor peak shapes, has been seen with the Thermo Hypersil Gold C18 1.9 μm stationary phase and for these reasons these materials (which have generated excellent results elsewhere) were not included in the evaluations reported here. As can be seen in Fig. 3, the most popular UHPLC stationary phase material, Waters Acquity BEH C18 1.7 μm, was observed to operate at >700 bar even with the column oven set at 65 °C (combined with an efficient mobile phase pre-heating device in-line prior to the UHPLC column, courtesy of Jasco, UK). When this column was used solely with the column oven set at 40 °C the backpressure was even higher, leading to concern regarding the robustness of the system for application in the successful conduct of thousands of analyses of extracted plasma samples. The Luna C18(2) HST 2.5 μm material was found in our hands to deliver excellent separations for the bromo-guanosine, labetalol, reserpine and SB243213A test mix, with a backpressure of approximately 525 bar but it was observed that this column typically lost chromatographic performance within 300–600 injections of plasma extracts (results not shown). This is not unexpected as the material is not specified by the manufacturers to be stable above 400 bar back pressure. An alternative material is the 3 μm Luna PFP (pentafluorophenyl) bonded silica material which exhibits a lower backpressure at the same flow rate, as would be expected for larger particles. This material generated a backpressure of approximately 320 bar for a 50 mm × 2.1 mm column at the chosen 1.1 mL/min flow rate, significantly below its pressure specification limit of 400 bar and offers a different selectivity based on multiple mechanisms of interaction (including hydrogen bonding, dipole–dipole and aromatic). Potentially the Luna PFP 3 μm stationary phase offers useful alternative selectivity for compounds for which adequate separation on C18-based materials proves unachievable. It is notable that the peak widths achieved on these columns were not dissimilar to those achieved on the 1.7 μm Acquity material, re-iterating that the "ballistic" gradient conditions employed do not permit demonstration of the extremely high efficiency and high resolving power of the sub-2 μm materials. For the Ascentis Express 2.7 μm C18 material (Fig. 3(d)), similar peak widths are observed at approximately 75% of the maximum specified backpressure for this column, even after

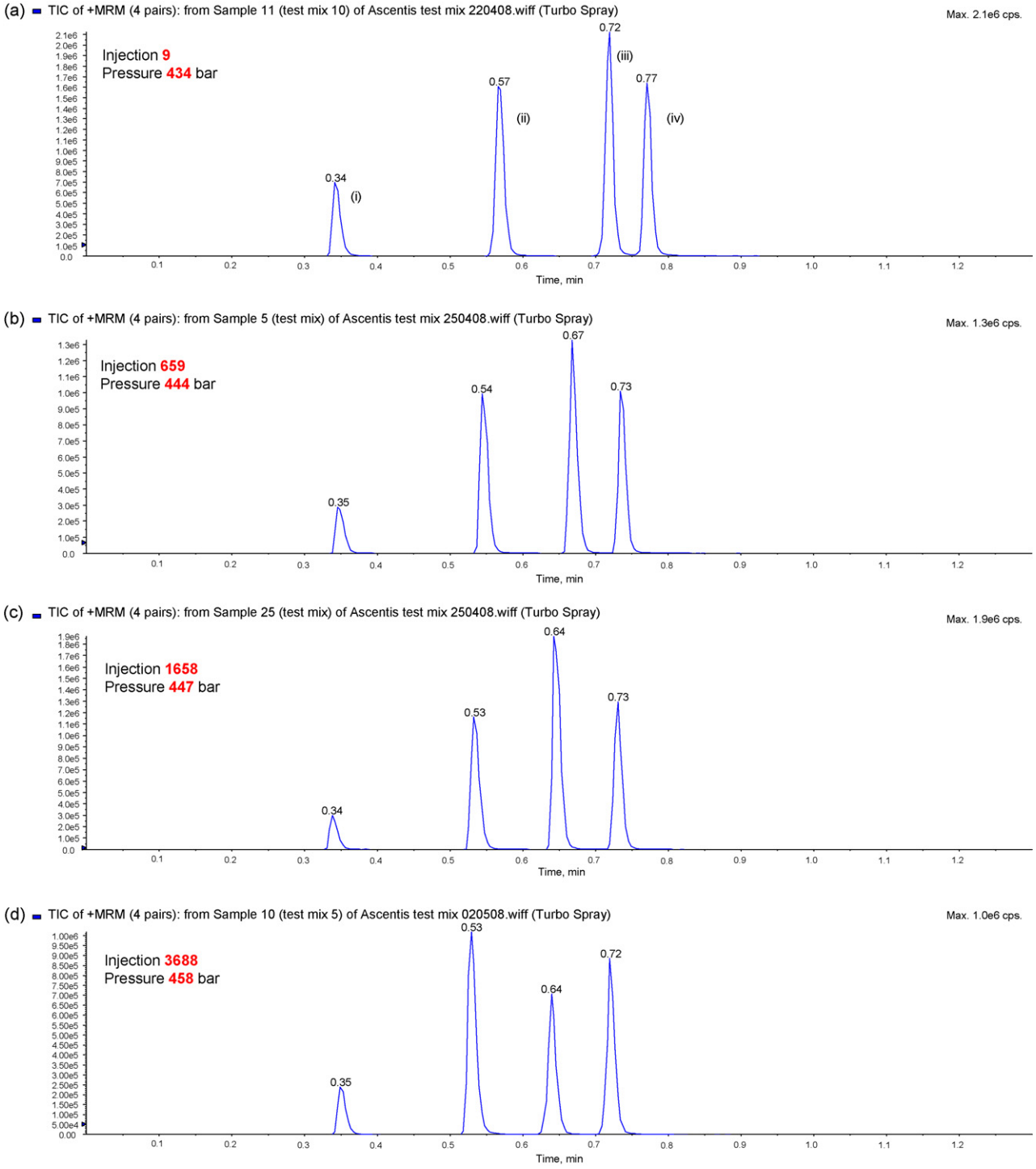


Fig. 2. Gradient UHPLC–MS/MS chromatograms on a 50 mm × 2.1 mm i.d. 2.7 μm Ascentis Express C18 column for (i) bromo-guanosine, (ii) labetalol, (iii) reserpine and (iv) SB243213A interspersed at occasion between the analysis of multiple sets of protein precipitated plasma extracts. The injection number relates to the total number of injections performed on the column. n.b. the change of relative analyte response visible in the bottom chromatogram is due to the preparation of a new batch of test mix solution and not due to instrumental or methodological changes.

>1500 injections of protein precipitated plasma extracts, and as has been shown earlier in this report, this material has demonstrated its capacity to successfully analyse many more plasma extracts beyond this. The inclusion of fourfold overlaid chromatograms in Fig. 3c and d further illustrates the excellent repeatability of the UHPLC conditions employed.

Fig. 4 further illustrates the stability and robustness of this column in application in pharmaceutical bioanalysis. As can be seen, there is virtually no change in either the retention or peak shape for the analyte GSK A over the course of the analysis of approximately 2500 plasma samples. It was determined that the mean retention time for GSK A was 0.523 min with a coefficient of variation (CV) of <1.1% (equivalent to ~0.3 s) over the course of 2687 injections of protein precipitated plasma extracts. The apparent variability visible in the overlaid chromatograms is accentuated by the very short time span of the *x*-axis. In fact the CV for the retention time for all

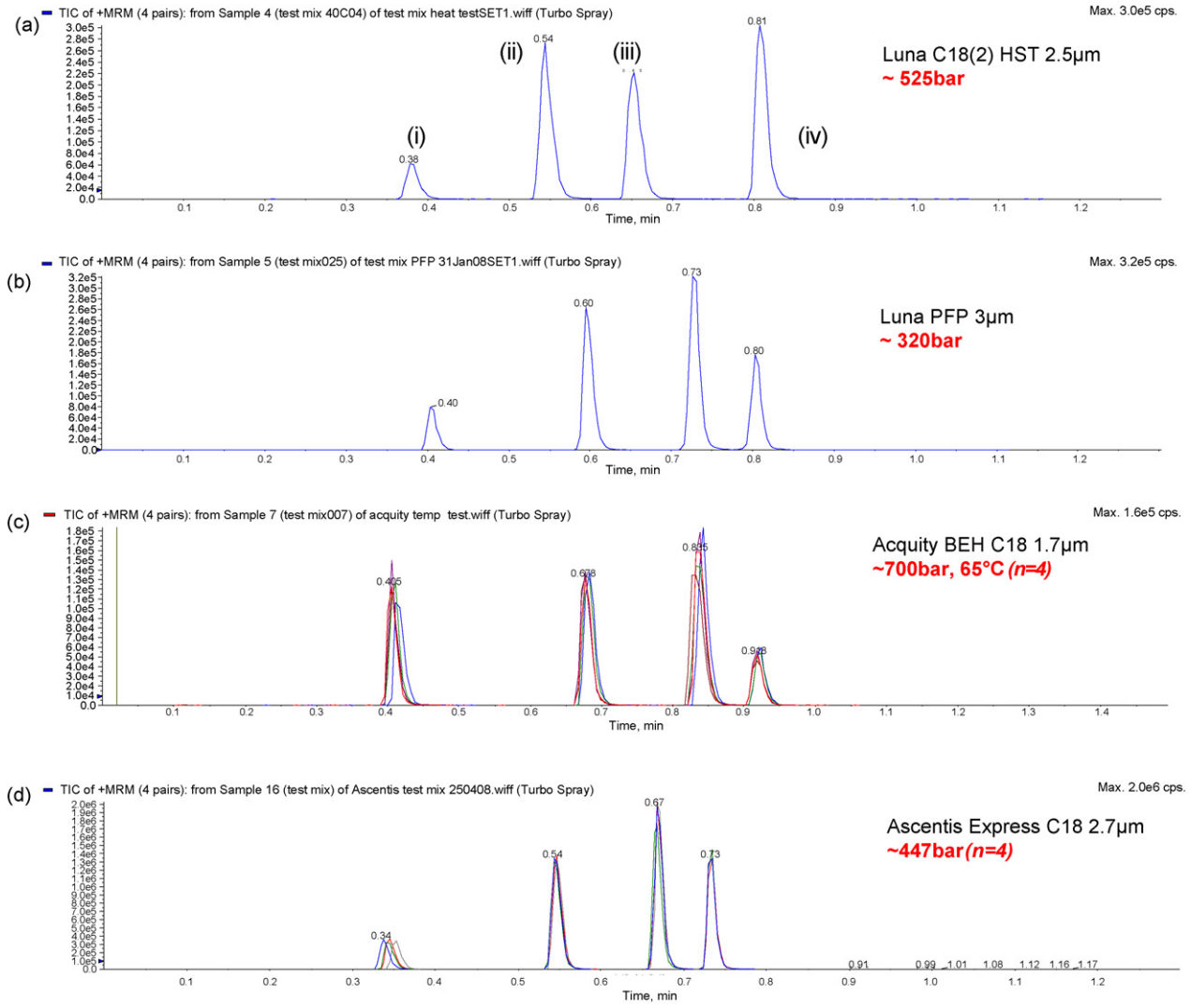


Fig. 3. Gradient UHPLC–MS/MS chromatograms of the (i) bromo-guanosine, (ii) labetalol, (iii) reserpine and (iv) SB243213A test mix on several possible columns for generic gradient UHPLC–MS bioanalysis. All columns 50 mm × 2.1 mm: (a) Luna HST C18(2) 2.5 μm, (b) Luna PFP 3 μm, (c) Acquity BEH C18 1.7 μm (n = 4 chromatograms overlaid) and (d) Ascentis Express C18 2.7 μm.

components was ~1% and the CV for the peak areas is ~5%, well within assay acceptability criteria for drug discovery bioanalysis.

The methodology is in routine, daily use on five quantitative UHPLC–MS/MS systems and the typical column lifetime is at least 2500–3000 injections.

3.2. Assay accuracy and precision

Whilst the emphasis of drug discovery bioanalysis is to generate information as rapidly as possible, both to ensure the rapid progression to subsequent testing of suitable molecules and to ensure the rapid attrition of unsuitable molecules, it is fair to say that these decisions cannot be made with confidence if the bioanalysts cannot demonstrate the integrity and validity of their measurements. Therefore, it is vital when assessing the potential of any new instrumentation, technique or methodology to demonstrate that the assay performs to an acceptable standard. No significant evidence of matrix effects was observed in the assay. Table 2 presents the intra-assay accuracy and precision data for the analysis of GSK A in plasma samples by UHPLC–MS/MS using a partially porous stationary phase following automated 96-well protein precipitation extraction of plasma samples after this column had previously been subjected to >1000 injections of similar extracts. It can be seen from Table 2 that across the range studied, 0.5–5000 ng/mL the assay exhibits suitable accuracy and precision for use in the drug discovery environment. The precision, as considered by the % coefficient of variation of the interpolated concentrations, is <20% at the limit of quantification and <15% across the rest of the calibration range, and the bias is <15% deviation from the nominal concentration across the entire calibration range.

3.3. Carryover

The carryover present in the assay of GSK A in plasma, as assessed by comparison of the apparent concentration values present in a blank plasma extract injected immediately following a 5000 ng/mL calibration standard, was determined to be <0.2%.

3.4. Generic application

The above results demonstrate that the application of the fused-core material in our methodology provides very reproducible

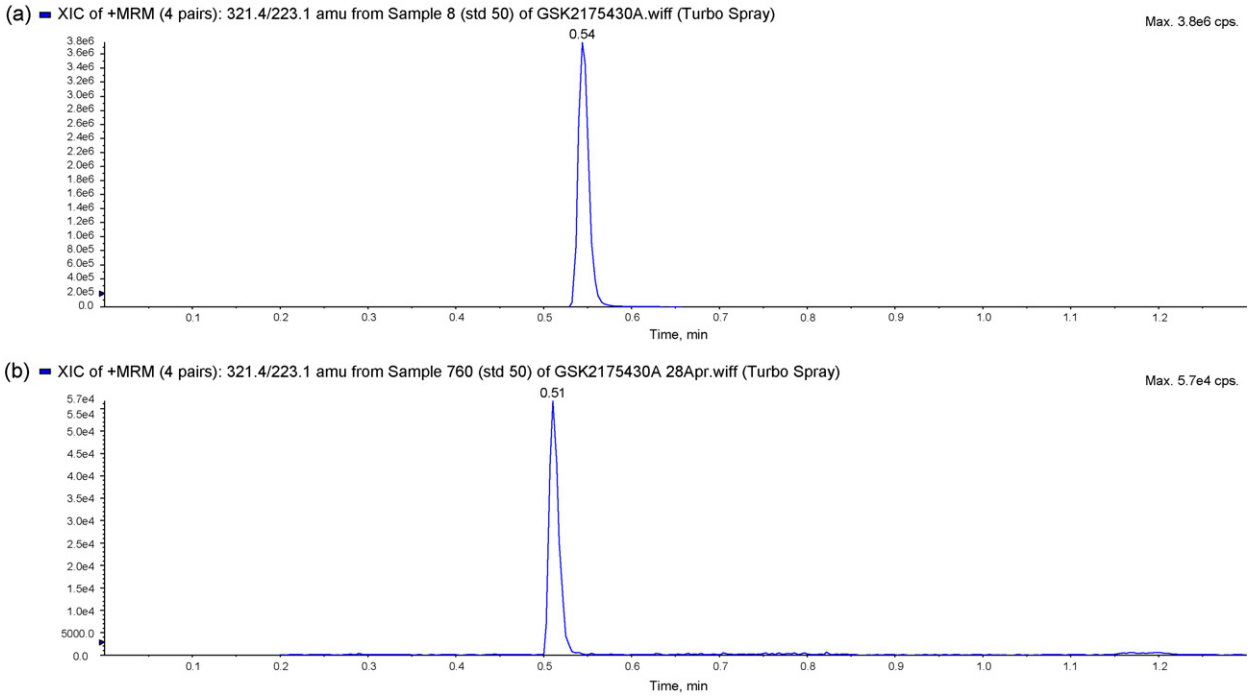


Fig. 4. Gradient UHPLC–MS/MS chromatograms of extracted plasma extracts of GSK A on an Ascentis Express C18 50 mm × 2.1 mm 2.7 μm column. (a) 50 ng/mL plasma GSK A calibration standard from the 1st calibration set (injection 28 on the column) and (b) from the 149th calibration set (injection 2419). n.b., change in response due to routine preventative maintenance of the mass spectrometer.

Table 2
Inter-assay accuracy and precision data for the analysis of GSK A by UHPLC–MS/MS following extraction from plasma samples by automated protein precipitation.

	0.5	1	2	5	10	20	50	100	200	500	1000	2000	5000
Cal line 37	0.520	1.01	1.45	5.26	9.66	19.5	49.0	101	195	529	1090	2140	4960
Cal line 38	0.451	0.903	1.63	5.22	10.4	19.3	50.2	107	213	577	1080	2120	5120
Cal line 39	0.308	0.910	1.63	4.21	8.51	21.4	53.0	104	233	586	1120	2400	5040
Cal line 40	0.492	0.901	1.85	5.07	9.04	20.1	52.3	105	203	532	1060	2210	4970
Cal line 41	0.422	0.939	1.98	4.80	10.9	17.5	51.2	92.8	189	498	973	2040	4650
Cal line 42	0.493	1.11	1.69	5.17	9.68	17.6	46.1	88.6	197	512	1010	2010	4380
Mean	0.448	0.962	1.71	4.96	9.70	19.2	50.3	99.7	205	539	1060	2150	4850
SD	0.077	0.083	0.186	0.4	0.870	1.50	2.51	7.38	15.9	35.2	54.5	141	282
% CV	17.1	8.65	10.9	8.08	8.97	7.78	4.99	7.40	7.78	6.54	5.15	6.53	5.80
% bias	−10.5	−3.78	−14.8	−0.90	−3.02	−3.83	0.60	−0.267	2.50	7.80	5.55	7.67	−2.93

chromatography for an in-house test mix and that, together with sample preparation by protein precipitation, forms the basis of an accurate and reproducible assay for the compound GSK A in plasma samples. A claim for a generic application clearly cannot be made on the application of the methodology to a single plasma assay. Following the development and implementation of the described assay for GSK A in plasma, this identical methodology has been applied with equal success to tens of compounds from different chemical series and across approximately 10 discovery programmes, derived from both the Respiratory and Immuno-inflammation areas. These varied therapeutic targets and the presence of multiple lead compound series for each target gives rise to considerable chemical diversity and it is the success of this methodology in plasma analyses across this broad spectrum that gives the authors a high confidence in the generic applicability of the fused-core material for high throughput, reversed-phase bioanalysis in support of drug discovery.

4. Conclusion

The modern discovery and development of new medicines is an extremely time and resource consuming endeavour. It is therefore essential that unsuitable compounds are "weeded out" as early as possible and resource is focussed on the progression of the molecules with the greatest chance of successfully achieving registration. This demands the ability for drug discovery programmes to be able to make reasoned, data-backed "go/no go" decisions regarding compounds in accordance with tight deadlines. The implication of this for the bioanalytical drug discovery laboratory is that samples must be analysed rapidly so that information adding value to the understanding of a compound's properties is generated in an appropriate timescale. Thus fast and reliable generic methods for pharmaceutical bioanalysis are prerequisite. Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) is gaining increasingly widespread use for this purpose, and a robust, generically applicable column is a necessity. In this report, data has been presented showing the robustness of a partially porous 2.7 μm diameter particle stationary phase and the accuracy and precision for the application of this material in a typical analysis of a pharmaceutical in plasma samples. The partially porous stationary phase material has demonstrated equivalent resolving power to sub-2 μm materials under the ballistic gradient chromatography conditions employed, and shown to exhibit excellent resilience and performance over the analysis of

thousands of protein precipitated plasma extracts, suggesting that this type of column is a valuable tool for pharmaceutical bioanalysts.

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