Turbulent Flow Chromatography: an Evolving Solution for Bioanalysis

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Bioanalysis has evolved considerably over the last ten years with the rapid implementation of three key technology levers: robotics for automated sample extraction, fast liquid chromatography coupled to mass spectrometry for high throughput trace analysis and finally Laboratory Information Management Systems (LIMS) for seamless data processing and data interpretation. This article describes how Turbulent Flow Chromatography (TFC), by allowing on-line extraction of crude biological matrices, has played an important role in addressing both old and new challenges of modern bioanalysis. After a brief reminder of TFC principles, the authors will focus on describing how the TFC platform has evolved to become the tool of choice for optimal analysis cost, speed and data quality. Practical considerations such as assay sensitivity and column robustness will be discussed in detail. In addition to some examples of current applications, future technology advances will also be presented as TFC adapts to an evolving Drug Metabolism and Pharmacokinetic (DMPK) environment.

Introduction

The determination of small drug molecules in biological fluids, mainly plasma and urine, has remained for a long time a very challenging task. This was due to both the complexity of the biological matrices requiring time consuming sample preparation and the need for long analytical runs, typically using reversed-phase liquid chromatography (RP-LC), to achieve the appropriate separations of remaining endogenous peaks. The recent implementation of automated off-line 96/384-well plate extraction (including protein precipitation, liquid-liquid or solid-phase extraction), or on-line extraction (including TurboFlow chromatography (TFC)) has allowed fast sample clean-up and partly removed the bottleneck associated with sample preparation. The routine usage of tandem mass spectrometers (MS/MS) for quantitation has provided a highly selective means of monitoring the peak of interest without the need to develop a lengthy RP-LC method. With short analytical run times (e.g. less than 2 minutes) and minimal sample preparation, the original bottlenecks associated with biological samples have gradually been addressed. Bioanalysis as we know it has become a mature analytical field. Nowadays it is not unusual for pharmaceutical companies to use a short suite of generic methods suitable to support most in-vitro and in-vivo DMPK samples. TFC/MS/MS is part of Pfizer's generic tool box for routine bioanalysis. A description of how the technique has evolved in our labs is provided. The advantages and limitations of the TFC platform are also discussed in detail.

In this context of well established and fit-for-purpose practices, the need for further investments and wide-spread implementation of new technologies could be perceived as challenging. However, this is without taking into consideration the constantly evolving opportunities arising in both the drug discovery and development arenas. The need to bring new drugs to patients even faster is raising the bar for all analytical applications in term of reduced cost, increased speed and data quality. Specific examples where TFC can greatly impact the drug development process are also discussed in this article.

TFC Principles

TurboFlow methods are based on the direct injection of biological samples without previous extraction or treatment onto a column packed with large particles. These large particles have an additional level of selectivity via the stationary phase chemistry added to them. After the sample is injected onto a TurboFlow column the high flow rate (cf. 1.5 – 5.0 mL/min) generates turbulent flow conditions inside the column. Since 100% aqueous mobile buffers are used, the small analyte molecules are retained via diffusion into the particle pores, while the proteinaceous material is washed to waste (Figure 1a). Once the compounds of interest are extracted from the biological matrix, they are eluted from the TurboFlow column onto the analytical column with a volume of solvent, which has been stored in a holding loop. The holding loop should have a volume at least ten times that of the TurboFlow column and is typically filled with organic mobile phase (for reversed stationary phase) or pH buffered solutions (for ion exchange phases). As the analytes are released from the TurboFlow column they are transferred with the pumping solvent (at a considerably lower flow rate than that used during loading) through the tee rotor-seal in the second valve and mixed with the pumping solvent from the analytical system. The analyte molecules

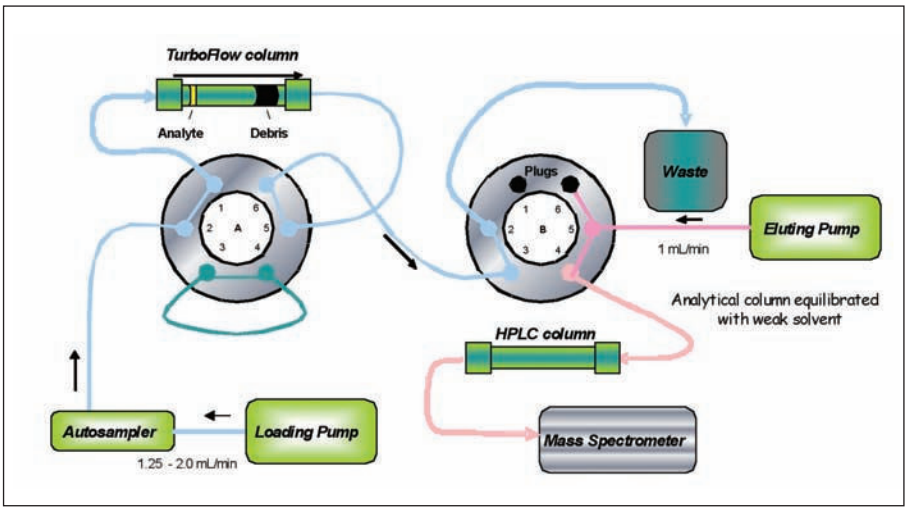

Figure 1a: Focus-Mode: Loading Step. Turbulent flow sweeps debris from the sample matrix through the Turboflow column while analyte(s) are retained.

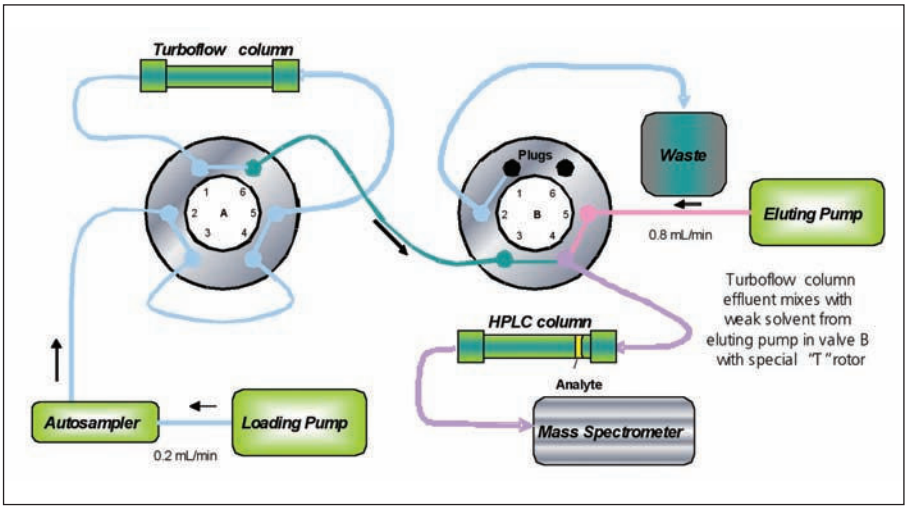

Figure 1b: Focusing/Transfer Step. The flow from both pumps is combined (and hence diluted) through the T rotor seal, thus allowing the loop contents to transfer the analytes retained on the Turboflow column into a stacked band on the analytical column

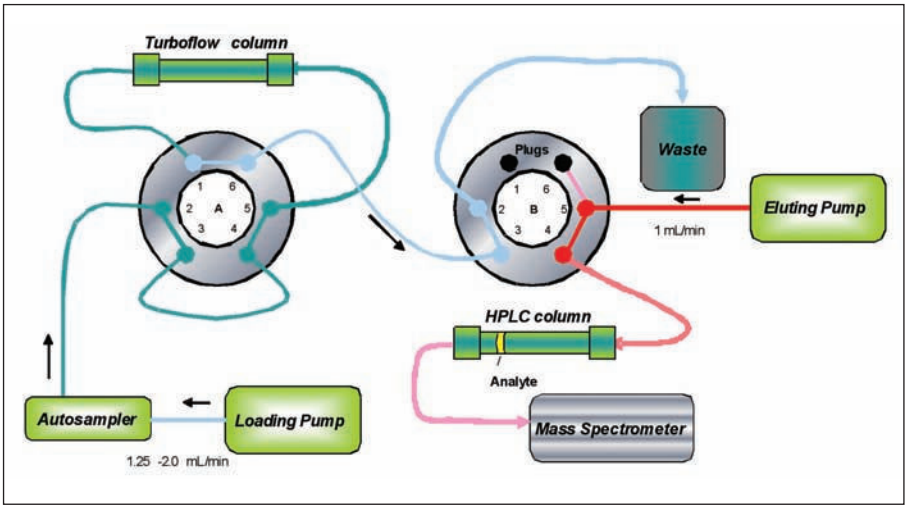

Figure 1c: Focus Mode - Eluting Step. The analytes are removed from the analytical column via isocratic or gradient elution. The Turboflow column is washed and the loop is filled and closed in preparation for the next injection.

are then focused into a sharp band at the head of the HPLC column (Figure 1b). When the transfer is completed, both valves turn to isolate each column, thereby permitting washing of the TurboFlow column and filling of the loop for the next injection. A 'regular' gradient or isocratic elution can then take place in parallel on the analytical column into the MS detector (Figure 1c).

Evolution of the TFC Platform

The theory of turbulent flow in open tubes has been discovered and studied for decades; however its application to LC packed columns was only patented in 1997 by Quinn and Takareski [1]. The challenge at the time was to design a chromatographic platform that would utilise turbulent flow properties to isolate small analytes from macromolecules present in complex matrices such as biological fluids. The study and understanding of the limitations associated with this initial setting were key to the learning's and innovative solutions behind the evolution of the commercially available platform as we know it today. The various ways in which the challenges associated with on-line extraction techniques in general were addressed through refining and re-designing the different components of the TurboFlow platform are discussed next.

On-line extraction systems are generally using a 2-D chromatography concept where the extraction happens in the first dimension and the chromatographic separation in a second dimension. The key challenge is to ensure the different chromatographic conditions of the two dimensions remain compatible in order to optimise the analyte transfer. Switching from a 5.0 mL/min extraction flow rate to a typical 1.0 mL/min chromatographic flow rate (50 x 4.6 mm i.d. analytical column) meant that refocusing on an analytical column post extraction could be difficult. For some applications where chromatographic resolution was not crucial, a dilute and shoot approach (Quick elute mode [2]) was used to redirect the flow, after splitting, towards the MS detector. Very quickly, ionisation effect issues were reported due to co-elution of the peak of interest and endogenous materials. Therefore, the need for a chromatographic separation or at least a refocusing step before entering the MS became pivotal to build up analysis quality. The focus mode approach (Figure 1a, 1b and 1c) was then introduced to allow the analyte to be transferred on a suitable HPLC column before detection. Further evolution of the column design, essentially a reduction of the column diameter to 0.5 mm i.d., allowed a decrease in the flow rate required to achieve turbulence (~ 1.5 – 2.0 mL/min). Typically a flow rate above 1.2 mL/min is sufficient to obtain clean extraction and good recovery. Lower extraction flow rate and smaller column dead volume facilitated the analyte transfer and resulted in lower peak dispersion. In addition to improving chromatographic resolution (Figure 2), this also considerably

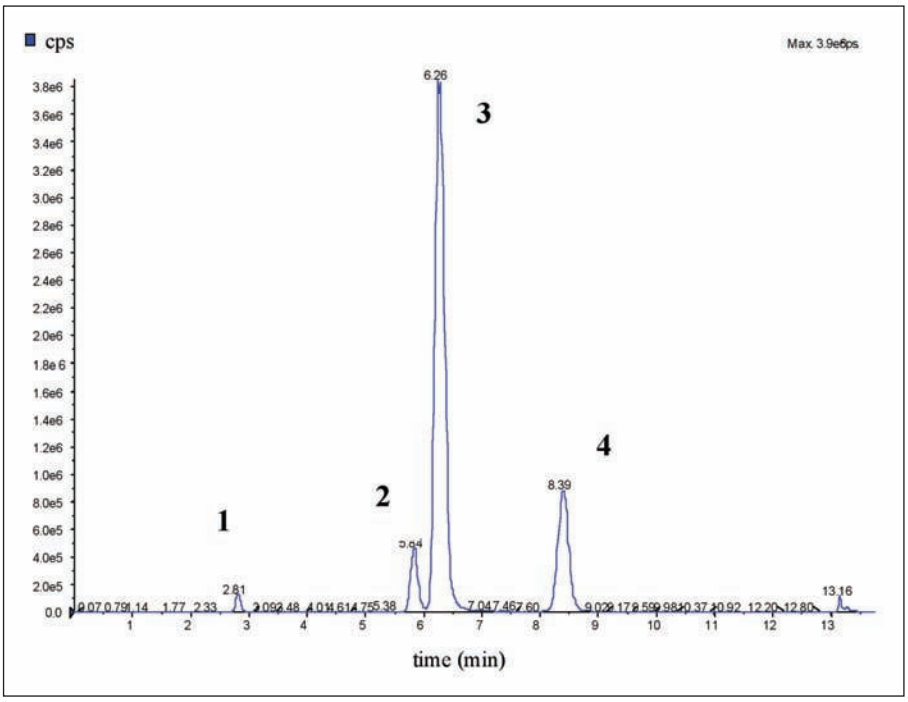

Figure 2: Chromatographic resolution and MS/MS detection of an active pharmaceutical ingredient (API) and its metabolites (1&2. Metabolites; 3. Proprietary API; 4. Internal standard) on a C18 Zorbax Extend column (150 x 4.6 mm i.d.; 5 micron particles; flow rate: 0.8 mL/min; mobile phase (isocratic): 50/50 v/v water/acetonitrile containing 0.01% trifluoroacetic acid) after turbulent flow extraction from human plasma.

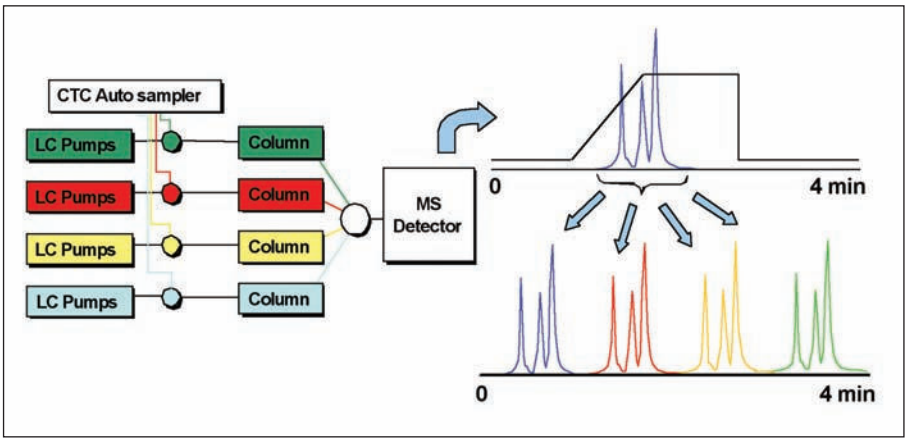

Figure 3: Multiplexing with Turboflow chromatography allows up to four LC systems to run into one MS.

reduced the solvent usage which was considered as a main drawback with initial 1.0 mm id columns.

Reduction of both flow rate and column i.d. had its limitations though, particularly for the injection volume. Typically 15 L of crude plasma (or 30 L diluted 1:1 with internal standard) could be loaded onto the system. This is a very small volume compared to the typical 500 L of plasma originally used for off-line extraction (LLE, SPE or protein precipitation) of early clinical samples where high assay sensitivity is key. Hence initial TurboFlow usage in our lab was mainly directed towards analytical support of toxicology studies where high systemic exposures only require average assay sensitivity (1 - 5 ng/mL). With the development of new generations of mass spectrometers over the past decades, sensitivity is now rarely an issue when using the TurboFlow platform. For a typical set of pharmaceutical compounds designed for oral administration (e.g. following the rule of 5 [3]), we routinely reached LLOQ values in the 0.1 - 0.5 ng/mL range with 15 L injection volume [4]. The possibility of extracting very small sample volumes allows the development of more robust pre-clinical pharmacokinetic and pharmacodynamic (PK and PKPD) models. On-line extraction also reduces considerably sample preparation and could prevent sample degradation. This is the case when labile metabolites and/or biomarkers analysis is carried out concurrently to the active compound.

New off-line robotic sample handlers (96- and 384-well format) coupled to short LC-MS/MS methods (typically 1.0 – 2.0 min) are nowadays the standard platforms to analyse a very large number of samples for high throughput screening, pre-clinical and clinical bioanalysis. Therefore, for on-line techniques to compete, system robustness is essential to bring down cost without compromising quality. To achieve this, the chemistry of TurboFlow extraction columns has evolved over time. More than a thousand plasma samples (15 L injection) are routinely analysed in our labs using the same polymeric column (Cyclone 50 x 0.5 mm i.d.) without observing any changes in peak shape or retention time. This is a significant cost reduction when compared with an off-line technique where ten 96-well SPE or protein precipitation blocks would have been needed. In addition to a significant increase in robustness, the polymeric sorbent used is very retentive for a wide range of chemistries, therefore reducing considerably the need for method development from one compound to the other.

In summary, the TurboFlow platform has reached a level of maturity through a step by step approach in addressing the issues highlighted throughout the years by the analytical scientists using this technique for day-to-day samples analysis. As a result, TurboFlow has now become in its own right the tool of choice for modern bioanalysis.

Applications

Once generic, validated TurboFlow, or indeed 'regular' analytical LC methods, are in place within a high throughput screening laboratory there can be two bottlenecks for sample throughput. The first is the time of the LC method gradient, where ironically, the more costly part of the system, the MS, is idle during the loading and wash stages of analysis. A solution that has gained wide use, particularly in the clinical field, to increase throughput on such systems is to stagger and channel multiple LC flows through the valve system to a single mass spectrometer and hence 'multiplex' the system. A schematic for such a system is presented in Figure 3. A practical example is provided in Figure 4. An example of pharmaceutical compounds that have been analysed via 4 separate LC channels onto a single MS is described by Berube [5] and for two LC channels by Chassaing et al [6]. In the work described by Berube, the sample batch would normally take 48 hours to analyse using one LC system, however,

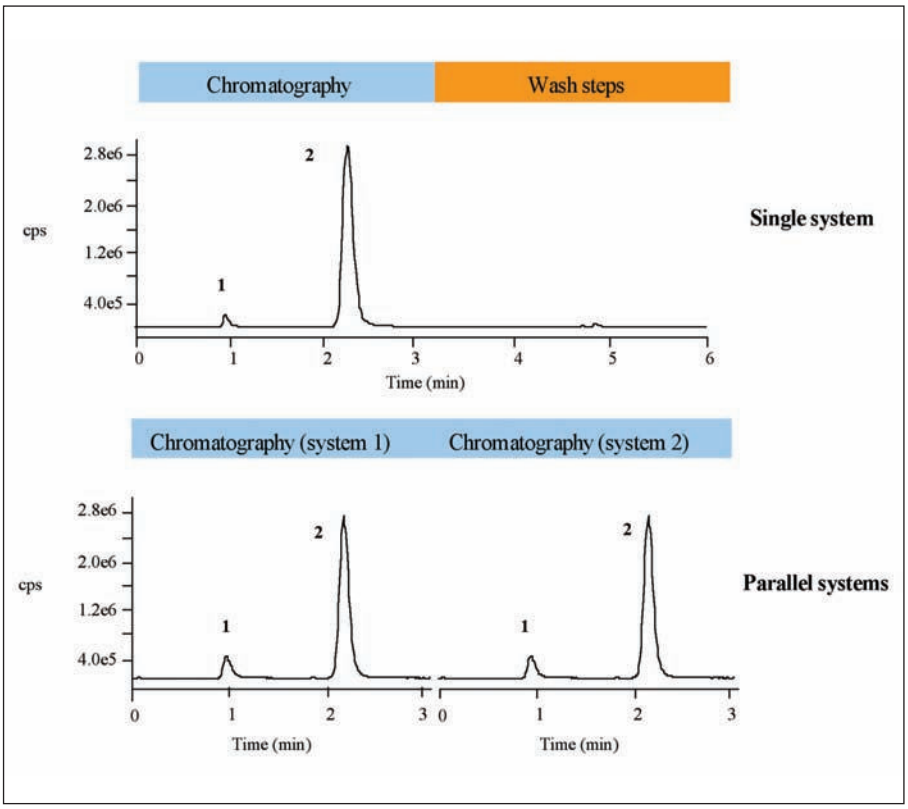

Figure 4: 2-fold increase in throughput using a parallel TurboFlow platform for the analysis of a lipophilic compound in human plasma (1. Metabolite; 2. Proprietary API); chromatographic resolution and MS/MS detection on a C18 Zorbax Extend column (50 x 4.6 mm i.d.; 5 micron particles; flow rate: 0.8 mL/min; mobile phase (isocratic): 50/50 v/v water/acetonitrile containing 0.01% trifluoroacetic acid) after turbulent flow extraction.

by channelling additional LCs the throughput is quadrupled taking just 12 hours to run the same batch. The second area of improvement comes in the compound optimisation step where an analyst would re-plumb the system in order to infuse each compound of interest and determine the optimum transitions, collision energy, and tuning before manually creating a new instrument method. This process can also be automated now using an algorithm in the software named QuickQuan. Instead of manual infusion of individual components a group of compounds is added to a database and the software selects an LC and autosampler method in order to submit a batch to the system. The autosampler can then pick up each solution, inject through the system, and hence, infuse into the MS so that optimal settings per compound can be saved and a report generated [5]. Manual compound optimisation for the ten compounds analysed was estimated at ~ 2 hours as compared to an approximate ten-fold time saving of thirteen minutes when utilising the automated optimisation algorithm.

In addition to the time savings achieved using automated sample optimization and multiplexing, further utilisation of the small injection volume required on the 0.5 mm i.d. columns has been exploited. For example a number of investigators have now reported the successful analysis of drug compounds such as immunosuppressants and antibiotics from low volume samples such as ocular fluid (tears) [7, 8]. Research laboratories are reporting the use of TurboFlow technology in their analyses of extremely complex matrices such as hemodialysates [9] and edible animal tissues [10]. Perhaps of more interest to the pharma industry is the emerging use of the TurboFlow technology in protein-ligand screening/affinity ranking experiments [11]. So far this method has been utilised to demonstrate the affinity selection of a small steroidal alkaloid library with the acetylcholinesterase and butyrylcholinesterase proteins. The fast isolation and generic retention of the protein/ligand complexes would suggest that it may become useful in the high-throughput screening of such compound mixtures in the future. Several other scientific fields not discussed here could benefit from TurboFlow technology. This is certainly the case within pharmaceutical development where the recovery of a drug substance from a complex drug product formulation can sometimes be very challenging.

TFC: The Future

Bioanalysis of oral drugs, e.g. generally defined by a set of physicochemical properties following the rule of 5 [3], has become a well understood field where most challenges have already been identified and dealt with successfully over the past ten years. However, the increased emphasis on both drug safety and translational biology, e.g. the need to understand how pre-clinical efficacy models are representative of human pharmacology, has considerably modified the expectations for what needs to be measured routinely in biological samples. Therefore, the historical design of bioassays is gradually evolving towards a higher degree of complexity. Nowadays, it is not unexpected to monitor, sometimes in the same sample, not only the drug levels but also its potential active/reactive metabolites as well as the biomarkers associated with the mechanism of action of the drug. Metabolites are obviously more polar and/or generally smaller than the drug itself and could present stability issues (e.g. acyl glucuronides, N-oxides). As for the chemical space for biomarkers, it could span from a very small and polar compound such as a neurotransmitter to a very large and hydrophobic entity like fatty acids. Amongst the key analytical challenges with biomarkers are generally the sampling procedure, the sample volume available and, again, the potential stability issues.

Adventuring outside the boundaries of a well defined 'rule of 5' box is requiring further thinking in the development process for bioanalytical assays. The TurboFlow approach already has some intrinsic capabilities that facilitate the analysis of biomarkers and metabolites. First, it provides high sensitivity assays without the need for high sample volume, typically 15 L injection volume could suffice. In addition, the on-line extraction approach removes the need for lengthy sample preparation procedure, hence reducing sample degradation issues frequently observed with biomarker analysis. Over the years, different types of chemistries have become available for the TurboFlow extraction columns, from polymer-based weak ion-exchangers to hydrophobic silica-based sorbents. Therefore, the need to extract a wide range of compounds from a wide range a matrices has been addressed through the diversity of extraction sorbents commercially available. A method development module is also available to provide a quick screen of columns and mobile phases best suited for a target application.

A concern still remains though when analysing and quantifying in the same run several labile compounds of both related and unrelated structures. Even when using MS/MS, chromatographic separation has to become a crucial component to raise assay quality. The recent implementation of Ultra High Pressure Liquid Chromatography in most bioanalytical labs has not only generated a significant increase in sample analysis throughput, but has also allowed high chromatographic resolution to come back into play in a field where the typical 5 cm chromatographic columns were merely used for peak refocusing. Coupling an on-line extraction approach allowing minimal sample preparation with a powerful analytical separation has to be the ultimate approach for bioanalysis. The new Transcend platform [12] is coming very close to provide an off-the-shelf technical solution to achieve just that. With Transcend, the analytical compartment of the typical TurboFlow platform has been upgraded with high pressure flux pumps able to deliver UHPLC resolution post extraction. Despite limited data available in the literature and the need for specialist hands to operate it, this one-size-fit-all platform could become an answer to emerging bioanalytical challenges.

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