Analyst

The Use of an Integrated Ceramic Micro-Fluidic Separations Device For The High Sensitivity LC/MS/MS Quantification of Drugs in Low Volume Samples from DMPK Studies

Journal:	*Analyst*
Manuscript ID:	AN-ART-04-2015-000646.R1
Article Type:	Paper
Date Submitted by the Author:	28-May-2015

Complete List of Authors:	Plumb, Robert; Imperial College, Surgery and Cancer; Waters Corporation, Health Sciences Murphy, James; Waters, Tomany, Mike; Waters, Wilson, Ian D.; Imperial College, ; Imperial College, Smith, Norman; Kings College, Evans, Christopher; GlaxoSmithKline, DMPK Nicholson, Jeremy; Imperial College London, Biomolecular Medicine Kheler, Jonathan; GlaxoSmithKline, DMPK Bowen, Chester; GlaxoSmithKline, DMPK Rainville, Paul; Water Corporation, Health Sciences

The Use of an Integrated Ceramic Micro-Fluidic Separations Device For The High Sensitivity LC/MS/MS Quantification of Drugs in Low Volume Samples from DMPK Studies

Paul D. Rainville[1,2], James P Murphy[1], Mike Tomany[1], Ian D Wilson[4], Norman W Smith[2], Christopher Evans[3], Jonathan Kheler[3], Chester Bowen[3], Robert S Plumb [4], and Jeremy K Nicholson[4]

1 Waters Corporation, Milford, MA, USA
2 Kings College, Micro Separation Group, Stamford St, London, UK
3 GlaxoSmithKline, DMPK Department, King of Prussia, PA, USA
4 Imperial College, Department of Surgery and Cancer, Exhibition Rd, South Kensington, London, UK

Abstract

An integrated capillary scale (300µm id) ceramic microfluidic LC system combined with MS/MS has been successfully employed for the quantitative analysis of pharmaceutical compounds in human plasma. The capillary ceramic microfluidic LC/MS/MS system showed an approximate 20-fold (range 11 -38-fold) increase in sensitivity compared with a standard 2.1mm scale UPLC/MS/MS system for a broad range of analytes. The loading capacity of the devices capillary separations channel allowed injection of 2µL of an aqueous solution, and up to 1.2µL of a typical protein- precipitated plasma sample, onto the reversed-phase chromatography system. The system also showed excellent chromatographic performance and robustness, with no deleterious effects on the chromatography observed over the course of 1000 injections of protein-precipitated plasma. The ability of the ceramic microfluidic LC/MS/MS system to deliver this level of sensitivity and performance enables the routine quantification of pharmaceutical compounds from small format samples, such as those obtained by dried blood spot or other blood microsampling approaches, to be performed.

Corresponding Author Paul_Rainville@waters.com

Key Words: Micro LC, Bioanalysis, DMPK, LC/MS/MS

Introduction

Providing high quality quantitative and qualitative data to support compound discovery, lead candidate selection, preclinical safety assessment and clinical trial support is the principal task of the DMPK (drug metabolism and pharmacokinetics) scientist. The adoption of technologies such as reversed-phase HPLC in the late 1970's and early 1980's [1], LC/MS in the early 1990's [2,3], LC-NMR [4,5] and accurate mass LC/MS [6] in the late 1990's into DMPK analysis has increased the speed, sensitivity, precision and quality of the pharmacokinetic and metabolic information generated in these pivotal studies. The introduction of sub 2μm LC systems in the early 2000's provided another opportunity for the DMPK scientist to improve both data quality and information content whilst reducing analysis time [7,8]. So e.g., Pedraglio *et al* showed that assay times could be reduced from 3.5 minutes to 1.5 minutes whilst assay sensitivity was increased by a factor of 5 by employing sub 2μm LC [9]. These authors also demonstrated that the observed increase in assay sensitivity enabled the pharmacokinetics of the candidate drug molecule to be better defined by allowing drug concentrations in samples obtained from the 24hr time point to be measured rather than their having to be estimated by extrapolation. This technology was also quickly adopted in drug metabolite identification studies resulting in both improved throughput [10] and the detection of metabolites at lower concentrations [11].

These LC/MS technologies have served the DMPK scientist well over the last 30 years where sample volumes from both pre-clinical and human volunteer studies have typically been in the order of 50-1000μL, with limits of quantification ranging from 10pg/mL to 1ng/mL. However, over the past 4 years there has been a revolution in the collection of blood-derived samples from animals used in pre-clinical trials. Thus, interest in, and the move towards, the adoption of dried blood spot/micro sampling technologies for sample collection [12,14] has lead to a dramatic rethink in animal study design. The collection of smaller samples, particularly from rodents, has allowed multiple blood samples to be taken from each animal during studies, reducing the number of animals required to define the pharmacokinetic properties of new drug candidates. This reduction in animal numbers, which also reduced the total amount of drug substance needed to perform such

studies, not only provides ethical and fiscal benefits, but also results in enhanced data quality as all of the toxicokinetic profiles are generated from a small number of individual animals. This contrast with the previous need to obtain composite profiles via the sacrifice of several animals at each time point to provide sufficient sample for analysis. Whichever of these microsampling approaches are finally adopted for DMPK studies, the challenge to the bioanalytical scientist remains the same; the available sample volume for analysis has been significantly reduced whilst the level of sensitivity required to support the assays has not changed. As illustrated by Spooner *et al* [15] the sensitivity of modern LC/MS/MS systems is more than enough to address the requirements of the majority of orally administered studies. However, there are occasions when the combination of low sample volumes and low systemic concentrations of drug substance require greater analytical performance to obtain the requisite pharmacokinetic data.

Capillary scale and nano scale LC has long been employed in the field of proteomics to analyse proteins isolated by gel electrophoresis or a similar technique. The chromatographic scale benefits of the 150 and 75μm internal diameter columns result in a more concentrated peak and hence improved sensitivity of detection. The use of micro scale LC is not new and many of the fundamental studies were performed in the early 1980's by scientists such as Novotny who, in his early work, demonstrated the sensitivity benefits of capillary scale LC (cLC) [15]. These sensitivity benefits were further exploited by Henion *et al* when combined with electrospray MS to deliver extremely high levels of sensitivity for protein and peptide analysis [17]. Although capillary and nano scale LC showed great potential to increase assay sensitivity these initial studies relied upon "home built" systems, which hampered the rapid adoption of the technology by the wider chromatographic community. This problem was alleviated by the advent of commercial cLC systems in the late 1990's early 2000's which provided the potential for more widespread reproducible and reliable micro and nano-scale LC [18]. However, the performance of these instruments still remained very much dependent upon the skill and experience of the user to make the required connections and to extract the best from the system. This was especially apparent at the nano-scale level, where small voids in column connections had a very deleterious effect on the observed LC performance. Despite such limitations these systems have been successfully employed for both

proteomics and DMPK applications [19]. In the DMPK area, for example, Dear *et al* showed how these micro scale LC systems could be employed for both quantitative bioanalysis of tail bled mice and also for the identification of drug metabolites using capillary LC combined with ion trap MS [20,21].

One obvious route to avoid these performance-damaging column connection voids is to co-locate the column, connections and MS emitter/spray tip in one device (such approaches are often referred to as "lab on a chip" or "chips"[22]). Much of the initial work on these micro-fabricated devices has employed glass or plastic supporting substrates. Whilst these materials are of sufficient mechanical strength for low efficiency separations using 3 or 5μm particle LC the higher back pressures produced by longer columns packed with sub 2μm porous particles require a more mechanically robust material as a support. We have previously described the use of such a micro-fluidic device for the elucidation of drug metabolites obtained from in *in vitro* microsomal incubation studies [23], and more recently in a metabonomic application to urine [24]. Here we describe, and evaluate, the use of an integrated ceramic-based micro-fluidic device for the analysis of drugs in plasma samples of the type that would result from low sampling volume DMPK studies.

Experimental

Chemicals:

Alprazolam, atenolol, pentamidine, metoprolol, antipyrine, dextromethorphan, diphenhydramine, erythromycin, danazol monobasic sodium phosphate, dibasicsodium phosphate, EDTA desmopressin and salmeterol xinafoate were all purchased from Sigma Chemical Co. (St. Louis, MO., US) Formic acid, methanol and acetonitrile were all purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). The deuterated alprazolam used as an internal stadard, and the alprazolam-hydroxy-metabolite were obtained from Cerilliant (Round Rock, TX, USA). Exendin-4 was supplied by GlaxoSmithKline R&D, (Upper Merion, PA, USA). Rat blood was obtained from Equitech-Bio (Kerrville, TX, USA) and EDTA was used as the anti coagulant. Water was obtained from an in-house milli Q filtration system Millipore (Billercia MA, USA). The blood was stored refrigerated prior to use and used within 5 days of delivery.

Construction of ceramic microfluidic device

High-temperature co-fired ceramics (HTCC) technology has been widely used in the production of multilayered electronic circuits. Unlike common printed circuit board (PCB) technology the HTCC fabrication process enables passive electronic components to be embedded with multiple layers on ceramic material resulting in a high density complex board in a very small scale. This same principle has been implemented to create a microfluidic device. Instead of using a multi-layered technique to embed electronic components, fluidic channels are created within the device. Thereafter the device may be packed with chromatography media creating a compact efficient chromatographic column.

The ceramic process begins with a raw material called *green tape* which is available in thin sheets. The tape consists of ceramic material cast in an organic binder. A precision UV micromachining system is used to create a microfluidic channel on a single sheet of tape. The thickness of the tape defines the ultimate height of the microfluidic channel whilst the width of the laser cut determines the channel width. The centre layer is sandwiched by two pairs of sheets closing off the channel creating either a square or

rectangular cross section. Vias are placed through the top two layers of green tape above the origin and the terminal end of the channel to serve as ports to direct fluid into and out of the device.

The five-layered stack of green tape is laminated under isostatic pressure and elevated temperature ensuring that all layers are in direct contact prior to firing. The firing process occurs in two steps: An initial low-temperature organic burnout is used to remove the organic binder. Following this stage, the furnace temperature is slowly ramped to the sintering temperature of the ceramic. During the sintering process the ceramic glass particles begin to melt and flow while the structure of the device and internal features are maintained. The densification which accompanies the sintering process results in a monolithic structure with an embedded channel able to withstand high pressures.

The upper pressure capability is inversely proportional to the channel width. The greatest stress concentration within the channel corners ultimately limits the device. In this prototype device a 300 μm channel was capable of withstanding 12-15 kpsi.

Analytical Chromatography Test Mixture

Alprazolam, atenolol, pentamidine, metoprolol, antipyrine, dextromethorphan, diphenhydramine, erythromycin, and danazol were individually dissolved in methanol at a concentration of 1mg/mL. The individual standards were then spiked into a 1:1 (v/v) mixture of methanol:water to create a text mix of concentration 1ng/mL for each analyte.

Sample Preparation

Dried Blood Spot Analysis

Exendin-4 authentic standard was accurately weighted and dissolved in a 1:1 (v/v) mixture of methanol: water to yield a concentration of 100μg/mL. This solution was further diluted in fresh rat blood to produce a calibration curve covering the range 1pg/mL to 10ng/mL.

Desmopressin was accurately weighed and dissolved in a 1:1 (v/v) mixture of methanol: water to yield a concentration of 100µg/mL. This solution was further diluted in fresh rat blood to produce a calibration curve covering the range 10pg/mL to 10ng/mL.

A 15µL aliquot of the spiked blood samples prepared for each of the above analytes was placed on a Whatman DMPK C DBS card (Whatman, USA). The blood samples were allowed to dry for 2hrs at room temperature. A 4mm core was then cut from the center of each blood spot. The sample "core" was dissolved in 200µL of methanol, vortex mixed for 5 minutes, allowed to stand for 20 minutes, and then centrifuged at 13,000 RCF for 10 minutes. The supernatant was then removed and a 10µL diluted with water, 90µL.

Plasma Analysis

Alprazolam, its deuterated internal standard, and its hydroxy-metabolite were dissolved in 1:1 (v/v) methanol: water (v/v) at 1mg/mL. Control rat plasma was spiked with the alprazolam and 4-hydroxy-alprazolam standards to produces calibration samples with final concentrations of 100pg/mL to 100ng/mL. A 50µL aliquot of the sample was protein precipitated with 100 µL of cold acetonitrile containing the alprazolam D5 internal standard at a concentration of 20ng/mL, vortex mixed and centrifuged at 13,000g for 5 minutes. The resulting supernatant was transferred to glass autosampler vials for analysis by LC/MS.

LC/MS/MS

For comparison purposes samples were run on two systems, either a conventional ACQUITY system for 2.1 mm scale chromatography or, for capillary scale LC on the ceramic microfluidic device, a nanoACQUITY UPLC system (Waters Corp, MA, USA). Conventional UPLC MS was undertaken as follows. A 1µL injection of the sample was then made onto the ACQUITY UPLC system with separations performed on a 2.1 mm x 100 mm column packed with 1.7 µm BEH C18 thermostatically controlled at 45.0˚ C. The analytes were eluted under a linear gradient using mobile phases consisting of either A: 0.1% formic acid (A) or acetonitrile (B). The gradient began at 5 % B rising to 95% in

3.0 minutes at a flow rate of 600 µL/min. The column effluent from the system was directed into a Xevo TQ mass spectrometer (Waters Corp, Wilsmlow, UK) operated in positive electrospray ionisation (ESI) mode with multiple reaction monitoring (MRM).

For the chromatographic separations on the ceramic microfluidic device injections from 0.1-2µL of the sample was then made into the nanoACQUITY UPLC system. As for the "conventional" UPLC separation the mobile phases consisted of 0.1% formic acid (A) and acetonitrile (B). The analytes were eluted using a linear gradient from 5 – 95 % B in 3.0 minutes at a flow rate of 10 µL/min. Separations were performed on a prototype ceramic microfluidic device with a 0.3 mm x 100 mm channel packed with a 1.7 µm BEH C18 stationary phase. The temperature of the device was thermostatically controlled at 45.0˚ C. Capillary scale column separations were performed on a 0.3 x 100mm fused silica column packed with 1.7 µm BEH C18 material using identical chromatographic conditions to those employed for the ceramic microfluidic device.

The Analytical text mixture was analysed on the same analytical system using a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C18 thermostatically controlled at 45.0˚ C. The compounds were analysed using gradient elution with an aqueous formic acid (0.1%): acetonitrile gradient from 5 – 95% over 10 minutes at 12µL/min. The detection was performed on a Xevo TQS mass spectrometer (Waters Corp, Wilmslow, UK) with a capillary voltage of 1Kv, a cone voltage of 30V and a collision energy of 15, with a nebuliser gas flow rate of 300L/hr. The following transitions were used for data acquisition atenolol 267.3 ⇒ 145.1, pentamidine 341.4 ⇒ 120.1, metoprolol 268.3 ⇒ 116.1, antipyrine 189.2 ⇒ 106.4, dextromethorphan 272.4 ⇒ 171.1, diphenhydramine 256.4 ⇒ 167.1, erythromycin 716.8 ⇒ 158.2, and danazol 338.4 ⇒ 145.

For the analysis of drugs in bloodspots/plasma the column effluent was directed into a Xevo TQ mass spectrometer operated in positive ESI and in MRM modes. The MRM transitions employed were: *m/z*309.2 ⇒ 281.0(alprazolam), *m/z* 325.0 ⇒ 297.0 (hydroxylated alprazolam), and *m/z* 314.2 ⇒ 286.0 (deuterated alprazolam). Desmopresin was monitored using the SRM transition of 535.5 ⇒ 328.2 with a cone voltage of 30 and collision energy setting of 20. Salmeterol was monitored using the transition SRM 416.5 ⇒ 232.2 employing a cone voltage of 28V, and collision energy

setting of 24ev. Exendin 4 was monitored using the transition SRM 838 ⇒ 948 employing a cone voltage of 20V, and collision energy setting of 22eV.

In all cases the quantitative analysis was performed using MassLynx, TargetLynx application manager (Waters Corp, Wilmslow, UK)

Chromatographic efficiency was determined for both the capillary scale column and microfluidic ceramic device in isocratic mode. Both the capillary column and separations channels were packed with 1.7 μm BEH C18 material the columns were eluted using a 1:1 methanol:water mobile phase at a temperature of 40ºC at a flow rate of 5μL/min. A 0.1μL injection of alprazolam at a concentration of 100ng/mL was made onto the column and the peak efficiency determined using the peak width at half height.

Results and Discussions

Chromatographic Performance

As with any chromatographic method the results obtained are dependent upon the overall performance of the system, not just the column efficiency, but also how well band broadening is controlled. This band broadening must be managed both pre-column as well as post column such that as much of the true chromatographic performance of the column is retained and available for analysis. This is particularly true at these very small chromatographic scales where peak volumes are on the scale of a few tens of nanolitres, such that even a small void volume, e.g., one microliter or less, can cause a disastrous overall reduction in chromatographic performance. Such voids etc., often manifest themselves as broad, tailing peaks. The data displayed in **Figure 1** illustrates the separation of the eight common pharmaceuticals used to prepare the text mixture analysed on the prototype ceramic microfluidic device using the generic reversed-phase gradient methodology. With the exception of pentamidine all of the analytes showed symmetrical Gaussian peaks, with a peak width in the region of 6 seconds at the base. These data show that the device was capable of delivering acceptable quality chromatographic performance providing, in this case, a chromatographic peak capacity in the region of 100. The MS response of the ceramic microfluidic device then compared to that of a "conventional" 2.1mm analytical UPLC system (Supplementary **Figure S1**). In this example a 1μL injection (100% water) of the therapeutic peptide, desmopressin (a synthetic analogue of vasopressin used to treat bed wetting in infants), was made onto both conventional 2.1 mm and ceramic microfluidic UPLC systems at the same concentration of 100pg/mL. The column eluents were monitored by a tandem quadrupole MS instrument operated positive ESI in MRM mode. To avoid any bias the same mass spectrometer was used in these experiments for both column geometries. Both separations were operated in gradient elution mode and the chromatography was scaled to give exactly the same gradient steepness with an identical number of column volumes defining the gradient on both columns. It was evident from the resulting data that the ceramic microfluidic device showed somewhat more tailing than the 2.1mm ID column (although it is arguable that this is an unfair comparison as the injection loading, when

viewed as a percentage of the column volume, is not the same and does not represent the real-life operating conditions of the system). The observed tailing may therefore be due to the relatively large loading volume of 1μL on the ceramic microfluidic device which represented one-seventh of the volume of the 300 μm separations channel but only ca. 1% of the volume of the conventional column. However, other factors could also contribute to the observation, such as dispersion within the system or as a result of a less efficient packing process with the narrow separations channel when compared to the conventional 2.1 mm analytical column. Thus, although the absolute chromatographic performance of the ceramic microfluidic device was lower than the analytical column it was still acceptable for the purposes of quantitative bioanalysis. The relatively later elution of the peak from the capillary column compared to the conventional UPLC separation (1.65 vs 0.85 min. respectively) results from the increased time required to displace (sweep) the intrinsic delay volume of the two chromatography systems. Hence, at a flow rate of 10μl/min even the small internal volumes of the dedicated capillary system can manifest themselves as a delay in the chromatography. In contrast, with the conventional system the "system volume" of 80μL takes less than ten seconds to be swept by the eluent. This is the case with even the most optimized of nano scale chromatography systems and will have an effect on comparative throughput for quantitative analysis. Although the ceramic microfluidic device showed increased tailing, and a slightly increased analysis time over the conventional system, the performance was more than acceptable for use in quantitative analysis.

Comparative Sensitivity of Conventional and Ceramic Microfluidic System

The most striking observation from the data shown in Figure 1a/b is that the capillary scale separation delivered by the ceramic microfluidic device gave a significantly greater peak response compared with that of the conventional UPLC system. In the desmopressin example, for the same injection volume and mass loaded onto both systems the ceramic microfluidic device showed an almost 40-fold increase in signal response (peak area). In order evaluate further the effect of the column geometry on peak response a series on chemically diverse pharmaceutical compounds were analysed using both column geometries. As in the previous experiment the data for both instruments were

acquired on the same mass spectrometer to avoid potential bias caused by different instrument responses (however, the instrument was optimized for nebulizer gas flow for each chromatographic system). The data obtained for this comparison is shown in **Table 1**, and show that the ceramic microfluidic system consistently showed a greater response, measured as signal-to-noise, than the conventional scale system. The magnitude of the increase in response varied from compound to compound, ranging from 11-fold with salmeterol xinafoate to 38-fold with desmopresin; this variation in response may be due to the hydrophilic/lipophilic nature of the compounds being tested or due to superior ionization characteristics at lower flow rates. The average increase in sensitivity for the series of compounds tested was determined to be 20-fold.

Loading Capacity

The increase in signal response for the ceramic microfluidic system was based upon the same volume (1μL), and therefore mass of analyte, being loaded onto both column geometries. However, the applicability of the capillary scale system for quantitative analysis is dependent upon the maximum load that can be applied to the column and the robustness of the column towards biologically derived samples. Whilst it is not always the case in complex mixture analysis, and it is not always a linear function, increasing the sample injection volume usually results in greater peak height/area and improved limits of detection. The loading capacity of the system was investigated for both aqueous solutions and in biologically derived samples. The data displayed in Supplementary **Figure S2** show the chromatographic response obtained from the analysis of the antidepressant alprazolam in an aqueous solution (10ng/mL). From the obtained data it was determined that with an aqueous injection solution and using a reversed – phase gradient a volume of up to of 2μL of drug-containing solution could be loaded onto the column without any deleterious effect on the chromatography. As can be seen from Figure S2 the increase in injection volume resulted in an almost linear increase in peak intensity.

The same experiment was then repeated with alprazolam, spiked into in rat plasma at 10ng/ml, following protein precipitation with cold acetonitrile at a ratio of 2:1 (solvent:plasma). The sample was vortex mixed, centrifuged and the supernatant removed for analysis, resulting in a solution that contained approximately ≈ 60% organic solvent. The sample was introduced onto the LC/MS system at various injection volumes ranging from 0.1 – 2.0μL, with results displayed in Figure 2. With these highly organic samples we observed that the maximum sample volume of protein precipitated plasma that could be injected onto the ceramic microfluidic device was 1.2μL. The peak shape and retention times remained constant up to this injection volume of 1.2μL, but larger injection volumes resulted in splitting of the analyte peak and a reduced retention time. This injection volume may appear to be small volume compared to the analytical scale LC separation, where 10-15μL injection volume is possible, but with the increased sensitivity resulting from the use of the capillary system an overall improvement in assay performance was still observed. In addition, when only a few microlitres of sample are available, such as is the case for e.g., with tail-bled mouse studies and paediatric or neurological samples, there is a clear advantage to this scale of chromatography as it delivers a significant increase in sensitivity. The maximum volume / mass loading onto the system could potentially be increased by the use of sample dry down and reconstitution or sample clean-up with, for example, solid phase extraction if required.

Tolerance Towards Plasma Derived Samples

As described above, the increased sensitivity of the separation observed using the ceramic microfluidic system format is particularly attractive to the bioanalyst. However, in order to be an effective analytical technique the system must be robust towards the injection of hundreds if not thousands of plasma extracts to enable routine day-to-day operation. The majority of bioanalytical assays require the analysis of ca. seventy to one hundred samples in a single batch (although longer runs are not unusual) with the expectation of performing a minimum of five hundred to one thousand analyses per column in order for the methodology to be economically viable. In order to evaluate the robustness of the ceramic micro-fluidic device towards plasma-derived samples rat plasma was spiked with alprazolam and 4-hydroxy-alprazolam at concentrations of

100pg/mL. These plasma samples were processed by protein precipitation with acetonitrile as previously described and then analysed on the ceramic microfluidic device in 10 separate batches of 100 injections after which the column performance was evaluated. The overlaid injection of the first and one thousandth injection are displayed in Supplementary Figure S3 and the similarity of the clearly traces show the column to be robust and reproducible over the whole of the 1000 sample analysis, with little to no reduction in column performance or increase in column backpressure. As well as being sufficiently robust towards biological samples and to be cost effective, chromatographic systems used for bioanlysis must also be reproducible over the long term. During the course of a safety assessment or clinical trial study thousands of samples will be analysed and, in order for this microscale approach to be applicable for routine use, it must be reproducible from column to column over the long term. The inter- and intracolumn performance of these prototype ceramic microfluidic devices were evaluated both in terms of reproducibility and column efficiency (measured in isocratic mode). The data displayed in Table 2 compared the column efficiency of six ceramic microfluidic devices chromatography systems and six 300µm capillary columns of the same dimensions. The results showed that the inter device reproducibility was high, as well as the fact that the column efficiency of the ceramic chromatography system was similar to that of the packed capillary column. The data displayed in Table 3 shows the retention time reproducibility of alprazolam and the 4-hydroxy metabolite from a plasma extract on four ceramic microfluidic device. The retention time RSD for alprazolam ranged from 0.0 to 0.94 and from 0.27 to 0.80 for the 4 hydroxylated metabolite. The inter-tile retention time variation was less than 0.03 mins. These results suggest that each of the columns delivered similar results in terms of both chromatographic performance and assay sensitivity and indicate that this micro column approach should be suitable for use in routine bioanalysis.

MS Compatibility and Interface

Nano and capillary scale LC has been predominantly exploited in the field of proteomics for the detection and characterization of peptides and intact low molecular mass proteins. The majority of the MS sources employed in for these applications can be classified as

"open-sources" and are not isolated from the general laboratory atmosphere. This is in contrast to the ion-spray sources (electrospray or turbo ion-spray) that are employed with conventional scale chromatography where the source is isolated from the laboratory environment. This "open-source" construction allows easy adjustment of spray position and optimization of response by the scientist. The higher mass-to-charge values monitored in proteomics, typically 600 *m/z* and above, means that the majority of the chemicals present the laboratory atmosphere, which are typically observed with signal response lower than *m/z* 500, do not interfere with the experiment being undertaken. However, with small molecule analysis these low molecular weight compounds present in the laboratory atmosphere can significantly impact assay performance. To evaluate the real effect of this a "capillary LC closed source" was developed that was purged with nitrogen and isolated from the laboratory atmosphere. A comparison of the background signals obtained with both the "open-source" construction and "closed-source" construction is shown in Figure 3. These results show that the "open-source" signal possessed a greater number of low *m/z* background signals than the "closed-source" construction. In the closed source there were relatively few ions above *m/z* = 300 and the *m/z* = 218 ion was dominant whereas, in the case of the open source configuration the *m/z* = 148.9 and *m/z* = 95 ions showed a significant response as did those at *m/z* = 354 and *m/z* = 370.9. This interference resulted in a ten-fold reduction in analyte response for the "open-source" compared to the "closed-source" configuration, in SRM mode. As a result of this interference all of the experiments conducted in this study were performed with the "closed source" only.

Sensitivity in DMPK Studies

The applicability of this integrated ceramic micro-fluidic separations device for quantitative DMPK analysis was investigated with two different classes of analytes; i) a small molecule benzodiazepine (alprazolam) in combination with its hydroxylated metabolite and ii) a large molecular weight candidate therapeutic peptide. This allowed the evaluation of the performance of the micro-fluidic system with both traditional small molecules and also a biotherapeutic peptide, which are becoming an increasingly important area of research in the discovery of novel drugs [26].

The short acting benzodiapine alprazolam and its hydroxylated metabolite were spiked into rat blood at concentrations ranging from 100pg/mL to 1000ng/mL and then spotted onto a DBS card and extracted as described above. A representative chromatogram obtained from the analysis of the analyte, internal standard and the metabolite are shown in Supplementary Figure S4. The alprazolam and its deuterated internal standard eluted with a retention time of 1.45 minutes with the hydroxylated metabolite eluting at 1.35 minutes. As can be seen from the extracted ion chromatograms the peak shapes were acceptable, with an average peak width of 6 seconds at the base and the hydroxy-metabolite well resolved from alprazolam. The resolution of alprazolam from the endogenous material in the sample analytes was monitored using the simultaneous full-scan capability of the mass spectrometer along with the MRM signal for the target analytes [25]. The results obtained are illustrated in Figure 4, where Figure 4a shows that the alprazolam peak (shown in RED) was clearly resolved from the background (shown in GREEN), endogenous, material (such as e.g., phospholipids) in the sample. This chromatographic resolution allowed a limit of detection of 100pg/mL, equal to 100fg on column, to be reached. The data displayed in Figures 4b & c shows the extracted ion chromatogram for the blank and 100pg/mL standard respectively. The blank signal has been magnified by a factor of 5 in the region where the alprazolam peak elutes to determine the analyte signal-to-noise value. These results show that at a 100pg/mL level the assay exhibited a signal-to-noise value of 5:1. A representative calibration line for the alprazolam test analyte is shown in Figure 4d, and showed a correlation coefficient of $r^2 = 0.9951$ using 1/x weighting and an intercept of -3.76. These data showed that the system was suitable for quantitative bioanalysis and could deliver acceptable levels of performance for use in the routine.

A similar approach was used for the evaluation of systems response with the candidate therapeutic peptide Exendin-4, a 38 amino acid peptide with a molecular weight of 4186 amu. The peptide DBS sample was prepared in a similar manner to that employed for alprazolam. The data obtained were compared to that obtained a conventional 2.1 x 50 mm scale separation using a 1.7µm C18 chromatographic system. The results displayed in Figure 5(a) illustrate the data obtained at the conventional scale, showing that the peptide peak eluted with a retention time of 1.2 minutes and exhibited a limit of detection

of 10ng/mL with a 20μL injection volume. The data in Figures 5 b & c show the results for the analysis of the same sample using a 1μL injection onto the micro-fluidic system where the peptide peak eluted with a retention time of 2.1 minutes. From Figures 5b & c the responses obtained with 100 & 250pg/mL extracted standards can be seen. These mass chromatograms show that the result obtained for the 250pg/mL standard on the ceramic microfluidic system delivered a similar, if not slightly superior, response to that obtained with the conventional scale system. The data obtained from the 100pg/mL sample on the ceramic microfluidic system revealed a slightly inferior signal to noise to the 10ng/mL standard on the analytical scale system, but represents the usable limit of detection of the system. These results suggest that with this particular biotherapeutic peptide the ceramic microfluidic system was capable of delivering an increase in assay sensitivity in the region of 40 to 100-fold that of the conventional chromatography system with 20-fold less sample injected onto the column. However, although the majority of the increase in sensitivity was due to the reduction in chromatographic scale a contribution from increased efficiency of ionization the peptide at low flow rates was also responsible for the observed improvement in response. The results obtained from the analysis of these two compounds shows that the capillary scale microfluidic device delivers increased assay sensitivity over conventional scale LC and also provides the specificity required for a bioanalytical assay. The increased signal response provided by the ceramic microfluidic device provides a significant advantage in terms of assay sensitivity with only a small increase in analysis time, in what is already a rapid method. The analysis time for exendin-4 was increased from 2 minutes to 3 minutes and by the same amount for the desmopresin assay. Whilst this amounts to a 50 % increase in analysis time, the overall analysis time is still acceptable for a bioanalytical assays. In terms of cost savings for the overall process of performing the study in animals, this approach allows serial bleeding therefore improving PK data, a reduction in the number of animals required, reduction in animal facility costs (including technical staff) , a reduction in solvent costs (>99%) and reduced sample transport cost.

Conclusion

Capillary scale LC/MS/MS shows great potential for the analysis of candidate pharmaceuticals and their metabolites in biological fluids resulting from low sample volume DMPK studies. The data reported in this study suggest that an increase in sensitivity in the 10 to 40-fold range can be achieved. The chromatographic performance of the system was sufficient to deliver a specific reproducible assay as illustrated with the alprazolam analysis. The use of an integrated microfluidic device removes the need to make complicated column connections or to precisely optimize the spray position, thus simplifying the routine application of this technology. Despite a relatively low column volume of just 7μL it was possible to load 2μL of aqueous sample, and up to 1.2μL of a protein precipitated plasma extract containing 60% organic solvent, onto the capillary column without degrading the chromatographic performance. The capillary column demonstrated its robustness towards the injection of extracted biological samples with greater than one thousand injections possible with no deleterious effect on the column performance. These results suggest that these ceramic microfluidic devices can be used in the routine bioanalytical environment to provide high sensitivity data. Whilst this technology may not be necessary for the majority of bioanalytical assays the increased sensitivity of capillary scale LC/MS may facilitate the development of micro-sampling technologies such as dried blood spots.

References

1. High-pressure liquid chromatographic method for the simultaneous quantitative analysis of propranolol and 4-hydroxypropranolol in plasma. Nation RL, Peng GW, Chiou WL. J Chromatogr. 1978 May 1;145(3):429-36
2. High-throughput quantitative bioanalysis by LC/MS/MS. Jemal M. Biomed Chromatogr. 2000;14(6):422-9.
3. Recent advances in use of LC/MS/MS for quantitative high-throughput bioanalytical support of drug discovery. Ackermann BL, Berna MJ, Murphy AT. Curr Top Med Chem. 2002;2(1):53-66
4. On-line liquid chromatography coupled with high field NMR and mass spectrometry (LC-NMR-MS): a new technique for drug metabolite structure elucidation. Burton KI, Everett JR, Newman MJ, Pullen FS, Richards DS, Swanson AG. J Pharm Biomed Anal. 1997;15(12):1903-1,

5. Application of directly coupled LC-NMR-MS to the structural elucidation of metabolites of the HIV-1 reverse-transcriptase inhibitor BW935U83. Shockcor JP, Unger SE, Savina P, Nicholson JK, Lindon JC. J Chromatogr B Biomed Sci Appl. 2000 1;748(1):269-79
6. Quantification and rapid metabolite identification in drug discovery using API time-of-flight LC/MS. Zhang N, Fountain ST, Bi H, Rossi DT. Anal Chem. 2000 15;72(4):800-6
7. Increasing throughput and information content for in vitro drug metabolism experiments using ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer. Castro-Perez J, Plumb R, Granger JH, Beattie I, Joncour K, Wright A. Rapid Commun Mass Spectrom. 2005;19(6):843-8
8. Development and validation of a fast and sensitive UPLC-MS/MS method for the quantification of six probe metabolites for the in vitro determination of cytochrome P450 activity. De Bock L, Boussery K, Colin P, De Smet J, T'jollyn H, Van Bocxlaer J. Talanta. 2012 30;89:209-16
9. New perspectives in bio-analytical techniques for preclinical characterization of a drug candidate: UPLC-MS/MS in in vitro metabolism and pharmacokinetic studies. Pedraglio S, Rozio MG, Misiano P, Reali V, Dondio G, Bigogno C. J Pharm Biomed Anal. 2007 27;44(3):665-73
10. UPLC-MS, HPLC-radiometric, and NMR-spectroscopic studies on the metabolic fate of 3-fluoro-[U-14C]-aniline in the bile-cannulated rat. Athersuch TJ, Castro-Perez J, Rodgers C, Nicholson JK, Wilson ID.Xenobiotica. 2010;40(7):510-23
11. High-throughput bioanalysis with simultaneous acquisition of metabolic route data using ultra performance liquid chromatography coupled with time-of-flight mass spectrometry. O'Connor D, Mortishire-Smith R. Anal Bioanal Chem. 2006;385(1):114-21
12. Facilitating pharmacokinetic studies in children: a new use of dried blood spots. Patel P, Mulla H, Tanna S, Pandya H. Arch Dis Child. 2010;95(6):484-7,
13. Incurred sample reanalysis comparison of dried blood spots and plasma samples on the measurement of lopinavir in clinical samples. Meesters RJ, Hooff GP, Gruters R, van Kampen JJ, Luider TM. Bioanalysis. 2012;4(3):237-40,
14. Dried blood spots as a sampling technique for the quantitative determination of guanfacine in clinical studies. Li Y, Henion J, Abbott R, Wang P. Bioanalysis. 2011;3(22):2501-14
15. Capillary HPLC: Columns and Related Instrumentation. J. Chrom. Sci. 1980 18(9): 473-478
16. Dried matrix spot direct analysis: evaluating the robustness of a direct elution technique for use in quantitative bioanalysis. Abu-Rabie P, Spooner N. Bioanalysis. 2011 ;3(24):2769-81
17. Determination of leucine enkephalin and methionine enkephalin in equine cerebrospinal fluid by microbore high-performance liquid chromatography

and capillary zone electrophoresis coupled to tandem mass spectrometry. Mück WM, Henion JD. J Chromatogr. 1989 27;495:41-59
18. Instrumental requirements for nanoscale liquid chromatography. Chervet JP, Ursem M, Salzmann JP. Anal Chem. 1996 1;68(9):1507-12
19. High-sensitivity nanoLC-MS/MS analysis of urinary desmosine and isodesmosine. Boutin M, Berthelette C, Gervais FG, Scholand MB, Hoidal J, Leppert MF, Bateman KP, Thibault P. Anal Chem. 2009 1;81(5):1881-7
20. The use of capillary high performance liquid chromatography with electrospray mass spectrometry for the analysis of small volume blood samples from serially bled mice to determine the pharmacokinetics of early discovery compounds. Fraser IJ, Dear GJ, Plumb R, L'Affineur M, Fraser D, Skippen AJ. Rapid Commun Mass Spectrom. 1999;13(23):2366-75,
21. The rapid identification of drug metabolites using capillary liquid chromatography coupled to an ion trap mass spectrometer. Dear GJ, Ayrton J, Plumb R, Fraser IJ. Rapid Commun Mass Spectrom. 1999;13(5):456-63
22. Monolithic integration of two-dimensional liquid chromatography-capillary electrophoresis and electrospray ionization on a microfluidic device. Chambers AG, Mellors JS, Henley WH, Ramsey JM. Anal Chem. 2011 1;83(3):842-9, Proteomic analysis of plasma membrane vesicles. Bauer B, Davidson M, Orwar O. Angew Chem Int Ed Engl. 2009;48(9):1656-9
23. Addressing the challenge of limited sample volumes in in vitro studies with capillary-scale microfluidic LC-MS/MS.Rainville PD, Smith NW, Wilson ID, Nicholson JK, Plumb RS. Bioanalysis. 2011;3(8):873-82.
24. Advances in liquid chromatography coupled to mass spectrometry for metabolic phenotyping. Rainville, P.D, Theodoridis, G, Plumb, R.S, Wilson, I.D. Trends Anal. Chem; 2014, 61, 181-191
25. A novel LC-MS approach for the detection of metabolites in DMPK studies. Plumb RS, Mather J, Little D, Rainville PD, Twohig M, Harland G, Kenny DJ, Nicholson JK, Wilson ID, Kass IJ. Bioanalysis. 2010 2(10):1767-78.
26. Market watch: Sales of biologics to show robust growth through to 2013 M Goodman Nature Reviews Drug Discovery 8, 837: 2009

Table 1: Comparison of Peak Response Area Ceramic Microfluidic Device and Analytical Scale LC/MS With 5 Model Compound

Compound	Analyte Peak Response		Fold Increase
	Capillary Scale	2.1mm Scale	
Alprazolam	650	40	14
4-Hydroxyalprazolam	223	10	22
Desmopressin	7008	184	38
Salmeterol Xinafoate	1025	97	11
Nafazodone	980	62	16

Table 2: Comparison of Chromatographic Efficiency Between Six Capillary columns and Six Ceramic Microfluidic Devices for

Column No	Capillary column Theoretical Plates	Ceramic micro-fluidic Theoretical Plates
1	8287	8689
2	10329	8892
3	11813	8995
4	11747	7289
5	8640	11250
6	10500	9114
Average N	10219	9038
Std Dev	1496	1273
% RSD	15	14

Table 3 Inter and Intra Device Comparison of Chromatographic Performance

N = 6 Injections	Tile 1	Tile 2	Tile 3	Tile 4
Retention Time Alprazolam	1.55	1.57	1.56	1.56
% RSD	0.33	0.00	0.94	0.35
Retention time Hydroxy Alprazolam	1.45	1.48	1.46	1.56
% RSD	0.28	0.55	0.80	0.27
Rs at 5% Peak Height	1.50	1.20	1.33	1.17

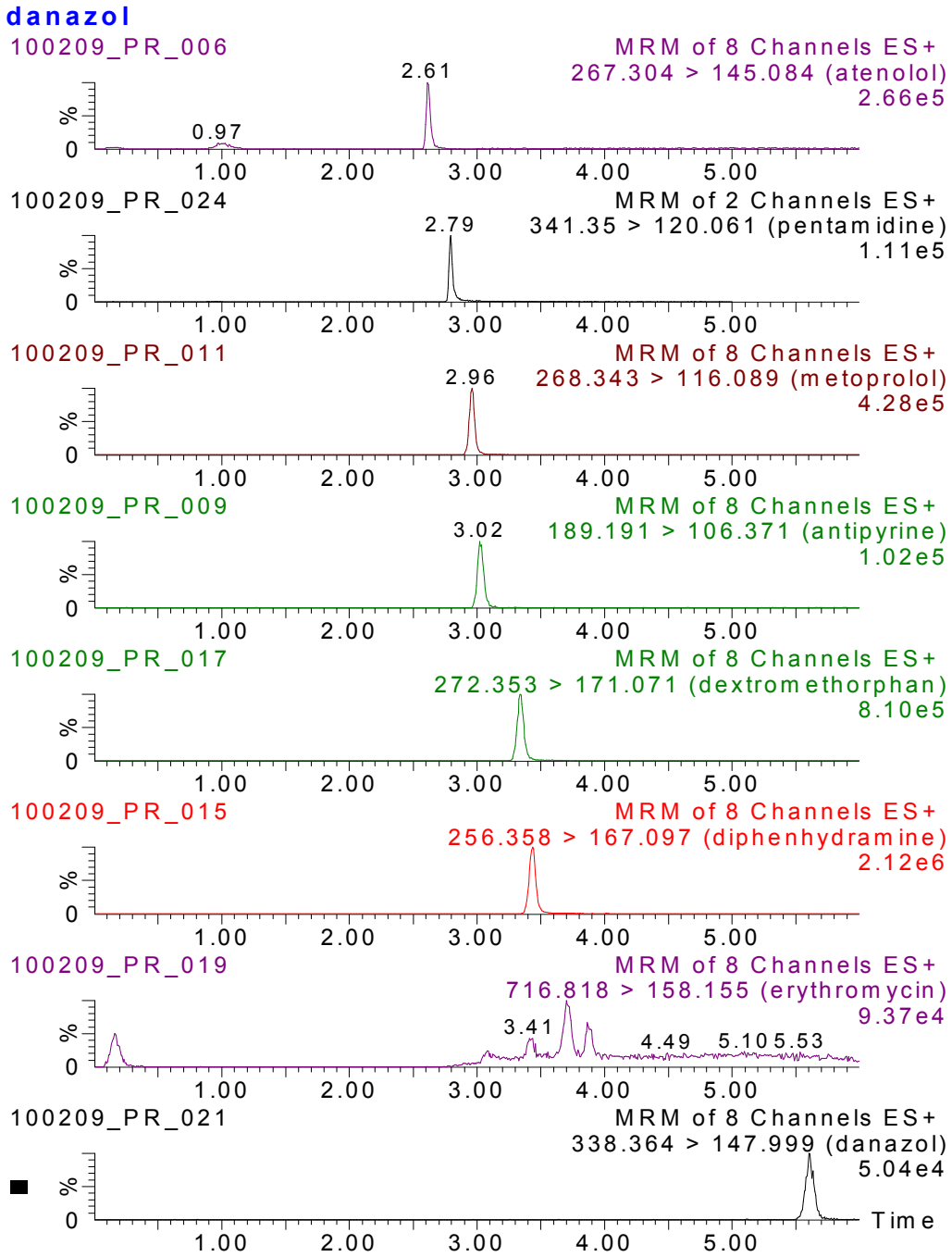

Figure 1: Analysis of 8 probe pharmaceutical compounds (compound identities indicated on mass chromatograms) on ceramic microfluidic device using a reversed- phase gradient (5-95% formic acid (aq): acetonitrile over 7) minutes at 12µL/min with detection by positive ion MRM mass spectrometry

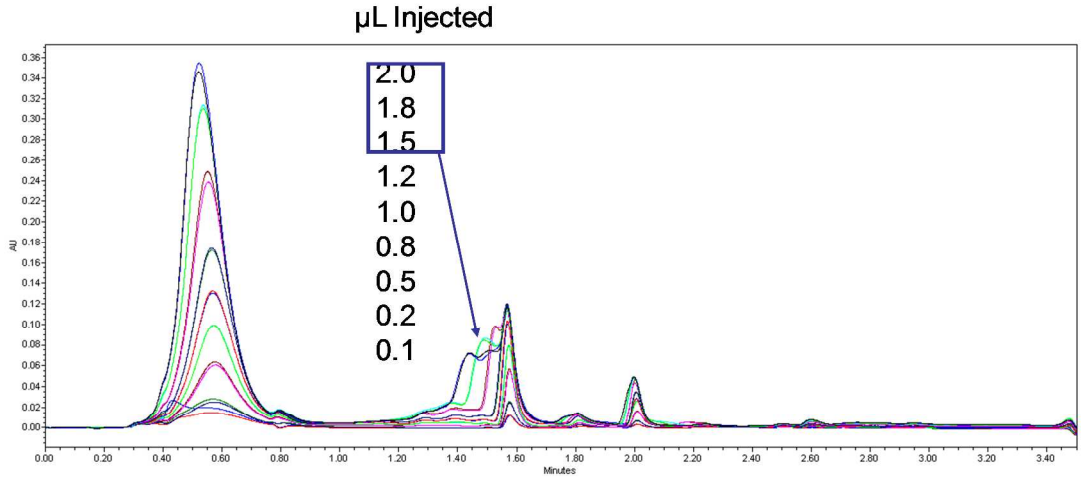

Figure 2: Overlaid Chromatographic Response For Alprazolam With Increasing Injection Volume Of Plasma Precipitated Sample. Injection of protein precipitated plasma containing alprazolam with increasing injection volumes from 0.1 to 2µL onto a 0.3 x 100mm ceramic microfluidic device packed with 1.7µm BEH C18 material and eluted a 5-95% formic acid (aq) acetonitrile gradient over 3 minutes at 12µL/min with detection by positive ion MRM. Maximum volume loaded onto the ceramic microfluidic device without deterioration of the peak shape was 1.2µL.

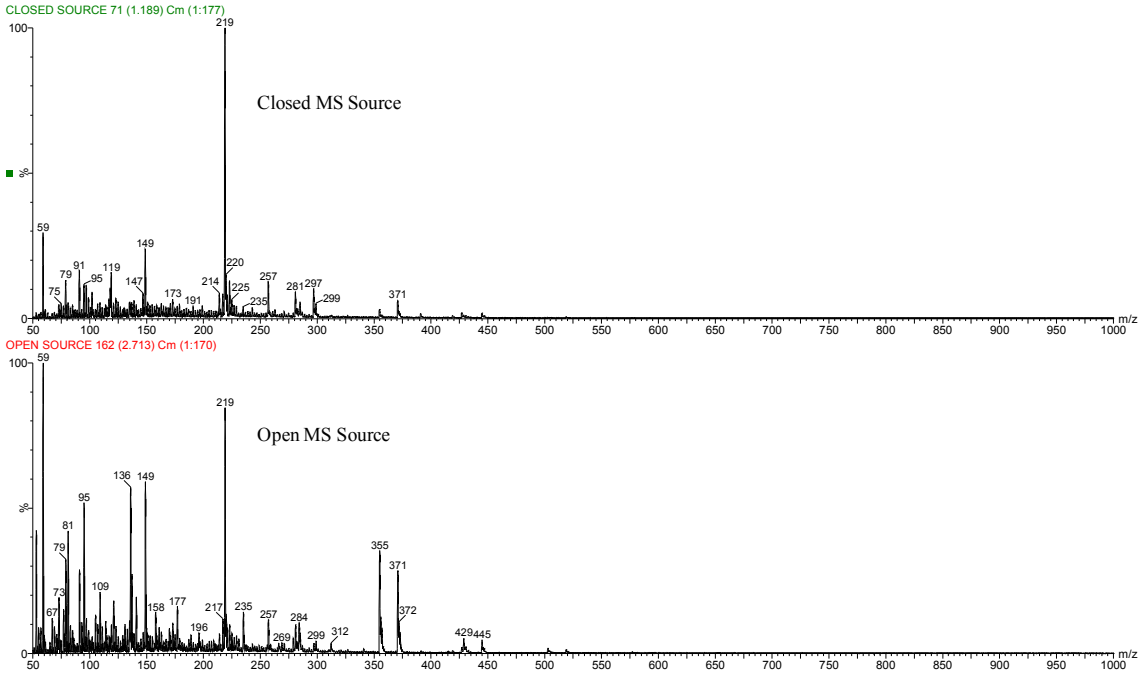

Figure 3: Comparison of the Ambient MS signal For The Closed and Open Capillary Scale MS Source. Positive ion full scan mass spectrum, 50-1000 *m/z*, of background solvent signal (formic acid (aq): acetonitrile) 50:50, using either the closed source (top) and open source (bottom)

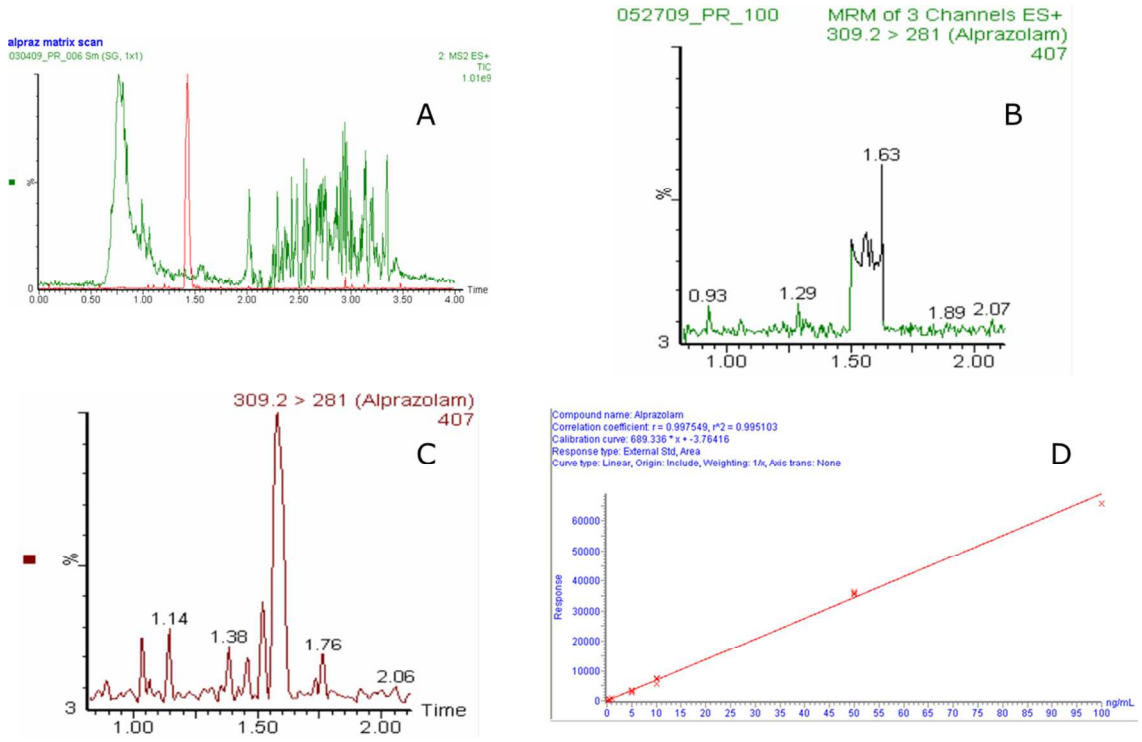

Figure 4: LC/MS analysis of alprazolam using a ceramic microfluidic device with a separation channel of dimensions 0.3 x 100mm device packed with a 1.7µm BEH C18 material eluted with a reversed-phased gradient and MS/MS detection in positive ESI mode. 4a Shows the MRM signal for alprazolam (red) & background MS signal (GREEN). 4b & c Show the extracted ion chromatograms for the blank and 100pg/mL standards respectively. 4d Is the calibration line from 0.1-100ng/mL

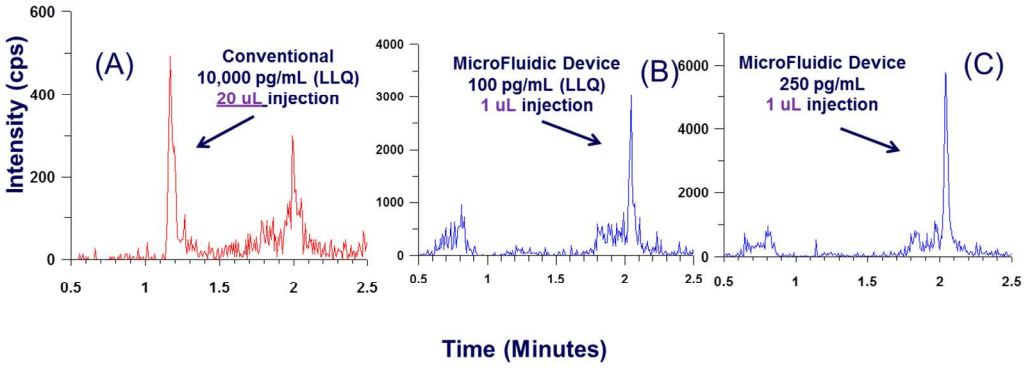

Figure 5: Comparison of the Exendin-4 peak response using a conventional scale LC/MS/MS and a ceramic microfluidic device LC/MS/MS system. The analytical separation (A) was performed on a 2.1 x 5cm 1.7µm BEH column and eluted under reversed-phase gradient conditions over 3 minutes with a 20µL injection of 10,000pg/mL Exendin-4 standard. The ceramic microfluidic separation was performed on a 0.3 x 100mm ceramic microfluidic device packed with 1.7µm BEH eluted under reversed-phase conditions over 3minutes, with a 1µL injection of either a 100pg/mL (B) or a 250pg/mL of Exendin-4 standard.