Applications of Capillary LC(–MS) to Drug Metabolism Studies

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Introduction
The usefulness of capillary liquid chromatography (LC) as a tool for improving the effective limit of quantification of analytical methods was shown 25 years ago (1). However, it is only recently that dedicated capillary LC instrumentation has been introduced allowing the routine use of this technique. Despite the obvious sensitivity advantages of capillary LC it has not been embraced by a majority of chromatographers for general use. This has, in part, resulted from a real and/or perceived unreliability of capillary LC columns, particularly when used for the analysis of “dirty” extracts of biological fluids. Recent years, however, have seen the introduction of an increasing range of robust, reproducibly packed capillary-scale (≤320 µm i.d.) columns from a variety of manufacturers. These columns have restored the trust of many analysts in capillary LC as a reliable and usable technique. This, together with the availability of robust, commercial capillary LC systems that can accurately and reproducibly produce gradients at capillary flow-rates (low µL/min), has increased enthusiasm for the technique. Capillary LC is now being applied more widely, including in our own area, the study of drug metabolism and pharmacokinetics.

In this article we will highlight some of our success with capillary LC and capillary LC–mass spectrometry (MS) for the analysis of pharmaceutical compounds in biological fluids. Applications of capillary LC to both quantitative and qualitative analysis will be shown. The performance of the methods will be discussed, in terms of speed, resolution and sensitivity. We will also briefly discuss the available instrumentation.

Quantitative Bioanalysis
An essential part of the drug discovery and development processes is establishing a detailed knowledge of the pharmacokinetic properties of candidate drugs. An understanding of these properties allows a comparison of systemic exposure to the test drug between humans and the animal species employed in pharmacology and toxicology studies. This is crucial for the determination of a safe and effective dosage regime for patient groups.

For accurate determination of the pharmacokinetic properties of a drug candidate, a quantitative assay for the compound in biological fluids is required. This assay must invariably be highly sensitive and must be specific for the analyte under investigation.

The current method of choice in quantitative bioanalysis is high performance liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) following preparation of the biological sample by solid-phase extraction (SPE) or protein precipitation (2, 3). Such methodology provides sensitive and specific assays. In addition, operation of the chromatography under a protocol whereby a gradient profile is rapidly generated and cycled (fast gradient LC), provides acceptably high sample throughput. Typically, such procedures, based on a 100–250 µL sample size, can achieve limits of quantification (LOQ) in the region of 1–0.1 ng/mL.

Table 1: Comparison of typical flow-rates and potential changes in sensitivity on reduction of column internal diameter.

<table>
<thead>
<tr>
<th>Column internal diameter (mm)</th>
<th>Equivalent flow-rate (µL/min)</th>
<th>Approximate increase in concentration for eluting peaks of equivalent width</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6</td>
<td>1000</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>189</td>
<td>5.3x</td>
</tr>
<tr>
<td>1</td>
<td>47.3</td>
<td>21.1x</td>
</tr>
<tr>
<td>0.5</td>
<td>11.8</td>
<td>84.7x</td>
</tr>
<tr>
<td>0.3</td>
<td>4.3</td>
<td>235x</td>
</tr>
<tr>
<td>0.18</td>
<td>1.5</td>
<td>667x</td>
</tr>
</tbody>
</table>

Figure 1: The structure of ondansetron.
The introduction of combinatorial approaches to the synthesis of potential drugs has refined the requirements of bioanalysis. First, a large number of potential drug candidates are often generated, requiring some means of ranking in terms of overall suitability for development. Such a decision will involve many factors, one of which will be a comparative evaluation of pharmacokinetic properties of the most promising candidates. Consequently, several compounds may enter pharmacokinetic studies at a time when only small amounts of drug compound have been synthesized. Thus, the number of animals that may be studied will be limited, as may the dosages. This will tend to generate low volumes of sample containing very low concentrations of drug. Second, high pharmacological potency is a desired property in a new drug candidate but this can lead to extremely low human dosages and the requirement for very high sensitivity assays with accurate determination at the low pg/mL level. One possible answer to these needs is capillary LC–MS.

Capillary LC columns (typically 180–320 µm i.d.) are, by definition, volumetrically smaller than conventional analytical columns (2–4.6 mm i.d.) of equivalent lengths. Thus, an equivalent linear mobile-phase flow can be generated from a flow-rate reduced in proportion to the column volume difference between the capillary and the conventional column; that is, in proportion to the square of the difference in the radius of the column internal diameter. This leads to dramatic reductions in flow-rates and, if the same peak efficiency (peak width) is achieved, a dramatic increase in the concentration of an analyte as it elutes from the column. For concentration-sensitive detectors, such as an electrospray mass spectrometer, this can translate into equally dramatic improvements in sensitivity (see Table 1).

The increased sensitivity achievable with capillary LC has been demonstrated in our laboratory by the analysis of ondansetron (Figure 1). The existing method of analysis uses LC–MS–MS and has an LOQ of 2 ng/mL from a 250 µL plasma sample (4). The use of a capillary LC system coupled to a triple quadrupole mass spectrometer resulted in a 200-fold reduction in the LOQ to 10 pg/mL from just 100 µL of plasma.

In this analysis 100 µL of plasma was mixed with 200 µL of acetonitrile and centrifuged, and the supernatant was removed and evaporated to dryness. The resulting residue was reconstituted in 25 µL of 10% aqueous methanol, and a 1 µL aliquot was injected onto a 10 cm × 300 µm column. The column was maintained at 40 °C and eluted with a 10–50% acetonitrile:aqueous formic acid gradient over 10 min at 10 µL/min. The column effluent was monitored by positive ion MS–MS using the transition 294 → 169.9.

Figure 2: A 1 µL aliquot of an extracted plasma blank injected onto a 10 cm × 300 µm, Higgins Targa C18, 5 µm column. The column was maintained at 40 °C and eluted with a 10–50% acetonitrile:aqueous formic acid gradient over 10 min at 10 µL/min. The column effluent was monitored by positive ion MS–MS using the transition 294 → 169.9.

Figure 3: A 1 µL aliquot of an extracted plasma 10 pg/mL ondansetron standard injected onto a 10 cm × 300 µm, Higgins Targa C18, 5 µm column. The column was maintained at 40 °C and eluted with a 10–50% acetonitrile:aqueous formic acid gradient over 10 min at 10 µL/min. The column effluent was monitored by positive ion MS–MS using the transition 294 → 169.9.
sensitivity of 10 pg/mL was equivalent to 40 fg on column. Such sensitivity from just 100 µL of sample serves to highlight the potential benefit of capillary LC. Indeed, as only 4% of the available sample was injected a further increase in sensitivity is possible; this will be discussed later in the article.

Capillary LC in pharmacokinetic studies:

To demonstrate how capillary LC–MS can benefit the drug discovery and development programme, it has been applied to the analysis of a compound in plasma samples generated in drug development support studies. During a standard rodent pharmacokinetic study, approximately 0.5 mL of blood is required from three animals at each time point to produce good quality data. This approach requires samples from 20–40 animals but uses significant amounts of candidate drug substance for dosing. In early drug discovery, a potential drug substance can be a very precious commodity.

Fraser et al. (5) demonstrated that capillary LC–MS–MS can be used instead of conventional LC–MS–MS to provide the same level of assay sensitivity with significantly reduced sample volume. In the revised protocol, rats were replaced with mice and just 30 µL of blood was removed from each mouse, at each time point. The assay involved precipitation of the blood samples with acetonitrile containing an internal standard, centrifugation and subsequent evaporation and reconstitution of the supernatant, an aliquot of which was then analysed by capillary LC–MS–MS (column i.d.180 µm). The assay had a sensitivity of 5 ng/mL, and good quality concentration versus time data were generated from just three mice dosed intravenously and three mice dosed orally. This study design reduced the number of animals required, by a factor of 10. The use of mice instead of rats, together with the reduction in rodents required, allowed the mass of compound necessary to perform the study to be reduced by a total factor of 100.

A comparison of the plasma time curves produced following intravenous or oral dosing is given in Figures 5 and 6. Either study design provided suitable data for pharmacokinetic analysis but it could be argued that the capillary LC data were more relevant, as all the data points in the concentration versus time profile were...
numbers of new and potentially interesting drug compounds. More new compounds generate more studies (and, therefore, samples), and the time available to the bioanalyst is now even more precious. One pragmatic approach to this challenge has been to compromise on the chromatographic separation efficiency using large–particle size stationary phases and rely on the MS–MS detection system to provide specificty. This methodology has limitations that will be addressed later. With such large particle sizes (typically 30–50 µm) the usual requirement for pretreatment of a biological fluid sample is removed, as the potential for column blockage is largely negated. Also, large–particle size materials imply an inherently low back pressure, enabling methodologies with very high flow-rates. So, biological fluid samples can be assayed without pretreatment and with very short chromatography times. This technique has been called turbulent flow or ultra-high flow-rate chromatography and has found increasing use over the past few years (6–8). In this technique, an aliquot of a plasma sample is injected into the chromatograph, the plasma proteins are unretained and are swept to waste whilst the analyte molecules are retained sufficiently to allow their separation from the bulk of endogenous components within the biological matrix. Subsequently, elution of the analyte occurs as the gradient develops, typically over a one minute period. We have used this technique in the support of a development programme for a potential drug candidate. Obviously, whilst assay throughput is greatly enhanced, the lack of sample preparation means that there is no opportunity for sample preconcentration and, therefore, the technique has been limited to a typical LOQ of approximately 5 ng/mL. However, as with any other form of chromatography, miniaturization of the column, and hence reduction of the flow-rate required to deliver an equivalent linear flow velocity, offers the potential for enhanced concentration of the eluting analyte peak (see Table 1). Indeed, we have demonstrated that this technique is applicable in the capillary scale (9) and have achieved a reduction in the LOQ to 0.5 ng/mL, based on a sub-5 µL sample, with a 2–2.5 min/sample cycle time.

This is a low-resolution technique and the typical peak shape achieved is shown in Figure 7. It, therefore, must be remembered that some drug metabolites (e.g., N-oxides, glucuronides) may elute from the column at the same retention time as the parent compound, be thermally unstable and, in a heated electrospray source, revert to the parent compound and lead to inaccurate quantification of the parent. It is perhaps reasonable, therefore, that the most suitable application for this technology is in the analysis of clinical samples from large studies that are conducted at a stage of the drug development process at which the metabolism of the candidate drug has been well characterized. Faced with several thousand clinical samples and a tight deadline the analyst would undoubtedly find benefit in this procedure.

A further development has been the introduction of multiplexed interfaces to the mass spectrometer, allowing the use of numerous columns in parallel. This approach offers significant gains in sample throughput. As the peak of interest elutes from each of the parallel columns and enters the multiplex interface, the interface rotates allowing the eluent from only one column at a time into the mass spectrometer. The data are collected into separate data files for each of the column eluents. A schematic of the parallel ultra-high flow-rate LC–MS–MS system is shown in Figure 8. In our laboratory we have adapted the capillary ultra-high flow-rate LC–MS–MS method described above to a system in which four capillary columns are operated in parallel, and have achieved a throughput of 96 samples per hour with an LOQ of 1 ng/mL (10). The calibration lines derived from each of the four channels were found to be equivalent and superimposable. A composite calibration line comprising data collected from all four channels is shown in Figure 9. It must be stressed that a multiplexed approach is not restricted to these ultra-high flow-rate methods.
Qualitative Bioanalysis

It is not only in the arena of quantitative analysis that capillary LC is affecting drug discovery. Capillary LC–MS is also enhancing qualitative analysis. An area in which this has been used to especially good effect is in the identification of drug metabolites. Capillary LC is particularly useful in the analysis of limited-volume samples, because of its inherent compatibility with small volumes, and, in studies conducted at low dosages, its increased sensitivity is obviously beneficial.

An example of this is the identification of a drug metabolite in plasma from a GlaxoSmithKline compound (11). The compound had been found to be metabolically unstable and in order to identify more stable structural analogues, it was necessary to identify the site of metabolism. Using conventional narrow-bore reversed-phase LC–MS it was only possible to obtain full-scan MS data, not MS–MS data. The MS data alone were not able to provide the necessary information needed to identify the site of metabolism.

Capillary LC connected to an ion-trap mass spectrometer was employed to increase the method sensitivity and so provide the required MS–MS data. A sample of plasma was prepared by protein precipitation using acetonitrile. The sample was centrifuged and the supernatant evaporated to dryness and reconstituted in 50 µL of 25% methanol (aq.). 1 µL of the sample was analysed on a 15 cm /H11003 300 µm column with MS–MS detection. The spectrum produced from the metabolite peak was compared with that obtained for the parent compound analysed in a similar manner. The extracted ion chromatograms of the parent compound and that of the metabolite are given in Figures 10 and 11. These figures also show the MS spectrum of the parent compound and the MS–MS spectrum of the m/z 582 ion of the metabolite. A comparison of the MS data indicated that the compound had undergone metabolic hydroxylation. The MS–MS data clearly indicated that the position of the hydroxylation was in a specific phenyl ring system, the m/z value of this ring-system had increased from 186 in the parent to 202 in the metabolite (Figure 12).

Thus, the application of the enhanced sensitivity offered by capillary LC was shown to provide information that was of benefit to synthetic chemists insofar as it clearly highlighted the moiety of the molecule that was metabolically unstable and could be usefully modified.

Large Injection Volumes

There is one major dilemma with capillary LC and that is, the low volumetric flow-rate! On the one hand this gives the technique the high sensitivity that is its key advantage, whilst on the other hand miniaturization can limit the volume of sample that can be loaded onto the column in a reasonable period of time. This is not an issue when working with very small sample volumes, for example, those generated in chemical bead synthesis (12), but it is in bioanalysis. Experience gained in our laboratories (13) has led us to the conclusion that the minimum volume that can be used to accurately and reproducibly reconstitute a dried SPE residue and transfer it to a vial is approximately 30 µL. With a capillary LC flow-rate of 5–10 µL/min this would take at least 3 min to inject. Larger volumes would take even longer. This is not acceptable in a high-throughput environment such as modern bioanalysis. Thus, injection volumes would need to be reduced, meaning that the majority of the sample would remain in the vial and, therefore, assay sensitivity reduced, removing the key advantage of operating in the capillary-scale.

To overcome this problem a second pump, switching valve and a short guard column have been employed. In this mode...
of operation, a large-volume sample 50–200 µL is injected onto a small guard column (1 cm × 300 µm), at a high linear flow-rate of 100–200 µL/min. This gives a relatively low back-pressure in the region of 80 bar. The sample is loaded in 1–2 min. The valves are switched and the sample is then eluted from the guard column onto the capillary column with a flow-rate of 5 µL/min. The effect of such a system can be seen in Figure 13. In this example the detector response is compared for a 2 µL direct injection of a mix containing amitriptyline and trimipramine with that achieved for a 50 µL injection, which was swept onto the guard column after having been “stacked” on a guard column. As can be seen the increase in peak height is substantial. Initial experiments using biological extracts have shown that the guard column is robust to at least 80 injections of 50 µL. The next step will be to investigate this using a real drug candidate assay. In this mode of operation we choose to work in the forward flush mode, to ensure that any extraneous particulate matter will be retained on the guard column and not back-flushed onto the top of the analytical column.

**Instrumentation**

At its simplest, all that is required to generate a capillary flow is a standard high performance liquid chromatography (HPLC) pump, a tee-piece and a piece of tubing leading to waste! The conventional pump delivers the flow, which is split in the tee-piece, and the bore and length of the tubing define the proportion of flow that is split to waste. In practice, however, the adjusting of such a simple system to accurately deliver a defined required flow-rate is not trivial. However, the principle of generating the flow at a conventional flow-rate and then performing a calibrated or controlled split to deliver a low µL/min flow-rate to the capillary column is sound and, indeed, it is incorporated into some of today’s commercially available systems. A system incorporating a calibrated split is the AccurateTM, or in its form as a fully automated system the UltiMateTM, from LC Packings (Amsterdam, The Netherlands). The pump in this system delivers a flow at a conventional (mL/min) flow-rate that passes through a splitter, which is precisely calibrated and delivers a micro-flow to the capillary column. A drawback of systems that use a passive split, is that if the back-pressure resulting from the column increases, possibly because of column contaminants from a biologically derived sample extract, then the proportion of the flow that goes to waste instead of through the column will be increased. This will cause lower flow through the capillary column and hence alterations in the retention times of analytes. A recent introduction is the Agilent 1100 Capillary LC system. This again involves generation of a flow at a relatively high flow-rate with subsequent splitting, but also incorporates an active flow monitoring device that monitors the flow-rate through the capillary column and adjusts the split ratio appropriately according to whether it senses the flow to be too high or too low.

Other systems have been developed whereby the pump is engineered to produce the initial flow at the required capillary flow-rate and no splitting is required. These include syringe-based systems, such as the Applied Biosystems ABI 1000A and systems using reciprocating

![Figure 11: A 1 µL aliquot of an extracted plasma injected onto a 15 cm × 300 µm, Hypersil Hypurity Elite C18, 3 µm column. The column was maintained at 25 °C and eluted with a 0–100% acetonitrile:aqueous formic acid gradient over 33 min at 5 µL/min. The column effluent was monitored by positive ion ESI automatic MS–MS over the range of 100–600 m/z.](image)

![Figure 12: Structures of m/z ions (a) 186 and (b) 202 derived from the metabolite or the parent compound as determined by capillary LC–MS–MS.](image)
pumps such as the MicroTech Ultra-Plus. Both of these approaches were successful but suffered from drawbacks such as the need to repressurize, which sometimes led to unacceptable run times. The need to repressurize at the end of syringe strokes has been addressed by the use of twin syringes per solvent channel as in the recently introduced Waters CapLC™. One might intuitively feel that a directly generated capillary flow is preferable to generation of a bulk flow with subsequent splitting, for example, to reduce solvent consumption, particularly for costly, deuterated solvents, such as those used in nuclear magnetic resonance spectroscopy, and to reduce waste disposal costs. However, it is fair to say that these systems may be limited in their ability to accurately mix low percentages of one solvent with high percentages of another at very low, approaching nanolitre per minute, flow-rates.

It is not the intention of the authors to recommend one or other of these systems here as a preference, as all have both merits and limitations.

**Conclusions**

Capillary LC is becoming more widely used in support of drug metabolism and pharmacokinetic studies. We have shown how it can be applied to achieve either higher sensitivity or comparable limits of quantification for low-volume samples. The latter fact has enabled study protocols to be redesigned so that serial bleeding and fewer animals per study are used. This means that pharmacokinetic data are obtained from low amounts of compound. In turn this allows these studies to be conducted earlier in the drug discovery process adding value to the information on which candidate selection decisions are based.

We have also shown that capillary LC techniques are compatible with high-throughput analysis, particularly when such columns are operated in parallel. The technique also offers benefits in the field of drug metabolite identification. In the example shown in this article the use of capillary LC allowed MS–MS data to be obtained where only full-scan MS data were produced from conventional analytical LC–MS.

It is the authors’ belief that capillary LC will continue to expand in bioanalysis and may within a few years become the dominant technique of choice, particularly for quantitative bioanalysis.

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**References**


The authors have between them a cumulative 40 years experience of the pharmaceutical industry, mostly within drug metabolism. Following the 1995 merger of Glaxo and Wellcome, a part of their role has encompassed the development and implementation of novel techniques for bioanalysis.

Gordon Dear and David Mallett can still be contacted at what is now GlaxoSmithKline R&D, Park Road, Ware, Hertfordshire, SG12 0DR, UK, whereas Robert Plumb has recently taken up an appointment with Waters Corp., 34 Maple Street, Milford, Massachusetts 01757, USA.

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**Figure 13:** (a) 2 µL direct injection onto a capillary column and (b) 50 µL injection stacked onto guard column and then eluted at a low flow-rate onto the capillary column.