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Short communication

Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS

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Abstract

The ability to rapidly detect and characterize drug metabolites in biological fluids often relies on a combination of a high quality chromatographic separation and sensitive high resolution mass spectrometry. Here, the performance of two high throughput LC/MS approaches, namely monolith columns and sub-2 μm particle Ultra Performance Liquid Chromatography (UPLC) columns is compared for the detection and identification of the human metabolites of acetaminophen in urine. The UPLC system produced approximately three times the sensitivity and detected more metabolites than the monolithic column approach. The sharp peaks produced by UPLC seem to be particularly advantageous when coupled to electrospray mass spectrometry, apparently reducing ion suppression leading to superior sensitivity and hence lower limits of detection.

Keywords: HPLC; LC/MS; UPLC

1. Introduction

The main reasons that compounds are eliminated from the drug discovery process are related to efficacy or toxicity [1,2]. Observed toxicity may be due to the dosed compound itself or a metabolite and, as part of any drug discovery activity, it is important to screen for the presence of such toxic metabolites [3]. Metabolite detection and identification is usually performed by LC/MS(MS) [4]. During such a screening process, speed and sensitivity are important factors, as there are typical tens to hundreds of compounds to be evaluated and the candidate compound may be dosed at very low levels to mice and rats. Both of these factor place significant strain on the analytical process. The rate of analysis depends on the ability of the chromatography system to reliably separate the components of interest in the sample. This ability is a measure of the peak capacity per unit time of the chromatography system [5]. There have been several successful approaches to increasing analytical throughput in drug discovery including; the use of short columns with rapid gradients [6–8], cassette dosing and analysis [9], monolithic column chromatography [10,11] and, more recently, Ultra Performance Liquid Chromatography (UPLC) [12].

Of these approaches the monolithic columns and UPLC are the most promising as they offer high throughput with good chromatographic performance. The monolithic columns give a chromatographic performance equivalent to 4 μm particle columns. They utilize the high permeability of the stationary phase allowing long columns to be employed with high linear velocity mobile phases, hence generating rapid separations with little loss in chromatographic performance. Ultra Performance Liquid Chromatography takes advantage of the flat nature of the van Demmter plot for sub 2 μm stationary phases to generate higher chromatographic performance. These UPLC materials can be operated at very high mobile phase linear velocities with no loss in chromatographic performance [12].

In order to compare the relative performance of these two chromatographic approaches, the human metabolism of the common analgesic compound acetaminophen (paracetamol) was investigated. Acetaminophen is an over the counter medicine used for the treatment of headaches and fevers that has been available for many years; however, it is not

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without side effects and every year many people die due to overdosing on the drug (either accidentally or deliberately). Acetaminophen is a minor analgesic and is very safe at dose levels of 500–1000 mg. The metabolism of acetaminophen is well understood [13–15], it is eliminated in man by two major routes: sulphation and glucuronidation. A minor metabolite is formed by oxidation of the molecule to form *N*-acetyl-*p*-bezoquinoneimine. This metabolite is toxic but it's effect is normally mitigated by reaction with glutathione to form a mercapturic acid metabolite [16].

In an overdose situation the metabolic balance is altered. The conjugation pathways are quickly saturated and cofactors, in particular sulphate, are depleted. Thus acetaminophen metabolism is directed to the oxidation pathway resulting in increased levels of the toxic metabolite produced in the liver. These increased levels rapidly deplete the available glutathione [16]. As a result the toxic metabolite reacts with the proteins in the liver to cause tissue damage which results in liver necrosis. The antidote involves the oral or intra venous administration of a glutathione precursor such as *N*-acetylcsyeine [17]. Here, we compare the detection of these metabolites of acetaminophen in human urine using monolithic columns and UPLC with Q Tof MS analysis.

2. Experimental

2.1. Chemicals

Acetonitrile (HPLC grade) was purchased from JT Baker (NJ, USA), ammonium formate and formic acid (spectroscopic grade) was purchased from Sigma–Aldrich (MO, USA). Distilled water was purified "in-house" using a MilliQ system Millipore (MA, USA). Leucine–enkephalin was obtained from Sigma–Aldrich (MO, USA).

2.2. Acetaminophen samples

Two 500 mg acetaminophen tablets (Tylanol) were dosed orally to a human volunteer. Urine was collected 1 h post dose. The sample was stored frozen (−20 °C) prior to analysis.

2.3. Chromatography

Chromatographic separations were performed either a 5 cm × 4.6 mm ChromSpeed monolithic column (Merck, Darmstadt, Germany) or a 5 cm × 2.1 mm ACQUITY^{TM} 1.7 μm column (Waters Corp, Milford, USA) using a ACQUITY^{TM} Ultra Performance Liquid Chromatography system (both separations were performed on the ACQUITY^{TM} system). The columns was maintained at 40 °C and eluted with a linear gradient of 0–40% B, where *A* = 0.1% formic acid and *B* = acetonitrile 0.1% formic acid. The gradient duration was either 10 min at a flow rate of 500 μL/min for UPLC and 2 mL/min for HPLC on the Monolithic column. The urine samples were diluted 1:5 in distilled water, a 10 μL injection of each sample was made onto the column. The column eluent was directly split such that approximately 150 μL/min was direct to the mass spectrometer.

2.4. Mass spectrometry

Mass spectrometry was performed on a Waters Q Tof micro (Waters MS Technologies, Manchester, UK) operating in positive ion mode. The nebulization gas was set to 300 L/h at a temperature of 250 °C the cone gas set to 0 L/h and the source temperature set to 120 °C. A capillary voltage and a cone voltage were set to 3200 V and 30 V, respectively. The Q Tof Micro acquisition rate was set to 0.3 s, with a 0.1 s interscan delay. Argon was employed as the collision gas at pressure of 5.3×10^{-5} Torr. The instrument was operated at two alternating collision energies, 5 eV and 25 eV, with the data being collected into two separate data channels.

All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine–enkephalin was used as the lock mass (m/z = 556.2771) at a concentration of 50 fmol/μL and flow rate 30 μL/min. Data were collected in centroid mode, the lockspray frequency was set at 5 s, and data were averaged over 10 scans.

3. Results and discussion

The positive ion TIC chromatograms for the monolithic and UPLC columns are given in Fig. 1. As can be seen, the UPLC chromatogram shows significantly more peaks, with the average peak width of the UPLC separation being approximately 3 s at the base and the monolithic column being 4.8 s. Both the UPLC and monolithic LC/MS systems were able to detect the major sulphate metabolite of acetaminophen, m/z = 232. However, the UPLC/MS system produced a peak approximately three times the height of that produced by the monolithic LC/MS system, Fig. 2. As the injection volumes of the analysis were scaled to take account of the different column geometries, these data suggest that the UPLC system produced a more sensitive analysis than the monolithic column approach, probably due to increased resolution and efficiency of the sub 2 μm particles The monolithic column operated with a back pressure of approximately 800 psi while the sub 2 μm UPLC columns generated a back pressure of approximately 5200 psi.

One of the benefits of the UPLC system is the extra chromatographic resolution generated the sub 2 μm particles. The data shown in Fig. 3 illustrates this extra chromatographic performance produced by the UPLC^{TM} system compared to that of the monolithic LC/MS system. In this example we can see that the UPLC system clearly detects three *S*-cysteine conjugates of acetaminophen whilst the monolithic column only detects two? Is this MSMS data? The extra peak detected is as a direct result of the extra sensitivity of the UPLC system. It can be observed that the signal intensity of the UPLC data is significantly better to that achieved by the monolithic LC/MS

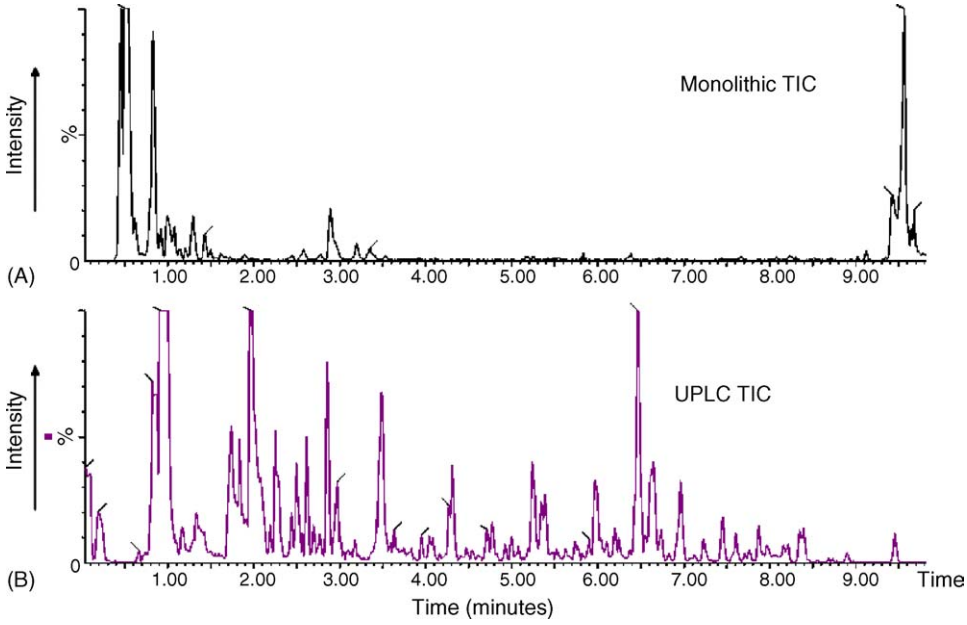

Fig. 1. Positive ion LC/MS TIC trace resulting from the injection of diluted human urine onto either (A) a 0.46 cm × 5 cm Speed Rod column or (B) a 0.21 cm × 5 cm ACQUITY^{TM} UPLC column. Both columns were eluted with a 0–40% aqueous formic acid–acetonitrile gradient over 10 min. The resulting column eluent was detected by positive ion electrospray TOF MS.

system; thus aiding the detection of the drug metabolites in the urine sample.

One of the major routes of elimination for acetaminophen is conjugation with glucuronic acid to form the ether glucuronide metabolite at the 4-position. The LC/MS analysis of the urine sample is shown in Fig. 4 along with the corresponding UPLC/MS analysis. As we can see from this data the UPLC system facilitates the resolution of three glucuronide metabolites ($m/z = 328$) of acetaminophen, these structure was confirmed by MS/MS giving the characteristic loss of 176 for glucuronic acid. A closer inspection of the structure of acetaminophen reveals there is the possibility of two sites of conjugation, the hydroxyl group and the amide nitrogen to form an *N*-glucuronide, the third glucuronide observed may be due to the presence of both α and β forms. The resulting MS spectrum obtained from the peak eluting with a reten-

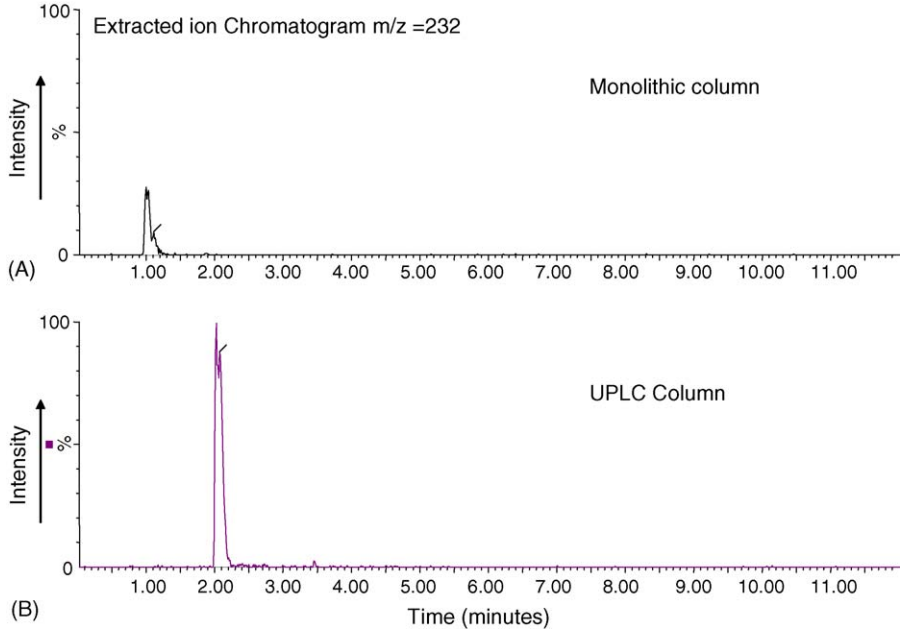

Fig. 2. Extracted ion chromatogram of acetaminophen-sulphate ($m/z = 232$), positive ion LC/MS TIC trace resulting from the injection of diluted human urine onto either (A) a 0.46 × 5 cm Speed Rod column or (B) a 0.21 cm × 5 cm ACQUITY^{TM} UPLC column.

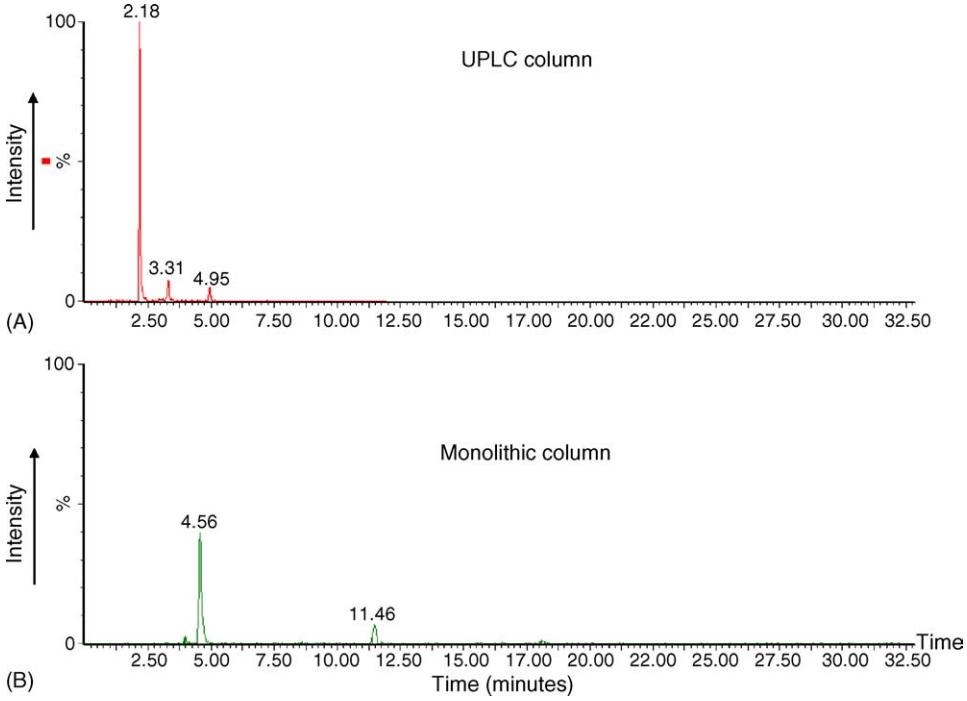

Fig. 3. Extracted ion chromatogram of acetaminophen-*S*-cysteine conjugate ($m/z = 271$), positive ion LC/MS TIC trace resulting from the injection of diluted human urine onto either (A) a 0.21 cm × 5 cm ACQUITY^{TM} UPLC column or (B) a 0.46 cm × 5 cm Speed Rod column.

tion time of 2.0 min is displayed in Fig. 5(A). The exact mass analysis of the glucuronide peaks gave a value of 328.1041, which resulted in an elemental composition of $C_{14}H_{18}NO_8$ with a mass error of 2.6 ppm. The high collision energy MS data of the glucuronide peak at 2.0 min gave valuable information on the fragment ions of the peak, the resulting MS spectra is given in Fig. 5(B). Here, we can see that that the major fragment ion produced was $m/z = 152.0705$ corresponding to acetaminophen and the loss of 176.0324. The mass accuracy of the Q Tof micro afforded great confidence that all three of the metabolites were indeed the glucuronides of acetaminophen. The detection of the two extra *S*-cysteine

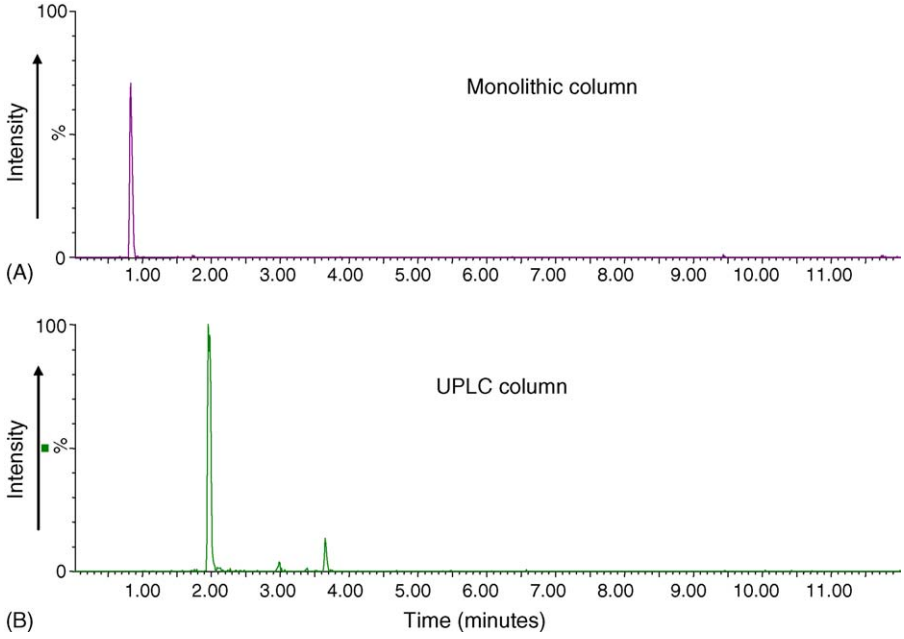

Fig. 4. Extracted ion chromatogram of acetaminophen-glucuronides ($m/z = 328$), positive ion LC/MS TIC trace resulting from the injection of diluted human urine onto either (A) a 0.46 cm × 5 cm Speed Rod column or (B) a 0.21 cm × 5 cm ACQUITY^{TM} UPLC column.

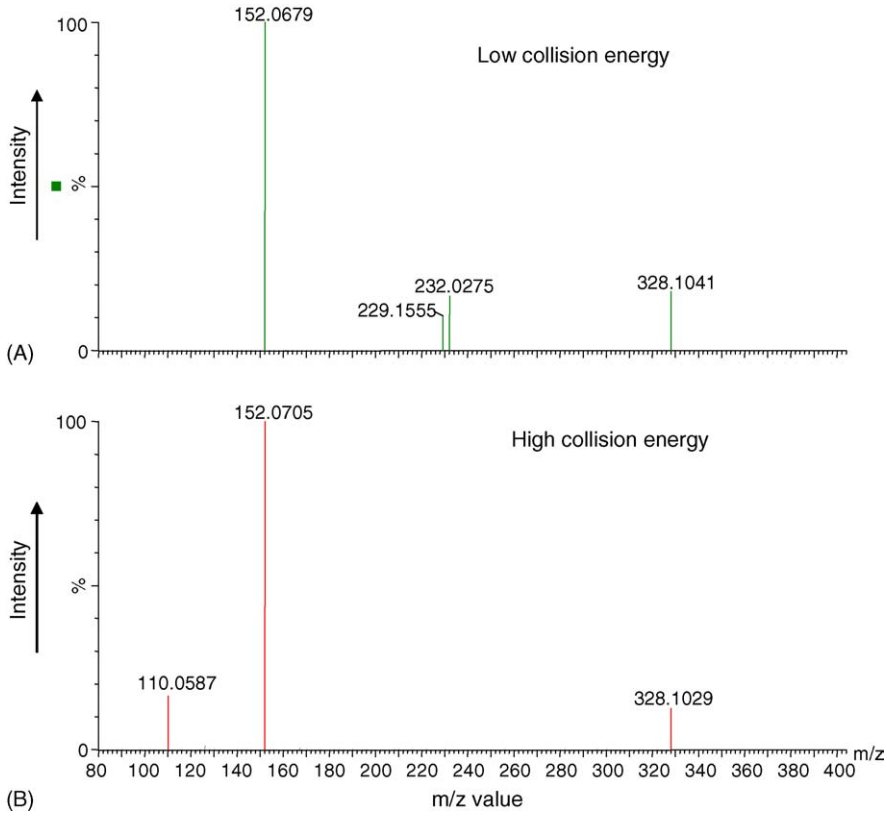

Fig. 5. MS spectrum from the (A) low and (B) high collision energy analysis of the UPLC acetaminophen glucuronide peak eluting with a retention time of 2.0 min.

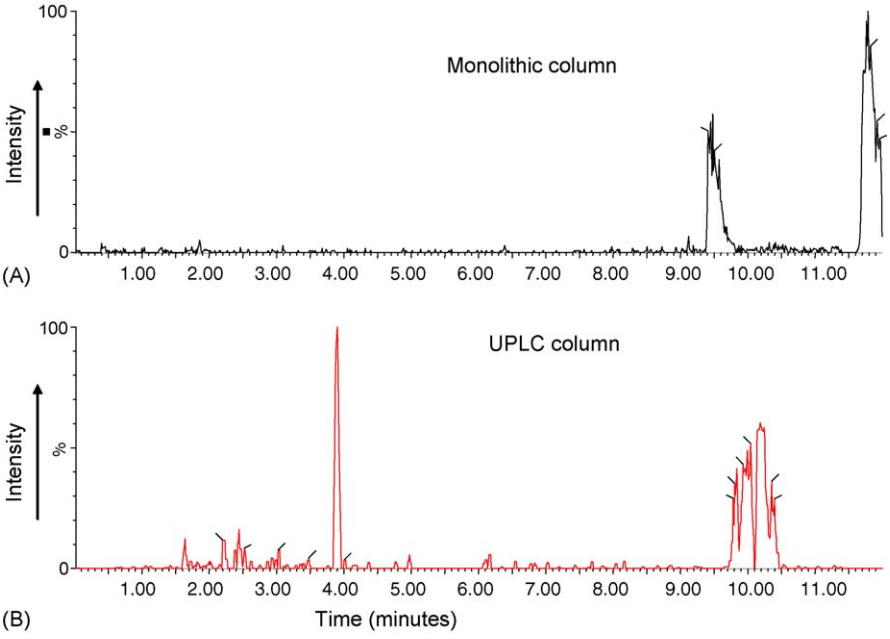

Fig. 6. Extracted ion chromatogram of acetaminophen–glutathione conjugate ($m/z = 427$), positive ion LC/MS TIC trace resulting from the injection of diluted human urine onto either (A) a 0.46 cm × 5 cm Speed Rod column or (B) a 0.21 cm × 5 cm ACQUITY^{TM} UPLC column.

and two extra glucuronide metabolites of acetaminophen by the UPLC/MS system compared to that of the monolithic HPLC/MS system again illustrates the extra sensitivity and resolution of the UPLC system.

The presence of a glutathione conjugate is often used as a marker of potential drug toxicity. Compounds susceptible to this form of metabolic clearance are often removed from the drug discovery process. The extracted ion chromatograms for the glutathione metabolite of acetaminophen ($m/z = 427$) for UPLC/MS and LC/MS is shown in Fig. 6. Whilst the excretion of glutathione conjugates in the urine is unusual the presence of this metabolites is confirmed by the exact mass MS data. With the monolithic column LC/MS system it is not possible detect the glutathione metabolites however, with UPLC/MS the glutathione metabolite is detected with a retention time of 3.9 min, again demonstrating the extra sensitivity of the UPLC/MS system. When designing the experiment care was taken to ensure that the mass loading on the columns were normalized for the column diameter, thus 10 μL of urine was injected onto the monolithic column and just 2 μL was injected onto the UPLC column. Thus the improved sensitivity observed for the UPLC analysis was due to the chromatographic process and not due to reduced analyte dilution as a result of the column diameter. Both of the chromatography systems were operated at the optimal linear velocities, both could be operated at higher linear velocities giving rise to faster analysis and narrower peaks in a time domain, but no more resolution. The extra resolution and peak sharpness, produced by the UPLC chromatographic system resulted in improved ionization efficiencies and hence improved detection limits.

4. Conclusion

Ultra Performance Liquid Chromatography is an ideal separation tool for complex mixture analysis. The UPLC/MS approach was approximately three times more sensitive than the monolithic column LC/MS approach, it also detected significantly more metabolite peaks. However, these sub 2 μm particle columns operate at pressure significantly greater than conventional HPLC and thus require a specially designed chromatography system to obtain maximum chromatographic performance. The sharp peaks produced by UPLC are of particular advantage when coupled to electrospray mass spectrometry, reducing ion suppression and leading to superior sensitivity and hence lower limits of detection.

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