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Journal of Chromatography B, 709 (1998) 243–254

JOURNAL OF CHROMATOGRAPHY B

Use of generic fast gradient liquid chromatography–tandem mass spectroscopy in quantitative bioanalysis

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Received 18 November 1997; received in revised form 6 February 1998; accepted 6 February 1998

Abstract

Short narrow analytical HPLC columns have been used successfully with high linear flow-rates and combined with mass spectrometric detection to produce a generic approach to quantitative bioanalysis. The approach has been used to validate several assays in the low ng/ml region and an example is given in this paper. When combined with a simple solid-phase extraction process the need for complicated, time consuming method development has been removed for the majority of pharmaceutical compounds. The approach takes advantage of not only the extra selectivity of the MS–MS detector but the excellent resolution and peak shape produced by gradient elution.

Keywords: Quantitative analysis

1. Introduction

A specific assay is required when determining the concentration of a drug candidate and or its metabolite in a clinical or toxicokinetic sample to ensure that good quality safety and pharmacokinetic data can be derived. Liquid chromatography with spectrophotometric or mass spectrometric detection is the technique of choice for quantitative work, and when combined with NMR it can also be applied to the unequivocal structural characterisation of pharmaceutical compounds and their metabolites [1–4].

Most current assays for pharmacokinetic or toxicokinetic analysis rely upon isocratic, reversed-phase chromatography of samples prepared by solid-phase extraction (SPE). Whilst this is an effective approach for sample analysis it often requires extensive, time consuming development of optimum sample preparation and chromatographic conditions.

The coupling of mass spectrometers with liquid chromatography and the introduction of atmospheric pressure ionisation interfaces have brought improvements in assay specificity and sensitivity with some reduction in method development times. The successful application of mass spectrometric methods for bioanalysis can be compromised if the MS source becomes contaminated with endogenous material present in the extracts from plasma or urine. Thus the gain made from reduced assay development and run times on MS is often dissipated by the deterioration in the instrument performance.

Careful attention to sample preparation can help in avoiding contamination of the MS source; off-line fully automated SPE has proved to be a robust and rapid method when used in the 96-well format [5]. On-line SPE–LC–MS–MS methods [6] have been

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0378-4347/98/$19.00
PII: S0378-4347(98)00074-7

developed successfully but sample run times are still in the 5–7 min range. Also this approach will not be successful when the compound of interest needs to be resolved from a coextracting and coeluting metabolite that thermally degrades to the parent compound in the MS source.

Automation of SPE has meant that for many assays the rate limiting step is now the chromatographic method development. Time saved in such development would bring further improvements and help analysts keep pace with the demands for high throughput methods for a diverse range of chemical structures.

Reversed-phase gradient HPLC has become the preferred method for the profiling of drug metabolites from sample urine and plasma extracts [7,8]. These systems offer excellent resolution and efficiencies but they are operated over a long time scale, typically 30–60 min, and are thus not compatible with high throughput analysis. The successful use of fast gradient, short column HPLC has been reported recently for the analysis of drug candidate compounds from combinatorial chemistry in organo–aqueous solutions [9]. This approach utilises high linear mobile phase velocities and HPLC columns of 3–5 cm in length with an internal diameter of 4.6 and 2 mm. The approach benefits from short analysis times, 3–5 min without a significant loss in chromatographic resolution.

In this paper the authors will present the use of a fast generic gradient LC system in biopharmaceutical analysis for the quantitative analysis of analytes in rat plasma by LC–MS–MS, following generic solid-phase extraction. The throughput, chromatographic resolution, column dimensions and instrument limitations will be critically reviewed.

2. Materials and methods

2.1. Instrumentation

The UV–Vis chromatography was performed using a model HP1100 system comprising of a binary gradient pump, autosampler, column oven, and diode-array detector (Hewlett-Packard, Waldbron, Germany). All LC–MS–MS experiments were performed on a model HP1090 binary pump and autosampler (Hewlett-Packard), coupled to a PE-Sciex API-III+ mass spectrometer (Sciex, Concord, Canada).

2.2. Chemicals

HPLC grade acetonitrile and methanol were supplied by Rathburn (Walkerburn, UK), HPLC grade water and formic acid were purchased from Fisons (Loughborough, UK). All other compounds were of analytical grade and purchased from Sigma (Poole, UK).

2.3. HPLC performance test mix

An 11 component test mix was produced by dissolving 100 mg of each of the following components in 250 ml acetonitrile–50 m*M* ammonium acetate (1:1, v/v); uracil, theophylline, acetofuran, acetanilide, *m*-cresol, acetophenone, propiophenone, benzofuran, butyrophenone, valerophenone, hexanophenone, heptanophenone and octanophenone. The sample test mix was stored refrigerated prior to use.

2.4. Analyte molecules for the example assay

The analytes for the example validated assay (Analyte I and Analyte II) were novel basic drugs and were synthesised by GlaxoWellcome Research and Development (Stevenage, UK).

2.5. Solid-phase extraction

SPE was performed using a Zymate (Zymark, Warrington, UK) automated 96-well SPE system, using 30 mg Waters Oasis HB 96-well SPE plates. A 100-μl aliquot of plasma sample was mixed with 100 μl of aqueous internal standard solution (stable isotope for Analyte II and stable isotope for Analyte I at 100 ng/ml) and added to an SPE well that had been previously primed with 400 μl of methanol, followed by 400 μl of water. The sample was drawn through under vacuum, then washed with 400 μl of water and finally the sample was eluted with 400 μl of methanol. The eluent was evaporated to dryness under nitrogen at 40°C, the residue was reconstituted in 50 μl of methanol, vortex mixed and diluted with 150 μl of water. The resulting solution was trans-

ferred to an autosampler vial for analysis by LC–MS–MS.

2.6. Chromatography

The chromatographic columns used in this study were supplied by Phenomenex, (Chesterfield, UK), Waters (Milford, MA, USA) and Capital HPLC (Edinburgh, UK).

2.7. Chromatographic conditions for bioanalysis

A 15-μl aliquot of the extracted sample was injected onto a 50×2 mm Phenomenex Magellen C_{18} 3-μm HPLC column, the column was maintained at 40°C and eluted with a gradient of aqueous formic acid (0.1%, v/v) and 95% acetonitrile–aqueous formic acid (0.1%, v/v) over 2 min returning to 100% aqueous formic acid by 2.1 min. The column was allowed to re-equilibrate for 0.9 min before the next injection. A volumetric flow-rate of 800 μl/min was employed with approximately 100 μl of the eluent being directed to the mass spectrometer and 700 μl split to waste. The split column eluent was directed to a TurboIonspray (TISP) interface operating in positive-ion mode, at 550°C using nitrogen as both the nebulizer (50 p.s.i.) and auxiliary gas (2 l/min). Analytes and internal standards were detected by tandem mass spectrometry (MS–MS) using selected reaction monitoring (SRM) of the transitions *m/z* M+1→M−273 and *m/z* M+1→M−204 for Analyte I, and its M+4 internal standard and *m/z* M+1→M−110 and *m/z* M+1→M−128 for Analyte II and its M+4 internal standard respectively. A dwell time of 150 ms per transition was employed for all analyses. Argon was used as the collision gas at an indicated target thickness of 200×1013 atm. cm^{-2} and a collision energy of 20 eV was used.

2.8. Intra-assay validation

The intra-assay precision was determined by assaying six replicate validation controls (VCs) of rat plasma spiked with a known amount of both Analyte I and Analyte II at five separate concentrations 1, 2, 75, 150 and 200 ng/ml. The VCs were quantified against two calibration lines containing both Analyte I and Analyte II with a concentration range of 1–200 ng/ml.

3. Results and discussion

3.1. Gradient formation

The accurate, reproducible production of a gradient from zero to 95% organic solvent in 3 min can be achieved with modern low pressure mixing gradient pumps, when using flow-rates of 3–4 ml/min and LC columns with dimensions of 50×4.6 mm. These flow-rates are not compatible, however, with the atmospheric pressure chemical ionisation sources used in modern mass spectrometers, so it is necessary therefore to use lower flow-rates e.g. 800 μl/min and thus LC columns with smaller internal diameters e.g. 2.1 mm. In order to produce gradients as described with volumetric flow-rates of 800 μl/min a high pressure binary pump is required. It is possible to define these gradients at low flow-rates when using a pump with a proportional mixing valve design; a comparison of two gradients produced by a high pressure binary pump and low pressure pump are shown in Fig. 1a and b.

The necessity for a high pressure mixing binary pump is derived from the need for a low delay volume between the outlet check valves of the two solvent pumps and the head of the column, including the autosampler. Low pressure mixing pumps have a mixing chamber with a typical volume of 400 μl plus and this can lead to delay volumes of 1 ml or more. The model HP1100 LC system used in this experiment was modified by removing the mixing (ballantini) column and replumbing with 0.005-inch capillary bore stainless steel tubing to reduce the delay volume to approximately 200 μl. The disadvantage of using capillary tubing is the significant increase in back-pressure introduced into the system. Indeed when operated at a flow-rate of 1–2 ml/min with a 5-cm long column, 30–40% of the back pressure experienced by the system is due to the tubing alone. Therefore, it is necessary to construct the LC system with column dimensions and flow-rate in mind, in order to minimise the delay volume whilst keeping the tubing back pressure to a minimum.

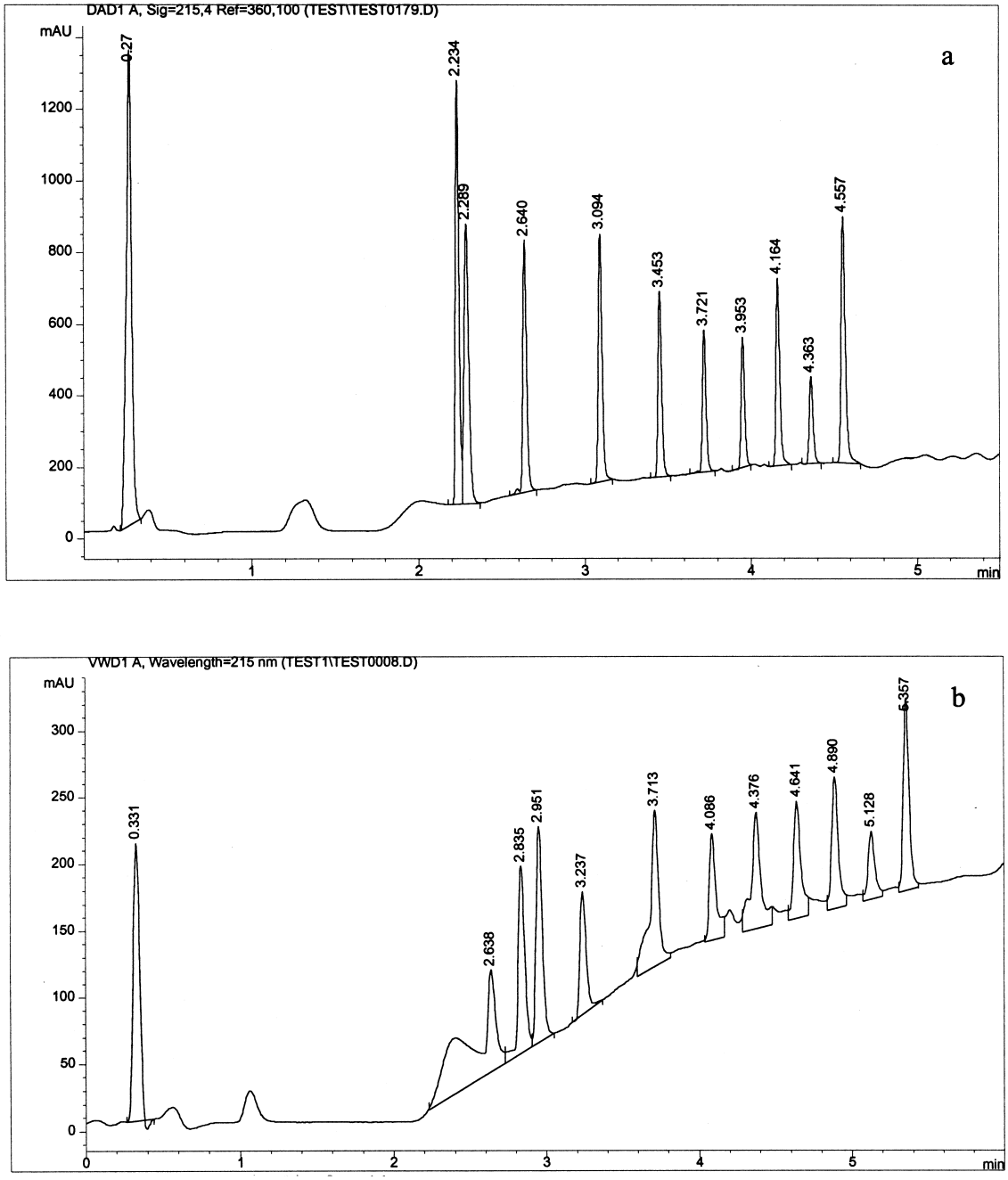

Fig. 1. (a and b) An injection (1 μl) of an 11 component test mix (containing uracil, theophylline, acetofuran, acetanilide, *m*-cresol, acetophenone, propiophenone, benzofuran, butyrophenone, valerophenone, hexanophenone, heptanophenone and octanophenone) onto a 50×2 mm Phenomenex Magellan C_{18} column. The column was maintained at 40°C and eluted with a 0–95% aqueous formic acid (0.1%) and acetonitrile gradient over 3 min following a 1-min isocratic phase of aqueous formic acid (0.1%) at a flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm. (a) Run on a high pressure binary pump, (b) on a low pressure proportionation valve mixing pump.

3.2. Column dimensions

The aim of these investigations was to produce a reproducible gradient with maximum resolution with the minimum run time. By employing a 50×2 mm C_{18} or C_8 alkyl-bonded silica column and a flow-rate of 0.8–1.2 ml/min, resulting in a back pressure of approximately 100 bar, a reproducible gradient can be produced in 2 min with acceptable chromatographic resolution, Fig. 2. A similar gradient can also be produced in 1 min by using a 20×2 mm column and a flow-rate of 3 ml/min, Fig. 3. However such a high volumetric flow-rate is not compatible with an MS detector, without employing a high percentage split to waste, which could result in a reduction in assay sensitivity.

Micro-bore and capillary bore HPLC has been used successfully in the analysis of peptide tryptic digests [10,11]; these are normally reversed-phase isocratic systems or very long gradient runs. Little work has been published on the use of micro-bore LC with short columns and fast gradients.

The column dimensions were reduced to 30×1 mm and packed with Inertsil ODS-3 3-μm material, a gradient of 100–5% aqueous formic acid–acetonitrile was run over 2 min. This produced a chromatogram with acceptable resolution and reproducibility, Fig. 4. A very high eluent flow-rate of 800 μl/min was required to construct the gradient in the time scale of 2 min. Although this is MS compatible it is no lower than the flow-rate used with the 2-mm column.

The combination of a 3 cm×1 mm column packed with 3-μm material and capillary tubing also results in a system back pressure of over 350 bar which is not practical to run routinely. The reason such a high volumetric flow-rate (800 μl/min) is required is due to the dead volume in the system; as columns with smaller and smaller internal diameters are employed, the dead volume in the HPLC system has a greater effect. For this reason we have used 50×2 mm and 30×2 mm columns for the remainder of the investigation.

3.3. Quantitative assay

A generic gradient LC system has been developed for use in conjunction with MS–MS detection for the

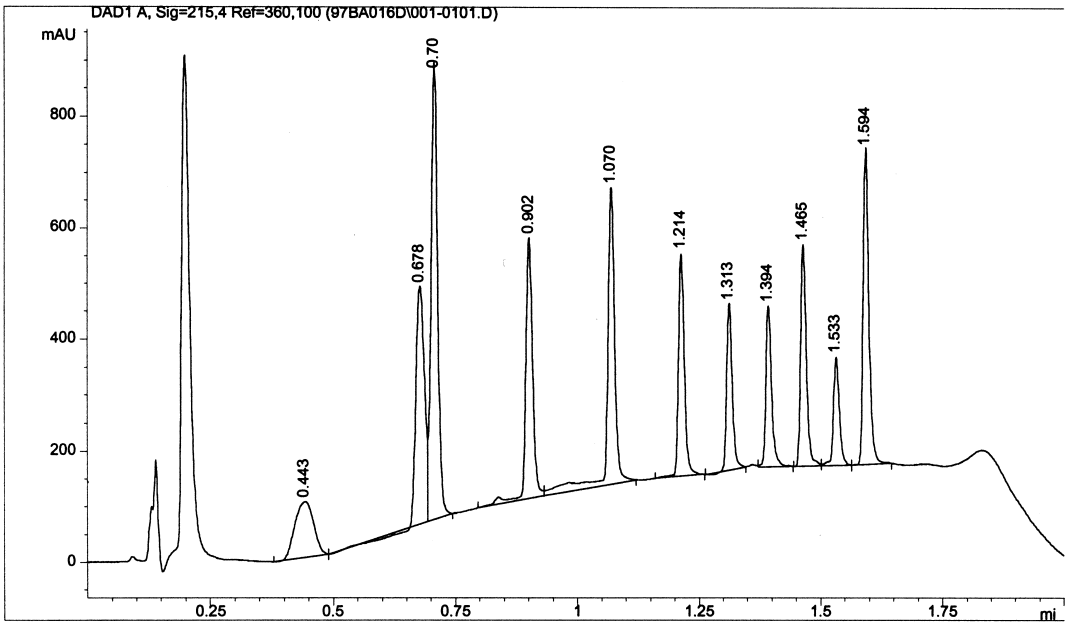

Fig. 2. Injection (0.5 μl) of an 11 component test mix onto a 50×2 mm Waters Symmetry Shield C_8, 3.5-μm column. The column was maintained at 40°C and eluted with a 0–95% aqueous formic acid (0.1%) and acetonitrile gradient over 2 min at flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

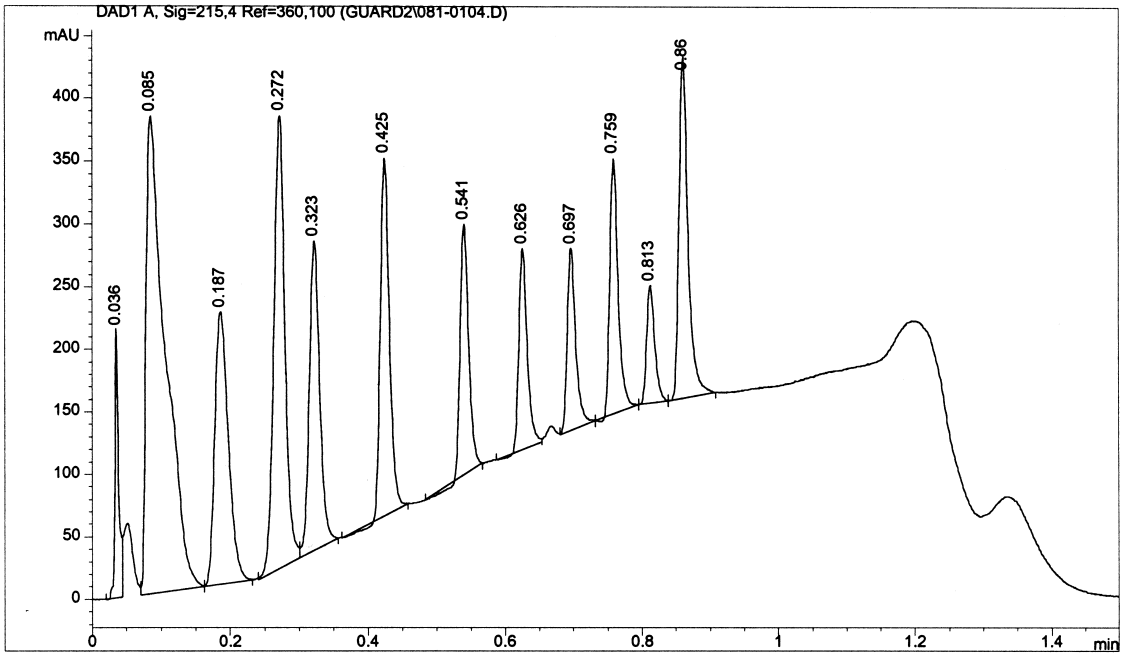

Fig. 3. Injections (0.5 μl) of an 11 component test mix onto a 20×2 mm Supelco ABZ-plus C_{18} 5-μm column. The column was maintained at 40°C and eluted with a 0–95 aqueous formic acid (0.1%) and acetonitrile gradient over 1 min at flow-rate of 3000 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

analysis of drug candidate molecules in plasma and serum, following solid-phase extraction. This method has been used successfully for the analysis of several chemically different drug candidate molecules in plasma and serum. A typical example assay is outlined in the remainder of this paper. The method will be used to discuss the advantages of this generic gradient approach to bioanalysis.

3.3.1. Analysis of Analytes I and II in rat plasma

The method for the simultaneous determination of Analyte I and Analyte II in rat plasma was validated successfully in the analytical concentration range of 1–200 ng/ml for both compounds. The intra-assay validation results are summarised in Table 1. A typical chromatogram of extracted blank plasma and the 1 ng/ml Analyte I and Analyte II standard are show below in Figs. 5 and 6, respectively. This assay required no method development with the exception of the optimisation of the MS–MS parameters, the choice of the internal standard concentration and determination of a suitable calibration range. It was therefore possible to complete the method development process in under 2 h. The method has been used successfully to support a toxicological safety study for both compounds.

There was no increase in column back pressure from the start to the end of the analytical run and the analytical column has been used for over 500 injections without replacement. There was also no evidence of any analytical carry-over between injections. These observations are probably a result of the gradient reaching an organic concentration of 95%; such a wash very effectively cleans the stationary phase prior to the next injection. The HPLC instrumentation used in this study employs a sampling needle that is washed continuously by the mobile phase and the use of a 0–95% aqueous formic acid (0.1%)–acetonitrile gradient ensures the needle is washed clean of the current sample prior to the next injection.

3.3.2. Throughput

The use of automated 96-well SPE and fast gradient HPLC has resulted in methodology that can extract and analyse 96 samples in under 7 h, one

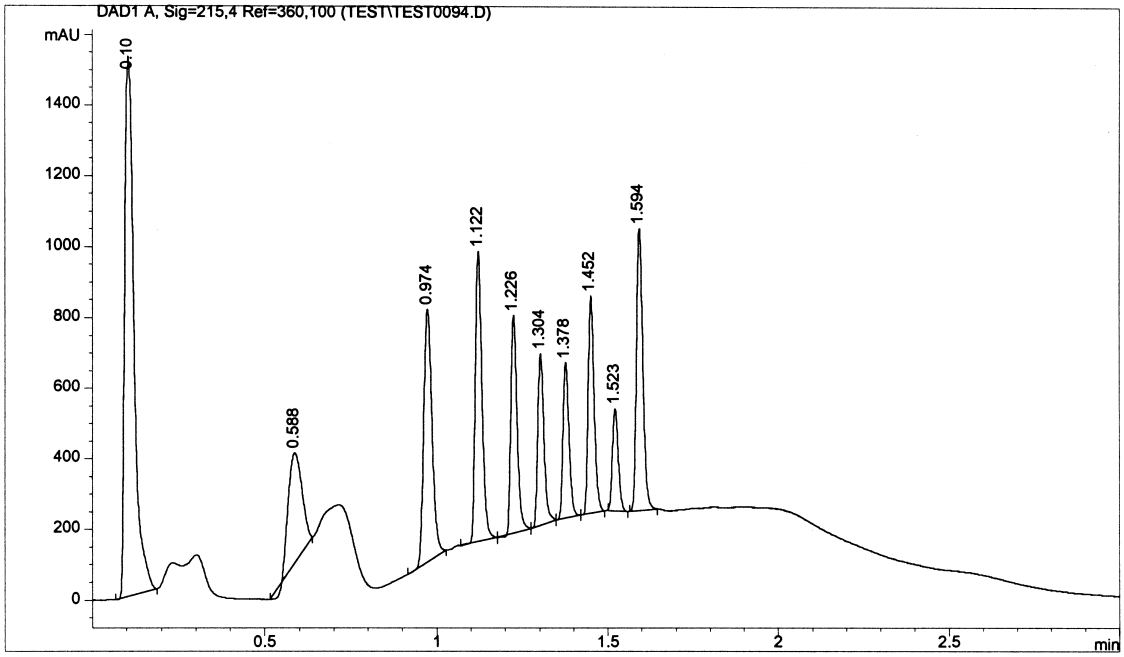

Fig. 4. Injection (0.5 μl) of an 11 component test mix onto a 30×1 mm Capital Inertsil ODS-3 C_{18} 3-μm column. The column was maintained at 40°C and eluted with a 0–95 aqueous formic acid (0.1%) and acetonitrile gradient over 2 min at flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

sample every 4.4 min. The automated SPE system employed in this analytical method has been described previously by Pleasance and Biddlecombe [12].

The SPE robotic system was able to extract 96 samples in 40 min using the methodology described above. The sample extracts were evaporated to dryness under a stream of nitrogen and reconstituted in 1.5 h, the analysis of the extracts by LC–MS–MS was completed in 4.8 h. Once the first 96 samples have been extracted the next 96 sample extracts will be ready for analysis immediately the LC–MS analysis of the first 96 is complete. Thus there is a 2.1-h period required to prime the system at the beginning of the working day, with the following samples taking 3.0 min each to analyse.

Table 1
Summary of the assay validation data

Sample	Nominal concentration (ng/ml) (1995)				
	1	2	75	150	200
Analyte I					
Mean	1.12	1.94	77.5	151	195
S.D	0.04	0.12	1.90	3.14	4.35
%C.V.	3.3	6.4	2.5	2.1	2.2
Accuracy	12.0	−3.2	3.3	0.6	−2.6
Analyte II					
Mean	0.93	1.80	75.3	145	189
S.D	0.06	0.06	0.36	2.49	3.31
%C.V.	6.7	3.3	0.5	1.7	1.8
Accuracy	−7.2	−9.8	0.4	−3.1	−5.4

3.3.3. Advantages of generic gradients in working practices

The drug development process at the ‘first time into man’ stage usually involves a safety and tolerability study in which dose escalation occurs every 2–3 days. This results in around 50 to 80 samples which need to be analysed and reported within 24 h before the next dosing regime can be embarked upon. Therefore, it is usually necessary to analyse around 80 to 100 samples (including standards and QCs) from several different compounds during a typical week. A significant amount of time is often required when changing from one specific assay to another. This involves changing the column

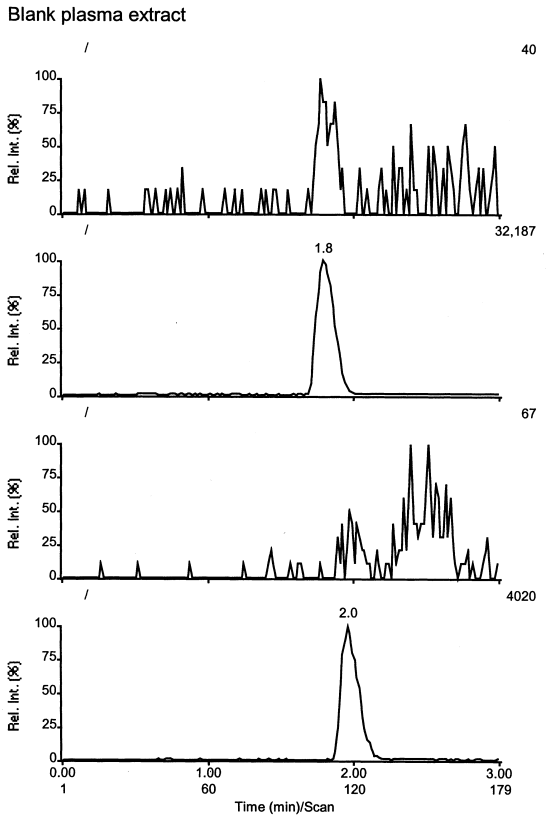

Fig. 5. Sample chromatogram of blank extracted plasma. Chromatographic conditions: injection volume 15 μl; LC column 50×2 mm 3-μm Phenomenex Magellen C_{18}. The column was maintained at 40°C and eluted with a 0–95 aqueous formic acid (0.1%) and acetonitrile gradient over 2 min at flow-rate of 800 μl/min. The column eluent was monitored by MRM mass spectroscopy.

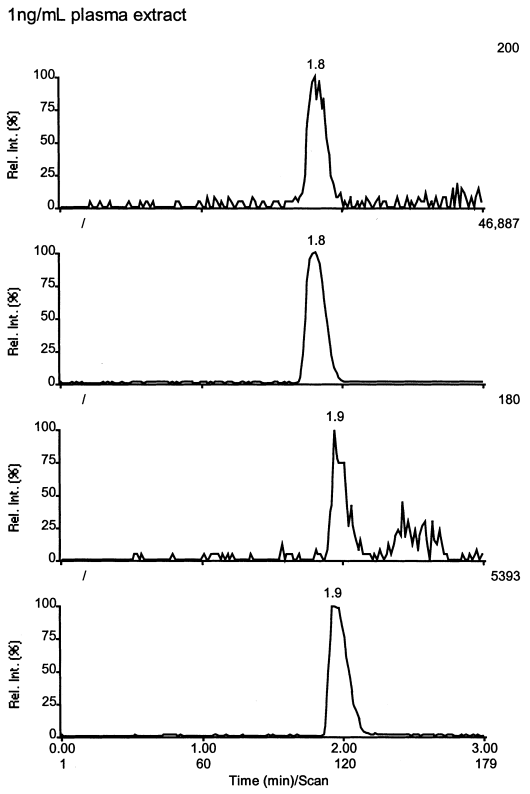

Fig. 6. Sample chromatogram of a 1 ng/ml extracted plasma standard of Analyte I and Analyte II. Chromatographic conditions: injection volume 15 μl; LC column 50×2 mm 3-μm Phenomenex Magellen C_{18}. The column was maintained at 40°C and eluted with a 0–95 aqueous formic acid (0.1%) and acetonitrile gradient over 2 min at flow-rate of 800 μl/min. The column eluent was monitored by MRM mass spectroscopy.

and mobile phase, allowing the system to equilibrate and then testing the chromatography. This can sometimes take upwards of 1 h.

Using a generic gradient LC–MS method we have been able to obtain satisfactory chromatography and produce validated assays so far on six new drug compounds with a wide range of chemical properties. Though the authors are well aware that it will not be possible to analyse every compound with this system, it is envisaged that this approach can be used for at least 80% of the compounds encountered. If each compound was chromatographed using the generic gradient system there would be no delay, except loading the mass spectrometer parameters, before starting the next analytical run.

3.4. Injection of organo–aqueous solutions

A significant proportion (20%) of the initial 7 h processing time is accounted for by the evaporation and reconstitution process. It would be of advantage, therefore, if this process could be eliminated. To that end the effect on the chromatographic performance of the direct injection of various volumes of methanol containing the two Analytes was investigated.

It was observed that a maximum volume of 25 μl of methanol, containing test compounds, could be injected without affecting the retention time or shape of the chromatographic peak. Above this volume the retention time of the compounds was inconsistent and the peak shape was unacceptable. The same

experiment was performed using the same compounds dissolved in methanol–water (50:50, v/v). The result of this experiment was that over 100 μl of sample could be injected onto the LC column with minimal effect on either the retention time or peak shape of both compounds, an example chromatogram of a 10- and 100-μl injection is shown in Figs. 7 and 8. This approach will, however not be suitable for very hydrophilic compounds which are poorly retained on C_{18} bonded silica columns.

These results indicate that the organic eluent of a SPE process can be diluted with an equal volume of water and then injected onto the LC–MS system. This would eliminate the need for an evaporation and reconstitution step prior to LC–MS analysis. In order to maintain acceptable assay sensitivity the SPE elution volume would need to be kept to minimum e.g. 100–150 μl. The use of low mass sorbent bed and membrane disk SPE for bioanalysis was discussed by Jones et al. [13] and it was shown that extraction efficiencies of greater that 80% can be achieved with elution volumes as low as 50 μl. The combination of fast gradient HPLC and low elution volume SPE will be investigated further by the authors.

3.5. Chromatographic resolution

The amount of chromatographic resolution required for quantitative analysis is dependent on the selectivity of the detection technique employed. When using MS–MS detection the need for baseline resolution between compounds is removed due to the extra selectivity of the detector, therefore a faster sweeping gradient can be employed and thus higher throughput can be achieved. When using single ion monitoring (SIM), mass spectrometry in conjunction with HPLC, greater chromatographic resolution is required as the instrument is less selective. Therefore a 50×2 mm, 3-μm C_{18} LC column with a chromatographic gradient time of 2 min was used for LC–MS–MS, and a 30×2 mm, 3-μm C_{18} LC column with a chromatographic gradient time of 4 min was employed for LC–MS (SIM). Typical chromato-

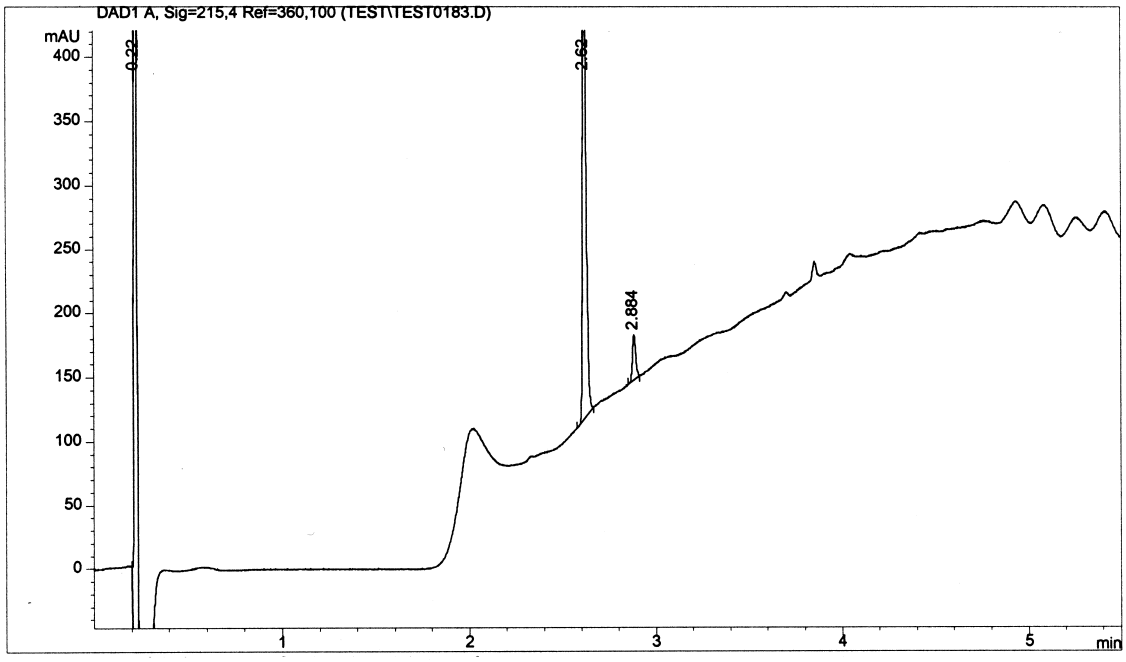

Fig. 7. A 10-μl injection of Analyte I and Analyte II in methanol–water (50:50, v/v) onto a 50×2 mm Phenemenex Magellen C_{18} 3-μm column. The column was maintained at 40°C and eluted with a 0–95% aqueous formic acid (0.1%) and acetonitrile gradient over 2 min at flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

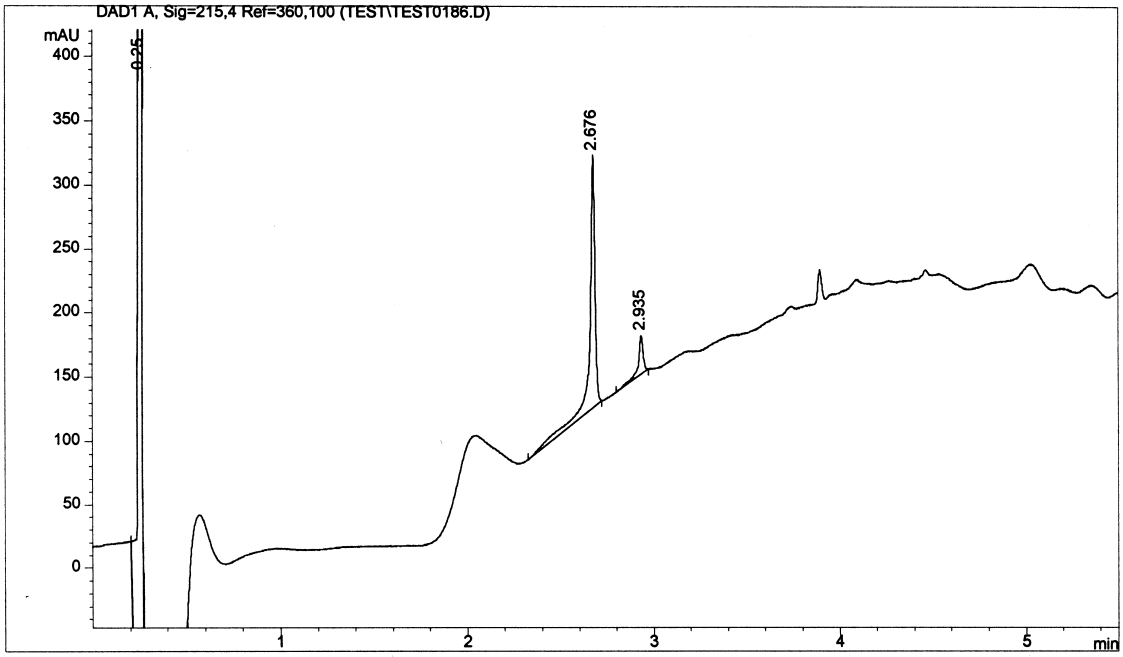

Fig. 8. A 100-μl injection of Analyte I and Analyte II in methanol–water (50:50, v/v) onto a 50×2 mm Phenemenex Magellen C_{18} 3-μm column. The column was maintained at 40°C and eluted with a 0–95% aqueous formic acid (0.1%) and acetonitrile gradient over 2 min at flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

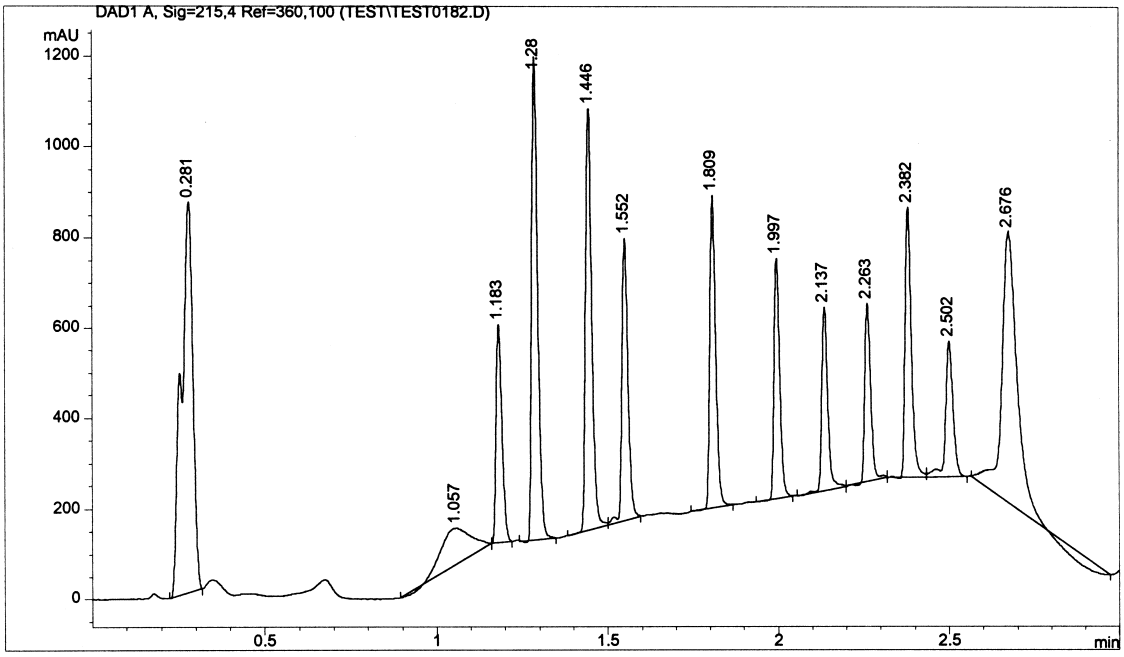

Fig. 9. An injection (1 μl) of an 11 component test mix onto a 50×2 mm Phenomenex Magellan C_{18}. The column was maintained at 40°C and eluted with a 0–95% aqueous formic acid (0.1%) and acetonitrile gradient over 2 min followed by a 1-min isocratic period of aqueous formic acid (0.1%) at a flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

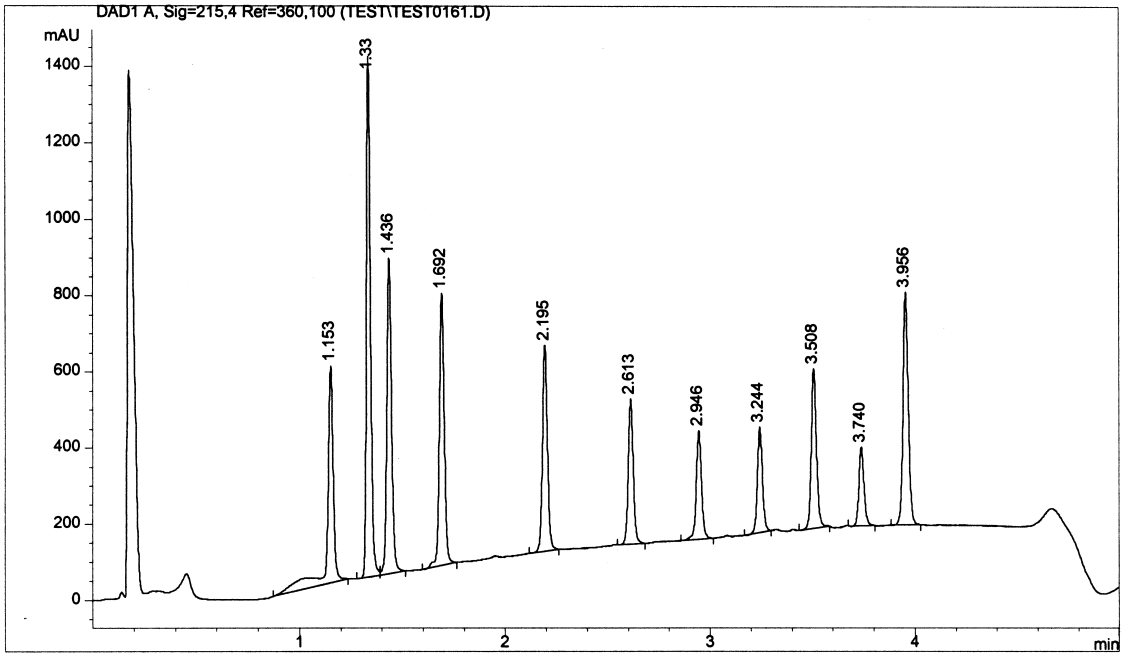

Fig. 10. An injection (1 μl) of an 11 component test mix onto a 30×2 mm Phenomenex Magellan C_{18} column. The column was maintained at 40°C and eluted with a 0–95% aqueous formic acid (0.1%) and acetonitrile gradient over 4 min followed by a 1-min isocratic period of aqueous formic acid (0.1%) at a flow-rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.

grams for each gradient profile are shown in Figs. 9 and 10. It can be clearly seen from these two chromatograms that the combination of a 30×2 mm column and 4 min gradient results in greater chromatographic performance.

4. Conclusion

Fast generic gradient HPLC has been combined successfully with MS–MS detection to provide a rapid generic approach to bioanalysis without compromising assay sensitivity. This approach has resulted in a significant increase in sample throughput, with one sample being analysed every 3 min. The use of fast generic gradient LC, has, for those compounds tested in our laboratory, removed the need for any chromatographic method development.

When combined with a generic SPE this approach has the potential to dramatically speed up the method development process for bioanalysis using LC–MS–MS and LC–MS. The ability to inject relatively large volumes of aqueous–organic solvent onto the LC system without degrading the chromatography will allow future methods to be developed without the need for an evaporation and reconstitution step.

Whilst it is recognised that this approach will not work for every compound that is encountered in the drug development process, it is likely to work for more than 80% of them. This should make a significant impact in applications where high throughput bioanalysis is sought.

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