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Comparison of fused-core and conventional particle size columns by LC–MS/MS and UV: Application to pharmacokinetic study

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ABSTRACT

The chromatographic performance of fused-core (superficially porous) HPLC packing materials was compared with conventional fully porous particle materials for LC–MS/MS analysis of two pharmaceuticals in rat plasma. Two commercially available antidepressants, imipramine and desipramine, were assayed using a conventional analytical C_{18} column (5 μm, 2.0 mm × 30 mm) and a fused-core C_{18} column (2.7 μm, 2.1 mm × 30 mm). Retention time, column efficiency, pressure drop, resolution, and loading capacity were compared under the same operating conditions. The fused-core column demonstrated reduced assay time by 34% and 2–3-fold increased efficiency (*N*). Loading capacity up to 25 μl of extract injected on column showed no peak distortion. The registered back-pressure from a flow rate of 1.0 ml/min did not exceed 3400 psi making it compatible with standard HPLC equipment (typically rated to 6000 psi). Two mobile phases were examined, and morpholine as an organic base modifier yielded a 2–5-fold increase in S/N near the limit of detection over triethylamine. The 2.7 μm fused-core column was applied to the analysis of imipramine and desipramine in extracted, protein precipitated rat plasma by LC–MS/MS. The calibration curves were linear in the concentration range of 0.5–1000 ng/ml for both imipramine and desipramine. Intra-run precisions (%CV) and accuracies (%bias) were within ±7.8% and ±7.3% at three QC levels and within 14.7% and 14.4% at the LOQ level for both analytes. Following a single method qualification run, the method was applied to the quantitation of pharmacokinetic study samples after oral administration of imipramine to male rats.

1. Introduction

The popularity of liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the analysis of pharmaceuticals in complex biological matrices is due, in part, to its high sample throughput, selectivity and sensitivity for the analytes of interest [1–4]. However, as with all aspects of the pharmaceutical analytical sciences, increasing speed and throughput are both desirable and necessary. In recent years, strategies to accomplish more rapid analysis have emerged, including use of fast linear gradients and monolithic columns [5–7]. Maintaining assay robustness (*e.g.*, for FDA CFR 21 part 58 compliant studies) in the analysis of complex matrices such as serum, plasma, urine, etc., while increasing speed can present significant challenges. Mass spectrometric analysis, particularly using electrospray ionization (ESI), is influenced by ion suppression (or matrix effects) which arise from charge competition with coeluting components in the biological sample [8–11]. Additional challenges include isobaric coeluting peaks and in-source fragmentation which can be undetected and contribute erroneously to the response of the analyte [12–14]. Strategies for overcoming these analytical issues often involve increasing chromatographic resolution.

Whether the goal is increasing analysis speed or resolution, manipulating efficiency (*N*) through use of smaller particle size is now a well employed strategy. Commercially available sub-2 μm particles have emerged which take advantage of this relationship, and have demonstrated the ability to maintain assay accuracy and robustness while reducing cycle times [15–19]. However, as pressure increases with the inverse square of the decreasing particle size, the use of these small particles often requires specialized HPLC systems, typically capable of tolerating back-pressures of up to 15,000 psi. For analytical approaches using conventional HPLC equipment, 3–5 μm porous particle columns remain the preferred choice [20,21]. The recent commercialization of fused-core particle technology presents a new option for HPLC bioanalysis. With a 1.7 μm solid silica inner core surrounded by a 0.5 μm porous silica shell [22], the material has a shortened diffusion path which allows for rapid mass transfer and thus reduced axial dispersion and peak broadening.

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The van Deemter equation describes the relationship between linear velocity (μ) and plate height (H), and is widely used in the evaluation of column efficiency:

$$H = A + \frac{B}{\mu} + C\mu$$

where the A, B, and C terms represent eddy diffusion, longitudinal diffusion, and axial dispersion (also termed resistance to mass transfer), respectively. The C term is the key contributor for the loss of column efficiency when flow rates are increased beyond the optimized linear velocity in order to achieve faster separations. Several recent literature reports comparing fused-core particles with fully porous particles show improved (lower) reduced plate heights when compared with larger size porous particles, including at higher flow rates where improved mass transfer in the thin porous layer provides for less band dispersion due to axial diffusion [23–25]. Cunliffe and Maloney report an efficiency of 77–88% that of a similar 1.8 μm packing with only 47% of the back-pressure [26]. Reports of reduced eddy diffusion (A term) due to the narrow particle size distribution and homogeneity of the bed packing are also listed as reasons for the improved efficiency [25]. Fused-core particle columns have drawn attention in the pharmaceutical bioanalytical realm with a few reported applications using fused-core columns coupled with LC–MS/MS and LC-Ion-Trap-FTMS for biological sample quantitation [27,28]. In this study we investigate the applicability of a fused-core particle column for the routine LC–MS/MS bioanalysis of two commercially available antidepressant drugs, imipramine and desipramine, in rat plasma. Several LC–MS and LC–MS/MS methods have been previously reported for the determination of imipramine and desipramine and other antidepressant drugs. Shinozuka *et al.* [29] reported a solid phase extraction (SPE) coupled LC–MS method for determination of imipramine and desipramine (out of a total of 20 antidepressant drugs) with quantitation limits of 100 and 150 ng/ml for imipramine and desipramine respectively. Castro *et al.* reported a SPE-LC–MS/MS method with an LOQ of 2.0 ng/ml [30] and a high throughput, on-line SPE-HPLC–MS/MS method with an LOQ of 10 μg/ml [31]. In the report presented here, the efficiencies of a 2.7 μm fused-core particle column and a porous 5 μm particle column were compared using both UV and mass spectrometric detection. Peak tailing was minimized by the addition of morpholine or triethylamine (TEA) as a mobile phase modifier. A method was developed that employed a simple protein precipitation extraction and a fused-core column coupled with LC–MS/MS detection to quantitatively detect imipramine and desipramine in rat plasma with LOQs of 0.5 ng/ml.

2. Experimental

2.1. Chemicals and reagents

Imipramine HCl, desipramine HCl, amitriptyline HCl (internal standard, IS), and naphthalene were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were of 98% or higher purity. Methanol, water, and acetonitrile, all of HPLC grade, were obtained from JT Baker (Phillipsburg, NJ, USA). Ammonium hydroxide solution (29.8% purity), and ammonium acetate (both ACS grade) were obtained from Mallinckrodt (Hazelwood, MO, USA). Morpholine (>99% purity) from Sigma–Aldrich and triethylamine (100% purity) from JT Baker Company were added to mobile phases to reduce secondary interactions and improve peak shape. Sodium chloride solution, 0.9% (Injection USP) was received from B. Braun Medical Inc. (Bethlehem, PA, USA). Pooled K_3EDTA rat plasma was purchased from Bioreclamation (Hicksville, NY, USA). Refer to Fig. 1 for the structures for imipramine, desipramine, amitriptyline, morpholine, and TEA.

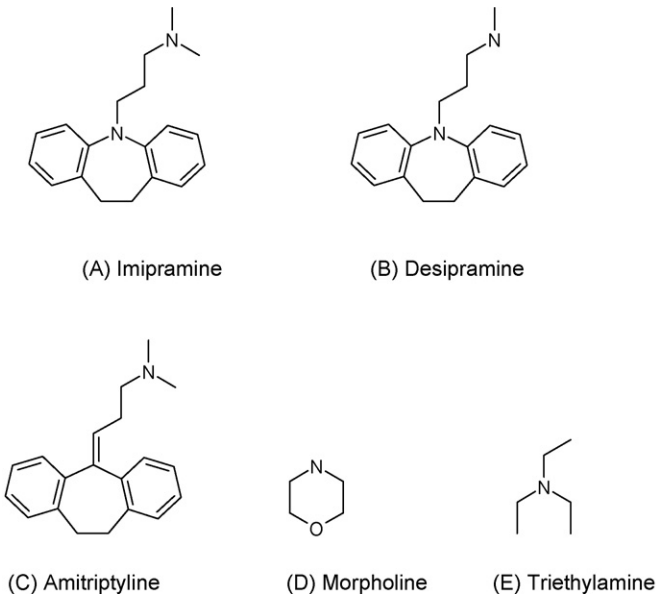

Fig. 1. Chemical structures of imipramine (A), desipramine (B), amitriptyline (C), morpholine (D), and triethylamine (E).

2.2. Instrumentation and chromatographic conditions

2.2.1. Instrumentation

A Prominence™ high performance liquid chromatographic system from Shimadzu (Columbia, MD, USA), was used throughout the study. The system consists of two gradient mixing pumps (LC-20AD), a 96-well plate compatible autosampler (SIL-20AC HT), degasser (DGU-20A5), integrated column switcher oven (CTO-20A), and communication bus module (CBM-20A). Outfitted with stainless steel plumbing, the system is rated to and can tolerate operating pressures of up to 9000 psi.

The HPLC system was coupled in series with a Shimadzu SPD-20A UV/Vis detector and a Sciex API 4000 LC–MS/MS (atmospheric pressure ionization triple quadruple mass spectrometer, Applied Biosystems, Foster City, CA, USA). The UV wavelength was set at 254 nm for the imipramine and desipramine experiments and at 274 nm for experiments using naphthalene. Both the ESI and APCI ionization modes were tested for the analysis of imipramine and desipramine and positive ion mode ESI gave a higher intensity (based on analyte response) than did APCI under conditions optimized for each ionization mode. For ESI, the ion spray voltage was set at 5000 V. Curtain, nebulizer, auxillary, and collision gases were set at 12, 32, 34, and 4 psi, respectively. The collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were set at 30, 10, and 10 V, respectively. The declustering potentials (DP) were set to 50, 30, and 50 V for imipramine, desipramine, and amitriptyline, respectively. Q1 and Q3 were set to unit resolution and the dwell time was 200 ms. Analytes were monitored via multiple reaction monitoring (MRM) employing the following precursor to product ion transitions [30]: imipramine, m/z 281.0 → 86.0, desipramine m/z 267.0 → 72.0, and amitriptyline, m/z 278.0 → 233.0. The analytical data were processed and integrated through Analyst software (Version 1.4.2). Data regression and concentration calculations were performed using the Watson Laboratory Information Management System (Version 7.2.0.03) (Thermo LabSystems, Philadelphia, PA, USA).

2.2.2. HPLC conditions for imipramine and desipramine

The chromatographic conditions, including the mobile phase composition, were optimized to achieve symmetric peak shapes for

imipramine and desipramine. Mobile phase A consisted of a mixture of 10 mM ammonium acetate and 0.1% NH_4OH in water (pH 8.5) and mobile phase B consisted of a mixture of 10 mM ammonium acetate, 0.1% NH_4OH, and 0.2% organic base (*i.e.*, morpholine or TEA) in acetonitrile. As basic amines often participate in secondary binding interactions with silanol groups on the stationary phase, they frequently require a mobile phase modifier to achieve adequate peak shape and retention [32]. To this end, addition of 0.2% (v/v) of a volatile organic base modifier, morpholine or TEA, in mobile phase B (MPB) was used to improve the peak symmetry (*i.e.*, reduced the peak tailing) presumably by reducing secondary interactions with the stationary phase. The 0.2% concentration was determined by successively increasing the base percentage in acetonitrile (MPB) until optimal peak symmetry was observed (data not shown). The mobile phase was mixed on-line at A–B (40:60, v/v) and delivered isocratically. For the van Deemter plot experiments, the flow rate was varied from 0.025 to 1.0 ml/min and for the PK sample analysis a flow rate of 0.4 ml/min was used. The injection volume was 10 μl for UV detection and 3 μl for mass spectrometric detection.

Column comparison experiments were conducted using a 2.1 mm × 30 mm Halo fused-core C_{18} column (MacMod, Chadds Ford, PA, USA) and a 2.0 mm × 30 mm Luna 5 μm porous $C_{18}(2)$ column (Phenomenex, Torrance, CA, USA). Chromatography was performed at ambient temperature for all work. Only the Halo fused-core column was employed in the method qualification and analysis of rat PK samples.

2.2.3. HPLC conditions for naphthalene

For the van Deemter experiments with naphthalene, two porous particle columns, a 2.1 mm × 30 mm, 3.5 μm ACE C_{18} column (Advanced Chromatography Technologies, Chadds Ford, PA, USA) and a 2.1 mm × 30 mm, 1.8 μm Acquity UPLC HSS C_{18} column (Waters, Milford, MA, USA) were also used in addition to the two columns listed in Section 2.2.2. Isocratic elution using unbuffered acetonitrile–water (60:40, v/v) at various flow rates from 0.025 to 1.4 ml/min was used. A 1.0 μg/ml solution of naphthalene in methanol was injected at 10 μl. The UV flow cell volume was 8 μl.

2.3. Stocks, calibration standards, and quality control samples

Fresh stock solutions containing imipramine and desipramine were prepared separately by dissolving each compound in acetonitrile–water (1:1, v/v) at a concentration of 1.0 mg/ml. Serial working solutions containing imipramine and desipramine were prepared in acetonitrile–water (1:1, v/v) at concentrations of 50, 10, 5, 4, 1, 0.5, and 0.05 μg/ml. Calibration standards were prepared at concentrations of 1000, 500, 250, 100, 50, 10, 1, and 0.5 ng/ml for both imipramine and desipramine by spiking an appropriate amount of the standard working solutions into blank K_3EDTA rat plasma. The calibration standards were assayed with QC samples, similarly prepared in blank K_3EDTA rat plasma at 750, 20, 1.5, and 0.5 ng/ml for imipramine and desipramine respectively. A stock solution of IS was prepared by dissolving amitriptyline in acetonitrile–water (1:1, v/v) at a concentration of 1.0 mg/ml. The working IS solution was prepared by appropriate dilution to 100 ng/ml in acetonitrile with 0.1% NH_4OH. A stock solution of naphthalene was prepared by dissolving the compound in methanol at a concentration of 1.0 mg/ml and a working solution of 1.0 μg/ml was prepared by appropriate dilution from the stock solution with methanol. All stocks and working solutions were stored in a glass vials at 4 °C. Plasma samples were prepared fresh the day of use.

2.4. Plasma sample preparation

Plasma concentrations of imipramine and desipramine were simultaneously determined using the LC–MS/MS method. All plasma calibration standards, QC samples, control plasma and study samples were briefly vortexed and an aliquot of 50 μl rat plasma sample was transferred into a 1.2 ml polypropylene well of a 96-well block. A 150 μl aliquot of 10.0 ng/ml working IS solution in acetonitrile containing 0.1% NH_4OH was added to each well. To the double blank well, 150 μl of acetonitrile containing 0.1% NH_4OH was added. The sample block was vortex-mixed for 1 min, followed by centrifugation at approximately 1643 × *g* for 10 min. A 100 μl portion of the supernatant was transferred to a 96-well injection block for injection onto the LC–MS/MS.

2.5. Method qualification

A method qualification run using the fused-core column with LC–MS/MS detection was conducted to assess method suitability for analysis of imipramine and desipramine in rat plasma. Plasma standard and quality control samples were prepared fresh. The specificity of the method was tested by screening three different lots of blank rat plasma. Each blank sample was tested for interferences in the MRM channels using the reported extraction procedure and LC–MS/MS conditions.

Sets of spiked calibration standards ($n = 8$) and QC samples ($n = 6$ at each concentration) were prepared and analyzed in one batch run to evaluate linearity, precision and accuracy. The intra-run precisions and accuracies were estimated by analysing six replicates containing imipramine and desipramine at four different QC levels, 750, 20, 1.5, and 0.5 ng/ml. The criteria for acceptability of the data follow the US FDA guidance [33] which specifies the limit for accuracy as within ±15% deviation from the nominal values and a relative standard deviation (RSD) within ±15%. Precision and accuracy were assessed at the lowest concentration of the standards (0.5 ng/ml), representing the LOQ for the assay, with an acceptance criteria of ±20% for precision and accuracy. The calibration curve was acceptable when a minimum of 75% of the total number of calibration standards fell within a back-calculated accuracy of ±15% of the nominal value (±20% at the LOQ). The coefficient of determination (r^2) was calculated for each calibration curve.

2.6. Pharmacokinetic study

Healthy adult male Carotid Artery Cannulation (CAC) Sprague–Dawley rats weighing 350–380 g (Charles River Laboratories, Portage, MI, USA) were housed in well ventilated cages and kept at room temperature on a regular 12-h light:12-h dark cycle. Animals were cared for in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals [34] for animal experimentation. The rats were acclimatized to the laboratory environment for at least 2 days before conducting the experiment and were not fasted at the time of dosing. An oral dosing formulation at a concentration of 15 mg/ml was prepared by accurately weighing imipramine and dissolving in 0.9% saline solution. The formulation was administered to the rats using a 3 ml syringe fitted with a cannula (oral gavage tube). The dose volume was 2 ml/kg for the test compound. Blood samples were collected from the CAC using an automated blood sampler (Accusampler™, DiLab, Inc., Littleton, MA, USA), into K_3EDTA polypropylene tubes at pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h post-dose. Plasma was separated by centrifugation at approximately 1643 × *g* for 10 min at 4 °C and stored at −20 °C prior to analysis.

3. Results and discussion

3.1. van Deemter plot for naphthalene with UV detection

Improving chromatographic efficiency through reduced particle size has long been understood as a viable strategy. Band dispersion from eddy and axial diffusion shows a dependence on particle size, with a reduction in particle diameter expected to yield significant reduction in plate height (*i.e.*, increased efficiency). Fused-core particles of 2.7 μm diameter with a 0.5 μm diffusion path (9 nm pore size) surrounding a 1.7 μm solid silica inner sphere allow for rapid diffusion. More rapid diffusion results in reduced axial dispersion while the narrow distribution of particle sizes (6% standard deviation) allows for reduced dispersion caused by eddy diffusion. Reported plate height and reduced plate height values for fused-core particles are less than values for porous particles of larger size and are comparable to (and in some cases less than) 1.7 μm porous particles [22,26].

The van Deemter plot of plate height as a function of linear velocity remains an important tool to evaluate chromatographic performance under specific laboratory and experimental conditions [35]. Conducting such an experiment with a simple system, such as naphthalene with UV detection, allows for direct comparison of performance relative to other porous particles and to prior literature reported results. Therefore, van Deemter plots were generated using the fused-core column and conventional porous particle columns for the analysis of a 1.0 μg/ml neat solution of naphthalene in acetonitrile–water (1:1, v/v) with UV detection at 274 nm. Three porous columns (5 μm Luna C_{18}(2) 2.0 mm × 30 mm; 3.5 μm ACE C_{18} 2.1 mm × 30 mm; 1.8 μm Acquity UPLC HSS C_{18} 2.1 mm × 30 mm) were tested along with a 2.7 μm fused-core Halo C_{18} 2.1 mm × 30 mm column using a mobile phase of acetonitrile/water (60:40, v/v) with flow rates of 0.025–1.4 ml/min. van Deemter curves of plate height, H (μm) as a function of linear velocity (mm/s) were plotted for each column yielding plot shape and efficiency trends similar to as previously reported (DeStefano *et al.*, [24]) using 4.6 mm × 50 mm columns in various particle types. Plate height was calculated using the following equation:

$$H = \frac{L}{N}$$

where L is the column length and N is the number of theoretical plates calculated by the peak width at half height method [36], *i.e.*,

$$N = 5.55\frac{t_R^2}{w_{1/2}^2}$$

The column efficiency, N, for the 2.7 μm fused-core particle column increased approximately 2–6-fold compared to 3.5 and 5 μm porous particle columns as shown by the plate height minimum. When compared to the 1.8 μm Acquity UPLC HSS column, the fused-core column had comparable performance and both showed relatively low plate height through the flow rates tested. These results are in keeping with manufacturer and literature reported data [22,26], which show similar experimental performance and van Deemter behavior between the 1.8 μm porous particles and the fused-core particles. As a result of the improved mass transfer kinetics for both these particle types, faster flow rates may be used without loss of column efficiency, thus effectively decreasing cycle time.

3.2. Column performance comparison using imipramine

3.2.1. van Deemter plot for imipramine using UV and MS detection

To compare the column performance under conditions more typically encountered in the small molecule bioanalytical laboratory, imipramine and desipramine, two tricyclic antidepressants with basic properties were used as test compounds for conducting column evaluations. The van Deemter plot for an imipramine solution (1.0 μg/ml in acetonitrile–water (1:1, v/v)) using a 2.7 μm fused-core particle column (2.1 mm × 30 mm) and a 5 μm porous Luna C_{18} (2) column (2.0 mm × 30 mm) was constructed using optimized mobile phase conditions including either morpholine or TEA. Although MS/MS detection is typically the method of choice in the bioanalytical laboratory, its use for constructing van Deemter plots is atypical due to concerns over peak distortion because of the

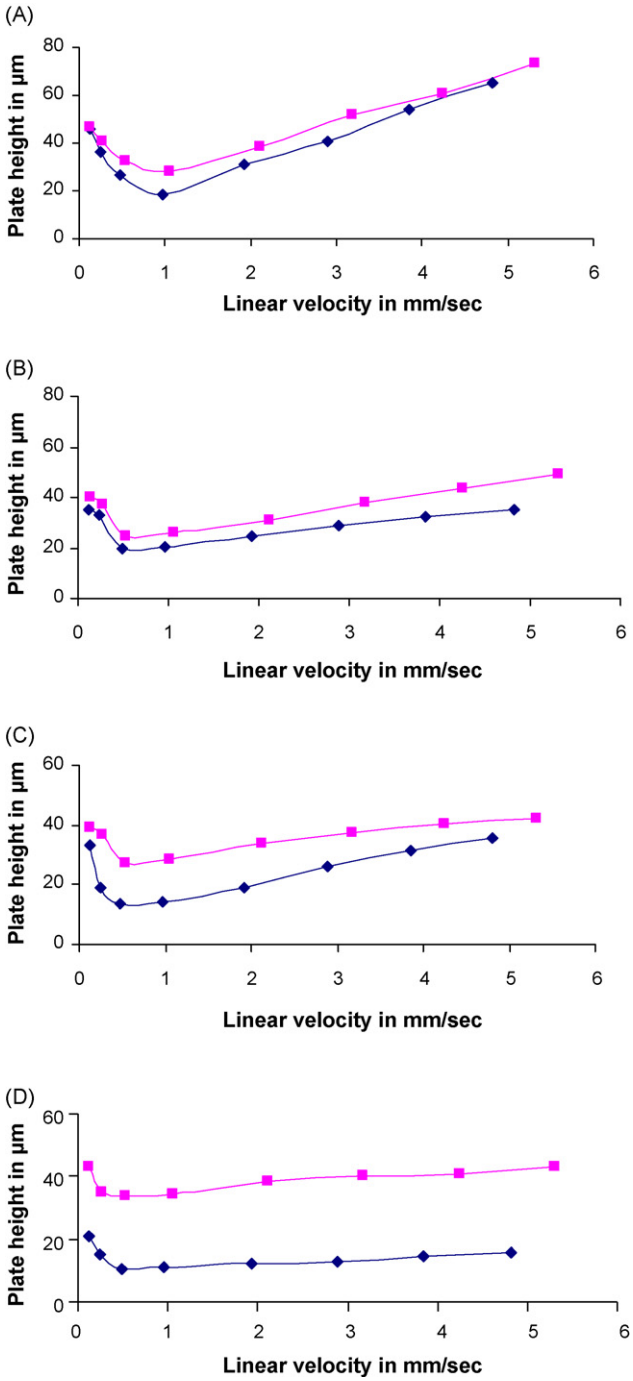

Fig. 2. Plate height (μm) versus linear velocity (mm/s) of imipramine (1.0 μg/ml) using UV and MS detectors in morpholine and TEA mobile phases with 2.7 μm fused-core particle (◆, 2.1 mm × 30 mm) and 5 μm porous particle C_{18} (2) (■, 2.0 mm × 30 mm) columns: (A) UV/morpholine, (B) MS/morpholine, (C) UV/TEA, and (D) MS/TEA.

Table 1
Fundamental parameters of 2.7 μm fused-core and 5 μm porous particle columns with morpholine and TEA mobile phases.

Organic base modifier	Column	Flow rate (ml/min)	N	H	h	k'	Resolution[a]	Pressure (psi)
Morpholine	2.7 μm fused-core	0.1	2737	10.96	4.06	7.41	1.97	280
		0.4	2396	12.52	4.64	1.93	0.93	1320
		1	1405	21.35	7.91	0.65	0.66	3310
	5 μm porous	0.1	1583	18.95	3.79	11.28	1.69	150
		0.4	1280	23.44	4.69	2.74	0.7	700
		1	748	40.09	3.02	1	0.54	1600
TEA	2.7 μm fused-core	0.1	3523	3.51	3.15	5.59	1.57	270
		0.4	2493	12.03	4.46	1.43	1.42	1310
		1	1793	16.72	6.19	0.33	0.47	3280
	5 μm porous	0.1	1773	16.92	3.38	8.49	1.85	140
		0.4	969	30.97	6.19	2.01	1.12	680
		1	628	47.78	9.56	0.72	0.45	1580

[a] Resolution of imipramine was calculated from desipramine.

source inlet volume. Additionally, the response is highly dependent on source conditions and could fluctuate through the flow range tested. Therefore, the plots were constructed separately using data produced by UV (254 nm wavelength) and ESI (Turboionspray) MS/MS detection for each column and with each mobile phase condition. An injection volume of 3 and 10 μl for MS/MS and UV detection, respectively, was used. Fig. 2 shows the van Deemter plots of imipramine using UV (top) and mass spectrometric (bottom) detection with mobile phase contained morpholine (Fig. 2A and B) and TEA (Fig. 2C and D).

In the morpholine case (Fig. 2A and B) the minimum plate heights were approximately the same for UV and MS/MS detection for each column. The overall efficiency was increased by approximately 1.5-fold in the UV case and 1.25-fold in the MS/MS case for the fused-core particle column versus the porous particle column. A slight difference in optimized flow rate at the van Deemter minimum (point of maximized efficiency, N) is observed at 1 mm/s (0.2 ml/min) versus 0.5 mm/s (0.1 ml/min) for UV detection and MS detection, respectively. In comparing the plot trends, they are found to be similar over the entire range of flow rate for both the 2.7 μm fused-core particle column and 5 μm porous particle Luna C_{18} (2) columns. However the slopes of the C-term dominated portion of the curves are notably different between UV and MS plots, with the UV data predicting greater efficiency loss as flow rate is increased beyond the optimal linear velocity for both column types. Thus the MS data is less reliable for prediction of the column efficiency, as compared with UV, at higher flow rates. When operating at flow rates near the optimized rate, the MS/MS evaluation serves as a reasonable detection mode for conducting the efficiency evaluation.

In the case of TEA added to the mobile phase (Fig. 2C and D) the optimized linear velocity for UV and MS/MS detection are matched at 0.5 mm/s (0.1 ml/min). However, unlike the morpholine case, the predicted efficiency gain with the fused-core particles shows some disparity at 1.7-fold for UV and 2.8-fold for MS/MS. The trend between UV and MS/MS detection also did not match and the MS data suggest only nominal loss of efficiency with increasing flow rate. In this case, MS/MS detection does not serve as a reasonable detection mode for assessing column performance and would be unreliable for predicting true column performance and efficiency. Further experiments using TEA showed the MS response to imipramine as highly sensitive to source conditions and required optimization at each flow rate (unpublished data). In an overall comparison between bases and particle types, the UV data show the 2.7 μm fused-core particle with TEA provided optimal N (plate height of approximately 15) at 0.5 mm/s (0.1 ml/min).

Chromatographic parameters other than N and H can be calculated to assess the performance of the fused-core particle columns against that of the porous particle columns. The calculated parameter resolution (R_s) is defined as

$$R_s = \frac{2[(t_R)_B - (t_R)_A]}{(w_A + w_B)}$$

where $(t_R)_A$ and $(t_R)_B$ are the retention time of desipramine and imipramine, and w_A and w_B are the peak width of desipramine and imipramine.

The retention parameter k' is defined as

$$k' = \frac{(t_R - t_M)}{t_M}$$

where t_R and t_M are the retention times of the test compound and the dead time of mobile phase. The reduced plate height h is defined as

$$h = \frac{H}{d_p}$$

and is usually plotted versus reduced linear velocity to yield a Knox plot [37]. The reduced plate height adjusts the observed plate height by normalizing by the particle size (dp). All of the chromatographic

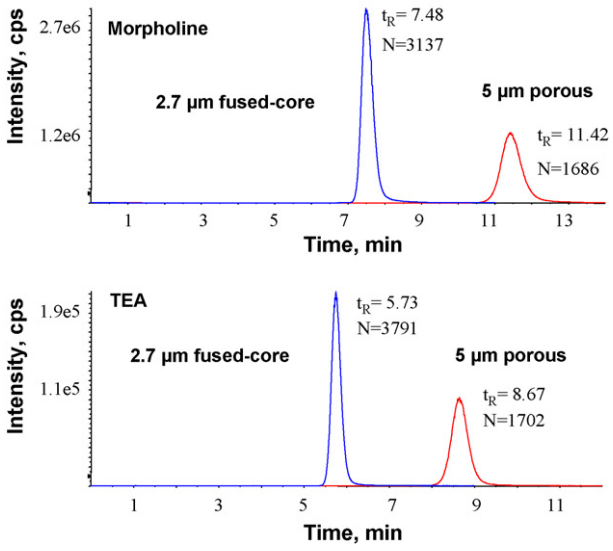

Fig. 3. Chromatographic performance: overlay of 2.7 μm fused-core particle and 5 μm porous particle columns with morpholine and TEA mobile phases and MS detection.

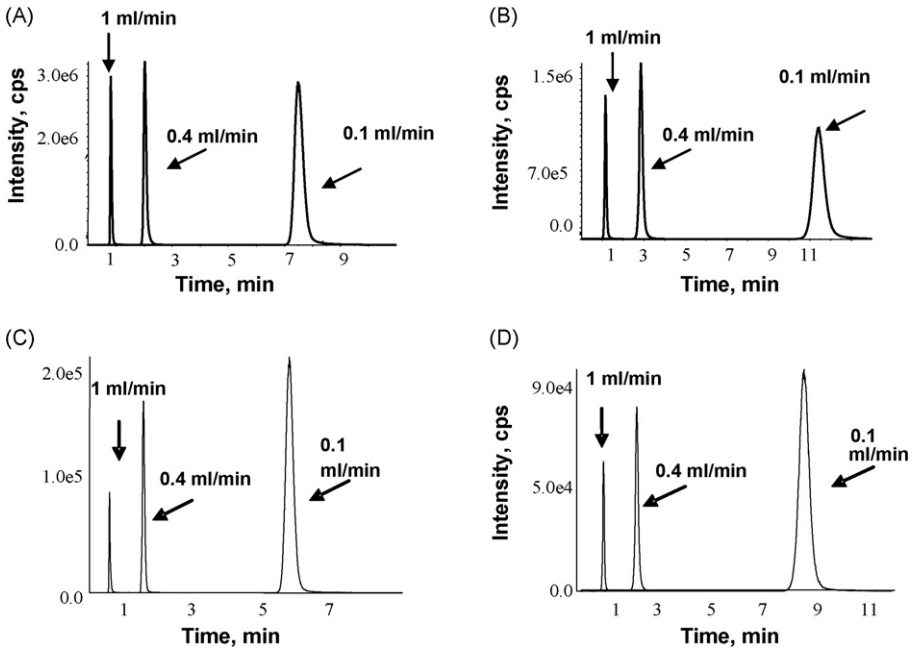

Fig. 4. Chromatographic overlay of imipramine at three flow rates in a 2.7 μm fused-core particle column and a 5 μm porous particle column using morpholine and TEA mobile phase with MS detection: (A) 2.7 μm fused-core/morpholine; (B) 5 μm porous/morpholine; (C) 2.7 μm fused-core/TEA; (D) 5 μm porous/TEA.

parameters are shown in Table 1 using MS/MS data for both morpholine and TEA at flow rates of 0.1, 0.4, and 1.0 ml/min.

A comparison of the 2.7 μm fused-core column with the 5 μm porous C_{18}(2) column, shows that N increases approximately 2–3-fold for both mobile phases at all flow rates. Overall resolution of imipramine from desipramine was highest (at approximately 2) at an optimized linear flow rate (0.1 ml/min) when using morpholine and the fused-core 2.7 μm particles. Back-pressures for the fused-core columns were moderate (less than 3400 psi at a 1.0 ml/min flow rate) and allowed the use of a conventional HPLC system (typical pressure limits of 6000 psi). The reduced plate height (h) showed no significant difference between the fused-core and 5 μm porous C_{18}(2) columns when using morpholine while h decreased for the fused-core column when using TEA as mobile phase. Although increased retention was observed for the 5 μm column as evidenced by the higher k' values, this was not considered an advantage due

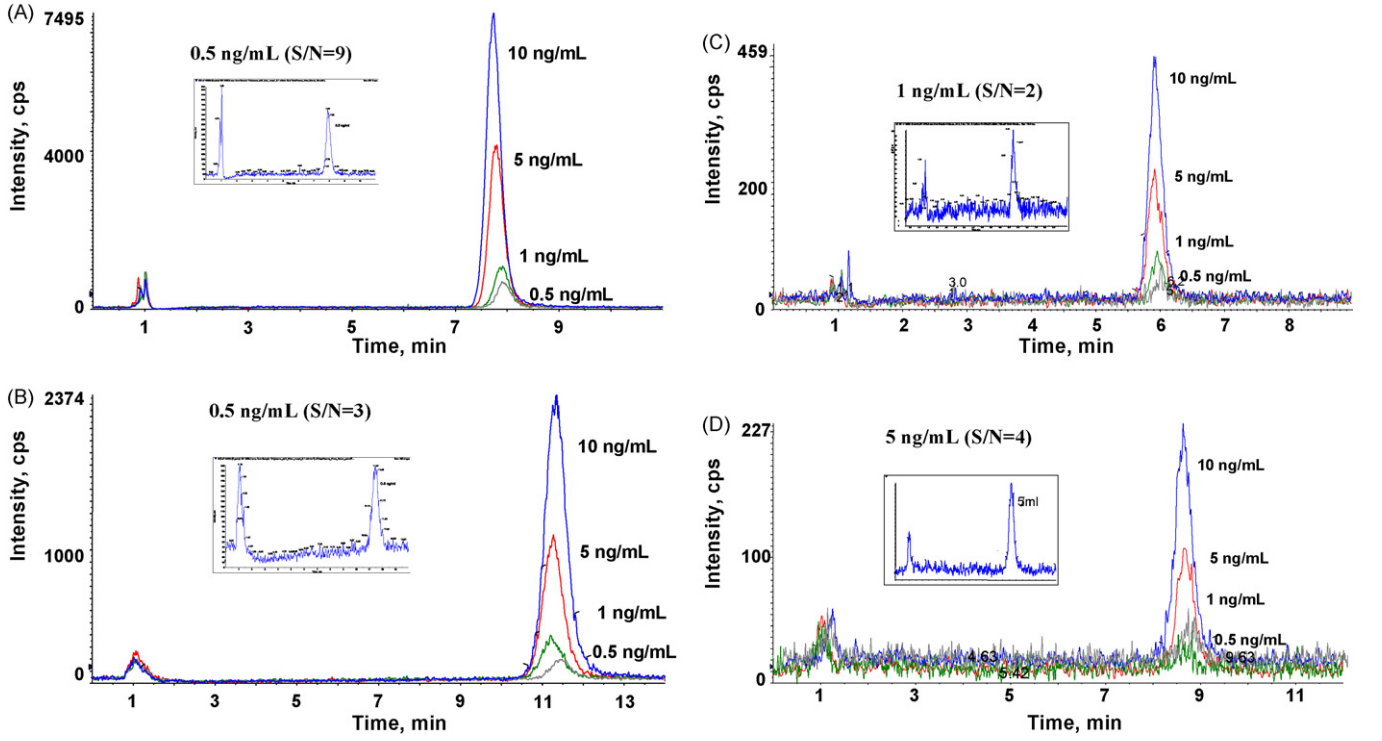

Fig. 5. LOD investigation in matrix samples: overlay of four concentration injections using 2.7 μm fused-core and 5 μm porous particle columns with morpholine and TEA mobile phases and MS detection: (A) 2.7 μm fused-core/morpholine; (B) 5 μm porous/morpholine; (C) 2.7 μm fused-core/TEA; (D) 5 μm porous/TEA. Insets show lowest concentration with S/N nearest 3.

Table 2
S/N ratio comparison between 2.7 μm fused-core and 5 μm Porous porous particle columns in the presence of morpholine and TEA.

Flow rate (ml/min)	Concentration (ng/ml)	Morpholine				TEA		
		2.7 μm fused-core	5 μm porous	S/N ratio between 2.7 μm fused-core and 5 μm porous	Mean S/N ratio between 2.7 μm fused-core and 5 μm porous	2.7 μm fused-core	5 μm porous	S/N ratio between 2.7 μm fused-core and 5 μm porous
0.1	0.5	8	3	2.7	3.0	ND	ND	NA
	1	11	4	2.8		2	ND	NA
	5	43	13	3.3		6	4.12	1.5
	10	76	24	3.2		10	6.33	1.6
0.4	0.5	6	2	3	2.7	ND	ND	NA
	1	10	3.5	2.9		ND	ND	NA
	5	49	19	2.6		3.57	2.3	1.6
	10	82	36	2.3		5.84	3.17	1.8
1.0	0.5	5	1.5	3.3	3.7	ND	ND	NA
	1	8	2.2	3.6		ND	ND	NA
	5	30	8	3.8		1.2	ND	NA
	10	59	15	3.9		2.9	1.77	1.6

to the accompanying increased band broadening (loss of efficiency) and longer cycle time with the 5 μm column.

3.2.2. MS/MS chromatograms of imipramine extracted from rat plasma

Aliquots of extracted rat plasma samples prepared following the procedure described in Section 2.4 were injected onto the 2.7 μm fused-core and 5 μm porous columns, eluted using the optimized linear flow rate of 0.1 ml/min, and analyzed by MS/MS. Fig. 3 shows overlaid chromatograms of the imipramine MRM transition from samples fortified at 5.0 μg/ml using the 2.7 μm fused-core and 5 μm porous columns. Comparing the fused-core 2.7 μm particle column to the porous 5 μm particle column performance, the retention times decreased by approximately 34% for both mobile phases, the number of theoretical plates (N) increased 1.9–2.2-fold, and peak intensity (cps) increased approximately 2–3-fold. Also, the overall response for morpholine was approximately 14× higher when compared to TEA, suggesting ionization suppression of the imiprimine response in the Turboionspray™ MS source with TEA.

Overlaid chromatograms of the same injected 5.0 μg/ml extracted sample for morpholine and TEA show the band dispersion and varying response observed with increasing flow rate from 0.1 ml/min to 1 ml/min (Fig. 4). As suggested by the disparity in TEA van Deemter curves (Fig. 2C and D), the TEA response (intensity, cps) appears to suffer from lack of source condition optimization and the signal degrades significantly with increased flow rate. Such response variability was not a significant factor when morpholine was used, as suggested by the similarity in van Deemter between UV and MS/MS (Fig. 2A and B), and a flow rate of 0.4 ml/min yields the best overall response for imipramine with morpholine in the mobile phase.

3.3. Limit of detection (LOD) investigation of matrix samples

To investigate the response near the limit of detection (LOD), rat plasma samples were prepared at 0.5, 1.0, 5.0, and 10 ng/ml

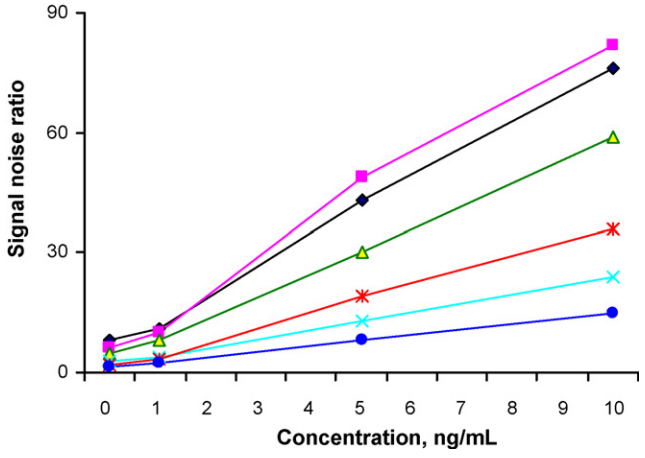

Fig. 6. Effect of the particle size and flow rate on the signal-to-noise ratio in morpholine mobile phase. 2.7 μm fused-core particle 2.1 mm × 30 mm column at (♦) 0.1 ml/min; (■) 0.4 ml/min, and (Δ) 1.0 ml/min flow rate; 5 μm porous particle C_{18} (2) 2.0 mm × 30 mm column at (X) 0.1 ml/min, (Ж) 0.4 ml/min, and (●) 1.0 ml/min.

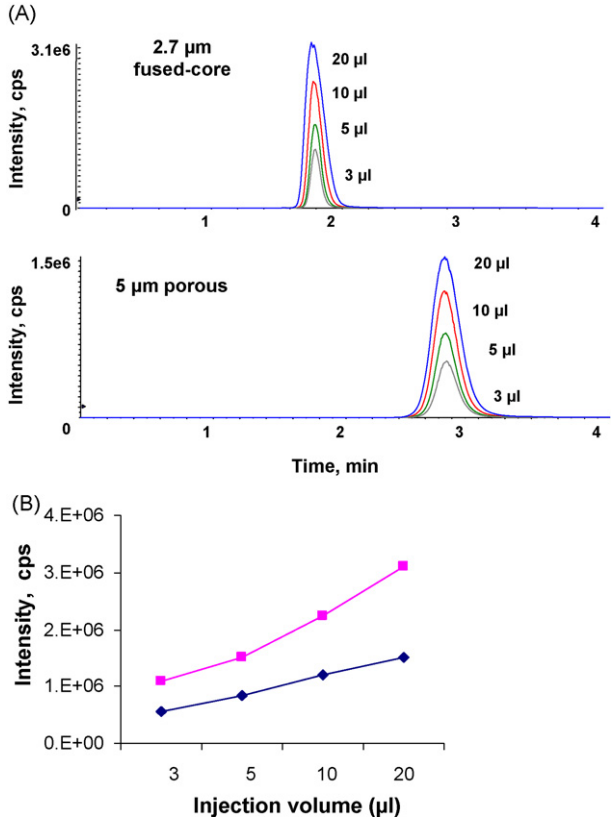

Fig. 7. Comparison of loading capacities between 2.7 μm fused-core and 5 μm porous particle columns with morpholine: (A) overlay of chromatograms with different injection volumes and (B) peak intensity versus injection volume (top line: 2.7 μm fused-core particle column; bottom line: 5 μm porous particle column).

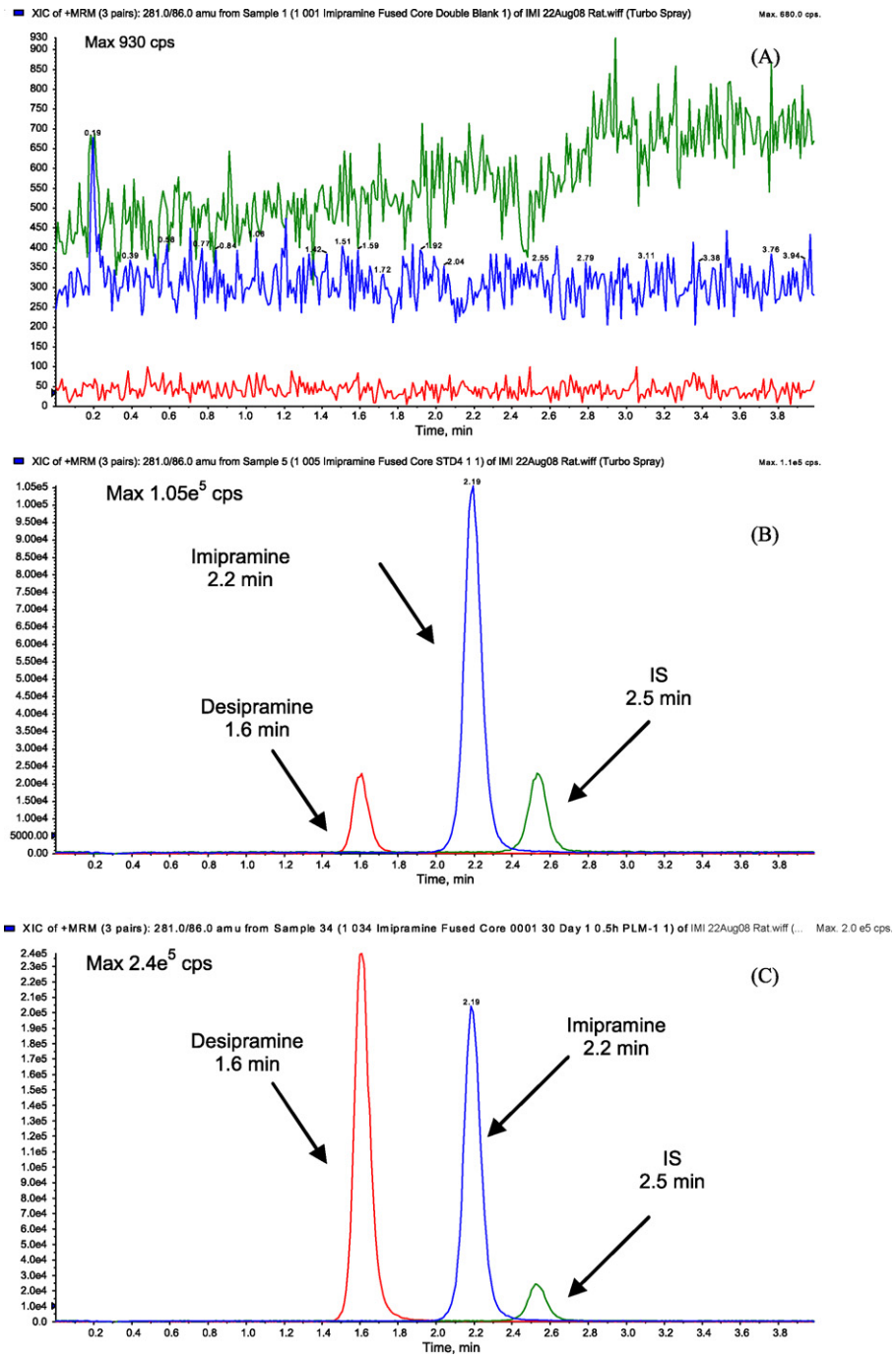

Fig. 8. Typical chromatograms of (A) blank plasma; (B) plasma spiked with imipramine, desipramine and amitriptyline (IS) at 50 ng/ml; (C) plasma sample for Rat 1 at 0.5 h post-oral dose of 30.0 mg/kg imipramine [desipramine (red), imipramine (blue); and amitriptyline (green)] using fused-core particle column and morpholine mobile phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

imipramine and were extracted using the simple protein precipitation method previously described (Section 2.4). A 3 μl aliquot of the supernatant was injected onto the 2.7 μm fused-core particle and 5 μm porous particle C_{18}(2) columns, respectively, using morpholine and TEA in the mobile phases. The compound was eluted using the optimized flow rate of 0.1 ml/min and analyzed by MS/MS. In Fig. 5 the chromatograms of the four concentration levels of plasma samples are overlaid for each column and each mobile phase condition. The concentration most closely approximating a reasonable LOD of approximately 3× the signal-to-noise (S/N) is shown in the insert. While in all cases the S/N increased with increasing sample concentration, the S/N of the 2.7 μm fused-core column was greater at all concentrations when compared to the 5 μm porous C_{18}(2) column under both mobile phase conditions. When TEA was used in the mobile phase (Fig. 5C and D), a lower response was observed, potentially due to ionization suppression, and the approximate LOD was raised to 1 and 5 ng/ml for the 2.7 μm fused-core and 5 μm porous columns, respectively. This compares with the morpholine case (Fig. 5A and B) where the approximate LODs of 0.5 ng/ml were realized for both particles, effectively lowering the LOD by 2–10-fold.

The S/N for the four concentrations tested near the LOD at flow rates 0.1, 0.4, and 1.0 ml/min were calculated and are summarized in Table 2. In all cases, the S/N for the same concentration measured

Table 3
Precision and accuracy of QCs using fused-core particle column.

Concentration (ng/ml)		Intra-assay	
Nominal	Mean measured (n = 6)	Precision (%CV)	Accuracy (%Bias)
Imipramine			
0.5	0.562	2.5	12.4
1.5	1.45	3	−3.3
20	20.1	1.5	0.5
750	766	1.5	2.1
Desipramine			
0.5	0.572	14.7	14.4
1.5	1.39	7.8	−7.3
20	20	2.8	0.0
750	781	1.4	4.1

%CV = SD/mean × 100.
%Bias = (mean measured − nominal)/nominal × 100.

on the 2.7 μm fused-core particle column was higher than for the 5 μm porous column. When the flow rate was increased beyond the optimized linear flow rate (0.1 ml/min), the S/N for TEA degraded significantly and effectively raised the LOD. The morpholine data, in contrast, showed better response near the LOD and S/N degradation is less pronounced with increasing flow rate. At high flow rates in excess of the optimized linear velocity, the improved mass transfer properties of the porous particles should allow for greater efficiency and S/N relative to the 5 μm particle column. This is suggested in the morpholine data by the increase in the mean ratio of S/N (2.7 to 5 μm) from approximately 3 for 0.1 and 0.4 ml/min to 3.7 for 1.0 ml/min. A plot of the morpholine S/N data with increasing concentration, Fig. 6, shows the maximum response is obtained at 0.4 ml/min for both columns. Although the raw intensity (cps) of the peaks are optimal at this flow rate, the quality of the separation, as determined by maximum N, is not optimal at this flow rate, but rather at 0.1 ml/min (Fig. 4A and B).

Based on this LOD data, morpholine was selected as the mobile phase base modifier for the loading capacity test and rat PK study because of its higher intensity and increased S/N near the LOD. A flow rate at 0.4 ml/min, though not the optimized flow rate, provided adequate peak response and was considered to have a practical cycle time for routine application.

3.4. Loading capacity comparison

To evaluate column loading capacities, an extract of 1.0 μg/ml imipramine in rat plasma was injected onto a 25 μl sample loop with varying injection volumes. The compound was loaded onto the 2.7 μm fused-core particle and 5 μm porous particle C_{18}(2) columns using the morpholine mobile phase system at a flow rate of 0.4 ml/min. Fig. 7A presents the overlaid chromatograms for injection volumes of 3, 5, 10, and 20 μl on each column separately. No peak distortion was observed with the increase of injection volume for either column. The peak intensity (cps) versus injection volume for each column plotted in Fig. 7B shows increasing response with increasing injection volume for both columns. The overall higher response profile observed for the 2.7 μm fused-core particle column is attributable to the reduced band broadening as compared with the 5 μm porous column.

3.5. Method qualification

A LC–MS/MS method for the simultaneous determination of imipramine and desipramine in K_3EDTA rat plasma was developed and tested using the 2.7 μm fused-core column with morpholine as the organic base mobile phase modifier. Prior to analysis of rat PK study samples, a single method qualification run was conducted to assess the method performance.

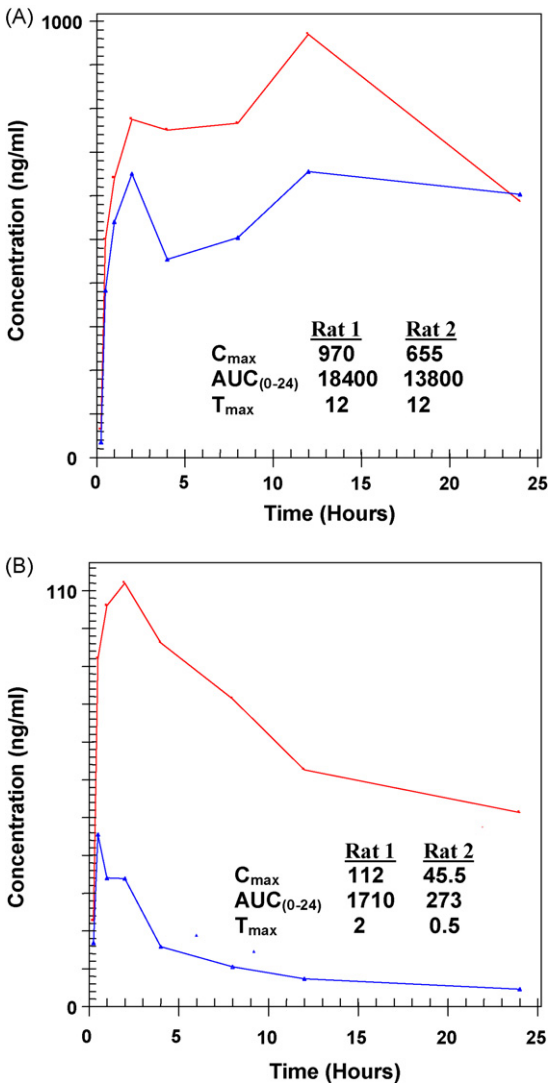

Fig. 9. Desipramine (A) and imipramine (B) individual plasma concentrations (ng/ml) versus time (h) after oral administration of 30 mg/kg imipramine to male rats ((■) rat 1, (▲) rat 2). Inserted data of C_{max} (ng/ml), $AUC_{(0-24)}$ (ng/ml h), and T_{max} (h).

3.5.1. Specificity and selectivity

Fig. 8 shows typical chromatograms of extracted blank control rat plasma, a plasma standard spiked with imipramine, desipramine, and a rat plasma sample from the oral PK study. The retention times of desipramine, imipramine, and the IS were 1.6, 2.2, and 2.5 min, respectively. No interferences in the MRM traces for imipramine, desipramine, or the IS were observed from endogenous substances in any of the three blank control rat plasma lots tested.

3.5.2. Calibration curve

Calibration curves were characterized over the range of 0.5–1000 ng/ml for both imipramine and desipramine using quadratic (1/x) regression. The r^2 for imipramine and desipramine were 0.9996 and 0.9997, respectively, showing good response and fit for both compounds. The back-calculated concentrations and their accuracy measured as %Bias from the nominal value for each standard were within ±15%.

3.5.3. Accuracy and precision

The precision and accuracy of the assay were assessed by the analysis of six replicates at each of the four QC levels. The QC results,

including the LOQ at 0.5 ng/ml, are summarized in Table 3 and show the precision (%CV) and accuracy (%Bias) values were within the acceptable range (±15%, ±20% at the LOQ) and the method was considered acceptable for sample analysis.

3.6. Pharmacokinetic study

Using the qualified method, rat plasma samples from the oral PK study were analyzed to generate concentration versus time profiles for imipramine and desipramine (Fig. 9). The maximum plasma concentration (C_{max}) with associated time (T_{max}) and $AUC_{(0-24)}$ (the area under the plasma concentration–time curve from 0 to 24 h) of imipramine and desipramine are also shown in Fig. 9 (see insert). These data correlate with prior published concentration–time profiles and exposure data [38,39].

4. Conclusions

Increasing the speed of analysis while maintaining or improving chromatographic resolution and assay robustness remain significant challenges in the bioanalysis of complex matrix samples. Decreasing particle size is one common strategy to increase speed and/or column efficiency as seen in reports of many methods and applications using sub-2 μm porous particles. However, the increased system back-pressure associated with such techniques requires specialized HPLC instrumentation. The introduction of fused-core particles provides another tool for consideration in terms of assay performance improvement which does not require specialized high pressure systems. In the work presented here, all pressures remained below 3400 psi. The 2.7 μm particle fused-core column showed efficiencies and overall van Deemter kinetics similar to previously published results for naphthalene with UV detection. Application to a more realistic scenario in the quantitation of imipramine/desipramine in rat PK study samples was successfully demonstrated in this work.

Prior to the analysis, performance characteristics were evaluated. In a direct comparison of the 2.7 μm particle fused-core column versus the 5 μm fully porous particle column, the efficiency was increased by 2–3-fold, retention (*i.e.*, cycle time) was decreased 34%, and a 3–4-fold increase in S/N was observed. The data further suggest that the overall loss of column efficiency for the 2.7 μm particle fused-core column is less severe at flow rates in excess of the optimal linear flow rate so cycle times could be further reduced through a higher flow rate. More significant to the application, these performance advantages allowed for a lower concentration for the LOQ of 0.5 ng/ml in the study assay of the PK study samples despite the "dirty" nature of the rat protein precipitated extracts.

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