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Ultra-short columns and ballistic gradients: considerations for ultra-fast chromatographic liquid chromatographic–tandem mass spectrometric analysis

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Abstract

Ultra-fast chromatographic separations has enabled fast chromatographic method development and rapid analysis for sample quantification. Decreasing over-all analytical time has become a factor of major importance for all aspects of drug discovery. However, merely decreasing chromatographic analysis time by decreasing k' can lead to inconsistent quantitative or qualitative results due to ineffective separations in complex matrices. We have found that by changing column length and gradient slope we can maintain chromatographic integrity of chemically diverse analytes and achieve the analytical speed required for bioanalytical drug discovery quantitative analysis. We have optimized method development strategy by performing separations on 2×20 mm HPLC columns at flow-rates of 1.5 ml/min to 2 ml/min with full linear gradients achieved in 1 min for the quantification of pharmaceuticals and their metabolites from biological matrices. This method development strategy can be readily adapted to other matrices. This paper will discuss the effects of column length and gradient time in ultra-fast chromatographic resolution. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ultra-short columns; Ballistic gradients; Gradient elution; Column length

1. Introduction

Fast chromatographic methods in tandem with mass spectrometry detection are becoming more prevalent for sample analysis in drug discovery [1–3]. This technique can be used for the determination of compounds in biological matrices, for the estimation of purity, and the confirmation of compound structure in a combinatorial chemistry laboratory. Analytical run time is dependent upon the retention characteristics of the analyte, mobile phase flow-rate, and column length. Decreasing analytical run time by the reduction of retention (k') can compromise resolution and the chromatographic integrity of the analysis. Increasing the flow-rate can result in a faster analysis but can result in prohibitive back pressure effects. However, increasing the flow-rate on short (50 mm or less) columns will decrease analysis time without a subsequent prohibitive increase in back pressure. Decreasing analytical run time also may be effected by decreasing column length which will cause a change in retention without

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changing selectivity, but can also cause a decrease in efficiency.

We have previously described LC–MS/MS methods that have been developed for quantification using rapid (''ballistic'') gradients on narrow bore, short HPLC columns (2×20 mm) that have resulted in 2 min or less analytical cycle times while maintaining chromatographic integrity [4–6]. This paper describes the ultra-fast gradient approach and the effects of reducing either column length or gradient time in order to reduce analytical run time without sacrificing chromatographic resolution. The data presented are derived from the use of a test mixture containing amitriptyline, diclofenac, enoxacin, fenofibrate, finasteride, indinavir, MK-869, pioglitazone and raloxifene. The choice of components in the test mixture were based on their physicochemical properties, namely their acidic, basic, and neutral characteristics, and also to ensure a distribution across the molecular mass range typically found in pharmaceutical analysis. All compounds within the mixture were monitored using positive ion detection. This test mixture was used to compare retention time, peak width and resolution at different gradient times on different column lengths. The effect of particle size (5 μm and 3 μm) on resolution and analysis time was also compared. Separations were performed on DASH BetaBasic C8, (5 and 3 μm) columns in 2×20, 2×10, and 2×5 mm configurations (ThermoHypersil Keystone Scientific Operations, Bellefonte, PA).

2. Experimental

2.1. HPLC Conditions

The HPLC system used was a Perkin-Elmer Series 200 system with two micro pumps and an autosampler fitted with a 20 μl injection loop. High pressure static mixing was done with a 250 μl mixing chamber. All HPLC tubing used was 0.005″ PEEK with fingertight PEEK fittings. The column eluent was split 1:5 into the MS using an Accurate flow diverter (LC Packings, San Francisco, CA).

The mobile phase used was A (95% H_2O/5% acetonitrile, v/v) and B (95% acetonitrile/5% H_2O, v/v). Both reservoirs contained 0.1% formic acid as a modifier.

Gradients were identical (starting concentration, initial hold, final concentration, return to initial conditions, and re-equilibration) except for the length of the linear slope. Linear velocity was held constant on all column lengths and gradient conditions.

2.2. MS Conditions

An API-3000 mass spectrometer (PE-SCIEX, Concord, Ontario, Canada) was used to obtain the LC–MS/MS data. All analyses were performed using the Turbo Ionspray source with typical settings as follows: nebuliser gas 7, curtain gas 10, CAD gas 4, electrospray voltage 4500 V, ring voltage 290 V, orifice voltage 60 V, and a drying gas temperature of 400°C.

Mass spectrometer conditions were optimized for the entire test mixture and MRM (Multiple Reaction Monitoring) transitions and collision energies were optimized for each analyte. Mass spectrometer cycle times were optimized to ensure a minimum of 12 data points across each component peak.

2.3. Test mixture

A solution containing a mixture of equal concentrations of finasteride, indinavir, MK-869 (Merck and Co., Rahway, NJ); amitriptyline, diclofenac, enoxacin, and fenofibrate (Sigma, St. Louis, MO); pioglitazone and raloxifene (Eli Lilly, Indianapolis, IN) was prepared in 90% H_2O/10% methanol (v/v) for analysis. The structures of the components of the test mixture and the MRM transitions used in the analysis are given in Table 1.

3. Results and discussion

The components of the test mixture eluted in the following order: (1) enoxacin, (2) pioglitazone, (3) indinavir, (4) raloxifene, (5) amitriptyline, (6) finasteride, (7) diclofenac, (8) MK-869 and (9) fenofibrate (Figs. 5 and 6). The flow-rate (1.5 ml/min), injection volume (1 μl), and the initial gradient hold time (0.2 min) were held constant, varying only the linear portion of the gradient on 2×20 mm (5 and 3

Table 1
Physical and analytical parameters of test analytes

Elution order	Analyte	Structure	MRM transitions
1	Enoxacin		321.2→234
2	Pioglitazone		357.2→134
3	Indinavir	Chiral	614.4→421
4	Raloxifene		474.1→112
5	Amitriptyline		278.3→233
6	Finasteride		373.2→317
7	Diclofenac		296.1→215

Table 1. Continued

Elution order	Analyte	Structure	MRM transitions
8	MK-869	F F F Chiral O O F F F N O N N N F	535.3→277
9	Fenofibrate	O O O O Cl	360.9→233

μm), 2×10 mm (5 and 3 μm), 2×5 mm (5 and 3 μm) DASH BetaBasic C8 HPLC columns as follows: 1.5 min, 1.0 min, 0.8 min, 0.5 min and 0.3 min.

System back pressures were less than 3000 p.s.i. regardless of particle size or column length at a flow-rate of 1.5 ml/min as shown in Table 2. The back pressure decreased with column length and increased with particle size as expected [7]. The back pressure was well within normal operating pressure limits of the HPLC pump and fingertight PEEK fittings.

In order to evaluate the effect of gradient time (t_G) on the overall chromatographic integrity of the analysis the following parameters were determined: k* (average value of k, sample retention, during gradient elution) Formula 1 [9]; G_s (gradient steepness parameter), Formula 2 [9]; and N (theoretical plates or column efficiency), Formulas 3a and 3b [8]. Experimental t_0 was determined by the injection of a non-retained salt solution under gradient conditions, on all column lengths. Experimental t_0 was found to be 0.10 min for the 2×20 mm column and 0.08 min for both the 2×10 and 2×5 mm columns. The system volume (time in the absence of a column) was determined to be 0.05 min. The duty cycle of the instrument and the volume of the system in relation to actual column volume indicate that experimental t_0 cannot be accurately measured. In order to estimate chromatographic parameters and to illustrate the differences between the varying column lengths, theoretical t_0, Formula 4 [9], was used in all calculations. All relevant formulas can be found in the literature cited and are shown in Table 3 [8,9].

Table 2
System back pressure (p.s.i.)

Particle size (μm)	Column dimensions (mm)		
	2×20	2×10	2×5
5	1400	1000	800
3	2600	1500	1100

It is known that varying organic concentration in an isocratic separation will cause k and α (selectivity) to change [9]. During gradient elution, changes to k and α are effected by changing gradient steepness (*Gs*). *Gs* is the defined as the % per min change in B per column volume of mobile phase [9]. As gradient steepness decreases the number of peaks that are resolved increases; this increase in resolution is accompanied by both an increase in peak width and an increase in run time. An increase in %/min for gradient conditions is equivalent to an increase in organic concentration for isocratic conditions, therefore, an increase in k* (gradient) is equivalent to an increase in k' (isocratic). Table 4 supports current theory by showing that with decreasing gradient time (t_G) and increasing *Gs* the efficiency of the column decreases, as reflected by decreasing N and k*.

To illustrate the resolution of the compounds with

Table 3
Gradient formulas

Formula 1:	$k^* = \frac{20t_G F}{Vm(\Delta\%B)}$	t_G = time of gradient Vm = column volume F = flow-rate $\Delta\%B$ = change in organic
Formula 2 (G_s or b):	$G_s = \frac{Vm(\Delta\%B)}{Ft_G}$	t_G = time of gradient Vm = column volume F = flow-rate $\Delta\%B$ = change in organic
Formula 3a:	$N = 16\left(\frac{(2.3b+1)Gt_0}{2.3bW}\right)^2$	G = gradient compression factor b = gradient slope W = peak width $t_0 = t_R$ for non-retained components
Formula 3b:	$G^2 = \frac{1+p+\frac{p^2}{3}}{(1+p)^2}$	(where $p = 2.3b$)
Formula 4:	$t_0 = \frac{Vm}{F}$	Vm = column volume F = flow-rate

changing gradient time, retention time is plotted as a function of gradient time as shown in Figs. 1–3. These figures indicate that enoxacin is inadequately retained on both 2×5 mm and 2×10 mm columns. Peak widths were found, in most cases, to be 0.03 to 0.05 min wide at $W_{10\%}$ (peak width at 10%) regardless of column length, particle size, gradient time or steepness; and all peaks were symmetrical based on asymmetry factors.

The retention characteristics of the first eluting peak (enoxacin) and the last eluting peak (fenofibrate) were most affected by changes in column length and gradient steepness. Enoxacin was present in all chromatograms, but demonstrated dramatic band broadening as the column length decreased and the gradient steepness increased characteristic of elution in the solvent front. As gradient steepness increased, regardless of column length or particle size, fenofibrate was not detected in the mass chromatogram. There was no evidence of fenofibrate co-elution with another analyte, separation is specific and selective with the use of MRM transitions. Fenofibrate is the most lipophilic analyte in the test mixture and is strongly retained so this analyte may be eluting in subsequent chromatograms. Very broad well-retained peaks may not be readily detectable in a short run time mass chromatogram.

Changes in selectivity (α) with the increased Gs are more pronounced on the longer column affecting the retention of both fenofibrate and MK-869, the last two eluting peaks. On a 2×20 mm column, a t_G of 0.8 or greater affords adequate retention for all

Table 4
Chromatographic parameters

t_G (min)	k^*	G_s			N		
		2×20 mm	2×10 mm	2×5 mm	2×20 mm	2×10 mm	2×5 mm
1.5	12.5	1.6	0.8	0.4	2200	1900	1800
1.0	8.3	2.4	1.2	0.6	1200	900	800
0.8	6.7	3.0	1.5	0.8	670	400	530
0.5	4.1	4.8	2.4	1.2	300	250	250
0.3	2.5	8.0	4.0	2.0	140	150	70

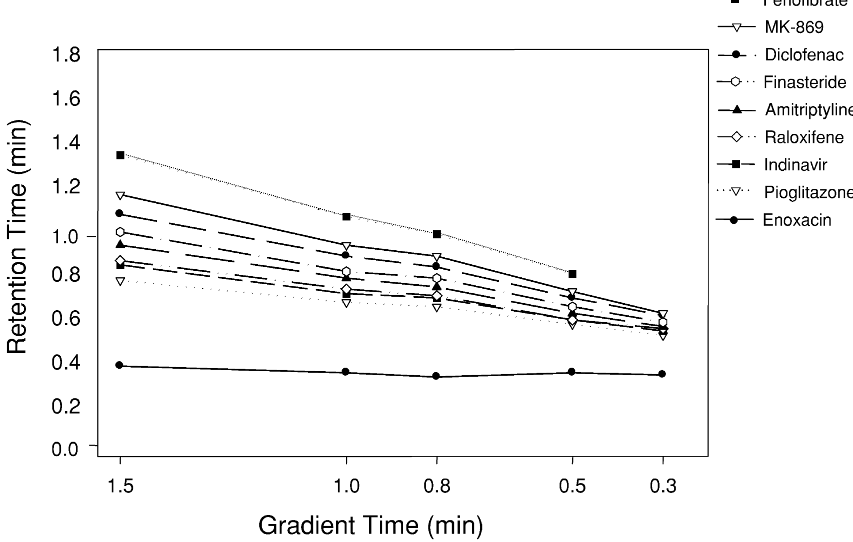

Fig. 1. Retention time vs. gradient time on 2×5 mm, 5 μm HPLC column.

compounds in this mixture. In addition, the greatest resolution is achieved at a t_G of 1.5.

Fig. 4 illustrates a comparison of data obtained using columns containing 3 and 5 μm particle size packing material (2×20 mm, 1.5 ml/min, t_G=1.5 min, Gs=1.6). Components 3 and 4 (indinavir and raloxifene, respectively) are resolved more effectively and the overall run time is shorter using the 5 μm column.

Fig. 5 shows five chromatograms obtained by varying only t_G with the subsequent changes in Gs. Gradient times were decreased from 1.5 min to 1.0, 0.8, 0.5 and 0.3 min. As the gradient steepness increases, the retention time decreases, and the

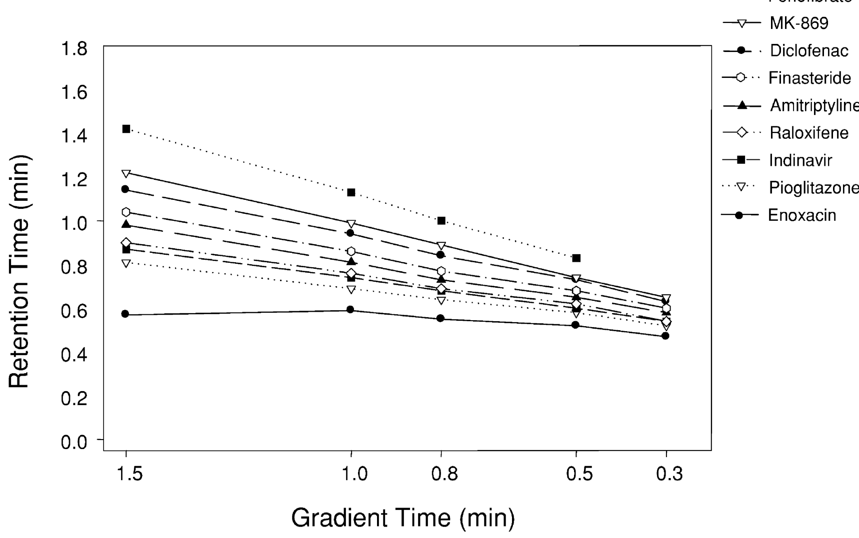

Fig. 2. Retention time vs. gradient time on 2×10 mm, 5 μm HPLC column.

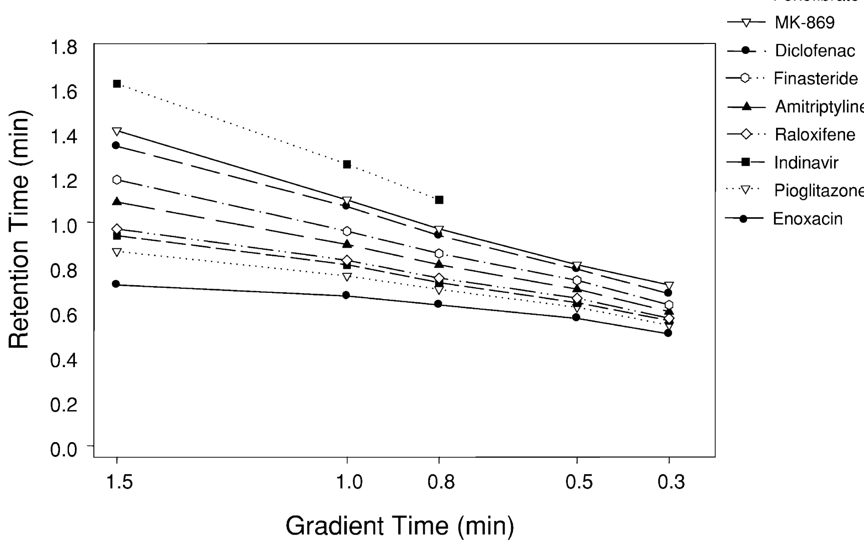

Fig. 3. Retention time vs. gradient time on 2×20 mm, 5 μm HPLC column.

resolution between peaks decreases. Peaks 1 and 8 (enoxacin and MK-869, respectively) afforded increased peak widths at t_G of 0.3 min (Gs of 8.0), demonstrating changes in α with gradient steepness.

We have demonstrated that changing gradient steepness in gradient elution may be the most effective way of changing selectivity (k^* and α) thereby decreasing overall run time. However, other factors, such as sample matrix, need to be considered. Chromatographic resolution becomes a primary factor of concern with samples in complex mixtures or samples containing salts, metabolites or other endogenous and exogenous material in the matrix. Decreasing analytical run time by decreasing gradient time will subsequently decrease resolution and compromise the analysis of complex mixtures. The presence of salts, isobaric components, and other endogenous material results in matrix effects that can cause ion suppression and may induce analytical variability, particularly if the matrix interferences are not reproducible from sample to sample [10–14].

Fig. 6 shows three chromatograms obtained using the same gradient time of 1.5 min where only the length of the column was varied. Resolution decreases between components 3 and 4 (indinavir and raloxifene, respectively) as column length decreases. Band width increases for component 1 (enoxacin) as column length decreases indicating elution in the solvent front. Overall run times decrease with decreasing column length, as evidenced by the shorter retention time of the last eluting peak (fenofibrate from 1.6 to 1.4 to 1.3 min). The run time decreases proportionally with decreasing column length, reflecting the decrease in residence time. Fenofibrate's retention time decreased from 1.6 to 1.4 min (12 s) by decreasing the column length by half (10 mm), and decreased again from 1.4 to 1.3 min (6 s) with another 5 mm reduction in length, a proportional decrease demonstrating that reduction of overall sample analysis time can be achieved easily by decreasing column length.

Extracolumn effects become a consideration with very short columns since the volume between the mixing chamber and the column has an important impact on fast gradient analysis and re-equilibration time. System volumes should be kept as low as possible, but still provide adequate mixing between the pumps and the column inlet. In order to minimize the effects of variable dwell volume on separation, consideration must be given to ensure that the sample and the gradient arrive at the column inlet at the same time by either using an initial isocratic step (0.2 min) or to inject the sample after the gradient has begun. We use this isocratic portion of the

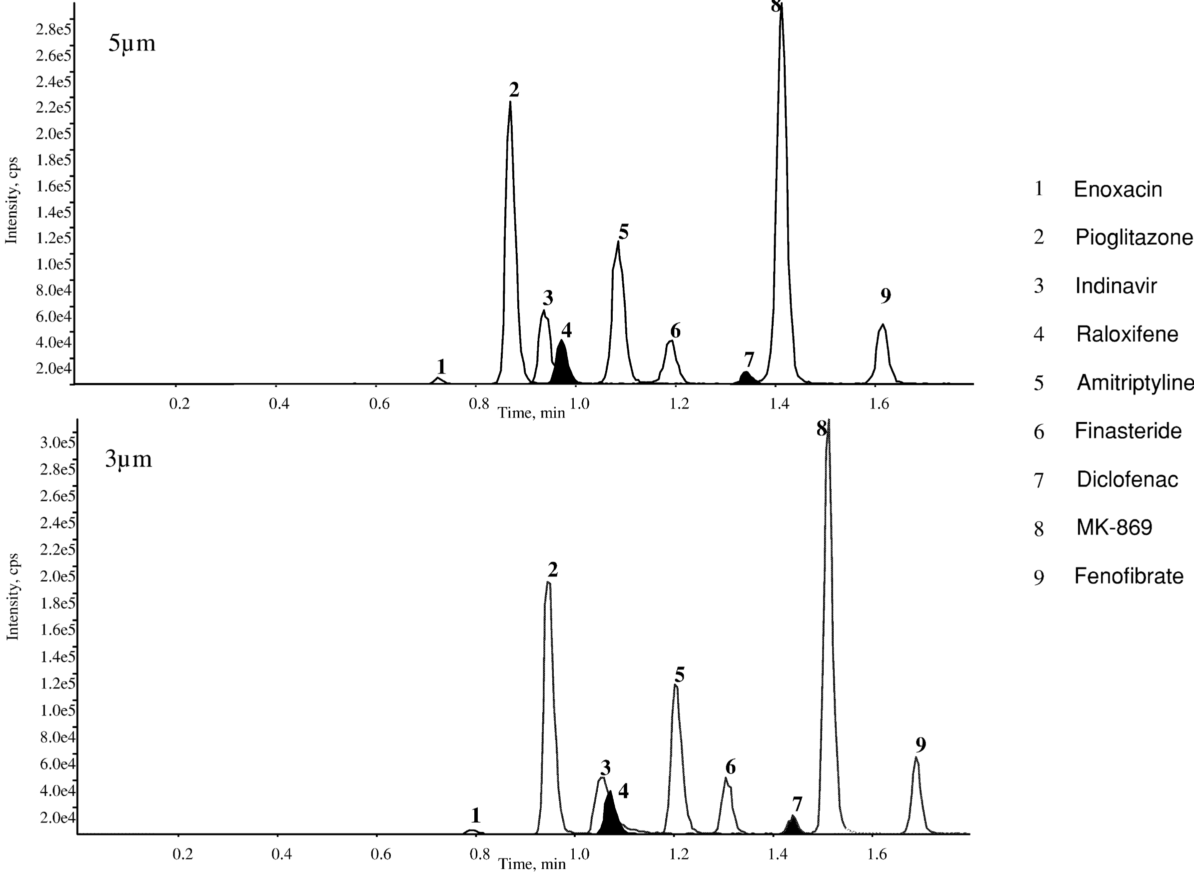

Fig. 4. Comparison of 5 μm and 3 μm on a 2×20 mm column with a 1.5 min gradient.

gradient profile to compensate between HPLC systems of varying dwell volumes by adjusting the initial isocratic time. We have minimized our system volumes by utilizing low-dead volume systems with high pressure, low volume mixing chambers (75 or 250 μl), by using small I.D. tubing (0.005″), zero dead-volume unions, and keeping sample path length as short as physically possible between the injector and the detector. In addition to extracolumn effects from the fluid path, extracolumn effects of the data acquisition system need to be considered. The sampling rate must be optimized to obtain enough samples across the chromatographic peak for accurate peak definition. Our HPLC instrumentation is standard and does not use any special fittings or system configurations, and our data acquisition parameters are set within the limitations of our mass spectrometer.

4. Conclusion

In order to effectively decrease sample analysis time while maintaining chromatographic resolution the column length and time of gradient should be optimized for k^*. If only column length is changed, the separation will be affected by N. We have observed changes in α with changing Gs. These changes in N and α are indicated by the elution and band characteristics of enoxacin, MK-869, and fenofibrate.

We have also observed that decreasing particle

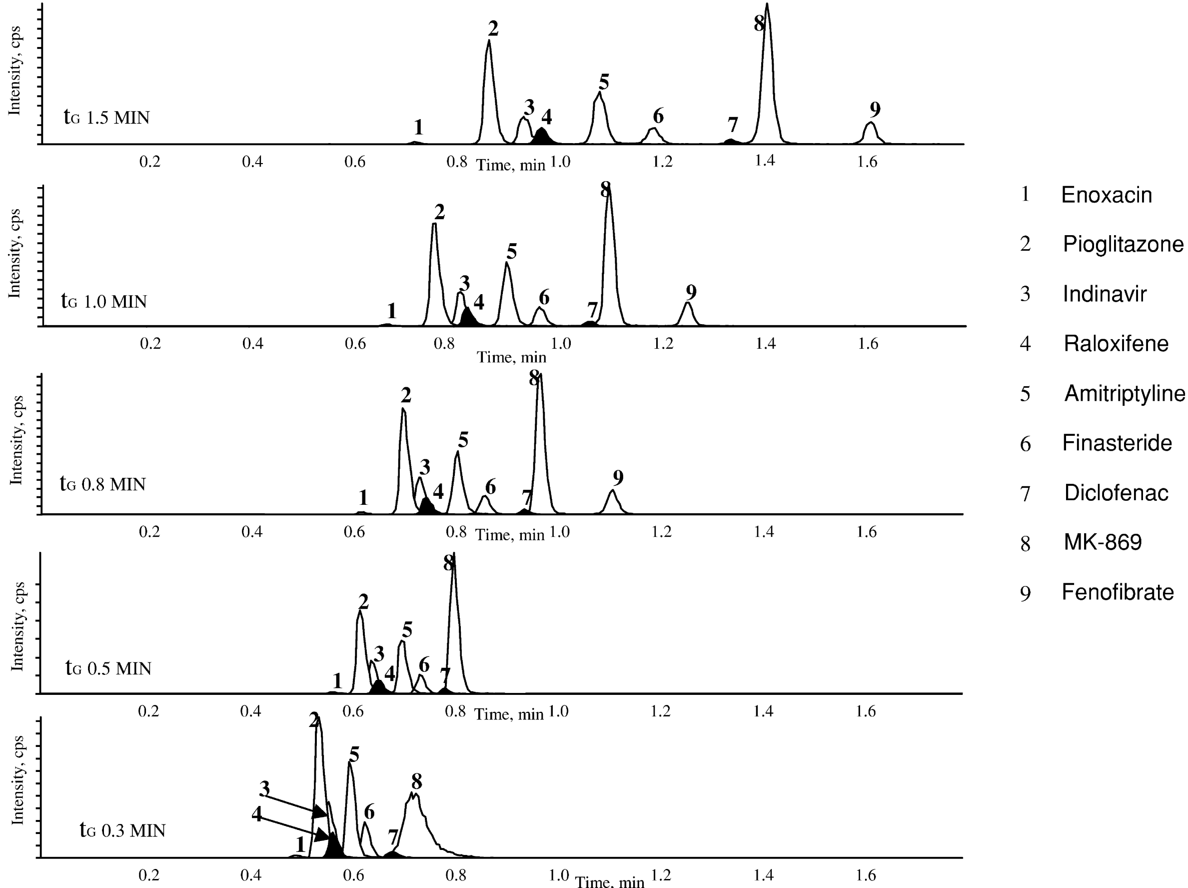

Fig. 5. Comparison of gradient times on a 5 μm, 2×20 mm column.

size with decreasing column length may not increase resolution as would be expected. No observable differences between the two particle sizes can be attributed to either post-column band broadening or distortion of the gradient by the HPLC system [8]. The major source of our post-column band broadening may be attributed to the use of a splitter and tubing within the TurboIonSpray™ source which are adding the majority of the post-column path length (minimum of 12″) and cannot be reduced.

In our laboratory, 2×10 mm and 2×20 mm, 5 μm columns with a t_G of 1.0 have been used effectively for the separation of pharmaceuticals and metabolites from biological matrices. Our typical injection volumes are 1–10 μl of supernatant from acetonitrile protein precipitations, or samples reconstituted with 50:50 (v/v) MeOH:ACN. Injection of small volumes of strong solvent into the weak eluent stream have not resulted in any distortions of peak shape or fluctuations in retention times. We have not observed any indication of solvent mixing anomalies as evidenced by reproducible (0.01 min) retention times and symmetrical chromatographic peaks. Band compression with gradient elution produces peaks with decreased peak widths and increased peak heights yielding the added benefit of improving analytical sensitivity by increasing the signal-to-noise ratio. This approach is currently used with HPLC equipment from more than one manufacturer and we have found method transfer between instruments and

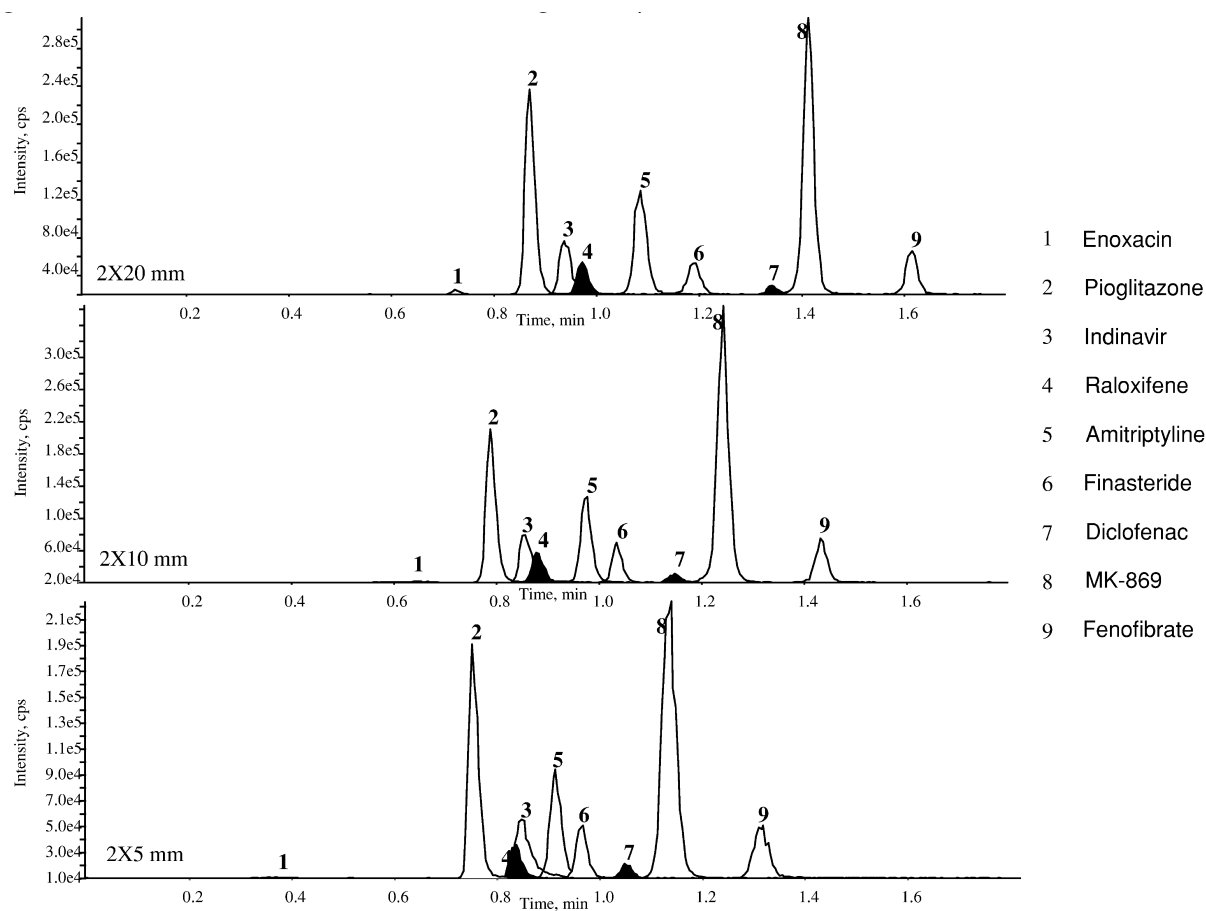

Fig. 6. Comparison of column lengths (5 μm) with a 1.5 min gradient time.

analysts to be trivial. To date we have quantified more than 50 000 samples with this approach and have demonstrated method reproducibility and ruggedness based on curve linearity and back-calculation of quality control standards [4–6].

Reduction of overall sample analysis time can be achieved easily by decreasing column length and/or by decreasing gradient time. A major factor in the reduction of analysis time is also the reduction of method development time associated with fast chromatography. We routinely optimize LC–MS/MS quantification methods based on column phase selectivity and gradient slope (t_G between 1.5 and 0.8 min) for novel compounds and complex mixtures within 15 min. However, consideration still must be given to the sample matrix and the overall requirements of the analysis. It is possible to compromise chromatographic integrity by either using a column that is too short or a gradient that is too steep.

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