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Development of a LC–LC–MS complete heart-cut approach for the characterization of pharmaceutical compounds using standard instrumentation

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Abstract

A total heart-cut LC–LC–MS technique is described for the analysis of pharmaceutical compounds. Utilizing ordinary LC–MS equipment, a standardized multi-dimensional, highly selective approach was developed. The system was developed on synthetic mixtures containing ten active pharmaceutical compounds. The methodology would be further deployed for the impurity characterization of batches of active chemical, process intermediates, and reference standards. Column-switching was employed to heart-cut sequential fractions from one set of LC conditions to another set of LC conditions. Two high efficiency columns, including the Merck Chromolith® C18, for fast LC–LC analysis were used. The system provides a highly automated, turnkey, multi-dimensional approach applicable to a variety of complex mixtures with little user intervention. It is envisioned that this idea could be viewed as a platform for other types of LC–LC analyses including peptide and achiral–chiral analyses.

Keywords: Three-dimensional; Total heart-cut; Chromolith®; LC–MS; Pharmaceuticals; Column switching

1. Introduction

Detection and identification of impurities in the active pharmaceutical ingredients (API's) and chemical processes is critical in the pharmaceutical industry. Improved analytical approaches are necessary to meet increasingly stringent regulatory requirements for the determination of impurities in API's. ICH guidelines indicate that impurities at or above 0.1% in the drug substance require identification [1]. In addition, an understanding of impurities in the chemical process is key to the production of high purity drug products [2].

Active pharmaceutical compounds are continuing to evolve to larger and more complex molecules. As a result, the number of structurally-related impurities has also increased. Because of this, the need for improved highly selective analytical methods to determine closely related structural impurities is critical. LC–MS has been widely employed in the pharmaceutical industry due to its high selectivity [3].

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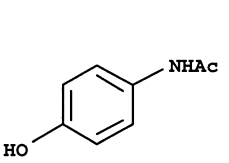

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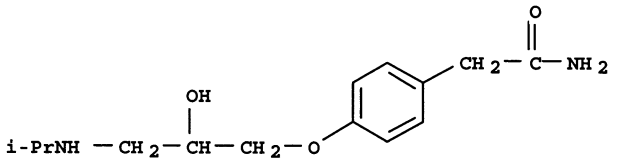

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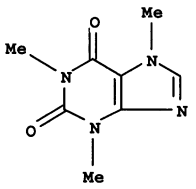
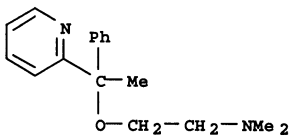


Caffeine **(3)**



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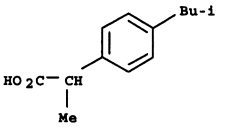
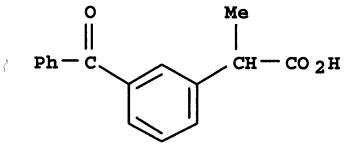


Ibuprofen **(5)**



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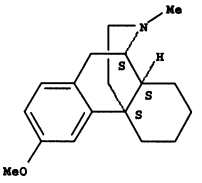
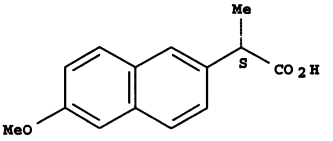
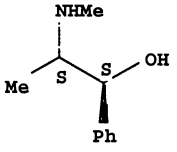


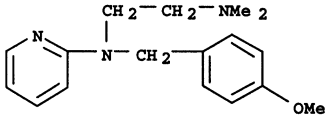
d-Methorphan **(7)**



d-Naproxen **(8)**



Pseudoephedrine **(9)**



Pyrilamine **(10)**

Fig. 1. These are the structures of the compounds used to develop the process.

Table 1
Mobile phases used in for two sets of LC conditions

Experiment	Eluent A	Eluent B
1	0.1% TFA in water	0.1% TFA in methanol
2	0.1% TFA in water	0.1% TFA in acetonitrile

Table 2
General HPLC conditions

Flow rate	2.0 ml/min
Detection	UV at 220, 280 mn, MS +ion scan
Columns (2)	Chromolith 4.6 × 100 mm, 5 μm
Column temperature	Ambient

Table 3
Gradient conditions

Time (min)	Type	Percentage of eluent A	Percentage of eluent B
0	Initial condition	100	0
5	Linear gradient	0	100
6	Isocratic	0	100

Table 4
General HPLC conditions

Flow rate	2.0 ml/min
Detection (analysis method only)	UV at 220, 280 mn, MS +ion scan
Columns (2)	Chromolith 4.6 × 100 mm, 5 μm
Column temperature	40 °C

LC–MS, however, does have a number of limitations with respect to detection of impurities. First of all, the impurity would need to ionize to be detected. Newer ionization techniques, such as atmospheric pressure photoionization, are reducing the number of 'un-ionizable' compounds, however, significant numbers still exist.

The ionization potential of coeluting impurities may play an important role in their detection. The ionization of the weaker compound could be significantly suppressed by that of the stronger.

The detection of coeluting impurities may also be obscured if unresolved spectrally. Isomer impurities are often difficult to detect even with LC–MS because the molecular ions are identical. Isobaric compounds would also present the same challenge for quadrupole type instruments. Even with Q-tof technology, structural information from fragmentation would be difficult without prior chromatographic resolution of the compounds. Without prior resolution, fragmentation spectra would be that of the combined isobaric compounds.

Other spectral interferences may obscure the detection of coelution impurities. Compounds can often produce a variety of ions. Adduct, fragmentation, and multiply charged ions could result in an overlooked impurity producing like ions. Furthermore, a low level impurity of 1 amu more

Table 5
Fraction collection conditions

Time (min)	Type	Percentage of eluent A	Percentage of eluent B
0	Initial condition	100	0
5	Linear gradient	0	100
6	Isocratic	0	100

Eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in methanol.

Table 6
Fraction analysis conditions

Time (min)	Type	Percentage of eluent A	Percentage of eluent B
0	Initial condition	100	0
5	Linear gradient	0	100
6	Isocratic	0	100

Eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile.

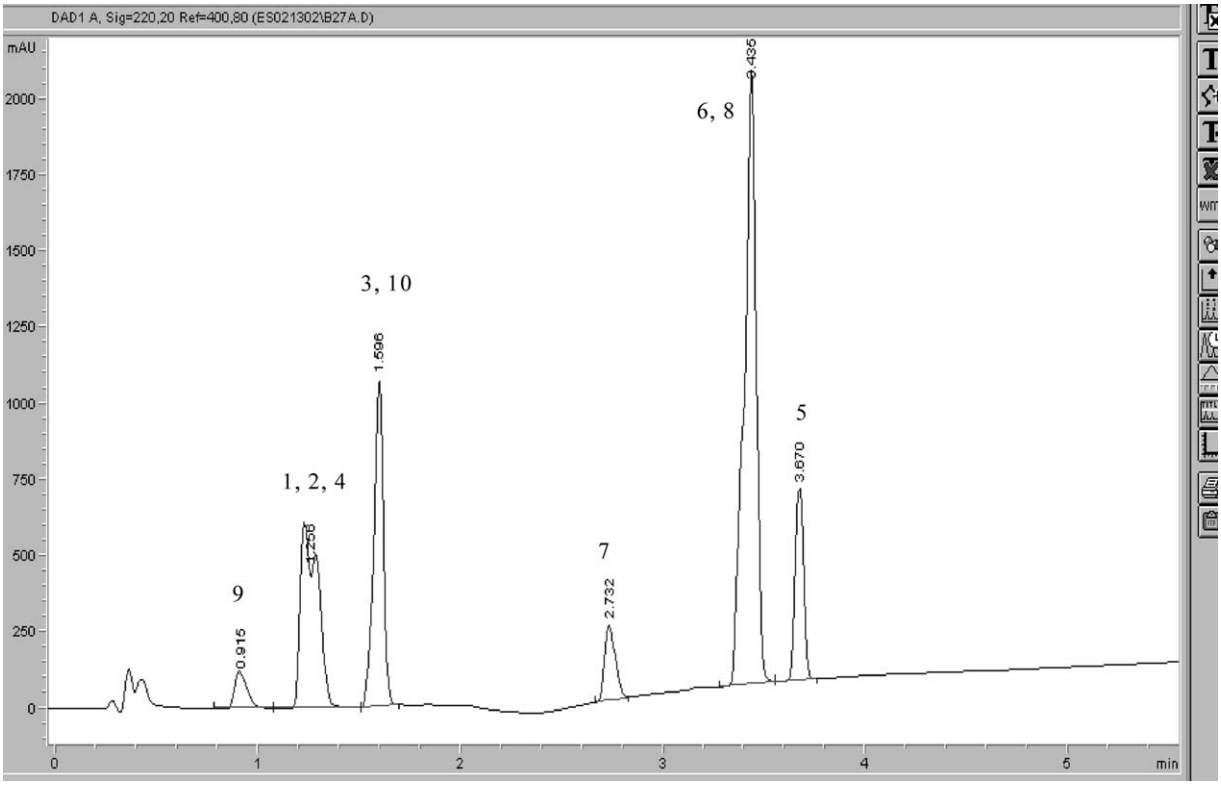


Fig. 2. The cyano column analysis of the analytical test mixture. Only three of ten compounds were resolved.

than the parent may be obscured by the C^{13} isotope ion.

An LC–LC–MS system was explored as a means of improving resolution via a multi-dimensional approach. Analytical techniques, such as TLC and CE, are well suited toward two-dimensional analyses. With HPLC, separations are essentially carried out in one-dimension making a completely integrated, continuous, two-dimensional approach unrealistic. However, a total two-dimensional LC–LC separation may be accomplished by taking a complete set of fractions from one LC analysis to another LC analysis. As the size of fraction segments approach zero, and the number of fractions approaches infinity, and a true and complete two-dimensionality is approached. This would also result in an infinite analysis time. By choosing fraction segments wisely, and using fast analyses, a fairly accurate two-dimensional analysis could be approximated and performed in a reasonable timeframe.

A two-dimensional LC approach would have intrinsic advantages over the typical single-dimensional approach. In theory, the peak capacity for the two-dimensional approach would be the product of peak capacities for each analysis [4,5]. Though selectivity of a one-dimensional analysis can be improved, method development for individual applications can be time consuming and require specific combinations of mobile phases and columns. The approach outlined here relies on a standardized two-dimensional technique that could be employed on a variety of pharmaceutical related analytes.

Most ‘orthogonal’ approaches in literature attempt to combine techniques as different as possible to improve selectivity. These applications include ion exchange–reversed phase [6], size exclusion–reversed phase [7], normal phase–reversed phase [8], and achiral–chiral [9]. Though the traditional approach may provide greater selectivity due to the application of different

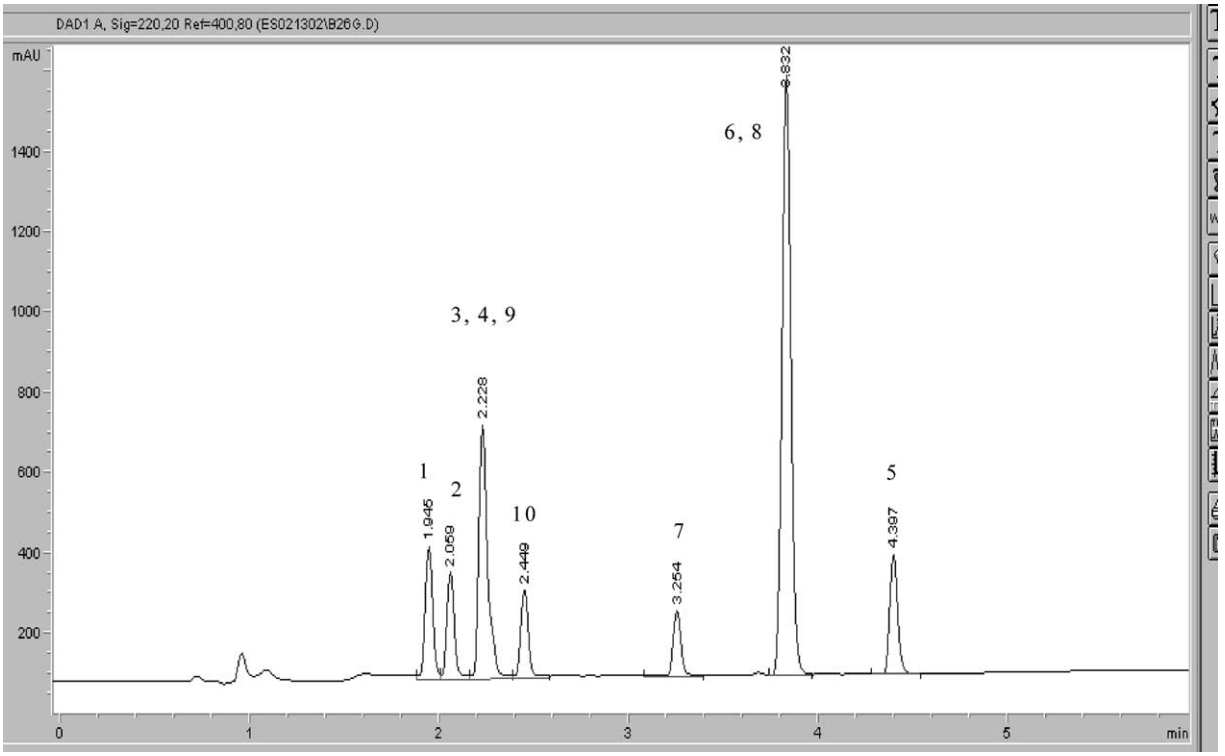


Fig. 3. The chromolith column analysis of the test mixture. Only five of ten compounds were resolved.

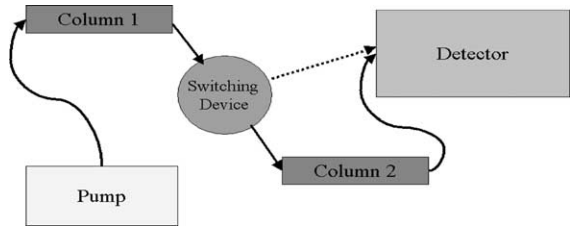


Fig. 4. The flow path is either though column 1 or column 1 and 2. The later path is used for the fraction analysis.

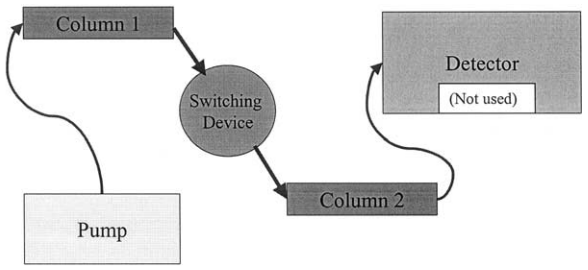


Fig. 5. The initial equilibration of both columns with 0.1% TFA in water.

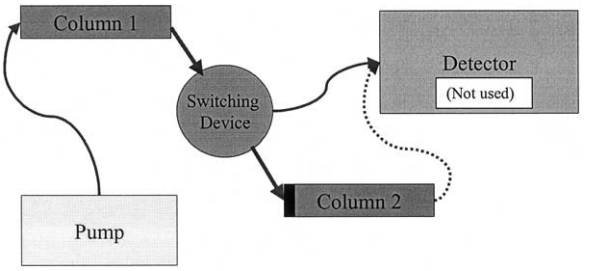


Fig. 6. A 15 s fraction is deposited to column 2. Prior to the fraction collection the mobile phase path is only through column 1.

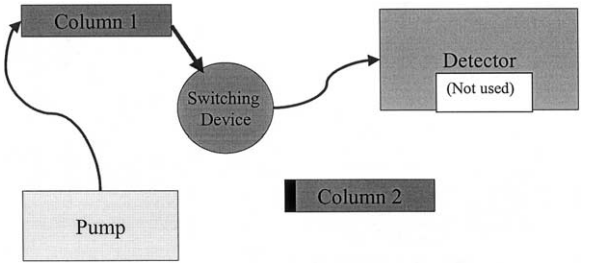


Fig. 7. After the deposit of the fraction, later elutors are quickly rinsed from column 1 using 100% organic eluent. Then column 1 is quickly reequilibrated with 0.1% TFA in water.

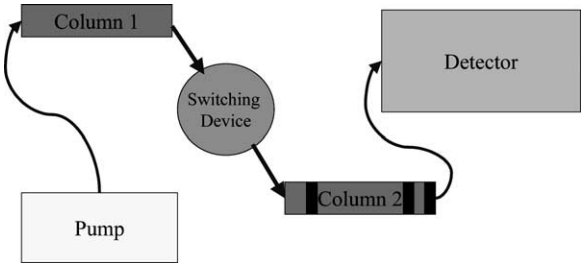


Fig. 8. The flow is redirected to column 1 and 2. The fraction components are eluted using LC conditions 2.

separation mechanisms, the goal was also to develop a 'universal' approach that could be applied to a wide variety of pharmaceutical compounds. For a standardized approach to be broadly effective, analytes must perform well on both sets of analytical conditions. For example, size exclusion may offer some enhancements in selectivity, but also result in poor efficiency nullifying any benefits. Therefore, reversed phase, the most widely used chromatographic technique in the pharmaceutical industry, was chosen.

A methodology was envisioned to utilize a complete heart-cut approach employing two reversed phase LC conditions. Though heart-cut approaches and column-switching techniques have been used in the past, their focus was almost exclusively to improve resolution for a particular analyte, usually from matrix interferences [10–12]. The approach described here involves the complete transfer of contiguous repetitive fraction analysis from one column on to a second column for analysis. The approach was envisioned to provide qualitative information, however, quantitation may be possible if supported by validation of a specific application. This approach has the advantage of determining several analytes in a complicated mixture.

Two fast, high efficiency columns were explored including the relatively new chromolith columns. By using two efficient columns, instead of a pre-column/analytical column combination, the peak capacity product would be maximized. Most comprehensive two-dimensional methods employ a standard analytical column utilizing a longer run time or column, followed by a short, two-column analysis in the second-dimension [13,14]. With this scenario, the fraction analysis needs to be completed before the transfer of the following fraction. The overall peak capacity product, discussed

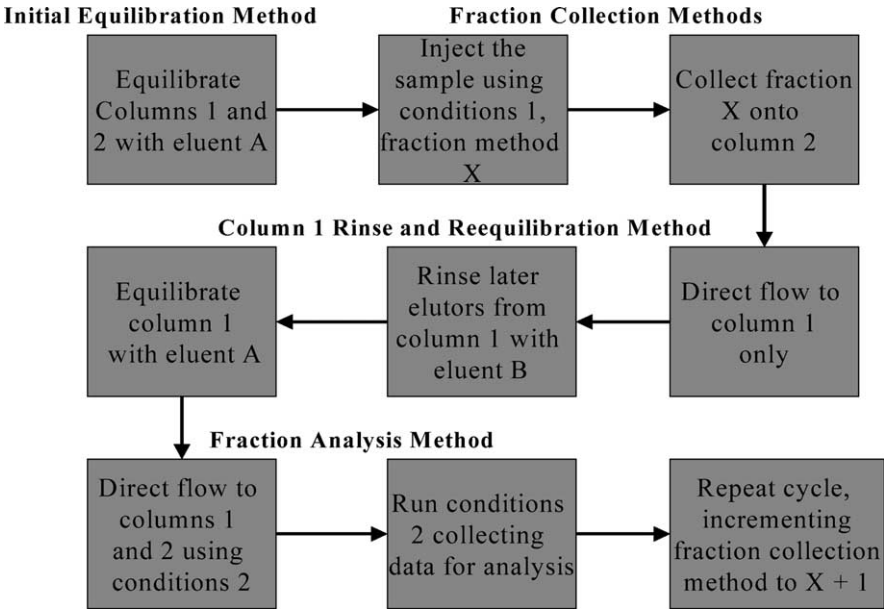


Fig. 9. This depicts the flowchart detailing the process. This is accomplished using four identical methods except for the fraction collection, which varies in collection time.

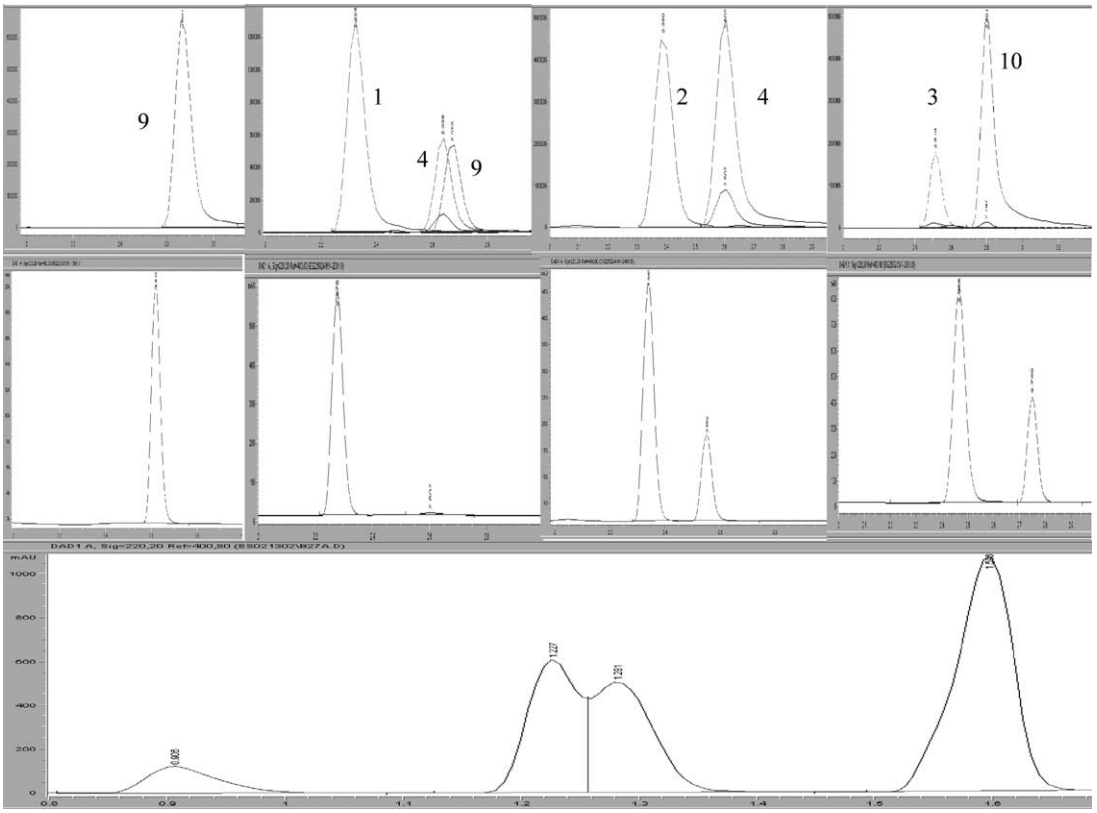


Fig. 10. Earlier eluting fractions containing analytes. From top to bottom, secondary analysis MS extracted ion, secondary analysis UV trace, and primary analysis UV trace. From left to right, eluting initially from the primary analysis, fractions 4 (0.75–1.00 min), 5 (1.00–1.25 min), 6 (1.25–1.50 min) and 7 (1.50–1.75 min).

previously, may be limited by the capacity of the secondary analysis, which needs to be accomplished typically within a 1 min time-frame.

The use of two columns with comparable diameters also has advantages with respect to sample load. Several examples in literature employ

Table 7
Near isocratic elution methods for the secondary analysis used for specific fractions according to Table 8

Secondary analysis method	Time 0 min % (0.1% TFA in) (acetonitrile/water)	Time 1.00 min % (0.1% TFA in) (acetonitrile/water)	Time 6.00 min % (0.1% TFA in) (acetonitrile/water)	Notes
2a	0/100	15/85	15/85	Fast gradient/ isocratic
2b	0/100	25/75	25/75	Fast gradient/ isocratic
2c	0/100	35/65	35/65	Fast gradient/ isocratic
2d	0/100	45/55	45/55	Fast gradient/ isocratic
2e	0/100	55/45	55/45	Fast gradient/ isocratic

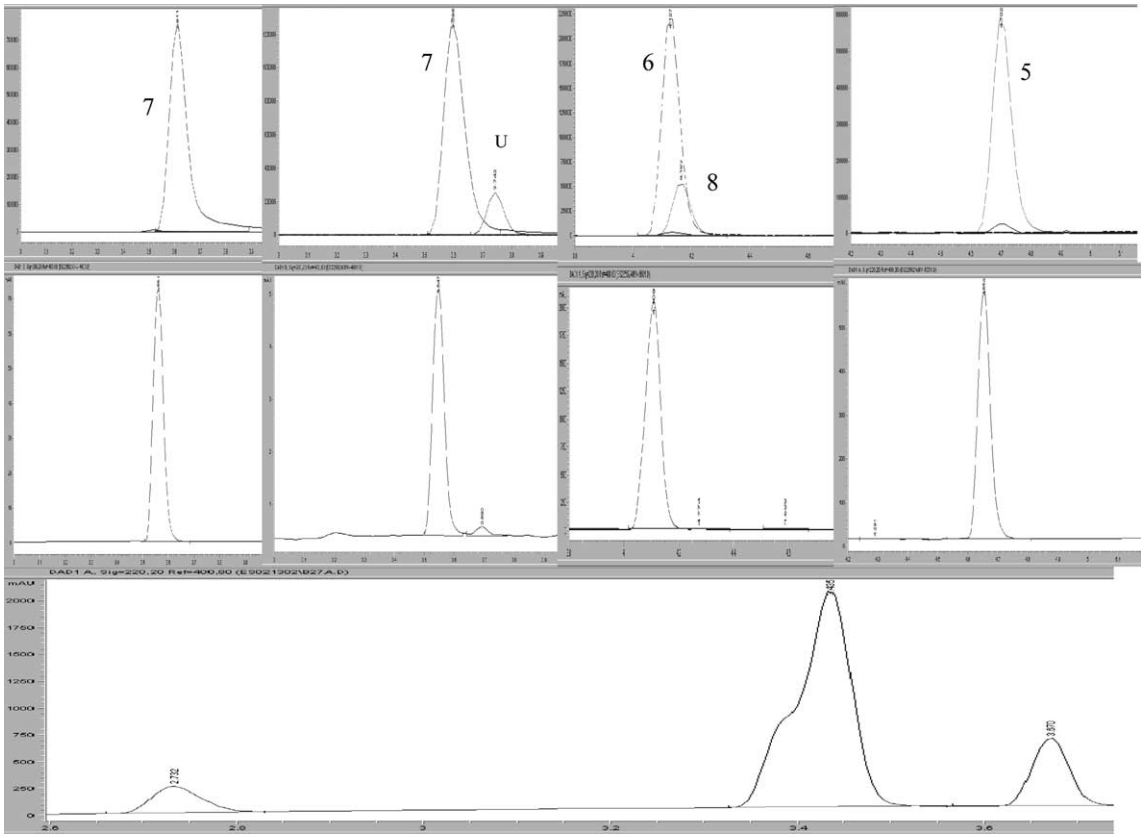


Fig. 11. Later eluting fractions containing analytes. From top to bottom, secondary analysis MS extracted ion, secondary analysis UV trace, and primary analysis UV trace. From left to right, eluting initially from the primary analysis, fractions 11 (2.50–2.75 min), 12 (2.75–3.00 min), 14 (3.25–3.50 min) and 15 (3.50–3.75 min).

microbore/conventional bore columns to avoid focusing problems [15]. This, however, could result in either excess loading of the microbore column or insufficient loading in the conventional column. Therefore, either selectivity or sensitivity would be sacrificed. The approach outlined here overcomes these difficulties.

Though other comprehensive two-dimensional approaches are possible, the goal of this work was to accomplish this with typical HPLC equipment including one pump and one column-switching device. A 'programming over plumbing' approach was used to overcome instrument limitations. Furthermore, a completely automated, logical approach of linking several methods in series and requiring minimal user intervention was developed. Though the total analysis would be longer that of conventional LC analyses, the significant time needed for method development for each application would be eliminated.

2. Experimental, results, and discussion

2.1. Equipment and reagents

All solvents used in the HPLC method were of analytical grade. Supplies of ten different common pharmaceutical compounds were purchased from Sigma-Aldrich. These were D-methorphan, doxylamine succinate, atenolol, D-naproxen, ibuprofen, pyrilamine maleate, pseudoephedeine HCl, acetaminophen, ketoprofen, and caffeine. The compounds had a variety of typical functional groups

including, primary, secondary, tertiary and aromatic amines as well as carboxylic acids (Fig. 1).

An Agilent (Hewlett–Packard) 1100 HPLC system equipped with a degasser, binary pump, autosampler, column switching device, and temperature controlled column compartment was used for this study. A Merck, Chromolith 4.6 × 100, and a Zorbax, Cyano, 3 μm 4.6 × 50 mm were used. The detector for this system was UV-diode array/HP G1946D mass detector. For MS, the flow was split so that approximately 30% of the flow entered the ESI source. A ChemStation was used for instrument control and automated data collection. Linking several methods in sequence was critical for generating results for several experiments rapidly.

Table 8
Fractions were obtained from the primary analysis using the cyano column and the conditions from Tables 4 and 5

Faction from the primary analysis (start time–end time, min)	Secondary analysis method (Table 7)
0.75–1.00	2a
1.00–1.25	2a
1.25–1.50	2a
1.50–1.75	2a
1.75–2.00	2b
2.00–2.25	2b
2.25–2.50	2b
2.50–2.75	2c
2.75–3.00	2c
3.00–3.25	2c
3.25–3.50	2d
3.50–3.75	2d
3.75–4.00	2e
4.00–4.25	2e
4.25–4.50	2e

The secondary analysis of the fractions was carried out according to Table 8 and general conditions from Table 4.

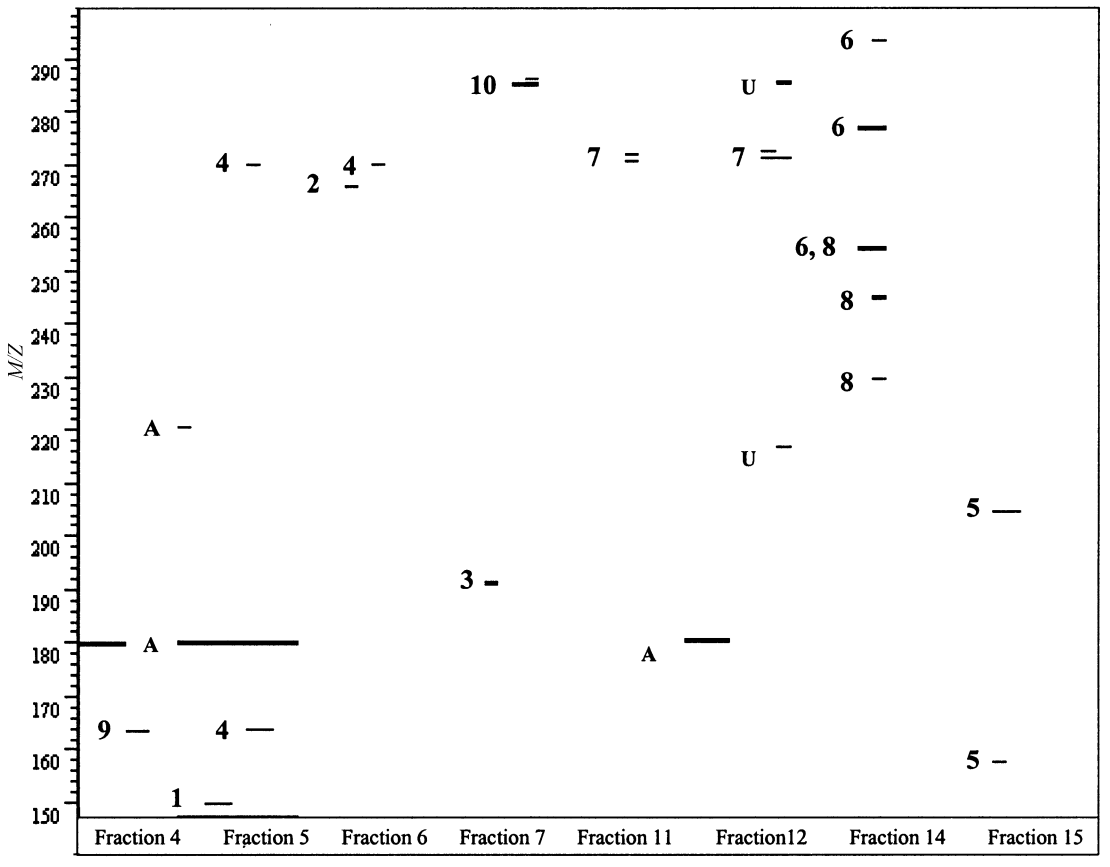


Fig. 12. Reconstructed fractions displayed in two-dimensional isoplot format. Compounds **1**–**10** are displayed including adducts and fragments. A low level unknown (U) and chromatographic artifacts (A) were also detected.

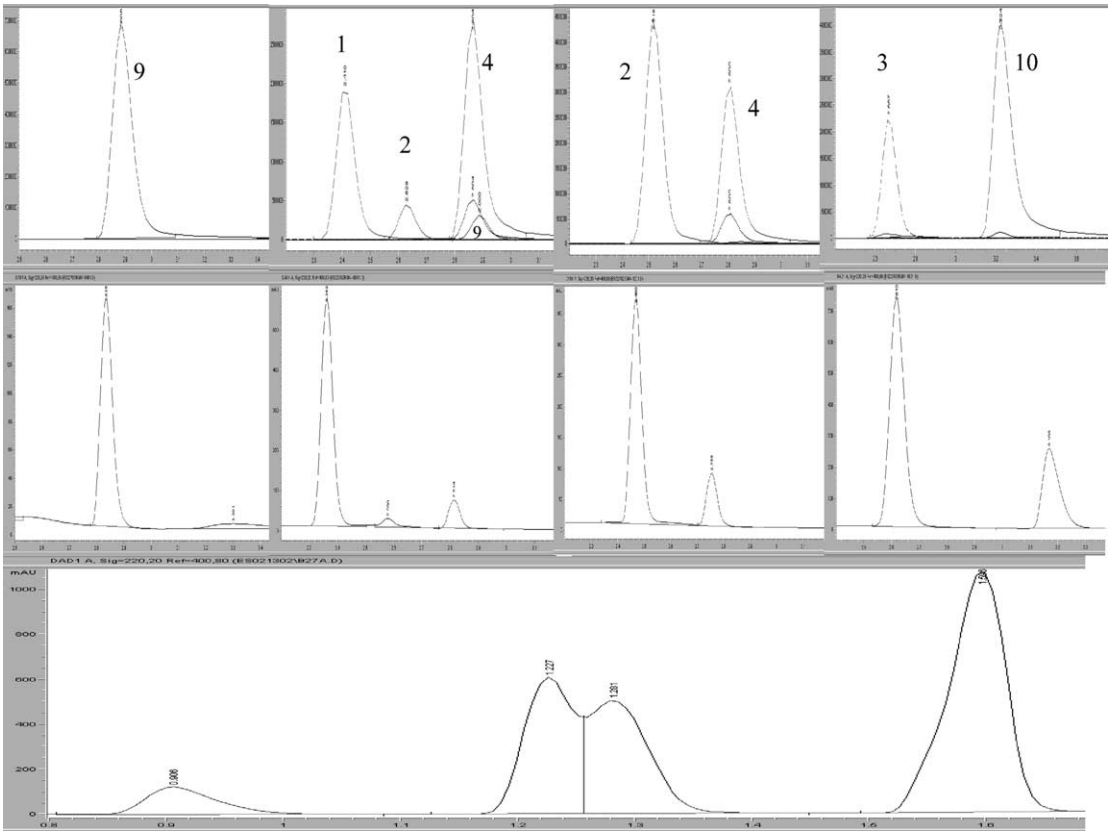


Fig. 13. Earlier eluting fractions containing analytes from the gradient–isocratic runs. From top to bottom, secondary analysis MS extracted ion, secondary analysis UV trace, and primary analysis UV trace. From left to right, eluting initially from the primary analysis, fractions 4 (0.75–1.00 min), 5 (1.00–1.25 min), 6 (1.25–1.50 min), and 7 (1.50–1.75 min).

2.2. Development of single column analysis conditions

The first objective was to find two sets of HPLC conditions which would individually produce good chromatographic peakshape, but not necessarily resolve all analytes. The initial goal was to develop different HPLC conditions to heighten selectivity, yet still produce good chromatography. A solution containing the ten randomly chosen pharmaceutical compounds was prepared at 0.5 mg/ml in 50/50: acetonitrile/water. After the initial single-dimensional screening of four mobile phases with the chromolith column, the studies were continued with two eluents (Table 1). A linear gradient of 0–100% B from 0 to 5 min and a 1 min hold at 100% B was utilized (Tables 2 and 3).

2.3. Combining LC conditions

The conditions from Tables 1–3 were used to create two sets of LC conditions; employing methanol for set 1 and acetonitrile for set 2. An equal volume of 0.1% TFA was added to the sample solution for sample/eluent pH consistency. The first attempt was made with two Chromolith® columns connected via a column-switching device but this was quickly abandoned due to poor analyte focusing.

The primary column was replaced with a Zorbax® SB CN, Rapid Resolution (4.6 × 500 mm) 3 μm column. Because the stationary phase is more hydrophilic than that of the C18 column, the analytes elute with less organic content. Fifteen second fractions were taken from the first column

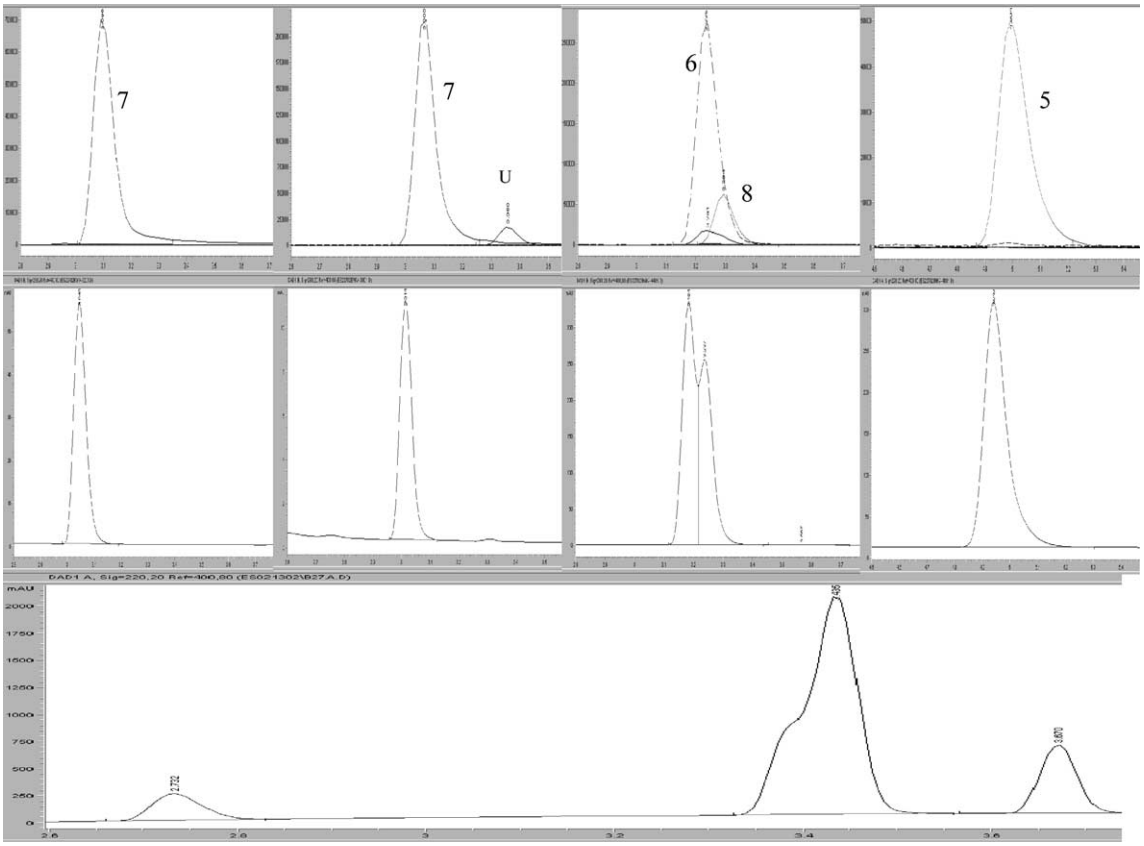


Fig. 14. Later eluting fractions containing analytes from the gradient–isocratic runs. From top to bottom, secondary analysis MS extracted ion, secondary analysis UV trace, and primary analysis UV trace. From left to right, eluting initially from the primary analysis, fractions 11 (2.50–2.75 min), 12 (2.75–3.00 min), 14 (3.25–3.50 min) and 15 (3.50–3.75 min).

Table 9
From the test mixture of ten analytes, the number resolved as a function of technique is displayed

Methodology	Number of resolved analytes	Analytes not resolved
LC	3	(Acetaminophen, atenolol, doxylamine succinate), (pyrilamine maleate, caffeine), (ketoprofen, naproxen)
LC–LC	8	Ketoprofen, naproxen
LC–LC–MS	10	–

to the second column. This appeared to deposit the analyte on the secondary column with good focusing. In addition, a greater degree of selectivity could be possible due to the different stationary phases. A 10 s fraction scenario may have been more beneficial at the cost of increased time.

Before undertaking the two-column scenario, each column was used individually for the analysis of the test mixture. This was done using the general conditions from Tables 4 and 5 for the cyano column, and Table 6 for the chromolithic column. Ignoring the void volume area, six and

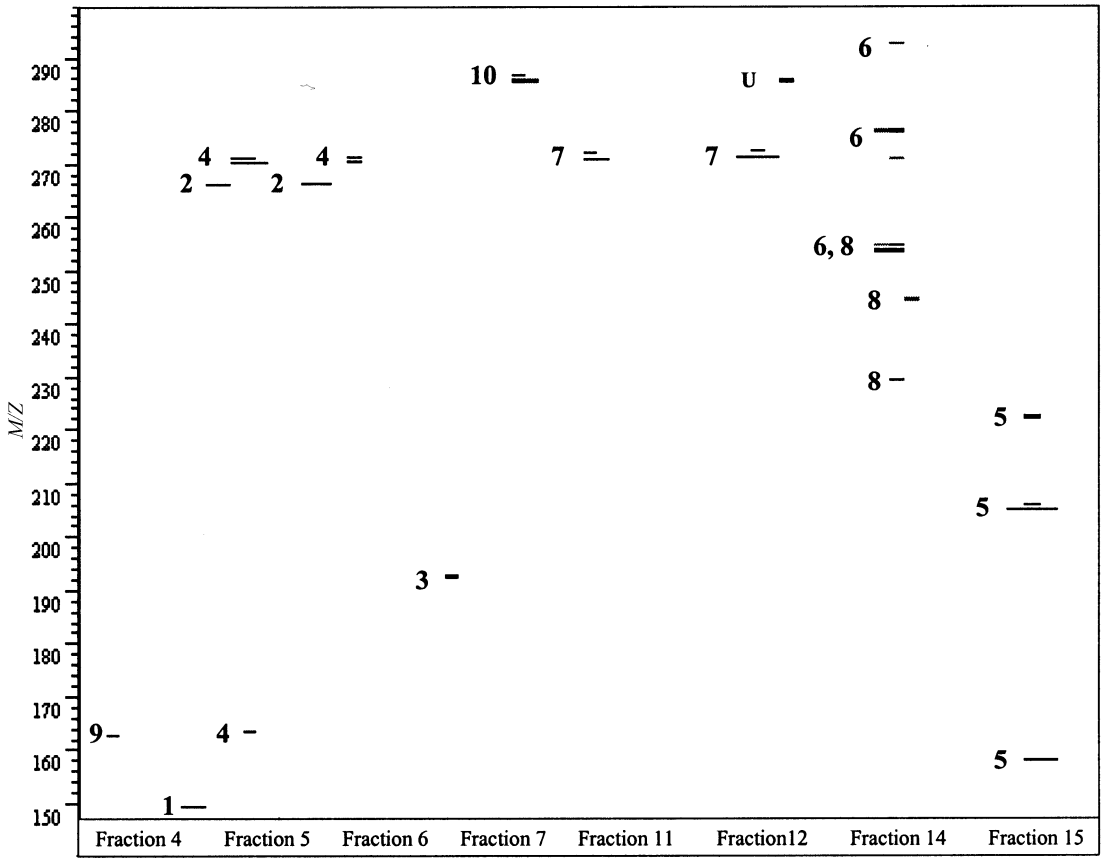


Fig. 15. Reconstructed fractions from the gradient–isocratic runs are displayed in two-dimensional isoplot format. Compounds **1**–**10** are displayed including adducts and fragments; **1** low level unknown was also detected (U). Artifacts previously noted in the gradient–gradient runs are virtually eliminated.

seven baseline resolved peaks were observed in the cyano and chromolith column analysis, respectively. The chromatograms are displayed in Figs. 2 and 3.

Analytes would elute from column 1 using the first set of LC conditions. A fraction of the column 1 eluent would be deposited on the head of column 2. This, in turn, would be run using a second set of LC conditions. The analysis would then be repeated with an incremented fraction. Because the standard HPLC included only one pump and column switching device, the flow path could only be directed to column 1 or to columns 1 and 2 (Fig. 4).

Analysis of a single fraction involves the linking of four methods in sequence. These include initial equilibration of both columns with 0.1% TFA in water (Fig. 5), injection of sample using column 1/conditions 1, deposit fraction of sample to column 2 (Fig. 6), quick wash/reequilibration of column 1 (Fig. 7), and redirect flow to column 2 using conditions 2 for the analysis (Fig. 8). The sequence is then repeated for subsequent fractions. The four methods remain identical with the exception of the fraction collection method, which is incremented to collect the succeeding fraction for the following cycle. The overall flowchart is depicted in Fig. 9.

It was observed that this approach resulted in a relatively narrow elution window within each fraction on the second column. This result was not totally unexpected since compounds eluting in the 15 s fraction should have comparable pola-

Sequence Table:

Method and Injection Info Part:

Sample Name	Method	Inj Volume (μl)
0 none	RMC1C2E	
57 none	RMC1C2E	
58 Vial 1	F-375C1E	10.0
59 none	FRC1E	
60 none	ANMC1C2E	
61 none	RMC1C2E	
62 Vial 1	F-400C1E	10.0
63 none	FRC1E	
64 none	ANMC1C2E	
65 none	RMC1C2E	
66 Vial 1	F-425C1E	**1.0**
67 none	FRC1E	
68 none	ANMC1C2E	
69 none	RMC1C2E	
70 Vial 1	F-450C1E	10.0
71 none	FRC1E	
72 none	ANMC1C2E	
96 none	ANMC1C2E	

Fig. 16. This is a partial view of the sequence table illustrating how a particular fraction containing the parent at high level could be reduced to 1 (μl). All other fractions were given a standard 10 (μl) injection for better detection of low level impurities.

rities. Only the fractions containing analytes are displayed (Figs. 10 and 11).

Isoplot two-dimensional data was gathered for each of the above fractions. The individual plots were reconstructed into a single isoplot (Fig. 12).

2.4. CN–C18, gradient–isocratic

To increase resolution in the second column, a near isocratic (a fast, 1 min gradient, then a 5 min isocratic hold) analysis was employed for the secondary analysis (Table 7) with the general conditions (Table 4). The intention was to elute the analytes during the isocratic portion of the secondary analysis. Groups of fractions were assigned different strength isocratic secondary methods (Table 8), i.e. early fractions containing more polar compounds were subsequently analyzed using a weaker isocratic eluent. A 3 min retention time was targeted in the second column.

The isocratic modification exhibited two advantages. The resolution of analytes was improved and gradient artifacts were reduced. The ketoprofen–naproxen pair (**6**, **8**) that had coeluted under gradient conditions exhibited a slight separation. The UV trace and extracted ion chromatograms of each compound for each fraction were overlayed to check for co-elution (Figs. 13 and 14). The performance of the LC, LC–LC, and LC–LC–MS was compared (Table 9).

Isoplot two-dimensional data was gathered for each of the above fractions. The individual plots were reconstructed into a single isoplot (Fig. 15).

The technique was applied for impurity characterization of proprietary reference standards. Here, the analyses were carried out on highly concentrated, 50 mg/ml sample preparations. This was up to 500-times more concentrated than typical HPLC preparations. Here, a 0.1% impurity would be equivalent to 0.5 ng on column. Because of this, there is a inherent enhancement to the working limit of detection, however, the actual limit of detection was not verified by spiking. Intuitively, the limit of detection would be dependent almost exclusively on the sample concentration.

One attractive feature of this approach is the easy modification of injection volume for a particular fraction(s) (Fig. 16). Because the injection volume for each fraction could be easily modified in the sample table, a significantly reduced injection volume could be applied to the fraction containing the parent. With this, impurity levels could be kept relatively high for superior response. At the same time, contamination of the MS source due to high parent load would be avoided.

3. Conclusions

A highly selective LC–LC–MS system was developed and demonstrated on test mixtures and real samples. The technique involved little modification of existing equipment; only a logical approach linking several methods. A fairly stan-

dardized methodology encompassing a total on-line fraction analysis was carried out with minimal user intervention. Once developed, sequence and method files could easily be electronically transferred to other HPLC systems.

Two general gradient methods were developed on a variety of pharmaceutical compounds. Both LC methods needed to be short for the approach to be practical. A gradient–isocratic approach was also further developed to enhance selectivity in the secondary analysis. The new high efficiency Chromolith C18 column was found to have excellent performance characteristics necessary to expedite the approach. The authors look forward to smaller diameter and alternate stationary phase versions for enhanced selectivity and sensitivity.

Mass spectrometry was key to both developing the system and its subsequent use. With MS, early setbacks in the chromatography such as peak splittings, were quickly recognizable. Non-working approaches were quickly abandoned. MS was also key in tracking analytes in the complicated LC–LC system without the need to inject individual markers. From these experiments, LC–MS appears to be a more selective tool than LC–LC. The LC–LC–MS approach is an unquestionable selectivity improvement to the individual approaches. We look forward to applying this to LC–LC–MS-MS.

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