

Application of a column selection system and DryLab software for high-performance liquid chromatography method development

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Abstract

This paper describes a strategy for the development of chromatographic methods for drug candidates based upon the use of simple MS compatible mobile phases and optimization of the chromatographic selectivity through variations of the stationary phase and mobile phase pH. The strategy employs an automated column selection system and a series of HPLC columns, varying in hydrophobicity and silanol activity, in combination with DryLab software to develop chromatographic methods for the separation of mixtures of bupivacaine and its metabolites; acidic, basic, and neutral compounds; and atenolol, nitrendipine, and their degradation products.

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1. Introduction

In pharmaceutical drug development there is a need to develop HPLC methods that are sensitive to changes in the related substance profile of a drug candidate due to modifications in the synthetic pathway and/or formulation, as the candidate drug progresses from early to late stage development. Therefore, it is not only necessary to develop HPLC methods efficiently, but there is also a need to maximize information about method specificity. Valuable information on chromatographic method specificity can be obtained by variations in the stationary phase, mobile phase composition and pH. Traditionally, strategies for the development of specific and robust HPLC methods have relied on the abilities of the chromatographer. The development time is dependent upon the chromatographer, but even for the most experienced it is a labor intensive and time-consuming process.

The process of HPLC method development generally uses a series of columns and selectivity is obtained through modifications of the mobile phase organic modifier, pH, and occasionally ion pairing reagents. While this can produce robust methods, it

can be time consuming and the mobile phase may not be compatible with mass spectrometric (MS) detection. For example, the addition of ion pairing reagents and many buffer salts are incompatible with LC/MS.

In an effort to improve the efficiency of method development and maximize information about method specificity, a number of computer simulation programs have become commercially available in the last 15 years. DryLab[®] software is one such program which, on the basis of a small well-defined number of experiments on a particular stationary phase, predicts the separation following changes in mobile phase composition, mode of elution (either isocratic or gradient), temperature, pH or column parameters (dimensions, particle size, flow rate). A number of reviews and applications of DryLab[®] software are reported in refs. [1–6].

A number of papers in the literature report the use of automated multi-column systems for the purpose of either column scouting or for obtaining specificity data for a given method. Mazzeo et al. developed a four-column approach for stability-indicating assays utilizing both stationary phase chemistry as well as mobile phase pH to alter the selectivity of a separation [7]. This approach was shown to be useful in finding degradation products of lansoprazole by chromatographing a degraded sample on four different columns at high and low pH.

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Pfeffer and Windt reported an automated method development system using a 12-column selection system with DryLab[®] software optimization [8]. They performed a single gradient trial experiment on a number of selected columns. After obtaining data for each column, the ‘best’ column was selected by visually comparing the resultant chromatograms. DryLab was then used to optimize the separation on the selected column.

This paper describes a strategy for the development of chromatographic methods for drug candidates based upon the use of simple MS compatible mobile phases and optimization of the chromatographic selectivity through variations of the stationary phase and mobile phase pH. The rationale for this approach is based on a number of interdependent factors; (1) the use of MS detection is preferred as this can aid in the determination of method specificity; (2) variation of the chromatographic selectivity during method development is necessary to optimize the specificity; (3) an information database is generated which can be accessed at any point in development; (4) as development proceeds from pre-clinical to marketing submission, a method will typically be transferred a number of times. Reducing the complexity of the mobile phase is beneficial for efficient transfer of the methods.

The current work describes the application of an automated column selection system using a series of HPLC columns varying in hydrophobicity and silanol activity, in combination with DryLab[®] software to efficiently develop robust, specific and MS compatible HPLC methods for the separation of mixtures of drugs and/or their metabolites or degradation products.

2. Experimental

2.1. Chemicals

Bupivacaine, 3'-hydroxybupivacaine, 4'-hydroxybupivacaine, desbutylbupivacaine, atenolol, and atenolol acid were provided by AstraZeneca (Wilmington, DE, USA). Lidocaine, mepivacaine, prilocaine, amitriptyline, prednisolone, naproxen, ibuprofen, *p*-hydroxyphenylacetamide, *p*-hydroxyphenylacetic acid, and nitrendipine were purchased from Sigma (St. Louis, MO, USA) and were used as received. Nitropyridine was formed by degrading a 1 mg/ml solution of nitrendipine in 50:50 (v/v) methanol: water under UV irradiation for 72 h. Methanol, acetonitrile, formic acid, and ammonium hydroxide were of HPLC grade or better, and were purchased from Fisher Scientific (Hanover Park, IL, USA).

2.2. HPLC columns

The HPLC columns used in this study, together with the associated hydrophobicity, steric selectivity, acidic, basic, and cation exchange parameters, are shown in Table 1. The columns were evaluated for each of these parameters using the Column Match evaluation program available at www.Rheodyne.com (Rheodyne, Rohnert Park, CA, USA). All columns had dimensions of 4.6 mm × 150 mm.

2.3. HPLC/MS conditions

An Agilent Technologies 1100 HPLC system equipped with a diode-array detector and an 1100 mass spectrometer (Agilent Technologies, Wilmington, DE, USA), and a LC Spiderling Deluxe column selection system (Chiralizer Services, LLC, Newton, PA, USA) were used in this study. The system dwell volume was 1.18 ml. A column temperature of 30 °C was maintained throughout. The initial experiments consisted of two gradient runs on each column using a mobile phase gradient of 5–100% acetonitrile in 20 and 60 min. The mobile phases were formed from mixing of acetonitrile and water, each containing 0.1% (v/v) formic acid (pH of 2.7) or 0.1% ammonium hydroxide (pH 10.5). A flow rate of 1 ml/min was used for all studies. Both UV (diode array) and mass spectrometric detection were used in all experiments. For MS detection, electrospray ionization was used with a dry gas flow rate of 10 L/min, fragmentation voltage of 80 V, capillary voltage of +2.8 kV, nebulizer pressure of 2.5 bar, and dry gas temperature of 300 °C. To aid in peak tracking, UV spectra (190–400 nm) along with UV detection at 230 nm was used for the atenolol/nitrendipine mixture and the acids, bases, and neutrals mixture, while detection at 210 was used for bupivacaine and its metabolites.

2.4. Method development strategy

In this work a sample was chromatographed on a series of columns varying in hydrophobicity and polarity (Table 1) using an automated column selection system with MS detection. For each sample, initial input experiments were performed on the columns and the resulting chromatographic data were entered into DryLab[®] software (Rheodyne, Rohnert Park, CA, USA). Peak identities were confirmed by the DryLab[®] software peak matching function, the UV spectra and molecular ion data.

A simulation of an isocratic separation on each column was performed in DryLab[®] software to determine if all peaks in the sample satisfied the criteria: $k' \geq 1.0$ and baseline resolution within 40 min. If an isocratic separation did not meet the criteria, DryLab[®] software was used to construct a gradient to meet the above criteria, which was then experimentally confirmed. The simulations that satisfied the above criteria were experimentally confirmed. The best separation was selected based on shortest run time and peak shape (tailing factor ≤ 2.0).

3. Results and discussion

The method development strategy was applied to the separation of bupivacaine and its metabolites (Fig. 1); a mixture of acidic, neutral and basic analytes (Fig. 2); and a mixture of atenolol, nitrendipine and their degradation products (Fig. 3).

3.1. Bupivacaine and metabolites

HPLC methods for the separation of bupivacaine and its desbutylbupivacaine, 3'-hydroxybupivacaine and 4'-hydroxybupivacaine metabolites have been previously reported

Table 1
Column selectivity parameters [15]

Column description	Column manufacturer	H^a	S^b	A^c	B^d	$C(2.8)^e$	$C(7.0)^f$
XTerra MS C8 3 μm	Waters Corporation, Milford, MA	0.660	0.057	-0.617	0.107	-0.186	-0.197
Symmetry Shield C8 3 μm	Waters Corporation, Milford, MA	0.750	0.024	-0.505	0.108	-0.592	0.169
Symmetry C8 3 μm	Waters Corporation, Milford, MA	0.894	-0.047	-0.204	0.020	-0.507	0.284
XTerra Pheny 3 μm	Waters Corporation, Milford, MA	0.690	-0.076	-0.374	-0.003	0.102	-0.033
Spherisorb ODS2 5 μm	Waters Corporation, Milford, MA	0.690	-0.076	-0.374	-0.003	0.102	-0.033
Spherisorb C8-2 5 μm	Waters Corporation, Milford, MA	0.763	0.090	-0.032	0.052	0.736	1.141
Prodigy C8 5 μm^g	Phenomenex, Torrance, CA						
Luna C18(2)	Phenomenex, Torrance, CA	1.003	-0.023	-0.121	-0.006	-0.269	-0.173
LiChrospher RP-18 5 μm	Phenomenex, Torrance, CA	0.969	0.057	0.266	-0.048	0.978	1.240
Inertsil C8-3 3 μm	Alltech, Deerfield, IL	0.830	-0.004	-0.265	-0.016	-0.333	-0.362
Hypersil BDS-C18 3 μm	Alltech, Deerfield, IL	0.993	-0.017	-0.095	-0.009	0.336	0.280
Adsorbosphere XL C8-B 5 μm	Alltech, Deerfield, IL	0.986	0.069	0.060	-0.050	1.492	1.679
Zorbax Extend C18 3 μm	Agilent Technologies, Wilmington, DE	1.098	-0.050	0.012	-0.041	0.030	0.016
Zorbax SB cyano 3 μm	Agilent Technologies, Wilmington, DE	0.502	0.108	-0.224	0.042	-0.146	1.047
Zorbax Eclipse XDB-C8 3 μm	Agilent Technologies, Wilmington, DE	0.918	-0.026	-0.221	-0.009	0.002	0.011
ACE C8 3 μm	Mac-Mod, Chadds Ford, PA	0.660	0.057	-0.617	0.107	-0.186	-0.197

^a H , column hydrophobicity.

^b S , accessibility of the stationary phase to insertion of "bulky" molecules ("steric interaction," similar to "shape selectivity").

^c A , column hydrogen-bond acidity due to non-ionized silanols.

^d B , column hydrogen-bond basicity due to basic groups in the stationary-phase (e.g., embedded polar groups).

^e $C(2.8)$, column cation-exchange activity due to ionized silanols (at pH 2.8).

^f $C(7.0)$, column cation-exchange activity due to ionized silanols (at pH 7.0).

^g Information not currently available for this column.

[9–12]. However, the separation of the 3'- and 4'-hydroxy isomers has been reported only once [13].

In this work the initial two input experiments were performed on a series of columns (Table 1) using a gradient mobile phase of acetonitrile/water containing 0.1% formic acid (pH 2.7). The data were imported into DryLab[®] software and an isocratic separation was predicted for eight of the columns (Fig. 4). The predicted separations were experimentally verified for these eight columns (Fig. 5). The actual (t_{exp}) and simulated (t_{sim}) retention times for each component in the mixture, together with their ratio ($r = t_{\text{exp}}/t_{\text{sim}}$), are given in Table 2. The ratio is a quantitative measure of the agreement between the experimentally determined separation and that predicted by DryLab[®] software. All ratios, with the exception of the bupivacaine peak on the XTerra MS C8 column, ranged in value from 0.72 to 1.08, thus indicating good agreement between the experimental and simulated results. Evaluation of these based on run time and

tailoring factor indicated that the best separation was achieved on the Symmetry Shield column.

3.2. Mixture of acidic, basic, and neutral compounds

This method development strategy was applied to a mixture of acidic, basic, and neutral compounds that is not typical of most pharmaceutical applications since it consists of compounds with significantly different $\text{p}K_a$ values. However, this artificial mixture represents a challenging scenario with which to test the method development strategy. The structures of the compounds in this mixture are shown in Fig. 2. The initial gradient input experiments were performed on the columns shown in Table 1 using a mobile phase pH of 2.7 and the data were imported into DryLab[®] software. An isocratic separation with baseline resolution was not predicted possible within 40 min but was predicted to be possible on four of the columns under gradient conditions at pH 2.7. The experimentally verified separations are shown in Fig. 6. All four columns showed good resolution of the compounds, however the XTerra MS column did not achieve baseline resolution of amitriptyline and prednisolon, while the best separation was achieved on the Symmetry column.

To determine the utility of a high pH mobile phase, initial gradient input experiments were performed on the Zorbax Extend C18, XTerra MS C8, XTerra Pheny columns using a mobile phase pH of 10.5 and the data were imported into DryLab[®] software. An isocratic separation with baseline resolution was predicted on all three columns at pH 10.5. The experimentally verified separations are shown in Fig. 7. All three columns showed good resolution of the compounds, however the XTerra MS C8 column exhibited the best peak shape.

The results obtained show that separations with baseline resolution of all compounds can be achieved at pH 2.7 and 10.5.

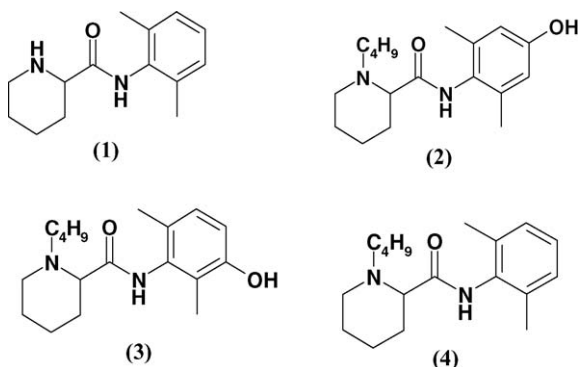


Fig. 1. Structures of desbutylbupivacaine (1), 4'-hydroxybupivacaine (2), 3'-hydroxybupivacaine (3) and bupivacaine (4).

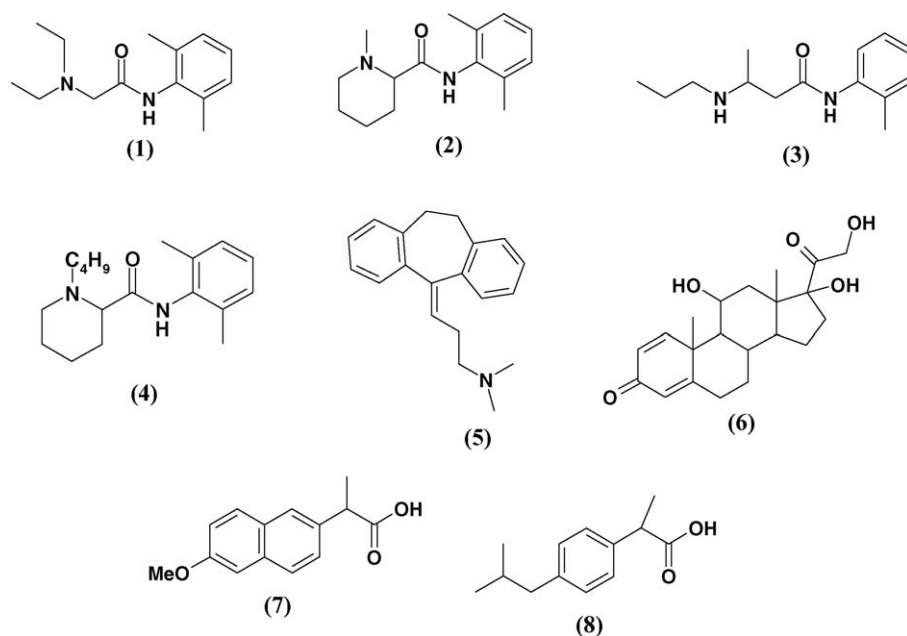


Fig. 2. Structures of lidocaine (1), mepivacaine (2), prilocaine (3), bupivacaine (4), amitriptyline (5), prednisolone (6), naproxen (7) and ibuprofen (8).

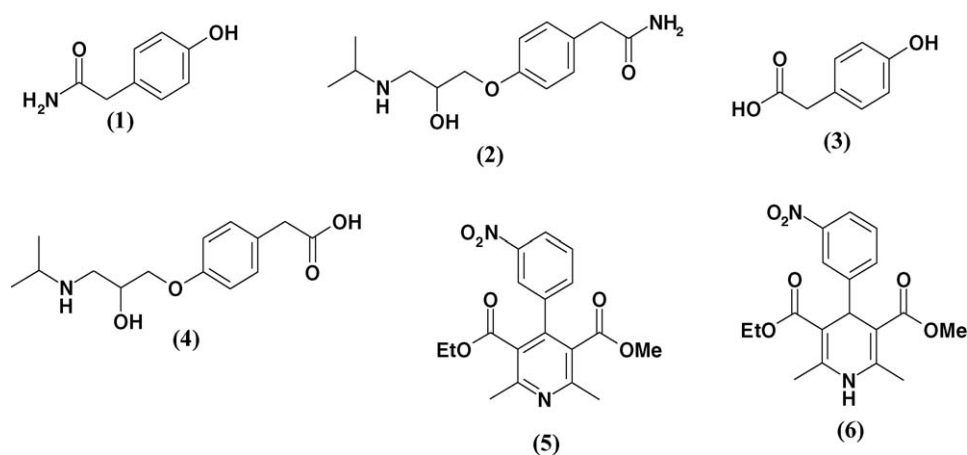


Fig. 3. Structures of *p*-hydroxyphenylacetamide (1), atenolol (2), *p*-hydroxyphenyl acetic acid (3), atenolol acid (4), nitropyridine (5) and nitrendipine (6).

Table 2

Retention time comparison [$r = t_{\text{exp}}/t_{\text{sim}}$] for simulated, t_{sim} , DryLab (Fig. 4) and experimentally, t_{exp} , verified (Fig. 5) separations of (1) desbutylbupivacaine, (2) 4'-hydroxybupivacaine, (3) 3'-hydroxybupivacaine and (4) bupivacaine. Column identifications (a–h) are as described in Fig. 4

	1			2			3			4		
	t_{sim}	t_{exp}	r	t_{sim}	t_{exp}	r	t_{sim}	t_{exp}	r	t_{sim}	t_{exp}	r
	Fig. 4	Fig. 5		Fig. 4	Fig. 5		Fig. 4	Fig. 5		Fig. 4	Fig. 5	
a	3.5	3.1	0.89	4.0	3.4	0.85	4.5	4.0	0.89	18.0	14.1	0.78
b	3.7	2.7	0.73	4.0	2.9	0.73	4.5	3.4	0.76	15.6	13.2	0.85
c	2.9	2.1	0.72	3.2	2.7	0.84	3.6	3.0	0.83	10.1	8.2	0.81
d	3.2	2.3	0.72	3.7	3.0	0.81	4.1	3.4	0.83	6.6	5.9	0.89
e	4.4	3.7	0.84	4.7	3.8	0.81	5.3	4.5	0.85	15.3	13.9	0.91
f	3.2	2.9	0.91	3.4	3.2	0.94	3.9	3.7	0.95	13.2	12.6	0.95
g	3.6	2.6	0.72	3.9	3.8	0.97	4.5	4.4	0.98	12.9	10.6	0.82
h	2.6	2.8	1.08	2.8	3.0	1.07	3.0	3.5	0.86	5.6	10.5	1.88

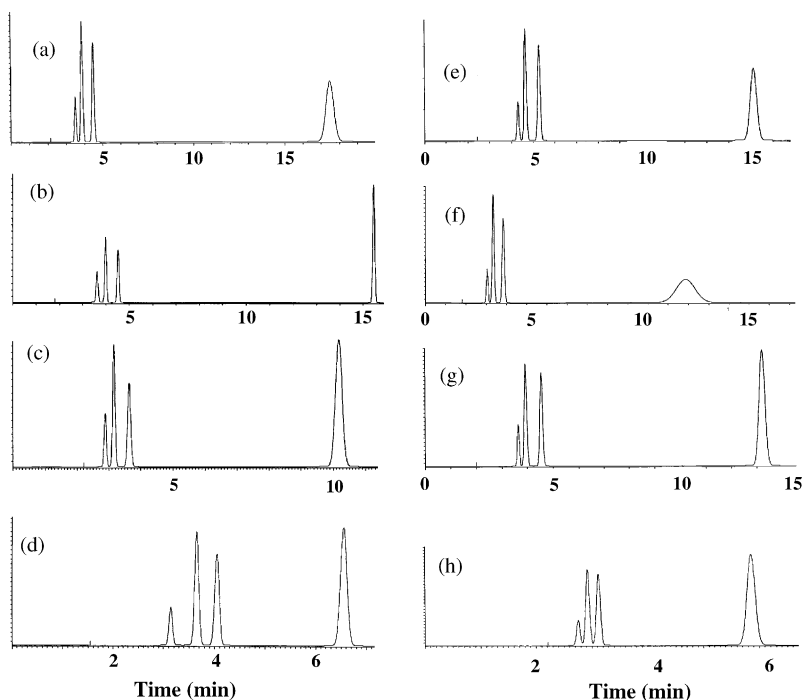


Fig. 4. Simulated DryLab chromatograms of bupivacaine and its desmethyl, 3'-hydroxybupivacaine and 4'-hydroxybupivacaine metabolites on the Prodigy (a), Symmetry (b), Inertsil C8-3 (c), Symmetry Shield (d), Ace C8 (e), Zorbax Eclipse XDB (f), Hypersil BDS (g) and XTerra MS C8 (h) columns.

It should be noted that the peak order changes significantly as a result of the mobile phase pH. This provides valuable information about the specificity of the method, giving the chromatographer greater confidence that no compounds may be co-eluting

with another. Although in the current example this has been performed on a known mixture, the utility of performing the separation will be more fully realized on a sample with an unknown number of compounds.

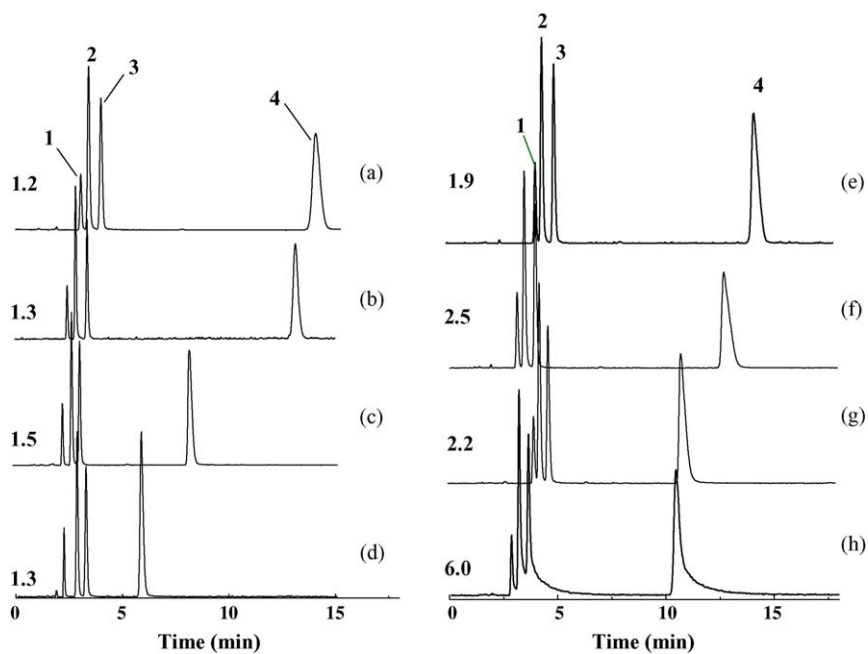


Fig. 5. Experimentally verified isocratic separation of bupivacaine and its desmethyl, 3'-hydroxybupivacaine and 4'-hydroxybupivacaine metabolites using the chromatographic conditions as predicted by DryLab on the Prodigy (a), Symmetry (b), Inertsil C8-3 (c), Symmetry Shield (d), Ace C8 (e), Zorbax Eclipse XDB (f), Hypersil BDS (g) and XTerra MS C8 (h) columns. Peak identities: desbutylbupivacaine (1), 4'-hydroxybupivacaine (2), 3'-hydroxybupivacaine (3) and bupivacaine (4). The USP tailing factor of bupivacaine is indicated on the left hand side of each chromatogram. Peak order is the same for all chromatograms.

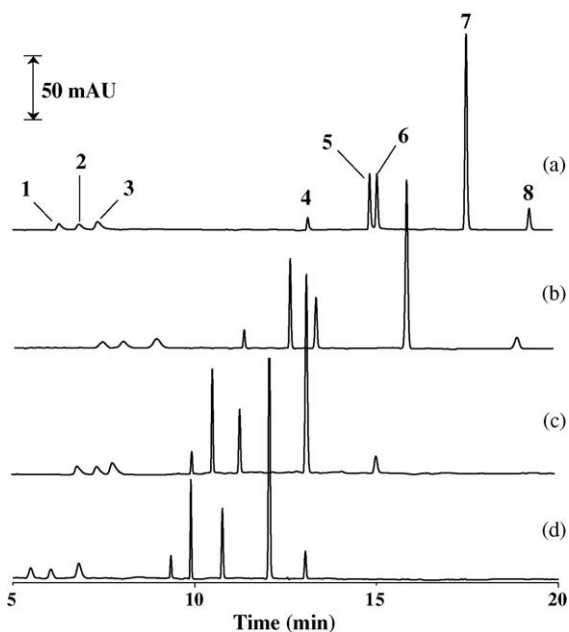


Fig. 6. Gradient chromatograms at pH 2.7 of lidocaine (1), mepivacaine (2), prilocaine (3), bupivacaine (4), amitriptyline (5), prednisolone (6), naproxen (7) and ibuprofen (8) on the XTerra MS C8 (a), Prodigy (b), Inertsil C8-3 (c) and Symmetry (d) columns. Peak order is the same for all chromatograms.

3.3. Atenolol, nitrendipine, and their degradation products

The structures of atenolol, nitrendipine, and their degradation products are shown in Fig. 3. An isocratic separation of this mixture has been previously reported using a complex five component mobile phase with a non-MS compatible buffer [14]. The

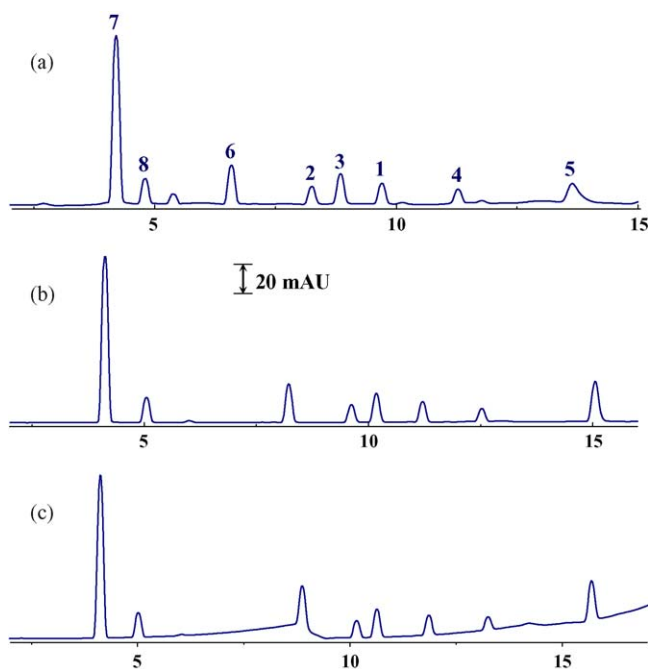


Fig. 7. Gradient chromatograms at pH 10.5 of lidocaine (1), mepivacaine (2), prilocaine (3), bupivacaine (4), amitriptyline (5), prednisolone (6), naproxen (7) and ibuprofen (8) on the Zorbax Extend C18 (a), XTerra MS C8 (b) and XTerra Phenyl (c) columns. Peak order is the same for all chromatograms.

method development strategy was employed to produce a MS compatible separation for this mixture. Although the selectivities of atenolol and nitrendipine are different this separation was particularly challenging because the selectivity of their respective degradation products were not significantly different and they had a tendency to exhibit poor peak shape. The method development strategy was used to make a less complex, MS compatible mobile phase, that would still give good peak shape and acceptable resolution of the compounds. Initial input gradient experiments were performed on the columns shown in Table 1 using mobile phases as described in the experimental section. While DryLab[®] software predicted that an isocratic separation was not possible within 40 min, it did predict that a gradient separation was possible on the Hypersil BDS and Mac-Mod ACE columns using a mobile phase of pH 2.7. The other columns required a long runtime or did not adequately separate the compounds. Chromatograms obtained using the gradient conditions predicted by DryLab[®] software are presented in Fig. 8. The segmented gradient programs for Fig. 8c (gradient programs 1–3, respectively) are given in Table 3.

To determine the utility of a high pH mobile phase, initial gradient input experiments were performed on the Zorbax Extend C18, XTerra MS C8, XTerra Phenyl columns using a mobile phase pH of 10.5 and the data were imported into DryLab[®] software. A gradient separation with baseline resolution was predicted on the Zorbax Extend C18 column using a mobile phase of pH 10.5. The experimentally verified separation is shown in

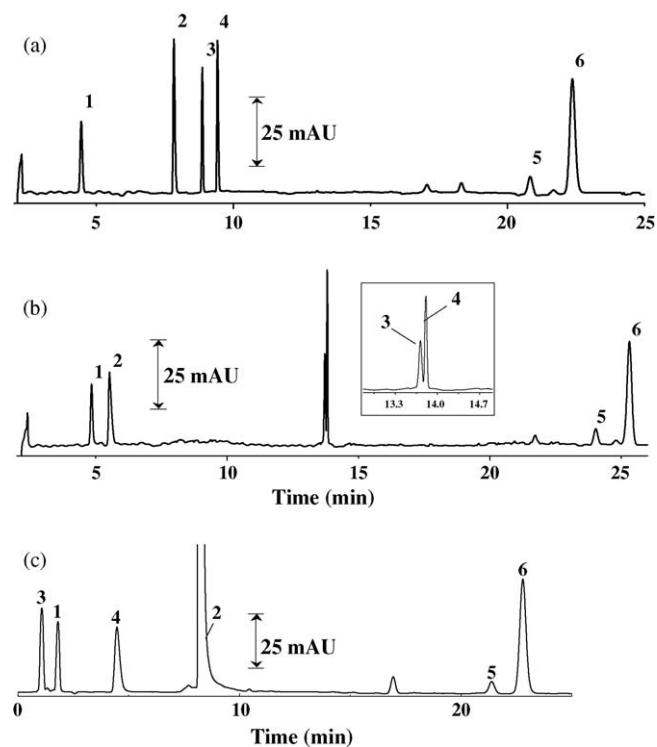


Fig. 8. Gradient chromatograms of atenolol, nitrendipine and their degradation products on a Hypersil BDS (a) and Ace C8 (b) columns at a mobile phase of pH 2.7 and on a Zorbax Extend C18 column at a mobile phase of pH 10.5 (c). Peak identities: *p*-hydroxyphenylacetamide (1), atenolol (2), *p*-hydroxyphenyl acetic acid (3), atenolol acid (4), nitropyridine (5) and nitrendipine (6).

Table 3
Gradient program for Fig. 8a–c

Time (min)	Gradient Program 1 ^a	
	%A	%B
0.00	94	6
3.9	94	6
8.7	58	42
30.0	58	42

Time (min)	Gradient Program 2 ^b	
	%A	%B
0.00	93	7
10.1	93	7
11.4	55	45
30.0	55	45

Time (min)	Gradient Program 3 ^c	
	%A	%B
0.0	94	6
5.0	94	6
7.1	61	39
35.0	61	39

Mobile phase for Fig. 8a and b: A = water containing 0.1% formic acid (pH 2.7) and B = acetonitrile containing 0.1% formic acid. Mobile phase for Fig. 8c: A = water containing 0.1% ammonium hydroxide (pH 10.5) and B = acetonitrile containing 0.1% ammonium hydroxide.

^a Fig. 8a.

^b Fig. 8b.

^c Fig. 8c.

Fig. 8. Adjusting the pH of the mobile phase to 10.5 did not produce a better separation than that at pH 2.7, but it did alter the elution order of the compounds. The separation at pH 10.5 gave greater confidence that all the degradation products of atenolol and nitrendipine were adequately separated.

It is evident that method development strategy was particularly useful for the atenolol/nitrendipine sample since it allowed the efficient development of an MS compatible separation using a simpler three component mobile phase.

3.4. Use of data base to modify separations

The application of the described method development strategy results in the efficient collection of a large amount of data. This data can be subsequently utilized to modify the separation in response to changes in the synthetic pathway and/or formulation or analytical demands on the method. An example of the latter is the determination of minor impurities in drug substances. In order to obtain acceptable quantitation limit for the degradation products, a high mass of the parent drug must be injected into the column. This has the effect of decreasing the resolution of peaks eluting on the tail of the parent peak as illustrated by the separation of atenolol, nitrendipine and their degradation products with atenolol at a concentration of 1 mg/ml. DryLab[®] software was used with previously obtained data to model the required adjustments to accommodate the high mass loading of atenolol in the method without additional laboratory work. The

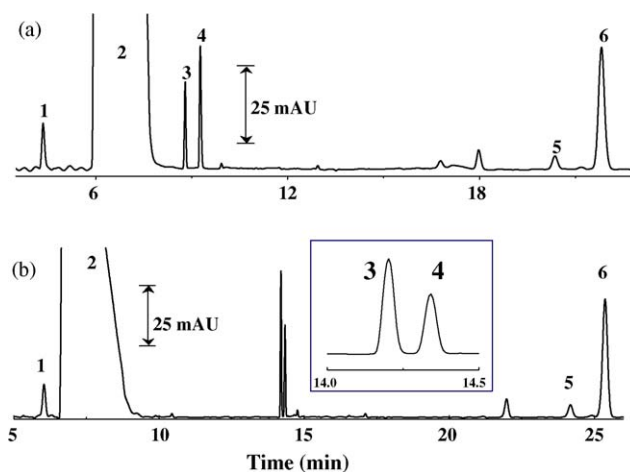


Fig. 9. Gradient chromatograms of atenolol (1 mg/ml), nitrendipine and their degradation products on a Hypersil BDS (a) and Ace C8 (b) columns at a mobile phase of pH 2.7. Peak identities: *p*-hydroxyphenylacetamide (1), atenolol (2), *p*-hydroxyphenyl acetic acid (3), atenolol acid (4), nitropridine (5) and nitrendipine (6).

simulated gradients were then experimentally verified on the Hypersil BDS and Mac-Mod ACE columns (Fig. 9).

4. Conclusions

A strategy has been described that uses an automated column switching system and a series of HPLC columns varying in hydrophobicity and silanol activity, in combination with DryLab[®] software, for the efficient development of robust, specific and MS compatible HPLC methods for mixtures of drugs and/or their metabolites or degradation products.

The main advantage of this column switching strategy over traditional method development practices lies in its ability to generate a large amount of data on several columns in a relatively short period of time. This body of data can be subsequently used with DryLab[®] to modify the separation without additional laboratory work. In addition, the strategy allowed for the efficient development of an equivalent method for the separation of atenolol, nitrendipine and their degradation products with a simplification of the mobile phase from the routine method, thereby improving efficiency of transfer of the method during drug development. In summary, use of the column selection system in conjunction with DryLab[®] software allows the chromatographer to develop a robust, specific and easily modified HPLC method more efficiently than the traditional manual approach.

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