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An automated multidimensional screening approach for rapid method development in high performance liquid chromatography

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Abstract

Despite enormous advancements in the area of high performance liquid chromatography (HPLC) in recent years, method development remains a major challenge. This is primarily due to the unknown nature of the matrix material which sometimes is difficult to characterize (e.g. biological matrices). To improve the efficiency of method development a multidimensional screening approach was presented. This approach was based on two major steps: (1) a matrix spiked with drug was eluted from a large number of columns, each under different mobile phase compositions, to provide the preliminary selectivity–separation information; (2) this information was then used to compose column switching pairs (each pair consisted of a preparatory column followed by an analytical column) and the elution profile was evaluated to determine the suitable clean up and quantitation conditions. An example was provided using ethyl 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (EEDA), an X-ray enhancement agent, in human plasma. Since the HPLC system was fully automated the data generation time, and consequently the method development time, can be significantly reduced. © 1997 Elsevier Science B.V.

Keywords: Rapid method development; HPLC; Pharmaceuticals; Human plasma; Drug

1. Introduction

High performance liquid chromatography (HPLC) as a major bioanalytical tool has seen enormous advancements in recent years [1]. These include sophisticated instrumentation, and a wide range of new stationary and mobile phases for a variety of applications. However, method development still remains a major challenge with respect to the efficiency by which the separation mode and experimental conditions are

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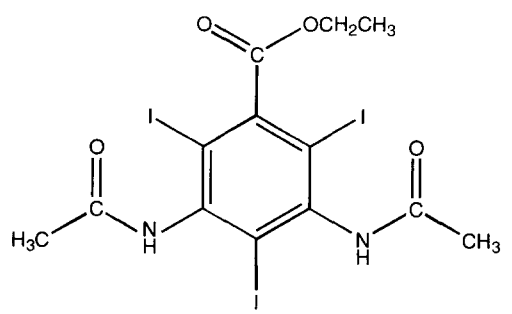
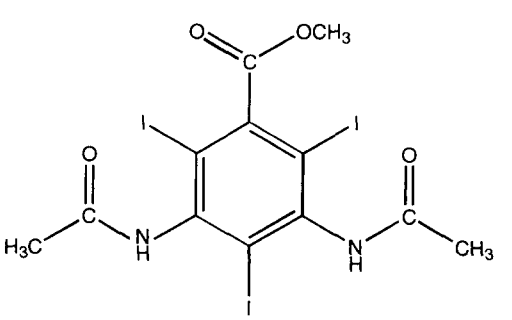
determined. Traditionally, method development relies heavily on modification of existing methods for compounds that have similar structures to the analyte(s) under investigation. However, the existing methods may not represent optimal conditions since many were developed without systematic evaluation of various combinations of experimental conditions. Furthermore, although there is a large body of information in the literature pertaining to a large number of known compounds it is often difficult to develop methods for new classes of compounds where little is known about the separation chemistry of the agents. Perhaps the most difficult element of method development, and probably the most important, is the characterization of matrix material in terms of its selectivity difference from the analyte as a function of experimental parameters.

There have been outstanding contributions from Snyder et al. [2] to systematic method development in various separation modes. These authors proposed detailed strategies to guide step-by-step method development activities. However, the determination of the separation mode, the first step in the method development, received limited attention which may reflect insufficient characterization of the matrix material. In addition, the proposed strategies operated on a sequential basis, i.e. evaluation of experimental parameters one at a time, resulting in low efficiency. Nevertheless, these strategies have been and will continue to be valuable in method development especially when a method is fine tuned. Recently, Kirkland reported a practical method development strategy for reversed-phase HPLC of ionizable compounds [3] in which the experimental variables and logistics are proposed in detail. Although intended for ionizable compounds the concept of the strategy is applicable to other classes of compounds. In addition to traditional wet chemistry, computer simulation [4,5] and chemometrics [6,7] are also used in method development to improve efficiency.

In this paper an automated multidimensional screening approach is presented for HPLC method development with an aim at a more systematic and structured process to rapidly target the suitable method conditions. This approach involves screening of columns with different mobile phase compositions (e.g. varying organic modifiers and pH). The resultant information is used to guide the selection of the sample clean up scheme, the analytical column, and other experimental conditions. The advantage of this approach is that it rapidly evaluates the characteristics of the matrix material and analyte under different chromatographic conditions. Therefore, a suitable method would be quickly obtained. An X-ray enhancement agent, ethyl 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (Fig. 1), was used to illustrate the basic steps involved in this approach. This example shows a relatively simple yet quick screening process. More parameters can be added to the screening process to obtain optimal methods.



(A)



(B)

Fig. 1. Structures of EEDA (A) and methyl 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (internal standard) (B).

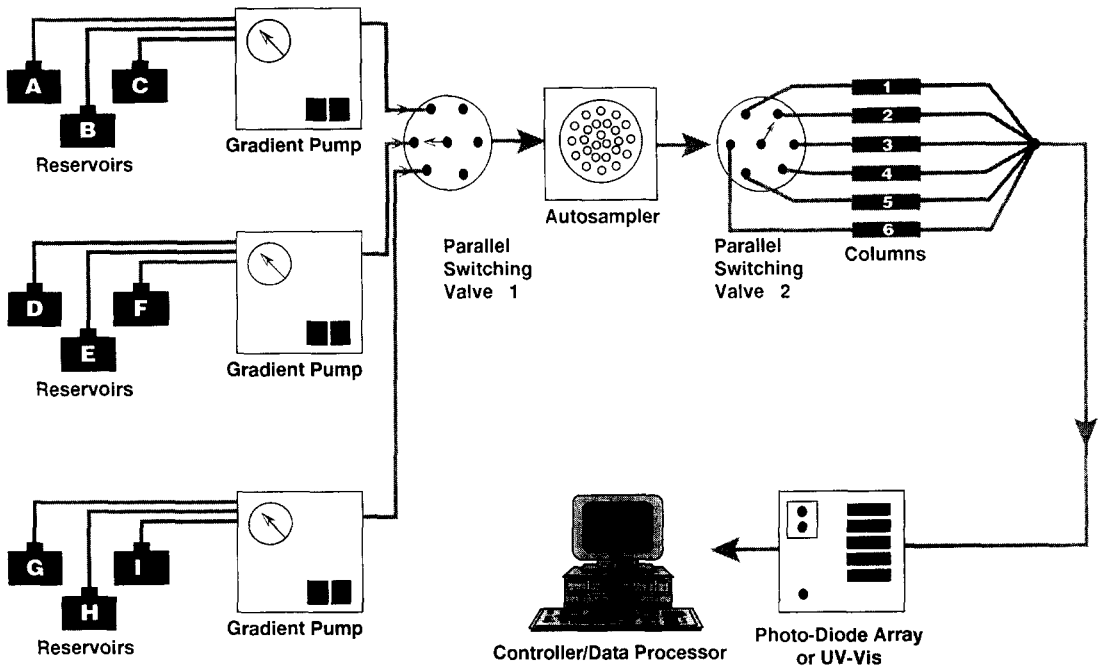


Fig. 2. Diagram of instrument layout for the initial screening of the column and mobile phase. The solid arrows point in the flow direction. The half solid arrows in the parallel switching valves point at the column that is connected to the flow path, and the pump that is selected to deliver a gradient. Columns 1–6 were tested by injecting human plasma spiked with EEDA and gradient elution with various compositions based on reservoirs A–I. See the text for more detailed descriptions of each component, the overall operation of the system and the study design.

The major components of the HPLC system are three gradient pumps, an autosampler, 2 parallel and 1 serial switching valves, a photodiode array detector (PDA), and a computer loaded with an operating software. The system can be configured in a variety of ways but there are two basic configurations (Figs. 2 and 3) corresponding to two major steps in the method development.

1.1. Step one: screening columns and mobile phase compositions

The instrument layout is shown in Fig. 2. Each gradient pump is provided with three mobile phase reservoirs, two of which are used to deliver a linear gradient after a given injection. Six columns are attached to a parallel switching valve, any one of which can be used for a given injection. When an injection is made by the autosampler into a selected column a gradient with desired composition is delivered through parallel switching valve 1 by the pump activated; the PDA monitors the elution profile and the data are recorded.

Typically several injections of drug-spiked matrix are made into each column under various mobile phase compositions to compare the mobile phase effect on separation. The column assembly can be expanded to accommodate additional columns (6 columns per additional valve) by adding parallel switching valves in series. Upon completion of the run the large number of chromatograms form a 'multidimensional map' from which the affinity (retention time) of the matrix material and analyte for different packing can be evaluated and compared under varying mobile phase compositions. In practice the elution profiles are compared as a function of one variable at a time. For example, chromatograms can be evaluated as a function of column packing while the

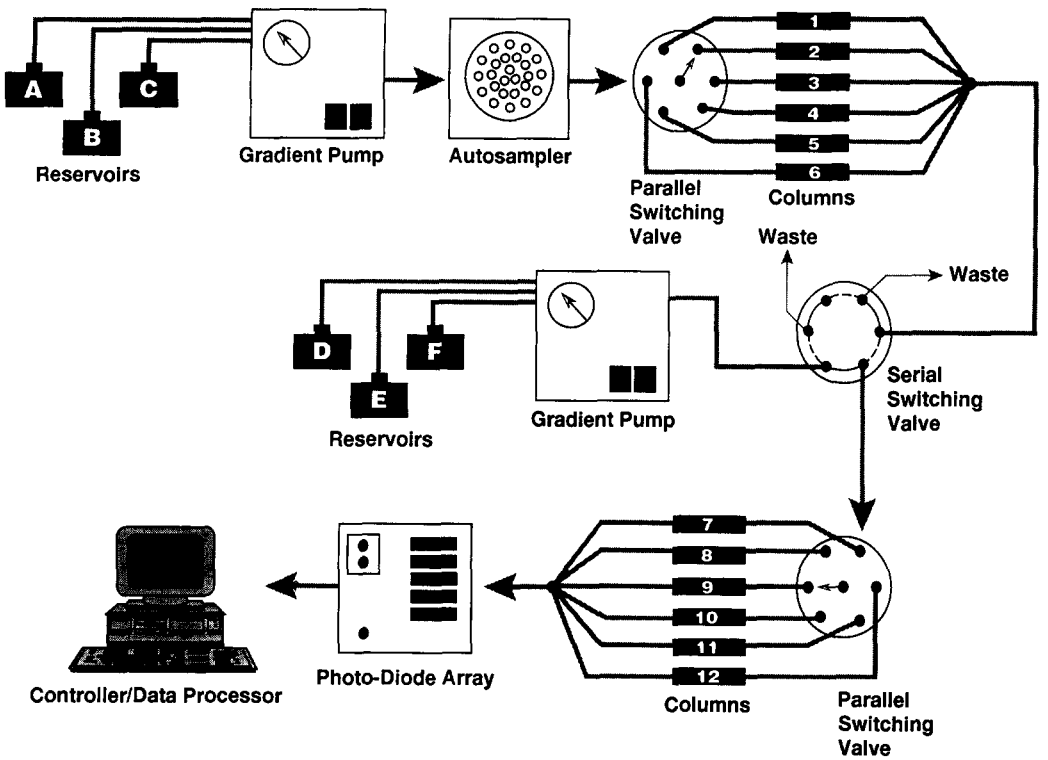


Fig. 3. Diagram of instrument layout for column switching screening. The solid arrows point in the flow direction. The half solid arrows in the parallel switching valves point at the columns that are connected to the flow path. Any column in the first group (1–6) could be paired with any column in the second group (7–12) to form a unique combination of column switching. See the text for more detailed information concerning the overall operation and study design.

mobile phase composition is fixed. Once the data are analyzed the candidates for the analytical column are selected based on the resolution, peak shape etc. The candidates for the preparatory column are identified based on their selectivity differences from the analytical columns and the amount of matrix materials in the analyte region; the mobile phase composition is determined for each candidate column based on selectivity and mobile phase compatibility between the analytical and preparatory columns.

1.2. Step two: screening combinations of column switching pairs

The instrumentation layout is shown in Fig. 3. Two groups of columns, preparatory (columns 1–6) and analytical (columns 7–12), are configured in series through a serial switching valve that directs the flow from the preparatory group to either waste or the analytical group. Within each group the parallel switching valve determines the column to be connected to the flow path for a given injection. Through computer control of these valves any column from the first group can be coupled with any one from the second group to form a unique column switching pair. The gradient pump before the autosampler delivers mobile phase solutions A–C to the preparatory group while another pump delivers D–F to the analytical group. The entire operation is synchronized by the computer. During the elution in a preparatory column the serial switching valve directs a slice of the elution profile contain-

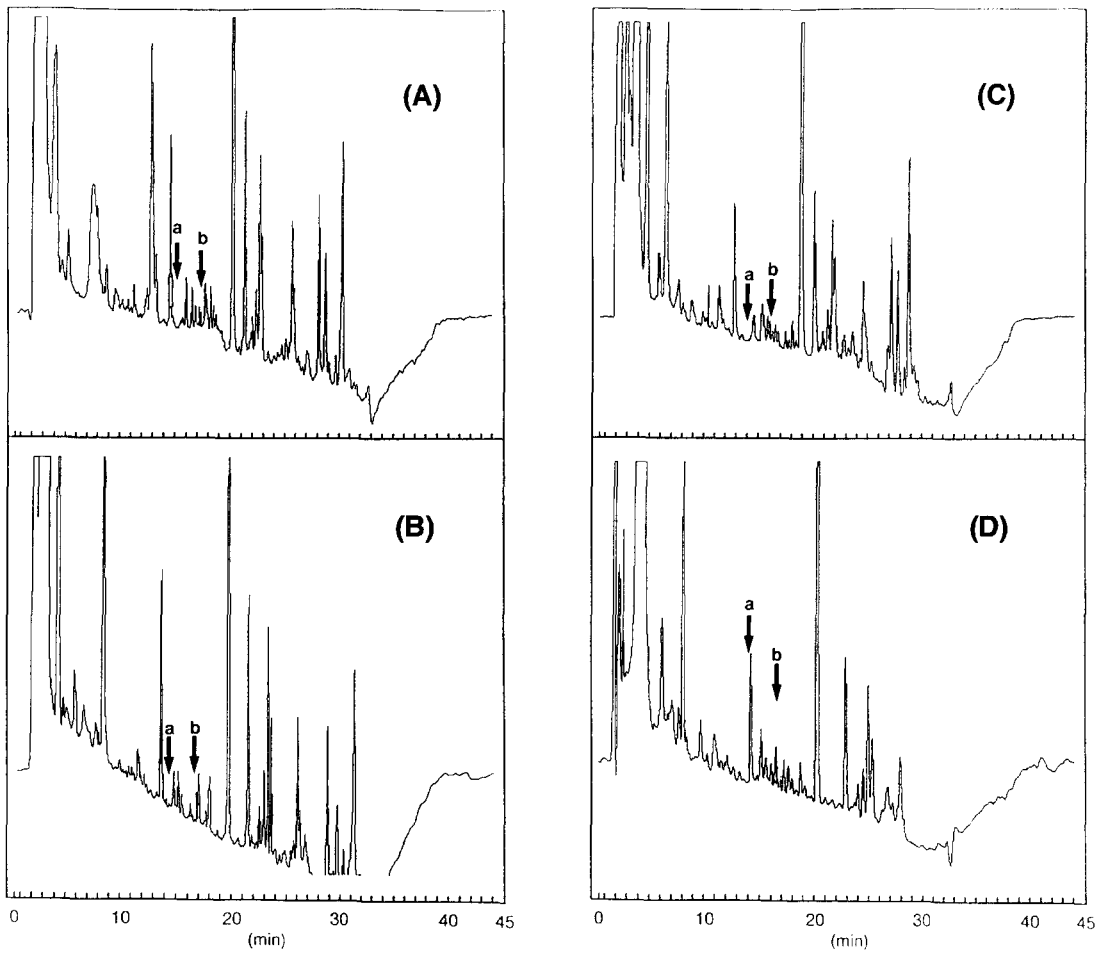


Fig. 4.

ing the analyte to an analytical column where another gradient elution takes place. The resultant chromatogram represents a clean up by the first column followed by an analytical elution in the second column. At the end of the run all the chromatograms corresponding to various switching pairs are compared to decide which combination offers the best clean up. Based on the gradient elution profile the suitable isocratic elution conditions (on both analytical and preparatory columns) are readily obtained.

2. Experimental

2.1. Equipment and materials

Tetrahydrofuran (THF), methanol (MeOH), acetonitrile (ACN), water, all HPLC grade, and sodium hydroxide (10 N), Baker analytical grade, were from J.T. Baker (Phillipsburg, NJ); phosphoric acid (85%) and formic acid (96%), both ACS reagent grade, were from Aldrich (Milwaukee, WI).

Spherex C-1, C-6 and CN columns, a Bondclone C-18 column, a Zorbax amino column and an

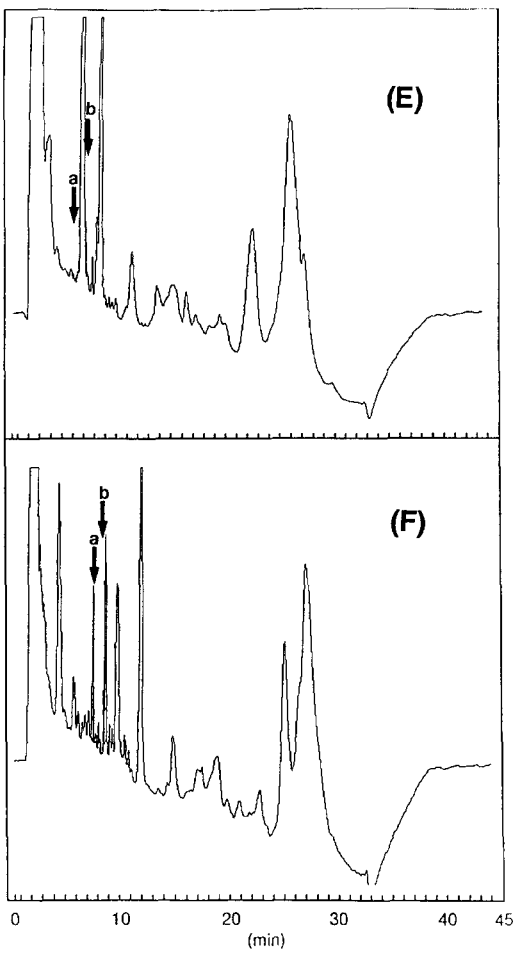


Fig. 4. Chromatograms (239 nm) of ACN extracted human plasma obtained under a linear gradient (5–50% ACN in 27 min, pH 3.0 formate as aqueous mobile phase) from the phenyl (A), C-6 (B), CN (C), C-18 (D), C-1 (E) and amino (F) columns. The full scale of absorbence (vertical axis) was identical for all the chromatograms. The arrows point to the elution positions of internal standard (a) and EEDA (b). The elution positions were identified based on additional injections of standards and matrix spiked with standards. See Section 2 for more details.

IB-SIL phenyl column, all 150 mm × 4.6 mm, 5 μm, were from Phenomenex (Torrance, CA).

The HPLC system consisted of three Varian 9010 gradient pumps, a Varian 9100 autosampler, a Varian 9065 Polychrom photodiode array detector, Rheodyne switching valves, and a Compaq 486 DX2 workstation running Varian Star software.

Control human plasma, with EDTA as the anticoagulant, was from Rockland (Gilbertsville, PA). Ethyl 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (EEDA) and methyl 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (internal standard, Fig. 1) were synthesized at Nycomed R&D, (Wayne, PA).

2.2. Study design

Three ml ACN were added to 0.5 ml of human plasma (already spiked with EEDA and internal standard) followed by centrifugation to remove the proteins. After the supernatant was evaporated, the residue was reconstituted with 150 ml of MeOH–water (1:5) prior to injection (100 μl) onto the HPLC system.

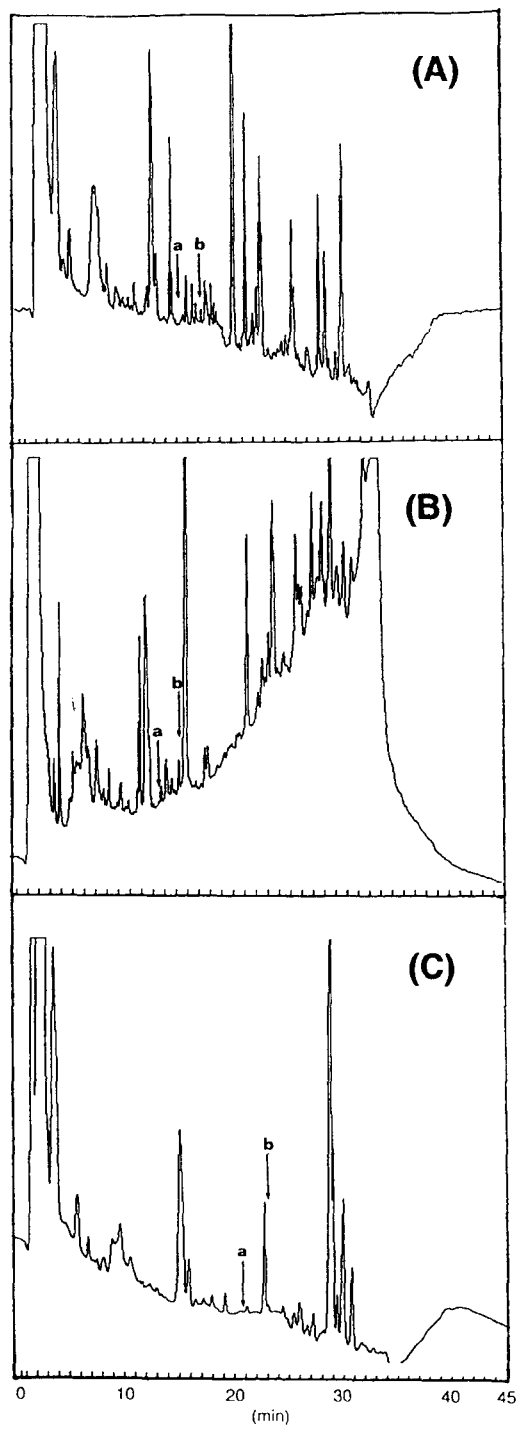


Fig. 5.

2.2.1. First run

The instrument configuration is shown in Fig. 2. Mobile phase reservoirs C, F and I were charged with ACN, MeOH and THF, respectively; reservoirs A, D, and G contained 25 mM formate buffer, pH 3.0; reservoirs B, E, and H were filled with 25 mM phosphate buffer, pH 7.2. The organic modifiers selected in this study exemplified the most common applications, while the pH values chosen here represented both acidic and neutral pH regions. Columns 1–6 were phenyl, C-6, C-1, amino, CN, and C-18, respectively, representing the most common sorbents. A standard binary linear gradient profile was used from 5 to 50% organic modifier in 27 min at a flow rate of 1 ml min^{-1}. The detection wavelength range was 190–390 nm. Each column was tested under the standard gradient profile with various mobile phase compositions, including different organic modifiers, and varying pH of buffer solutions. Under a given set of conditions (column, pH and organic modifier) three injections were made including: neat drug and internal standard, matrix alone, and matrix spiked with drug and internal standard, in that order, to locate the elution position of the drug and internal standard with respect to the matrix. Altogether there were 108 injections made in this run: 3 injections (replicates to confirm reproducibility) × 6 columns × 3 organic modifiers × 2 pH values.

2.2.2. Second run

Based on the results obtained from the first run, C-1 and amino columns under MeOH and pH 3.0 (formate) were identified as the preparatory column candidates and phenyl and C-6 columns

Fig. 5. Chromatograms (239 nm) of ACN extracted human plasma obtained from a phenyl column under linear gradient elutions from 5 to 50% ACN (A), THF (B) and MeOH (C) with pH 3.0 formate, in 27 min. The full scale of absorbence (vertical axis) was identical for all the chromatograms. The arrows point to the elution positions of internal standard (a) and EEDA (b). The elution positions were identified based on additional injections of standards, and matrix spiked with standards. See Section 2 for more details.

under ACN and pH 3.0 as the analytical column candidates. The instrument configuration was virtually the same as that shown in Fig. 3 with the exception that only 2 columns were included in each column group. The standard gradient profile was used for both preparatory and analytical columns. There were 4 switching combinations: C-1 to C-6, C-1 to phenyl, amino to C-6 and amino to phenyl.

2.2.3. Isocratic run

Based on the gradient elution profiles the mobile phase composition for isocratic elution was determined. In this study amino and phenyl columns were selected to be the preparatory and analytical columns, respectively. The mobile phase compositions were 40% MeOH in pH 3.0 formate buffer for the amino column and 30% ACN in pH 3.0 formate buffer for the phenyl column, both at 1 ml min^{-1}. During the elution on the analytical column the amino column was washed with 80% MeOH for 3 min followed by a 5 min equilibration to the initial mobile phase conditions. The detection wavelength was 239 nm.

2.2.4. Validation run

A linear calibration curve consisted of 50, 100, 200, 400, 600, 800 and 1000 ng ml^{-1} was used, with three replicates at both ends and singlets in between.

Peak area ratio (EEDA to internal standard) was plotted versus nominal concentration. A weighting of $1/Y^2$ was used. Samples were analyzed at 50, 100, 250, 500, 800 and 1000 ng ml^{-1}, each with six replicates.

3. Results and discussions

3.1. First run

The objective of this run was to compare the difference in selectivity between the matrix material and analyte as a function of column chemistry and mobile phase composition. To consolidate the large number of chromatograms generated in this run (total 36 chromatograms) into a manageable group of figures, and to avoid redundant presentations, only the representative figures that illustrate the important points will be presented in this paper. The effect of column chemistry was first examined using a given mobile phase composition (pH 3.0 aqueous, ACN as organic modifier) as an example. The chromatograms are shown in Fig. 4. The columns, in terms of the analyte affinity, can be divided into two groups: phenyl, C-6, CN and C-18 as the higher affinity group with retention times in the 14–18 min range and C-1 and amino as the lower affinity group with retention times in the 6–9 min range. Within each group the general features of the elution profile were similar, based on PDA analysis of the major peaks, but the profiles were significantly different between the two groups. The resolution was comparable within each of the following pairs: phenyl and C-6, CN and C-18, and C-1 and amino.

The drug peak height ratio of (phenyl or C-6):(CN or C-18):(C-1 or amino) was 2.4:1.9:1.0. Virtually the same observations were made when the column chemistry was examined under other mobile phase compositions (THF, MeOH, as organic modifier, pH 7.2 aqueous). Secondly the effect of organic modifier on separation was evaluated using a phenyl column at pH 3.0 as an example. MeOH eluted much less matrix material in the chromatogram (Fig. 5) which makes it an attractive choice for the preparatory column. On the other hand, the peak height of drug eluted by MeOH was about 50% shorter (due to a broader elution band) than that by ACN and THF, which would compromise sensitivity in an analytical column. The baseline drift at 239 nm was found to be more sensitive to the THF gradient. The same trend was also found with other columns under pH 7.2. Finally, the effect of pH on the separation was determined using a phenyl column and ACN as an example. Neutral pH seemed to compress the profile toward void volume (Fig. 6) making the chromatogram more crowded. A relatively smaller amount of matrix material was eluted at pH 3.0, as shown in the chromatogram (Fig. 6). Similar results were obtained from other columns and organic modifiers as well. Based on these observations C-6 and phenyl columns were selected as the candidates for the analytical column due to their higher resolution and larger

peak height; C-1 and amino were selected as the candidates for the preparatory column because of the relatively weak mobile strength required for elution which offered the benefit of peak focusing when the analytes were switched to the analytical column. In addition, C-1 and amino showed significantly different elution profiles compared to the rest of the columns suggesting the greatest selectivity difference desired for column switching. MeOH was selected as the organic modifier in the preparatory column as a result of a relatively cleaner elution profile while ACN was chosen for the analytical column based on peak shape; both were coupled with pH 3.0 formate buffer for the gradient. Based on the results from the first run there were other viable choices as well, but it was important at this point to balance between the large number of options and the manageable size of the experiment.

Design of the first screening run is important to the discovery of the preliminary selectivity difference between matrix material and analyte under different conditions. Although in this study representative organic modifiers and pH values were evaluated other selections of mobile phase compositions are certainly feasible depending on the compound and matrix under investigation and the columns to be evaluated. By the same argument a variety of columns can be tested in addition to the ones used in this study. In principle it is desirable to evaluate as many columns and mobile phase compositions as possible provided the run is within a controllable size.

The gradient profile of 5 to 50% organic modifier in 27 min used in this study was equivalent to a 60 min gradient from 0 to 100% organic modifier. Experience has shown that this gradient slope would separate most of the multi-components (drug in plasma) into at least shoulders or split peaks if they were practically separable. This likelihood was suitable for initial evaluation of the selectivity in biological matrices. Caution should be taken when large molecules are analyzed or proteins are not removed prior to chromatography because the selectivity of these compounds are more dependent on gradient slope [8]. Under these circumstances typically more than one gradient slope would be used [8] to probe selectivity. The gradient offers a more complete elution profile including the portion of matrix material that otherwise would be absorbed in the column under isocratic conditions. Thus, the late eluters can be identified early on and dealt with in the method development. However, the final concentration of organic modifier should be limited to a certain level so that the run time can be reduced.

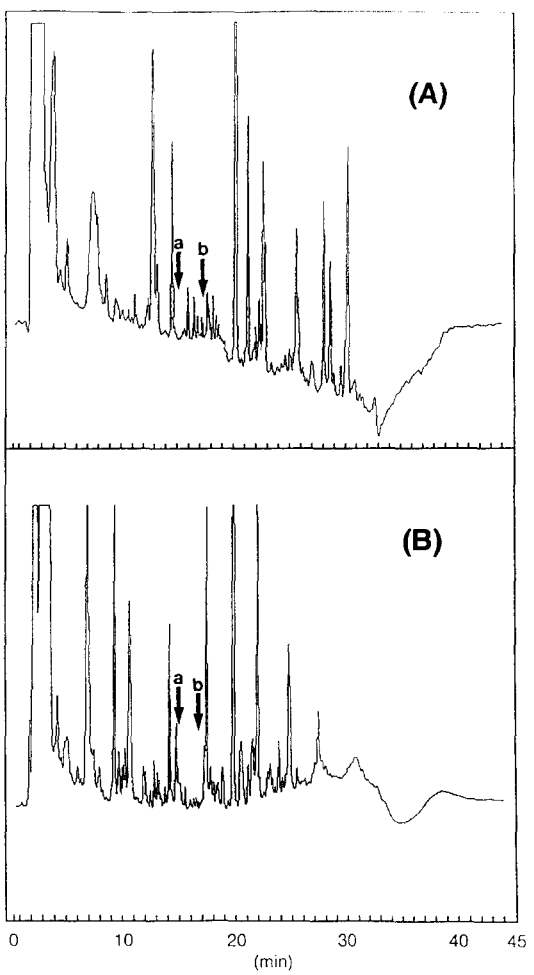


Fig. 6. Chromatograms (239 nm) of ACN extracted human plasma obtained from a phenyl column under linear gradients from 5 to 50% ACN in pH 3.0 formate (A), and pH 7.2 phosphate (B) buffers, in 27 min. The full scale of absorbence (vertical axis) was identical for all the chromatograms. The arrows point to the elution positions of internal standard (a) and EEDA (b). The elution positions were identified based on additional injections of standards, and matrix spiked with standards. See Section 2 for more details.

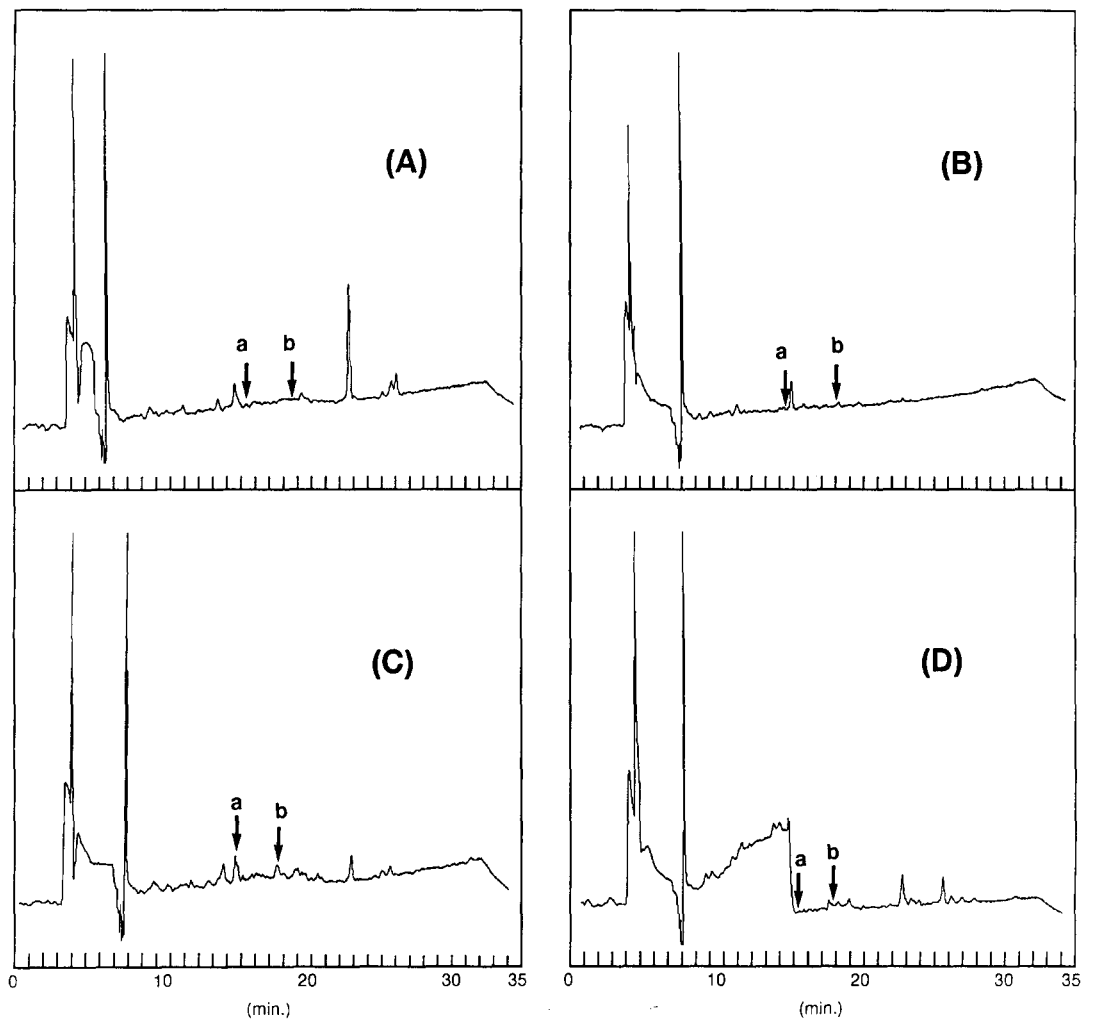


Fig. 7. Chromatograms (239 nm) of ACN extracted human plasma obtained from column switching pairs: amino to phenyl (A), C-1 to C-6 (B), amino to C-6 (C) and C-1 to phenyl (D). The linear gradients were delivered from 5 to 50% MeOH (preparatory column) or ACN (analytical column) in pH 3.0 formate buffer, in 27 min. The full scale of absorbence (vertical axis) was identical for all the chromatograms. The arrows point to the elution positions of internal standard (a) and EEDA (b). The elution were identified based on additional injections of standards and matrix spiked with standards. See Section 2 for more details.

3.2. Second run

Based on the results from the first run four switching combinations were proposed, including C-1 to C-6, C-1 to phenyl, amino to C-6 and amino to phenyl (experimental conditions were described in Section 2). The chromatograms obtained based on these combinations are shown in Fig. 7. Significant interfering peaks were found to overlap with the internal standard and drug when amino to C-6 was employed while in the cases of C-1 to C-6 and C-1 to phenyl relatively smaller interfering peaks were found to overlap with the drug. The best combination was amino to phenyl where there was virtually no interference in the drug region and little or no interference in the internal standard region. Therefore the amino to phenyl was selected as the switching configuration.

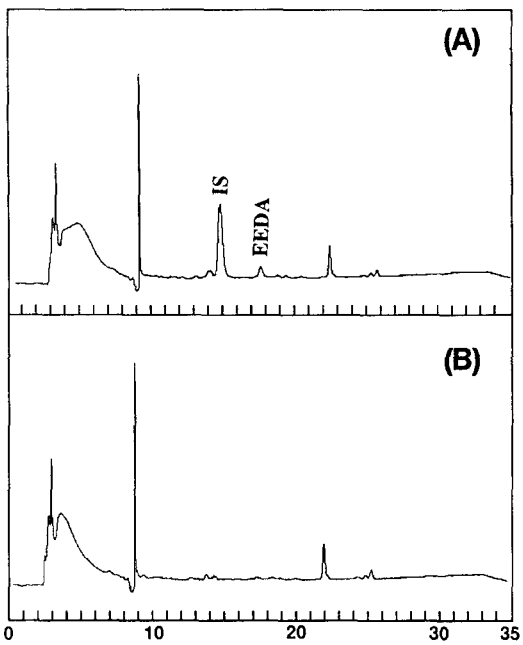


Fig. 8. Chromatograms of human plasma spiked with EEDA (50 ng ml^{-1}) and the internal standard (IS) (200 ng ml^{-1})(A) and human plasma blank (B). Elution was isocratic. Other experimental conditions are described in Section 2.

It was anticipated that when isocratic elution was applied some of the other switching combinations might also become practically feasible due to improved separations. However, the selection process needed to be narrowed down in order to effectively reach a definitive end point. Other combinations certainly could serve as back up options.

Table 1
Accuracy and precision of the validation run (EEDA in human plasma)

Nominal (ng ml^{-1})	Measured (ng ml^{-1})	M%N[a] ($n=6$)	%CV[b] ($n=6$)
50	48.6	2.8	7.6
100	93.5	6.5	5.3
250	243	2.8	3.8
500	482	3.6	5.4
800	786	1.8	2.8
1000	1008	0.8	3.9

[a] Mean % difference from nominal.
[b] Coefficient of variance.

3.3. Isocratic run

Organic strength was chosen for both amino and phenyl columns based on the gradient elutions earlier (estimated percent of organic modifier corresponding to analyte elution) and further fine tuning (several isocratic elutions varying percent of organic modifier). Suitable chromatograms based on isocratic elutions from amino followed by phenyl are shown in Fig. 8. No significant interference was observed in the analyte regions. The actual run time was about 19 min but the chromatograms in Fig. 8 were extended to show late eluters. The late eluters did not interfere with the next injection because they came off the column (approximately 7 min post injection) well ahead of the elution of the internal standard (approximately 15.5 min).

3.4. Validation run

Accuracy and precision were assessed based on human plasma spiked with EEDA and the internal standard. A linear calibration curve in the range 50–1000 ng ml^{-1} was used for quantitation. The regression coefficient was 0.9999. The quantitation data is given in Table 1. The coefficient of variation (CV%) and mean difference from nominal were less than 8%.

Information from the first and second runs can also be used to select initial conditions for sample preparation using solid phase extraction although the option was not pursued in this study.

In addition to the example provided in this paper, the multidimensional screening approach for method development has been successfully applied to several different methods. The time required to develop a method varies depending on factors such as minimum quantifiable level (MQL) required, structural similarities between the analyte and the matrix interference, experience level of the scientist, etc. In general a reduction of greater than 50% in method development time is anticipated based on our experience.

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