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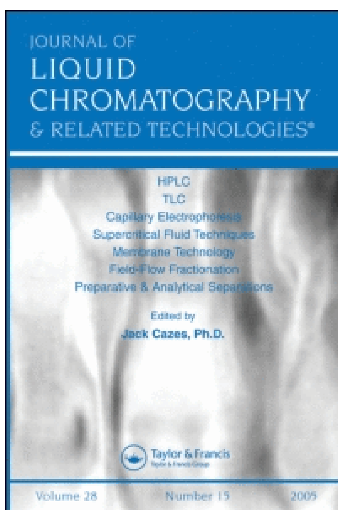

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Versatile Multidimensional Chromatographic System for Drug Discovery as Exemplified by the Analysis of a Nonpeptidic Inhibitor of Human Leukocyte Elastase

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VERSATILE MULTIDIMENSIONAL CHROMATOGRAPHIC SYSTEM FOR DRUG DISCOVERY AS EXEMPLIFIED BY THE ANALYSIS OF A NONPEPTIDIC INHIBITOR OF HUMAN LEUKOCYTE ELASTASE

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ABSTRACT

A direct injection method for the determination of nonpeptidic inhibitors of human leukocyte elastase in plasma, using column switching techniques, has been developed. In the technique, a heart-cut fraction is collected from a size exclusion column to separate the drug and metabolites from the plasma protein. Peak compression, concentration and additional purification are performed by a collection column. The components are flushed forward onto an analytical column for the separation of the drug component from the metabolites. The parallel processing of the technique gives it a number of advantages over conventional extraction procedures and many column switching procedures in terms of speed and effort required in the analysis of samples. The technique's advantages and the precision and accuracy of the assay are discussed.

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INTRODUCTION

In the development of pharmaceuticals, an analysis for the drug in plasma is generally required. High-performance liquid chromatography (HPLC) is one of the most common methods for analyzing these drugs. The organic modifiers in most conventional HPLC mobile phases require the removal of proteins from the plasma or serum before analysis, in order to avoid the precipitation caused by denaturing the protein. Commonly, a pretreatment step is performed using liquid or solid phase extraction procedures to remove the protein before HPLC analysis. These procedures are laborious and introduce additional sources of errors. Efforts to automate solid phase extraction techniques have met with some success using robotics and commercial instrumentation.[1] However, the expense, set-up time and labor involved has limited the general application.

Column switching techniques that allow for direct analysis of plasma or serum without a separate pretreatment step are being used more frequently. There have been a number of review articles that discuss applications and configurations for column switching.[1-4] Problems encountered with the direct injection of plasma onto HPLC columns are rapid clogging, accumulation of proteinaceous materials and long equilibration times. These problems often require frequent column changes or larger column particles that deteriorates performance.[5-8]

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Probably the most common column switching configuration is the use of alkyl-bonded phases for the pre-column. The drug is adsorbed onto the column and the proteinaceous material is allowed to pass through the column. This approach generally results in the need for higher concentrations of organic modifiers to remove the drug (increasing the probability of protein precipitation) or requires back-flushing (which can increase the equilibration times). Using this approach, proteinaceous material can accumulate on the column resulting in the deterioration of the column's performance.

Hagestam and Pinkerton[9] introduced internal surface reversed-phase (ISRP) supports having a hydrophobic internal surface and a hydrophilic outer surface. The concept was that the proteinaceous material would be excluded from the internal surface avoiding the adsorption problem and allowing for the direct analysis of plasma samples on a single column. There are limitations on the amount and types of organic modifiers that can be used with the ISRP approach. This may be one reason why the ISRP technique has not been more widely used. Shintani[10] used an ISRP column as a pre-column in a multidimensional chromatography system. Back-flushing was still necessary to recover the drug, but the accumulation of proteinaceous material was not reported.

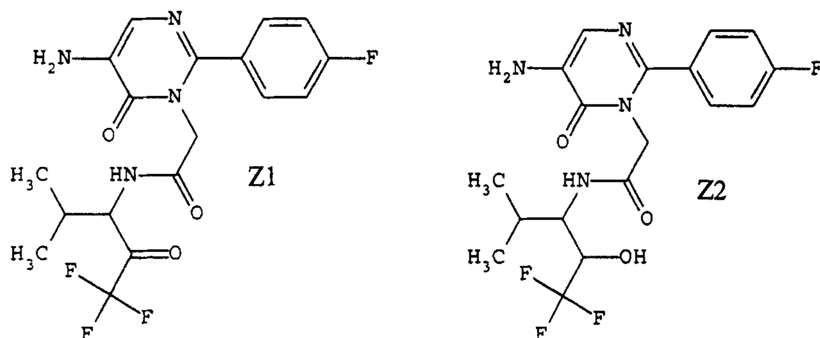

Figure 1. Structure of parent compound (Z1) and its major metabolite (Z2).

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Few papers[11,12] have been found where size exclusion columns (SEC) were used as a pre-column in multidimensional chromatography systems. The use of SEC to separate proteinaceous material from drugs in plasma would avoid many of the problems previously discussed. The author of this paper has used SEC as a pre-separation column for a number of development drugs.

In this paper, the use of SEC as a pre-separation technique in the assay of a development drug will be discussed. The column switching configuration to be discussed is very versatile and has been used for a number of different types of exploratory drug candidates. This configuration was used extensively in the evaluation of the nonpeptidic inhibitors of human leukocyte elastase (NIHLE).[13-15] This paper illustrates the column switching configuration and exemplifies its performance with one of the NIHLE compounds evaluated. The example compound is presented in Figure 1 and the synthesis has been previously reported.[15]

EQUIPMENT AND MATERIALS

Chemicals

Z1 and a mixture of the erythro and threo alcohols, Z2 (major metabolite), were obtained from the Medicinal Chemistry Department of ZENECA Pharmaceuticals (Wilmington, DE, USA). Acetonitrile, methanol and water were all HPLC grade and obtained from J. T. Baker (Phillpsburg, NJ, USA). The potassium phosphate, monobasic was Ultrapure Pure and the potassium hydroxide (for pH adjustment) was ACS reagent grade; both were obtained from J. T. Baker (Phillpsburg, NJ, USA). Tetra butyl ammonium phosphate (TBAP)

was prepared in-house from a 40% solution of tetra butyl ammonium hydroxide obtained from Aldrich Chemical Company (Milwaukee, WI, USA). A 0.25 M solution of tetra butyl ammonium hydroxide was adjusted with 0.2 M phosphoric acid (ACS reagent grade J. T. Baker, Phillpsburg, NJ, USA) to a pH of 7.5. The TBAP solution was purified by passing the solution through an ODS column at 1 mL/min.

Instrumentation

The main HPLC system consisted of a Hewlett-Packard (HP)1090M with ChemStation and column switching valve. Additional equipment used included two HP 1050 programmable HPLC pumps, a 1050 variable UV detector (set at 306 nm), a second six port switching value (Rheodyne Inc., Cotati, CA, USA) and a HP 3350 data acquisition system. A Hewlett-Packard 1090L HPLC, HP 1050 variable UV detector (set at 306 nm) and HP 3350 data acquisition systems were used for the non-column switching comparison. All Hewlett-Packard equipment was obtained from Hewlett-Packard Company (Wilmington, DE, USA).

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The following columns, as indicated in Figure 2, were used: Two optional conditioning columns (C1 & C5) 5 µm, 150 mm x 4.6 mm I.D., C_{18} Rainin Instrument Co. (Ridgefield, NJ, USA), one collection column (C4) 7 µm, 15 mm x 3.2 mm I.D., RP-18 Brownlee (San Jose, CA, USA), one size exclusion column (C3) 10 µm, 300 mm x 7.5 mm, TSK-125 BioRad (Richmond, CA, USA) with a guard column (C2) 7 µm, 15 mm x 3.2 mm I.D., diol Brownlee (San Jose, CA) and one analytical column (C6) 5µm, 250 mm x 4.6 mm, LC-18 Supelco, Inc. (Bellefonte, PA, USA). However, a number of substitute columns can be and have been used with this technique.

METHOD

Column-Switching Procedure

The interconnection of the apparatus is illustrated in Figure 2. One of the HP 1050 pumps (P1, Bio pump) delivers a constant flow of size exclusion mobile phase through the conditioning column (C1), injector (I), guard column (C2) and size exclusion column (C3). The effluent from this column is directed by switching valve (V1), which is controlled from the 1090M time table as contact # 2. A detector is generally connected (optional manual switching valve may be installed) to waste 1 during method development to establish the cut times from the size exclusion column. The other HP 1050 pump (P2, wash pump) delivers a wash solution through conditioning column (C5) to collection

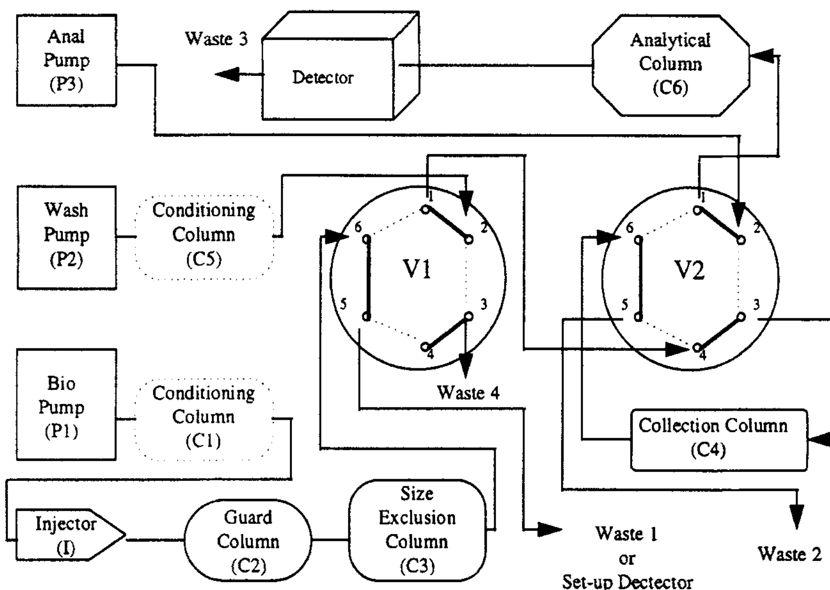

Figure 2. Column switching configuration and interconnections.

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column (C4). The flow is programmed using the controls on the HP 1050 pump (P2). Typically, the flow is 0.2 mL/min except when the collection column (C4) is being actively washed, then the flow is 2.0 mL/min. The flow is directed through column (C4) via valves (V1) and (V2). The mobile phase for the analytical column (C6) is supplied by the 1090M pumping system (P3, Anal pump). The sample is transferred from collection column (C4) to the analytical column (C6) via valve (V2), which is controlled from the time table as event "column".

HPLC Parameters

The analysis parameters for Z1 and Z2 are as follows: The size exclusion mobile phase was 0.025 M potassium phosphate (pH 6.8) with 5% (v/v) acetonitrile and the flow rate was 1.5 mL/min. The wash mobile phase was 1% methanol (v/v) and flow rate was 0.2 mL/min. The flow rate was increased linearly to 2 mL/min over a 1 minute period for the active wash cycle then decreased to 0.2 mL/min over a 1 min period. The analytical mobile phase was 0.01 M tetra butyl ammonium phosphate at pH 7.5 with 31.5% acetonitrile (v/v). The analytical column flow rate was typically 1.2 mL/min and the column temperature was maintained at 28 °C using the 1090M column heater.

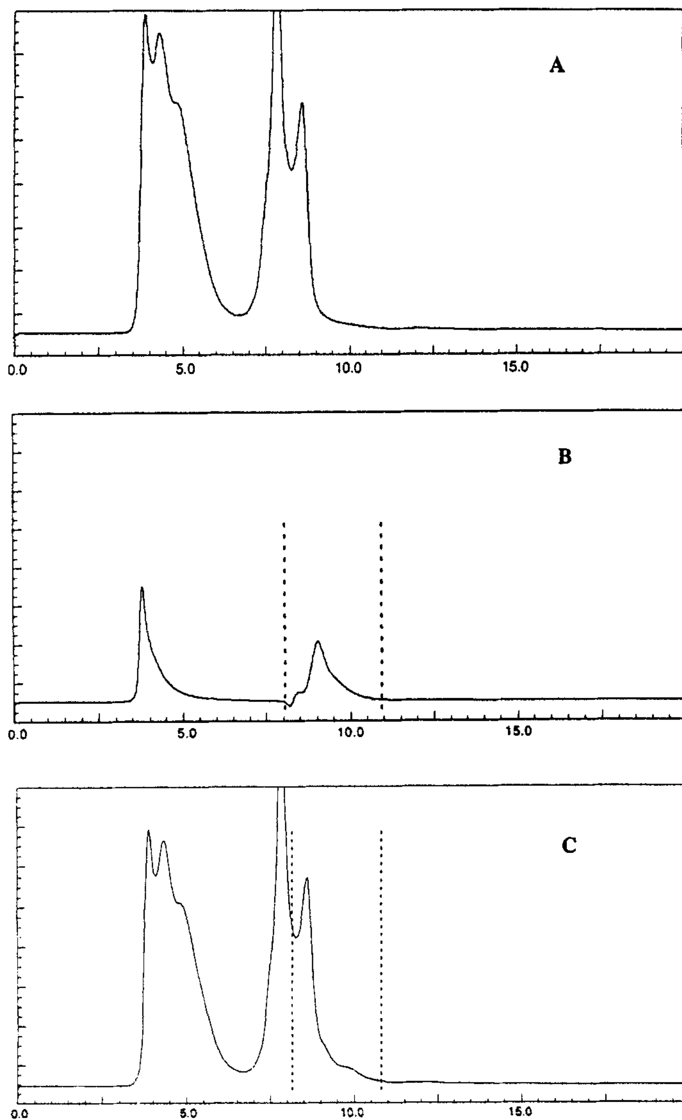

Figure 3. (a) Chromatogram of blank plasma. (B) Chromatogram of a 10 μg/mL aqueous standard of Z1. (C) Chromatogram of a 10 μg/mL standard of Z1. The chromatograms were obtained from the effluent of the size exclusion column (C3). The dotted lines show the approximate times of the heart cut from the size exclusion column.

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Sample Analysis Procedure

Plasma samples (100-200 µl) were filtered by centrifugation through a 0.45 µm Utrafree®-MC filter, Millipore Corp. (Bedford, MA, USA) using an Eppendorf micro centrifuge model 5415C, Brinkmann Instruments, Inc. (Westbury, NY, USA). The time and speed are not critical and are dictated by the conditions needed to obtain a filtrate. The filtered plasma sample is injected (typically 50 µL) onto the size exclusion column. The proteins elute first, then the parent (Z1) and metabolites (Z2) elute together in the 8-11 minute zone. At 8 minutes (V1) is switched to the activate position (dotted lines) and the components from the size exclusion column are transferred to the collection column. At the end of 11 minutes, (V1) is returned to the non active position. Starting at 10 minutes the wash flow rate was increased linearly over a one minute period to achieve a flow rate of 2 mL/min at 11 minutes. The collection column is then washed for 1 minute at 2 mL/min. At 12 minutes, (V2) is activated (dotted lines) and the collection column and analytical column are in series. The analytical mobile phase transfers the compounds from the collection column to the analytical column. Contact #4 on 1090M is activated on/off for 0.01 minutes to start the electronic data acquisition. The collection column remains in series with the analytical column for 1 minute and is then returned to the non active position (solid lines) and is now being washed at 0.2 mL/min. The next sample is injected; thereby, the processing of the second sample is started while the first sample is eluting from the analytical column. Three chromatograms illustrating the separation on the size exclusion column are given in Figure 3. In the plasma containing chromatograms (A and C), the earlier peaks represent the higher molecular weight proteins. The second set of major peaks is endogenous components, some of which are trapped and eliminated in the next purification step.

A typical elution time for Z1 is about 8 minutes on the analytical column. The erythro alcohol (Z2) elutes about 1 minute before Z1 and the threo alcohol elutes about 1 minute after Z1. The alcohol metabolites are the most difficult separation and create the need for the long elution time of Z1. A chromatogram of a plasma standard with 2.5 µg/mL of Z1 and 7.5 µg/mL of the alcohol mix (Z2) is illustrated in Figure 4. The separation between the peaks can be increased by decreasing the acetonitrile concentration, which will increase the elution time. However, good quantitative results can be achieved with the electronic area measurement for these peaks

Preparation of Standards

A series of aqueous standards (30% acetonitrile) was prepared by weighing 10 mg of Z1 into a 10 mL volumetric flask and dissolving in acetonitrile. This solution was serially diluted to obtain the following standards 10,000, 5,000,

1000, 500, 100 and 50 ng/mL. A rat plasma standard series was prepared by evaporating one of the intermediate standards (100,000 ng/mL) with a stream of nitrogen and quantitatively diluting it with rat plasma. The spiked plasma standard was then diluted serially with rat plasma to obtain the following standards 10,000, 5,000, 1,000, 500, 100 and 50 ng/mL. The plasma samples were filtered through 0.45 micron filters before injecting into the size exclusion column.

Determination of Assay Linearity and Precision

The area of the Z1 peak was measured using the HP 3350 data acquisition system with the Genie integrator software. The linearity and precision of the aqueous standard (30% acetonitrile) series were first analyzed on the HP 1090L by injecting directly onto an analytical column without column switching. This establishes the linearity and precision of the method without column switching. Each experiment represents the 5 standards stated above and 3 experiments were run each day. The same series was re-evaluated in the same manner on with the 1090M column switching configuration. The plasma series was then analyzed on the 1090M with column switching. Three sets of the five plasma standards were analyzed for two days.

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Determination of Assay Recovery and Accuracy

An important evaluation is the effect that plasma has on the recovery of the components of interest. A comparison of standards prepared with plasma and standards containing no plasma will allow for the effects of plasma on the system to be determined. Therefore, standards prepared in plasma and the corresponding aqueous standards were analyzed and compared. The system was calibrated to yield the prepared aqueous standards concentrations. The corresponding plasma standards were analyzed and the concentration determined by comparing to the respective aqueous calibration.

RESULTS

The linearity was evaluated using non weighted linear regression analysis. The slope is the standard's concentration divided by the chromatographic area counts. The intercept is concentration. The results from the direct analysis of the aqueous series are tabulated in Table 1. The results from the column switching analysis of the aqueous standards are tabulated in Table 2. The analyses of the plasma standards using the column switching analysis are tabulated in Table 3. The correlation coefficient was similar for all three

Table 1

Linearity of Aqueous Standards Without the use of Column Switching (50 ng/mL to 10,000 ng/mL)

Experiment	Slope	Intercept	Correlation Coefficient
1	0.007387	-0.1571	0.99999
2	0.007365	11.40	0.99998
3	0.007353	10.86	0.99998
4	0.007271	-2.820	0.99999
5	0.007446	33.74	0.99996
6	0.007589	-0.4131	0.99997
7	0.007504	12.93	0.99999
8	0.007516	5.776	0.99997
9	0.007492	-18.34	0.99998
mean	0.007436	5.886	0.99998
SD	0.000104	11.71	0.00001
RSD %	1.40	199	0.00105

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Table 2

Linearity of Aqueous Standards using Column Switching (50 ng/mL to 10,000 ng/mL)

Experiment	Slope	Intercept	Correlation Coefficient
1	0.006485	21.53	0.99999
2	0.006495	22.73	0.99999
3	0.006425	36.11	0.99998
4	0.006709	51.13	0.99991
5	0.006673	51.58	0.99993
6	0.006777	51.22	0.99996
7	0.006660	71.21	0.99996
8	0.006687	48.45	0.99995
9	0.006698	54.53	0.99998
mean	0.006623	45.39	0.99996
SD	0.000122	15.95	0.00003
RSD %	1.84	35.1	0.00276

Table 3

Linearity of Plasma Standards using Column Switching (50 ng/mL to 10,000 ng/mL)

Experiment	Slope	Intercept	Correlation Coefficient
1	0.006899	35.10	0.99996
2	0.006905	33.60	0.99997
3	0.007002	29.95	0.99992
4	0.006744	41.46	0.99990
5	0.006614	32.49	0.99996
6	0.006582	40.32	0.99995
mean	0.006791	35.49	0.99994
SD	0.000171	4.23	0.00003
RSD %	2.51	11.9	0.00273

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Table 4

Precision and Accuracy of Plasma Standards using Column Switching (n=6)

Aqueous Standards Concentration (ng/mL)	Mean Plasma Assay Concentration (ng/mL)	SD	Mean Percentage Recovery	SD
10000	9647	175	96.5	1.76
5000	4859	66	97.2	1.33
1000	946	14	94.6	1.35
500	456	7	91.1	1.39
100	102	6	102	5.63
50	52	3	104	6.24

determinations (Tables 1-3) and was greater than 0.9999. The percentage recoveries for plasma samples are tabulated in Table 4. The average recovery was 97.6 % for the series. There is no obvious difference in the peak shape or the separation of the metabolites between the switching and the non-switching chromatograms.

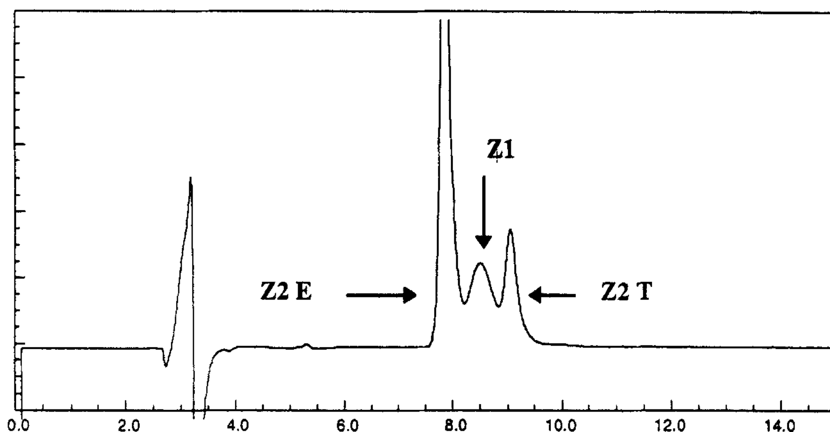

Figure 4. Chromatogram of 50 μL plasma standard (2.5 μg/mL of Z1 and 7.5 μg/mL Z2 mix, erythro and threo) processed by the multidimensional chromatographic system and detected eluting from the analytical column (C6).

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A comparison of a 10 μg/mL aqueous standard peak injected directly onto an analytical column and the same standard processed through the multidimensional system are illustrated in Figure 5.

DISCUSSION

The use of a size exclusion column to separate proteins from drug components simplifies the development of new analyses. Generally, the adjustment of the pH is not a critical factor nor the percentage of organics needed to achieve enough solubility for the components to elute. The pH is maintained between 3 and 7.5 and the level of organics is keep below 15 % in order to avoid the precipitation of proteins. The use of a high salt content mobile phase, as is frequently used with size exclusion columns, was not found to be necessary. The achievement of a true size separation mechanism is not necessary. The retention time observed for Z1 eluting from the size exclusion column is greater than would be predicted for a true size separation mechanism. Presumably, the separation is enhanced by the presence of some reverse-phase interaction.

The use of the collection column solves many of the problems associated with multidimensional systems. A number of investigators[7] (including this author) believe the clogging problem many investigators have experienced using direct plasma injections has been caused by the high percentage of organics in the analytical mobile phase being switched onto the pre-separation

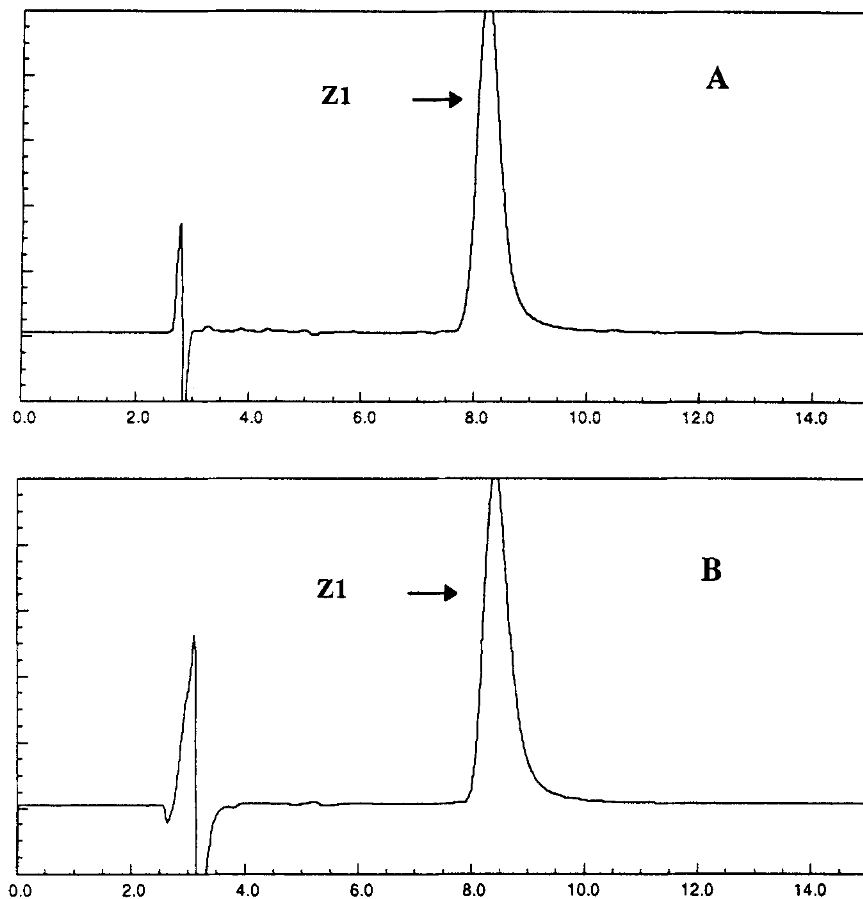

Figure 5. (A) Chromatogram of a 10 μg/mL aqueous standard injected directly onto the analytical column. (B) Chromatogram of a 10μg/mL aqueous standard processed through the multidimensional system.

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column. This may cause residual proteins on the pre-separation column to be precipitated. The collection column assures that the size exclusion column mobile phase and the analytical mobile phase do not come into contact. Clogging of the size exclusion and collection column has not been a problem over the numerous years that this technique has been in use, even when using 5 micron size exclusion columns. The collection column de-couples the size exclusion column and the analytical column allowing a wide range of mobile phases to be used and the separation on both columns to be optimized. It compresses the broad peak from the size exclusion column; thereby, yielding

peak widths similar to non-switching techniques (see Figure 5). This column also makes it possible to inject large sample volumes unto the size exclusion column and allows for the concentration of samples by collecting multiple injections before switching to the analytical column. This technique makes the processing of two samples at the same time possible. This keeps the analysis time similar to off line sample preparation techniques. Additional processing of the sample by washing the collection column with different solutions is also possible using this technique.

The collection column should be as small as possible, as the volume of the column becomes the injection volume and the guard column for the analytical column. The packing material is generally the same or of a weaker column strength than the analytical column. These columns do not clog, but the retention capacity decreases after about 200 injections. This is probably caused by a combination of switching abruptly between different mobile phases and the small amount of packing material present in the 15 mm guard columns normally used for this purpose.

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In the configuration presented, the collection column is washed with a neutral mobile phase when not in use and unfortunately impurities are concentrated from the mobile phase. The conditioning columns (C1 and C2) and the flow programming of the wash mobile phase are all being utilized to minimize the concentration of impurities.

The three modular parts of this system allow each component to be optimized individually. This greatly speeds up method development time. The analytical separation from non-switching HPLC methods can generally be used with very few modifications. Once the system is operational, the only sample preparation required is the filtration of the plasma through a 0.45 μm filter. The system is normally operated overnight unattended. The unattended operation and ease of sample preparation have made a significant contribution to the number of analyses that can be accomplished by an individual operator. Pharmacokinetic evaluations for Z1 in hamster, rat and dog have previously been reported[15] using this column switching technique.

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