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# Analysis of carbamazepine and its 10,11-epoxide in serum by direct sample injection using surfactant containing eluents and column switching*

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**Abstract**: Column switching is used in conjunction with surfactant containing mobile phases and traditional reverse phase LC columns to provide a highly reproducible and accurate analytical procedure for carbamazepine and its 10,11-epoxide in serum. This approach eliminates the tedious sample preparation steps commonly used in the analysis of drugs by HPLC, while providing a high degree of protein removal prior to the final analysis. Various pre-columns were investigated and a pellicular reverse phase column was found suitable for optimum concentration of drugs and removal of serum proteins. A variety of standard reverse phase columns could be used for the analytical separation. The separation of the drugs could be accomplished with a high degree of reproducibility. Tandem pre-column operation was demonstrated to give a sample throughput of 10 $h^{-1}$.

**Keywords**: *Liquid chromatography; column switching; carbamazepine; therapeutic drug monitoring; plasma; direct injection.*

## Introduction

Direct injection is desired in the liquid chromatographic analysis of complex serum samples in pharmaceutical and clinical research [1–4] as it eliminates tedious and time consuming sample preparation processes which can also frequently introduce error. Also, direct serum injection can provide a higher throughput of samples, making it attractive for routine analytical studies (e.g. in pharmaceutical and clinical laboratories). However, before using direct sample injection, two critical problems must be addressed. First, proteins should not interfere with the analysis of the compounds of interest; and, second, the direct injection of serum samples should not reduce the lifetime of the analytical column. Approaches to address these issues have included the use of special packing materials [5, 6], micellar containing eluents [7–9], surfactant containing eluents [10, 11] and column switching techniques [3, 5, 12–18]. Each approach has its own advantages and shortcomings.

Recently, an approach has been developed for direct sample injection, which insures that proteins are solubilized by using surfactant containing eluents in which the surfactant concentration may be above or below the critical micelle concentration (CMC) [10, 11]. This finding has broadened the range of eluents that can be used with reverse phase columns in direct serum injection applications. In fact, methods development for sub-CMC LC is similar to conventional paired-ion chromatography. Organic solvents can be used as eluent additives at relatively high concentrations [10, 11], thus avoiding a limitation of micellar LC eluents, which are predominantly aqueous. The adjustment of the organic component of the mobile phase provides a more familiar means of controlling resolution. In addition, sub-CMC mobile phases are easier to prepare (i.e. less foaming) and have a less deleterious effect on valve seals than a mobile phase containing a surfactant at a concentration significantly above the CMC.

When using any direct sample injection, either with a specialty phase column or a standard reverse phase column with a mobile phase containing a surfactant, the presence of a large protein peak eluting at the void volume

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can adversely affect the detection of the compounds of interest [11]. An excellent example of this is the analysis of carbamazepine (CBZ) and its 10,11-epoxide (CBE). These compounds must be detected in the short UV region (e.g. 214 nm), where serum proteins absorb strongly. As a result, the peaks of interest occur on the tail of a large protein peak. Because of the difficulties in integration on a tailing peak, relatively poor precision is observed for the peak area of drugs at therapeutic concentrations (8% for CBE and 10% for CBZ) [11].

## Experimental

*Instrumentation*

The modular liquid chromatograph (Waters Chromatography Division of Millipore Corporation, Milford, MA) consisted of two Model 510 Solvent Delivery Systems, a Model 712 WISP autoinjector with cooling unit and a Model 440 Absorbance Detector equipped with an Extended Wavelength Module. The detection wavelength of 214 nm was used throughout this work. An Eluent Stabilization System (Waters) was pressurized with helium and used in the 'blanketing' mode. A Waters Automated Valve Station, (WAVS, Waters) provided the valves and associated electronic controls for the column switching experiments. Control of the instrument and column switching was provided by a Model 840 Chromatography Control Station (Waters). Analogue data were monitored on an SE 120 Dual Channel Recorder (Waters) and digital data was auto-archived to a Model 860 Networking Chromatography Station (Waters) for processing and storage.

The column switching configuration which has been described elsewhere [11], used a six port valve and required two solvent delivery systems. A 'loading pump' delivered the eluent containing the aqueous surfactant, and an 'analysis pump' supplied the analysis eluent. The autoinjector was positioned between the loading pump and port 2 of the switching valve. Switching times used in the loading step varied from 1.0 to 10.0 min in the various methods. The flow rates for both the loading and the analysis pump were 1.0 ml $min^{-1}$.

In certain column switching experiments two pre-columns were employed as shown in Fig. 1. One pre-column was used to isolate the drugs from the serum (using the loading eluent) during which time the other pre-column was a part of the analytical system. After the conclusion of an analytical run, the valve positions were switched so that the pre-column that was used to sorb the serum analytes was switched into the analysis mode while the other pre-column was switched into the loading mode. This tandem approach was used to demonstrate the increased productivity which can be achieved.

*Columns, eluents, reagents*

The following analytical columns (Waters) were used in this work: μBondapak Phenyl, Nova Pak C18, Resolve C18, Delta Pak C18 and Nova Pak CN. Some experiments used a guard column which was dry packed with Corasil/C18 bulk packing (Waters). The particle size of the Corasil/C18 packing was 37–50 μm. In other cases, a Guard Pak (Waters) was used containing μBondapak C18, μBondapak Phenyl, Resolve C18, or Nova Pak Cyano Guard Pak inserts. An inline pre-column filter kit containing a 2 μm filter assembly was positioned between the injector and column throughout this work.

Purified and filtered water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA). HPLC grade methanol and 2-propanol were obtained from J.T. Baker (Phillipsburg, NJ) and sodium dodecylsulphate (SDS) (BioChemika) was obtained from Fluka (Ronkonkama, NY).

Mobile phases containing SDS were prepared by dissolving the required quantity of the reagent in the desired solvent (either water or a water–alcohol blend). Since foaming was never severe, these mobile phases were filtered under vacuum through a 0.45 μm cellulose acetate filter (HAWP, Millipore Corp.) housed in a Solvent Clarification Kit (Waters). The eluents were degassed by ultrasonication.

The sample matrix was adult bovine serum (triple 0.1 μm, sterile filtered) from HyClone Laboratories (Logan, UT). When received, the bovine serum was divided into 10 ml aliquots and stored frozen until needed. Spiked bovine serum samples were prepared by adding small quantities of 1 mg $ml^{-1}$ methanolic solutions of carbamazepine (Sigma, St Louis, MO) and carbamazepine-10,11-epoxide (Alltech-Applied Sciences, Deerfield, IL) to aliquots of the adult bovine serum. In an

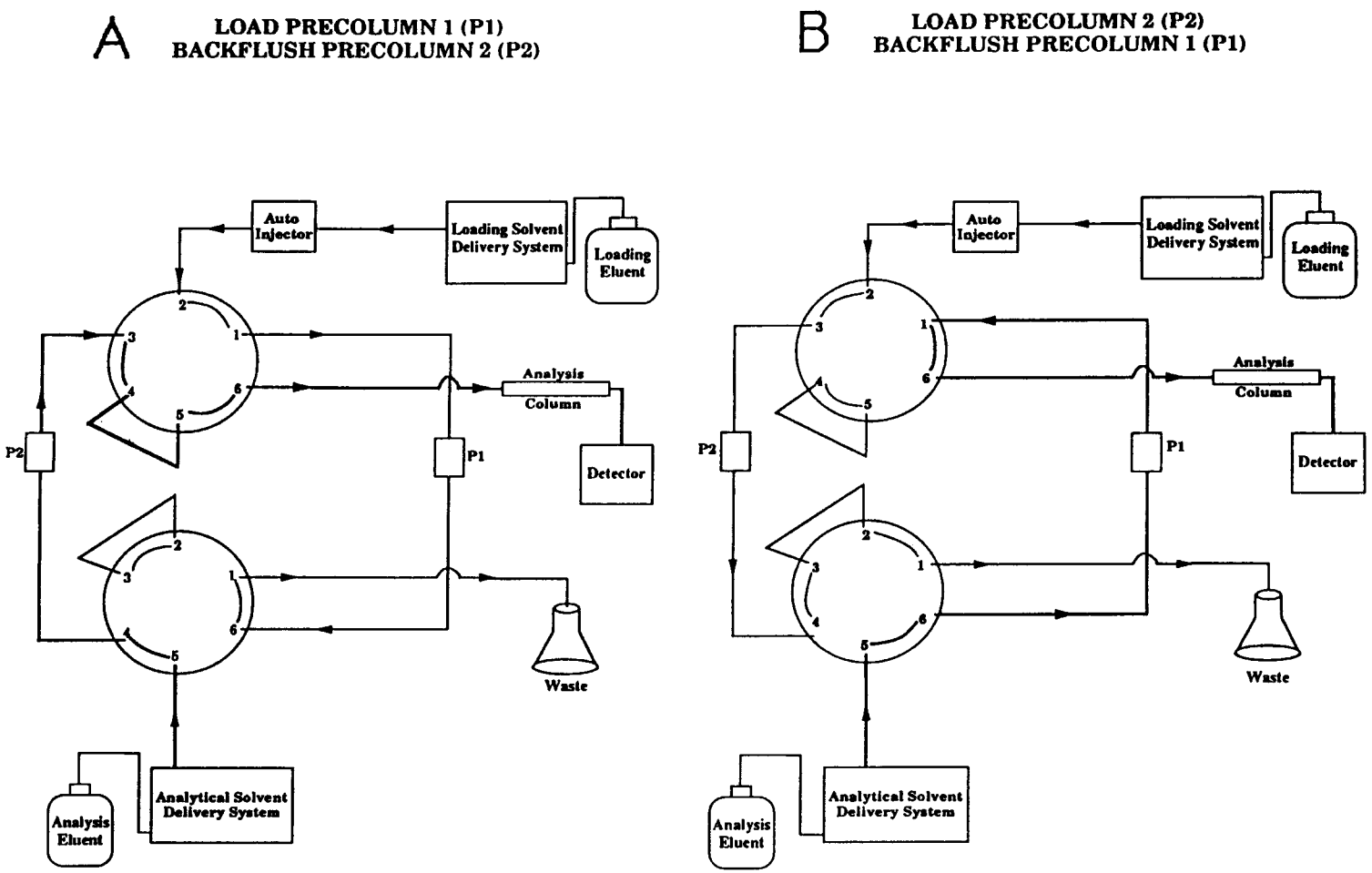


**Figure 1**
Tandem pre-column switching system.

identical manner, spiked aqueous samples were obtained. The concentration of all spiked serum and spiked water samples were at therapeutic concentrations (2.0 μg $ml^{-1}$ for CBE and 4.8 μg $ml^{-1}$ for CBZ).

## Results and Discussion

Based on previous experience [10, 11], the analysis of carbamazepine and its 10,11-epoxide was selected for this work. Carbamazepine is commonly used as an anticonvulsant and has a relatively narrow therapeutic concentration range of 4–10 μg $ml^{-1}$ in serum. *In vivo*, the drug is transformed into its 10,11-epoxide, which itself is considered to be pharmacologically active. Because there is a poor correlation between dosage and serum concentration, it is necessary to monitor both compounds. Generally LC is used to determine the concentration of both compounds in a single experiment. It should be noted that concentrations of CBE greater than 5 μg $ml^{-1}$ will create cross reactivity in immunochemical procedures and lead to an overestimate of CBZ concentration [19]. This effect is, of course, not observed in analyses performed by HPLC, since the two compounds are separated prior to quantitation.

In this paper, the development of an assay for CBZ and CBE using column switching techniques is described. There are usually two steps in a column switching method, a loading step and an analysis step (Fig. 1). In the loading step, the sample is injected onto a pre-column using an aqueous surfactant containing mobile phase. Serum proteins are not retained by the pre-column and are eluted to waste, while the analyte(s) are concentrated on top of the pre-column. In the analysis step, the drugs are backflushed with a mobile phase that has a higher organic content than the loading eluent. This strips the analyte(s) from the pre-column and effects the chromatographic separation. In the development of a column switching assay for carbamazepine and its 10,11-epoxide, each of these steps is discussed separately, followed by a description of work done to optimize the overall procedure.

### *Selection of the pre-column*

There are a number of criteria to be considered in the selection of the pre-column for this analysis. The size of the pre-column should be small enough to minimize the loading time. At the same time the pre-column must have a sufficient capacity to retain the drugs of interest. In addition, it should be no more retentive than the analysis column so that the more lipophilic components of the serum sample are not strongly retained. Finally, from a practical point of view, the pre-column should have a long lifetime.

In the experiments to select the appropriate pre-column and loading eluent for the first step (refer to Fig. 1), the analytical eluent and analytical column were kept constant [methanol:water (30:70, v/v) containing SDS (50 mM) μBondapak Phenyl, 150 × 3.9 mm]. This was the analytical eluent and column described previously [11]. Efforts to optimize the analysis step are described later. Using this analysis eluent and column, preliminary experiments with a loading eluent of 5 mM SDS indicated that the width of the serum protein peak at the void volume was relatively large with the μBondapak C18 and the Resolve C18 pre-columns. This was undesirable because the unwanted serum components must be eluted to waste as quickly as possible during the loading step. The retention of the drugs on the Nova Pak Cyano was very low. It was found that μBondapak Phenyl and Corasil/C18 pre-columns retained the drugs and still provided relatively narrow peak widths for the serum components. These two were considered as appropriate candidates for the pre-column.

### *Selection of the loading eluent and loading time*

In the ideal loading step, there will be complete elimination of the serum proteins with total retention of the compounds of interest. Because the loading step is a function of both the pre-column and the loading eluent, it is necessary to investigate the effect of varying both the composition of the loading eluent and the loading time for the pre-column. Initial studies involved 5 mM SDS in water for 2.5 min with the μBondapak Phenyl pre-column. For a set of 180 analyses of CBZ and CBE at therapeutic concentrations, precision (RSD) of peak areas was 8/9%, similar to that obtained from the direct serum injection [11]. (For a typical chromatogram see Fig. 8 in ref. 11.) After 175 injections, the separation began to degrade. An increase in the area of the serum peak was observed and the disturbance between the CBZ and CBE peaks grew to the point where it began to interfere with the quantitation of the drug

peaks. When spiked water samples were studied in place of spiked bovine serum, peak area precision (RSD) was approximately 3%, thus the problem in reproducibility is apparently due to contributions from the serum.

To determine if all the drugs for the final analysis were retained while minimizing the serum peak (i.e. identifying the optimum trade-off between serum expulsion and drug retention), the effects of increasing the time of the loading step and increasing the SDS concentration in the loading eluent were investigated. To do this the loading step was varied from 1.0 to 10 min (1.0, 1.5, 2.0, 5.0 and 10.0 min) at two different SDS concentrations (5 and 20 mM). When a μBondapak Phenyl pre-column was used with 5 mM SDS and a 10 min loading time, 97% of the serum was eluted to waste and only 3% appeared in the chromatogram. Further experiments showed that a loading time of only 2 min was sufficient for the Corasil/C18 pre-column with 5 mM SDS. Increasing the concentration of SDS with that loading time had little effect on the level of protein which was sent to the analytical column. This latter result is in agreement with the earlier observation that eluents containing sub-CMC concentrations of SDS can be used effectively for direct serum injection [10]. It was found, however, that higher concentrations of SDS could cause premature elution of CBZ and CBE from the pre-column during the loading process. For example, if 20 mM SDS was used for 2 min, there was little change in the amount of protein which was sent to the analytical column but we observed a drug recovery of less than 75%. With a 5 min loading time, all of the drugs were sent to waste. If we used a 1.5 min elution time, all the drugs were retained on the pre-column, but a significant amount of protein was sent to the analytical column. Due to the difficulty in maximizing drug retention and minimizing protein retention on this pre-column with 20 mM SDS, it was decided that this loading eluent was not suitable and standardized the loading eluent at 5 mM SDS.

It should be noted that a very small amount of protein was eluted from the pre-column onto the analytical column with this loading condition. However, when too much protein comes through, the useful lifetime of the analysis column was shortened. In order to determine the lifetime of the pre-column, a study which involved injecting 10 μl of serum containing therapeutic concentrations of the drugs was carried out using a loading solvent of 5 mM SDS and the analytical eluent described above. When μBondapak Phenyl was used in the pre-column, it was found that the area of the serum peak was small. However, the area of the serum peak increased with the number of injections, such that in one situation after 180 injections the serum peak increased five fold in peak height. Further, it was not possible to obtain the original peak area, even after replacing the pre-column. In addition, with increasing injection number the baseline became more and more unstable, and a disturbance or 'system peak' was observed. This system peak did interfere with the analysis of CBZ and CBE when using the μBondapak phenyl pre-column, but did not create a problem with the Corasil/C18 pre-column.

*Cause of the system peak*

It was found that the system peak occurred at different retention times using different types of pre-columns. Comparing the chromatograms obtained from the injection of water with those obtained for the serum blank suggested that the system peak was not serum related. A possibility is that the system peak was caused by a disturbance of the equilibrium of the SDS coating in the pre-column relative to the analytical column [20, 21]. To investigate this possibility, the same mobile phase was used for both the loading and the analysis eluent and column switching was carried out. This provided a very flat baseline. This observation supported the hypothesis that the disturbance was not caused by the valve switching process itself, but was due to a disturbance caused by valving of the pre-column's contents (largely loading eluent) into the analytical column.

*Lifetime of the pre-column*

Figure 2(A) and (B) indicate the results of the pre-column lifetime experiments. When a μBondapak Phenyl pre-column was used, the serum peak increased in area. After about 40 injections the serum peak area became significant, relative to that of the drugs and efforts with this column ceased. On the other hand, when using Corasil/C18, the serum peak area remained constant in size for over 200 injections. This agrees with previous findings

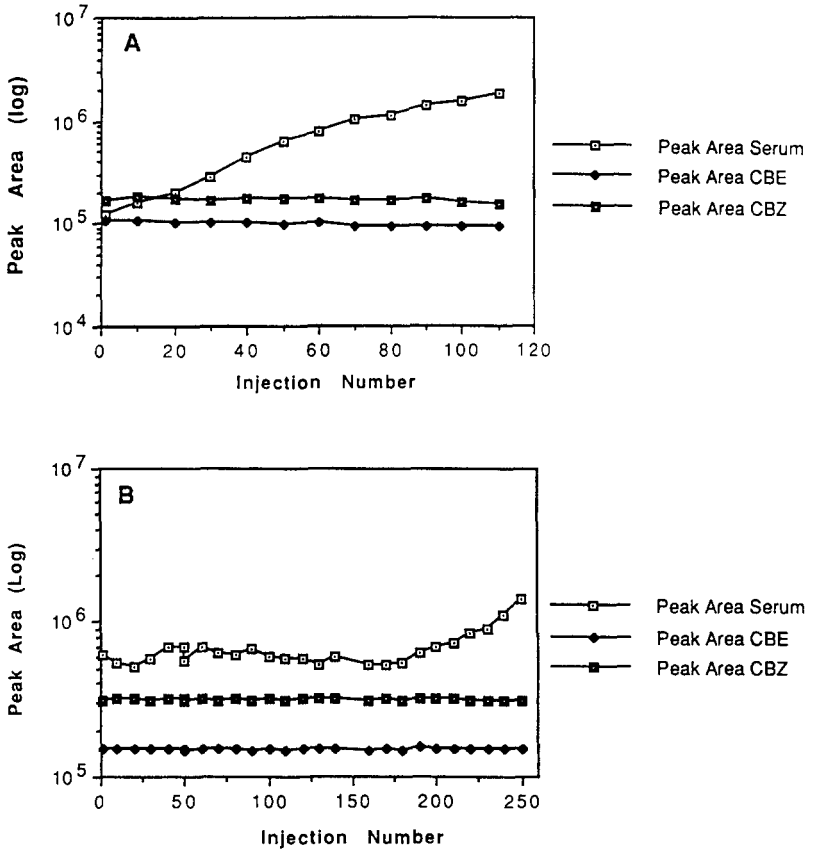


**Figure 2**
Peak area of the serum peak as a function of injection number. (A) Loading eluent: 5 mM SDS; analysis eluent: 30% MeOH/70% $H_2O$/50 mM SDS; analysis column: μBondapak Phenyl; pre-column: μBondapak Phenyl guard pak; loading time: 2.5 min. (B) Loading eluent: 5 mM SDS; analysis eluent: 28% IPA/72% $H_2O$/10 mM SDS; analytical column: resolve C18; pre-column: Corasil/C18; loading time: 1.5 min.

that pre-columns with a large particle size have a longer lifetime [22]. In addition, the suitability of the Corasil/C18 pre-column may also be due to the fact that this material has a relatively low surface area (nominally 12 $m^2$ $g^{-1}$) with a very low pore volume and a low carbon load (1/2% C). These factors may contribute to low protein binding for this stationary phase. The results of the lifetime studies revealed that a loading pre-column containing Corasil/C18, an SDS concentration of 5 mM and a loading time of 1.5 min was very suitable with regard to long term stability and constancy of the area of the serum peak.

*Selection of the analysis column and mobile phase*

The initial selection of the analytical column was a μBondapak Phenyl column. This was based upon our previous work on direct sample injection [11] and resulted in a very acceptable chromatogram for the separation of CBZ and CBE using an analytical eluent of methanol: water (30:70, v/v) containing 5 mm SDS. When a Resolve C18 or Nova Pak C18 column was used to separate the drug and its metabolite, a significantly larger methanol concentration (e.g. 45%/55% MeOH/water, 5 mM SDS) was required in the mobile phase to effect the separation of CBZ and CBE. This analytical column–mobile phase combination could possibly be used for the separation: however, if any protein did elute from the guard column, the high concentration of methanol (which is known to be a poor solvent of protein) in the mobile phase might cause protein precipitation. To test this hypothesis a 10 μl serum sample was added to 10 ml of methanol–water containing 5 mM SDS. Clouding of the solution occurred, suggesting

that some component of the serum sample had limited solubility in this mobile phase. It was felt that this could create difficulty in the long term use of the chromatographic system and the use of another organic modifier was considered.

Addition of serum to 2-propanol–water mixtures containing 5 mM SDS (up to 40% 2-propanol) did not cause protein precipitation. When the 2-propanol–water ratio was varied between 25 and 30% 2-propanol (with a constant 5 mM SDS), it was determined that an optimum separation was obtained at 28% 2-propanol on a Resolve C18 column which was somewhat better than that obtained on the phenyl column. Since the main goal was to demonstrate the utility of column switching with a mobile phase containing sub-CMC levels of surfactants, the analytical column was used for the remainder of the study.

*Efficiency of the separation*

It is known that when surfactants are added to the mobile phase, the intrinsic efficiency of the column decreases [11]. This becomes very significant when micellar mobile phases are used [23]. In order to determine if the efficiency of the analytical column could be improved, a series of experiments were performed to determine an optimal concentration of SDS with 2-propanol–water (72:28, v/v). When the mobile phase contained 5 mM SDS, the column exhibited 2200 theoretical plates for CBZ and 2300 for CBE (15 cm column) and these values decreased as the SDS concentration was increased. In the absence of SDS, these values were 5200 and 5800, respectively, and a very satisfactory separation could be obtained (Fig. 3).

Since removal of SDS from the analysis eluent provided a more efficient separation, the question was whether it could be used in the column switching approach to the separation of the complex drug mixture. Since the serum sample was injected in a 5 mM SDS eluent and the analysis eluent always contains a trace level of SDS which results from the partial removal of the SDS coating on the pre-column, it was hypothesized that SDS binding to protein might maintain solubilization of the small amount of serum components which could reach the analytical column, thus ensuring an adequate life span.

*Lifetime studies of the complete assay*

Using the Corasil/C18 pre-column with a 5 mM SDS loading eluent and a Resolve C18 analysis column with a mobile phase consisting of 2-propanol–water (72:28, v/v), lifetime studies were performed. There was no degradation seen in the chromatographic performance during a series of 500 consecutive assays

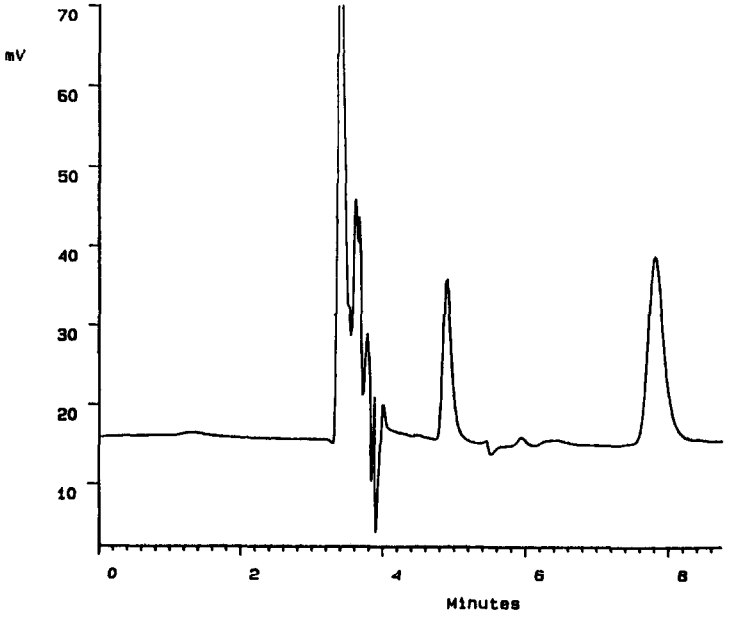


**Figure 3**
Injection of a 10 μl spiked bovine serum sample. Loading eluent: 5 mM SDS; analysis eluent: 28% IPA/72% $H_2O$; analytical column: resolve C18; pre-column: Corasil C18; loading time: 2.5 min. First peak (5 min) = CBE, second peak (8 min) = CBZ.

(changing the Corasil pre-column after 250 injections) (Fig. 4). A plot of the area of the drug peak as a function of injection number demonstrates the excellent reproducibility of the system. The RSD over 500 CBZ samples (using every 10th injection for the calculation, $n = 50$) was less than 1.5%. When the Resolve C18 column was used with SDS in both the loading eluent and the analytical eluent (5 mM SDS), the RSDs were likewise again less than 1.5% over a series of 450 consecutive analyses (using every 10th injection for the calculation) (Fig. 5).

*Tandem pre-column operation*

In order to demonstrate a way to increase

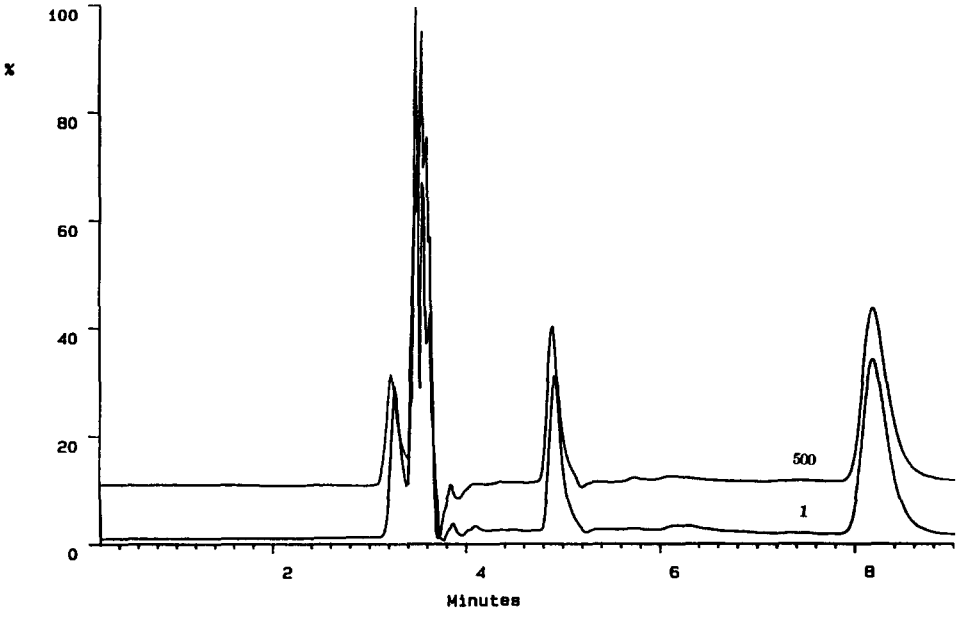


**Figure 4**
Chromatogram of the first and 500th injection of a 10 μl spiked serum sample (2.0 μg $ml^{-1}$ for CBE and 4.8 μg $ml^{-1}$ for CBZ). Loading eluent: 5 mM SDS; analysis eluent: 28% IPA/72% $H_2O$/5 mM SDS; analytical column: resolve C18; pre-column: Corasil C18; loading time: 1.5 min.

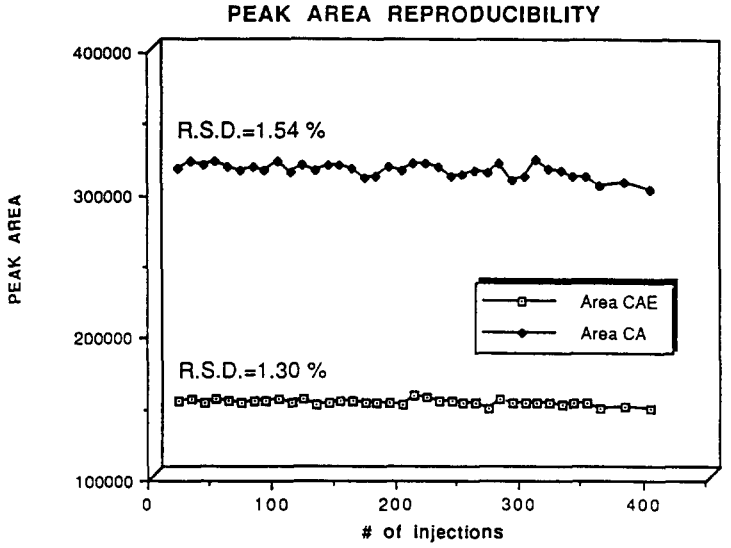


**Figure 5**
Peak area reproducibility of 450 HPLC assays ($n = 45$). Injection of 10 μl of spiked serum sample of clinical level (2.0 μg $ml^{-1}$ CBE; 4.8 μg $ml^{-1}$ CBZ). Loading eluent: 5 mM SDS; analysis eluent: 28% IPA/72% $H_2O$/5 mM SDS; analytical column: resolve C18; pre-column: Corasil C18; loading time: 1.5 min.

the analysis throughput, two pre-columns were used. While one pre-column was loaded with sample to expel the proteinaceous material and to concentrate the drugs, the other was eluted by the analytical mobile phase. The separation of the compounds of interest took 6 min, so the column switching valves were operated at that interval. It should be noted that tandem pre-column operation subjected the pre-column to a loading process of 6 min, which is significantly longer than the 1.5 min that was previously used. Although a lower flow rate for the pre-column with this loading time could have been used it was decided to use the same flow rate as used for a 1.5 min loading time. The additional volume of loading eluent was believed to reduce the level of retained protein and would therefore lengthen the lifetime of the pre-column. In this regard, it was found that each pre-column had a lifetime of 400 injections using a 6 min loading time. Experiments verified that there was no loss of drugs due to the longer loading step, and a very complete protein elution occurred as shown in Fig. 6. To demonstrate the tandem pre-column operation, the same analytical system as described for single pre-column operation was chosen. This resulted in 10 $h^{-1}$, instead of the 6 $h^{-1}$ with the single valve system. Over a period of 400 injections, the RSDs for peak area were 1.5% for CBZ and 1.2% for CBE (using every 10th injection for the calculations).

## Conclusions

These investigations indicate that the use of column switching with sub-micellar concentrations of surfactants can be a powerful tool in the development of an assay by LC. In this work, an automated assay for CBZ and CBE which is well suited for routine analytical work was developed. The procedure takes 6 min per sample, has an excellent RSD of less than 1.5%, and may be applicable to many existing LC analyses. Column switching with an appropriate loading eluent (e.g. 5 mM SDS) and pre-column clearly provides a simple, reliable and effective means for the removal of serum proteins prior to the analysis of drugs and is being used to monitor human subjects (L.A. Bowers, personal communication).

One of the critical issues was the concentration of the surfactant in both the loading step and the analytical step. While the surfactant was required to solubilize the proteins in the loading step, this work indicated that it is very worthwhile to carefully investigate the appropriate concentration of the surfactant in each part of the assay. The observation that very reproducible results were obtained, even in the absence of surfactant in the analysis step

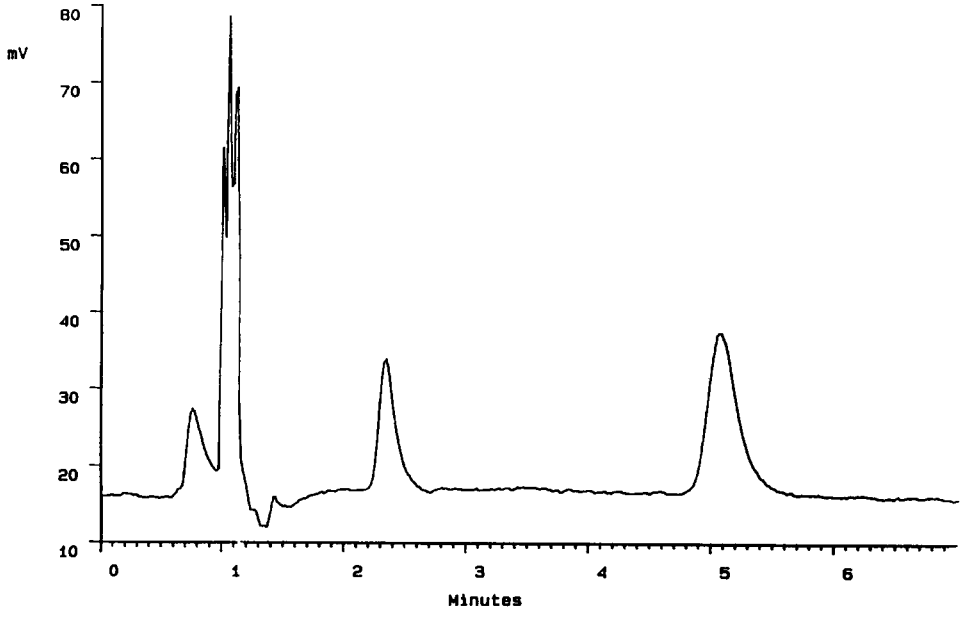


**Figure 6**
Typical chromatogram obtained by the tandem column switching method. Loading eluent: 5 mM SDS; analysis eluent: 28% IPA/72% $H_2O$; analytical column: resolve C18; pre-column: Corasil C18; loading time: 6 min.

suggests that a study into the effect of each component of the mobile phase in an analysis may prove very fruitful to the analyst. If the major concern is the efficiency of the separation, the SDS should be deleted. There are, however, benefits to the use of the surfactant, including an increase in the selectivity and a decrease in the retention times of the analytes which could lead to a shorter analysis. Additionally, the presence of SDS in the analytical eluent provides a safety margin in situations where too much protein would be transferred into the analytical column from the column switching step.

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