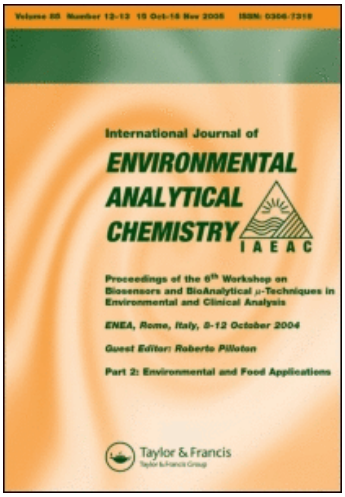

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Pre-Column Switching Techniques for the Determination of Drugs and Metabolites in Body Fluids in Research and Routine Analysis

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Pre-Column Switching Techniques for the Determination of Drugs and Metabolites in Body Fluids in Research and Routine Analysis†

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The use of a column switching system for direct injection of samples and of a sample clean-up on reversed phase pre-columns is described. The pre-columns were filled with spherical C-18 silica gel of particle size 30 μm.

Two applications are reported on: (1) the direct injection of serum samples for the simultaneous analysis of nine antiepileptic drugs and metabolites and (2) the determination of phenytoin and of carbamazepine in serum ultra-filtrates.

The purge liquid for the sample clean-up was diluted phosphoric acid, and the eluent mixture for the chromatographic separation was water/acetonitrile. The analytical column (length 12.5 cm) was filled with C-18 silica gel of particle size 5 μm. A gradient elution was chosen for the first application, while the second application was carried out using isocratic chromatographic conditions.

KEY WORDS: HPLC, pre-column switching, direct injection, serum, ultra-filtrates.

INTRODUCTION

Several methods for the analysis of antiepileptic drugs (AEDs) in body fluids using liquid–solid extraction as sample pre-treatment have been described in the last decade. Activated charcoal,[1,2]

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kieselguhr,[3] divinylbenzene cross-linked polystyrene,[4] or reversed phase silica gel[5,6] were used as solid phases for the adsorption of the drugs. In the last years some column switching techniques using reversed phase pre-columns for the isolation of drugs from serum, plasma, urine and saliva have been published.[7-10] One team[7,8] recommended a method with an alternating double valve switching of two pre-columns (see Figure 1). The other authors[9,10] used only a single valve and a single pre-column for the sample clean-up procedure (see Figure 2). In our papers dealing with the analysis of AEDs and metabolites (MBs) in urine[11] and serum[12] we described double pre-column switching techniques according to the method of Roth[7] and Beschke.[8] Using this method with alternately switched pre-columns we observed the following disadvantage. The sample clean-up with two different pre-columns often leads to varying retention times on the analytical column.

The authors,[7,8] who prefer the use of two pre-columns in an antiparallel mode, point out that avoidance of time loss for re-equilibrating the pre-column and loading of the next sample is an advantage of this method.

Saving of time for pre-column preparation can also be achieved using only one switching valve and one pre-column.

As only a short time is required for the elution of the adsorbed drugs from the pre-column to the analytical column, the valve can be switched back after a few minutes. During the remaining time of the chromatographic run, the pre-column can be re-equilibrated, the next sample can be injected, and the sample matrix can be washed out by the purge liquid (see Figure 3).

1. ANALYSIS OF AEDs AND MBs WITH DIRECT INJECTION OF SERUM

Our recently published column switching method for the analysis of AEDs and MBs in serum[12] was slightly modified. For the separation on reversed phase columns acidified mixtures of eluents were required, when aliquots of a methanolic solution of the AEDs were injected,[13] in order to avoid uncontrolled shifts of the retention times of phenobarbital. Therefore, we initially used for the column switching method also an eluent mixture containing an acidic phosphate buffer solution (pH 4). On the other hand, an acidic purge

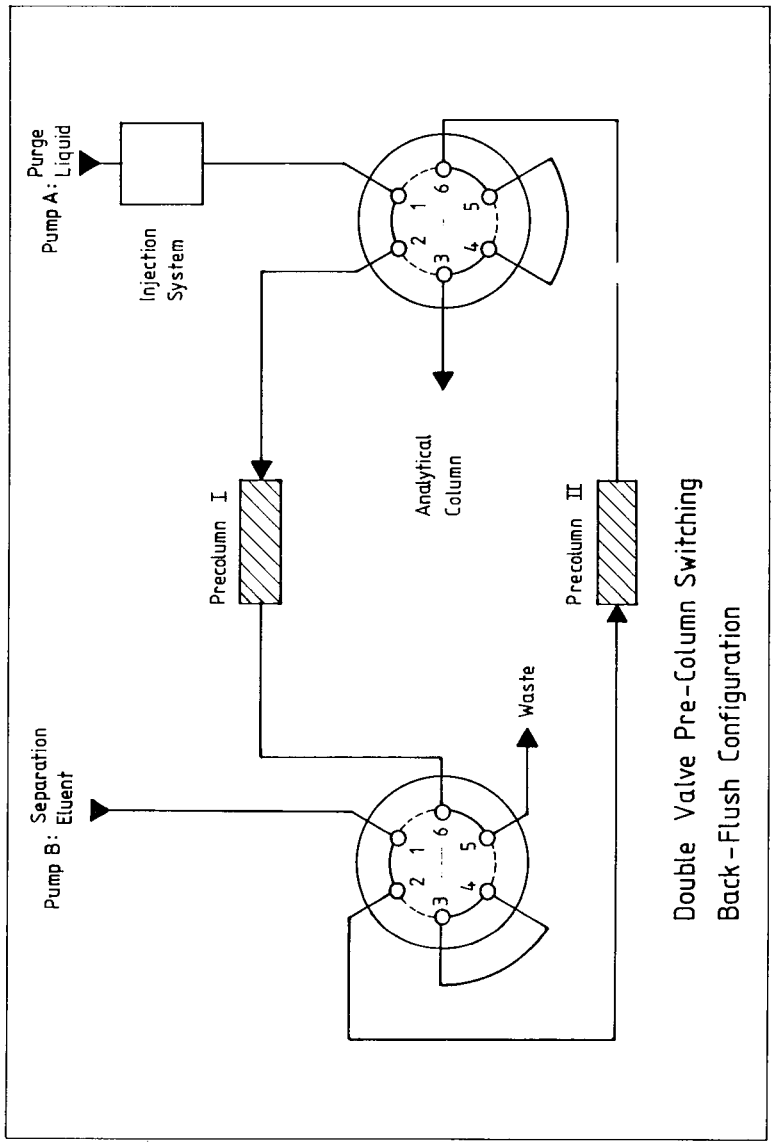

FIGURE 1 Scheme for a double valve switching system.

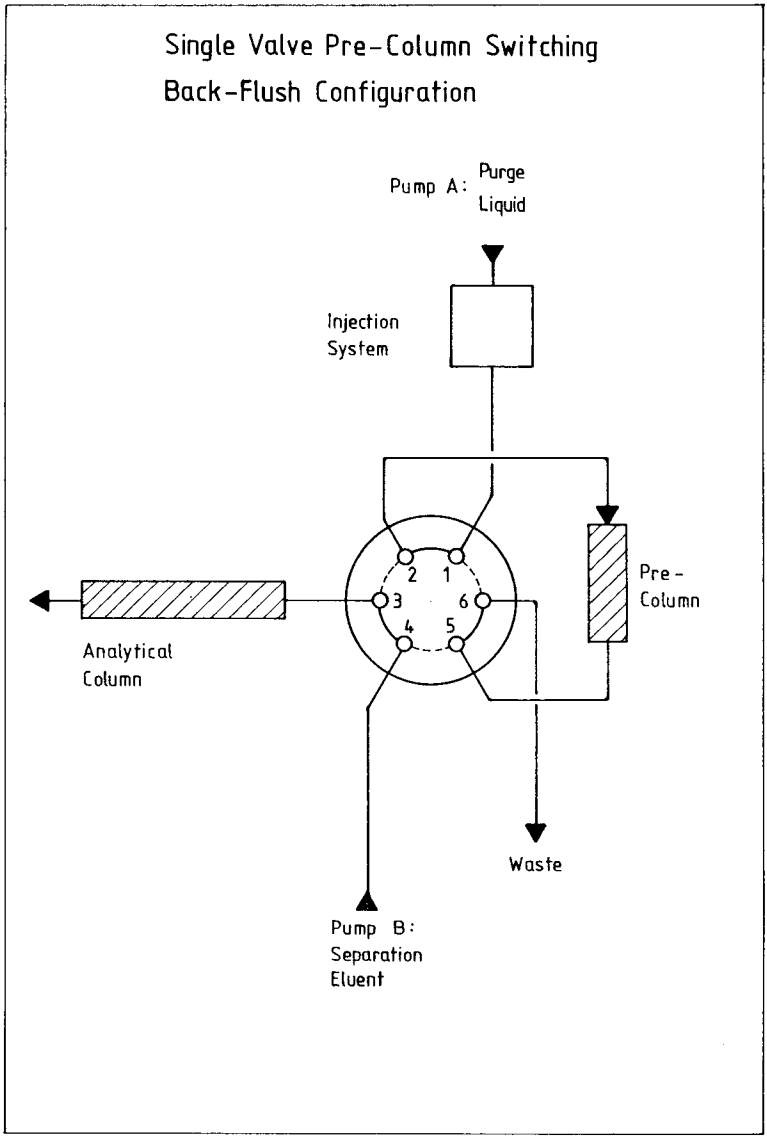

FIGURE 2 Scheme for a single valve switching system.

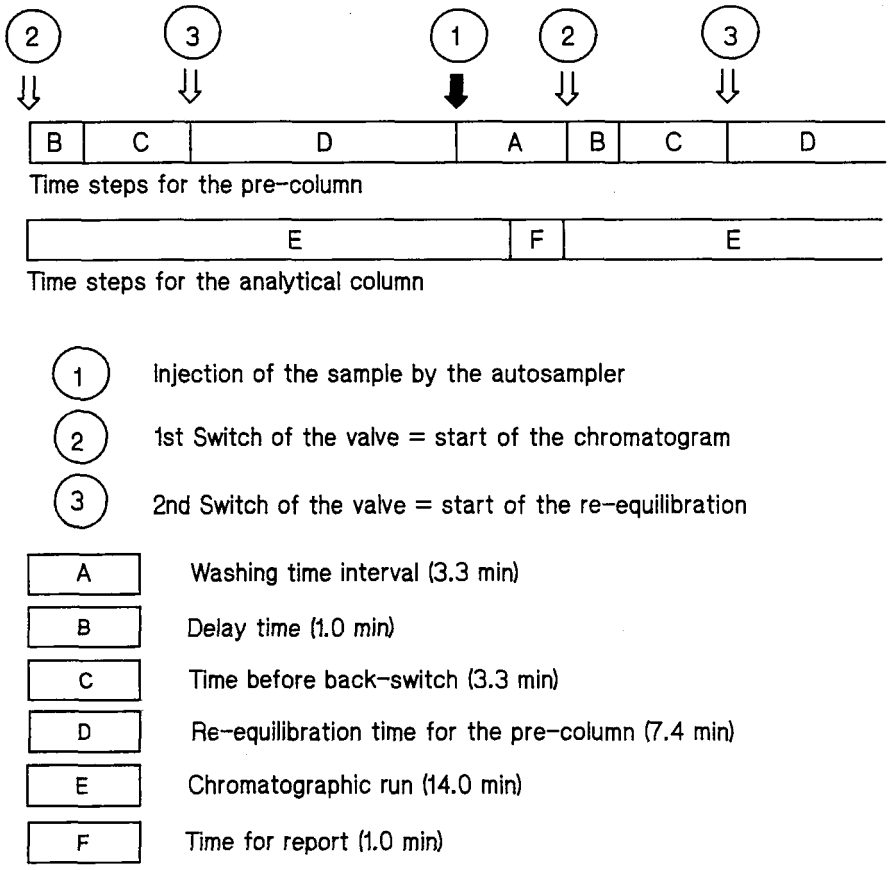

FIGURE 3 Time steps and events in the column switching cycle.

liquid (pH 3) is required in the column switching method using RP-18 pre-columns to get a complete recovery of phenobarbital. When the valve is switched over for the elution of the adsorbed AEDs onto the analytical column, a pre-column volume of the acidic purge liquid is transferred to the analytical column. This amount of acid seems to be sufficient to avoid retention time shifts of phenobarbital, even if only water/acetonitrile is used as eluent mixture for the chromatographic separation.

Further modifications of the published column switching method using direct injection of serum samples were made. Instead of an

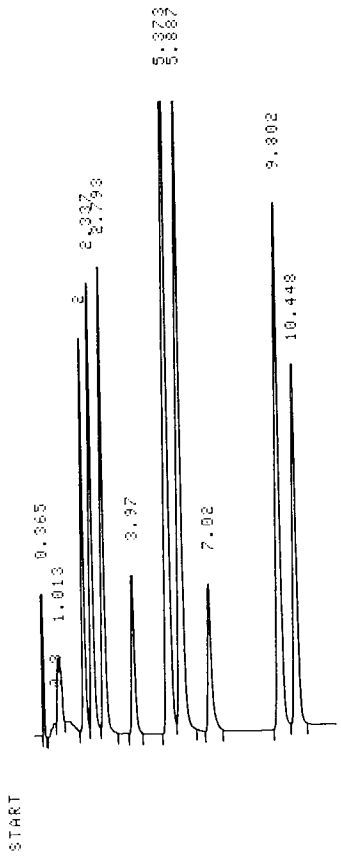

FIGURE 4 Chromatogram of a calibrated sample; *RT*: 2.000 PE 25.0 [μg/ml], 2.337 ET 100.0, 2.793 PR 25.0, 3.970 DIOL 5.0, 5.373 PB 50.0, 5.887 DM 50.0, 7.020 EPO 5.0, 9.802 PT 25.0, 10.448 CBZ 15.0

analytical column with 25 cm length a 12.5 cm column with a 1 cm guard cartridge is used.

The sample clean-up is carried out using 1 cm pre-column cartridges instead of 0.5 cm cartridges because some of the 0.5 cm cartridges showed incomplete recovery of ethosuximide.

In addition to the modifications of the chromatographic parameters the production of the calibration samples was improved. The stability of the initially used calibration solution of AEDs and MBs

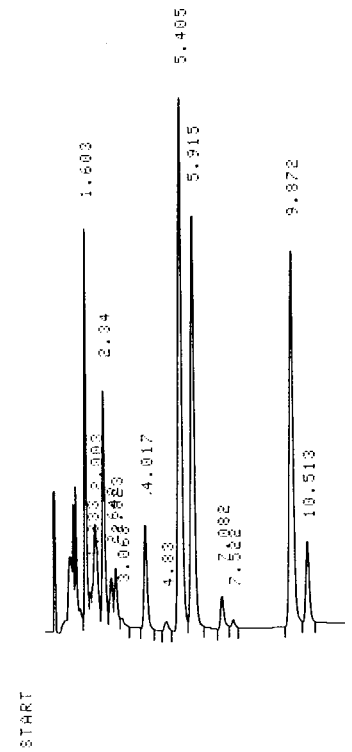

FIGURE 5 Chromatogram of a patient sample: *RT*: 2.340 ET 51.5 [μg/ml], 4.017 DIOL 3.2, 5.405 PB 33.6, 5.958 DM 31.0, 7.082 EPO 1.1, 9.872 PT 18.7, 10.513 CBZ 3.5.

in water/acetonitrile (90:10 v/v) was limited. Therefore we produced calibration samples for the analysis of AEDs with direct serum injection which are dissolved in bovine albumine solutions [see Materials and Methods (1)]. These albumine solutions can be stored at −18°C.

Materials and methods (1)

Apparatus

The equipment was obtained from the following firms: automatic pipetter/diluter from Corning/Gilford, Düsseldorf (F.R.G.); vortex

evaporator from Buchler Instruments, Searle Analytic Inc., Fort Lee, N.J. (U.S.A.); autosampler WISP 710 B from Waters, Koenigstein (F.R.G.); column switching module SE-1, HPLC low-pressure gradient-former 2500, high precision pump model 300 B, constant flow pump model 600/200, spectrophotometer SP-4, Shimadzu integrator C-R3A and Spark Holland HPLC column thermostat SpH 99 from Gynkotek, Munich (F.R.G.); analytical column (125 mm × 4.6 mm I.D.) and guard-cartridge (10 mm × 4.6 mm I.D.) filled with Shandon–ODS Hypersil® (5 μm) and pre-column cartridges (10 mm × 4.6 mm I.D.) filled with Nucleosil® 30 C-18 from Bischoff-Analysentechnik, Leonberg (F.R.G.).

Chemicals

The chemicals were obtained from the following firms: primidone (PR), ethosuximide (ET), phenytoin (PT), carbamazepine (CBZ) and carbamazepine-10,11-epoxide (EPO) form Desitin-Werk/Carl Klinke, Hamburg (F.R.G.); 2-ethyl-2-phenyl-malonediamide (PE) and N-desmethyl-methsuximide (DM) from Aldrich-Chemie, Steinheim (F.R.G.); phenobarbital (PB) from Bayer, Leverkusen (F.R.G.); 10,11-dihydro-10,11-dihydroxy-carbamazepine (DIOL) from Ciba-Geigy, Basle (Switzerland); albumine, bovine fraction V from Paesel, Frankfurt a.M. (F.R.G.); acetonitrile ChromAR® from Promochem, Wesel (F.R.G.); the water was selfmade with a ORGANICpure-HPLC-Wassergeraet® from Wilhelm Werner, Koeln (F.R.G.); all other chemicals were of analytical reagent grade and obtained from Merck, Darmstadt (F.R.G.) or Baker Chemicals, Deventer (The Netherlands).

Calibration samples

One-hundred μl quantities of a stock solution containing 25 mg PE, 100 mg ET, 25 mg PR, 5 mg DIOL, 50 mg PB, 50 mg DM, 5 mg EPO, 25 mg PT and 15 mg CBZ in 100 ml acetonitrile were given into 10 ml screw-capped centrifuge tubes by an automatic pipetter/diluter. The solvent was evaporated off and the residues were redissolved in 1,000 μl quantities of a solution of bovine albumine in bi-distilled water (5%). The tubes were shaken for 30 minutes at 37°C and then frozen at −18°C.

For external standardisation in the AED analysis by direct sample injection the calibration samples were thawed out and treated in the same manner as the patient samples.

Chromatographic parameters

Temperature of the analytical column: 70°C. Injection volume: 50 µl. Detection wavelength: 205 nm. Purge liquid: 0.01 vol. % phosphoric acid (pH 3). Flow rate of the purge liquid: 0.5 ml/min. Duration of washing: 200 sec. The solvents for the separation with gradient elution are pre-mixed to avoid degassing problems. Mixture A: water/acetonitrile (90:10 v/v). Mixture B: water/acetonitrile (40:60 v/v). Flow rate for the analytical column: 1.5 ml/min. Gradient program: from the start 2 min with 90%A/10%B (=15% acetonitrile), in 10 min to 60%A/40%B (=30% acetonitrile).

2. DETERMINATION OF PHENYTOIN AND CARBAMAZEPINE IN SERUM ULTRA-FILTRATES

In serum a relatively high amount of the AEDs phenytoin and carbamazepine is bound to serum proteins. The degree of protein binding is reported to be 87–94% for PT and 72–76% for CBZ.[14]

It is supposed that only that part of the AEDs which is not bound to proteins is able to penetrate the blood-brain-barrier to be efficient in the brain. Therefore it can be concluded that the unbounded concentrations of PT and CBZ in the serum give a better correlation with the clinical effects and side effects than the total amount of drugs in the serum.[15] Systems for the ultrafiltration of serum are commercially available which are able to retain more than 99% of the serum proteins.[14] A sample enrichment using liquid–solid extraction by pre-columns is especially suitable for the analysis of the ultra-filtrates because the concentration of CBZ is reduced to 25% of PT to ca. 10% in the filtrate, compared with the original total concentration in the serum.

An ultra-filtration of 500–1,000 µl serum gives the yield of 230–300 µl filtrate, from which duplicate injections of 100 µl can be made. The samples were concentrated by the sample washing step and no band broadening can be caused by the high injection volume of 100 µl.

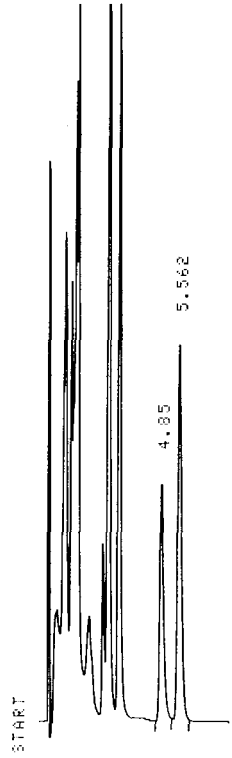

FIGURE 6 Chromatogram of a calibration sample for serum filtrates; *RT*: 4.850 CBZ 1.5 [μg/ml], 5.562 PT 2.5.

The chromatogram of the ultra-filtrate (see Figure 7) shows a late peak at a retention time of about 18 minutes. This unknown unpolar compound can only be observed when serum filtrates are injected. Obviously, this compound is extracted from the ultra-filter membrane.

If the HPLC pump is connected with a gradient former, this unknown compound can be eluted earlier. If only the isocratic conditions [see Materials and Methods (2)] can be used, the run time for the chromatogram of serum filtrates should be prolonged to 20 minutes to avoid an interference of the following separations.

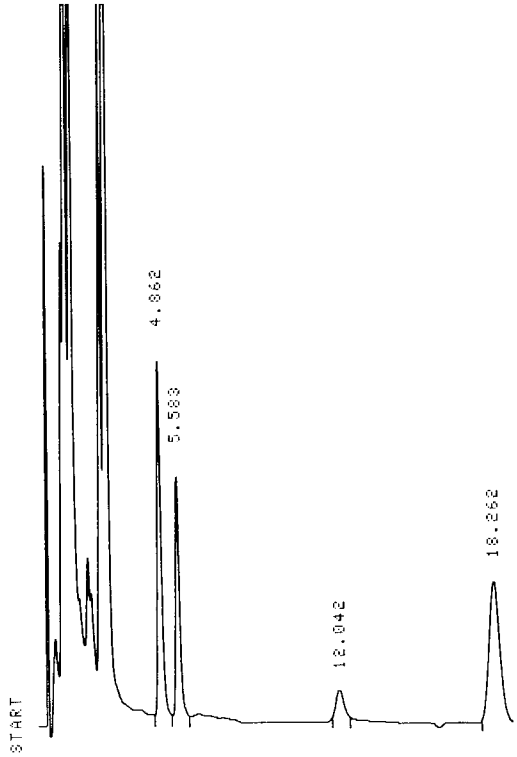

FIGURE 7 Chromatogram of a filtrate of a patient sample; *RT*: 4.862 CBZ 2.23 [μg/ml], 5.583 PT 1.59.

Materials and methods (2)

Apparatus and chemicals

The automatic superspeed centrifuge Sorvall® SS-3 with an angle-head rotor was obtained from Ludwig Hormuth, Heidelberg (F.R.G.); the EMIT® FreeLevel (TM) System I including one-way tubes with ultra-filter membranes were obtained from Syva/Merck, Darmstadt (F.R.G.); the remaining equipment and chemicals were the same as for the direct injection of serum samples.

Calibration samples

The calibration samples for the analysis of the free level of PT and CBZ in serum were the same as those used for the direct injection of serum samples. As the PT concentration in the ultra-filtrates is expected to be about 10% of the original concentration in the serum, the albumin calibration samples are diluted with a sodium acetate solution (1%).

A quantity of 100 µl of the thawed calibration sample and 900 µl of the sodium acetate solution are mixed to give concentrations of 2.5 µg/ml PT and 1.5 µg/ml CBZ in the diluted samples. Quantities of 100 µl of this diluted calibration samples as well of the ultra-filtrates are injected to carry out external standardisation.

Sample centrifugation

500–1,000 µl quantities of serum were pipetted into an ultra-filtration tube and centrifuged for 20 minutes with 2,000 × g in a centrifuge with an angle-head rotor. The interior temperature of the rotor must be 25 ± 3°C because temperature affects drug binding to proteins.[14]

Chromatographic parameters

Temperature of the analytical column: 20°C. Injection volume: 100 µl. Solvent mixture for the isocratic separation: water/acetonitrile (70:30 v/v). The remaining chromatographic parameters are the same as for the direct injection of serum.

Acknowledgement

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