Influence of Mobile Phase Conditions on the Clean-Up Effect of Restricted-Access Media Precolumns for Plasma Samples Injected in a Column-Switching System

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Key Words
Column liquid chromatography
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Mobile phase composition

Summary
Various mobile phases including phosphate buffer, pure water and five kinds of biological buffers (pH around 7) were systematically studied in terms of their ability to clean-up plasma matrix on precolumns of restricted-access media in a column-switching system. The necessary washing time, buffer pH, type and content of organic modifier were evaluated with respect to plasma elution profiles on restricted-access media precolumns. The influence of different mobile phases on the recovery of plasma matrix from alkyl-diol silica precolumns was studied by means of a scanning spectrophotometer. Our results show that phosphate buffer near physiological pH with small amounts of 2-propanol or acetonitrile was preferable for direct injection of large plasma volumes (500 μL). More than 93 % of the proteins in a plasma matrix can be recovered within 3 min from the alkyl-diol silica C18 column (25 mm x 4 mm I.D.) as measured at 280 nm for all selected mobile phases except for tris (hydroxymethyl) aminomethane buffer, from which only about 88 % was obtained.

Introduction
The column-switching technique, utilizing restricted-access media (RAM) as precolumns, has been shown to be an ideal direct injection technique for analysis of small molecules in biological fluids, because sample pretreatment and analytical separation were integrated in the same system [1–11]. The major advantages of such systems over other direct injection techniques, e.g. single column LC mode, such as the use of RAM columns or applying micellar [12] or surfactant [13, 14] eluents for conventional reversed-phase columns, is its re concentration ability, low detection limit and flexibility in combining different separation mechanisms. Furthermore, RAM precolumns have revealed much longer lifetime than that of conventional precolumns [7, 9, 10, 15, 16], since the majority of proteins and endogenous compounds are eluted within the void volume of RAM columns.

Plasma and serum samples are known to be the most complex biological matrices. Proteins and other potential interferences possess different physical and chemical characters and are present in matrices at high levels (μg mL⁻¹–mg mL⁻¹) [17]. Such samples are susceptible to environmental changes, such as storage time, freeze-thaw cycles, and furthermore the compound concentrations in matrices may vary individually. On the other hand, separation of analytes from matrices and detection at ng mL⁻¹ levels are generally required for an analytical method applied in pharmacokinetic and biopharmaceutical studies. When the drug of interest neither has selective functional moieties (visible chromophore, fluorophor or electrochemical properties) nor can easily be derivatized, increasing the injection volume and setting the ultraviolet detection at the short wavelength region, e.g. 210 nm, are prevailing ways to enhance the detectability [9, 10]. However, problems may arise. Firstly, increase of injection volume simply means increasing the risk of adsorption of protein related compounds on the RAM packings. Secondly, considerable high background absorbance and fluctuating baseline caused by the matrices could make the detection at a nonselective wavelength, e.g. 210 nm, impossible.

The role of the mobile phase would be to create such a chemical environment that effectively solvates proteins and endogenous compounds to make them elute without being retained on the column; to extend the RAMs lifetime; to be compatible with the selected detection principle. Therefore, the selection of mobile phase conditions for RAM precolumns should take the following factors into account: (1) Maintenance of proteins in their native state. (2) Elution of proteins and other
endogenous compounds to waste as quickly as possible. (3) Retaining analytes properly and removing proteins and endogenous compounds to the utmost to minimize the possible interferences under nonselective detection, ideally UV 210 nm. (4) Obtaining high recovery for analytes. (5) Matching with the separation mobile phase to eliminate interfering system peaks.

The mobile phase pH and the organic modifier content have been considered as critical parameters in direct injection techniques [9, 18-27]. No systematic studies on the evaluation of appropriate mobile phases for RAM precolumns in the column-switching systems have yet been reported. In this work, different mobile phase systems including phosphate buffer, water and biological buffers were applied on precolumns of either semipermeable surface (SPS) or alkyl-diol silica (ADS) types. The effect of washing time, buffer pH, type and content of organic modifier were studied by connecting the precolumn to a UV detection via a column-switching device. The evaluation of the mobile phase systems in terms of recoveries of plasma matrix was based on scanning of fractions collected from the precolumn at different time periods using a UV spectrophotometer.

**Experimental**

**Chemicals**

Methanol and acetonitrile (LiChrosolv), 2-propanol (gradient grade), tris(hydroxymethyl)aminomethane (TRIS), 1 M 9956 titrisol sodium hydroxide solution and ortho-phosphoric acid were purchased from Merck (Darmstadt, Germany). 2-[2-Amino-2-oxoethyl) amino] ethanesulfonic acid (ACES), N,N-bis[2-hydroxyl] glycine (BICINE) and 3-[N-morpholino] propanesulfonic acid (MOPS), were from Sigma (St. Louis, USA). N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic] (HEPES) was provided by Janssen Chimica (Geel, Belgium). Tetrabutylammonium hydrogen sulfate (TBA) was obtained from Fluka (Buchs, Switzerland). All were of > 99 % purity.

**Plasma Sample Preparation**

Pooled blank plasma obtained from the Blood Centre, Academic Hospital (Uppsala, Sweden), was stored at ~20 °C. Prior to injection, the blank plasma was thawed under room temperature and filtered through 0.22-μm Millex-AP/GS syringe filter units (Millipore S.A., Molsheim, France) with no further dilutions.

**Precolumns and Column Packing Procedures**

SPS precolumns with a C18-ligand as the internal surface, 5 μm, 10 × 10 mm I.D., were purchased from Regis (Morton Grove, IL, USA). ADS columns of C8 and C18, 25 μm, 25 × 4 mm I.D., were dry packed, sealed with sieves and PTFE nets (Institute of Clinical Chemistry, University Hospital, Munich). All columns were conditioned in 2-propanol.

**Chromatographic Systems and Conditions**

Two chromatographic systems were employed in the study. One was composed of two pumps, pump 1 (LKB HPLC 2150, Bromma, Sweden) and pump 2 (Beckman 114 M, Berkeley, CA, USA), both of them equipped with SSI pulse dampers, a 10-port valve (Valco, Houston TX, USA) with a 500 μL loop (Valco Europe, Switzerland), a LDC 3100 variable wavelength UV monitor (Riviera Beach, FL, USA) reading at 210 or 280 nm and a recorder (Kipp & Zonen BD 112, Holland). The precolumn was connected to the UV detector via a switching device in a back-flush mode similar to that used in ref. [9]. Plasma samples were firstly directed to waste for certain volumes using the studied mobile phase, and was then transferred for detection. The precolumn was washed with water and methanol before introducing a new mobile phase. At least 50 mL of mobile phase passed through the column prior to injecting plasma. Another chromatographic system consisted of the above described pump 2, a Rheodyne injector 7125, equipped with a 500 μL loop (Berkeley, CA, USA) and the precolumn. The injection volume for both systems was 500 μL. The systems were kept at ambient temperature.

**The Recovery of Plasma Matrix**

The recoveries of plasma matrix using different mobile phases were studied in the second system by scanning absorbance spectra on a spectrophotometer (Cecil 3000 series, Cambridge, England). The spectrophotometer was controlled by a personal computer (Goldstar Co. Ltd., Seoul, Korea). Plasma fractions eluted from the precolumn were collected at three periods at three-minute intervals. References related to each studied mobile phase for calculating the total plasma proteins was made in such a way that a 500 μL plasma was identically injected into the loop with no column in place and collected for 3 min. Each injection with and without the column was, at least, performed in triplicate. All first fractions collected from 0-3 min were diluted 50-fold prior to scanning on the spectrophotometer. Respective mobile phase was stored as baseline. The path length of the radiation through the sample was 1 cm.

**Results and Discussion**

**Effect of Mobile Phase Conditions on the Elution of Plasma Matrix**

Phosphate buffer, pH ~ 7.4, was the first buffer of choice, since it is a physiological buffer. This buffer would supply an appropriate environment to stabilize most of plasma proteins. Its pH value is suitable for retaining most of reasonably hydrophobic neutral and weak basic substances on reversed-phase columns. Furthermore, an ionic strength around 0.05 would give high solubility for most plasma proteins and acceptable buffer capacity for analytical objects.