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Alkyl diol silica: restricted access pre-column packings for fast liquid chromatography-integrated sample preparation of biological fluids

Christian Schäfer*, Dieter Lubda

Merck KGaA, Frankfurter Strasse 250, D-64271 Darmstadt, Germany

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Abstract

A silica-based pre-column packed with a 'restricted access material' was used for fully automated solid-phase extraction of drugs and metabolites from untreated biological fluids via a simple valve-switching method. The bifunctional sorbent employed, alkyl diol silica, is characterised by a different chemical modification of the outer surface (diol groups) and the pore surface of the particles (alkyl chains: C_4, C_8 or C_{18}). This allows for a fast and efficient clean-up of complex biological samples, such as blood, serum, urine or milk, with exclusion of the macromolecular matrix (proteins, nucleic acids) in less than 1.5 min.

Keywords: Restricted access media; Extraction methods; Sorbents; Alkyldiol silica; Automation

1. Introduction

The ever increasing number of samples, together with pressure for higher laboratory efficiency, requires the development of high-throughput, fully automated, analytical methods. In the HPLC analysis of low-molecular-mass compounds (drugs, metabolites) from biological fluids (hemolysed blood, plasma, serum, gastric juice, milk, fermentation broth, supernatants of cell cultures, tissue homogenates), macromolecular matrix components present a challenge for sample preparation: proteins and nucleic acids have to be removed before chromatographic separation. Larger amounts of organic solvents in the mobile phase, and non-specific interactions with the residual silanol groups on the surface of chromatographic supports lead to denaturation and irreversible adsorption of the macromolecular matrix constituents. Because of their size, proteins are preferentially bound to the outer surface of a porous particle, where they block access to the pores for smaller analytes. In this way, they reduce the amount of adsorption sites available for chromatographic interactions. The result is a remarkable loss in column capacity, a decrease in selectivity (through surface modification by protein adsorption) and an irreversible increase of column back-pressure, which adversely affects the life-time of the HPLC column. In order to minimise these effects, acids (e.g., perchloric acid, chloroacetic acid) are commonly added to precipitate proteins and nucleic acids prior to HPLC analysis. However, some drugs and metab-

*Corresponding author. Tel.: +49-6151-72-5477; fax: +49-6151-72-8548.

E-mail address: chris.schaefer@merck.de (C. Schäfer).

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olites are known to bind to proteins and are lost due to co-precipitation upon the addition of acid, which leads to a loss of sensitivity and reproducibility of the analytical method.

Macromolecular sample components can be simply and effectively removed by restricted access materials (RAMs), tailor-made solid-phase extraction (SPE) sorbents that allow for direct and repetitive injection of untreated biological fluids [1–6]. The sample clean-up (fractionation) of the sample into matrix and target analytes with these porous, mostly, silica-based sorbents relies on the simultaneous occurrence of two chromatographic separation mechanisms: selective reversed-phase interaction or ion-exchange chromatography of low-molecular-mass analytes and size exclusion chromatography (SEC) for the macromolecular sample constituents. However, this requires, in addition to a well-defined accessibility to the pores, a special topochemistry of the particle surface. Therefore, RAM sorbents are bifunctionally modified particles. The adsorption centres for small analytes are localised exclusively at the inner pore surface (e.g., *n*-alkyl groups, ion-exchange groups), while the outer particle surface, which is in contact with macromolecules such as proteins and nucleic acids, carries biocompatible functional groups. Many different RAM sorbents have been developed in recent years and were discussed in detail in a comprehensive review by Boos and Rudolphi [4]. A very efficient way to obtain a hydrophilic and non-participating particle surface is by derivatisation with diol groups. The resulting alkyl diol silica (ADS; e.g., LiChrospher RP ADS, Merck, Darmstadt, Germany) is electroneutral and has non-denaturing properties with respect to proteins and nucleic acids. The macromolecular matrix components will not adsorb on the particle surface; instead, they will remain in the void volume between the particles. A pore size of 6 nm allows access to the pores only for analytes with a molecular mass below 15 000 (SEC). The proteins can thus be eluted with the void volume and can be transferred directly into the waste. Smaller analytes, however, such as drugs and metabolites from body fluids, pesticides or hormone residues from milk or animal tissue samples are able to enter the pores and interact with the alkyl chains or ion-exchange groups bound to the inside of the pores (Fig. 1). A RAM sorbent with RP-4, RP-8 or RP-18 modification can be selected, to perfectly match the polarity of the target analytes. In this way, the macromolecular fraction can be quantitatively removed from a native biological fluid without the need for pH- or solvent-induced precipitation of the proteins, leading to the disadvantages described above. If the RAM sorbent is filled into a short pre-column (e.g. 25×4 or 25×2 mm for LC–MS), the column can be integrated into the existing HPLC setup via a simple valve-switching procedure and can, under optimal conditions, be reused for up to 2000 fractionation cycles. The experimental arrangement and the working principle of the system setup are described in the following section.

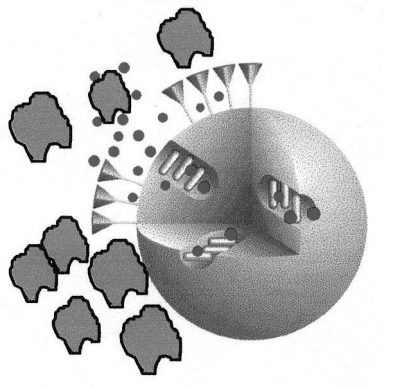

Fig. 1. Schematic picture of a restricted access material.

2. Experimental

Fig. 2 shows the valve-switching setup for coupling the RAM pre-column to the analytical HPLC system. In addition to the RAM column, a standard six-port valve and an extra (isocratic) pump are required. In the first step, the native biological fluid is fractionated via the RAM pre-column. After the sample matrix has been quantitatively eliminated, the six-port valve is switched into the second position, for desorption of the analytes and transfer to the analytical HPLC column (Fig. 3). It is important to note that the analytes are desorbed in the ‘backflush’

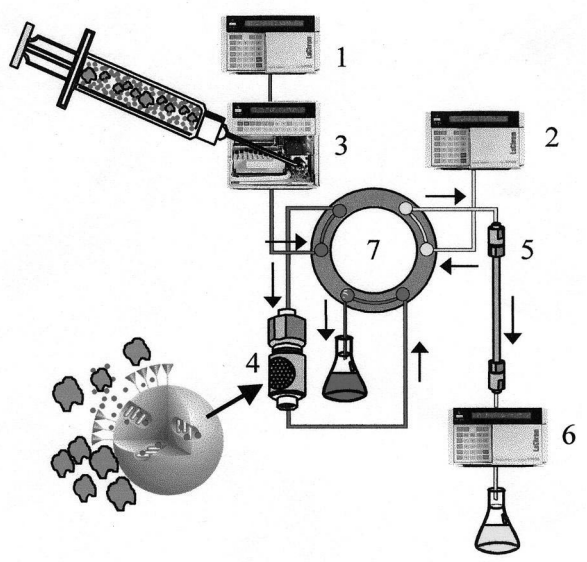

Fig. 2. Column-switching setup for LC–integrated sample preparation; initial valve-switching position (1): fractionation of the sample (exclusion of protein matrix). 1=Pump (isocratic); 2=HPLC pump; 3=Sample injector; 4=RAM pre-column; 5=Analytical column; 6=Detector; 7=Six-port switching valve.

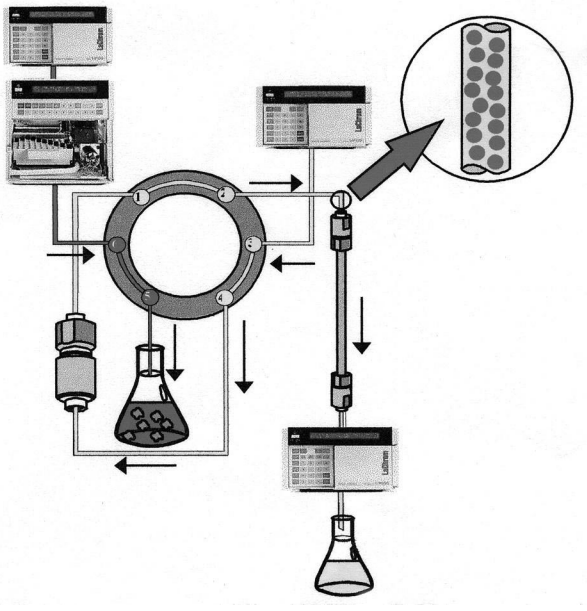

Fig. 3. Valve-switching position (2); desorption and transfer of the analytes to the HPLC column.

mode by the solvent that is delivered from the gradient pump of the HPLC system. Analyte elution in the reverse direction leads to fast regeneration of the RAM pre-column; the gradient pump allows the use of any mobile-phase composition required for optimal analyte transfer. Since, at this stage, the macromolecular matrix components have already been quantitatively removed from the system, a higher fraction of organic modifier (methanol, acetonitrile) in the mobile phase can be used to speed up the desorption process of the trapped analytes. However, the content of organic modifier in the mobile phase should be selected carefully; it should be high enough to allow for rapid desorption of the analytes from the RAM pre-column, but should still be low enough to prevent the start of the chromatographic separation process on the HPLC column. In this way, the analytes are focused into a small sample zone at the head of the analytical column (in-line peak compression), which leads to better peak shapes and improved detection sensitivity [7]. Once the analyte transfer is complete, the six-port valve is switched back to its initial position (Fig. 2). While chromatographic separation of the first sample occurs on the HPLC column, the second sample can be fractionated on the RAM pre-column (Fig. 4). By controlling the sequence of the sample fractionation step (pre-column) and the analytical separation (HPLC column), the total analysis time can be reduced significantly [8].

3. Results

With the analytical method presented here, biological fluids carrying a high load of proteins (plasma, serum, hemolysed whole blood, fermentation broth, supernatants of cell cultures or tissue homogenates) can be directly, i.e., in the native state, fractionated after a simple centrifugation step. Other application fields for RAM phases are the determination of residues in animal or human milk (pesticides, antibiotics, hormones). The enormous potential of the on-line column-switching method for the analysis of biological fluids lies, not only in the convenient fractionation of complex samples into matrix and target analytes, but also in the indepen-

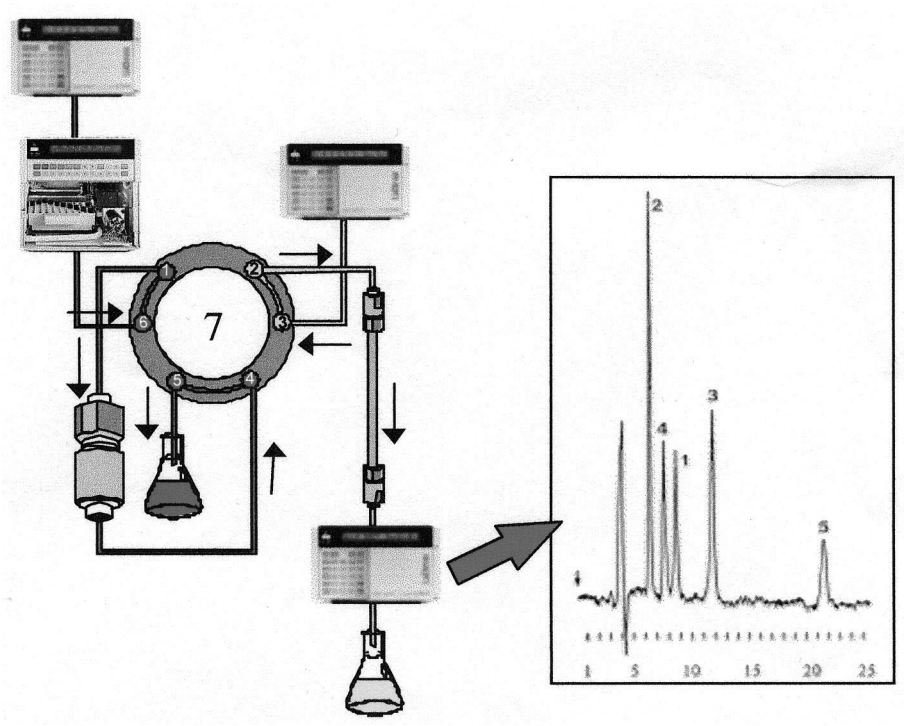

Fig. 4. Simultaneous chromatographic separation (HPLC column) and fractionation of the next sample (RAM pre-column).

dent choice of the stationary and mobile phases used for sample clean-up and chromatographic separation, which offer more flexibility. For example, for the extraction of epirubicin and metabolites from a human plasma sample, aqueous conditions and a RAM column with RP-4 modification have been used for the fractionation step, while the chromatographic separation was carried out on a C_8- modified HPLC column (LiChrospher 60 RP Select B, Merck) with 30% organic modifier in the mobile phase (Fig. 5) [9]. A large number of applications on RAM sorbents, with experimental setup and method description are summarised in a comprehensive collection [10].

In order to keep pace with the ever increasing number of samples in many fields of bioanalysis, automation and integration of sample preparation into the analytical separation system is only one step. Another important part is the coupling to fast and selective detection methods, such as mass spectrometry (MS) and even to multi-stage MS detectors (MS^n) [11]. However, most of the time, the high-throughput level of a MS–MS system (sample injection every 2–5 min) cannot be achieved due to

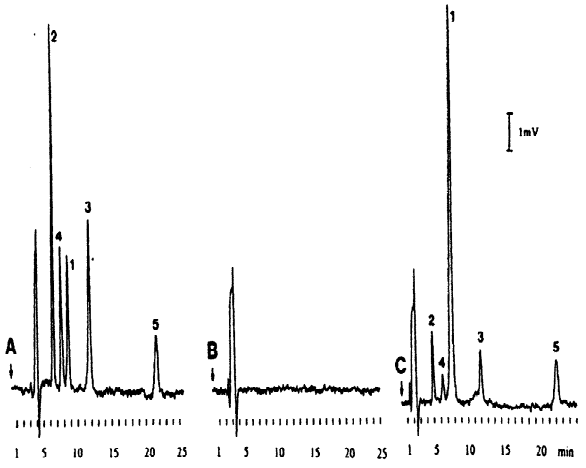

Fig. 5. Epirubicin and metabolites from human plasma: (A) standard, (B) human plasma and (C) human plasma 15 min after liver tumor chemoembolisation. Conditions: RAM pre-column, LiChrospher RP-4 ADS, 25 μm, 20×4 mm I.D.; analytical column, LiChrospher 60 RP select B, 250×4 mm I.D.; sample fractionation, water–methanol (95:5, v/v); flow-rate, 1 ml/min; analyte transfer and separation, acetonitrile–water (30:70, v/v); (0.1% TEA, pH 2.0, with TCA), flow-rate, 1 ml/min; detection, fluorescence (excitation, 445 nm; emission, 560 nm); injection volume, 50 μl; peak identification: (1) epirubicin; (2) epirubicinol; (3) epirubicin-aglycon; (4) epirubicinol-aglycon; (5) 7-deoxy-epirubicinol-aglycon (from [9] with permission).

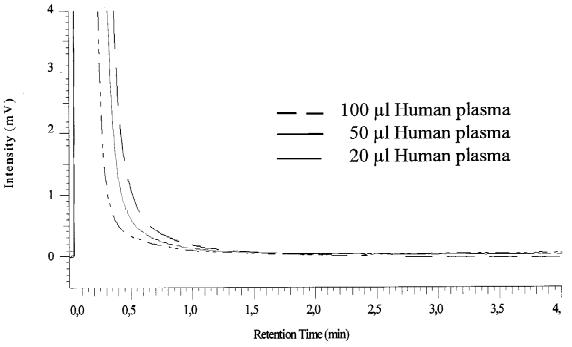

Fig. 6. Matrix elution profile for high-speed on-line sample fractionation. RAM pre-column, LiChrospher RP-18 ADS, 25 µm, 25×2 mm I.D.; detection, UV at 284 nm; mobile phase, water; flow-rate, 4 ml/min; sample, 20 µl human plasma (Figure courtesy of C. T. Fleischer, Ludwig-Maximilians-University, Munich, Germany).

the sample-preparation step, which generally constitutes the bottleneck of the analytical method. By implementing the fast, on-line SPE concept using RAM phases in a coupled column mode, it is possible to reduce analysis times for drugs from plasma or urine by 75% by applying flow-rates of 4 ml/min for the fractionation of biological fluids into a macromolecular matrix and low-molecular-mass analytes on a RAM pre-column. In that way, it is possible to fractionate 20 µl of human plasma in less than 1.5 min in a simple and reproducible way (Fig. 6). Once sample fractionation is completed, the analytes are transferred via valve-switching to the LC–MS–MS system [12]. Depending on the injection volume (typically 20–50 µl), the time required for one analytical cycle can be reduced to 2–5 min, leading to a sample throughput of 300–600 samples per day.

4. Conclusions

The use of RAMs is a powerful method to fully automate the sample preparation of biological fluids (hemolysed blood, plasma, serum, gastric juice, milk, fermentation broth, supernatants of cell cultures, tissue homogenates). By applying these materials to pre-columns in a valve-switching mode, fast and efficient sample clean-up is possible in less than 1 min, using high flow-rates for sample fractionation. Due to the long life time of silica-based RAM phases with biocompatible outer surfaces, this method provides maximum cost efficiency for SPE. Pre-columns packed with non-RAM-type sorbents, such as polymers, have recently been suggested for similar use in pre-columns (e.g., Oasis HLB, Waters, Milford, MA, USA). However, due to their non-specific binding characteristics, polymer sorbents are subject to protein adsorption, which reduces the lifetime of these columns tenfold compared to the diol-modified RAMs, which can be used for up to 2000 analytical cycles with injection volumes of 50 µl (Table 1).

Method development can be done manually [8] or using new software [13] that optimises all sample preparation steps (choice of RAM and HPLC column, selection of mobile phase for sample fractionation and analyte transfer, calculation of valve switching times) based on the structures of the target analytes. Quantitative and matrix-independent elimination of macromolecular components leads to stable baselines without peak interferences, giving maximum flexibility in the choice of the detector; UV detection below 240 nm and electrochemical detection are possible without interference from abundant proteins, allowing for easy peak identification and quantitation. Once the method has been developed, manual assistance or supervision from

Table 1
Comparison of sorbents for high-speed on-line sample preparation

Product	Basic support	Surface chemistry	Swelling/ shrinking	Column dimensions	Column change after
Oasis HLB (Waters)	Macroporous copolymer, 25 µm	Divinylbenzene/ *N*-vinylpyrrolidone	Possible	50×1 mm 20×2.1 mm	200 plasma injections of 50 µl each
LiChrospher RP-ADS (Merck)	Spherical silica gel, 25 µm	Outer surface: diol pore surface: C_4, C_8 or C_{18}	Not possible	25×2 mm 25×4 mm	2000 plasma injections of 50 µl each

laboratory personnel is no longer required, which significantly improves the quality of the working environment, especially if highly infectious samples (e.g., blood from patients with human immunodeficiency virus or hepatitis infection) have to be handled. Integrating sample preparation into the fully automated HPLC system enables the analyst to process large sample series overnight or over the weekend and, thus, increase laboratory efficiency significantly. As was demonstrated in this paper for the first time, RAMs are also suitable for rapid, high-throughput analyses, by performing sample fractionation at high flow-rates and coupling to fast detection methods (MS–MS). Flow-rates of 4 ml/min or even higher do not impair the quality of the analysis.

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