Using restricted-access materials and column switching in high-performance liquid chromatography for direct analysis of biologically-active compounds in complex matrices

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In the bioanalytical field, sample preparation is often considered the time-limiting step. Indeed, extraction techniques (e.g., liquid–liquid extraction (LLE) and solid-phase extraction (SPE)) are commonly used off-line for biological matrices.

To perform high-throughput analysis, there have been efforts to develop a faster sample-purification process. Special extraction sorbents, such as restricted-access materials (RAMs), allow direct, repetitive injection of complex biological matrices onto these supports. Coupling RAMs to column-switching high-performance liquid chromatography (HPLC) systems is a very attractive approach to biological sample preparation. This technique leads to automation, simplification and speeding up of the sample-preparation process.

In this article, we review coupling of RAMs to column-switching systems and give particular attention to commercially available supports. These RAMs are used in single-column or column-switching configurations for direct analysis of compounds in various biological fluids.

Keywords: Biological matrix; Column switching; High-performance liquid chromatography; HPLC; RAM; Restricted-access material; Solid-phase extraction; SPE

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1. Introduction

Restricted-access materials (RAMs) have been appearing in the scientific literature in the past two decades. RAMs are used mainly for the analysis of substances with low molecular mass (e.g., drugs, endogenous substances, and xenobiotics) in complex matrices containing high-molecular substances (most frequently proteins). RAMs enable direct injection of the biological sample into flow-analysis systems without previous sample treatment. They have several different structures, but their mechanism of separation is identical: a hydrophilic barrier enables the small molecules to permeate through the hydrophobic part of the stationary phase, and, at the same time, it excludes the macromolecules (by physical or chemical means, or a combination). Two principles are therefore applied:

- gel chromatography is used to exclude macromolecules (i.e. separation is based on molecular size); and,
- separation of the smaller molecules by adsorption or ion-exchange chromatography (i.e. separation is based on the way that small molecules interact with the stationary phase (see Fig. 1)).

The barrier preventing the entry of macromolecular substances into the hydrophobic part of the stationary phase can be of various types. The pores of the external stationary phase (their size is mostly 60 Å) have been recognized as the physical barrier for macromolecular substances excluding most of the serous and plasmatic proteins. Hydrophilic functional groups on the surface of a sorbent or polymer network, which are bound to the surface of stationary phase by covalent

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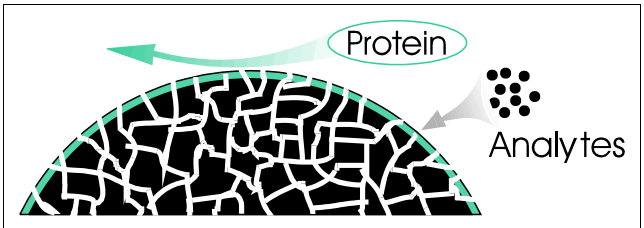

Figure 1. Structure of restricted-access material.

bonding, might also provide a chemical barrier for macromolecules. The polymer network also prevents the proteins from precipitating.

In 1985, Hagestam and Pinkerton [1] published the first paper on the subject of RAMs. Since then, not only has the number of papers on the subject grown but also more types of RAM based on different principles have been developed. There are five basic types of RAM, divided by group according to the nature of the barrier and the surface structure of the sorbent:

- mixed-functional phases and dual-zone materials;
- internal surface reversed-phase packings;
- shielded hydrophobic phases;
- semi-permeable surfaces; and,
- polymeric materials.

2. Types of RAM

2.1. Mixed-functional phases and dual-zone materials

Both the outer and internal surfaces of these materials show identical properties, which are provided by covalent bonding of two different functional groups or by one functional group with two possible interactions.

Silica gel, which has small pores (up to 55 Å), uses randomly distributed hydrophilic groups [2] to ensure the removal of macromolecules from the surface.

Another type of dual-zone stationary phase utilizes two different active centers belonging to one functional group – the hydrophilic part on the surface and hydrophobic chain inside the functional group [3]. Diol stationary phase can be considered as an example of such sorbents. The ethandiol endings of functional groups, which form a hydrophilic layer, preclude access of proteins to the internal stationary phase, whereas metoxypropyl chains covered by a diol surface retain low-molecular mass analytes.

In 1994, Kanda et al. [4] introduced this type of material, which was commercialized under the trade name Capcell Pak MF. Both outer and internal surfaces comprise a mixture of hydrophilic polyoxyethylene and hydrophobic styrene groups bound to a silicone polymer coated with porous silica gel (8 nm). The access of macromolecules is restricted by the long polyoxyethylene chains. Apart from styrene groups, C8, phenyl, and strong cation exchange (SCX) are also available as hydrophobic groups. Small analytes are retained by the interaction with hydrophobic or ion-exchange groups. These materials have a shorter lifetime than other RAMs, being able to cope with a total volume of only a few ml of biological fluids; however, Capcell Pak MF and Capcell Pak SCX materials have been recognized as a suitable approach for the direct determination of drugs in biological fluids.

2.2. Internal surface reversed phases

Internal surface reversed phases (ISRPs) are the oldest RAMs, introduced by Hagestam and Pinkerton [1] in 1985. These materials are characterized by two types of surfaces, two types of bound functional groups. The hydrophilic phase covers the outer surface of a sorbent, whereas the hydrophobic or ion-exchange phase [5] links to the internal surface of sorbent pores. The internal pore diameter functions as a physical barrier separating macromolecules from low-molecular-mass analytes. The separation mechanism is therefore a combination of reversed-phase and gel chromatography. Small molecules are able to diffuse into pores (60 Å) and are separated according to the hydrophobic interactions with the internal surface. However, the size of the pores does not allow the macromolecular substances to enter, so it excludes them from interaction with hydrophobic chains that are covalent-bound at the internal surface. Glass-fiber filter (GFF) sorbents and alkyl-diol-silica (ADS) are embedded in ISRP materials. GFF sorbents comprise porous silica gel. Their outer surface is covered by hydrophilic diol-glycine groups and the internal hydrophobic surface is formed by tripeptide glycine-phenylalanine-phenylalanine [6].

The retention mechanism is mainly caused by π-electron interactions. Moreover, the free carboxyl-group end of phenylalanine shows weak ion-exchange functionality. The ISRP materials may withstand several thousand plasma (or serum) injections, the total volume equivalent to 6–7 ml. GFF materials are suitable for the direct determination of drugs and drug metabolites in biological matrices, and for direct analysis of endogenous substances in serum and peptides from complex extracts. ADS together with GFF sorbent belongs to the group of the most popular RAMs; both have been produced since the 1990s. The structure of ADS material is very similar to GFF particles. The hydrophilic groups – glyceryl-propyl or diol are bound to the outer surface of particles. The ADS materials [7] are characterized by the different types of reversed phases (butyryl-C4, capryloyl-C8, or stearoyl-C18) on the internal surface. New ADS materials are formed by the sulphonic acid groups bound to the internal surface of particles. Ion exchangers, so-called XDS (exchange diol silica), are recognized as a suitable approach for the direct analysis of endogenous substances and pharmaceuticals in biological fluids (plasma, serum, urine, microdialysate, saliva, liver

homogenate, intestinal aspirate, cell cultures, bronchial secrets, maternal milk, and tissues). An ADS pre-column might withstand injection of 80–100 ml of plasma. LiChroCART (25 × 4 mm) filled with LiChrospher ADS RP-18, RP-8 and RP-4, particle size 25 μm (Merck, Darmstadt, Germany), is the most frequently used pre-column.

The preparation of these materials (e.g., alkyl-diol ISRPs) is based on the enzymatic degradation of hydrophobic functional groups (bound at the outer surface of silica gel), while the internal surface is protected against the enzyme thanks to the small pore size. The hydrophilic phase formed by glyceryl-propyl (diol) groups is bonded at the silica gel (pore size of 60 Å).

2.3. Shielded hydrophobic phases

In 1988, Gisch et al. [8] introduced a new type of RAM, named shielded hydrophobic phase (SHP), which is a stationary phase with a chemical barrier preventing the proteins from gaining access to the functional groups that are responsible for separating low-molecular-mass analytes. It is a hydrophilic polyethylene-glycol (or polyethylene-oxide) that forms the embedded hydrophobic phenyl group network within the polymer network and the whole unit is covalently bound onto the silica-gel carrier. The hydrophilic polyethylene-glycol network contains shielding hydrophobic phenyl groups that prevent protein penetration (hydrophilic shielding). Small molecules can still permeate through the polymer layer and interact with hydrophobic groups. The material is produced out of 5-μm silica gel with a pore size of 100 Å. This type of material is commercially available as Hisep SHP (Supelco, USA). The expected lifetime of the sorbents is one of the lowest for all these RAMs – only about 16 ml of serum can be passed through. They are used mainly for pre-concentrating and separating compounds containing phenyl groups [9,10].

2.4. Semi-permeable surfaces (SPSs)

This type of RAM has a typical hydrophilic polymer chemical barrier excluding the proteins from access to the surface. It has both external and internal moieties independently synthesized and, in most cases, covalently bound onto the surface of the silica particles. The outer surface (hydrophilic polyoxyethylene polymer) repels large molecules (such as proteins), while the internal surface, comprising a different type of hydrophobic reversed-phase (e.g., nitrile, phenyl, C8 and C18), retains small analytes that penetrate through the polymer layer.

Initial work in this field used the non-covalent coating of hydrophobic chains achieved with surface-active polymers, tenzides (e.g., Tween and Brij). However, there was a problem with the gradual elution of the polymer layer and regeneration of stationary phase was frequently required. Later, it was replaced by the polyethylene-glycol chain covalently bound directly onto the surface of the stationary phase (C4, C8, C18, CN and phenyl) [11]. This material is commercially available as SPS (Regis Technologies, USA).

Desilets et al. [12] discovered that the polymer (most frequently of a polyoxyethylene nature) bonded to the surface of a reversed-phase (e.g., C8 or C18) forms a semi-permeable hydrophilic layer that can restrict access of proteins to the underlying hydrophobic stationary phase.

Commercially-available SPS materials differ mainly in the functional groups forming the reversed-phase of the internal surface (e.g., nitrile, phenyl, C8 and C18 are the most common). The lifetime of SPS materials is quite similar to ADS materials and they can cope with being loaded with an amount equivalent to 50 ml of plasma. SPS material has successfully been applied to the analysis of small molecules in biological fluids.

One of the newest types of RAM comprises porous silica gel, the outer surface of which is coated with a human-plasma protein, α1-acid glycoprotein (AGP), which is covalently bound to the C18 stationary phase. AGP is a hydrophilic human protein, which is stable in both its natural and immobilized form. Its stability in the presence of organic solvents in the mobile phase is also quite good. The separation principle is similar to the previously described SPS type, and it enables the quantitative removal of proteins from the sample. It functions on the same principle as a chemical-diffusion barrier, apart from the fact that, in this case, the protein network forms the outer hydrophilic surface instead of the polymer. This extraction sorbent was introduced by Hermansson and Grahn in 1994 [13] and commercialized as BioTrap. It makes the external surface of the particles compatible with a proteinaceous sample that cannot penetrate into small pores (10 nm). Hydrophobic groups (C8 or C18) at the internal surface are responsible for interaction with small analytes. BioTrap can tolerate more than 30 ml of biological fluids, so its lifetime and performance are similar to the materials mentioned above. An advantage of this new material is the wider pH working range (2–10 for BioTrap MS). By comparison, common silica-gel materials have pH within the working range 2.5–7.5. This new RAM has been used for the analysis of (e.g., ibuprofen, naproxen, propranolol, carbamazepin, and phenytoin) in human plasma. BioTrap is produced in two variations, both having the same outer surface but with different internal surfaces:

- BioTrap Amine C18 is produced for the extraction of the basic drugs; and,
- BioTrap Acid C18 is for the analysis of the acid drugs.

2.5. Polymeric materials

Columns packed with polymeric materials are used for pre-concentrating hydrophobic and hydrophilic analytes and for removing high-molecular-mass substances (e.g., proteins) in column-switching systems. These extraction

columns are packed with various types of polymers and their producer (Shimadzu) divides them into the following four basic types: MSpak PK series; MSpak GF-4A; MSpak GF-310 or 320 series; and, Asahipak ODP-51 4B.

MSpak PK columns are packed with hydrophilic copolymers that contain N-vinyl acetamide. They show not only high performance in removal of high-molecular weight substances, such as proteins, but also high adsorption of both hydrophilic and hydrophobic analytes. The recovery rates of drugs from these columns were found to be in the range 90–110%. They are suitable for pre-concentrating trace amounts of different substances and also for on-line sample pre-treatment during the analysis of drugs and metabolites in biological fluids. The particle size is 30 μm and the optimal flow rate depends on the column diameter (0.8–3.0 ml/minute). The column can be used over a large pH range (2–12) and they can cope with mobile phases with organic solvents (e.g., methanol or acetonitrile) and buffers up to a concentration of 0.3 mol/l. The maximum pressure on the column should not exceed 10 MPa.

MSpak GF-4A columns remove high-molecular-weight substances using size exclusion. As they are packed with polyvinyl alcohol, they are much more efficient than the PK series as far as the removal of high-molecular-weight substances is concerned; nevertheless, if we compare them to PK series, they are less capable of retaining the hydrophilic substances. They are therefore not useful for the pre-concentrating hydrophilic substances, such as caffeine. The particle size is 9 μm.

Asahipak ODP-51 4B columns are packed with polymer-based gel bound with C-18 groups. Compared with the pre-treatment columns that are packed with C-18 bonded silica gel (e.g., ODS columns), ODP-51 4B can be used in a wider pH range (2–12). The particle size is 5 μm and the number of theoretical plates for these pre-columns exceeds 2000.

3. RAMs for direct analysis of biological samples

As explained previously, columns and pre-columns filled with RAMs have been recognized as a suitable tool for the direct injection of biological samples that contain proteins (entirely biological material), directly into the flow-analysis system. In the most basic chromatographic system using these packings, the sample is loaded directly into the mobile phase. In this type of system, there is both separation of the analytes from the proteins and separation of the analytes themselves. However, it is essential to use a mobile phase showing non-denaturizing properties (i.e. with the content lower than 25% of acetonitrile, 20% of isopropanol and 10% of tetrahydrofuran, respectively). These percentage values characterize the beginning of protein denaturation; however, in practice, no more than 20% organic phase is used. This is a real disadvantage as, under certain conditions, the analyte must have suitable retention on the column and the restrictions concerning the mobile phase significantly decrease the scale of applicability.

RAMs are much more frequent in multi-column chromatography systems using the column-switching technique. These systems enable simultaneous protein removal and analyte pre-concentration on the RAM pre-column, whereas the separation of low-molecular-mass analytes takes place on the analytical column with a common sorbent type. Restrictions concern only the mobile phase into which the biological sample is loaded. Of the several modes that can be used, back-flush column-switching is used most. Nevertheless, high demand on the devices (2 pumps and selection switching valve with synchronization unit) is considered a disadvantage.

RAM pre-columns could also be integrated into the ''non-separative'' flow systems of flow-injection analysis (FIA) or sequential-injection analysis (SIA). However, due to back pressure of RAM columns, their integration into FIA systems (using a peristaltic pump) is practically impossible. The syringe pump used for the commercial SIA analyzer reaches a pressure that allows the flow of mobile phase through a short RAM pre-column where the sorbent particle size is 20 μm or higher. The newly developed SIA-RAM technique could be used for simple screening analysis of drugs in biological material [14,15].

As mentioned above, RAMs have been developed as suitable tools for direct, repetitive injection of untreated biological samples into the analytical system, so they are ideal for automation, purification, and pre-concentration. Two approaches have already been described:

- direct mode or single column; and,
- column switching.

In the first type, the RAM column is directly connected to the detector. In this case, the support is used for extraction and separation. In the second type, the RAM pre-column extracts only and a switching valve connects it with an analytical column, where the separation is performed.

3.1. Direct mode

In the direct-mode configuration, the analytical procedure involves three steps:

1. sample extraction;
2. analyte elution; and,
3. re-equilibration of the extraction support.

First, the biological fluid is injected onto the extraction pre-column with an appropriate mobile phase. During this extraction step, analytes are retained by extraction onto sorbent, while endogenous components (mainly proteins) are eluted from the pre-column. Afterwards, analytes are eluted from the support to the detector. Finally, the pre-column is washed and the sorbent is

re-equilibrated with mobile phase, so that the pre-column is ready for the injection of another sample.

In accordance with the type of the detection, two different approaches have been used. RAM columns emerged in the middle of the 1980s when the UV spectrophotometer was by far the most commonly used detector for this type of analysis. Given the relatively low selectivity of UV detection, special attention has to be paid to the chromatographic effectiveness of these extraction supports.

More recently, MS detection has been used more widely for biological analysis; however, direct connection of a RAM column to MS detection is still quite rare.

3.2. Column switching

In recent decades, the column-switching configuration has proved useful for the determination of substances in biological matrices. The extraction support, used for the extraction and/or pre-concentration of the sample, is coupled to an analytical column via a selection valve, and it separates analytes before detection, for which an additional pump and switching valve are required (Fig. 2).

The switching valve is in position A during the extraction step. The sample is injected into the extraction pre-column with a stream of extraction mobile phase. Concurrently, the analytical column is adjusted for elution of the mobile phase. The valve is switched to the position B after elution of the matrix. Analytes are eluted from the extraction pre-column either in the back-flush or straight-flush mode using the analytical mobile phase and are transferred onto the analytical column. Afterwards, the valve is switched to its initial position (position A). Analytes are separated on the analytical column prior to detection. Simultaneously, the extraction pre-column is re-equilibrated by loading with mobile phase so that the system is ready for the next sample injection.

Column-switching configurations can contain various numbers of pre-columns, switching valves and pumps. Fig. 2 shows a simple column-switching configuration.

Recently, RAMs have been widely used in the column-switching configuration. Independent of the extraction support, this configuration offers increased selectivity and sensitivity while simultaneously decreasing analysis time.

3.2.1. Straight-flush and back-flush modes. The straight-flush mode is the simplest mode applied to processing of biological samples using the column-switching configuration. First, the sample is injected on the pre-column, where undesirable components are directly discharged to waste. By rotating the six-port selection valve, the fraction (containing the analytes that are being analyzed) is transmitted onto the analytical column and the analytes are separated. This configuration is called straight-flush mode and has been used for the analysis of drugs (e.g., oxiracetam, ofloxacin, aminopyrin, or adriblastin in various types of samples [16]).

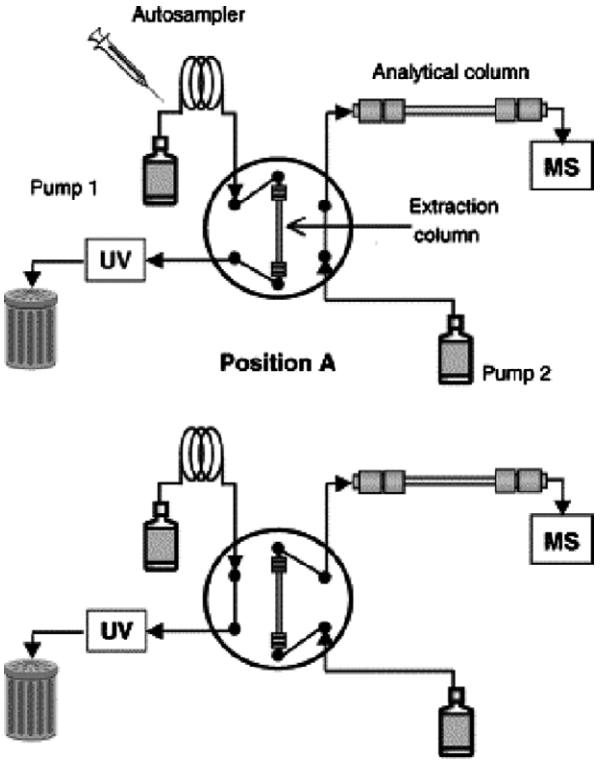

Figure 2. Column-switching system with back-flush configuration (reprinted from [43] with permission from Elsevier).

The back-flush mode is carried out in direction of flow opposite to that of the analytical mobile phase through the pre-column. In this way, analytes retained on the pre-column front are directly transferred to the analytical column. After removing the fraction of interest, the powerfully retained components of the matrix may also be removed from the pre-column by reverse flow. In this approach, the analytical column is protected from contamination of components from the matrix, which are eluted later. The back-flush mode also minimizes peak broadening. To achieve this, an additional pumping system usually provides the stability required, as disturbance of the sorbent packing in the pre-column can occur by changing the direction of flow of the mobile phase. The back-flush mode has been used for separating and determining several antiarhythmics, gastrointestinal medicines, antihypertensive drugs and antidepressants, as well as their related substances [17].

4. Sample preparation, types of samples and binding to proteins

Sample preparation remains the most serious problem for automating HPLC of biological samples due to the

high amounts of proteinaceous content that require pre-treatment. The analysis of untreated raw sample leads to irreversible adsorption of proteins to the column surface and mostly to their denaturation. Consequently, it considerably reduces both the efficiency of the chromatographic system and the lifetime of the analytical column.

It is therefore essential to treat the biological sample before injection into HPLC. The treatment mainly concerns the removal of the proteins present in the sample. For example, liquid-liquid extraction (LLE) has been used as a traditional off-line method for biological sample pre-treatment. However, this method is time consuming, inaccurate and hard to automate. In addition, consumption of organic solvents is high. LLE has therefore been replaced with solid-phase extraction (SPE), which uses cartridges restricted to one use only. Nevertheless, SPE has not proved to be sufficient. That is why the number of HPLC methods, including on-line sample pre-treatment and using the column-switching, have been growing in recent years.

Table 1 compares three types of sample pre-treatment:

- classical LLE;
- SPE; and,
- column switching.

It is apparent that the traditional preparation techniques include a great number of steps that can lead to considerable losses of analytes. Column-switching methods minimize the number of manual steps, and that increases the accuracy and the precision of the method. Moreover, it significantly shortens the time necessary for sample processing, so it also shortens the overall time for analysis.

Compared to the traditional sample-treatment techniques, column-switching is also advantageous as an internal standard is unnecessary because the accuracy and the precision of the method are increased. However, an internal standard can still prove useful if it is necessary to process a long sequence of the samples. Column-switching also protects light-sensitive analytes, as there is no light exposure due to sample pre-treatment. Table 2 summarizes all the main advantages and the disadvantages of using a column-switching system for analysis of biological materials.

Practically all liquid biological samples that have had suspended particles removed can be injected directly into the column-switching system, while solid samples need to be dissolved and homogenized. The matrix composition and the amount of injected sample determine the column lifetime. Biological fluids are

Table 1. Comparison of methods for preparing samples of biological material (LLE, SPE and column-switching technique)

Liquid-liquid extraction (LLE)	Solid-phase extraction (SPE)	Column switching
Sample dosing	Sample dosing	Possible centrifugation,
Internal standard addition	Internal standard addition	Filtration and/or sample
Organic solvent addition	Cartridge equilibration	dilution
Shaking	Sample application	Injection
Centrifugation	Matrix elution	Valve switching
Mixture partition	Analyte elution	
Possible re-extraction	Solvent vaporization	
Solvent vaporization	Re-dissolution	
Re-dissolution	Filtration	
Filtration	Injection	
Injection		

Table 2. Advantages and disadvantages of column-switching system during biological sample preparation

Advantages	Disadvantages
Minimal sample adjustment	Switching valves, extraction RAM columns and pumps required
On-line sample preparation	Compatible mobile phases required
Significant decrease in total analysis time	Regular pre-column exchange required
Possibility of full automation	
Higher accuracy and precision	
Improvement of the selectivity in combination with various chromatographic modes	
No necessity of internal standard	
Photo-labile analyte protection	
Low consumption of organic solvents	

Table 3. Usage of restricted-access materials (RAMs) in HPLC column-switching

Field of application	Matrix	Analyte(s)	Restricted-access material (RAM), producer	Ref.
Analysis of drugs and other biologically active substances in biological fluids	Human whole blood	Benzodiazepines and metabolites	LiChrospher RP-18 ADS (25 × 4 mm), particle size 25 µm, Merck, LiChrospher 60 XDS (25 × 4 mm), 25 µm, Merck	[18]
	Human plasma	Cyproterone acetate	LiChrospher RP-4 ADS (25 × 2 mm), 25 µm, Merck	[19]
	Human urine	Verapamil and its metabolites	LiChrospher RP-8 ADS (25 × 4 mm), 25 µm, Merck	[20]
	Human plasma			
	Human plasma	Sotalol	LiChrospher 60 XDS (25 × 4 mm), 25 µm, Merck	[21]
	Human plasma	Meloxicam	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[22]
	Human plasma	Cloxacillin	LiChrospher 60 XDS (DEAE/diol) (25 × 4 mm), 25 µm, Merck	[23]
	Human serum	Furosemide	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[15]
	Human plasma	Rofecoxib	LiChrospher 60 RP-18 ADS (25 × 4 mm), 40–63 µm, Merck	[24]
	Human serum	Voriconazole	LiChrospher RP-8 ADS (25 × 4 mm), 25 µm, Merck	[25]
	Human plasma	Caffeine and metabolites	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[26]
	Human plasma	Cocaine and benzoylecgonine	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[27]
Determination of antidotes	Human plasma	Atropine	LiChrospher 60 XDS (SO_3/diol) (25 × 4 mm), 25 µm, Merck	[28]
Veterinary analysis	Horse plasma	Ketoprofen enantiomers	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[29]
Identification of human plasmatic peptides with molecular weights up to 20 kDa	Human whole blood	Angiotensin 1 Angiotensin 2	LiChrospher 60 XDS (SO_3/diol) (25 × 4 mm), 25 µm, Merck, LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[30]
	Human whole blood Human urine	Peptides Drugs	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck, LiChrospher 60 XDS (25 × 4 mm), 25 µm, Merck	[31]
	Human plasma Human urine	Enzymes Proteins Drugs	SCX-RAM (SO_3/diol) (25 × 2 mm) and (25 × 4 mm), 25 µm (LSP MDA, Merck)	[32]
Development of new drugs	Sheep serum Sheep plasma	Inhibitor of matrix metalloproteinases (MMPs)	LiChrospher RP-8 ADS (25 × 4 mm), 25 µm, Merck	[33]
Purification of plasmid DNA	Cell lysate	Plasmid DNA (gene vector)	Q-Sephacryl S-500 HR, –	[34]
Environmental monitoring	Human urine	1-, 2-naphthol	LiChrospher RP-8 ADS (25 × 4 mm), 25 µm, Merck	[35]
	Human plasma	Organophosphorus triesters	LiChrospher RP-18 ADS (25 × 4 mm), 25 µm, Merck	[36]
	Human urine	Five major metabolites of di-(2-ethylhexyl) phthalate (DEHP)	LiChrospher RP-8 ADS (25 × 4 mm), 25 µm, Merck	[37]
	Drinking and surface water River water River sediment Wastewater	Steroid sex hormones Drugs Alkylphenolic surfactants	LiChrospher RP-4, RP-8 or RP-18 ADS (25 × 4 mm), 25 µm, Merck	[38]

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Table 3 (*continued*)

Field of application	Matrix	Analyte(s)	Restricted-access material (RAM), producer	Ref.
Food analysis	Fruits Water Soil	Pesticides Herbicides	Hisep SHP (50 × 4.6 mm), 5 μm, Supelco SPS-5PM-S5-100-C18 (50 × 4.6 mm), 5 μm, Regis Tech	[39]
	Apple juice	Patulin	-	[40]
	Egg yolk	Cholesterol	BioTrap C18, protein-coated RP-18 pre-column, ChromTech	[41]
	Milk	Polyamines	LiChrosorb C18 (50 × 4.6 mm), 10 μm, Agilent	[42]

most problematic when they contain a high fraction of proteins and cells (blood, plasma and serum). Cerebrospinal and interstitial fluids, as well as urine, are generally more compatible with LC because they have lower protein content, so simple filtration can provide suitable pre-column stability. Other ways considered to prolong column lifetime include centrifugation, dilution, sample filtration, use of in-line filters and off-line analyte extraction. Proteins can also be precipitated and removed prior to injection to the chromatographic system. Lower efficiency than expected might be found where the drug is strongly bound to proteins. In such cases, significant discrepancies in recovery can be observed during analysis of identical kinds of samples. The main ways to exclude the drug from the binding site of protein is by diluting the sample or by adding organic solvent prior to injection onto the pre-column. The amount of the organic solvent added depends on the polarity and the nature of the denaturation of the solvent.

5. RAMs in analytical instrumentation

In practice, RAMs have been used for only a few years. Most publications on RAMs [18–27] concern the determination of drugs and other biologically-active substances in body fluids. Almost 70% of all cases refer to plasma analysis, followed by serum (13%), urine (3.3%) and whole blood (3.3%). Other types of biological material (e.g., saliva, hair, microdialysate, and liver tissue) are used only rarely.

Other publications have dealt with the determination of antidotes in biological material [28], veterinary determinations of drugs [29], identification of human-plasma proteins (approx. 9% of publications) [30–32], research into new drugs [33] or DNA-plasmid cleaning [34].

Environmental monitoring has seen the second greatest use of RAMs, with approximately 21% of published articles [35–38]. Pollutants are either determined directly in the environment (i.e. drinking water, river water or wastewater and soil (54%)) or in body fluids (mostly in plasma and urine, 46%) of people who had previously been exposed to contaminants.

Food analysis forms around 12% of publications, with determination of contaminants in wines, milk, juices, sea fish, fruit and vegetables.

Several examples of the uses of RAMs are presented in Table 3 [18–42], which includes the field of application, the type of matrix, the analyte determined, the type of RAM that was used, the producer and references.

Pre-columns of LiChroCART, filled with RAM material LiChrospher RP-18 ADS, were extraction pre-columns used most in analyzing drugs and other biologically-active substances in body fluids. These pre-columns are suitable for the analysis of plasma, serum, whole blood, urine or saliva samples. Only a few drugs were extracted on ion-exchange XDS pre-columns.

Ion-exchange pre-columns were the most often used in analyzing human-plasma proteins in sizes up to 20 kDa.

LiChroCART with LiChrospher RP-18 ADS material as sorbent was also used most often in the pre-column in environmental analysis.

In food analysis, less common pre-columns packed with sorbent with C18 functional groups were used.

6. Advantages and disadvantages of RAMs

To compare the performance of RAM sorbents with common SPE sorbents, we can apply several parameters, such as lifetime, separation efficiency, protein and analyte recovery, as well as the influence of LOD or mistakes occurring during off-line sample adjustment.

The lifetime of the sorbent is the most important factor for sorbent comparison, and, when comparing common SPE sorbent with RAM sorbent, RAM lifetimes are extremely high. The price of RAM columns is usually high and frequently exceeds the price of a common analytical column; however, most of the RAM sorbents have a theoretical lifetime of injection of 100 ml of human plasma, so, if 50 μl is injected, it means that it is possible

to achieve 2000 analyses without any change in recovery, separation performance and back-pressure, whereas commercial SPE cartridges are designed for one use only.

Analysis using RAM sorbents is also cheaper than analysis with traditional pre-treatment. In traditional extraction techniques, the costs for experienced laboratory staff capable of dealing with the complicated procedures must be included; these procedures are time consuming and consume organic solvents. However, it is essential to have more a complex chromatographic system with higher demands on instrumentation and staff available for on-line preparation and analysis of the sample.

The separation efficiency of RAM columns mainly depends on their type. For example, SPS columns have the highest efficiency (up to 60,000 theoretical plates). However, alky-diol ADS stationary phases (particle size 25 μm) have significantly lower efficiencies.

Protein and analyte recovery of RAM sorbents is practically 100%. Protein recovery means the percentage of protein injected that is eluted from the column. Where on-line RAM coupling is used in a system (i.e. direct injection of the sample), the analytes are not lost during pre-treatment. However, partial loss of analytes can be observed with drugs strongly bound to plasmatic proteins, especially to albumin.

The LOD might be decreased if the amount of the injected sample is increased. The amount of the sample in a simple chromatographic system is restricted by the capacity of the chromatographic column, whilst the amount of the sample in the column-switching system is virtually unlimited and depends only on the technical parameters of the system. With direct injection of raw sample, the errors caused by people during sample manipulation are significantly reduced. At the same time, ''safe'' manipulation of dangerous or infectious samples is surely a great advantage of this method.

7. Conclusion

The long lifetime, fast analysis, easy automation and simplification of the whole analytical procedure are major points in favor of using RAMs in HPLC analytical systems. Some of the most frequently published analytical work these days is on the analysis of drugs in biological material (e.g., plasma, serum, urine, and liquor). Using traditional manual techniques (LLE and SPE), it is often extremely difficult and time consuming to remove proteins and avoid the loss of analytes; and, large series of samples are almost impossible. RAMs as extraction pre-columns in a column-switching mode for HPLC offer the best prospects for the future, as this allows direct injection of previously unprepared biological material into the HPLC system. Apart from removing bio-matrix from the sample, the RAM allows isolation and pre-concentration of analytes, and, as the sample preparation is carried out on-line, there is virtually no loss of analytes.

RAM systems are especially useful for high-throughput sampling in biochemistry, and environmental and food analysis, where automation is essential. As no manual sample treatment is needed, the analysis is rapid and the work of an analyst is simplified and safer (especially in the cases of potentially infectious samples of biological material).

Compared to traditional SPE sorbents, RAM sorbents have many advantages, including longer lifetime, higher separation efficiency, higher analyte recovery, reduced analyte losses, lower organic waste production, lower total costs per analysis, and a lower risk of error by laboratory staff.

It is obvious that RAMs bring considerable advantages and that their usage in analytical practice will certainly extend in the future.

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