Features of chromatographic separation on heteropaternal sorbents

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Abstract. The possibility of using a short 20x4 mm chromatographic column with a heterosurface sorbent for direct determination of theophylline and caffeine in blood plasma and a 120x4 mm column for determination of tetracycline antibiotics in cow's milk was studied. Complete separation of tetracycline peaks was not achieved. Determination of caffeine and theophylline was performed successfully in plasma filtrates without long and time-consuming stages of preliminary preparation of the sample without protein deposition. Before backwashing, unfiltered blood plasma can be injected in an amount equivalent to 50 mg of dry matter. Retention characteristics of caffeine and theophylline are practically stable: the retention factor of theophylline for k = 4,0 ± 0,1 for caffeine k = 8,5 ± 0,4 eluent in composition of 0.02 M sodium hydrogen phosphate (pH = 6,0) – acetonitrile (90:10).

Heteropaternal sorbents (HS) allows one to quickly separate the interfering macromolecular sample components (e.g., proteins) from low molecular weight components of interest [1] due to the different interaction with macromolecules of proteins and small size components of samples. Most HS are advanced chemically modified silica. Their most significant chromatographic property consists in different interactions with the components of the chromatographic sample depending on their size. This can be achieved in two different ways. The first method uses "multiphase", formed by molecules of two types with different lengths of "legs", fixing them to the surface of silica. In this case, only modifier molecules with a longer "leg" are available for macromolecules of the sample (for example, proteins). Both types of molecules immobilized on the silica surface are available for small sample molecules, such as drug components [1, 2]. With this modification the surface of the modifier molecules of different types are distributed over the surface of the silica carrier randomly. The HS implements the concept of spatial separation of surfaces of two different types [3]. It is more complicated to manufacture HS with non-overlapping zones of the type of Pinkerton classic sorbent and easier to manufacture HS with overlapping zones are made [3]. In the simplest method of creating such HS, conventional ODS sorbents are used as a basis, on a part of the surface of which a modifying agent of the second type is applied sandwich like [4]. In addition, it is possible to classify TOS by the way of formation of heteropaternal surface. The most obvious and simple is the method of dimensional blocking, when the agent which is unable to penetrate deep into the pores of the sorbent due to the size of its own molecules is used to create a structure of the "sandwich" type. The kinetic-diffusion method uses difficulties with the penetration of the modifier deep into the pores, while the kinetic method is based on a different rate of interaction with the surface of the two modifiers [3, 4].

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In general, chromatographic separation of the analyzed substances can occur both by adsorption, distribution, ion exchange and by exclusive mechanism. As a result, the sample components move with different deceleration relative to the mobile phase. HS are designed in such a way that macromolecules do not penetrate into the pores and contact only with the "external" inert surface of the sorbent grains. This ensures the exclusion of biological macromolecules from the sample without separation at a rate comparable to that of the mobile phase. Low molecular components of the sample, on the contrary, penetrate into the pores of the sorbent grains, and interact with the groups grafted there (for example, alkyl), which leads to the separation of the components of the sample. This allows us to restrict the sample preparation by simple filtration of the samples and to carry out chromatographic analysis without removal of proteins. However, the efficiency of columns with HS is always lower than columns filled with the sorbent on the basis of which the HS is synthesized, since the protective coating of the HS slows the diffusion of low-molecular components of the samples into the pores and from the pores of the sorbent. Such sorbents based on silica matrices with grafted alkyl groups can be easily synthesized independently in the laboratory [2, 4].

With the direct introduction of the blood serum in a chromatographic column with ODS-phase there is a noticeable decrease in the column efficiency and the increase in hydrodynamic pressure due to deposition present in the sample protein components of the filter column and on the surface of the sorbent. The most common way to avoid this is sample preparation. The standard scheme includes the extraction of lipids solvent, lipid removal by centrifugation, the addition of a solution of a precipitating agent for protein precipitation and re-centrifugation [3]. An alternative to this long and time-consuming sample preparation is the use of HS. However, the efficiency of columns packed with these sorbents is below traditional, so the schemes with short preliminary GS-column or with prior use of HS-filter cartridge are successfully competing with one-column schemes. Below we consider it in more detail.

Objective: to assess the possibility of using heteropaternal sorbent for direct determination of tetracycline antibiotics in milk and use the column with heteropaternal sorbent on the basis of reversed phase sorbent (RPS), shielded crosslinked albumin, for direct determination of caffeine and theophylline in human blood plasma.

Materials and methods. Silica with a narrow pore size distribution and a maximum around 6 nm and a particle size of 5-7 µm was used as a matrix for the synthesis of HS. The surface of silica was activated, instilled hexamethyldisilane group in toluene for 16 hours in the presence of a catalyst. The outer surface of the sorbent was screened with human serum albumin, globules were crosslinked with glutaraldehyde and azomethine bonds were restored with sodium borohydride solution. HS was packed by suspension method in a stainless steel column with a diameter of 4 mm and a length of 2 or 12 cm.

For chromatography a gradient pump Spectra Physics P4000, a two-wave scanning UV detector UV2000 (Thermo Electron Corp., USA) and the loop metering crane "Rheodyne-7125" with a loop of 25 µl were used. The separations were performed at a mobile phase flow rate of 1 ml/min, detection was carried out at a wavelength of 254 nm.

The GS column was connected to a two-position crane (Figure 1). In the "loading" position (Figure 1 A), the eluent from the pump passed through the first metering valve and the HS column, after which it was sent to the drain. During the analysis, the sample was introduced into the system by means of a loop metering crane, the protein components at the outlet of the column were removed into the drain, then the crane was switched from the "loading" to the "input" position (Figure 1 B). Then the eluent with the analyzed components of the sample was directed into an analytical column for separation. After the output of the target components from the column, it was turned off by turning back the crane. At the same time it was possible to wash the GS column with a second pump. The scheme of backwash GS column is shown in Figure 2.
Figure 1. Scheme of organization of eluent flows in the mode of separation of protein components of the sample (A) and in the analysis of target components (B).

Figure 2. Scheme of back-flushing HS column

Results and discussion. For Figure 3 a chromatogram of a model mixture of tetracyclines in cow's milk is presented. HS is used for separation. But to achieve a complete separation of tetracycline peaks is impossible due to the low efficiency of the column. Casein, lactoalbumin and lactoglobulin come out as a single peak before the peaks of tetracyclines.
Figure 3 Chromatogram of the model mixture of tetracyclines in milk

Due to the fact that the hydrophobicity of crosslinked albumin molecules is less than the hydrophobicity of grafted alkyl groups, the retention time of drugs on the HS is slightly less than on the OFS. In addition, when working in acidic eluents with a pH below 4, protonated amino groups of protein globules of the on the outer surface of the HS are capable of absorbing negatively charged sorbates [5]. With the correct choice of geometric parameters of silica matrices for HS, the surface fraction available for protein components of the samples does not exceed a few percent, so when it is screened, the decrease in hydrophobicity in the best HS samples is small. However, the absorption of acidic sorbates at low pH of the eluent is observed in this case, too which makes it impossible to quantify them.

The problem of acid sorbates determination can be solved by using a chromatographic system with a short column filled with HS, a mobile phase flow switch and a standard column with RPS. In this case the separation of protein components of the sample on the column with HS is made at pH 4, and the separation on the RPS column – at the required pH value. The use of the system, which includes short column with the HS, together with the RPS under the scheme "chromatographic magnifier" instead of one column with HS allows to increase the separation efficiency of the target components.
In many cases, such a chromatograph scheme helps to avoid gradual contamination of the HS column with components from the analyzed samples that can be sorbed on the protein coating of the HS, due to the possibility to regenerate the HS column by backwash with an additional pump produced after the transfer of the target components to the main RPS column [5].

The obtained dependences of the retention of analytes of acidic and basic nature on the pH, ionic strength and concentration of the organic solvent in the eluent are similar for GS and OFS. Increasing the content of acetonitrile in the eluent 0.02 M sodium phosphate-acetonitrile (pH = 6.5) from 10 to 15% reduces the retention factors of theophylline and caffeine on the HS by a third. With a further increase in the content of acetonitrile to 20% retention factors do not change. Reducing the concentration of acetonitrile to 5% increases the retention factor of theophylline and caffeine in two and 3.3 times, respectively. This is due to the amplification of non-specific interactions of drugs with grafted hexadecylamine groups of GS. Changing the concentration of acetonitrile in the eluent allows to separate the target components of the sample from proteins in an acceptable time. If the eluent contains less than 10% acetonitrile, it is necessary to take measures against biodegradation of the sorbent.

For eluent sodium phosphate buffer solution (pH = 6.5) – acetonitrile (90:10) the variation of phosphate concentration in the buffer solution from 0.02 to 0.2 M does not affect the retention of benzoic acid, theophylline and caffeine. Consequently, the contribution of ion exchange interactions to the retention of sorbate molecules on HS is insignificant. Retention factor for benzoic acid $k = 1.4 \pm 0.1$; for theophylline $k = 3.2 \pm 0.1$ and for caffeine $k = 7.3 \pm 0.3$.

Due to the polyampholyte nature of HSA variation of the eluent pH alters the degree of protonation of ionogenic groups and affects ion-exchange properties of GS. With a change in the pH of the eluent 0.02 M sodium phosphate buffer solution – acetonitrile (95: 5) in the range from 3.0 to 6.0 retention of theophylline and caffeine, having a basic nature, slightly increased. Retention factor for theophylline $k = 4.0 \pm 0.2$ for caffeine $k = 9.1 \pm 0.4$. By increasing the pH to 7.5 theophylline retention factor monotonically decreases up to $k = 2.7$. The factor of retention of caffeine at the same time remains unchanged. The retention factor of benzoic acid in the pH range from 7.5 to 5.0 is constant ($k = 1.3$), with a decrease in pH to 4.5 it increases significantly ($k = 7.5$), with pH = 4.5 it increases even more ($k = 14$). At pH<4, benzoic acid is completely absorbed by the sorbent due to its interaction with protonated CHSA amino groups. Thus, eluent with pH from 5.0 to 7.5 is applicable for the determination of all the solutes, eluent with pH< 4.0 is unsuitable for the separation of substances of acid nature on the HS column.

Filter cartridge with heteropaternal sorbent can help us to significantly reduce the time of sample preparation in simple routine analyses in the absence of special equipment. The sample is dosed in the initial section of the cartridge, and attached to a disposable syringe with an eluent. Sample preparation consists in passing two portions of eluent through the cartridge. The first is equal to the volume of the cartridge. Generally, the interfering high-molecular components of the sample leave the cartridge together with the" dead " volume of the eluent and are disposed of. Next, an ultraviolet detector is connected to the output connector of the cartridge and the low-molecular components to be determined are eluted in series with the second portion. If the sample contains substances that vary greatly in retention time, or if one particular component with known retention time is of interest, the exit from the cartridge of the next component of the sample is determined quite clearly, and each of them can be collected in a separate vial. The total time of sample preparation does not exceed a few minutes. In the future, it is possible to identify the components of the sample by infrared spectroscopy or chromatography, for example, by TLC or GC.

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