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Influence of various biological matrices (plasma, blood microdialysate) on chromatographic performance in the determination of β-blockers using an alkyl-diol silica precolumn for sample clean-up

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Abstract

A HPLC column-switching system with LiChrospher RP-8 ADS precolumn was applied for the determination of beta-blockers (atenolol, pindolol, propranolol) in human plasma. The influence of biological matrices on the changes of the chromatographic parameters such as retention time, peak symmetry, area and selectivity were investigated. After injection of 5 ml plasma a decrease of retention times of the analytes was observed of up to 25% and an increase of asymmetry factors of up to 5%. Peak areas and selectivities were not changed. The observed effect could indicate changes of chromatographic performance caused by contributions of the analytical column or the ADS precolumn. The experiments with microdialysis excluded the contribution of the analytical column. A detailed investigation of experiments have been discussed in this paper.

Keywords: β-Blockers; Alkyl-diol silica

1. Introduction

Macromolecular compounds (such as proteins) have to be removed from a sample prior to high-performance liquid chromatographic (HPLC) analysis of drugs in biological samples because of their precipitation by large amounts of organic solvents in the mobile phase, non-specific and irreversible binding at the surface of the packing material or denaturation by residual silanol groups at the surface of the chromatographic support. These phenomena usually result in irreversible increase of pressure and loss of the column capacity and selectivity. On-line column-switching devices combined with advanced separation media technology represent a powerful and reliable solution to the automated clean-up and trace enrichment of drugs in complex biological samples with a consequent improvement in the analytical process [1]. During recent years several packing materials have become available. So called restricted-access media (RAM) express limited accessibility of macromolecular sample compounds to the ad-

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sorption sites of porous supports due to their molecular size. Besides size exclusion, which plays the dominant role in the restricted retention of proteins, irreversible protein binding and accumulation is prevented by the generation of a protective, non-adsorptive, hydrophilic outer packing surface by a protein [2–4] or polymer coating [5], special bonding chemistry [6] and enzyme-catalyzed hydrolysis [7,8]. The different types of RAM have been classified and reviewed in some publications [9–13].

Boos and Rudolphi [12] compared several RAM packings used in a single- and a coupled-column mode and discussed the advantages and disadvantages of the two approaches for the determination of epirubicin and its metabolites in human plasma.

Alkyl-diol silica (ADS), the internal surface reversed-phase support, was introduced by Boos et al. [7] and has been specially developed for use as precolumn packings and for on-line liquid–solid extraction (LSE) and enrichment of hydrophobic compounds in proteinaceous fluids by classical C_4-, C_8- or C_{18}-reversed-phase or ion-pair chromatography. It possesses two chemically different surfaces. The outer surface (glycerylpropyl, i.e. diol moieties) of the support is hydrophilic, electroneutral and non-adsorptive towards proteins. This means that the protein matrix of a biological sample can be directly flushed into the waste as the applied precolumn excludes the macromolecules in the void volume. Meanwhile, the analyte fraction, having free access to the binding centers at the hydrophobic internal surface of the porous particles is selectively extracted and enriched at the stationary phase of the precolumn. LiChrospher RP–ADS has a pore diameter of approximately 6 nm (physical diffusion barrier) and excludes macromolecules (e.g. proteins) having a molecular mass larger than 15 kDa in the void volume of a packed column. The lifetime of the ADS precolumn (expressed as the total injected volume of untreated plasma) as recommended by Merck (100 ml) was determined by the sample material used (e.g. protein content, total volume injected) and also by the selected chromatographic parameters (e.g. pH, proportion of organic modifier).

The broad hydrophobic retentive capability of LiChrospher ADS precolumn packings permits chromatographic determinations of a wide variety of compounds in biological matrices, such as propafenone and its metabolite 5-hydroxypropafenone in human serum [14]; enantiomers of atenolol in urine and plasma samples [15]; ketoprofen in horse plasma [16,17]; propranolol in rat blood microdialysate [18]; bupivacaine [19] and 8-methoxypsoralen [20] in human plasma. HPLC-integrated sample clean-up employing a LiChrospher RP-18 ADS precolumn was tested for several drugs and metabolites (talinolol, celiprolol, metoprolol, oxprenolol, triamterene, trimethoprim, tiracizine, articaine, detajmium, ajmaline, lamotrigine) in various biological fluids, such as serum, urine, intestinal aspirates and supernatants of cell cultures [21].

In our study, different analytes (atenolol, pindolol and propranolol) according to their lipophilicity and hydrophilicity were selected as model β-blockers. Beta blockers are clinically important drugs which are used in the treatment of disorders such as hypertension, angina pectoris and arrhytmia. A comprehensive overview on this subject was given by Egginger et al. [22]. The evaluation and characterization of behavior of LiChrospher RP-8 ADS precolumn and Separon SGX C_{18} analytical column in the HPLC column-switching system with focus to the mobile phase conditions, protein recovery, chromatographic behavior during plasma injections, contamination of proteinaceous compounds on the packing are presented in this work. The aim of the study was the investigation of changes of basic HPLC parameters during the course of direct injection of large volumes of different biological samples (human plasma, blood microdialysate) for determination of chiral drugs.

2. Experimental

2.1. Chemicals and reagents

Propranolol hydrochloride (AI-13,468-A) was supplied by Ayerst Laboratories (New York, NY, USA). Pindolol and atenolol were obtained from Sigma (St. Louis, MO, USA). Anhydrous potassium dihydrogen phosphate, HPLC-grade acetonitrile, methanol, phosphoric acid were purchased from Merck (Darmstadt, Germany). Buffer solutions were prepared using water from a Milli-Q® Reagent water system (Milford, MA, USA). All other chemicals and solvents

were of analytical grade and were used without further purification.

2.2. Instrumentation

The liquid chromatographic set-up consisted of a HP 1100 system (Hewlett-Packard, Waldbronn, Germany) equipped with a quarternary pump with on-line vacuum degasser, an autosampler, the thermostated column compartment equipped with Peltier cooling elements, a diode-array detector and an electrically controlled internal six-port column switching valve. Data acquisition and analysis was achieved by HP 3D ChemStation (Hewlett-Packard).

2.3. Chromatographic conditions

2.3.1. Procedure A

The column-switching system was presented schematically in a previous paper [18]. The LiChrospher® RP-8 ADS (25×4.0 mm I.D., 25 μm, Merck) was applied as a clean-up precolumn. The Separon SGX C_{18} [7 μm, 150×3 mm ID., compact glass column (CGC), Tessek, Prague, Czech Republic] was used as an analytical column. PEEK In Line Filter Kit (Peek Alloyed with Teflon, 5 μm; Watrex, Bratislava, Slovak Republic) was placed between the autosampler and the clean-up precolumn. The mobile phases: Mobile phase X — water with 5% acetonitrile at the flow-rate 1.5 ml min^{-1} was used for the clean-up step and mobile phase Y — 10 mM phosphate buffer, pH 3.0–acetonitrile–methanol (70:15:15, v/v/v) at a flow-rate of 1 ml min^{-1} for the analytical separation. Blank human plasma sample was spiked by the β-blockers in the concentration range 10–125 ng ml^{-1} for atenolol, 5–60 ng ml^{-1} for pindolol, 50–500 ng ml^{-1} for propranolol; the injection volume was 20 μl. Chromatographic parameters were measured after every 200 μl of spiked plasma volume injected.

2.3.2. Procedures B and C

The single instrumental set-up without the precolumn with direct injection (injection volume: 20 μl) of samples onto the analytical column was used in these experimental parts. Both types of experiments were performed using the same mobile phase Y and applying a new SGX C_{18} column from the same batch as in procedure A. For procedure B, methanolic standard solutions with concentration 25, 10, 50 ng ml^{-1} for atenolol, pindolol and propranolol, respectively were injected on the analytical column. Chromatographic parameters were measured after every 200 μl injected volume (10×20 μl).

For procedure C, rat blood microdialysate was spiked with 10–125 ng ml^{-1}, 5–60 ng ml^{-1}, 50–500 ng ml^{-1} of atenolol, pindolol and propranolol, respectively. Chromatographic parameters were measured after every 200 μl volume of standard methanolic solutions as well as spiked microdialysate. Microdialysis sampling was described and discussed in detail in the previous paper [18].

2.4. Plasma sample preparation

Blood was collected at the Institute of Preventive and Clinical Medicine from healthy volunteers into Heparin-Vacutainer tubes and immediately centrifuged at 10 000×*g* for 10 min. Aliquots of the plasma samples were stored at −20°C until analysis. Prior to the injection, the plasma samples were thawed under room temperature, vortexed and centrifuged for 5 min at 7000×*g* for separating coagulated proteins from the plasma matrix. Standard samples were prepared by adding known amounts of atenolol, pindolol and propranolol to an exact volume of plasma.

2.5. Determination of protein recovery

For determination of the elution profile of the plasma matrix (t_M), an ADS precolumn was directly connected to the UV detector. The volume of plasma injected on the ADS precolumn was 50 μl and the signal was measured at 210, 240 and 280 nm for 10 min. Various types of extraction mobile phases X were tested: Water with organic modifiers: 5% of isopropanol, 5% and 10% acetonitrile, 10% methanol and phosphate buffer (pH 6.9) with 10% acetonitrile and 10% methanol.

2.6. Chromatographic parameters

Peak retention time, asymmetry factor, selectivity and area were selected as chromatographic parame-

ters according to the standard literature [23]. The asymmetry factor (As) equals the ratio of the lengths of straight lines BC and AB in 10% of peak height. Selectivity alpha was calculated using the formula: alpha = $k'_{(b)}/k'_{(a)}$, where $k'_{(x)}$ is the retention factor for peak x calculated using the following equation: $k' = (t_R - t_o)/t_o$.

3. Results and discussion

3.1. Recovery of proteins

A plasma sample is known to be the most complex commonly-analyzed biological matrix [24]. Proteins (albumin, α_1-acid glycoprotein, lipoproteins) and other potential interferences (immunoglobulins) are present in matrices at high concentration levels and they are competing with analytes for adsorption on the internal reversed-phase surface of the sorbent. The mobile phase composition and the flow-rate as well as wavelength of the detector play a very important role during the determination of the elution profile of sample matrix (t_M). Absorbance at 280 nm is typical for almost all plasma proteins. A high background of absorbance and fluctuation of the baseline caused by the matrix at 210 and 240 nm made measurements at these wavelengths impossible. The selection of an appropriate mobile phase system for clean-up step in column-switching system using RAM precolumns has been described in detail by Yu and Westerlund [24]. In our study, various types of extraction mobile phases listed in Experimental section were tested. More than 95% of plasma proteins could be excluded in all selected mobile phases within 3 min, changes in organic modifier were not significant for protein recovery. In order to avoid undesired precipitation and irreversible adsorption of proteins within the sorbent bed of the ADS precolumn, the content of the organic modifier should be limited to: 20% methanol, 10% acetonitrile and 5% isopropanol (v/v) [21,25,26]. The optimal extraction mobile phase was chosen as water with 5% acetonitrile. It provided a relatively high recovery of the protein and some of the cleanest chromatogram in the column-switching (CSW) mode concerning interfering peaks eluted with a retention time close to pindolol (see the chromatograms in Fig. 1A and B). However, the content of 5% isopropanol in water was not sufficient for removing the interfering compounds eluting close to the peak of pindolol whereas phosphate buffer with organic modifier (methanol, acetonitrile) caused premature elution of atenolol because of its more hydrophilic character and a relatively low retention factor as compared to propranolol.

3.2. Validation of method

In general, a validated analytical method means that it gives reliable and reproducible results. We investigated the determination of linearity, precision, accuracy and robustness of the method during the validation process. The main validation parameters are shown in Table 1.

3.2.1. Linearity

Calibration curves were prepared in the range of 10–125 ng ml^{-1} for atenolol, 5–60 ng ml^{-1} for pindolol and 50–500 ng ml^{-1} for propranolol in plasma. The parameters of the calibration curves and their correlation coefficients show good linearity in the aforesaid concentration ranges (Table 1) and cover concentration ranges for the pharmaceutical substances usually achieved during the drugs' administration.

3.2.2. Precision and accuracy

Due to the automation and integration of the sample clean-up, the HPLC-integrated sample preparation system proved to be highly reproducible. Intra-day precision and accuracy of the method were evaluated by replicate analyses ($n = 4$) of the plasma calibration standards. Inter-day precision and accuracy were determined by assaying calibration standards at four separate days within 1 week. Respective values of coefficient of variation (C.V.) for precision and relative errors (RE) for accuracy were calculated. RE and C.V. values were expressed as the estimates of standard and absolute deviations calculated for files with the number of samples less than seven.

3.2.3. Repeatability

Injection repeatabilities were calculated at two different concentration levels of the analytes in

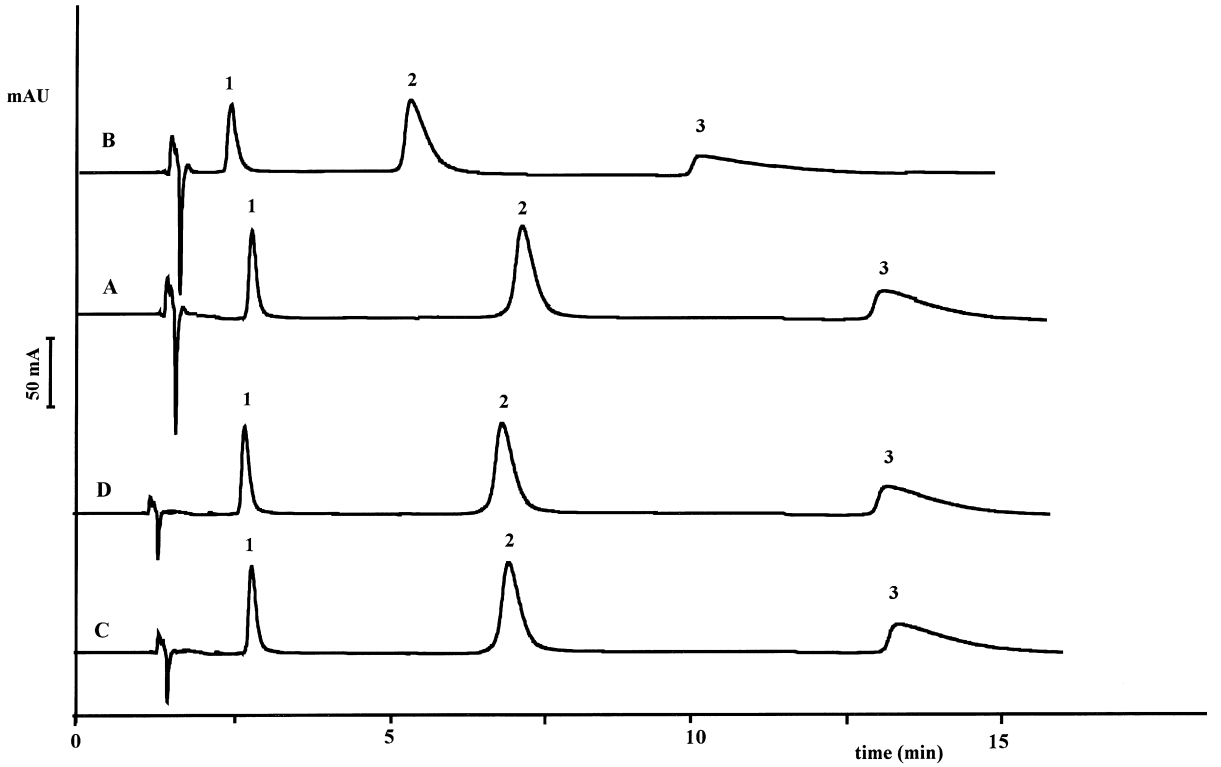

Fig. 1. The chromatographic separation of 100 ng ml^{-1} atenolol (1), 50 ng ml^{-1} pindolol (2) and 400 ng ml^{-1} propranolol (3). (A) and (B) Chromatograms obtained after injection of 20 μl of spiked human plasma and 5 ml of spiked human plasma applying the column switching system with ADS clean-up precolumn. (C) Chromatogram obtained after injection of 20 μl of methanolic solution on the analytical column. (D) Chromatogram obtained after the passage of 6.25 l of mobile phase through the analytical column. Chromatographic conditions: Analytical column: Separon SGX C_{18}, 150×3 mm I.D., 7 μm. Precolumn: LiChrospher RP-8 ADS, 25×4 mm I.D., 25 μm. Mobile phase X — water with 5% acetonitrile, flow: 1.5 ml min^{-1}. Mobile phase Y — 10 m*M* phosphate buffer, pH 3.0–acetonitrile–methanol (70:15:15, v/v/v), flow: 1 ml min^{-1}. Detection: DAD, 280 nm.

spiked plasma samples. The repeatabilities of retention times were determined from 20 injections of the standard and blank plasma samples. RE values were lower than 5% for all analytes in both cases.

3.2.4. Determination of limit of detection (LOD) and quantitation (LOQ)

The limit of quantitation was set at the concentration of the lowest calibration standard ($S/N=10$). The limit of detection using a 20 μl plasma injection was calculated by the comparison of the three-fold variation of baseline noise ($S/N=3$) obtained from analyses ($n=10$) of the blank plasma samples and signals of plasma samples spiked with known concentrations of (*R*)-propranolol·HCl or (*S*)-propranolol·HCl.

3.2.5. Robustness of method

The robustness of a method is its ability to remain unaffected by small changes in the parameters such as the percentual organic modifier content and the pH of the mobile phase, buffer concentration, temperature and injection volume [27]. An example of the robustness criteria is that the effects of the following changes in chromatographic conditions can be determined: Content of organic modifier in mobile phase adjusted by ±2%, mobile phase pH adjusted by ±0.1 pH units, etc. In our study, the pH of mobile phase was changed about +0.1 pH unit as well as

Table 1
Validation parameters for HPLC column-switching system using LiChrospher RP-8 ADS for sample pretreatment

Parameters	Atenolol 10–125 ng ml^{-1}	Pindolol 5–60 ng ml^{-1}	Propranolol 50–500 ng ml^{-1}
Intra-assay[a]			
RE%	0.75–2.43	0.56–3.98	0.57–4.95
C.V.%	0.59–1.91	0.41–4.12	0.41–4.21
Inter-assay[b]			
RE%	0.47–4.00	0.61–3.63	0.84–4.49
C.V.%	0.36–2.93	0.41–2.78	0.59–4.01
Repeatability			
RE%			
–of retention time[c]	0.06	0.18	0.95
–of peak area[d]			
E1	4.79	2.85	1.47
E2	1.19	0.59	0.99
Linearity			
–intercept	6.36±0.63	30.73±0.55	3.59±0.56
–slope	7.72±3.21	21.80±3.18	8.02±4.70
–correl. coefficient	0.9996	0.9995	0.9996
LOD [ng/ml]	5	3	20
Recovery (%)±RSD			
E1	97.80±2.60	98.40±2.20	98.30±2.30
E3	98.30±3.20	99.30±2.40	99.50±2.50
E4	99.20±3.50	100.30±3.20	100.50±3.10

[a] Intra-day precision and accuracy of the method were evaluated by replicate analyses ($n=4$) of the plasma calibration standard.
[b] Inter-day parameters were determined by calibration standards assays at four separate days ($n=4$) within 1 week.
[c] Relative errors (RE) (expressed as an estimation of relative standard deviation) were calculated for 20 injections of the standard and blank plasma samples at concentration level E2.
[d] RE values were calculated from 20 injections of the analytes in spiked plasma samples at two different concentration E1, E2. E1 — Experiments were carried out by injection of 10, 5 and 50 ng ml^{-1} of atenolol, pindolol and propranolol, respectively. E2 — For injections of 100 ng ml^{-1} of atenolol, 50 ng ml^{-1} of pindolol and 400 ng ml^{-1} of propranolol. E3 — For injections of 50 ng ml^{-1} of atenolol, 25 ng ml^{-1} of pindolol and 200 ng ml^{-1} of propranolol. E4 — For injections of 125 ng ml^{-1} of atenolol, 60 ng ml^{-1} of pindolol and 500 ng ml^{-1} of propranolol.

the content of organic modifier being decreased by about 2%. The retention time and asymmetry factor for atenolol, pindolol and propranolol were measured after 500 μl of plasma volume up to 5 ml. The values of C.V. and RE were lower than 4%.

3.2.6. Recovery of analytes

The recoveries of atenolol, pindolol and propranolol from spiked samples, determined at three different concentrations, were calculated by comparing the obtained peak areas with those from methanolic standards. The values demonstrate good recovery of the substances using ADS precolumn (as shown in Table 1). Recovery of analytes from the plasma was measured by calculating the percentage difference between the peak areas of extracted standards and those of the authentic (unextracted) standards in the relevant concentration range.

3.3. The evaluation of the stability of chromatographic parameters in model plasma experiments

For the determination of the optimal precolumn lifetime, the example of the determination of phenobarbital in human plasma (injection volume: 500 μl)

was chosen. The mobile phase for sample clean-up consisted of water, 0.01% phosphoric acid (85%), adjusted to pH 5.0 with triethylamine, 2% (v/v) methanol [26]. During our long-time use of the ADS precolumn, we observed problems with its performance and pressure increase after 200 repetitive injections of 20 μl plasma (representing total volume 4 ml of plasma). Although the recommended pretreatments and procedures for an optimal column lifetime were maintained (such as sample centrifugation, addition of organic modifier to the mobile phase, installation of a replaceable in-line filter between sample injector and precolumn), problems with a change of retention times or increase of the precolumn back pressure occurred, as can be seen in Figs. 2 and 4. This was one of the reasons to make a detailed investigation of precolumn behavior. A similar effect was described by Lamprecht et al. [15] for the enantioselective HPLC analysis of atenolol in human urine and plasma samples. The ADS column performance was reduced dramatically after several injections of 50 μl plasma using 0.01 *M* Tris buffer in the first dimension. They considered the reason to be the penetration of plasma matrix components below the exclusion limit into the pores, where they underwent adsorption, thus changing the property of the ADS material substantially and make it finally more polar, resulting in a decrease of retention time of atenolol.

The cleaning effect of the ADS precolumn was tested by examining the HPLC parameters of the analytes on the analytical column. Measurements were performed with procedure A. It was found that the retention times decreased from 2.4; 7.4; 13.4 min to 2.3; 7.2; 13.1 min for atenolol, pindolol and propranolol, respectively, which represents a relative decrease of about 2–3%. This trend is illustrated in histograms 2 A,B,C. For an evaluation of only the analytical column contribution, corrected retention time (t_R) was used. Corrected t_R was obtained by the

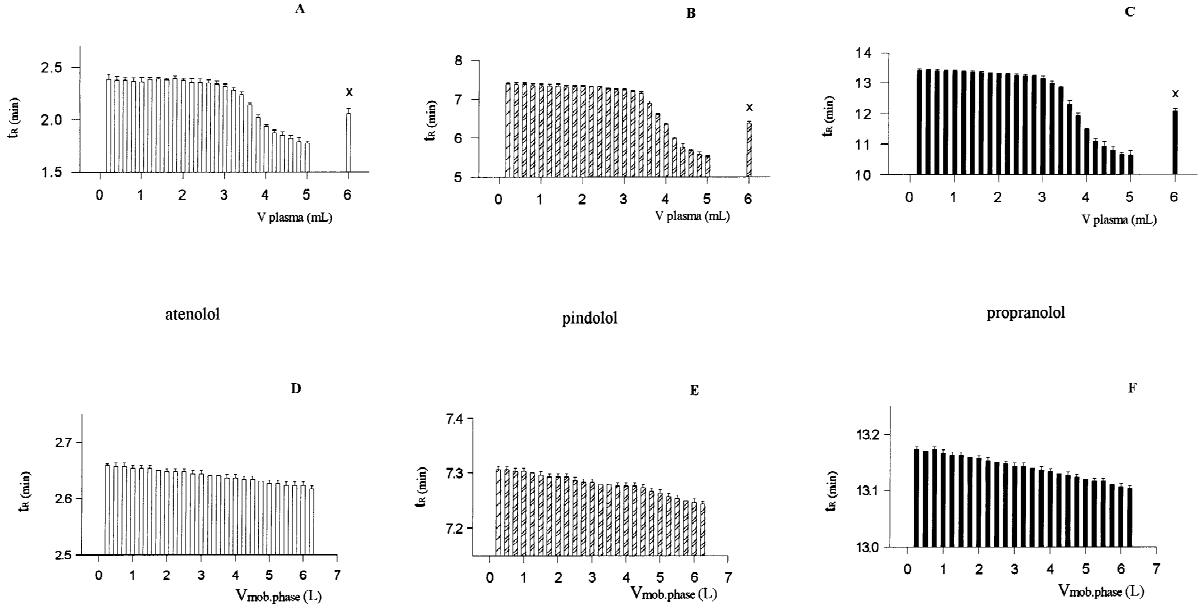

Fig. 2. The dependences of peak retention times of atenolol, pindolol and propranolol on total volume of plasma injected (5 ml) and mobile phase (6.25 l) passed through the analytical column. Chromatographic conditions: Analytical column: Separon SGX C_{18}, 150×3 mm I.D., 7 μm. Precolumn: LiChrospher RP-8 ADS, 25×4 mm I.D., 25 μm. Mobile phase X — water with 5% acetonitrile, flow: 1.5 ml min^{-1}. Mobile phase Y — 10 m*M* phosphate buffer, pH 3.0–acetonitrile–methanol (70:15:15, v/v/v), flow: 1 ml min^{-1}. Detection: DAD, 280 nm. (A,B,C) t_R versus volume of injected plasma, used procedure A. (D,E,F) t_R versus volume of mobile phase passed through the analytical column, used procedure B. x– represents the value observed after the washing procedure (with Pepsin enzyme and EDTA) described in the Discussion. Histograms represent the average value from 10 measurements with relative standard deviation.

subtraction of t_M in column-switching system. As shown in Fig. 3, the asymmetry factors increased by about 1.5% (3a) while peak areas (3b) and selectivities (3c) remained constant within the injection of 3 ml of plasma. After an additional injection of 2 ml there was a decrease of retention times to 25% observed (chromatograms in Fig. 1A and B) and an increase of asymmetry factors by 5%. The observed effect could indicate a decrease in the number of active hydrophobic centers for the analytes in the sorbent pores of the analytical column or a contribution of the ADS precolumn concerning the non-efficient removal of proteins. Since peak areas and selectivities (quantitative parameters) were constant it could be supposed that the changes of retention mechanism had not occurred. Moreover, experiments with the ADS precolumn showed that after 3 ml total volume of plasma, the pressure abruptly increased (Fig. 4). For elimination of the influence of the separation column, additional experiments were also carried out with a new SGX C_{18} column.

3.4. Influence of the mobile phase on the analytical column stability

This part of the study was performed using procedure B described in the Experimental section. Fig. 2D–F and chromatograms in Fig. 1C and D showed that after injection of 5 ml of methanolic standards corresponding to a total volume 6.25 l of mobile phase passed through the analytical column during all experiments (for 250 injections of 20 μl sample; 25 ml of mobile phase per sample considering flow-rate 1 ml min^{-1} and analysis time 25 min),

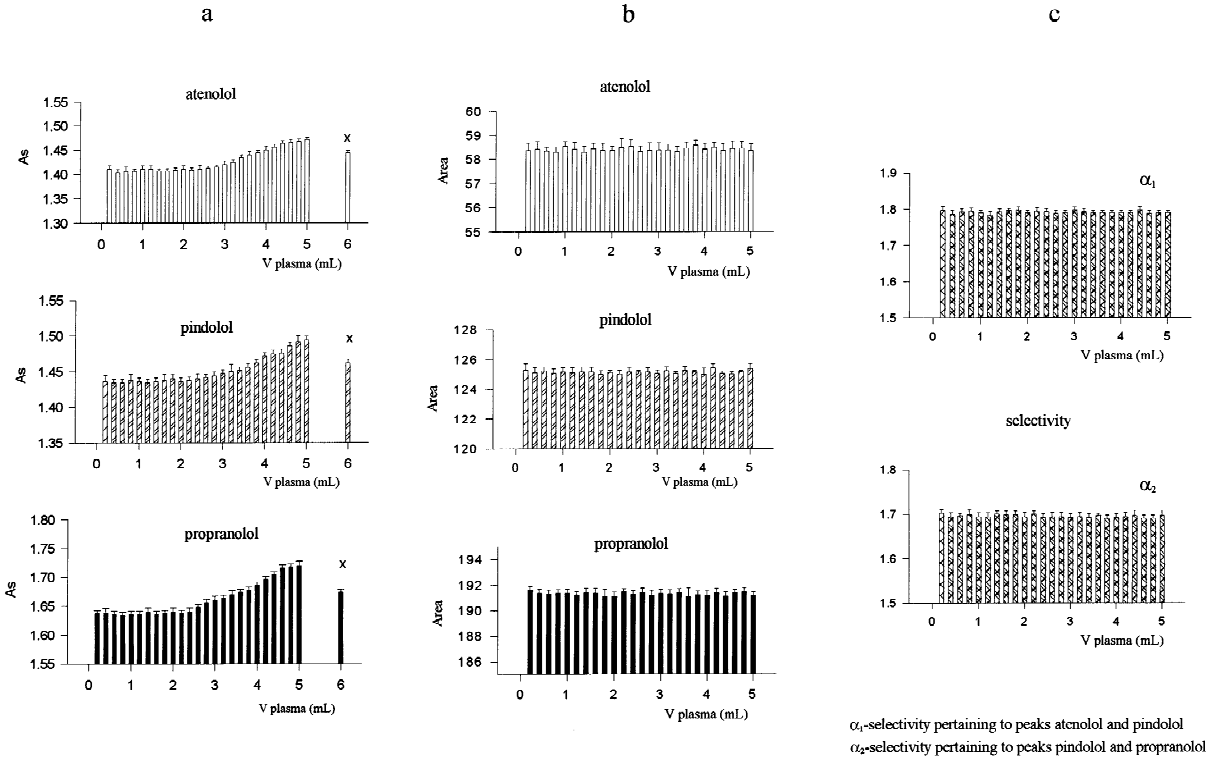

Fig. 3. The dependences of asymmetry factors (a), peak areas (b) and selectivities (c) of atenolol, pindolol and propranolol on the total volume (5 ml) of human plasma injected. Analytical column: Separon SGX C_{18}, 150×3 mm I.D., 7 μm. Precolumn: LiChrospher RP-8 ADS, 25×4.0 mm I.D., 25 μm. Other chromatographic conditions as in Fig. 2. x– represents the value observed after the washing procedure described in the Discussion.

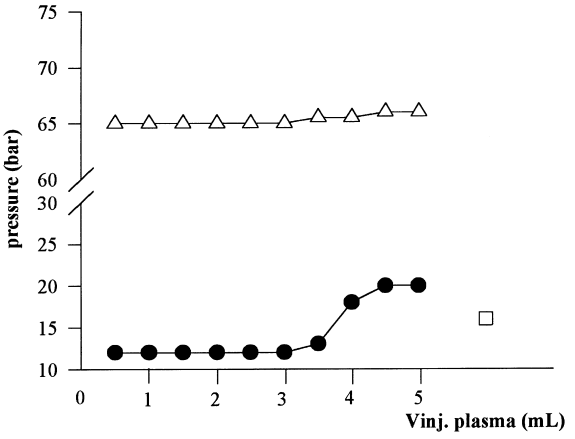

Fig. 4. Change of back pressure on the LiChrospher RP-8 ADS (●) and Separon SGX C_{18} (△) after repetitive injections of spiked human plasma (total volume of 5 ml) in the column-switching system. For chromatographic conditions see Fig. 2. □ the back pressure after the washing procedure as described in the Discussion.

there were only slight changes (about 2%) of retention times observed. This is related to changes in the surface of the stationary phase caused by the mobile phase. After long-term use of the analytical column there is a possibility that this effect may be observed. The differences between R_t values of analytes in Fig. 2 are related to individual behavior of analytical columns Separon SGX C_{18} due to different batches of the sorbent. These experiments showed that changes of the chromatographic parameters during plasma analysis were not caused by the degradation of the analytical column but probably by the changed properties of the ADS precolumn and the contribution of the plasma matrix. For an appreciation of whether matrix protein molecules (high and low-molecular mass) were responsible for this effect, microdialysis as an off-line sample pretreatment technique was used and tested in the same way as the plasma.

3.5. Microdialysis

We already dealt with the off-line microdialysis of plasma as sample pretreatment for the determination of propranolol enantiomers in detail in previous work [18]. A major advantage of microdialysis is that it is a selective sampling technique in the sense that no plasma proteins or other compounds with large molecular mass (in our case>30 kDa) enter the perfusion fluid. Therefore, from the analytical point of view microdialysate is a protein-free matrix allowing direct injection into the chromatographic system. Moreover, the microdialysis is adaptable to the on-line approach [28,29].

The present experiments were carried out using procedure C. Only slight changes of the basic chromatographic parameters (retention time, asymmetry factor, peak area and selectivity) were observed even after injection of 5 ml of microdialysate e.g. 250 injections (data not shown). Moreover, the microdialysis pretreatment made it possible to double the total volume of injected sample to 10 ml without major changes of parameters in comparison to untreated plasma as well. Therefore, it was possible to assume that the problems observed probably related also to the other influences rather than the protein molecules itself and/or the combined effect of several weakly controlled variables. However, it could be useful to consider the fact that the exclusion limit of the ADS precolumn as well as MWCO of microdialysis probe are 15 and 30 kDa, respectively. It follows that protein macromolecules having a molecular mass lower than 30 kDa could penetrate into the pores of C_{18} silica (pore diameter of bare silica 10 nm, C_{18} reversed-phase accessible pore diameter 5–8 nm corresponds to inclusion of molecules with molecular mass 20–30 kDa).

Moreover, we considered that the source of the problems could lie in metal (mainly Fe) leaching from the chromatographic system (in situ), which could give rise to the generation of complexes with the remaining silanol groups and/or adsorbed sample constituents which could occupy the surface of the analytical column. For confirmation of this assumption, additional experiments were performed. The ADS precolumn was washed with 0.1% solution of Pepsin enzyme in 10 m*M* HCl, pH=2 (for removal of potentially adsorbed protein molecules) for 24 h followed by 1 mM EDTA, pH 6 (for removal of metals). After this washing procedure, the chromatographic parameters (retention time, asymmetry factor) as well as the precolumn back pressure were improved about 12% (x-values in Figs. 2–4).

4. Conclusion

As conventional sample preparation is time-consuming and expensive, the use of the novel precolumn packing material ADS enables the development of HPLC-integrated sample clean-up for the determination of a variety of drugs as well as drug enantiomers in biological matrices. No manual sample clean-up is necessary allowing direct injection of untreated biological plasma samples. The precision, accuracy and sensitivity of the assay improved, due to the minimal manipulations of the biological sample. Because of the total elimination of the protein matrix, the recovery of the analyte is quantitative and no internal standard is required.

In our study, some problems with the lower lifetimes of ADS precolumns as compared to the manufactures specified value occurred during the analysis of selected analytes with different lipophilicity and hydrophilicity. Investigation of this problem pointed to the fact that the limiting factor was the number of injections onto the analytical column. This could be related to strongly adsorbed compounds from the sample which were irreversibly adsorbed, and their concentrations and properties contributed to the break after 3–4 ml of plasma observed in the histogram. It was not possible to eliminate the cumulative effect of heavy metals introduced by chemicals constituted by the mobile phase and/or leached by the mobile phase from the chromatographic system; i.e. originating from the mobile phase and some compounds in plasma. These factors influenced the retention times and the shape of the dependence of retention time on the total injected plasma volume was very similar to the breakthrough curve. It might point to an increase of the hydrophilicity inside the pores. In spite of this, the dependence of retention on the volume of the mobile phase passed through the analytical column followed a slow linear decrease.

The process of the precolumn treatment employing the cleavage effect of proteolytic (protein-degrading) enzyme Pepsin in combination with release of metals by EDTA complexation was tested. After this procedure, retention times and asymmetry factors showed an improvement of about 12%. This procedure might offer a solution to the above mentioned problems, but it demands more detailed investigation and could be the object of further study.

Under chosen conditions, we were able to separate the beta blockers in human heparinized plasma with matrix elimination using an ADS precolumn. However, biological sample clean-up procedures on the ADS precolumn cannot be considered as a general process, but the optimal conditions for sample pretreatment must be investigated and adjusted for each individual case which need not be in accordance with the standard procedure.

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