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Sequential injection extraction based on restricted access material for determination of furosemide in serum

Jitka Huclová[a], Dalibor Šatínský[a,*], Tiago Maia[b], Rolf Karlíček[a], Petr Solich[a], Alberto Nova Araújo[b]

[a] *Department of Analytical Chemistry, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic*
[b] *Department of Physical Chemistry, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4050 Porto, Portugal*

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Abstract

Restricted access material (RAM) column containing 25 μm C_{18} alkyl-diol support was integrated into the sequential injection analysis (SIA) manifold and the SIA–RAM system was tested for direct determination of furosemide in serum. LiChrospher® ADS column based on restricted access material is proposed to direct injection of biofluids. The integration of RAM material into SIA enabled creation of a comprehensive on-line sample clean-up technique combined with fluorescence quantitation of analyte.

Centrifuged and diluted serum sample was aspirated into the system and loaded onto the column using acetonitrile–water (2:98), pH 2.7. The analyte was retained on the column while proteins contained in the sample were removed to the waste without precipitation and clogging the column. Interfering substances complicating the detection were washed out by acetonitrile–water (15:85), pH 2.7 in the next step. The extracted analyte was eluted by means of acetonitrile–water (25:75), pH 2.3 to the fluorescence detector (emission filter 385 nm). The whole procedure comprising sample pre-treatment, analyte detection and column reconditioning took 20 min. The recoveries of furosemide from serum lay between 101.4 and 103.4% for three concentrations of analyte.

Keywords: Furosemide; Sequential injection analysis; Restricted access materials; Serum

1. Introduction

Sequential injection analysis (SIA) has been proposed by Růžička and Marshall as an efficient tool for automated liquid handling, especially performing of derivatisation reactions [1]. The technique is based on forward and reverse movement of a piston of a syringe pump, which together with a multi-position selection valve enables precise sampling of chemicals into the system and propelling of the sequenced zones to the reactors and detector [2]. Automation, velocity of the analysis and low consumption of sample and reagents are the most important features that favour the SIA technique for application in many fields of analysis, primarily by monitoring of long lasting processes [3–5].

Possibility of automated liquid handling is also advantageous by performing of more complicated operations such as sample pre-treatment. Nowadays, solid-phase extraction (SPE) represents the favourite sample pre-treatment method with many attempts to its automation and increase of sample throughput [6]. On-line coupling of SPE with SIA is one of the possibilities to meet these requirements [7–10]. SIA–SPE can be applied in analysis of such complex samples as bio fluids. Few SIA–SPE determinations in biological material were found in literature [11–14]. Removing of interfering sample matrix was achieved with urine samples while the SIA–SPE system based on modified silica gel sorbents was found to be limited in case of serum analysis [13].

In recent years, special SPE supports possessing restricted access properties have been developed to allow the direct injection of untreated biological samples into on-line SPE liquid chromatography (LC) systems [15–17]. These sorbents

* Corresponding author. Tel.: +420 495067274; fax: +420 495518718.
E-mail address: satinsky@faf.cuni.cz (D. Šatínský).

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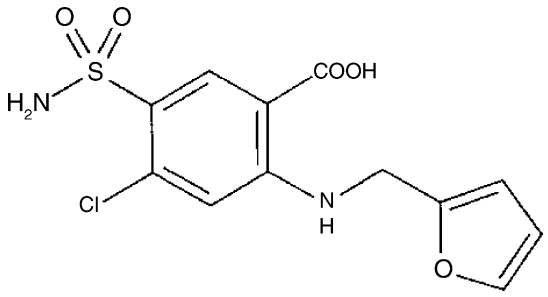

Fig. 1. Formula of furosemide.

called restricted access materials (RAMs), combine size exclusion of proteins (without destructive accumulation) and other macromolecular matrix components with the simultaneous enrichment of low-molecular analytes, which can be retained and extracted selectively.

The low-molecular-mass analytes are retained by conventional retention mechanisms such as hydrophobic, ionic or affinity interactions at the inner surface of the sorbent particles. The access of proteins is prevented by a physical diffusion barrier or a chemical barrier. In the majority of the applications described in the literature, RAMs are used in on-line coupled-column SPE–LC systems. These systems require highly sophisticated apparatus, higher operating cost and expensive instrumentation (column switching system, programs equipment and two pumps). Coupling of SPE based on restricted access materials with SIA was described as an alternative for direct determination of drugs in biological samples allowing decreasing expenditures on analysis. First attempt to on-line coupling of RAM and SIA was performed by determination of potential antileucotrienic quinlucast in serum and provided satisfactory results [14] that encouraged following studies of the hyphenation. The aim of the presented work was to examine further possibilities of the SIA–RAM system for direct analysis of serum with another drug and fluorescence detection. Furosemide, structure of which is shown in Fig. 1, was chosen as analyte possessing fluorescence capability and representing more polar substances than mentioned quinlucast. Being a strong diuretic furosemide is widely used in clinical practice in the treatment of hypertension and different kinds of oedema. Monitoring of furosemide in bio fluids is important not only in the clinical practice by treatment of patience but also in the field of doping control [18].

Several electrochemical methods for determination of furosemide in plasma and urine have been developed [19–21], however chromatographic procedures represent the method of choice in this case. HPLC techniques with spectrophotometric [22–29], fluorimetric [30,31] and amperometric [32] detection were proposed. Urine samples were analysed after dilution and filtration in some cases [25,26,30], while sample pre-treatment for removing of interfering sample matrix involving liquid–liquid extraction (LLE) [22–24,27,31] or SPE [33] was necessary when plasma or serum were analysed. The sample preparation step was mostly carried out in off-line mode, however Okuda et al. reported on-line coupling of SPE with HPLC and proved the advantages of such a configuration of the analytical procedure [34]. Method for direct determination of furosemide in serum comprising on-line sample preparation based on SIA–RAM hybrid technique has been proposed and advantages and disadvantages of such a connection are reported in the presented paper.

2. Experimental

2.1. Apparatus

2.1.1. Sequential injection system and restricted access material column

A commercially available instrument FIAlab® 3500 system (FIAlab® Instruments Inc., Bellevue, USA) with a syringe pump (syringe reservoir 5.0 ml) and an 8-port selection Cheminert valve (Valco Instrument Co., Houston, USA) was used. The manifold was equipped with fiber-optic fluorimetric detector PMT-FL (Ocean Optics Inc., Dunedin, USA) with UV light source D-1000-CE (Analytical Instrument Systems Inc., Flemington, USA). The fluorescence signal was adopted by secondary filter (385 nm, Edmund Industrie Optik, GmbH, Karlsruhe, Germany). The fluorescence detector was replaced by UV–VIS diode array detector USB 2000 (Ocean Optics Inc.) with a 10 mm laboratory-made flow cell for monitoring the elution profile of the macromolecular sample matrix. The whole SIA system was controlled by the latest version of program FIAlab for Windows 5.0. Flow lines were made of 0.75 mm I.D. PTFE tubing. On-line sample preparation was performed on RAM alkyl-diol silica (ADS) column (25 mm × 4 mm) packed with LiChrospher® RP-18 ADS 25 μm, a silica pore diameter 60 Å (Merck, Darmstadt, Germany). The RAM column was placed between the selection valve and flow cell of the detector (Fig. 1). A replaceable in-line filter (2–5 μm sieve, Merck) was installed prior to the column for column protection. A scheme of the sequential injection extraction system with the RAM column is depicted in Fig. 2.

2.1.2. HPLC apparatus

Preliminary experiments concerning the composition of mobile phase for analyte elution from the RAM column were performed on HPLC system consisting of a pump (Ecom LCP 4100, Prague, Czech Republic), autosampler (Waters 717, Milford, USA), UV detector (Waters 486) and data processing software CSW v. 1.7. Furosemide standard solution (30 μl, 10 μg ml^{-1}) was injected onto LiChrospher® RP-18 ADS column (25 μm, 25 mm × 4 mm), mobile phases of different composition were tested at flow rate 0.6 ml min^{-1} and the detector was set on 240 nm.

The same system with a Discovery® (Sigma–Aldrich, Prague, Czech Republic) C_{18} column (125 mm × 4 mm I.D., 5 μm), an injection volume of 100 μl of the eluting zone containing extracted furosemide after SIA–RAM procedure and

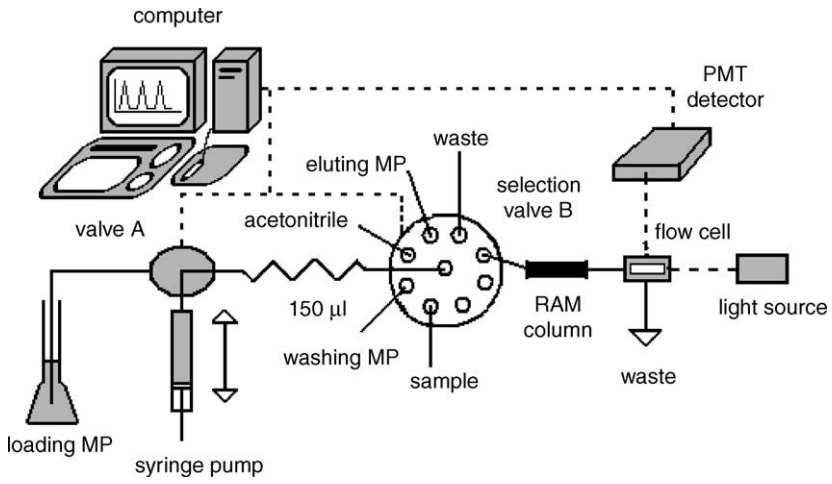

Fig. 2. Scheme of SIA–RAM system.

flow rate 1.0 ml min^{-1} was used for confirmation of the cleaning effect of the ADS column at 240 nm.

2.2. Reagents

All chemicals used were of analytical grade quality. Furosemide and organic modifiers for mobile phases preparation were obtained from Sigma–Aldrich. The standards of control sera Lyonorm U were purchased from Lachema Brno (Brno, Czech Republic). Millipore Milli-Q RG (Millipore s.r.o., Prague, Czech Republic) ultra pure water was used for preparing the solutions. Mobile phases were degassed by helium before use.

2.3. Preparation of spiked serum and standard samples

A stock solution of furosemide (500 µg ml^{-1}) was prepared by dissolving the substance in methanol. The flask was stored in the refrigerator protected to light for two months without stability problems. Fresh working standard solutions were prepared daily by appropriate dilution of the stock solution in water. Fast decomposition was observed when the solutions were stored without light protection. The standards of control sera (lyophylisate) for clinical chemistry were reconstituted and spiked with diluted solutions of furosemide to get the final concentration of furosemide (3.2–80 µg ml^{-1}) in five times diluted serum. The samples were spiked before analysis, incubated 1 h at 37 °C and then centrifuged for 10 min at 1750 × *g*. The supernatant was used for the analysis.

2.4. Optimisation of the steps of the sequential injection extraction

A procedure based on sequential aspiration of mobile phases of increasing content of organic modifier and their propelling through the column was proposed. The analytical cycle involved three main steps: (1) loading the sample onto the column and removing the proteinaceous ballast material, (2) washing the column and removing the more polar interfering substances complicating the detection and (3) elution and detection of the analyte. The composition of mobile phases used for particular steps was optimised separately.

The experiments concerning the composition of the mobile phases started by searching the optimum elution solvent. First experiments were carried out with HPLC system containing the LiChrospher® RP 18 ADS column. The optimum composition of the eluting mobile phase found on HPLC—acetonitrile–water (25:75), pH 2.3—was transferred to the SIA system. The retention behaviour of furosemide was checked making small changes in the composition of the eluting mobile phase and observing the changes in the retention time and shape of the peak of furosemide. The main task of these experiments was to obtain a symmetric peak of furosemide free of the noise of dead volume of the system.

The composition and volume of the mobile phases for the other steps of the procedure, the order of the required steps and the flow rates were optimised using the SIA system with fluorescence detection. Standard solution of furosemide (50 µl, 20 µg ml^{-1}) was loaded onto the column during these experiments and the eluting mobile phase found in the previous HPLC tests was employed. The other optimised parameters of the particular steps were adjusted to the composition of the chosen eluting mobile phase in order to design a procedure for extracting the analyte by contemporary removing of the proteinaceous matrix and other interfering components of the sample and efficient elution and quantitation of the analyte.

2.5. Design of the proposed analytical procedure

First, the syringe pump was filled with loading mobile phase acetonitrile–water (2:98), pH 2.7 (adjusted by 85% phosphoric acid) via the left position of the double position valve A. The sample (standard solution or spiked serum solution, 50 µl) was aspirated via port 5 of the selection valve

B (switching the valve A to the right position) to the connecting tubing leading from the middle port of the valve B to the pump. The sample was then propelled through port 8 of the selection valve B to the RAM column by reverse movement of the piston pump using the flow rate 0.6 ml min^{-1}. Furosemide was extracted on the column while proteinaceous matrix of the sample was washed to the waste. In the next step, washing mobile phase acetonitrile–water (15:85), pH 2.7 (adjusted by 85% phosphoric acid) was aspirated via port 4 of the selection valve B and pushed through the column washing fluorescing interfering substances to the waste (flow rate 1.2 ml min^{-1}). Finally, eluting mobile phase acetonitrile–water (25:75), pH 2.3 (adjusted by 85% phosphoric acid) was aspirated via port 2 of the selection valve B and used for elution of extracted furosemide to the detection point (flow rate 1.8 ml min^{-1}). It was necessary to wash the column with acetonitrile and recondition it with the loading mobile phase (both flow rates 1.2 ml min^{-1}) prior to aspiration of the next proteinaceous sample. The latter two steps were integrated at the beginning of the controlling program to ensure the column to be prepared for the following cycle.

3. Results and discussion

3.1. Composition of the particular mobile phases

The priority of our study was to optimise the composition of all the mobile phases to obtain the peak of the analyte that is differentiated from the matrix of serum and disturbing peak of the dead volume of mobile phase after the change of the phases in the flow cell. The mobile phases used in the particular steps influenced retention of the analyte and matrix during the whole procedure and the optimisation of their composition was a complex process. The optimisation of the single steps discussed in the following text was based on finding the optimum composition of the eluting mobile phase and adapting the composition of the other mobile phases to these conditions.

The loading mobile phase is proposed for propelling of the sample to the column and contemporary removing of proteins from the column. The content of organic phase is limited because of the risk of proteins denaturation and clogging the system. However, addition of low amount of organic solvent is recommended to achieve the release of the drug from the binding sites of serum proteins and to enhance the selectivity and sample clean up [35]. Acetonitrile–water (5:95) was tested but using this loading mobile phase in connection with eluting mobile phase acetonitrile–water (25:75) yielded in poor resolution of the peaks of furosemide and dead volume in the elution step. Finally, acetonitrile–water (2:98) was employed as loading mobile phase with good results concerning both resolution and recovery of the analyte, although furosemide is strongly bound to the proteins (97–98%) [22].

Washing step using the washing mobile phase had to be inserted between loading of the sample onto the column and elution of the analyte to the detector in order to remove some interfering fluorescing substances to the waste prior to the analyte detection and quantitation. A compromise between the sufficient strength of the washing solution for removing the interfering components and low impact of this solution on the retention time of furosemide during the elution step had to be found. Acetonitrile–water (15:85) was ascertained to be the strongest washing mobile phase that did not negatively influence retention of furosemide. Washing mobile phase of this composition enabled removing of the interferences from five-times diluted serum. Since it did not provide complete sample clean-up by injection of more concentrated serum samples, the samples had to be diluted to the mentioned concentration prior to the analysis.

It was necessary to adjust the composition of the eluting mobile phase to get convenient conditions for retention of furosemide at the column and its elution in form of a symmetric peak separated from the peak of dead volume after changing the mobile phases in the flow cell. Due to the limited length of the RAM column (25 mm) and high particles size (25 μm) there was the tendency for peak broadening and tailing with lower content of organics in mobile phase while increase of the amount of organics in mobile phase led to co-elution of furosemide with the dead volume. Various types of organic modifiers (methanol, acetonitrile, isopropanol, tetrahydrofuran) in different ratios in water were tested. Triethylamine and nonylamine were added to the mobile phase in attempt to restrict the tailing of the peak without sufficient results. Finally mobile phase acetonitrile–water (25:75), pH 2.3 (adjusted by 85% phosphoric acid) provided best conditions for elution of the analyte with respect to the retention time of furosemide and peak width.

Acetonitrile was used for column clean-up and conditioning between the single analytical cycles. It was necessary to recondition the column with loading mobile phase prior to the injection of the next sample to prevent the denaturation of the serum proteins in the column.

3.2. pH conditions

Due to the acid/base properties of furosemide (pK_a 3.9), it was necessary to decrease the pH of the eluting mobile phase to values at which ionisation of furosemide is suppressed and the analyte is well adsorbed to C_{18} chains of the sorbent on the inner surface of the column. Sufficient extraction of furosemide was achieved with eluting mobile phase at pH 2.3. Low pH was crucial for the shape of the peak of the eluted analyte. pH of the loading and the washing mobile phase was adjusted to 2.7 for the same purpose.

3.3. Flow rates and volumes of the mobile phases in the particular steps of the procedure

SIA is generally characterised by short time of the analysis and high sample throughput. Depending on the principal of the determination sample throughput going over 100

samples per hour can be achieved [36]. However, in case of SIA–RAM extraction the whole analytical procedure is more complex comprising sample pre-treatment and analyte quantitation and thus sample throughput is substantially reduced. Flow rates used with this technique are limited by back-pressure of the RAM column especially by loading the viscous proteinaceous sample.

Flow rate of 0.6 ml min^{-1} was proposed for the step of sample loading and removing the macromolecular ballast to the waste to prevent overloading of the syringe pump and to provide enough time for interactions of the analyte with the sorbent. Volume of the loading mobile phase is a crucial parameter because of the necessity to remove all proteinaceous matrix from the column prior to aspiration of the washing mobile phase with higher content of organic phase and thus denaturating properties. The contamination of the column with precipitated proteins would lead to an irreversible increase in back pressure and decrease of capacity and selectivity of the column. Removing of the proteinaceous ballast was monitored using the UV spectrometric detector at 280 nm, which corresponds to the absorption maximum of serum proteins [37]. The first step of the procedure (loading of the sample and removing the proteins from the column) was considered to finish when the detector had reached the baseline. A total volume of 4.2 ml of the loading mobile phase corresponding to time 420 s after injection was found to be sufficient for complete removing of the proteinaceous matrix after injection of 50 μl of undiluted serum. The volume of 4.8 ml of the loading mobile phase was used in the final procedure to ensure complete sample fractionation. Elution profiles of the macromolecular sample matrix using UV and fluorescence detection are presented in Fig. 3a and b, respectively. The fluorescence signal after injection of 50 μl of undiluted serum does not reach the baseline within time ascertained for fractionation step, which indicates presence of some low-molecular interfering substances that have to be removed in the next step.

Flow rate was increased to 1.2 ml min^{-1} for the washing step, which was a compromise between the speed of analysis and washing efficiency. The washing mobile phase (4.0 ml) enabled complete removal of the interfering substances without influencing the retention of the analyte.

The elution mobile phase was propelled through the column at the flow rate of 1.8 ml min^{-1} without any problems concerning the back-pressure. The higher value of flow rate also resulted in a narrower and higher peak of analyte comparing to lower flow rates. The lowest volume ensuring the complete elution of the analyte (4.9 ml) was applied.

3.4. Adsorption capacity of the ADS column

The adsorption capacity of the ADS column was studied. Standard solution of furosemide (50 μl, 40 μg ml^{-1}) was successively injected onto the column using the loading mobile phase acetonitrile–water (2:98), pH 2.7 as carrier. After 12 injections corresponding to total amount of 24 μg of furosemide the analyte started to leak from the column. The effect of the injected volume (50, 100, 250, 500 and 1000 μl) of furosemide solution (20 μg ml^{-1}) on the fluorescence intensity was studied and a linear relation characterised by calibration equation $I = (321.65 \pm 10.30)v + (32{,}509.86 \pm 5258.78)$ (I is the intensity of fluorescence, v the volume of furosemide solution) was found.

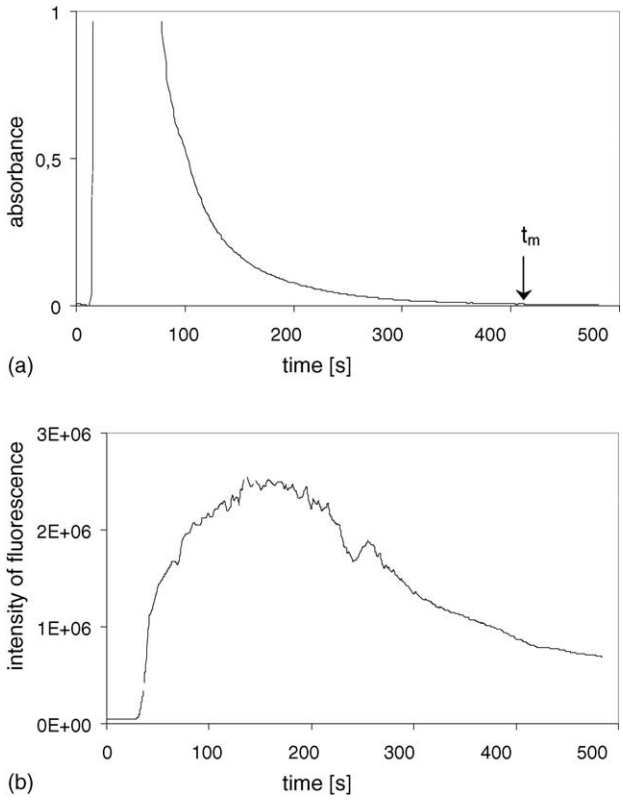

Fig. 3. (a) Elution profile of serum proteins, 50 μl of sample, acetonitrile–water (2:98), pH 2.7, flow rate 0.6 ml min^{-1}, UV detection 280 nm. T_m indicates the time corresponding to complete elimination of proteins, (b) elution profile of serum proteins, 50 μl of sample, acetonitrile–water (2:98), pH 2.7, flow rate 0.6 ml min^{-1}, fluorescence detection.

The extraction capacity of the column enables the increase of the injected sample volume and thus decreasing of the limit of detection. However, bigger sample volume would probably require higher volume of loading and washing mobile phases for removing the interfering matrix, which would be time consuming.

3.5. Validation

The method was validated with respect to the linearity, precision, accuracy, selectivity and sensitivity in order to evaluate the reliability of the results provided by the method.

3.5.1. Calibration and sensitivity

The calibration curve was established by measuring the fluorescence signal of six solutions of various concentrations of furosemide in serum. The linear relation between

Table 1
Intra- and inter-day precision of the proposed method

Furosemide concentration ($\mu g\,ml^{-1}$)	Intra-day precision (%RSD)		Inter-day precision (%RSD)	
	Standard solution	Serum	Standard solution	Serum
10	2.83	3.90	6.02	6.56
16	2.51	3.53	5.90	6.34
40	1.89	2.76	4.85	5.52

$n = 6$.

the fluorescence intensity and concentration of furosemide was found in the range 10–80 $\mu g\,ml^{-1}$ and was described by the following equation: $I = (1,\ 013.24 \pm 15.97)c + (33,\ 784.26 \pm 644.71)$ (I means the intensity of fluorescence, c the concentration of furosemide), the correlation coefficient being 0.9995.

The limit of detection and limit of quantitation were calculated by the comparison of the three-fold (3σ) and 10-fold (10σ) variation, respectively, of baseline noise and signals of serum samples spiked with known concentrations of furosemide. The detection limit was 3 $\mu g\,ml^{-1}$; the limit of quantitation was estimated to be 10 $\mu g\,ml^{-1}$ for 50 μl five times diluted serum injection. The low sensitivity of the system is blamed on extensive diffusion of the zones in the column due to relatively high particle size and column diameter [38].

3.5.2. Precision

Precision of the proposed procedure was characterised by parameter of repeatability, which was calculated for six consecutive measurements at three concentration levels (10, 16 and 40 $\mu g\,ml^{-1}$) of the analyte in 20% methanol solution and in spiked serum samples. The inter-day reproducibility was followed at the same concentrations for three consecutive days. The results in form of RSD are summarized in Table 1.

3.5.3. Accuracy

Accuracy of the method was estimated using the parameter of recovery. The recovery was calculated at three concentration levels (10, 16 and 40 $\mu g\,ml^{-1}$) by comparison of the responses of furosemide from spiked serum samples with those found by injection of the standard solutions at the same concentrations. The mean absolute recovery lay between 101.4 and 103.4% for furosemide and extraction efficiency was relatively constant over the range mentioned above (SD 2.09%).

3.5.4. Selectivity

The absence of interfering endogenous components of serum during the elution was demonstrated in Fig. 4, which shows typical record obtained during elution step by analysis of serum spiked with furosemide (8 $\mu g\,ml^{-1}$) and a blank serum (in both cases five times diluted).

The purity of the peak of furosemide obtained with SIA–RAM technique was confirmed using HPLC method by the following procedure. The zones of the analyte (standard and spiked serum) were entrapped after elution from the RAM column to vials and samples obtained in this way were injected into the HPLC system. This step of entrapping the analyte zones is possible due to the very precise syringe pump and stopped-flow technique. The volume of one analyte fraction was 4.9 ml and 100 μl of this volume were injected onto the Discovery® C_{18} analytical column. The cleaning effect of the ADS column, stability of the baseline and peak of the analyte after injection of "clean up" standard fraction and spiked serum fraction were confirmed. The length of the analysis corresponded to treble of the retention time of furosemide and no peaks of interfering substances were found in the "clean up" fraction.

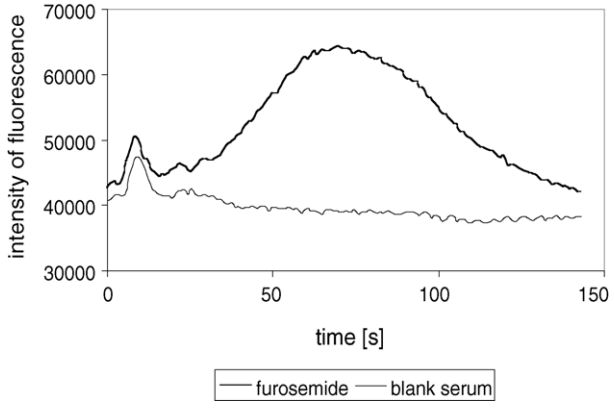

Fig. 4. Record of blank serum (20%) and peak of furosemide (8 $\mu g\,ml^{-1}$) in five times diluted serum, 50 μl of sample, acetonitrile–water (25:75), pH 2.3, flow rate 1.8 ml min^{-1}, fluorescence detection.

4. Conclusion

A hybrid technique involving SIA liquid handling and solid-phase extraction based on restricted access material was presented. The SIA–RAM system was tested for pre-concentrating, screening and direct determination of furosemide in human serum. The total analysis time within 20 min was achieved. The developed methodology has the potential to fill the gap between the traditional HPLC and manually performed sorbent extraction. The proposed method involving sample preparation can be simply automated and shows the possibility of restriction of manual sample handling. Repeated injections of untreated bio fluids are possible; the procedure ensures quantitative removal of protein matrix and on-column enrichment of the analyte. Minimum manipulation of the biological sample results in improved

precision and accuracy, shorter analysis time and lower costs per analysis. In contrast to the HPLC column switching technique, the SIA–RAM technique is based on non-continuous flow, which enables reduction of volume of consumed organic mobile phases and produced organic waste.

The main disadvantage of the proposed SIA–RAM method is low sensitivity. This is probably caused partly by diffusion of the zones in column of high diameter and particle size and partly by construction of the detection cell used in SIA. Studies with column of smaller diameter and other type of detector, respectively, could bring better results in future research. The SIA–RAM extraction procedure is convenient for determination of one drug, not for separation of complicated mixtures and simultaneous determination of a number of substances due to the limited separation efficiency of the RAM column. In summary, the proposed biological sample clean-up procedure based on the SIA–RAM system cannot be considered as a general process, but the optimal conditions of mobile phases for sample pre-treatment and analyte determination must be investigated for each individual case.

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