Evaluation of a novel anion-exchange restricted-access sorbent for on-line sample clean-up prior to the determination of acidic compounds in plasma by liquid chromatography

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Abstract

A new kind of silica-based restricted-access material (RAM) with anionic properties has been tested in pre-columns for on-line solid-phase extraction of acidic compounds from directly injected plasma samples prior to their determination by reversed-phase liquid chromatography (LC), using the column-switching technique. The outer surface of the porous RAM particles contains hydrophilic diol groups while diethylaminoethyl (DEAE) groups are bound to the internal surface which gives the sorbent the properties of a weak anion exchanger towards low-molecular-mass compounds. Due to an appropriate pore diameter (about 6 nm), macromolecules, such as proteins, are physically excluded from the pores and flushed directly out during the sample clean-up process, while small compounds have access to the inner surface and can be retained mainly by electrostatic interactions. The retention capability of this novel packing material has been tested for some hydrophilic acidic compounds such as aspartic acid, glutamic acid, ascorbic acid and acetylcysteine as well as for some more hydrophobic drugs such as naproxen, ibuprofen and diclofenac, used as model compounds. The influence of the composition of the washing liquid on the retention of the analytes in the pre-column has been investigated. The efficiency of the sorbent to clean-up complex matrices was also tested using human plasma and urine samples. A generic washing liquid composition was then selected in order to obtain efficient and selective sample clean-up as well as a high recovery of the acidic analytes.
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1. Introduction

In bioanalysis, sample preparation prior to any chromatographic analysis remains the major challenge for the determination of drugs and their metabolites in complex matrices, such as the biological fluids. In order to eliminate proteins and other matrix macromolecules, numerous laborious and time consuming off-line sample clean-up procedures based on liquid–liquid extraction (LLE) or solid-phase extraction (SPE) have been reported. Nowadays, due to the increasing demand for automation and high throughput analysis, on-line sample preparation procedures are increasingly used and in particular column-switching systems in which a pre-column packed with restricted-access material (RAM) is coupled to the analytical liquid chromatography (LC) column via a switching valve in order to permit the direct injection of protein rich samples, such as plasma.

A family of restricted-access sorbents, namely alkyl diol silica (ADS), belonging to the group of internal surface reversed-phase (ISRP) supports, was introduced by Boos et al. a few years ago [1,2]. The access restriction is obtained by use of silica particles (25 μm) with an appropriate pore diameter (about 6 nm), yielding a molecular mass cut-off of approximately 15 000. Macromolecules, such as proteins, have no access to the inner surface, to which butyryl (C_4), capryloyl (C_8) or stearoyl (C_{18}) moieties are bonded, and are then eluted directly from the pre-column. Low-molecular-mass compounds, such as the analytes of interest, can penetrate into the pores and be retained. Moreover, the outer surface of these porous RAM particles contains hydrophilic and electroneutral diol groups which prevents the adsorption and denaturation of proteins. Such supports have been

applied successively for on-line sample clean-up prior to the determination of compounds in different biological matrices [3–17].

An alternative technique recently introduced for the direct analysis of biological fluids is turbulent flow chromatography (TFC) by applying a high flow-rate to a short microbore LC column (1 mm i.d.) packed with large porous particles (50 μm) [18,19]. TFC allows the rapid passage of large macromolecules through the column with simultaneous retention of small analytes on C_2, C_8, C_{18}, phenyl or polymeric chains inside the pores [20].

However, due to too weak interactions with the inner surface of the extraction sorbent, the hydrophilic compounds could not be retained sufficiently. Besides, a lack of selectivity could be observed in the case of non-selective retention of matrix endogenous components that might interfere during the chromatographic analysis.

Two new kinds of silica-based RAM with ion exchange properties, namely exchange diol silica (XDS) sorbents, have been recently developed to extract more efficiently the hydrophilic compounds from directly injected biological fluids and to ensure more selective sample clean-up. These supports exhibit the same biocompatible outer surface as that of ADS material. The mean pore diameter of the silica particles (25 μm) is also about 6 nm. Due this physical diffusion barrier, the molecular mass cut-off is approximately 15 000. On the other hand, sulfonate or diethylaminoethyl (DEAE) groups are bonded to the inner surface of the porous sorbent, which gives it the properties of a strong cation or a weak anion exchanger towards low-molecular-mass compounds.

The retention capability and the efficiency of sample clean-up of the cationic exchange pre-column for the determination of some basic hydrophilic drugs in plasma has been evaluated by our research group and compared to RP-18 ADS material [21]. The retention capability towards hydrophilic cationic compounds is high and the gain in selectivity has been demonstrated. Moreover, a fully automated method was also developed and validated for the LC determination of sotalol in plasma using this kind of restricted-access sorbent for sample clean-up [22].

The aim of the present work is to test the retention capability of the novel anion-exchange RAM, namely XDS–DEAE/Diol sorbent, for some hydrophilic acidic compounds, such as glutamic acid, aspartic acid, ascorbic acid and acetylcysteine as well as for some more hydrophobic acidic drugs, such as naproxen, ibuprofen and diclofenac, used as model substances. To our knowledge, only naproxen and ibuprofen have been determined in plasma by coupling a pre-column packed with a particular kind of restricted-access sorbent to LC [23]. The latter consisted of uniform-sized molecularly imprinted polymer modified with hydrophilic external layer. For the other compounds, no method using RAM for sample pre-treatment has been reported.

The primary objective is to investigate the most important parameters likely to influence the retention of the analytes on the anion-exchange sorbent. Different factors related to the composition of the washing liquid, such as the nature and the concentration of the competing ion, the pH and the organic modifier content were studied. From the elution profiles of the different compounds and the biological matrix, the most suitable times for the rotation of the switching valve were then determined in order to ensure efficient and selective clean-up and complete transfer of the analytes from the pre-column to the LC column. The second objective consists of selecting generic conditions for sample clean-up prior to the LC determination of the compounds of interest in plasma.

2. Experimental

2.1. Chemical and reagents

Aspartic acid, glutamic acid, ascorbic acid, acetylcysteine, naproxen, ibuprofen and diclofenac were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. The structures of these compounds are presented in Fig. 1. Potassium dihydrogenphosphate, sodium perchlorate, sodium acetate, sodium carbonate,
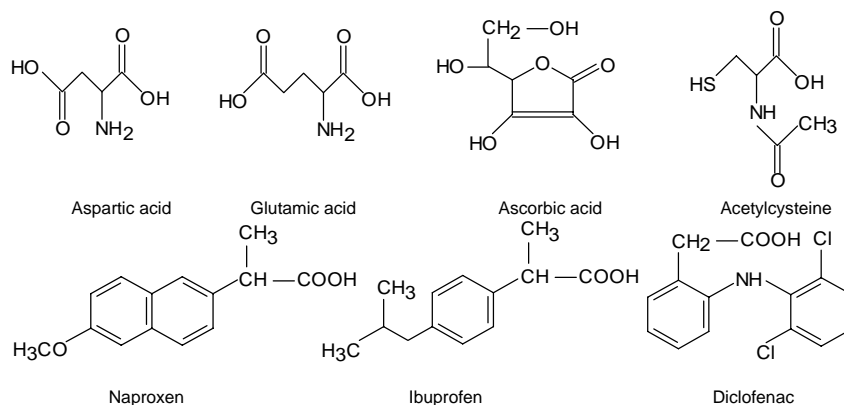

Fig. 1. Chemical structures of the acidic compounds tested.

sodium phosphate, sodium chloride, ammonium acetate were purchased from Merck (Darmstadt, Germany) and were of analytical grade. 1-Octanesulphonic acid sodium salt was supplied by Sigma. Acetic acid, phosphoric acid (85%), formic acid and perchloric acid were obtained from Merck. Methanol and acetonitrile were LiChrosolv LC gradient grade solvents purchased from Merck. The water used in all experiments was purified by means of a Milli-Q system (Millipore, Bedford, MA, USA).

The pre-columns were pre-packed with LiChrospher 60 XDS (DEAE/Diol) (supplied as research samples) (particle size: 25 μm) from Merck.

The analytical column pre-packed with LiChrospher 60 RP-Select B (particle size: 5 μm) was obtained from Merck.

2.2. Apparatus

The LC-integrated sample clean-up system was composed of a model 422 LC pump from Kontron Instruments (Schlieren, Switzerland) (pump 1) and the following units from Merck–Hitachi: a model L-6200 A pump (pump 2), a model AS-2000 A autosampler equipped with a 100 μl injection loop and a model L-4250 UV–Vis detector. A schematic representation of this column-switching system was shown elsewhere [24].

The LiChroCART pre-column (25 × 4 mm i.d.) packed with restricted-access material from Merck was fitted to a Valco model VICI AG six-port switching valve (Valco Europe, Schenkon, Switzerland).

The analytical column was a LiChroCART column (125 × 4 mm i.d.) from Merck and was thermostated at $25 \pm 0.1\,°C$ in a model L-5025 programmable column oven (Merck).

The different modules were connected through an interface (D-6000, Merck) with an IBM compatible computer (PC-AT; CPU type Pentium) on which the D-7000 HPLC manager software was loaded for the control of the analytical system and data collection. The model 422 pump from Kontron (pump 1) was controlled manually.

2.3. Chromatographic conditions

Isocratic separation was performed at 25 °C using a constant flow-rate of 1.0 ml/min. The composition of the different LC mobile phases is presented in Table 1. Before use, these mobile phases were degassed for 15 min in an ultrasonic bath. The analytes were monitored photometrically at 220 nm.

2.4. Standard solutions

2.4.1. Stock solutions

Stock solutions of each analyte were prepared in methanol at a concentration of 1.0 mg/ml and were stored in a refrigerator at 4 °C when not in use.

Table 1
Composition of the different mobile phases for the LC separation of the compounds

Compound	Organic modifier	O.M. (%, v/v)	Buffer pH	Conc. of OS⁻ (mM)
Aspartic acid	MeOH	2	2.9	2
Glutamic acid	MeOH	2	2.9	2
Acetylcysteine	ACN	5	3.0	–
Ibuprofen	ACN	50	3.0	–
Diclofenac	ACN	60	3.5	–

Mobile phase: 25 mM phosphate buffer–organic modifier (O.M.) containing or not octanesulfonate (OS⁻). ACN: acetonitrile; MeOH: methanol.

2.4.2. Solutions used for method development

Different diluted standard solutions were made up by diluting 1.0 ml of stock solutions with water to obtain a concentration of 50 μg/ml and were prepared daily.

2.4.3. Plasma samples

Human plasma samples were obtained from the Blood Transfusion Centre of Liège (Liège, Belgium) and were stored under −20 °C. Before use, the plasma samples were thawed at room temperature and centrifuged at $4500 \times g$ for 10 min. Aliquots were spiked with the diluted standard solutions and these plasma samples were prepared daily.

2.5. On-line sample clean-up and chromatographic separation

2.5.1. Loading and washing of sample matrix

One hundred microlitres of spiked plasma sample were directly injected by the autosampler into the pre-column. The biological matrix was washed out with the washing liquid consisting of a mixture of 0.005% acetic acid (pH 5.0)–methanol (97:3, v/v) for aspartic acid, glutamic acid, ascorbic acid and acetylcysteine and 2 mM sodium acetate adjusted to pH 5.0–methanol (97:3, v/v) for ibuprofen, diclofenac and naproxen. The washing liquid was delivered by pump 1 at a flow-rate of 1.0 ml/min for 8 min. During this step, the analytical column was re-equilibrated with the LC mobile phase delivered by pump 2 at a flow-rate of 1.0 ml/min.

2.5.2. Desorption and transfer

Eight minutes later, the switching valve was turned to position B and the pre-column was coupled to the analytical column, the analytes were then desorbed in back-flush mode and transferred to the top of the analytical column by the corresponding LC mobile phase (cf. Table 1) delivered by pump 2 at a flow-rate of 1.0 ml/min for 2 min.

2.5.3. Conditioning of the pre-column and chromatographic separation

After 2 min, the switching valve was turned back to position A allowing the re-equilibration of the pre-column with the washing liquid before the next injection. Simultaneously,

the analytes transferred to the analytical column were separated and quantified.

3. Results and discussion

3.1. Factors influencing the retention capability of the XDS (DEAE/Diol) sorbent for acidic compounds

Since the XDS (DEAE/Diol) sorbent presents the properties of a weak anion exchanger, the retention of the anionic analytes is mainly due to electrostatic interactions. Consequently, it is important that the DEAE groups bonded to the inner surface and the acidic compounds are fully ionised.

3.1.1. Influence of the washing liquid pH on breakthrough volume

The effect of the washing liquid pH on the breakthrough volume (V_b) which corresponds to the beginning of the elution of the analyte from the pre-column was tested by using solutions of 2 mM sodium acetate as washing liquids, the pH varying from 2.5 to 7.5. Practically, the breakthrough volume can be estimated visually from the elution profiles. However, in some cases, it is difficult to measure it by this way. It is then preferable to determine it as follows [25]:

$$V_b = (1 + k)\left(1 - \frac{2.3}{\sqrt{N}}\right) V_M$$

where k is the retention factor, V_M the void volume of the pre-column and N the number of theoretical plates.

As shown in Fig. 2, at pH values lower than 3.5, ibuprofen is mainly in undissociated form, while the DEAE groups are fully ionised. The breakthrough volumes were low. On the other hand, the increase of the washing liquid pH until 5.0 gave rise to an increase in the breakthrough volume due

Table 2
Influence of the nature of the co-ion in the washing liquid on the breakthrough volume and the retention factor of acetylcysteine

Washing liquid	Breakthrough volume (ml)	Retention factor (k)
Sodium perchlorate	1.5	9.9
Sodium acetate	1.8	11.5
Sodium carbonate	1.1	6.8
Sodium phosphate	1.2	9.6
Sodium citrate	0.5	2.4
Ammonium acetate	1.6	12.1
Sodium chloride	0.7	3.0

Sample: aqueous solution of acetylcysteine (conc.: 100 μg/ml); injection volume: 100 μl; washing liquid: 1 mM solution adjusted to pH 5.0–methanol (97:3, v/v); flow-rate: 1.0 ml/min; detection: UV at 220 nm; temperature: 25 °C; other conditions: see Section 2.

to the ionisation of ibuprofen. Under these conditions, the strongest electrostatic interactions were obtained. At higher pH values, the analyte remained ionised, but the DEAE groups were less and less ionised, which resulted in a decrease in breakthrough volume. Since the highest retention of ibuprofen on the anion-exchange sorbent was observed at pH 5.0, a washing liquid at this pH value was considered as adequate. The influence of this parameter has not been studied for the more hydrophilic acidic compounds, since they were not retained under the above conditions.

3.1.2. Effect of the composition of the washing liquid on the breakthrough volume and the retention factor of the analytes

As expected in ion exchange chromatography, the nature, the charge and the concentration of the co-ions in the washing liquid play an important role in the retention mechanism. Different washing liquids containing co-ions at a concentration of 1 mM were tested. As can be seen in Table 2, the
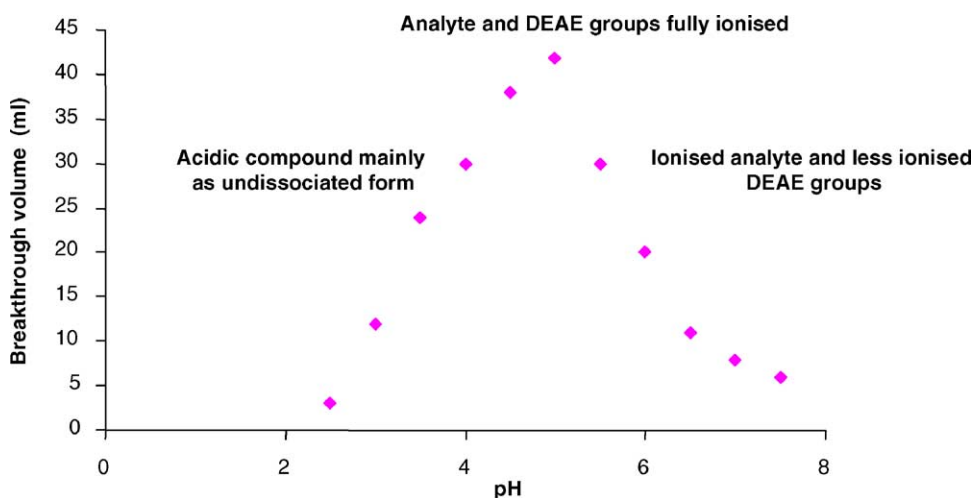

Fig. 2. Influence of the washing liquid pH on the breakthrough volume of ibuprofen. Sample: aqueous solution of ibuprofen (conc.: 100 μg/ml); injection volume: 100 μl; washing liquid: 2 mM sodium acetate–methanol (97:3, v/v); flow-rate: 1.0 ml/min; detection: UV at 254 nm; temperature: 25 °C; other conditions: see Section 2.

Table 3
Breakthrough volume of the hydrophilic compounds using diluted solutions of different acids as washing liquids

Analyte	Breakthrough volume (ml)			
	H_3PO_4 (0.003%)	HCOOH (0.004%)	CH_3COOH (0.005%)	$HClO_4$ (0.001%)
Aspartic acid	9	11	21	13
Glutamic acid	8	9	19	12
Acetylcysteine	7	8	18	10
Ascorbic acid	6	7	12	11

Sample: aqueous solution of each compound (conc.: 100 μg/ml); injection volume: 100 μl; washing liquid: diluted solution of acid containing 3% (v/v) of methanol (pH 5.0); flow-rate: 1.0 ml/min; detection: UV at 220 nm; temperature: 25 °C; other conditions: see Section 2.

breakthrough volume and the retention factor of acetylcysteine were low due to competition effects from the co-ions. Similar breakthrough volumes were observed with the other hydrophilic acidic compounds. Table 3 shows the results obtained when using diluted solutions of different acids as washing liquids. The pH of these solutions were about 5. Due to the reduction of the concentration of competing ions, the breakthrough volumes of the hydrophilic analytes were higher. Since the strongest affinity of the tested compounds was observed with 0.005% acetic acid (pH 5.0) as washing liquid, the latter was considered as adequate for sample clean-up.

Moreover, in the column-switching systems coupling restricted-access material to LC, the addition of a limited amount of organic modifier, such as methanol, acetonitrile or 2-propranol, to the washing liquid is important to enhance the extraction selectivity, to release the analyte from the binding sites of the plasma proteins and to obtain high recoveries. Therefore, the influence of the addition of methanol to the washing liquid consisting of 0.005% acetic

acid was tested. As illustrated in Fig. 3, the increase of the proportion of methanol in the washing liquid gives rise to a decrease in the breakthrough volumes of the hydrophilic acidic analytes. However, small changes were observed when the methanol content was lower than 5% (v/v). Consequently, a washing liquid containing 3% (v/v) of methanol was finally selected. Under these conditions, the analytes were not eluted from the pre-column.

3.2. Determination of generic conditions for on-line sample clean-up

The efficiency of the sorbent to clean-up complex matrices was then tested with human plasma and urine. One hundred microlitres of biological samples were injected into the pre-column directly connected to a UV detector by using a mixture of 0.005% acetic acid–methanol (97:3, v/v) as washing liquid. The UV absorbance was monitored at 280 nm for the plasma sample, this wavelength corresponding to the maximum of absorption for the plasma proteins, and at 230 nm for the urine sample. The fractionation step was considered complete when the detector signal reached the baseline. The washing time (T_w), i.e. the time for the complete elimination of the biological matrix, was determined from the elution profiles illustrated in Fig. 4A and B and corresponded to the first time for the rotation of the switching valve. As shown in these figures, the washing times for plasma and urine were estimated at 8 and 10 min, respectively. The presence of numerous ionic endogenous components in urine prolong the time needed for the complete elution of this matrix.

During the clean-up step, it is essential that no elution of the analytes occur. Consequently, the breakthrough volumes should be sufficiently high. According to the results obtained, a suitable composition for the washing liquid was
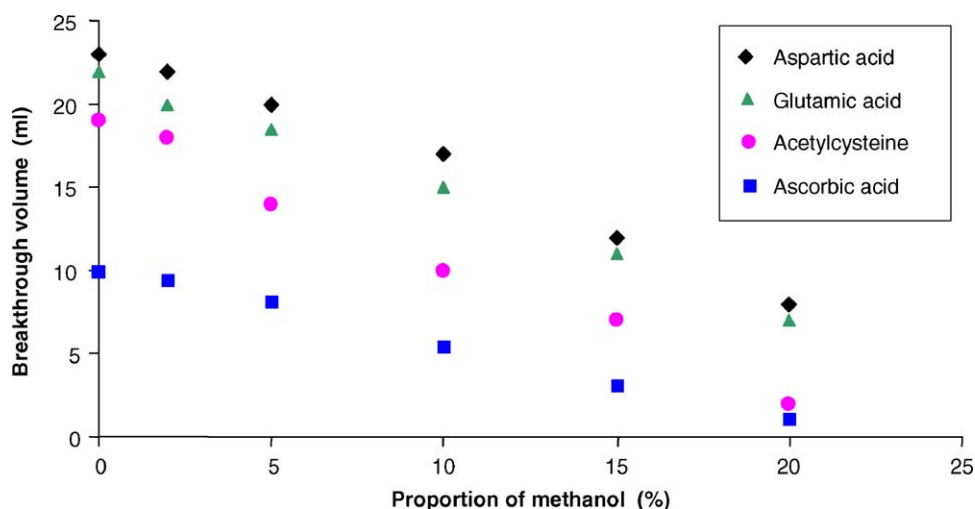

Fig. 3. Effect of the addition of methanol to the washing liquid on breakthrough volume. Sample: aqueous solution of each analyte (conc.: 100 μg/ml); injection volume: 100 μl; washing liquid: 0.005% acetic acid (pH 5.0)–methanol; flow-rate: 1.0 ml/min; detection: UV at 230 nm; temperature: 25 °C; other conditions: see Section 2.
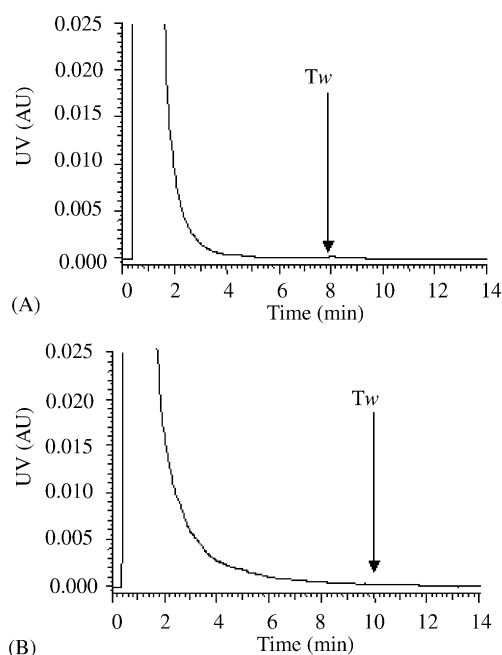
Fig. 4. Typical elution profiles of blank plasma and urine samples. (A) Elution profile of a blank plasma sample. (B) Elution profile of a blank urine sample. Injection volume: 100 μl; washing liquid: 0.005% acetic acid (pH 5.0)–methanol (97:3, v/v); flow-rate: 1.0 ml/min; detection: UV at 280 nm (plasma) and 230 nm (urine); temperature: 25 °C; other conditions: see Section 2. T_w represents the time for the complete elution of the sample matrix.

selected. For the hydrophilic compounds, the washing liquid was constituted of a mixture of 0.005% acetic acid (pH 5.0)–methanol (97:3, v/v), while for the hydrophobic drugs, a mixture of 2 mM sodium acetate adjusted to pH 5.0–methanol (97:3, v/v) could be used as washing liquid. The breakthrough volume and the retention factor of each compound obtained under these conditions are presented in Table 4. As can be seen, the retention of the analytes is sufficient, even for the more hydrophilic compounds.

The next step consisted of determining the time needed to elute the tested analytes from the pre-column in the back-flush mode and to transfer them to the analytical col-

Table 4
Breakthrough volume and retention factor of the acidic analytes using the suitable composition of the washing liquid for sample clean-up

Analyte	Breakthrough volume (ml)	Retention factor (k)	Washing liquid composition
Acetylcysteine	18	67	(A) 0.005% Acetic
Glutamic acid	19	70	acid (pH 5.0)–
Aspartic acid	21	72	methanol (97:3, v/v)
Ascorbic acid	12	45	
Ibuprofen	41	92	(B) 2 mM Sodium
Diclofenac	51	158	acetate adjusted to pH
Naproxen	65	224	5.0–methanol (97:3, v/v)

Sample: aqueous solution of each compound (conc.: 100 μg/ml); injection volume: 100 μl; flow-rate: 1.0 ml/min; detection: UV at 220 nm; temperature: 25 °C; other conditions: see Section 2.

umn with the adequate LC mobile phases (cf. Table 1). The determination of this transfer time (t_T) was performed by connecting directly the UV detector with the switching valve. Due to the high ionic strength of the different LC mobile phases, a time period of 2 min was sufficient to perform the complete transfer of each compound from the pre-column to the analytical column. Ten minutes after sample application, the switching valve returned to its initial position, allowing the sorbent to be re-equilibrated with the washing liquid.

Under the described operating conditions, this anion-exchange RAM has been applied successfully for on-line sample clean-up prior to the LC determination of acidic compounds in plasma. More than 300 injections of 100 μl of plasma have been performed without observing any increase in back pressure neither changes in the retention factor or the breakthrough volume of the tested analytes.

Fig. 5A and B illustrate typical chromatograms obtained after the analysis of a plasma sample spiked with aspartic acid and glutamic acid at a concentration of 5 μg/ml and a blank plasma sample. The chromatographic separation was carried out by using a mobile phase consisting of a mixture of 25 mM phosphate buffer pH 2.9–methanol (98:2, v/v) containing octanesulfonate at a concentration of 2 mM [26]. Although the UV detection was performed at 220 nm, the
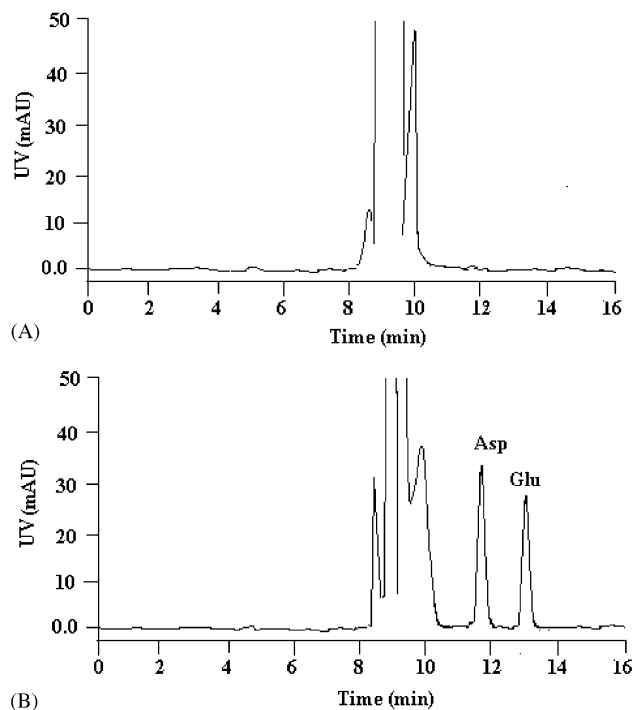

Fig. 5. Typical chromatograms obtained after on-line coupling of the anionic exchange pre-column XDS (DEAE/Diol) to LC for the automated determination of aspartic acid and glutamic acid in plasma. (A) Chromatogram of a blank plasma sample. (B) Chromatogram of a plasma sample spiked with aspartic acid and glutamic acid (concentration: 5 μg/ml). Operating conditions given in Section 2. Peaks: Asp, aspartic acid; Glu, glutamic acid.
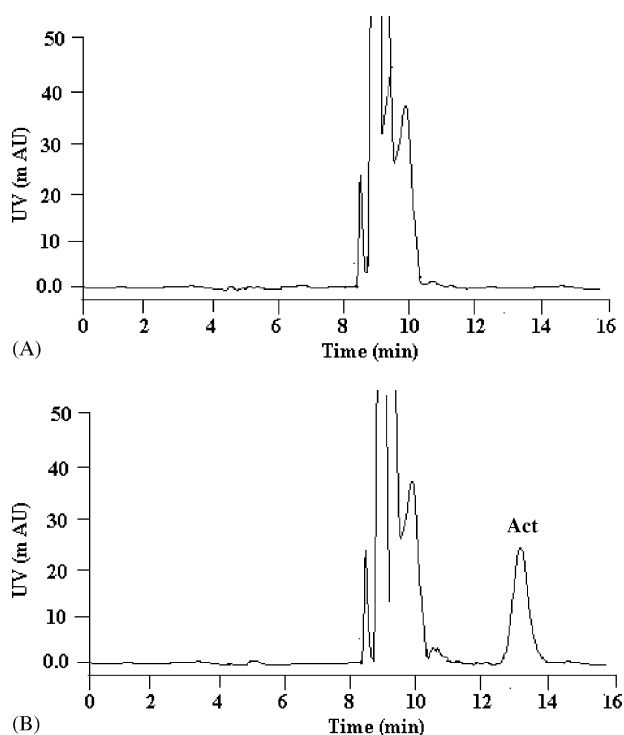
Fig. 6. Typical chromatograms obtained after on-line coupling of the anionic exchange pre-column XDS (DEAE/Diol) to LC for the automated determination of acetylcysteine in plasma. (A) Chromatogram of a blank plasma sample. (B) Chromatogram of a plasma sample spiked with acetylcysteine (concentration: 1 μg/ml). Operating conditions given in Section 2. Peak: Act, acetylcysteine.

solvent front was reduced and no interfering endogenous components of plasma were observed at the retention times of the two compounds, which demonstrated the excellent selectivity of this method. Due to the efficient sample matrix removal, similar chromatograms were observed after on-line coupling of the XDS (DEAE/Diol) pre-column to LC for the analysis of a plasma sample spiked with acetylcysteine at a concentration of 1 μg/ml and a blank plasma sample (cf. Fig. 6A and B).

Moreover, as can be seen in Table 5, satisfactory results were obtained for extraction efficiency. Indeed, the absolute recoveries were about 90% and the relative standard deviation values varied from 2.1 to 5.1% ($n = 5$), which demonstrated the extraction constancy. In order to prove the reliability of the developed methods, the complete valida-

Table 5
Extraction efficiency

Analyte	Concentration (μg/ml)	Mean recovery (%, $n = 5$)	R.S.D. (%, $n = 5$)
Aspartic acid	5.0	90	5.1
Glutamic acid	5.0	91	3.3
Acetylcysteine	1.0	89	2.1
Ibuprofen	0.5	91	4.5
Naproxen	0.5	93	3.2

Sample: spiked plasma; other conditions: see Section 2.

tion of a method based on this sample preparation technique will be performed and the full results will be presented.

4. Conclusions

The retention capability of a novel anion-exchange restricted-access material, namely XDS (DEAE/Diol) sorbent, has been tested for some hydrophilic acidic compounds, such as glutamic acid, aspartic acid, ascorbic acid and acetylcysteine as well as for some more hydrophobic acidic drugs, such as naproxen, ibuprofen and diclofenac. Since this sorbent exhibits the properties of a weak anion exchanger towards low-molecular-mass compounds, the retention of the acidic analytes depends on the pH of the washing liquid. Indeed, the compounds of interest and the DEAE groups bonded to the inner surface have to be ionised. In this study, the maximum retention capability was found to be at around pH 5. Besides, the nature, the charge and the concentration of the co-ions as well as the content of methanol in the washing liquid could influence the breakthrough volumes of the compounds.

The efficiency of this sorbent for clean-up of complex matrices was then demonstrated by injecting directly plasma and urine samples into the pre-column. The times for the complete elimination of the plasma and urine matrices were estimated at 8 and 10 min, respectively.

Generic conditions for sample preparation prior to the LC determination of the compounds of interest in plasma were also deduced. An appropriate composition of the washing liquid was selected and the most suitable times for the rotation of the switching valve were determined in order to ensure good sample clean-up and complete transfer of the analytes from the pre-column to the LC column. By coupling these two columns for the on-line determination of aspartic acid, glutamic acid and acetylcysteine in plasma, method selectivity was demonstrated towards endogenous components of plasma.

Consequently, the use of this new kind of packing material in a column-switching system seems to be a novel approach for on-line solid-phase extraction of acidic analytes from directly injected biological fluids, such as plasma. The advantages were clearly demonstrated, like the retention capability towards hydrophilic anionic compounds and the gain in selectivity.

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