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Sensitive determination of aspirin and its metabolites in plasma by LC–UV using on-line solid-phase extraction with methylcellulose-immobilized anion-exchange restricted access media

Eiichi Yamamoto[a,*], Susumu Takakuwa[a], Takashi Kato[a], Naoki Asakawa[b]

[a] *Analytical Research Laboratories, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan*
[b] *Drug Development Technology, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan*

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Abstract

We describe a sensitive determination of aspirin (ASA) and its three metabolites (salicylic acid [SA], 2,3-dihydroxybenzoic acid [2,3-DHBA], and 2,5-dihydroxybenzoic acid [gentisic acid (GA)]) in rat plasma. Analysis was carried out by on-line solid-phase extraction (SPE) using a methylcellulose-immobilized-strong anion-exchanger (MC-SAX), followed by liquid chromatography (LC) coupled with UV detection. The lower limits of quantitation for ASA and SA were 60 ng/mL in 100 μL of plasma, respectively. This method was validated with respect to intra- and inter-day precision, accuracy, and linearity up to concentrations of 20,000 ng/mL for ASA, SA, 2,3-DHBA and gentisic acid, respectively. The method was successfully applied to an analysis of the pharmacokinetics of ASA and SA in rats.

Keywords: Restricted-access media; Plasma; Anion-exchanger; Solid-phase extraction; Aspirin; Pharmacokinetics

1. Introduction

Liquid chromatography (LC) has been widely employed for the separation and quantitative analysis of a wide variety of compounds. Because the separation efficiency and effectiveness of LC depend largely on the chromatographic material used in the system, there is a great deal of interest in the development of novel chromatographic supports. Restricted-access media (RAM) [1] is one such support, and it has advantageous synergistic separation capabilities in that its macromolecular size exclusion characteristics are based on properties of the hydrophilic outer surface of the silica resin particles, while its diverse retention characteristics for smaller molecules originate from variable modifications on the inner surface of the silica [1–10]. The properties of RAM solid-phase extraction (SPE) columns and their application to biological fluid analysis have been examined in several reviews [11–14]. An On-line SPE system that incorporates a RAM column have been used for the isolation of small analytes (such as drugs and their metabolites) in biological samples and provides an alternative to traditional off-line approaches, and these SPE–LC system enables us the sensitive determination of trace analytes in biological samples such as blood plasma.

We have developed a novel type of RAM using methylcellulose (MC)-immobilized silica materials: a porous silica (particle size, 50 μm; pore size, 12 nm) was bonded to octadecylsilanized silica (ODS) ligands, and methylcellulose was immobilized on the surface silica gel by hydrogen bonding and van der Waals effects. Size exclusion columns constructed with the MC-immobilized silica materials had a molecular mass cut-off of approximately 14,000 Da with the physical diffusion barrier of the pore of the silica gel. The MC-octadecylsilanized silica (MC-ODS) SPE-column has been proved to be useful in the on-line sample pretreatment of a broad spectrum of drugs (anionic, cationic, and neutral) in plasma in the SPE–LC system [15,16].

Most of the RAM columns that have been developed so far have the retention mechanism of hydrophobic interaction against small analytes. Therefore, the weak retention of the hydrophilic analytes with these RAM columns remains problematic. To overcome the problem in the analysis of hydrophilic analytes in

* Corresponding author. Fax: +81 29 847 5771.
E-mail address: e-yamamoto@hhc.eisai.co.jp (E. Yamamoto).

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plasma, the ion-exchange RAM columns were developed, and several reports describe the analysis of drugs.

The ion-exchange diol silica RAM in conjunction with either a cation-exchanger [17] or an anion-exchanger [18] have also been applied to the quantitative analysis of drugs in plasma. We have also reported the use of MC-strong cation-exchanger (MC-SCX) for the analysis of cationic drugs and metabolites in plasma and in bile [19–21]. These columns allow for an automated analysis of small analytes in plasma that is rapid, simple, and accurate. As a branch of the MC-ion exchanger, MC-strong anion-exchanger (MC-SAX) which has trimethyl ammonium groups on the pore surface of the MC silica, has also been prepared for the on-line extraction of anionic analytes in biosamples such as plasma.

Acetylsalicylic acid (ASA), which commonly known as aspirin, is an analgesic, anti-inflammatory, and antipyretic drug and is one of the most widely used anionic drugs in the world. Once ingested, ASA is rapidly hydrolyzed in the body to produce salicylic acid (SA), which is the compound that is primarily responsible for the pharmacological activity of ASA. SA is further metabolized to gentisic acid (GA), salicyluric acid (SUA), and other conjugates [22]. A number of methods have been reported for the analysis of ASA and its metabolites in plasma samples, including LC [23–38], GC [39–42], and capillary electrophoresis [43]. Most of these methods utilized an off-line sample procedure involving solvent extraction and therefore required evaporation and reconstitution steps. Since evaporation leads to the loss of ASA and/or SA because of sublimation [24,32,35,44–47], the use of such procedures generally results in reduced recovery of the compounds being analyzed.

To overcome these problems, an on-line extraction procedure using a C18 extraction column in SPE–LC was developed for the quantitation of ASA and SA in plasma samples [36]. This method achieved the highest sensitivity among the methods previously mentioned, and its quantitation limits for ASA and SA in 200 μL of plasma sample were 100 and 250 ng/mL, respectively. However, even this improved method was still suboptimal because the recovery of SA tended to be reduced by the protein-binding to plasma protein, and the durability of the C18 extraction column was not sufficient for the procedure.

In the present study, our goal was to develop an effective on-line sample pretreatment procedure for quantitation of anionic compounds using MC-SAX in a column-switching LC system. We report the establishment of a simple, rapid, durable, and sensitive analytical method for the simultaneous quantitation of ASA and its three metabolites in plasma. A further benefit of this method is that quantitation may be accurately carried out without the use of an internal standard.

2. Experimental

2.1. Chemicals and reagents

Ammonium acetate, ammonium formate, dihydrogen potassium phosphate, hydrogen dipotassium phosphate, acetonitrile, ASA, and SA were purchased from Wako Pure Chemicals (Osaka, Japan). 2,3-Dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (GA), and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from TCI (Tokyo, Japan). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA). Dimethylsulfoxide (DMSO) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). All reagents were analytical grade and were used without further purification. Water was deionized and purified by a Milli-Q® purification system from Millipore (Bedford, MA, USA).

2.2. Animals

Male Sprague-Dawley (SD) rats (seven weeks, 285.1–300.3 g) were purchased from Charles River Laboratories Japan (Yokohama, Japan).

2.3. Sample preparation

Sprague-Dawley rat plasma was obtained by centrifugation of rat blood after anti-coagulation treatment with heparin. After dilution with an equal volume of 0.6% acetic acid aq. solution, the centrifuged plasma was used as "blank" plasma. A primary stock solution of a mixture of ASA, SA, 2,3-DHBA, and GA was prepared in acetonitrile; the final concentration of all compounds was 1 mg/mL each). A secondary stock solution (100 μg/mL) was prepared by diluting the primary stock solution in water–acetonitrile (70:30, v/v) containing 0.3% phosphoric acid. The drug-containing SD rat plasma samples were prepared by diluting the secondary stock solution with blank plasma at concentrations of 30, 60, 100, 500, 1000, and 10,000 ng/mL. The calibration standard solutions were prepared in a mixture of water and acetonitrile (70:30, v/v) containing 0.3% phosphoric acid at the same concentration as the spiked plasma samples. In the above preparations, calibration standard solutions and plasma samples were acidified to prevent the degradation of ASA.

2.4. Instrumentation and chromatography

For LC–UV, the Shimadzu (Kyoto, Japan) liquid chromatograph was equipped with two LC-10ADvp dual pumps, a DGU-10 degasser, an SIL-10ADvp auto-injector, a CTO-10AD column oven, an SPD-10Avp UV detector, and an SCL-10Avp system controller. Class VP version 5.1.2 software (Shimadzu) was used for liquid chromatograph control, data acquisition, and data analysis. The MC-SAX (Shimadzu Shim-pack MAYI-SAX) column (10 mm × 4 mm I.D., 50 μm particle, 12 nm pore size) was used as an LC column.

For LC–UV analysis, 10 μL of standard solution (1 μg/mL) was injected at 1 mL/min, and chromatography was performed on the MC-SAX column at 35 °C. Chromatograms were obtained at a UV detection wavelength of 235 nm.

For SPE–LC–UV, we used the Shimadzu column-switching LC system illustrated in Fig. 1. The system consisted of two LC-20AD pumps that were used for extraction LC, an LC-20AB (dual pumps) that was used for analytical LC, an SIL-20AC auto-injector, a DGU-20 degasser, a CTO-20A column oven,

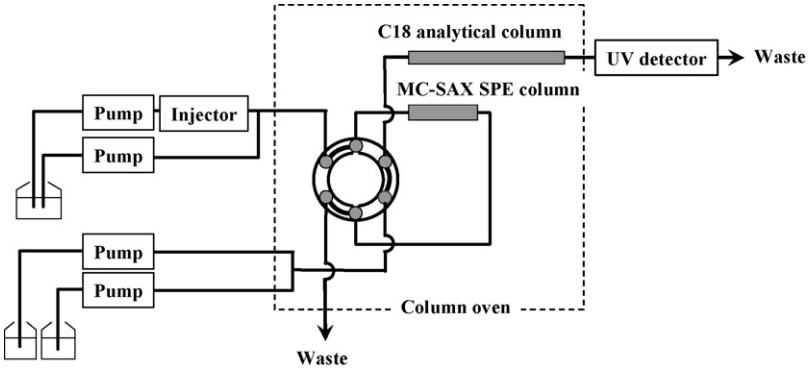

Fig. 1. Schematic diagram of the SPE–LC–UV system.

an FCV-20AH six-port valve, an SPD-20A UV detector, and a CBM-20A communication base module. The LC solution software was used for liquid chromatograph control, data acquisition, and data analysis. The MC-SAX SPE-column was used as an extraction column and a YMC (Kyoto, Japan) hydrosphere C18 (150 mm × 4.6 mm I.D., 5 μm particle, 12 nm pore size) was used as an analytical column.

For SPE–LC–UV analysis, the injected samples were delivered at 0.2 mL/min from the injector and on-line dilution was made with a dilution flow of the same mobile phase at 2.8 mL/min to enhance the compound recovery from plasma by increasing the ratio of free compound from protein-binding; solutes were concentrated on the MC-SAX SPE-column for 2 min. After the removal of plasma proteins and other matrix components, the target compounds enriched on the MC-SAX SPE-column were transferred to the analytical column by switching a six-port valve under the gradient elution mode at a flow rate of 1.0 mL/min at 35 °C. Eluant was detected by a SPD-10Avp UV detector at a wavelength of 235 nm. The mobile phases consisted of (A) a mixture of water and acetonitrile (1000:10, v/v) containing 0.2% TFA and (B) a mixture of water and acetonitrile (100:900, v/v) containing 0.1% TFA. The initial concentration of mobile phase (B) was 0% and was then increased to 30% for 18 min, after which the concentration was held at 30% for 2 min. After each run, both the SPE-column and the analytical column were flushed with mobile phase (B).

2.5. Evaluation of retention characteristics of the MC-SAX SPE-column

To investigate the effectiveness of on-line sample extraction for anionic compounds, we used the LC–UV system to examine the effect of ion species (and of their subsequent concentration in the extraction mobile phase) on the retention factor of ASA. Chromatography using the MC-SAX SPE column was carried out using isocratic elution of a mobile phase of water/acetonitrile (95:5, v/v) containing 1–50 mmol/L of phosphate buffer (pH 6.8), ammonium acetate (pH 6.5), or ammonium formate (pH 6.0) at a flow rate of 1.0 mL/min. The evaluation was carried out using three independent injections of the standard solution (1000 ng/mL).

We also tested the effect of organic solvent (acetonitrile) on compound recovery, and used the SPE–LC–UV system to evaluate any changes induced by using acetonitrile in the extraction mobile phase of the MC-SAX SPE-column. In the extraction LC, a mixture of water and acetonitrile (100:0, 98:2, 95:5, and 90:10, v/v) containing 5 mmol/L of ammonium acetate was delivered at 3.0 mL/min for 2 min. Retained compounds on the MC-SAX SPE column were transferred to analytical LC, and the peak areas of ASA and SA were evaluated. The evaluation was carried out with three independent injections of standard solution (1000 ng/mL).

2.6. Anionic drug analysis using the SPE–LC–UV system and validation of the method

Reproducibility was assessed by six independent injections of the calibration standard and of plasma samples, both containing 1000 ng/mL of compound. We also assessed the linearity of the quantitation of the standard solutions and plasma samples at compound concentrations of 30, 60, 100, 500, 1000, and 10,000 ng/mL (corresponding to actual plasma concentrations of 60, 120, 200, 1000, 2000 and 20,000 ng/mL, $n = 3$). Intra-day precision and accuracy were determined at every concentration level stated above ($n = 3$). Inter-day precision and accuracy were determined at 30, 100, and 10,000 ng/mL ($n = 6$). Precision was determined by a back-calculation method using a calibration plot of standard samples. Accuracy was determined by the degree of quantitative recovery of compounds from the plasma sample.

2.7. Pharmacokinetics of ASA and SA in rats

ASA dosing solution (containing DMSO:Tween 80:saline at ratios of 6.5:3.5:90, v/v/v) was injected intravenously (at 2.5 mg/kg body weight) into the right jugular vein of three SD rats, who had not been fed for 14 h prior to the procedure. Blood samples (∼250 μL) were drawn from the left jugular vein using a heparinized syringe at 5, 10, 15, 30 min, 1, and 1.5 h after compound injection. After collection, blood was centrifuged to obtain plasma and was stored at −20 °C until analysis. The frozen plasma was thawed and immediately diluted with an equal volume of 0.6% phosphoric acid aq. solution and centrifuged (1000 rpm for 10 min) to produce a plasma sample for analysis.

For quantitation, calibration curve samples for ASA, SA, 2,3-DHBA, and GA were prepared at concentrations of 100, 1000, and 10,000 ng/mL (corresponding to plasma concentration of 200, 2000 and 20000 ng/mL).

3. Results and discussion

3.1. Evaluation of MC-SAX material and the MC-SAX SPE-column

A previous report suggested that the MC-immobilized material can prevent adsorption of plasma protein to the stationary phase and that this phenomenon occurs throughout a broad pH range of 2.0–7.0 [15]. The recovery of plasma protein from the MC-ODS column (10 mm × 4 mm I.D.) was assessed by the following procedure. One hundred microliters of blank plasma was injected into an MC-ODS SPE column with 20 mmol/L phosphate buffer solution (pH 2.0, 4.0 and 7.0), or 20 mmol/L ammonium acetate solution with or without 10% acetonitrile at 2 mL/min using conventional HPLC. Under each mobile phase condition, the initial 4.0-mL fraction of the extraction mobile phase was collected in duplicate. The collected fractions were applied to quantitative absorbance measurement of plasma protein. The protein exclusion ability of the MC-ODS SPE column was assessed by the recovery of protein in the collected fractions.

To develop an effective extraction procedure for ASA and its metabolites, we evaluated the influence of mobile phase additives on retention factors with regard to the MC-SAX SPE-column. The mobile phases examined included water/acetonitrile (95:5, v/v) containing 0–50 mmol/L of phosphate buffer (K/K, pH 6.8), ammonium acetate (pH 6.8), or ammonium formate (pH 6.0). We did not include a separate pH control for the mobile phase in this evaluation because the pK_a of ASA and SA are 3.5 and 3.0 [48], respectively, and the pK_a of the trimethylammonium group on MC-SAX is predicted to be over 10. Thus, at pH 5.5–7, these drugs would be predicted to show sufficient electrostatic interaction with the trimethylammonium group on the MC-SAX.

Results of the analysis of the retention factor of ASA on the MC-SAX SPE-column are shown in Fig. 2. The retention factor of ASA decreased as the co-ion (phosphate ion, acetate ion, or formate ion) concentration increased, demonstrating that the retention mechanism of MC-SAX was based primarily on anion-exchange. Because the largest retention factor for ASA was observed when ammonium acetate was used as a source of the co-ion, we used 5 mmol/L of ammonium acetate as a mobile phase additive in this extraction LC system in order to promote effective on-line purification, with strong retention of target compounds on MC-SAX.

A previous report has shown that when using extraction LC with a RAM column, a small amount (<10% vol.) of an organic modifier such as methanol and acetonitrile facilitates the release of compounds from plasma proteins, which may often bind up the compound(s) [16]. We evaluated the effect of organic solvent in the extraction mobile phase by using the SPE–LC–UV system to assess the recovery of ASA and SA from the MC-SAX

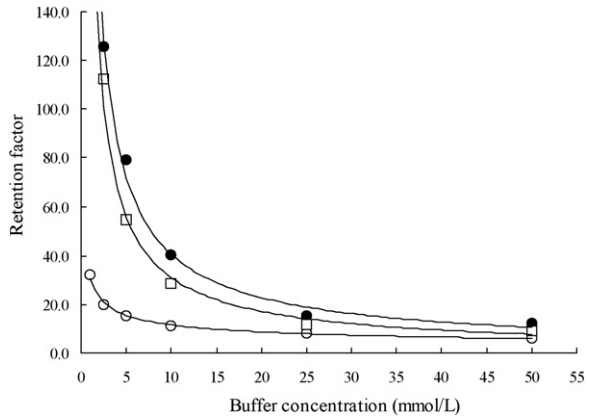

Fig. 2. Effect of mobile phase additive on the retention factor of ASA on a MC-SAX column in the LC–UV system. (◯) Phosphate buffer (pH 6.8 with 5% acetonitrile); (●) ammonium acetate (pH 6.8 with 5% acetonitrile); and (□) ammonium formate (pH 6.0 with 5% acetonitrile).

SPE-column, and tested 5 mmol/L of ammonium acetate solution plus acetonitrile at concentrations of 0, 2, 5, and 10% (v/v). Results showed that acetonitrile had little effect on compound recovery from the MC-SAX SPE column when using a standard elution solution. However, because SA is known to bind avidly to various proteins [47], we also tested the use of an elution solution containing 5 mmol/L of ammonium acetate in a mixture of water–acetonitrile (90:10, v/v) as the mobile phase in extraction LC to determine if it facilitated more efficient extraction of compounds from the plasma samples.

In this case, as for analytical LC in the SPE–LC system, we used the strong acid TFA as the mobile phase additive. TFA is primarily useful in that the TFA anion facilitates the desorption of ASA and of its acidic metabolites (such as SA) from MC-SAX in order to promote their elution from the analytical LC column. TFA also lowers the pH of the analytical mobile phase, which results in the conversion of anionic compounds to a non-ionic form that is amenable to the use of organic-solvent-rich mobile phases.

Next, we evaluated the influence of the volume of the extraction mobile phase on compound recovery from the MC-SAX SPE-column in the SPE–LC–UV system. An increase in mobile phase volume of up to 30 mL did not affect the recovery of SA, although we did observe a significant reduction in ASA recovery when the mobile phase volume exceeded 9 mL. This phenomenon might be explained by differences in retention strength for the SAX ligand, which might be caused by either the acidity or hydrophobicity of the compounds. Based on these results, we carried out extraction LC using a 2-min on-line extraction with a flow rate of 3 mL/min.

3.2. Analysis of ASA and its metabolites and validation of methods

Analysis of ASA and its metabolites (SA, 2,3-DHBA, and GA) in plasma was carried out by using an MC-SAX SPE-column in the SPE–LC–UV system; the resultant eluates were compared with standard samples. Representative HPLC chromatograms of blank plasma, spiked plasma samples, and

standard samples are shown in Fig. 3. In the chromatogram of the plasma sample, ASA and its metabolites were separated from the background peaks, and it was evident that this chromatogram was nearly identical to the chromatograms for the standard samples for ASA and SA. However, there was more variation between the chromatograms for 2,3-DHBA and GA and those of the corresponding standard samples. This difference is probably due to the broadening of the elution profiles for 2,3-DHBA and GA on the MC-SAX column; this broadening most likely resulted from the binding of the compounds to plasma proteins. This phenomenon is illustrated by the relationship between the dilution factor used in the extraction LC and the resultant peak shape in the analytical LC chromatogram. In fact, the larger the dilution factor in the extraction LC for plasma samples with SA and its metabolites (especially 2,3-DHBA and GA), the higher the theoretical plate for peaks.

The calibration curves ($n=3$) obtained by plotting peak area versus the compound concentration were linear for ASA and SA over the range of 30–10,000 ng/mL (actual concentration in plasma: 60–20,000 ng/mL), and the linear range for 2,3-DHBA and GA was 100–10,000 ng/mL (actual concentration in plasma: 200–20,000 ng/mL). The correlation coefficients (r) were $\geq$0.999 for every compound. The equations for the calibration plot were: $y=261.4x-3639.4$ for standard ASA; $y=275.8x-5353$ for ASA in plasma; $y=376.8x-5802.3$ for standard SA; $y=382.7x-16.7$ for SA in plasma; $y=226.1x+1953.6$ for standard 2,3-DHBA; $y=221.4x-20091.1$ for 2,3-DHBA in plasma; $y=275.2x+240.3$ for standard GA; and $y=273.8x-1111.7$ for GA in plasma.

The intra- and inter-day precision and accuracy of the methods at different concentrations are shown in Tables 1–4. Precision and accuracy were not determined for 2,3-DHBA and GA below 60 ng/mL in plasma because of the interfering effect of the background peak originating from the plasma. The intra-day precision ($n=3$) for the plasma sample ranged between 0.1 and 5.8% (CV) for the six concentrations, while inter-day precision ($n=6$) ranged between 0.2 and 11.4% at three concentrations. The accuracy value for the plasma sample was calculated as the percentage of the compound recovered. Results of precision and accuracy testing showed that the SPE–LC method performed acceptably well in this regard. Overall, results indicated that the analysis of anionic compounds with the MC-SAX SPE-column provided a reproducible method

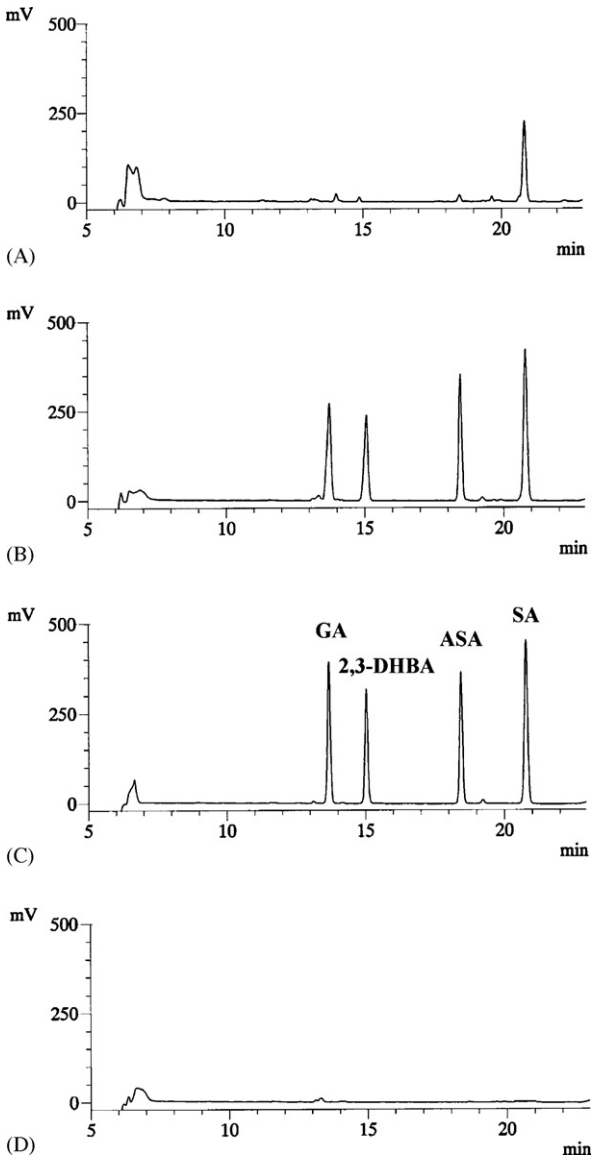

Fig. 3. Representative LC–UV chromatograms of ASA, SA, 2,3-DHBA, and GA in the SPE–LC–UV system. (A) Pharmacokinetic sample (5 min after intravenous injection); (B) spiked plasma sample (10 μg/mL); (C) calibration standard solution (10 μg/mL); and (D) blank plasma (predosing). LC conditions are described in the text.

Table 1
Intra-day precision and relative error of the method for the determination of GA, 2,3-DHBA, ASA, and SA in plasma samples ($n=3$)

Concentration (ng/mL)	GA		2,3-DHBA		ASA		SA	
	Precision (ng/mL)	CV (%)	Precision (ng/mL)	CV (%)	Precision (ng/mL)	CV (%)	Precision (ng/mL)	CV (%)
30	ND	ND	ND	ND	42.9 ± 0.3	0.8	27.3 ± 0.5	1.9
60	ND	ND	ND	ND	70.4 ± 1.3	1.9	47.6 ± 2.8	5.8
100	121.5 ± 0.3	0.2	80.0 ± 0.5	0.6	101.8 ± 0.5	0.5	94.7 ± 1.3	1.4
500	507.8 ± 2.7	0.5	454.0 ± 0.3	0.1	563.9 ± 11.2	2.0	519.6 ± 1.3	0.3
1000	968.1 ± 1.6	0.2	844.0 ± 0.7	0.1	965.3 ± 3.7	0.4	1016.1 ± 1.4	0.1
10000	10002.6 ± 23.9	0.2	9992.9 ± 5.5	0.1	9473.4 ± 22.4	0.2	9998.2 ± 45.9	0.5

ND, Not determined. Concentration: nominal concentration in sample after two-fold dilution of plasma with 0.6% acetic acid aq.

Table 2
Intra-day accuracy and relative error of the method for the determination of GA, 2,3-DHBA, ASA, and SA in plasma samples ($n = 3$)

Concentration (ng/mL)	GA		2,3-DHBA		ASA		SA	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
30	ND	ND	ND	ND	104.0 ± 1.5	1.4	97.6 ± 1.9	1.9
60	ND	ND	ND	ND	96.0 ± 2.5	2.6	98.1 ± 5.7	5.8
100	99.9 ± 0.2	0.2	100.3 ± 0.3	0.3	99.7 ± 0.6	0.6	100.9 ± 1.4	1.4
500	92.6 ± 0.5	0.5	103.4 ± 0.0	0.0	111.8 ± 2.3	2.1	102.7 ± 0.3	0.3
1000	101.9 ± 0.2	0.2	96.0 ± 0.1	0.1	106.6 ± 0.4	0.4	112.0 ± 0.2	0.1
10000	99.4 ± 0.2	0.2	98.6 ± 0.1	0.1	99.8 ± 0.2	0.2	101.7 ± 0.5	0.5

ND, Not determined. Concentration: nominal concentration in sample after two-fold dilution of plasma with 0.6% acetic acid aq.

Table 3
Inter-day precision and relative error of the method for the determination of GA, 2,3-DHBA, ASA, and SA in plasma samples ($n = 6$)

Concentration (ng/mL)	GA		2,3-DHBA		ASA		SA	
	Precision (ng/mL)	CV (%)	Precision (ng/mL)	CV (%)	Precision (ng/mL)	CV (%)	Precision (ng/mL)	CV (%)
30	ND	ND	ND	ND	39.7 ± 3.5	8.9	27.8 ± 1.6	5.7
100	135.6 ± 15.5	11.4	1016.8 ± 0.5	0.5	109.9 ± 9.0	8.2	101.9 ± 8.0	7.9
10000	10003.8 ± 21.2	0.2	9999.4 ± 10.5	0.1	9570.1 ± 107.0	1.1	9999.0 ± 35.9	0.4

ND, Not determined. Concentration: nominal concentration in sample after two-fold dilution of plasma with 0.6% acetic acid aq.

Table 4
Inter-day accuracy and relative error of the method for GA, 2,3-DHBA, ASA, and SA in plasma samples ($n = 6$)

Concentration (ng/mL)	GA		2,3-DHBA		ASA		SA	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
30	ND	ND	ND	ND	102.8 ± 2.5	2.4	102.3 ± 7.5	7.4
100	98.8 ± 1.1	1.1	103.5 ± 3.1	3.0	98.8 ± 1.1	1.1	103.5 ± 3.1	3.0
10000	98.2 ± 1.8	1.8	100.9 ± 0.9	0.9	98.2 ± 1.8	1.8	100.9 ± 0.9	0.9

ND, Not determined. Concentration: nominal concentration in sample after two-fold dilution of plasma with 0.6% acetic acid aq.

for the simultaneous quantitation of ASA and its metabolites in plasma, and the method did not require that any internal standard be present during the chromatography. This SPE–LC method also had high sensitivity for the isolation of compounds in plasma, which was achieved by the MC-SAX media's ample retention capacity for anionic compounds and also by its effective size exclusion and macromolecule-separating capability.

3.3. Pharmacokinetics of ASA and SA in rats

To test the applicability of the SPE–LC method for the detection and isolation of ASA, SA, 2,3-DHBA, and GA, we injected mice intravenously with an ASA dosing solution as described in Section 2. Plasma samples were then collected from three male SD rats, and after sample preparation, were chromatographically analyzed. A sample chromatogram is shown in Fig. 3A. The chromatogram displayed several peaks suspected to be metabolites; however, we unfortunately could not conclusively determine the presence of 2,3-DHBA and GA in the sample tested in this study. Therefore, we were limited to obtaining pharmacokinetic profiles for ASA and SA. Our pharmacokinetic analysis of ASA and SA was in general agreement with previous findings [29], indicating that the MC-SAX SPE-column in a column-switching LC system can be successfully applied to pharmacokinetic studies directed at quantifying levels of ASA and SA in plasma.

3.4. Stability of the MC-SAX column and durability of the system

Because routine analysis often entails processing a large number of samples over time, it is important to evaluate both the stability of the RAM columns and the general durability of the analytical system. We have previously reported that the MC-immobilized reversed-phase column is very stable [15,17]. In the present study, which encompassed over 80 injections of both plasma and standard samples over a two-week period, we did not observe any decrease in the SPE–LC performance or any significant pressure build-up in the LC system. These results suggest that the analysis of drugs and metabolites in plasma using the MC-SAX SPE-column in the column-switching LC system could be successfully applied for routine use for the analysis of drugs and metabolites in plasma, as well as for other applications.

4. Conclusion

We evaluated the MC-SAX SPE-column for analysis of ASA and SA in plasma using a column-switching system and found

that it was an effective method for the on-line enrichment of anionic drugs and metabolites in plasma. Our results indicate that the MC-SAX SPE-column will be useful for the pharmacokinetic evaluation of anionic drugs and metabolites in plasma samples, and we are currently carrying out further investigations to assess the utility of the MC-SAX SPE system for other applications.

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