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Novel liquid chromatographic–tandem mass spectrometric methods using silica columns and aqueous–organic mobile phases for quantitative analysis of polar ionic analytes in biological fluids

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Abstract

Use of silica stationary phase and aqueous–organic mobile phases could significantly enhance LC–MS–MS method sensitivity. The LC conditions were compatible with MS detection. Analytes with basic functional groups were eluted with acidic mobile phases and detected by MS in the positive ion mode. Analytes with acid functional groups were eluted with mobile phases at neutral pH and detected by MS in the negative ion mode. Analytes poorly retained on reversed-phase columns showed good retention on silica columns. Compared with reversed-phase LC–MS–MS, 5–8-fold sensitivity increases were observed for basic polar ionic compounds when using silica columns and aqueous–organic mobile phase. Up to a 20-fold sensitivity increase was observed for acidic polar ionic compounds. Silica columns and aqueous–organic mobile phases were used for assaying nicotine, cotinine, and albuterol in biological fluids. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Silica columns; Nicotine; Cotinine; Albuterol

1. Introduction

Rapid growth of using liquid chromatography with tandem mass spectrometry (LC–MS–MS) in pharmaceutical laboratories has been driven by the demand for speed at various stages in drug development, from high throughput screening of drug candidates, and rapid data generation at preclinical studies to fast analysis of clinical samples. Issues in LC–MS–MS that need to be addressed during method development include analytical column and mobile phase compatibility with the LC–MS interface [1,2], and inconsistent matrix effects from various lots of the biological matrices on biased quantitation of analytes [3–6].

Reversed-phase chromatography with ultraviolet (UV) or other types of detectors has been ubiquitously used for analysis of compounds in biological fluids. Reversed-phase columns demonstrate excellent stability, column efficiency and versatility with mobile phases of various compositions for the application of many varieties of compounds. However, reversed-phase separation in conjunction with MS may be problematic, particularly for polar ionic compounds. Ionization of polar analytes will decrease column-retention which is important to avoid

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matrix effects on LC–MS. To retain polar ionic compounds, highly aqueous mobile phase [7–12] or ion-pair chromatography [13] are needed. However, in order to achieve spraying conditions at the LC–MS interface necessary for adequate sensitivity, mobile phases containing highly aqueous solutions should be avoided [14]. Many drugs have basic functional groups, and MS in the positive ion mode often detects these drugs as protonated ions. Protonation is the most important means of ionization in positive ion electrospray mass spectrometry [15,16]. Acidic mobile phases are often used to ensure that these analytes are in their protonated forms [17], but such charged analytes will have even poorer retention on reversed-phase columns. Under these conditions, the analytes are eluted with the extraneous compounds at or near the solvent front, which often result in matrix suppression. Matrix suppression could also occur during the analysis of acidic compounds (detected as negatively charged ions in MS) when a mobile phase with a pH higher than the analyte pK_a is used.

The desired LC conditions for LC–MS–MS of polar compounds would be those that provide good analyte retention, and a mobile phase of relatively high organic content to optimize spraying conditions at the MS interface. These conditions are generally not met by most reversed-phase LC methods for polar compounds. An alternative is normal-phase liquid chromatography (NPLC). NPLC uses a stationary phase that is relatively more polar than the mobile phase with mobile phases consisting of a very non-polar solvent such as hexane and small amounts of polar organic solvent such as isopropanol. Traditional NPLC on silica is mainly applied to the analysis of hydrophobic compounds. The trace amount (in the ppm range) of water in the mobile phase had to be strictly controlled to ensure reproducible analyte retention [18]. Because of the difficulties of achieving consistent water content in the NPLC mobile phase and because biological fluids are aqueous in nature, NPLC has been rarely used for biological sample analysis [19–22].

In this article, the use of LC–MS–MS on silica columns with aqueous–organic mobile phase to analyze polar ionic analytes in biological fluids is presented. Quantitative LC–MS–MS methods for analysis of hydromorphone, morphine, morphine-3-glucuronide, and morphine-6-glucuronide have been previously reported [23,24]. Factors influencing the analyte retention were investigated. Basic compounds were eluted with an acidic mobile phase to create cations for electrospray and ion detection. Ionic analytes with acid functional groups were eluted with neutral pH mobile phases and detected by MS in the negative ion mode. Acidic analytes such as 2-thiophenecarboxylic acid (2-TCA), 3-methyl-2-thiophenecarboxylic acid (3-MCA), 2-thiopheneacetic acid (2-TAA), and basic compounds such as nicotine (NIC), cotinine (COT), albuterol (ALB), and bamethan (BAM) were used for study of the parameters affecting the retention. The chemical structures of the test compounds are shown in Fig. 1. Methods were validated for ALB in human serum,

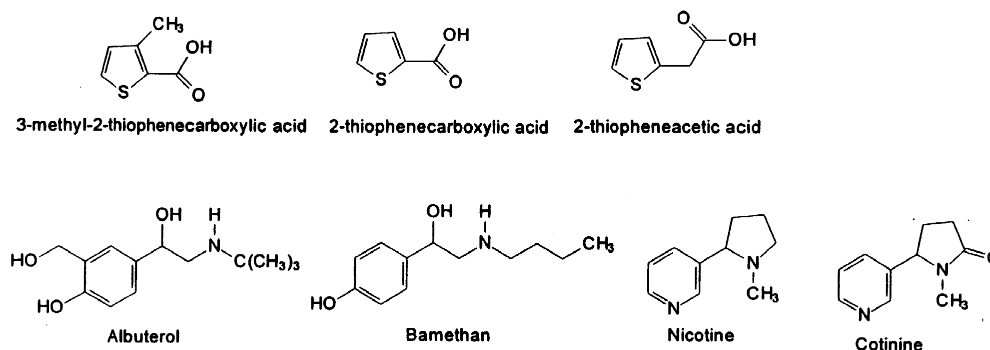

Fig. 1. Chemical structures of the test compounds. Abbreviations: ALB, albuterol; BAM, bamethan; NIC, nicotine; COT, cotinine; 3-MCA, 3-methyl-2-thiophenecarboxylic acid; 2-TAA, 2-thiopheneacetic acid; 2-TCA, 2-thiophenecarboxylic acid.

NIC and COT in human EDTA plasma. The silica column showed excellent peak symmetry, resolution power, and column stability.

2. Experimental

2.1. Chemicals and reagents

2-TCA (purity 99%) and 3-MCA (purity 98%) were available from Acros Organics (Pittsburgh, PA, USA). 2-TAA (purity 98%) was from Aldrich (Milwaukee, WI, USA). NIC (purity 99%), COT (purity 98%), and their internal standards (I.S.s) NIC-d_3 salicylate salt (purity 99%), COT-d_3 (purity 98%) were from Sigma (St. Louis, MO, USA). ALB (purity 99%) and its I.S., BAM sulfate salt (purity 99%), were also from Sigma. Formic acid (FA) and acetic acid (AA) were from Aldrich. Trifluoroacetic acid (TFA) was from Fisher (St. Louis, MO, USA). Ammonium hydroxide was from Sigma. Water, methanol, acetonitrile, chloroform and isopropanol, all of HPLC grade, were from Fisher. Control human serum, EDTA (K_3) was obtained from Biochemed (Winchester, VA, USA). Bond Elut Certify solid-phase extraction (SPE) cartridges (1 ml, 100 mg) were from Varian (Harbor City, CA, USA).

2.2. LC–MS–MS

The LC–MS–MS system consisted of a Shimadzu series 10ADVP HPLC system (Kyoto, Japan), and Perkin-Elmer Sciex API-3000 tandem mass spectrometer detectors with electrospray interface (Toronto, Canada). Sensitivities of multiple reaction mode (MRM) were optimized by testing on an infusion of 1 μg/ml each of the analytes in a mixture of methanol–water (50:50, v/v). For method development, analytes were dissolved in the mobile phases and injected onto silica or C_{18} analytical columns. Several brands of silica or C_{18} columns of the same size 50×2 mm I.D., 5 μm, all from Keystone Scientific (Bellefonte, PA, USA) were used. The columns were maintained at ambient temperature. Positive or negative ions were monitored in the MRM mode when acidic or neutral pH mobile phases were used, respectively. For basic amine analytes, mobile phases contained acetonitrile–water–FA [x:(100−x):0.2, v/v], where x is the variable ranging from 0 to 100. For acidic analytes, mobile phases were acetonitrile–water [x:(100−x), v/v], containing 5 m*M* ammonium acetate. Once the mobile phases were selected, analytes in various solvents were injected onto the column to select injection solvent. The column hold-up volume was measured by injecting 5 μl of an extracted blank biological sample under Q1 full scan mode (m/z 50–1000). The first deflection point on the chromatogram was used to calculate the hold-up volume [t_0 (min)×flow-rate (ml/min)]. The capacity factor (k') was then calculated as $(t_a-t_0)/t_0$ where t_a is the retention time of the analyte.

2.3. LC–MS–MS conditions for analysis of analytes in biological fluids

Analytical columns from three different batches have been used and consistent, similar chromatographic performance was observed. After 5–10 min of equilibration with the mobile phases, the brand new silica columns showed consistent retention times of the analytes.

2.3.1. NIC and COT in human ETDA plasma

Analytical column: Keystone Inertsil Silica 5 μm, 50×3 mm I.D., 5 μm, at room temperature; mobile phase: acetonitrile–water–TFA (90:10:0.05, v/v); flow-rate: 0.5 ml/min; injection volume: 20 μl; run time: 2 min; retention time (min): NIC 1.1, NIC-d_3 1.1, COT 1.2, COT-d_3 1.2; mass spectrometer: PE Sciex API 3000; ionization: positive ion electrospray (+ESI); mode: MRM, NIC 163 →84, NIC-d_3 166→87, COT 177→80, COT-d_3 180→101; drying gas flow-rate: 8 l/min; capillary voltage: 2 kV; orifice voltage: 26 V, source temperature: 400°C; ring voltage: 200 V; Q1 energy: −1 eV; collision energy: −28 eV.

2.3.2. ALB in human serum

Analytical column: Keystone Betasil Silica 5 μm, 50×3 mm I.D., 5 μm, at room temperature; mobile phase: acetonitrile–water–TFA (95:5:0.05, v/v); flow-rate: 0.5 ml/min; injection volume: 15 μl; run time: 3.5 min; retention time (min): ALB 3.1, BAM (I.S.) 2.6; mass spectrometer: PE Sciex API 3000; ionization: positive ion electrospray (+ESI); mode:

MRM, ALB 240→148, BAM (I.S.) 210→136; drying gas flow-rate: 8 l/min; capillary voltage: 3 kV; orifice voltage: 21 V, source temperature: 400°C; ring voltage: 200 V; Q1 energy: −1 eV; collision energy: −26 eV.

2.4. Procedures of sample extraction from biological fluids

2.4.1. NIC and COT from human EDTA plasma

A volume of 0.25 ml of plasma samples containing NIC/COT (1/10 to 200/2000 ng/ml) was spiked with 25 μl I.S. acetonitrile solution (100/1000 ng/ml NIC-d_3/COT-d_3) by using a Packard MultiPROBE II (Meriden, CT, USA). To this, 0.1 ml of 10% (v/v) ammonium hydroxide in water was added. Diethyl ether, 2.5 ml, was added to extract the analytes of interest by vortexing for 5 min. After freezing the aqueous layer, the upper layer was decanted, evaporated to dryness under nitrogen, reconstituted in 0.2 ml of acetonitrile, and injected onto the LC–MS–MS system.

2.4.2. ALB in human serum

A volume of 0.4 ml of serum samples containing ALB (0.05 to 10.0 ng/ml) was spiked with 50 μl I.S. water solution (10 ng/ml BAM) and 0.40 ml of 5% (v/v) acetic acid in water by using the Packard MultiPROBE II. The solution was then applied to the Bond Elut Certify SPE cartridge, which had been conditioned with methanol and water. After drawing the sample through the bed, the cartridge was washed with 1 ml of 5% acetic acid in water, followed by 1 ml of methanol. The analytes of interest were then eluted with two volumes of 0.75 ml of 2% (v/v) ammonium hydroxide in chloroform–isopropanol (80:20, v/v). The eluent was evaporated to dryness under nitrogen, reconstituted in 0.2 ml of acetonitrile–TFA (100:0.05, v/v), and injected onto the LC–MS–MS system.

2.5. Conduct of validation

A standard curve was defined by on set of calibration standards, randomized through the entire run. Three standard curves assayed over three consecutive days determined the inter- and intra-day reproducibility. Quality controls (QCs), randomized through the run, were also assayed together with the standard curves in achieving the inter-, and intra-day data. Each run also included a double blank, positioned immediately after the highest standard, to examine carry-over of the autosampler and the Packard MultiPROBE II. For one standard curve as many as 109 samples for NIC/COT and 114 samples for ALB were processed, which mimicked intended curve length for sample analysis. Stability of the analytes in biological fluids and through the analytical process was established with QCs.

2.6. Analytical data treatment

Chromatograms were measured using a MacQuan data system. A weighted [($1/x^2$) where x is the concentration of the analyte] linear regression was used to determine slopes, intercepts, and correlation coefficients. The resulting parameters were used to calculate concentrations:

$$\text{Concentration} = [\text{Ratio} - (y - \text{intercept})]/\text{Slope}$$

where ''ratio'' is the ratio of the analyte peak area to the I.S. peak area.

3. Results and discussion

3.1. Parameters affecting retention of polar ionic analytes on silica columns with aqueous organic mobile phases

Chromatographic methods on silica columns using a mobile phase of an organic solvent, predominantly methanol, and an aqueous buffer at neutral to alkaline pH have been reported for hydrophobic basic compounds [25–31]. Complex retention mechanisms involved ion-exchange, ion-pairs, salting-out, and reversed-phase retention by the siloxane. The hydrophobic basic compounds contain amine moieties, capable of ion-exchange with silanol groups on the silica surface. In order to understand the retention mechanism of polar ionic analytes on silica column using aqueous–organic mobile phase, the influence of the composition of mobile phase on the retention of polar acidic compounds was investigated. In mobile phases at pH 6–8, which are higher than their pK_a values, the negatively charged acids do not

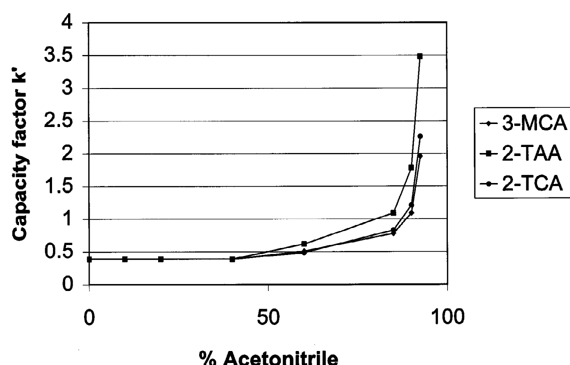

Fig. 2. Influence of acetonitrile concentration in mobile phase on k' of acidic compounds. Column: Hypersil silica, 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water [x:(100−x), v/v], containing 5 mM ammonium acetate, where x varied from 0 to 92.5.

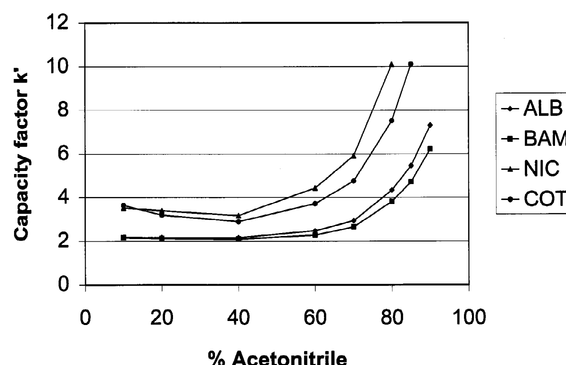

Fig. 3. Influence of acetonitrile concentration in mobile phase on k' of basic compounds. Column: Inertsil silica, 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water–FA [x:(100−x):0.2, v/v], where x varied from 10 to 90.

ion-exchange with the silanol groups. To demonstrate an ion-exchange effect with the silanol groups on the silica, the analyte must possess functional groups such as amines that interact with silanol. Acidic compounds used in this report obviously lack such a functional group. Increasing water concentration in mobile phase decreased analytes k'. Results obtained on a Betasil silica column are shown in Fig. 2. Similar results were obtained Hypersil and Inertsil silica columns. These results imply that the retention mechanism of polar ionic analytes on silica column with aqueous–organic mobile phase could be the normal phase, although more detailed study is needed to fully understand the retention mechanism.

Variability of chromatographic performance with different silica column brand was also investigated. As shown in Table 1, with a neutral mobile phase consisting acetonitrile and ammonium acetate buffer, similar k' values of the analytes were obtained for all the silica columns tested. The elution order of the analytes was the same on all columns.

Basic compounds such as ALB, BAM, NIC and COT also showed good retention on the silica column. Increase of acetonitrile in the mobile phase resulted in increased retention time (Fig. 3). Changing the FA concentration from 0.1 to 1% or replacing FA with AA did not alter the retention time. In order to improve peak shape, replacing FA with TFA in mobile phase is sometimes required. This resulted in significant decrease of the analyte retention on the silica column. Ion-pair between polar basic analytes and TFA is more hydrophobic than analyte itself, resulting in retention reduction and lower sensitivity. To compensate for these, higher acetonitrile and lower TFA (usually 0.01–0.05%) contents in mobile phase were needed. Although TFA may suppress signal of the analytes, the higher organic content in mobile phase seemed also compensate for this effect

Table 1
Chromatography of 2-TCA, 3-MCA, and 2-TAA on three brands of silica column[a]

Column (50×2 mm)	Capacity factor (k')			Plates/m ($N_{2\text{-TCA}}$)	Peak symmetry ($S_{2\text{-TCA}}$)
	2-TCA	3-MCA	2-TAA		
Hypersil	2.3	2.0	3.5	34 000	1.0
Inertsil	3.3	2.7	5.1	30 280	1.0
Betasil	3.3	2.7	5.1	52 000	1.0

[a] Mobile phase: acetonitrile–water (92.5:7.5, v/v) containing 5 mM ammonium acetate.

by introducing a more favorable spray condition. Therefore, the overall signal and sensitivity was not adversely affected by replacing FA with TFA.

3.2. Comparison of silica and reversed-phase C_{18} columns

Figs. 4 and 5 compared the chromatograms obtained on silica columns with a rich organic mobile phase and those on C_{18} columns with rich aqueous mobile phase, for basic and acidic compounds, respectively. For polar ionic basic compounds, the sensitivity obtained on the silica columns was 5–8-fold higher than those on C_{18} columns. For polar ionic acidic compounds, the sensitivity obtained on the silica was 20-times better than that on the reversed-phase column.

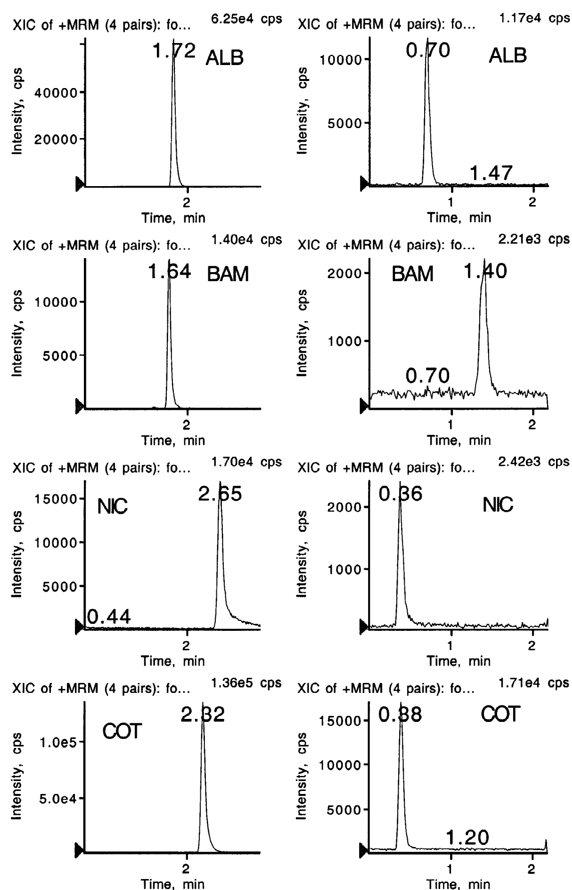

Fig. 4. Comparison of LC–MS–MS of NIC, COT, ALB and BAM on silica and C_{18} columns. Left panels: column: Hypersil silica 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water–FA (70:30:0.2, v/v); flow-rate: 0.5 ml/min; injection volume: 5 μl; sample: NIC 50 ng/ml, COT 50 ng/ml, ALB 1 ng/ml, BAM 1 ng/ml in acetonitrile–water–FA (70:30:0.2, v/v); detection: +ESI. Right panels: column: Hypersil BDS C_{18} 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water–FA (10:90:0.2, v/v); flow-rate: 0.5 ml/min; injection volume: 5 μl; sample: NIC 50 ng/ml, COT 50 ng/ml, ALB 1 ng/ml, BAM 1 ng/ml in acetonitrile–water–FA (10:90:0.2, v/v); detection: +ESI.

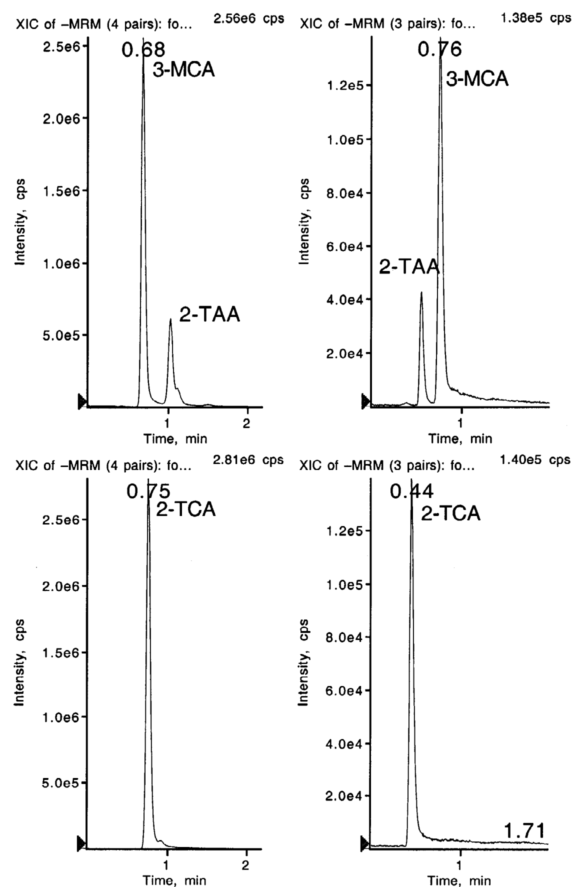

Fig. 5. Comparison of LC–MS–MS of 3-MCA, 2-TAA and 2-TCA on silica and C_{18} columns. Left panels: column: Hypersil silica 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water (92.5:7.5, v/v) containing 5 m*M* ammonium acetate; flow-rate: 0.5 ml/min; injection volume: 2 μl; sample: 100 ng/ml of 3-MCA, 2-TAA, and 2-TCA in acetonitrile–water (92.5:7.5, v/v) containing 5 m*M* ammonium acetate; detection: −ESI. Right panels: column: Hypersil BDS C_{18} 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water (5:95, v/v) containing 5 m*M* ammonium acetate; flow-rate: 0.5 ml/min; injection volume: 2 μl; sample: 100 ng/ml of 3-MCA, 2-TAA, and 2-TCA in acetonitrile–water (5:95, v/v) containing 5 m*M* ammonium acetate; detection: −ESI.

3.3. Choice of an injection solvent

Injection solution could significantly affect the peak shape and column efficiency [32,33]. Figs. 6 and 7 show the influence of injection solvent on chromatography for basic and acidic compounds, respectively. The sharpest peak and the highest sensitivity were obtained on silica columns when using acetonitrile as the injection solvent. This increase of column efficiency can be explained by the sample focusing effect obtained upon injecting acetonitrile, a weaker elution solvent, onto a silica column with acetonitrile–water as the mobile phase. Due to the sample focusing effect, the retention time was slightly longer with acetonitrile as the injection solvent than with the mobile phase.

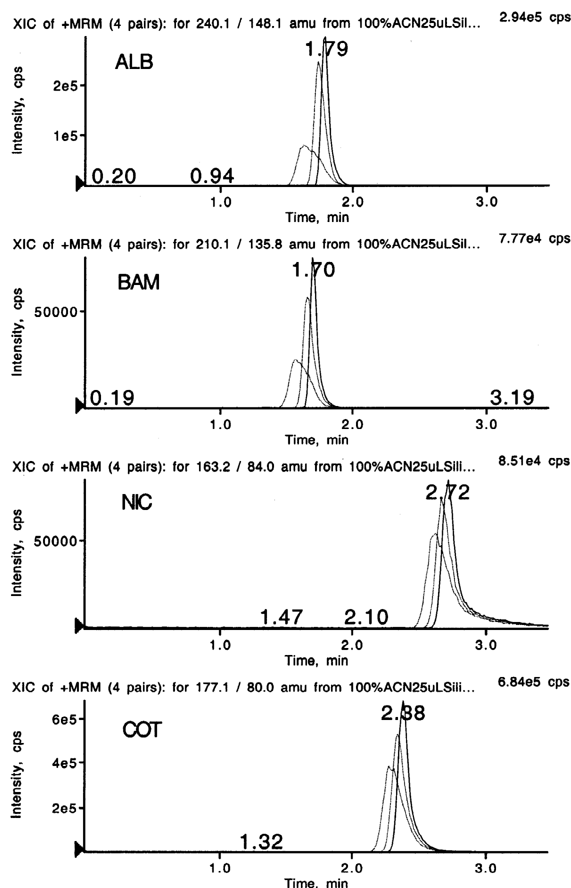

Fig. 6. Influence of injection solvent on LC–MS–MS of NIC, COT, ALB and BAM. Column: Hypersil silica, 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water–FA (70:30:0.2, v/v); flow-rate: 0.5 ml/min; injection volume: 25 μl; sample: NIC 50 ng/ml, COT 50 ng/ml, ALB 1 ng/ml, BAM 1 ng/ml in (solid lines with the highest peak height) acetonitrile–FA (100:0.2, v/v) or (dotted lines with intermediate peak height) acetonitrile–water–FA (60:40:0.2, v/v) or (dotted lines with the lowest peak height) acetonitrile–water–FA (20:80:0.2, v/v); detection: +ESI.

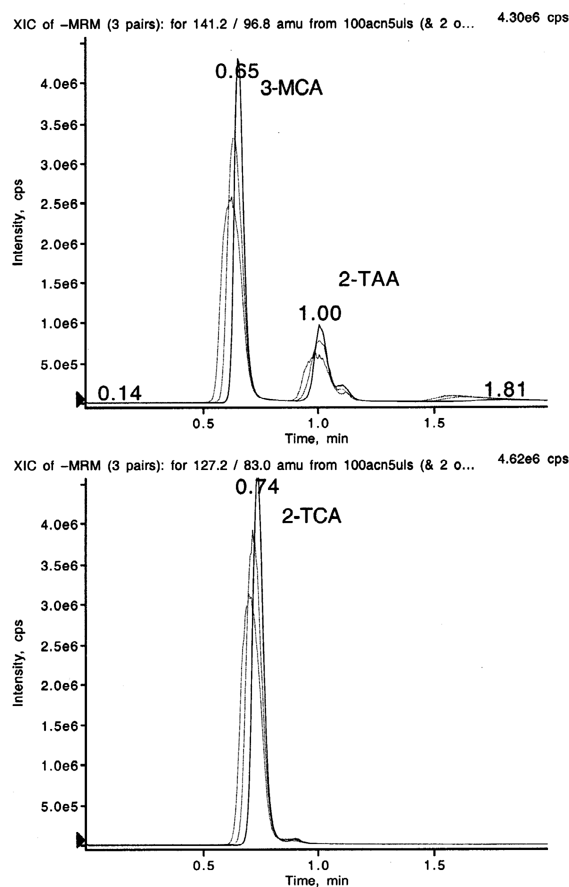

Fig. 7. Influence of injection solvent on LC–MS–MS of 3-MCA, 2-TAA and 2-TCA. Column: Hypersil silica, 50×2 mm I.D., 5 μm; mobile phase: acetonitrile–water (92.5:7.5, v/v) containing 5 m*M* ammonium acetate; flow-rate: 0.5 ml/min; injection volume: 5 μl; sample: 100 ng/ml of 3-MCA, 2-TAA, and 2-TCA in acetonitrile (solid line with the highest peak height) or acetonitrile–water (90:10, v/v) containing 5 m*M* ammonium acetate (dotted lines with the intermediate peak heights) or acetonitrile–water (85:15, v/v) containing 5 m*M* ammonium acetate (dotted lines with the lowest peak heights); detection: −ESI.

3.4. Quantitative analysis of polar analytes in biological fluids

LC–MS–MS detection of ALB in biological fluids using C_{18} columns has been reported [34–38]. None

of these methods was validated to measure as low as 50 pg/ml of ALB in human serum. LC–MS–MS methods on C_{18} columns were also reported for NIC and COT [39,40]. Measurement of as low as 1 ng/ml of NIC in human plasma was reported [40]. However, in order to achieve this sensitivity, as much as 8 ml of methylene chloride was used to extract the analytes from 1 ml of plasma. LC–MS–MS methods using silica columns have been developed and validated for quantitative analysis of ALB in human serum, NIC and COT in human plasma.

3.4.1. Sensitivity and selectivity

Figs. 8 and 9 show the LLOQ and blank chromatograms of ALB, NIC and COT, respectively. Matrix effects bias for different lots of biological fluids, which have been identified as one of the major potential problems for quantitative LC–MS–MS, were evaluated. Known amounts of analytes at the lower limit of quantitation (LLOQ) were in single spiked into each of at least six lots of biological fluids. LLOQs were also spiked in six replicates into one lot of biological fluid. These samples, together with blank samples (double blank) and blank samples with I.S., were run together with one set of calibration standards extracted from one lot of the biological fluid. Results are shown in Table 2 for ALB, and Table 3 for NIC and COT. Six out of six lots of blank serum were free from interference for ALB. For NIC and COT, there are two out of twelve plasma lots showing significant amount of NIC and COT, probably from smokers. There are

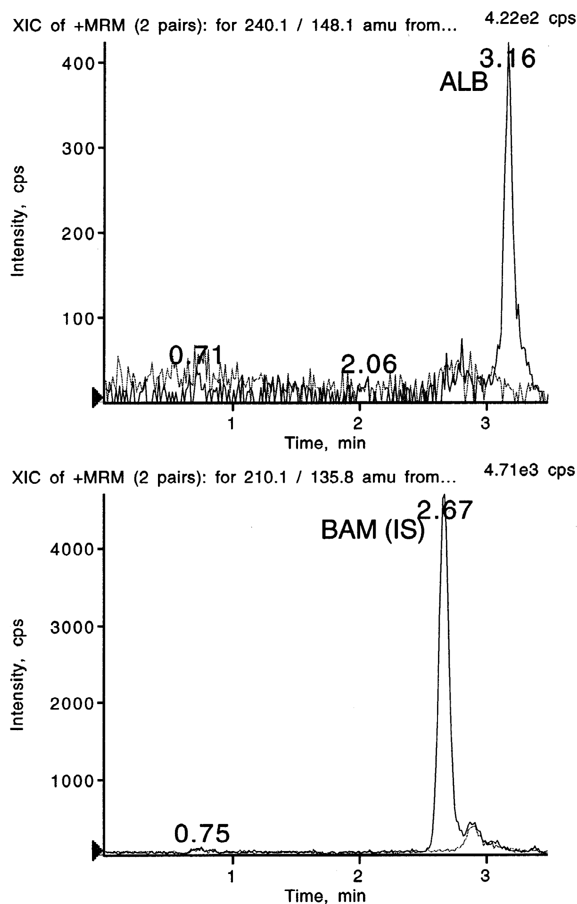

Fig. 8. LC–MS–MS of ALB (0.05 ng/ml) in human serum (solid line) and blank human serum (dotted line).

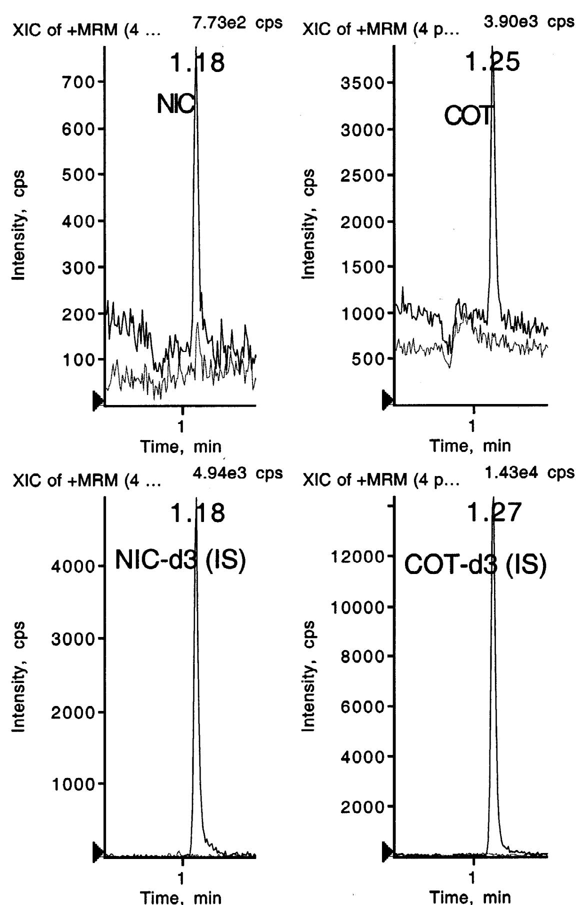

Fig. 9. LC–MS–MS of NIC (1 ng/ml) and COT (10 ng/ml) in human plasma (solid lines) and blank human serum (dotted lines).

Table 2
Within- and between-lots reproducibility of measuring ALB at LLOQ

ALB in human serum	Within-lot		Between-lots	
Spiked concentration (ng/ml)	0	0.050	0	0.050
Measured concentration (ng/ml)	ND	0.053	ND	0.049
		0.054	ND	0.057
		0.050	ND	0.049
		0.048	ND	0.057
		0.054	ND	0.045
		0.058	ND	0.049
Mean		0.053		0.051
RSD (%)		6.0		8.8
RE (%)		6.0		2.0

ND, Not detected (<20% LLOQ).
RSD, Relative standard deviation.
RE, Relative error.

two other lots showing small amounts of COT. Excluding the results from smokers, all of the spiked samples have calculated concentration close to the nominal values. The between-lots variability and within-lot variability are similar. The relative standard deviation (RSD) values and the mean results were analyzed by an F-test [41]. In no instance was the F-test significant. This indicated the lack of matrix effect difference among the tested lots.

3.4.2. Recovery

The recovery was determined by comparing the area counts of the QC samples vs. the area counts of the samples prepared by spiking the same amounts of analytes into extracted blank controls. The recovery results are shown in Table 4. In comparison with NIC, the recoveries for COT were relatively low but were very consistent as indicated by the low RSD values. Use of COT-d_3 as the internal standard is needed in this case.

3.4.3. Accuracy and precision

Accuracy and precision were established from three analytical runs for standards (Table 5) and QCs

Table 3
Within- and between-lots reproducibility of measuring NIC and COT at LLOQ

NIC and COT in human plasma	NIC				COT			
	Within-lot		Between-lots		Within-lot		Between-lots	
Spiked concentration (ng/ml)	0	1.00	0	1.00	0	10.0	0	10.0
Measured concentration (ng/ml)	ND	0.952	ND	1.02	ND	10.3	ND	10.1
		0.983	ND	1.20		10.8		
		1.01	26.5[a]	26.4	ND	11.1	ND	11.8
		0.904	ND	1.19		10.2		
		1.12	ND	1.15		10.6	218[a]	245
		0.995	ND	1.08		10.1		
			ND	1.17			4.73[a]	15.6
			ND	1.14				
			26.3[a]	26.4			ND	10.7
			ND	1.09				
			ND	1.22			ND	10.2
			ND	1.10				
							ND	10.8
							ND	10.2
							205[a]	238
							4.40[a]	16.0
							ND	9.40
							ND	10.8
Mean concentration (ng/ml)		0.994		1.14		10.5		10.5
RSD (%)		7.3		5.2		3.7		6.3
RE (%)		−0.6		+14		+5.2		+5.0

[a] Excluded from the calculation.
ND, Not detected (<20% LLOQ).

Table 4
Recoveries of ALB, NIC, COT and internal standards from biological fluids

	ALB ($n=6$)			BAM ($n=18$)
Nominal concentration	0.150 ng/ml	0.750 ng/ml	7.50 ng/ml	1.25 ng/ml
Recovery (%)	62.9	64.5	70.9	67.5
RSD (%)	3.5	6.2	5.8	6.8
	NIC ($n=6$)			NIC-d_3 ($n=18$)
Nominal concentration	3.00 ng/ml	30.0 ng/ml	150 ng/ml	10 ng/ml
Recovery (%)	54.3	48.0	58.0	59.6
RSD (%)	9.3	12.7	10.8	11.7
	COT ($n=6$)			COT-d_3 ($n=18$)
Nominal concentration	30.0 ng/ml	300 ng/ml	1500 ng/ml	100 ng/ml
Recovery (%)	15.9	13.0	14.3	15.4
RSD (%)	2.7	10.5	4.8	9.8

(Table 6). The standard curve was linear over the concentration range 0.050–10.0 ng/ml for ALB, 1.00–200 ng/ml for NIC and 10.0–2000 ng/ml for COT. Acceptable precision and accuracy were obtained for all three analytes.

3.4.4. Sample stability

Stability tests of the analyte in biological fluids and in sample extracts were established. The results are shown in Table 7. The analytes were stable during storage, sample extraction process and chromatography.

3.4.5. Silica column stability

A common perception regarding silica columns is that they are not stable when used for biological sample analysis, as polar ionic endogenous compounds are strongly retained and eventually deteriorate the column. This is probably true during classical normal-phase HPLC where very non-polar solvents are used as the mobile phase. However, it was found

Table 5
Accuracy and precision of calibration standards

ALB (ng/ml) in human serum								
Nominal concentration	0.0500	0.100	0.200	0.500	1.00	4.00	8.00	10.0
Calculated mean concentration	0.0512	0.0987	0.182	0.527	0.996	3.90	7.99	10.6
RSD (%)	4.0	9.9	4.0	4.8	11.1	4.6	6.3	3.8
RE (%)	+2.5	−1.3	−9.2	+5.4	−0.4	−2.6	−0.1	+6.3
NIC (ng/ml) in human plasma								
Nominal concentration	1.00	2.00	5.00	10.0	50.0	100	160	200
Calculated mean concentration	0.989	2.09	5.00	9.72	46.7	99.1	175	194
RSD (%)	0.9	2.2	4.7	1.5	6.2	6.8	2.3	2.6
RE (%)	−1.1	+4.3	0	−2.8	−6.6	−0.9	+9.4	−3.2
COT in human plasma								
Nominal concentration	10.0	20.0	50.0	100	500	1000	1600	2000
Calculated mean concentration	10.3	19.2	50.5	94.6	469	1003	1780	1967
RSD (%)	2.5	3.4	5.3	1.5	6.8	4.8	3.4	2.4
RE (%)	+2.5	−4.0	1.0	−5.4	−6.2	+0.3	+11.3	−1.7

Table 6
Accuracy and precision of quality control samples

	Inter-day ($n=18$)			Intra-day ($n=6$)		
ALB (ng/ml) in human serum						
Nominal concentration	0.150	0.750	7.50	0.150	0.750	7.50
Calculated mean concentration	0.145	0.740	7.58	0.132	0.781	8.03
RSD (%)	11	8.9	9.4	5.3	5.7	3.8
RE (%)	−3.3	−1.3	+1.0	−12	+4.1	+7.1
NIC (ng/ml) in human plasma						
Nominal concentration	3.00	30.0	150	3.00	30.0	150
Calculated mean concentration	3.10	30.2	143	3.06	30.5	143
RSD (%)	6.0	4.9	6.7	5.6	6.0	6.3
RE (%)	+3.2	+0.7	−4.4	+1.8	+1.8	−4.6
COT (ng/ml) in human plasma						
Nominal concentration	30.0	300	1500	30.0	300	1500
Calculated mean concentration	30.4	297	1391	30.6	307	1443
RSD (%)	8.1	8.0	8.1	6.3	8.2	10
RE (%)	+1.4	−0.9	−7.3	+2.0	+2.4	−3.8

Table 7
Stability of analytes in biological fluids and sample extracts

	ALB		NIC		COT	
	Time period	Percentage of nominal	Time period	Percentage of nominal	Time period	Percentage of nominal
Re-injection at room temperature	24 h	99–106	24 h	96–106	24 h	93–97
Bench-top at room temperature	24 h	100–105	24	103–105	24 h	104–107
Freeze–thaw	3 cycles	103–107	3 cycles	108–110	3 cycles	109–112
Sample storage (−20°C)	190 days	102–108	120 days	111–114	120 days	105–110

that LC–MS–MS with aqueous organic mobile phase on a silica column was compatible with the common sample-processing procedures such as protein precipitation, liquid–liquid extraction, and SPE. Column stability is shown in Table 8. No deterioration of the column performance was observed. No column washing was needed between the analytical runs. The excellent column stability is attributed to the use of polar mobile phase, which washes off polar endogenous compounds, thus eliminating their accumulation on the column.

Table 8
Stability of silica columns

	Injection No.	k'	Injection No.	k'
NIC	1	1.78	109	1.73
COT	1	1.16	109	1.14
ALB	1	6.70	114	7.20
BAM	1	5.53	114	5.83

3.4.6. Robustness test

In order to test the robustness of the method, validation curves were separately run on two LC–MS–MS instruments with two separate columns. The results for QCs injected on the two different silica columns are shown in Table 9. The RSD values and the mean results were analyzed by an F-test. In no instance was the F-test significant.

4. Conclusion

LC–MS–MS on silica columns with aqueous–organic mobile phase was found to be useful for the analysis of polar ionic compounds in biological

Table 9
Method robustness

	LC–MS–MS system A, column A			LC–MS–MS system B, column B		
ALB						
Nominal concentration (ng/ml)	0.150	0.750	7.50	0.150	0.750	7.50
Calculated concentration (ng/ml)	0.132	0.781	8.03	0.137	0.752	7.78
RSD (%)	5.3	5.7	3.8	4.5	3.2	3.2
RE (%)	−12	+4.1	+7.1	−8.9	+0.2	+3.7
NIC						
Nominal concentration (ng/ml)	3.00	30.0	150	3.00	30.0	150
Calculated concentration (ng/ml)	3.06	30.5	143	3.06	31.1	148
RSD (%)	5.6	6.0	6.3	4.3	7.3	5.5
RE (%)	+1.8	+1.8	−4.6	+1.9	+3.5	−1.3
COT						
Nominal concentration (ng/ml)	30.0	300	1500	30.0	300	1500
Calculated concentration (ng/ml)	30.6	307	1443	29.8	316	1485
RSD (%)	6.3	8.2	10	7.9	7.8	8.8
RE (%)	+2.0	+2.4	−3.8	−0.7	+5.4	−1.0

fluids. The sensitivity of polar ionic compounds was improved because of increased amount of organic solvent and enhanced analyte ionization in mobile phases. Better retention of the polar ionic analytes on silica columns also alleviated matrix effects, a phenomenon associated with reversed-phase LC–MS–MS analysis for polar ionic analytes. The silica column demonstrated excellent stability and resolution power. Quantitative LC–MS–MS methods of using silica column and aqueous–organic mobile phase for assaying albuterol, nicotine, and cotinine in biological fluids have been successful developed and validated according to the pharmaceutical industry guidelines [42].

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