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Review

# Bioanalytical liquid chromatography tandem mass spectrometry methods on underivatized silica columns with aqueous/organic mobile phases

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**Abstract**

This review article summarizes the recent progress on bioanalytical LC–MS/MS methods using underivatized silica columns and aqueous/organic mobile phases. Various types of polar analytes were extracted by using protein precipitation (PP), liquid/liquid extraction (LLE) or solid-phase extraction (SPE) and were then analyzed using LC–MS/MS on the silica columns. Use of silica columns and aqueous/organic mobile phases could significantly enhance LC–MS/MS method sensitivity, due to the high organic content in the mobile phase. Thanks to the very low backpressure generated from the silica column with low aqueous/high organic mobile phases, LC–MS/MS methods at high flow rates are feasible, resulting in significant timesaving. Because organic solvents have weaker eluting strength than water, direct injection of the organic solvent extracts from the reversed-phase solid-phase extraction onto the silica column was possible. Gradient elution on the silica columns using aqueous/organic mobile phases was also demonstrated. Contrary to what is commonly perceived, the silica column demonstrated superior column stability. This technology can be a valuable supplement to the reversed-phase LC–MS/MS.


*Keywords:* Reviews; Silica columns; Aqueous/organic mobile phases

**Contents**



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## 1. Introduction

Bioanalytical methods using silica columns and aqueous/organic mobile phases have appeared in the literature and have been used for the analysis of various types of polar compounds. To the best of this author's knowledge, a review article has not been written to summarize the applications. A timely review on this subject is deemed necessary to describe advances in this field. This review article only covers journal articles published in English. Many poster presentations on this subject at scientific meetings (such as AAPS and ASMS) over the last 5 years are not included in this review article since the posters may not have the same technical details as the journal articles.

Polar compounds are poorly retained on a reversed-phase column even with high aqueous mobile phases. For bioanalytical LC–MS/MS applications, especially under electrospray ionization (ESI) mode, poor analyte on-column retention may result in detrimental matrix effects due to the ion suppression to the analyte, from coeluting matrix components. Matrix effects have been identified as one of the major reasons why bioanalytical LC–MS/MS methods fail [1]. High aqueous content mobile phases are also not conducive to achieving the good spray conditions that are critical for sensitivity. Many drugs have basic functional groups, and acidic mobile phases are used and MS in the positive ion mode detects these drugs as the protonated ions. Ionization of polar analytes decreases the analyte on-column retention on a reversed-phase column.

To overcome this fundamental mismatch between reversed-phase LC and MS detection, scientists are looking into alternative approaches to achieve better sensitivity and better on-column retention. A desirable mobile phase would not only contain high organic content for better sensitivity but also have good on-column retention for polar ionic compounds. Hydrophilic interaction chromatography (HILIC) using bare silica or derivatized silica and low aqueous/high organic mobile phase has drawn the attention of LC–MS/MS practitioners. Although the name HILIC was coined by Alpert in 1990 on a derivatized silica column [2], this approach was first used by Jane in 1975 on an underivatized silica column [3]. HILIC is similar to normal phase LC in that the elution is promoted by the use of polar mobile phase. However, unlike classic normal phase LC where the water in the mobile phase has to be kept in minimal but constant (usually less than hundreds of ppm) levels, the presence of a significant amount of water (usually 5%) in the mobile phase for HILIC is crucial for maintaining a stagnant enriched water layer on the surface of the stationary phase into which analytes may selectively partition. HILIC also uses water-miscible polar organic solvents such as acetonitrile (ACN) or methanol instead of water-immiscible solvents like hexane and chloroform. Using HILIC, polar ionic analytes can be well retained on the column. A wide variety of stationary phases can be used for HILIC. Silica columns, which possess both HILIC and ion-exchange properties as will be discussed below, have long been used with aqueous/organic mobile phases [3–29]. The other frequently used HILIC columns include silica derivatized with amino [30–32], diol [33,34], amide [35–37], polysulfoethyl aspartamide and polyhydroxyethyl aspartamide [2], mixed-mode cation exchange [38,39], mixed-mode anion–cation exchange [40,41], pentafluorophenylpropyl [42], and even C18 [43]. LC–MS/MS using HILIC on derivatized silica has been used for the analysis of small polar analytes [35–37,40,42,43]. Underivatized silica columns with aqueous/organic mobile phases were found to be also an attractive alternative to reversed-phase columns because of the high organic nature of the mobile phases and better match between mobile phase and MS detection (basic compounds being eluted with acidic mobile phases).

## 2. Retention mechanism on silica columns with an aqueous/organic mobile phase

Underivatized silica surface contains functional groups like siloxanes, silanols, and minute quantity of surface and internal metals [28,29]. Silica has very weak reversed-phase characteristic due to the siloxane groups. Silanols can be present on the silica surface in single, germinal or vicinal forms and different silanol groups have different reactivity and different adsorption activity. Silica materials from different manufacturers may contain different amount of silanol groups and the ratio of the types present can also be different. Prior to the LC–MS era, chromatographic methods on silica columns using aqueous/organic mobile

phases have been used extensively for various types of compounds [3–29]. Complicated retention involved hydrophilic interaction [2], ion-exchange [15], and reversed-phase retention by the siloxane [22]. This multimodal retention is useful for achieving unique selectivity but is not always desirable. Depending on the relative strength of the various adsorption forces, multimodal retention can result in abnormal peak shape, especially when analyte is retained by more than a single dominant retention mechanism. Therefore, it is critical that all retention mechanisms are controlled during the analysis so that analytes primarily interact with stationary phase through one discrete mechanism. Increasing water content in the acidic mobile phase would initially increase the retention due to the reversed-phase retention on the silaxone groups. Further increasing the water content would decrease the retention because water is a stronger elution solvent on the silica column under HILIC mode, a primary retention mechanism for basic analytes with acidic mobile phases [2]. Fig. 1 shows the influence of acetonitrile concentration in the mobile phase on capacity factor ($k'$) of basic compounds. At neutral and slightly basic mobile phase pH, the retention of basic compounds is more complicated and seems to be dependent upon the compound due to the aforementioned multimodal retention [29,44,45]. For procainamide, benzylamine and nortriptyline, the respective retention time due to hydrophilic interaction between the compounds and the silanols remained constant between pH 2.7 and 4.5 [45]. The retention time doubled when pH was increased to 7.6 as the separation mechanism becomes predominantly ion-exchange in nature. Above pH 9.3 the retention decreases, as the bases become increasingly non-ionized. These results were similar to what had been previously reported [15]. Retention of diphenhydramine, on the other hand, remained constant between pH 2.7 and 11.5, probably due to the multimodal retention and lack of a discreet retention force. It should be noted that underivatized silica should not be used for long period with a basic mobile phase above pH 9.3 because of the loss of silica backbone, albeit such a loss is slower in a high organic mobile phase. For acidic compounds, 2-thiophenecarboxylic acid and 3-methyl-2-thiophenecarboxylic acid, retention was independent of the mobile phase pH and remained constant between pH 5 and 9 [46]. Since these two acidic compounds lack basic functional groups that could interact with silanol, ion-exchange may not play a dominant role in their retention. It is postulated that like basic compounds in acidic mobile phase, the hydrophilic interaction once again is the driving force for the on-column retention of these acidic compounds. Increasing the buffer concentration in the mobile phase (pH 7.6) decreased the retention for a group of basic compounds [45] but increased the retention for a group of acidic compounds [46]. As expected, increasing ionic strength in the mobile phase would weaken the ion-exchange, a dominant retention force at pH 7.6, leading to a decreased retention. The reason for the retention increase of acidic compounds upon increasing ionic strength is unclear and should be further investigated.

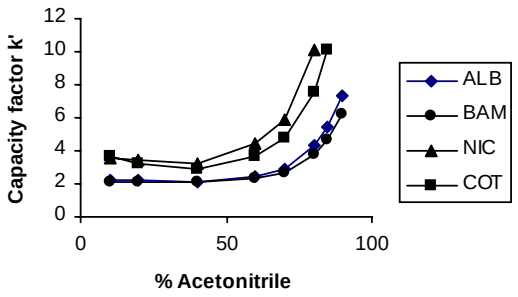


Fig. 1. Influence of acetonitrile concentration in mobile phase on $k'$ of basic compounds. Column, Inertsil silica, 50 mm × 2 mm, i.d. 5 μm; mobile phase, acetonitrile/water/formic acid ($x$:(100 − $x$):0.2 (v/v)), where $x$ varied from 10 to 90. ALB: albuterol; BAM: bamethan; NIC: nicotine; COT: cotinine. Reprinted from [44], with permission from Elsevier.

In summary, hydrophilic interaction between the compound and the silica stationary phase is the driving force for on-column retention of basic compounds in acidic mobile phase and acidic compounds in neutral mobile phase. Increasing water content in the mobile phase would decrease retention. Because of the low aqueous/high organic mobile phase and the favorable solution chemistry for electrospray ionization mode (acidic mobile phase for basic compounds and neutral mobile phase for acidic compounds) that ensures analyte is in the ionized form in solution, sensitive LC–MS/MS detection is therefore possible. Besides the HILIC LC–MS/MS on derivatized or underivatized silica columns, another very useful approach, albeit not used for bioanalytical LC–MS/MS method

Table 1
LC–MS/MS analysis of polar compounds in biological fluids

| Compound | Sample | Extraction | Stationary phase | Mobile phase/flow rate | MS | LLOQ (ng/ml) | Reference |
|---|---|---|---|---|---|---|---|
| Acyclovir | Rat plasma, amniotic fluid, placental tissue, and fetal tissue | PP with acetonitrile (ACN) | Spheri-5 silica, 5 μm, 100 mm × 4.6 mm | ACN/10 mM ammonium formate (80:20, pH 3 (v/v)), 0.7 ml/min | Micromass Quattro LC (+ESI), SRM | 50 | [48] |
| Albuterol | Human serum | Certify SPE (mixed-mode) | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (95:5:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.05 | [44] |
| Arginine, *N*,*N*-dimethyl arginine, *N*-monomethyl arginine, and *N*,*N*'-dimethyl arginine | Human plasma | PP with ACN | Spheri-5 silica, 5 μm, 100 mm × 4.6 mm | ACN/methanol/water (475:475:50 (v/v)), containing 0.1% formic acid and 10 mM ammonium acetate, 0.3 ml/min | Micromass Quattro II (+ESI), SRM | 1: Arginine, *N*,*N*-dimethyl arginine, and *N*,*N*'-dimethyl arginine; 2.5: *N*-monomethyl arginine | [49] |
| Choline and its metabolites | Mouse liver, rat liver, rat brain, cooked chicken, cooked beef, raw carrot, raw cabbage | LLE with chloroform | Solvent miser silica, 5 μm, 150 mm × 2.1 mm | Gradient elution: ACN/water/ethanol/1M ammonium acetate/acetic acid (800:127:68:3:2 to 500:500:85:27:18 (v/v)), 0.4 ml/min | ThermoQuest LCQ-DECA (+ESI), SIM | In ng/ml range | [50] |
| Clonidine | Human serum | LLE with ethyl ether | Betasil silica, 5 μm, 50 × 3 mm | ACN/water/formic acid (80:20:1 (v/v)), 0.7 ml/min | Sciex API 3000 (+ESI), SRM | 0.01 | [51] |
| Ethambutol | Human plasma, bronchoalveolar lavage fluid, alveolar cells | PP with ACN | Hypersil silica, 5 μm, 50 mm × 4.6 mm | ACN/water (8:2 (v/v)), containing 0.1% TFA and 4 mM ammonium acetate, 0.8 ml/min | Sciex API III+ (+ESI) and Micromass Quattro LC (+ESI), SRM | 50 for plasma and 5 for others | [52] |
| Ethionamide | Human plasma, bronchoalveolar lavage fluid, alveolar cells | PP with ACN | Hypersil silica, 5 μm, 50 × 4.6 mm | ACN/water (9:1,v/v), containing 0.06% TFA, 1 ml/min | Sciex API III+ (+ESI) and Micromass Quattro LC (+ESI), SRM | 50 for plasma and 5 for others | [53] |
| Fentanyl | Human plasma | 96-well certified SPE (mixed-mode) | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (92.5:7.5:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.05 | [54] |
| Fluconazole | Human plasma | 96-well LLE with methyl-*tetra* butyl ether (MTBE) | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (95:5:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.5 | [55] |
| Fluoxetine and norfluoxetine | Human plasma | LLE with MTBE | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (94:6:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.5 | [56] |
| Heroin, 6-monoacetylmorphine, morphine, morphine-3-glucuronide, morphine-6-glucuronide, and codeine | Mice serum | Ethyl SPE | Supelcosil silica, 5 μm, 250 mm × 2.1 mm | Methanol/ACN/water/formic acid (59.8:5.2:34.65:0.35 (v/v)), 0.23 ml/min | Sciex API I (+ESI), SIM | 0.5: heroin; 1: morphine-3-glucuronide; 4: 6-monoacetylmorphine, morphine, morphine-6-glucuronide, and codeine | [57] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hydrocodone and hydromorphone | Human plasma | 96-well certified SPE (mixed-mode) | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (92:8:0.01 (v/v)), 0.7 ml/min | Sciex API 3000 (+ESI), SRM | 0.1 | [58] |
| Hydromorphone | Human plasma | LLE with MTBE | Inertsil silica, 5 μm, 50 mm × 2 mm | ACN/water/formic acid (80:20:1 (v/v)), 0.2 ml/min | Sciex API III+ (+ESI), SRM | 0.05 | [59] |
| Levovirin | Rat and monkey plasma | PP with ACN | Inertsil silica, 5 μm, 50 mm × 3 mm | ACN/0.05% TFA (95:5 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 10 | [60] |
| Loratadine and descarboethoxy-loratadine | Human plasma | LLE with hexane | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (90:10:0.1 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.01: loratadine; 0.25: descarboethoxy-loratadine | [61] |
| Midazolam, 1′-OH midazolam and 4-OH midazolam | Monkey plasma | LLE with ethyl ether/hexane | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (95:5:0.05 (v/v)), 4.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.1 | [62] |
| Morphine, morphine-3-glucuronide and morphine-6-glucuronide | Human plasma | C18 SPE | Inertsil silica, 5 μm, 50 mm × 3 mm | ACN/water/formic acid (90:10:1 (v/v)), 1 ml/min | Sciex API 365 (+ESI), SRM | 0.5: morphine; 10: morphine-3-glucuronide; 1: morphine-6-glucuronide | [63] |
| Morphine, morphine-3-glucuronide and morphine-6-glucuronide | Human plasma | 96-well C18 SPE | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (91:9:0.01 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.5: morphine; 10: morphine-3-glucuronide; 1: morphine-6-glucuronide | [64] |
| Nicotine and cotinine | Human plasma | LLE with ethyl ether | Inertsil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (90:10:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 1: nicotine; 10: cotinine | [44] |
| Omeprazole, metoprolol, and fexofenadine | Human plasma | 96-well Oasis HLB SPE | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/formic acid (72.5:27.5:1 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 5 | [65] |
| Paroxetine | Human plasma | LLE with MTBE | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (94:6:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.05 | [66] |
| Pseudoephedrine | Human plasma and human urine | 96-well C18 SPE | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/formic acid (95:5:1 (v/v)), 0.6 ml/min | Sciex API 3000 (+ESI), SRM | 5 | [65] |
| Ribavirin | Rat and monkey plasma | PP with ACN | Inertsil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (95:5:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 10 | [67] |
| Ribavirin | Human plasma and serum | 96-well PP with ACN | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (95:5:0.05 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI) and Sciex API III+, SRM | 10 | [68] |
| Ritonavir and naltrexone | Human plasma | LLE with MTBE | Betasil silica, 5 μm, 50 mm × 3 mm | Gradient elution: ACN/water/formic acid (90:10:1 to 50:50:1 (v/v)), 0.5 ml/min | Sciex API 3000 (+ESI), SRM | 0.05: ritonavir; 0.2: naltrexone | [69] |
| Sildenafil and desmethylsildenafil | Human plasma | 96-well certified SPE (mixed-mode) | Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (94:6:0.05 (v/v)), 0.4 ml/min | Sciex API 3000 (+ESI), SRM | 1 | [70] |

yet, is to explore the ion-exchange property of the silica stationary phase by using low aqueous/high organic mobile phase at a neutral or slightly basic mobile phase where both the basic analyte and the acidic silanols are expected to be in their respective ionized forms.

## 3. Bioanalytical LC–MS/MS analysis of polar compounds on silica columns

As shown in Table 1, LC–MS/MS methods on silica columns with aqueous/organic mobile phases have been used to analyze compounds of various chemical structures, extracted from biological fluids and tissues using protein precipitation (PP), liquid/liquid extraction (LLE) and solid-phase extraction (SPE). Most of the methods used a selective reaction monitoring (SRM) detection instead of single ion monitoring (SIM) mode. In the SRM mode, ions of a selected mass (precursor ions) are transmitted by the first mass analyzer (Q1) into the collision cell (Q2) where they collide with the neutral atoms of an inert gas and fragment into product ions. The product ions are transmitted through a second mass analyzer (Q3). Usually, for a bioanalytical method the lowest concentration in the linearity range is defined as the limit of quantitation because these concentrations were adequate to define the elimination profile of the analytes for the intended pharmacokinetic studies. They may not be the lowest concentrations that can be quantified reliably. The mobile phases usually contained a high percentage of organic solvent, mostly acetonitrile, and a low percentage of an acidic aqueous solution containing an acid (formic acid or trifluoroacetic acid, TFA) and/or a buffer (ammonium acetate or ammonium formate). Even though TFA has been reported to suppress electrospray signals due to its ion-pair activity in the gas phase with the analyte ions [47], the gain in sensitivity by going to higher organic content is so large that the mobile phase containing small amounts of TFA (usually $<0.05\%$) still gives the best signal-to-noise ratio (and peak shape) for the applications on the silica columns. For the tested analytes such as sildenafil, desmethylsildenafil, fluconazole, nicotine, cotinine, midazolam and isoniazid, the author has found that the ionization suppression due to TFA can be avoided through the addition of 1% acetic acid in the mobile phase [71]. Chromatographic peak shape and retention were preserved under these conditions. Methods using silica columns and acidic aqueous/organic mobile phases demonstrated good chromatographic resolution power. Polar analytes can be easily retained and resolved on the silica column with an isocratic elution for arginine and three methylated arginines [49] and a gradient elution for choline and its five metabolites [50].

## 4. Comparison of sensitivity between silica and C18 LC–MS/MS methods

Because of the higher organic content in the mobile phase and the more favorable solution chemistry for ionization (acidic mobile phase for basic compounds and neutral mobile phase for acidic compounds), sensitivity improvement was observed for every compound, albeit the improvement is compound dependent. The comparison results are summarized in Table 2. The comparison study was always performed on the same instrument on the same day by the same chemist. Since the background noise was approximately the same for methods using silica and C18 columns, the absolute MS signals were compared to obtain the values for sensitivity improvement. To avoid the potential matrix suppression from biological samples, analytes in neat solutions were used for the comparison. From Table 2, it is obvious that low organic content mobile phases were used to retain polar analytes on the C18 columns. On the silica column, high organic content mobile phases were used, resulting in higher sensitivity as well as higher retention. Figs. 2 and 3 show the chromatograms of fluconazole on a silica column and on a C18 column, respectively, with mobile phase of different composition. On the silica column, increasing acetonitrile concentration in the mobile phase not only increased fluconazole on-column retention, but also enhanced its signal intensity due to the more favorable spraying condition. On the C18 column, with the increase of acetonitrile the retention for fluconazole decreases, as predicted by the reversed-phase mechanism. When similar on-column retention was achieved for fluconazole (90% acetonitrile on silica column and 21.5% acetonitrile on the C18 column), the signal intensity on the silica column is about 10-fold higher than that on the C18 column.

Table 2
Comparison of LC–MS/MS sensitivity improvement using silica stationary phase over reversed-phase stationary phase

| LC–MS/MS on silica columns | | LC–MS/MS on reversed-phase columns | | Sensitivity improvement | Retention time (min) | | Reference |
|---|---|---|---|---|---|---|---|
| Stationary phase | Mobile phase | Stationary phase | Mobile phase | | On silica | On C18 | |
| Hypersil silica, 5 μm, 50 mm × 2 mm | ACN/water/formic acid (70:30:0.2 (v/v)) 0.5 ml/min | Hypersil BDS C18, 5 μm, 50 mm × 2 mm | ACN/water/formic acid (10:90:0.2 (v/v)) 0.5 ml/min | Albuterol: 5.3 | 1.7 | 0.7 | [44] |
| | | | | Cotinine: 8.0 | 2.3 | 0.4 | |
| | | | | Bamethan: 6.3 | 1.6 | 1.4 | |
| | | | | Nicotine: 7.0 | 2.7 | 0.4 | |
| Hypersil silica, 5 μm, 50 mm × 2 mm | ACN/water (92.7:7.5 (v/v)), containing 5 mM ammonium acetate 0.5 ml/min | Hypersil BDS C18, 5 μm, 50 mm × 2 mm | ACN/water (5:95 (v/v)), containing 5 mM ammonium acetate, 0.5 ml/min | 3-MCA: 19 | 0.7 | 0.8 | [44] |
| | | | | 2-TCA: 14 | 0.8 | 0.4 | |
| | | | | 2-TAA: 20 | 1.0 | 0.5 | |
| Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (92.5:7.5:0.05 (v/v)) 0.5 ml/min | Hypersil BDS C18, 5 μm, 50 mm × 3 mm | ACN/water/TFA (37.5:62.5:0.05 (v/v)) 0.5 ml/min | Fentanyl: 7.0 | 1.7 | 1.6 | [54] |
| Betasil silica, 5 μm, 50 mm × 3 mm | Gradient ACN/water/formic acid (90:10:1 (v/v)) for 1 min then (90:10:1 to 50:50:1 (v/v)) in 1 min; 0.5 ml/min | Inertsil ODS3 C18, 5 μm, 50 mm × 3 mm | Gradient ACN/water/formic acid (10:90:1 to 70:30:1 (v/v)) 0.5 ml/min | Albuterol: 1.4 | 2.3 | 0.8 | [69] |
| | | | | Clonidine: 2.3 | 2.2 | 1.2 | |
| | | | | Fentanyl: 2.0 | 2.1 | 1.9 | |
| | | | | Naltrexone: 2.0 | 2.2 | 1.3 | |
| | | | | Loratadine: 2.9 | 2.1 | 2.1 | |
| | | | | Ritonavir: 120 | 0.9 | 2.9 | |
| Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (90:10:0.05 (v/v)) 0.5 ml/min | Inertsil ODS3 C18, 5 μm, 50 mm × 3 mm | ACN/water/TFA (21.5:78.5:0.05 (v/v)) 0.5 ml/min | Fluconazole:10 | 1.4 | 1.4 | [55] |
| Betasil silica, 5 μm, 50 mm × 3 mm | ACN/water/TFA (96:4:0.05 (v/v)) 0.25 ml/min | Inertsil ODS3 C18, 5 μm, 50 mm × 3 mm | ACN/water/formic acid (50:50:0.1 (v/v)) 0.25 ml/min | Paroxetine: 2.7 | 2.6 | 2.0 | [66] |

3-MCA: 3-methyl-2-thiophenecarboxylic acid; 2-TCA: 2-thiopheneacetic acid; 2-TAA: 2-thiophenecarboxylic acid.

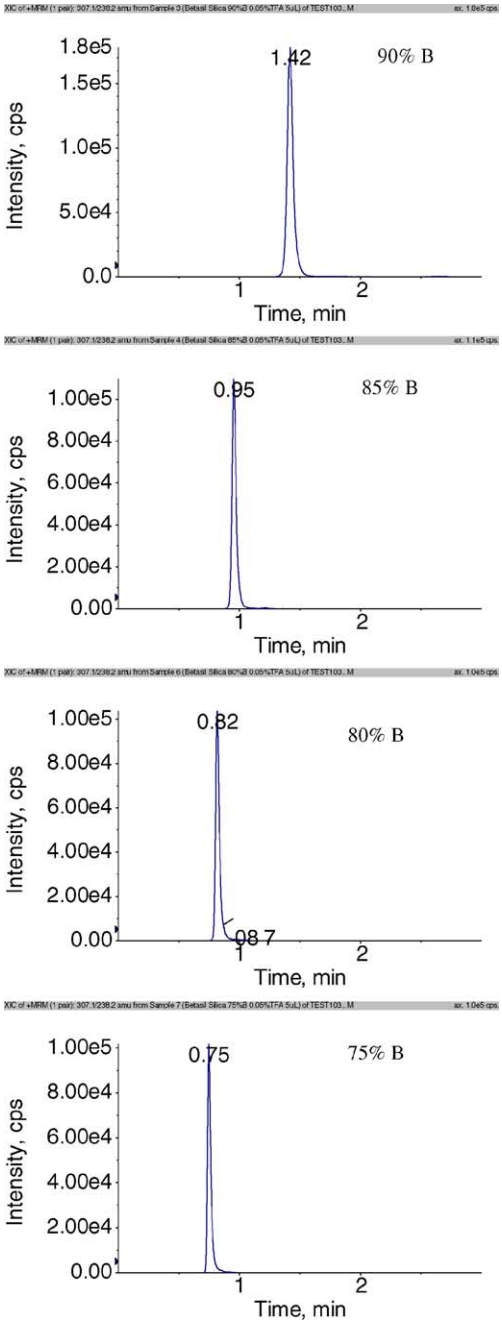


Fig. 2. Chromatograms of fluconazole on a silica column with aqueous/organic mobile phases. Column, Betasil silica 50 mm × 3 mm, i.d. 5 μm; mobile phase A, water/TFA (100:0.05 (v/v)); mobile phase B, acetonitrile/TFA (100:0.05 (v/v)); flow rate, 0.5 ml/min; injection volume, 5 μl of 100 ng/ml of fluconazole. Reprinted from [55], with permission from Elsevier.

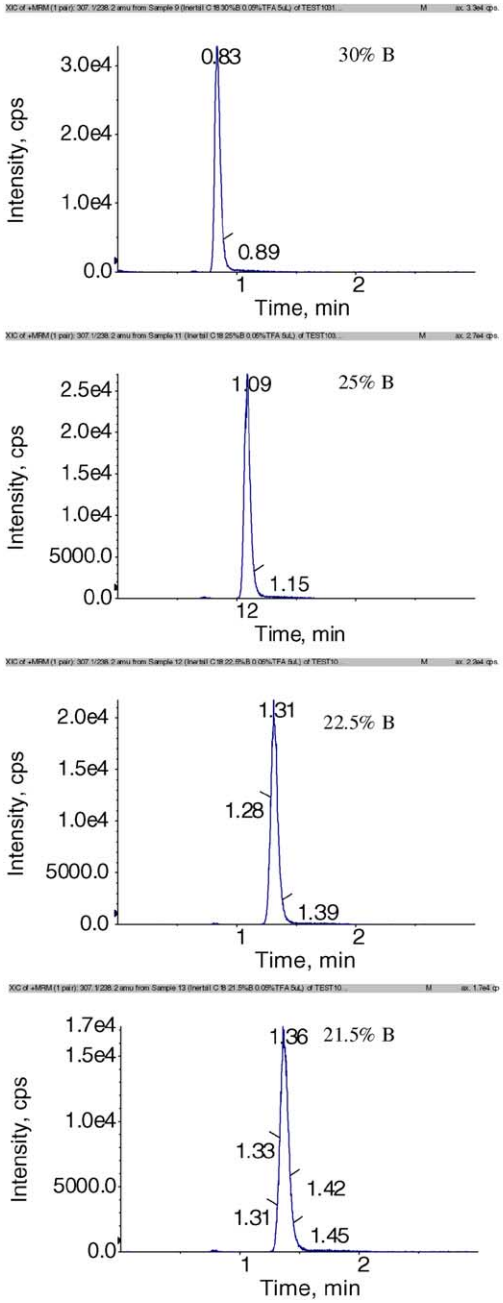


Fig. 3. Chromatograms of fluconazole on a C18 column with aqueous/organic mobile phases. Column, Inertsil ODS-3 C18 50 mm × 3 mm, i.d. 5 μm; mobile phase A, water/TFA (100:0.05 (v/v)); mobile phase B, acetonitrile/TFA (100:0.05 (v/v)); flow rate, 0.5 ml/min; injection volume, 5 μl of 100 ng/ml of fluconazole. Reprinted from [55], with permission from Elsevier.

## 5. Direct injection of reversed-phase SPE extracts onto silica column

While the commonly used SPE elution solvents (i.e. ACN) have stronger elution strength than a mobile phase on reversed-phase chromatography, they are weaker elution solvents on the silica column with an aqueous/organic mobile phase and therefore can be injected directly. Thus, the tedious manual steps of evaporation and reconstitution are eliminated, resulting in saving 50% of total time for a 96-well SPE extraction [65]. Pseudoephedrine was extracted from human plasma and urine using 96-well C18 SPE with 1% formic acid in acetonitrile as the elution solvent [65]. This elution solvent is weaker than the mobile phase, i.e. 1% formic acid in acetonitrile/water (95:5), and therefore was injected directly injected onto the silica column. Fig. 4 shows chromatograms of blank urine and pseudoephedrine extracted from urine. As already mentioned by van der Merwe et al. [72], loss of pseudoephedrine during the evaporation step was also observed in our laboratory. Direct injection of the eluents onto the LC–MS/MS system solved this problem. A similar approach has also been taken to analyze isoniazid in plasma using 96-well Oasis HLB SPE [73]. The organic eluent (acetonitrile) was injected directly onto the silica column. Otherwise, isoniazid would be almost entirely lost during the evaporation step. Ruterbories and Negahban presented an elegant approach of using HILIC LC–MS/MS bioanalysis of basic analytes to eliminate evaporation and reconstitution steps for 96-well liquid/liquid extraction [74]. The low aqueous/high organic mobile phase allows water-immiscible solvents to be injected directly without detrimental effects on chromatography.

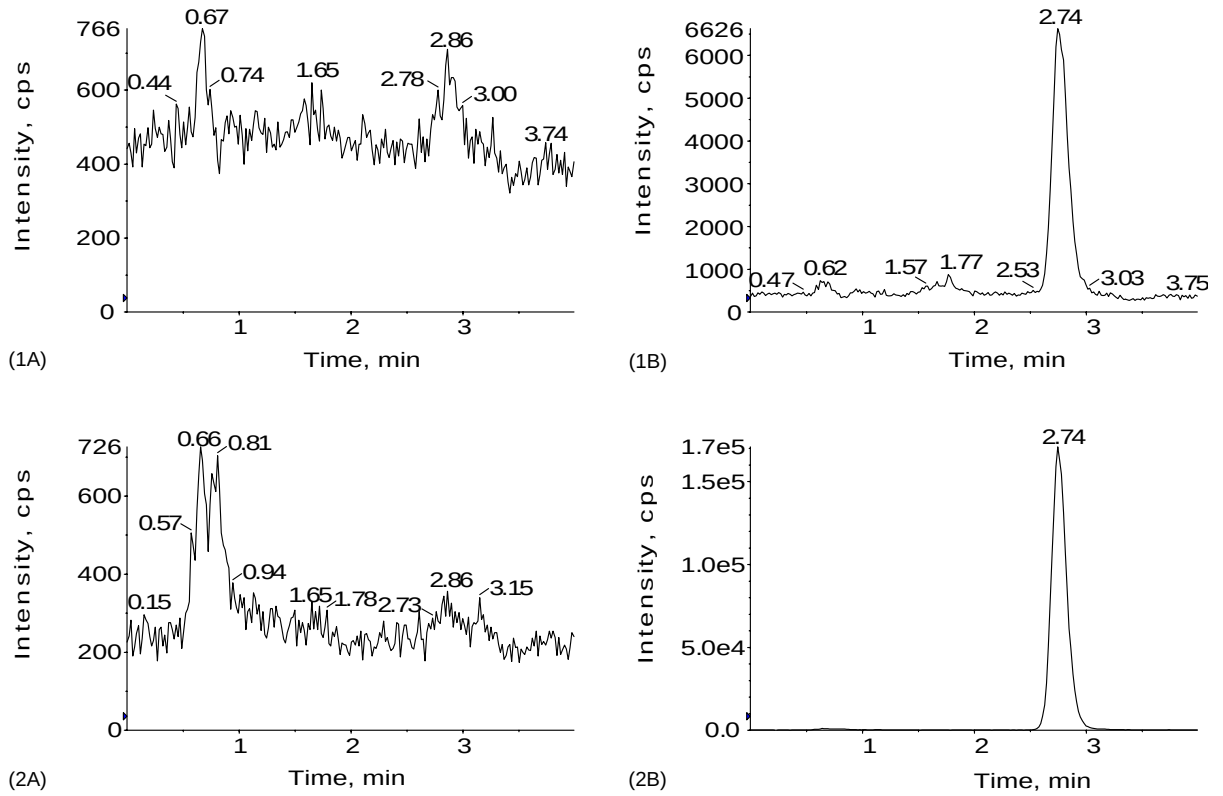


Fig. 4. Chromatograms of pseudoephedrine and internal standard from (A) an extracted blank urine and (B) LLOQ at 5 ng/ml. 1A: pseudoephedrine in blank urine (0 ng/ml); 2A: internal standard (pseudoephedrine-d3) in blank urine (0 ng/ml); 1B: pseudoephedrine in urine (5 ng/ml); 2B: internal standard in urine (150 ng/ml). Reprinted from [65], with permission from Wiley, New York.

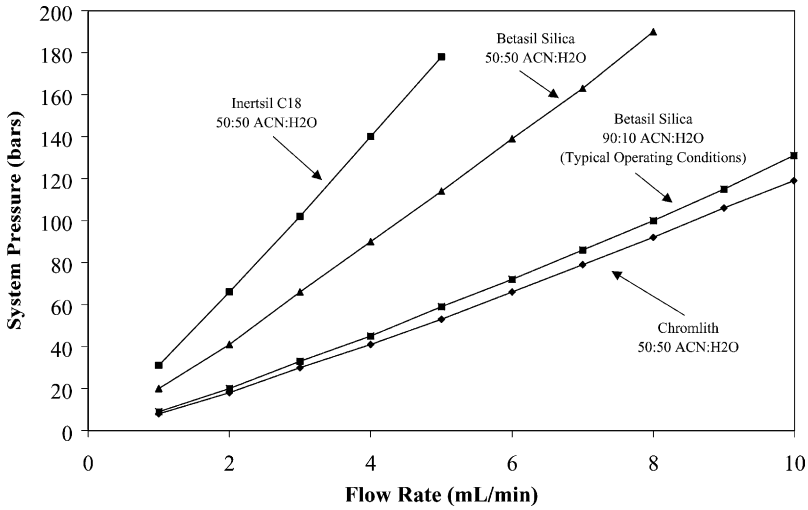


Fig. 5. Backpressure comparison between a Betasil silica column, an Inertsil ODS II C18 column and a monolithic column (Chromolith SpeedROD RP-18e). All columns were new 50 mm × 4.6 mm, i.d. 5 μm (except for the Chromolith C18 SpeedROD RP-18e). Reprinted from [62], with permission from Wiley, New York.

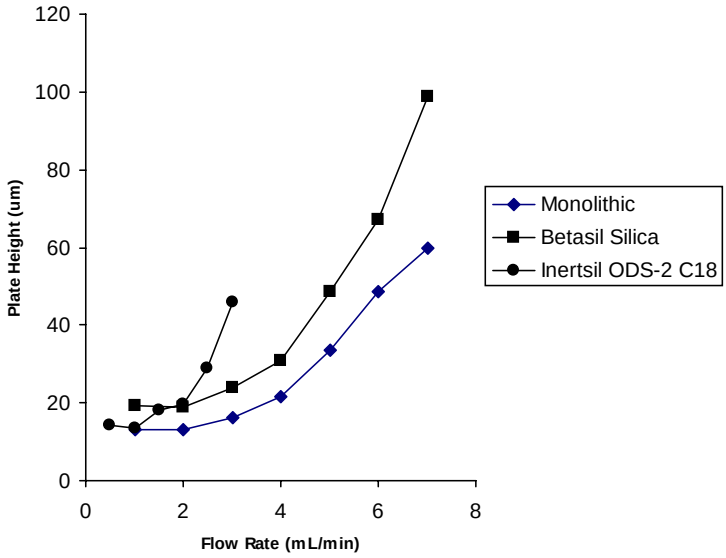


Fig. 6. Influence of column type on the optimal flow rate for column efficiency. All columns used were 50 mm × 4.6 mm, i.d. 5 μm (except for the Chromolith SpeedROD RP-18e). The compound used was fluconazole. The mobile phase used was 0.1% formic acid as A and 0.1% formic acid in acetonitrile as B. For the Inertsil and Chromolith SpeedROD RP-18e, the mobile phase was 25% B and the injection solvent was 100% water. For the Betasil silica, the mobile phase was 85% B and the injection solvent was 100% acetonitrile. The capacity factor ($k'$) was approximately 2 for fluconazole all columns. The plate number ($N$) was calculated by $N = 5.545(t_r/w_{1/2})^2$ where $t_r$ is the retention and $w_{1/2}$ is the peak width at the half peak height.

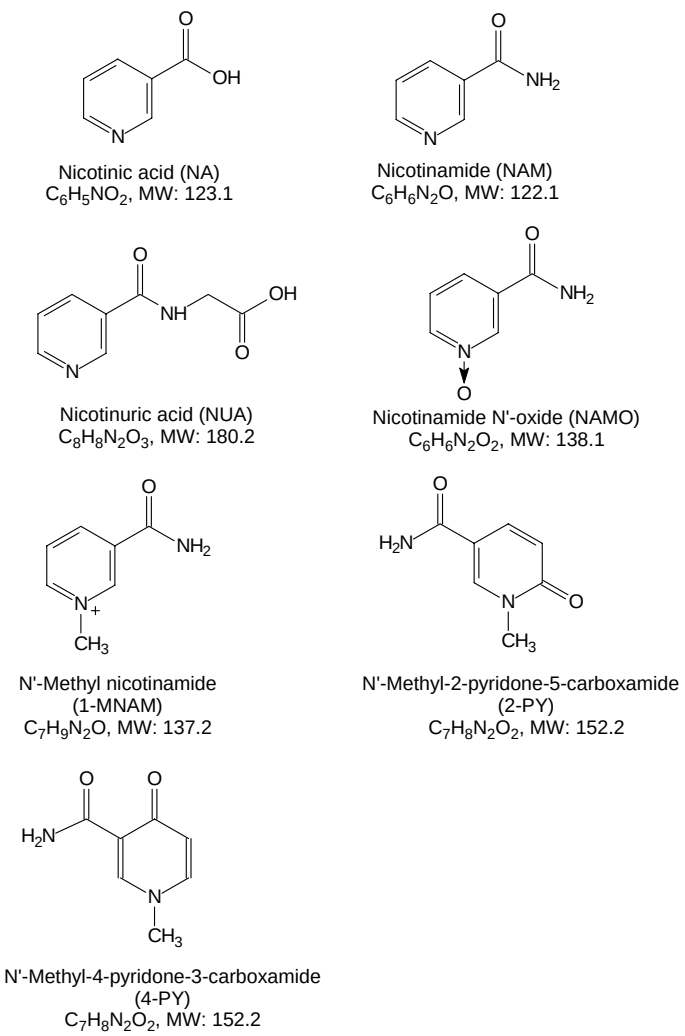


Fig. 7. Chemical structures of nicotinic acid and its six metabolites.

## 6. Ultrafast LC–MS/MS on silica column

Because of low backpressure generated with low aqueous/high organic mobile phases, silica columns can be operated at flow rates comparable with those used in ultrafast LC–MS/MS bioanalysis utilization of monolithic column [64]. This is illustrated in Fig. 5. It is obvious from this figure that while operated at a mobile phase composition of 50:50 acetonitrile/water, the silica column has significantly lower backpressure than the Inertsil ODS II C18 column. Furthermore, silica columns are typically operated at very high organic mobile phase and, under such conditions, the backpressures on packed silica columns (operated at 90:10 acetonitrile/water) are actually very similar to those of monolithic columns operated at 50:50 acetonitrile/water. Monolithic columns allow the use of high flow rates without significantly sacrificing column efficiency. For silica columns in the particulate form, efficiency might be slightly sacrificed at high flow rates and this loss of efficiency is not as significant as on a reversed-phase column

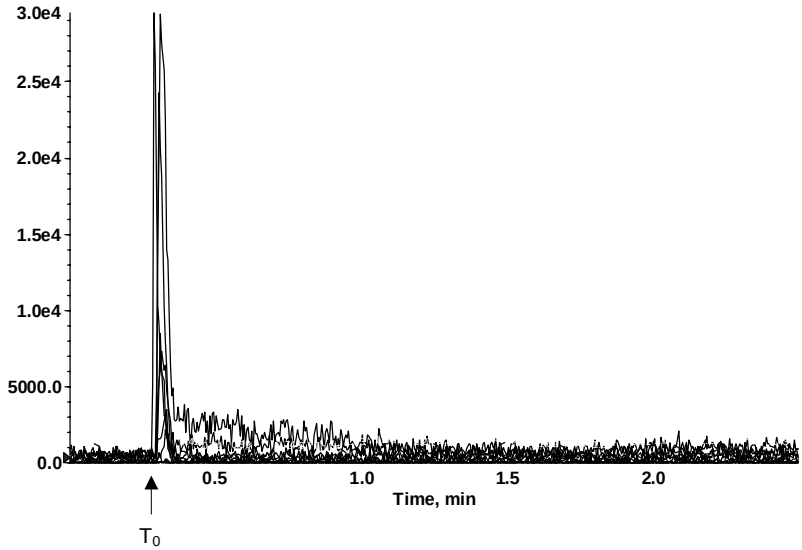


Fig. 8. A representative reconstituted extracted ion chromatogram (XIC) mass chromatogram of nicotinic acid and metabolites on C18 column. Column, Chromolith SpeedROD RP-18e 50 mm × 4.6 mm, i.d.; mobile phase, acetonitrile/water/formic acid (5:95:1 (v/v)); flow rate, 2.5 ml/min (split about 1–4 prior to the API 3000 TurboIonspray source); injection volume, 5 μl.

(Fig. 6). This is probably due to the contribution of viscosity of the diffusion and mass transfer factors, as already demonstrated by Gray [75]. Baseline resolutions between morphine, morphine-6-glucuronide (M6G), and morphine-3-glucuronide (M3G) in human plasma extracts were achieved within 30 s on a 50 mm × 3 mm Betasil silica column operated at 4 ml/min using isocratic elution [64]. Recently, we also applied this technique to analyze nicotinic acid and metabolites in plasma and urine. Nicotinic acid and its six metabolites are extremely polar in nature (see Fig. 7 for chemical structures) and do not retain on reversed-phase columns at all. Fig. 8 shows a representative reconstituted extracted ion chromatogram (XIC) mass chromatogram of nicotinic acid and metabolites obtained on a monolithic C18 column using a flow rate of 2.5 ml/min. Even with a very low organic mobile phase of acetonitrile/water/formic acid (5:95:1 (v/v)), all of the compounds elute near the solvent front. Using a Waters Atlantis HILIC Silica$^{TM}$ column and a gradient elution at 2 ml/min, nicotinic acid and metabolites are well retained on the column (Fig. 9). The gradient elution starts with a mobile phase of higher organic content (95% acetonitrile) and gradually changes to a mobile phase of lower organic content (50% acetonitrile). The re-equilibration time (0.2 min) on the silica column is very similar to that on a monolithic C18 column. The earliest eluting peak has a $k'$ of approximately 2. 2-PY and 4-PY are structurally similar and share common SRM transitions, therefore they have to be chromatographically separated. To avoid isotopic contribution from NAM to NA, NAM also needs to be chromatographically resolved from NA. 1-MNAM is a quaternary amine and the Waters Atlantis HILIC Silica$^{TM}$ shows satisfactory peak shape for this very polar compound.

## 7. Column stability of the silica column

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 that LC–MS/MS with aqueous/organic mobile phase on a silica column was compatible with the common sample-extraction procedures such as protein precipitation, liquid/liquid extraction, and SPE. Good ribavirin peak shape and baseline separations from

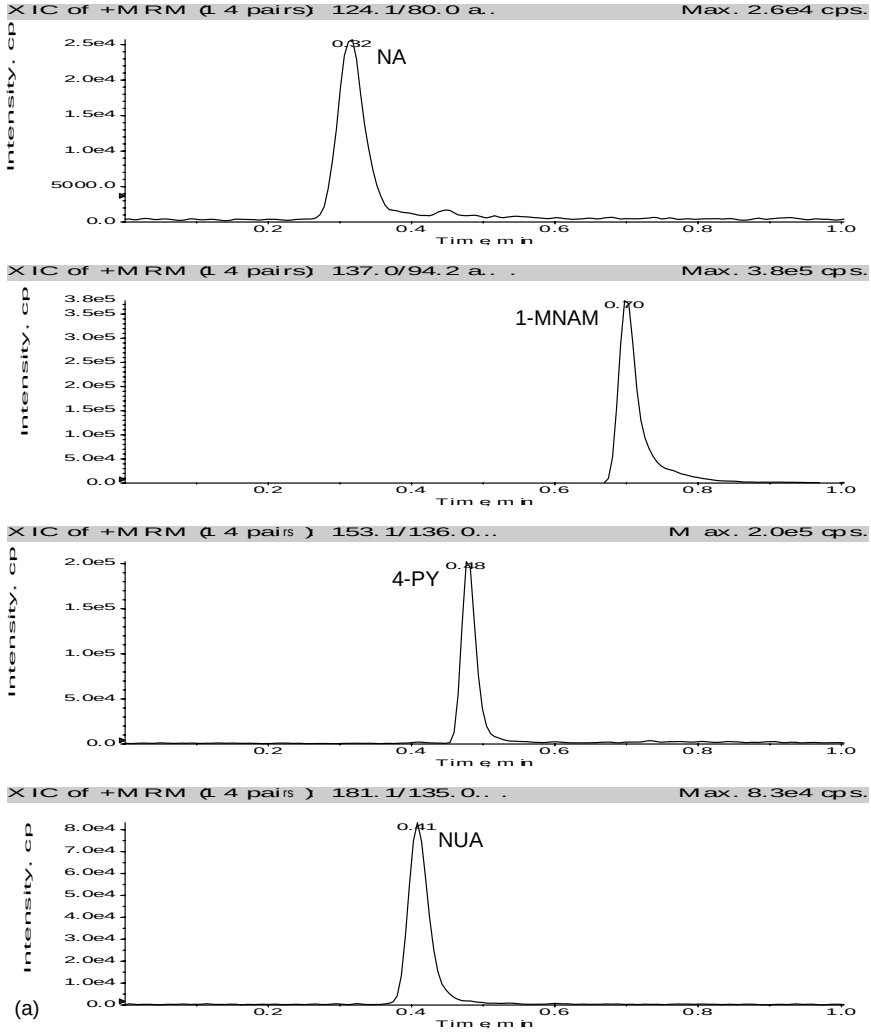


Fig. 9. Chromatograms of nicotinic acid and its six metabolites on silica column. Column, Waters Atlantis HILIC Silica$^{TM}$ 50 mm × 3 mm, i.d. 5 μm; mobile phase, acetonitrile/water/formic acid (95:5:0.2 to 65:35:0.2 (v/v)) in 0.25 min and then to (50:50:0.2 (v/v)) in 0.65 min; flow rate, 2.0 ml/min (split about 1–4 prior to the API 3000 TurboIonspray source); injection volume, 20 μl.

an endogenous interference peak were all well maintained for at least 350 injections of extracted samples, using protein precipitation [69]. The same is true for morphine and metabolites in human plasma for which SPE clean up is used [64]. The retention times and chromatographic separation remained stable throughout the analysis, as shown in Fig. 10. The peak shape of M6G and M3G was slightly worse in injection #376 than that in injection #4. However, this did not affect the peak integration and quantitation. Furthermore, the backpressure remained constant at 122 bar throughout the analysis of biological samples. No column washing was needed between the analytical runs. The good column stability is attributed to the use of a polar mobile phase, which washes off polar endogenous compounds, thus eliminating their accumulation on the column. This hypothesis has been substantiated by a post-column infusion study of assessing the ionization suppression for acyclovir and ganciclovir after a simple protein precipitation

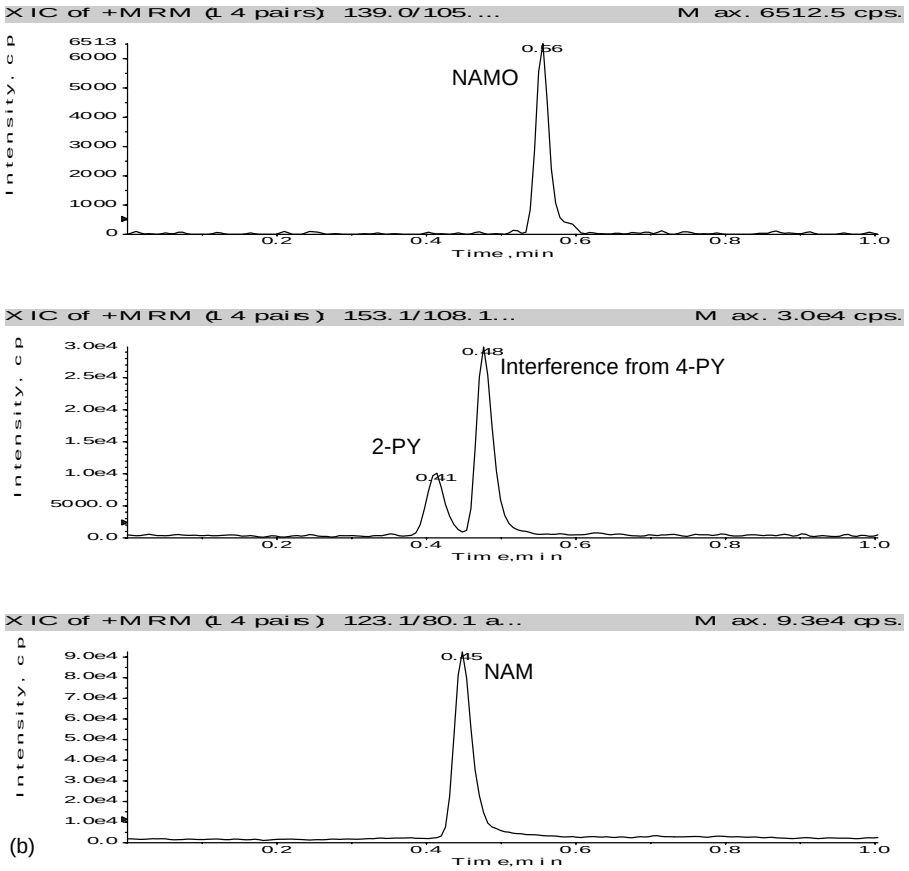


Fig. 9. (*Continued*).

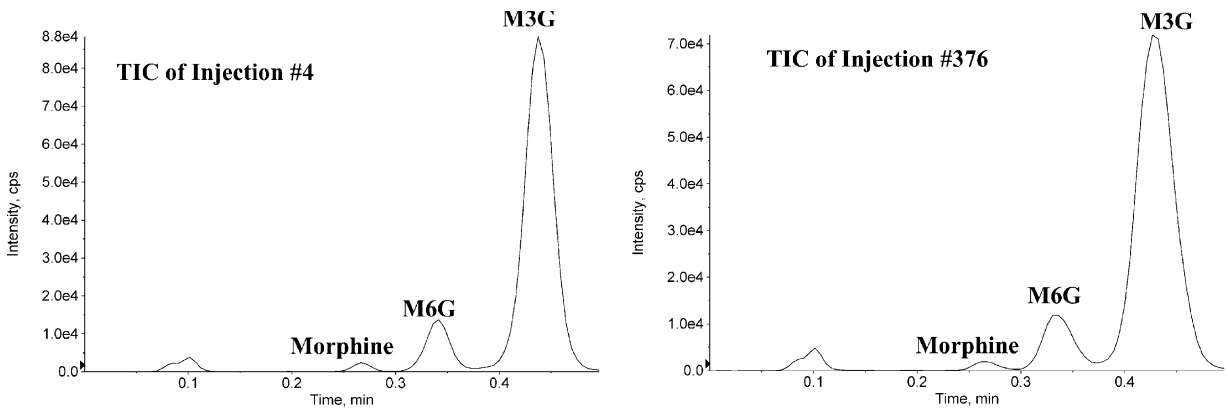


Fig. 10. Total ion chromatograms of injection #4 and #376, both of a morphine/M6G/M3G calibration standard (5:10:100 ng/ml), during the continuous analysis of 384 extracted samples in human plasma. Reprinted from [62], with permission from Wiley, New York.

[48]. Almost all of the ionization suppression regions were very close to the solvent front and were well resolved from the analytes of interest. The silica column stability was also demonstrated for liquid/liquid extraction [55]. Even with a mobile phase at pH 7.8, stability of 636 injections on a silica column was demonstrated for a six basic analyte mixture [45]. No column deterioration was observed.

## 8. Conclusion and future perspective

Bioanalytical LC–MS/MS methods on silica columns with low aqueous/high organic mobile phases have been successfully applied for the analysis of various types of polar compounds. Because of the high organic content in the mobile phase, significant sensitivity improvement over LC–MS/MS on a conventional reversed-phase column can be achieved. Due to the very low backpressure, a high flow rate LC–MS/MS on the silica column is possible. This technique is also compatible with commonly used sample extraction methods including protein precipitation, liquid/liquid extraction, and solid-phase extraction. The silica columns demonstrated excellent stability operated under these conditions.

Recently at Pittsburgh Conference 2003, Waters (http://www.waters.com) and Microsolv (www.microsolvtech.com) introduced their underivatized HILIC silica stationary phases under the trade names Atlantis HILIC Silica$^{TM}$ and Cogent Type-C Silica$^{TM}$, respectively. According to these column manufacturers, excellent peak shape, retention and resolution power may be achieved on these two new stationary phases. Monolithic silica columns, recently available commercially, would allow the use of high flow rates without significantly sacrificing column efficiency.

Much remains to be done. More bioanalytical applications should be explored, particularly on the Atlantis HILIC Silica$^{TM}$ and Cogent Type-C Silica$^{TM}$ columns. The retention mechanism on silica columns operated under aqueous/organic mobile phase conditions needs to be further elucidated. Characterization of silica columns from different manufacturers by chromatographic tests using aqueous/organic mobile phases would further increase our understanding on the silica chemistry and would further assist scientists to select the appropriate silica columns for their intended applications.

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