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Simple means to alleviate sensitivity loss by trifluoroacetic acid (TFA) mobile phases in the hydrophilic interaction chromatography–electrospray tandem mass spectrometric (HILIC–ESI/MS/MS) bioanalysis of basic compounds

Wilson Z. Shou *, Weng Naidong

Covance Laboratories Inc., 3301 Kinsman Boulevard, Madison, WI 53704, USA

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Abstract

Trifluoroacetic acid (TFA) is a commonly used additive in HPLC and LC–MS analysis of basic compounds. It is also routinely added to aqueous–organic mobile phases utilized in the hydrophilic interaction chromatography–electrospray tandem mass spectrometry (HILIC–ESI/MS/MS) technique used in our laboratories for bioanalysis. However, TFA is known to suppress the ESI signals of analytes due to its ability to form gas-phase ion pairs with positively-charged analyte ions. The most common method to overcome this problem involves the post-column addition of a mixture of propionic acid and isopropanol. However the post-column addition setup requires additional pumps and is not desirable for continuous analysis of large amounts of samples. In this paper we present a simple yet very effective means of minimizing the negative effect of TFA in bioanalysis by direct addition of 0.5% acetic acid or 1% propionic acid to mobile phases containing either 0.025 or 0.05% TFA. A factor of two- to five-fold signal enhancement was achieved for eight basic compounds studied. Furthermore, chromatography integrity was maintained even with the addition of acetic acid and propionic acid to existing TFA mobile phases. This method has been successfully applied to the HILIC–ESI/MS/MS high-throughput analysis of extracted biological samples to support pre-clinical and clinical studies.

Keywords: Trifluoroacetic acid; TFA; Electrospray; Liquid chromatography–mass spectrometry; LC–MS; LC–MS/MS; Hydrophilic interaction chromatography; HILIC; Silica column

1. Introduction

Liquid chromatography–mass spectrometry (LC–MS), equipped with atmospheric pressure ionization (API) sources, has become the method of choice for both quantitative and qualitative analyses of compounds in biological matrices [1,2]. While API interfaces such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI) have matured into robust techniques over the last decade, there still exist limitations inherent to the ion generation and transmission processes within these interfaces. For instance, only volatile mobile phase additives commonly used in conventional HPLC can be used with electrospray ionization. Trifluoroacetic acid (TFA) has traditionally been used in the HPLC analyses of basic compounds extensively [3]. It not only controls the pH of the mobile phases, but also acts as an ion-pair agent to improve peak shapes of basic compounds on silica-based columns. TFA is volatile and therefore can be used in LC–MS, and it has been used in the LC–MS analyses of peptides [4] as well as small molecules [5]. Our research group has also been using bare silica column operated under hydrophilic interaction chromatography (HILIC) mode

* Corresponding author. Tel.: +1 608 242 2712x7247; fax: +1 608 242 2735.
E-mail address: wilson.shou@covance.com (W.Z. Shou).

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with TFA-containing mobile phases for the LC–MS analyses of small molecules in biological matrices [6–11]. The major drawback of using TFA in LC–MS, however, is that TFA is known to suppress the ESI signals of analytes and reduce assay sensitivity [12,13]. This is primarily due to the ability of TFA to form gas-phase ion pairs with positively-charged analyte ions [14].

The most common method to overcome this problem involves the post-column addition of a mixture of propionic acid and isopropanol [14]. This was referred to as "TFA-Fix". The main mechanism of reducing TFA-related suppression is that the large excess of weak acid protonates TFA anions back to neutral TFA molecules, thus freeing up the analyte ions from being paired with TFA anions. However, the post-column addition setup requires additional pumps and is not desirable for the continuous analysis of large amounts of samples. In this paper, we present a simple yet very effective means of minimizing the negative effect of TFA in bioanalysis by direct addition of acetic acid (AA) or propionic acid (PA) to the TFA-containing mobile phase. Model compound sildenafil, desmethyl sildenafil, fluconazole, isoniazid, ethionamide, pyrazinamide, nicotine and cotinine were used to demonstrate the applicability of the simple technique. Comparison between TFA-only and TFA–AA or –PA mobile phases were conducted in terms of model analytes' sensitivity and chromatography. The comparison was performed on silica columns operated under HILIC mode, as well as on reversed-phase columns. Finally, real world examples from extracted biological samples will also be presented.

2. Experimental

2.1. Chemicals and reagents

Sildenafil (purity 99.9%) and desmethyl sildenafil (purity 97.6%) were purchased from Custom Synthesis Services (Madison, WI, USA). Fluconazole (purity 100%) was kindly supplied by Pfizer (Sandwich, UK). Isoniazid (purity 99%), ethionamide (purity 100%), pyrazinamide (purity 100%), nicotine (purity 99%) and cotinine (purity 98%) were purchased from Sigma (St. Louis, MO, USA).

Trifluoroacetic acid (TFA, 97%), formic acid (FA, >88%), glacial acetic acid (AA, >99.7%) and propionic acid (PA, >99.5%) were also purchase from Sigma. HPLC grade water and acetonitrile were from Fisher (St. Louis, MO, USA).

Mobile phases were prepared on a "volume-to-volume" basis. For instance, a mobile phase of acetonitrile–water–TFA–acetic acid (92:8:0.025:1, v/v/v/v) was prepared by combining 920 mL of ACN, 80 mL of water, 0.25 mL of TFA and 10 mL of acetic acid. Also, a mobile phase of 0.05% TFA in water (v/v) was prepared by combining 0.5 mL of TFA with 1 L of water. All mobile phases were sonicated for 5 min prior to use. No filtration or pH adjustment was made in either the aqueous or organic mobile phases.

2.2. Sample preparation

The sample preparation procedures for sildenafil and desmethyl sildenafil in human plasma and ultrafiltrate were similar to those described in a previous paper [15]. Briefly, a 0.350 mL volume of plasma or ultrafiltrate sample was loaded onto a 25 mg Certify^{TM} mixed mode solid phase extraction cartridge (Varian Sample Preparations, Walnut Creek, CA, USA) previously conditioned by 0.5 mL of methanol and 0.5 mL of 5% acetic acid in water. The cartridge was washed with 0.5 mL of 5% acetic acid in water and then 0.5 mL of methanol. The elution was carried out by two portions of 0.35 mL of 2% ammonium hydroxide in acetonitrile. The eluent was dried under a stream of nitrogen at room temperature and the residue was reconstituted in 0.2 mL of 0.05% TFA in acetonitrile.

2.3. LC–MS/MS method

The LC system used was a Shimadzu (Shimadzu, Columbia, MD, USA) series 10AD VP equipped with binary pumps, a degasser and an SIL-HT autosampler. Either a Betasil silica column (50 mm × 3 mm, 5 μm) from Keystone Scientific (Bellefonte, PA, USA), or a SpeedROD C18 column (50 mm × 4.6 mm) from Merck KgaA (Darmstadt, Germany) was used. The flow rate used was 0.5 mL/min for the silica column and 1 mL/min for the monolithic column. When the flow rate was 1 mL/min, the flow splitter on the LC–MS/MS system was used to ensure that approximately 0.5 mL/min went into the source. Separation was performed at ambient temperature.

All experiments were conducted on an API 3000 triple quadrupole mass spectrometer (Applied Biosystem, Foster City, CA, USA) equipped with its TurboIonSpray^{TM} interface operated under positive mode. The TurboIonSpray needle was maintained at 3.0 kV. The turbo gas temperature was 450 °C and the auxiliary gas flow was 8.0 L/min. Nebulizing gas, curtain gas and collision gas flows were at instrument settings of 10, 10 and 12, respectively. The mass spectrometer was operated under selected reaction monitoring (SRM) mode with a dwell time of 50 ms for each pair. The SRM transitions and their corresponding ion path parameters are shown in Table 1. These parameters could

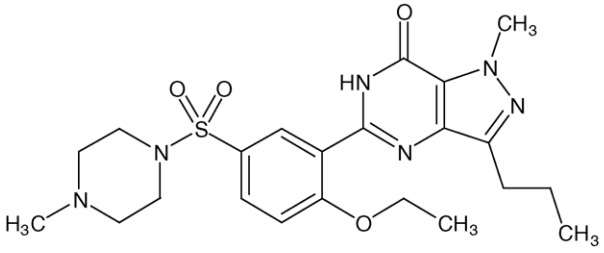
Table 1
SRM transitions and ion optics parameters for the test compounds

	SRM transition	Declustering potential (V)	Collision energy (eV)
Sildenafil	475 → 283	40	53
Desmethyl sildenafil	461 → 283	40	51
Fluconazole	307 → 238	36	23
Isoniazid	138 → 121	32	22
Ethionamide	167 → 107	28	38
Pyrazinamide	124 → 79	28	24
Nicotine	163 → 130	30	20
Continine	177 → 80	45	30

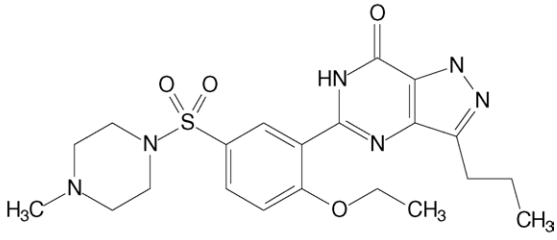
change slightly when a different mass spectrometer was optimized. Both resolving quadrupoles were maintained at unit resolution (0.7 mass unit at half height). Optimizations of the mass spectrometric conditions were carried out by infusing 100 ng/mL solutions of the analytes dissolved in 1:1 mixture of acetonitrile and water at 10 μL/min using a Harvard '22' syringe pump (Harvard Apparatus, South Natick, MA, USA). A Windows NT™ (Microsoft, Redmond, WA, USA) workstation running Analyst™ (version 1.1) was used for data acquisition and processing.

3. Results and discussion

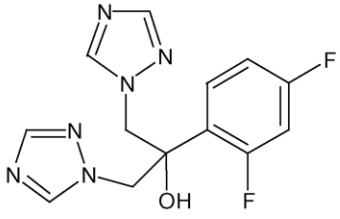
In our laboratory we routinely use silica columns operated under HILIC conditions for the analysis of polar,



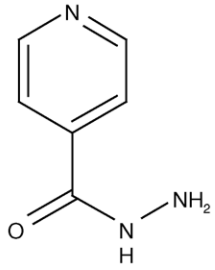
Sildenafil



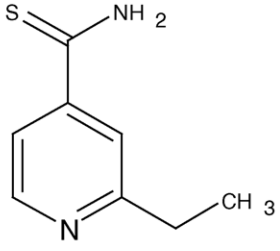
Desmethyl Sildenafil



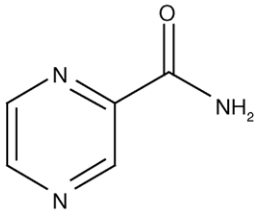
Fluconazole



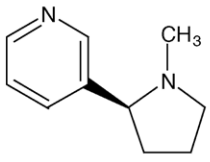
Isoniazid



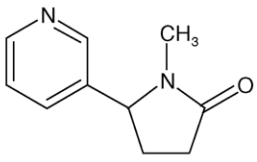
Ethionamide



Pyrazinamide



Nicotine



Cotinine

Fig. 1. Test compound structures.

basic compounds. TFA has been used extensively in the HILIC–MS/MS methods at concentrations between 0.01 and 0.05%, mostly due to its ability to obtain excellent peak shapes even from extracted biological samples. However, the reduction in sensitivity caused by TFA oftentimes precluded its use in assays that require high sensitivity. Therefore, we have been trying to investigate methods to minimize the suppression effect of TFA in the HILIC–MS/MS bioanalysis. The so-called "TFA-Fix" approach originally developed by Kuhlmann et al. involved the infusion of propionic acid and isopropanol post-column and before the ion source [14]. PA disrupted the ion pair between TFA and analytes by preferentially pairing with TFA, thus alleviating the suppression. Furthermore, this technique effectively de-coupled the electrospray ionization process from the liquid phase separation process. Therefore, chromatographic performance was not compromised. The disadvantages of this method, however, included the dilution of column effluent (which resulted in reduced peak concentration), as well as the setup requirement of an additional infusion pump. The latter requirement made this approach especially difficult to be used in the continuous, unattended bioanalysis of a large amount of samples (as in the case of our laboratory), mostly due to the volume limitations of conventional infusion pumps.

In light of these limitations, we have attempted to simplify the "TFA-Fix" approach by moving away from the post-column infusion setup. Instead, acetic acid or propionic acid was directly added to the TFA-containing mobile phases. In this work, neat solutions containing eight basic compounds were used to demonstrate the applicability of the approach. The structures of these compounds are shown

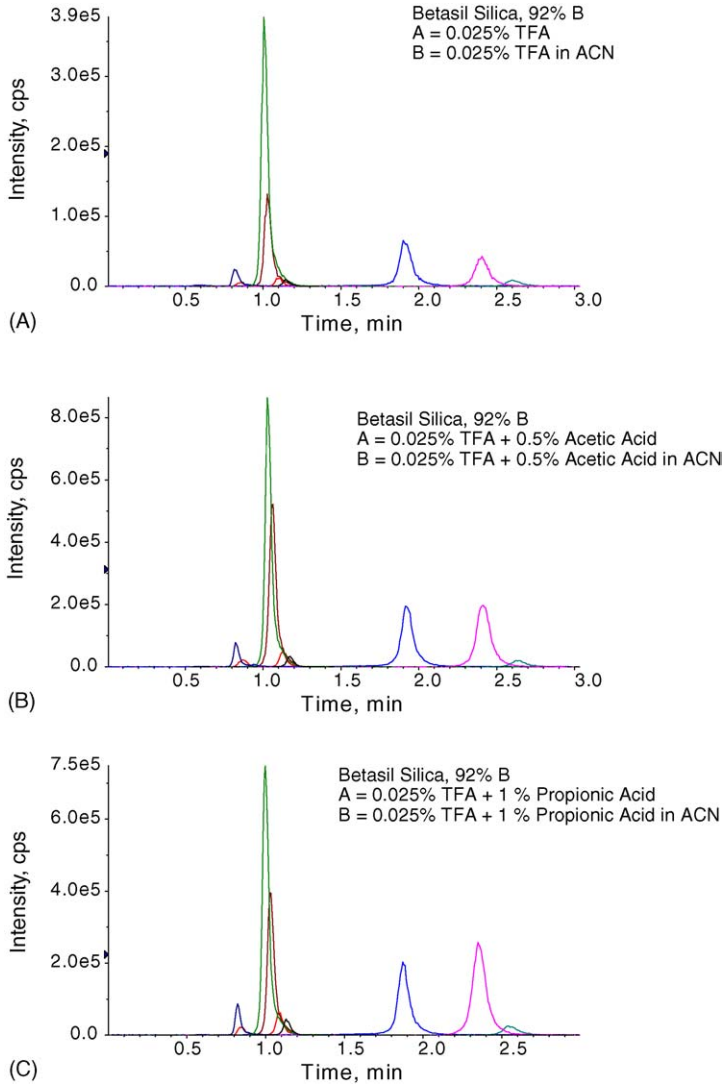


Fig. 2. Extracted mass chromatograms of test compounds on a Betasil silica column with different mobile phase additives: (A) 0.025% TFA; (B) 0.025% TFA + 0.5% acetic acid; and (C) 0.025% TFA + 1% propionic acid. The isocratic mobile phase used was 92:8 acetonitrile:water with additives (TFA, acetic acid, etc.) in both aqueous and organic portions of the mobile phase. Elution order (from earliest to latest): pyrazinamide (dark blue trace), ethionamide (green trace), fluconazole (brown trace), desmethyl sildenafil (red trace), sildenafil (black trace), isoniazid (blue trace), cotinine (pink trace) and nicotine (teal trace).

Table 2
Summary of analyte peak areas obtained (expressed as the percentage of the original analyte peak areas when TFA-only mobile phase was used) when weak acids were added to 0.025% TFA mobile phase

	0.025% TFA only	TFA + 0.1% AA	TFA + 0.5% AA	TFA + 1% AA	TFA + 0.1% FA	TFA + 0.5% FA	TFA + 1% FA	TFA + 0.1% PA	TFA + 0.5% PA	TFA + 1% PA
Sildenafil	100	233	343	230	96	68	64	145	131	420
Desmethyl sildenafil	100	255	381	267	81	76	72	153	263	493
Fluconazole	100	165	215	169	88	51	49	112	224	293
Isoniazid	100	210	248	125	102	77	76	119	183	264
Ethionamide	100	310	489	316	86	79	92	217	575	585
Pyrazinamide	100	210	299	177	71	62	64	139	251	298
Nicotine	100	250	375	249	104	94	82	153	206	285
Continine	100	149	211	118	77	59	48	73	146	185

TFA: trifluoroacetic acid; AA: acetic acid; FA: formic acid; PA: propionic acid.

Table 3
Summary of analyte peak areas obtained (expressed as the percentage of the original analyte peak areas when TFA-only mobile phase was used) when weak acids were added to 0.05% TFA mobile phase

	0.05% TFA only	TFA + 0.1% AA	TFA + 0.5% AA	TFA + 1% AA	TFA + 0.1% PA	TFA + 0.5% PA	TFA + 1% PA
Sildenafil	100	179	248	314	250	400	398
Desmethyl sildenafil	100	189	258	321	266	409	409
Fluconazole	100	238	329	383	307	350	321
Isoniazid	100	271	364	410	370	463	454
Ethionamide	100	187	253	283	270	311	300
Pyrazinamide	100	202	205	187	230	244	238
Nicotine	100	167	257	247	219	325	351
Continine	100	274	410	486	402	621	620

TFA: trifluoroacetic acid; AA: acetic acid; PA: propionic acid.

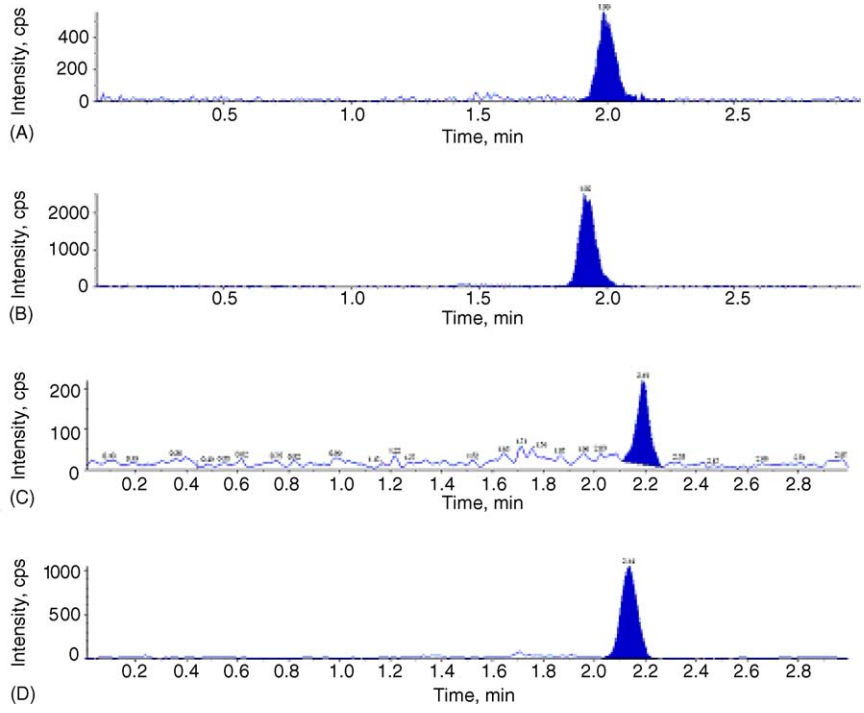


Fig. 3. Mass chromatograms from an extracted LLOQ calibration standard (A) sildenafil (1 ng/mL) in human plasma; (B) desmethyl sildenafil (1 ng/mL) in human plasma; (C) sildenafil (0.2 ng/mL) in human ultrafiltrate; and (D) desmethyl sildenafil (0.2 ng/mL) in human ultrafiltrate. Column: Keystone Betasil silica, 5 μm, 50 mm × 3.0 mm. Mobile phase: acetonitrile–water–TFA–acetic acid (92:8:0.025:1, v/v/v/v). Flow rate: 0.3 mL/min.

in Fig. 1. They were quite different in terms of functional groups and polarity. All experiments were conducted under positive electrospray ionization mode because it gave the best sensitivity for basic compounds. Fig. 2A shows the extracted mass chromatograms of a 100 ng/mL mixture of the compounds, obtained on a Betasil silica column with a TFA mobile phase (92% B isocratic where A was 0.025% TFA in water and B was 0.025% TFA in ACN). Mobile phases containing individual volatile acids, including acetic acid, formic acid and propionic acid at concentrations of 0.1, 0.5 and 1%, along with 0.025% TFA were made and the sample mixture was re-injected. Table 2 summarizes the results by normalizing the peak areas of the compounds obtained by TFA–AA, TFA–FA or TFA–PA mobile phases against those obtained from TFA-only mobile phase. First of all, it is evident that the addition of formic acid, regardless of its concentration, did not alleviate the suppression exerted by TFA mobile phase. In fact, as high as a 51% further reduction in peak areas was observed for fluconazole with a 0.025% TFA + 1% FA mobile phase, compared with a 0.025% TFA-only mobile phase. Similar trends were also observed from the other seven compounds. Similar results were obtained by Kuhlmann et al. when formic acid was added post-column [14]. On the other hand, the addition of AA or PA to TFA mobile phase improved the sensitivity significantly. The biggest improvement occurred when 0.5% AA or 1% PA was added to 0.025% TFA in the mobile phase. With the addition of 0.5% AA, peak area increase ranged from 111% for cotinine to 389% for ethionamide. With the addition of 1% PA, peak area improvement ranged from 90% for ethionamide to 490% for cotinine. Fig. 2B and C show the mass chromatograms obtained with 0.025% TFA + 0.5% AA and 0.025% TFA + 1% PA, respectively. From the comparison of Fig. 2B and C to A, it is evident that while assay sensitivity was significantly improved over TFA-only mobile phase, the peak shape, retention order and resolution were all maintained. Therefore, although our approach did not involve the de-coupling of the ionization process from the separation step, chromatographic performance was not altered or compromised. It was postulated that the reason for this was because AA and PA are both weak acids, therefore the addition of them did not change significantly the pH or ionic strength of the TFA mobile phase.

Similar results were obtained when TFA concentration in the mobile phase was increased from 0.025 to 0.05%, as shown in Table 3. The addition of 0.5% AA or 1% PA generally gave the biggest increase in peak area counts. The increases in peak areas ranged from 105 to 310% for 0.5% AA, and ranged from 138 to 520% for 1% PA. It is also interesting to note that in this case the addition of 1% AA also gave comparable results, with peak area increases ranged from 87 to 386%. Chromatographic performance remained unchanged (data not shown).

This approach has been successfully implemented in the validations and sample analyses of several HILIC–ESI/MS/MS methods in our laboratories. These assays included (1) sildenafil and desmethyl sildenafil in human plasma and plasma ultrafiltrate; (2) fluconazole in human plasma; (3) isoniazid in dog, monkey and ferret plasma; and (4) nicotine and cotinine in human plasma. Fig. 3 shows the mass chromatogram of extracted human plasma and ultrafiltrate samples that contained sildenafil and desmethyl sildenafil, acquired with a 0.025% TFA + 1% AA mobile phase on a Betasil silica column. The lower limit of quantitation (LLOQ) for the ultrafiltrate method was 0.2 ng/mL, five-fold lower than what has been previously reported [15].

While many basic compounds can be analyzed with HILIC–MS/MS, others are better analyzed with reversed-phase columns. Therefore, we also investigated the

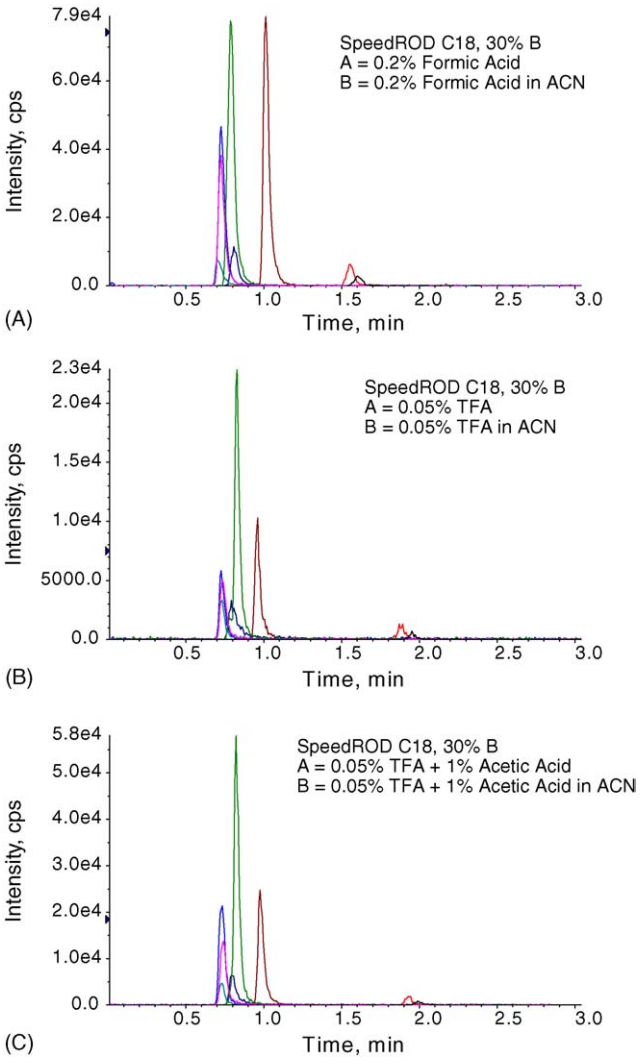


Fig. 4. Extracted mass chromatograms of test compounds on a SpeedROD column with different mobile phase additives: (A) 0.2% formic acid; (B) 0.05% TFA; and (C) 0.05% TFA + 1% acetic acid. The isocratic mobile phase used was 70:30 water:acetonitrile with additives (TFA, acetic acid, etc.) in both aqueous and organic portions of the mobile phase. Elution order (from earliest to latest): isoniazid (blue trace), cotinine (pink trace), nicotine (teal trace), ethionamide (green trace), pyrazinamide (dark blue trace), fluconazole (brown trace), desmethyl sildenafil (red trace) and sildenafil (black trace).

applicability of this approach on a reversed-phase column. Fig. 4A shows extracted mass chromatograms of a mixture of these compounds obtained on a monolithic C18 column (SpeedROD C18) with a mobile phase of 0.2% formic acid in water and 0.2% formic acid in acetonitrile (ACN). Fig. 4B shows the chromatograms when 0.05% TFA mobile phases were used instead of 0.2% formic acid, demonstrating the suppression by TFA. When 1% acetic acid was added to the mobile phase and the sample re-injected, as shown in Fig. 4C, the suppression effect by TFA was definitely alleviated, albeit to a lessor degree compared to results obtained by HILIC–MS/MS. The reason for this was attributed to the higher water content (70%) in the reversed-phase experiment compared to the HILIC experiment (8%). It was reported that one possible reason for the TFA suppression effect was actually the high conductivity and high surface tension of TFA-containing mobile phases [14]. Therefore, the high aqueous content of mobile phase in this case was thought to be the culprit. Nevertheless, sensitivity loss by TFA in the reversed-phase mobile phase was still alleviated, and the chromatographic separation was not disrupted, as shown by Fig. 4C.

4. Conclusions

The addition of 0.5% acetic acid or 1% propionic acid to mobile phases containing either 0.025 or 0.05% TFA enhanced the HILIC–ESI/MS/MS signal strength by a factor of two to five for the basic compounds studied. Sensitivity improvement was observed for not only HILIC–MS/MS, which operated under a mobile phase of ∼90% organic solvent, as well as RPLC–MS/MS, which operated at a lower organic (25–30%) mobile phase. Chromatographic resolution and selectivity were not disrupted by the addition of acetic acid and propionic acid to existing TFA mobile phases, as demonstrated by the same elution order and similar retention times. This method has been applied to the HILIC–ESI/MS/MS high-throughput analysis of extracted biological samples from pre-clinical and clinical studies.

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