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Evaluation of volatile ion-pair reagents for the liquid chromatography–mass spectrometry analysis of polar compounds and its application to the determination of methadone in human plasma

Songmei Gao [a], Siddhartha Bhoopathy [b], Zong-Ping Zhang [a,b,**], D. Scott Wright [b], Rand Jenkins [b], H. Thomas Karnes [a,*]

[a] *Department of Pharmaceutics, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980533, Richmond, VA 23298-0533, USA*
[b] *PPD Inc., Richmond, VA 23220, USA*

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Abstract

A liquid chromatography method using volatile ion-pairing reagents and tandem mass spectrometry was developed to obviate observed matrix effect for ionizable polar compounds. The present study investigated the addition of volatile ion-pair reagents to the reconstitution solution instead of the mobile phase to enhance the efficiency of chromatographic separation and minimize the sensitivity loss due to the formation of ion-pairs. The volatile ion-pair reagents used were perfluorinated carboxylic acids with *n*-alkyl chains: heptafluorobutanoic acid (HFBA), nonafluoropentanoic acid (NFPA), tridecafluoroheptanoic acid (TDFHA) and pentadecafluorooctanoic acid (PDFOA). The model analytes evaluated were *N*-methylnicotinamide (MNA) chloride, *N*-methyl 2-pyridone 5-carboxamide (2PY) and phenylephrine. The effects of alkyl chain length and the concentrations of the ion-pair reagents on the retention of analytes were studied, as well as the effect of pH on the retention of phenylephrine. The volatile ion-pair reagents in the reconstitution solution showed significant effect on the retention of the ionizable polar compounds, and the sensitivity of detection was improved for plasma samples through decreasing the matrix effect. This methodology was successfully applied to establish a quantitative assay for the polar drug substance methadone in human plasma with a concentration range from 0.1 to 50 ng/mL. Ion-pair reagents not only shifted the retention time but also reduced the carry-over peak for methadone.

Keywords: LC–MS; Polar compounds; Sensitivity enhancement; Volatile ion-pairing reagents

1. Introduction

Small organic molecules are commonly separated by reverse-phase liquid chromatography (RP-LC). However, conventional reverse-phase liquid chromatography often lacks the ability to adequately retain small polar molecules. The drawbacks of inadequate retention when using high performance liquid chromatography coupled with mass spectrometry (HPLC/MS) are significant: not only can polar interference peaks co-elute with the analyte; but unresolved endogenous species from the sample matrix can lead to ion suppression and unreliable quantitation [1]. In order to increase the retention of polar molecules, the compounds may require time-consuming derivatization procedures or the use of hydrophilic interaction chromatography (HILIC). HILIC, which employs polar stationary phases (such as silica, cyano, diol or amino column) and aqueous-organic mobile phases; although HILIC is frequently used in bioanalytical applications recently [2], poor retention and peak shape sometimes can be observed. Another alternative approach

* Corresponding author. Tel.: +1 804 828 3819; fax: +1 804 828 8359.
** Corresponding author.
E-mail address: tom.karnes@vcu.edu (H.T. Karnes).

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to effectively increase the retention of ionizable polar compounds is ion-pair chromatography. Traditional ion-pair chromatography uses either alkyl sulfonates for increased retention of protonated bases and other cations, or tetra alkyl ammonium salts for the increased retention of ionized acids and other anions [3–6]. However, these ion-paring reagents are not volatile and are therefore not compatible with mass spectrometry (MS). Extensive use of HPLC/MS for a wide variety of pharmaceutical separations suggests the importance of using volatile ion-pairing reagents for the separation of small ionizable polar molecules. Triethylamine, dibutylamine, tetrabutylammonium acetate and other agents [7–9] have been used as ion-pairing reagents for native or chemically modified oligonucleotides and other anions, such as sodium borocaptate, in LC/MS in the negative ion-detection mode. Perfluorinated carboxylic acids are ion-pairing reagents for cations and have been applied to the analysis of underivatized small peptides [10–12]. Due to the formation of ion pairs between the analyte and ion-pairing reagent, ionization efficiency can be impaired and poor detection sensitivity might be observed. A well-known instance is that severe ion suppression is noticed when using trifluoroacetic acid (TFA) to improve the separation of amino acids and some small peptides. Post-column infusion of isopropanol and propionic acid has been used to address this problem, which is referred to as the 'TFA Fix' [13]. Ion suppression was also observed for other alkyl chain perfluorinated carboxylic acids [14]. Kwon and Moini used atmospheric chemical ionization MS, which relies on gas-phase reactions rather than solution ionization, to overcome this problem when using NFPA as a mobile-phase additive [15]. Keever et al. employed a post-column infusion of propionic acid to minimize the electrospray signal suppression of HFBA for the quantitative determination of ceftiofur in milk [16].

Most of the applications involving volatile ion-pairing reagents with LC/MS have been limited to biomolecules such as oligonucleotides and amino acids. Applications to polar pharmaceuticals have not been extensively investigated, although it is necessary to develop approaches to achieve appropriate retention that allows sensitive quantification in biological matrices. In the present study, various perfluorinated carboxylic acids, HFBA, NFPA, TDFHA and PDFOA, were evaluated as ion-pairing reagents. *N*-Methylnicotinamide (MNA) chloride, *N*-methyl 2-pyridone 5-carboxamide (2PY) and phenylephrine (Fig. 1) were selected as the model analytes and analyzed in biometric. MNA, a metabolite of niacin [17], is a quaternary ammonium that is ionized in the mobile phase at pH 1–13; by contrast, its methabolite 2PY is a weak acid that is not ionized in the normal pH range. It is necessary to detect MNA and 2PY simultaneously to understand the pharmacokinetic profile of niacin. Phenylephrine, which is used for the temporary relief of sinus congestion caused by allergies or colds, is an amphoteric compound with dissociation constants of 8.9 (–OH) and 10.1 (–NH). In acidic conditions, the secondary amino group can be ionized. When using the typical RP separation, the retention times of these polar compounds were less than 1.2 min and ion suppression matrix effect was observed. Although reverse-phase ion-pair HPLC analysis of MNA and phenylephrine has been reported [18–20], these separation methods

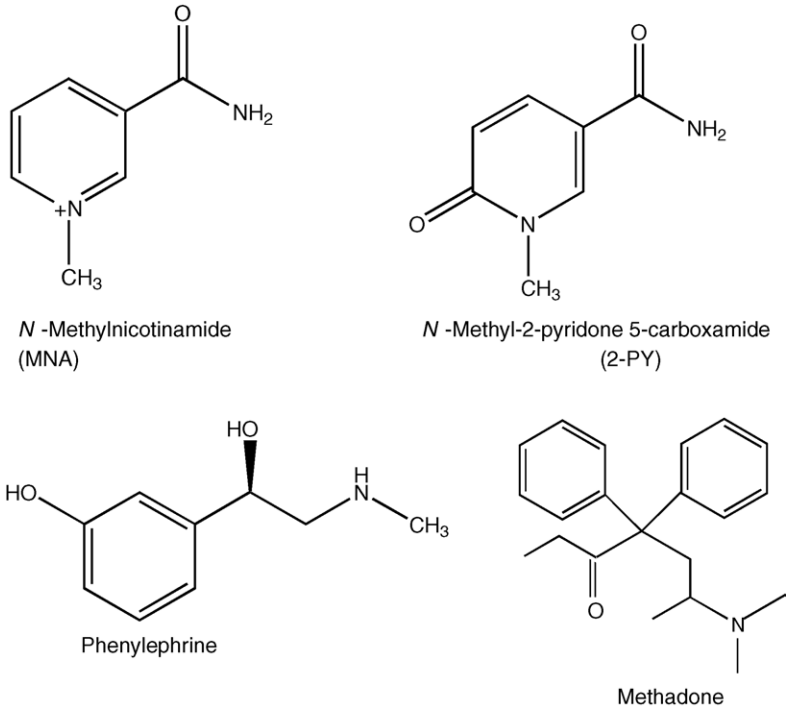

Fig. 1. Chemical structure of the three model compounds and methadone.

were generally coupled with ultraviolet (UV) detection and used non-volatile ion-pairing reagents, such as alkylsulfonic acid. We investigated the feasibility of ion-pair chromatography/MS for the analysis of these ionizable polar compounds in specific fluids. In order to reduce ion suppression, the ion-pairing reagents were added to the extract reconstitution solution. In contrast to traditional ion-pair chromatography [21], which employs the ion-pairing reagents in the mobile phase, column equilibration is not required in this method. To our knowledge, this is the first investigation of ion-pairing reagents in the reconstitution solution. Detection levels of the three analytes in human plasma extracts were compared using the ion-pairing reagents as mobile-phase and reconstitution additives. The distribution of the ion-pairing reagents in the column was studied and a possible retention mechanism was proposed. Finally, the chromatographic methodology was successfully coupled with MS to establish a quantitative method for the amine-possessing opioid-agonist methadone (Fig. 1) in human plasma, with a limit of detection (LOD) of 10 pg/mL. Ion-pairing reagents not only shifted the retention time but also reduced the carry-over peak for methadone.

2. Experimental

2.1. Reagents

The probe drugs *N*-methylnicotinamide chloride, 2PY and phenylephrine were obtained from Interchim (Montlucon, Cedex, France), Scientific Exchange, Inc. (Center Ossipee, NH, USA) and TCI, America (Portland, OR, USA), respectively. Methadone and its isotopically labeled internal standard (IS) methadone-d9 were purchased from Cerilliant (Austin, TX, USA). Ammonium acetate (99.99%) and the four volatile ion-pairing reagents HFBA (99%), NFPA (97%), TDFHA (99%) and PDFOA (96%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (88%, AR) was purchased from Mallinckrodt (Paris, KY, USA). HPLC-grade methanol was purchased from Burdick & Jackson (Muskegon, MI, USA). Ammonium hydroxide (28.0–30.0%) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was obtained from a ModuLab® MouPure Plus™ Reagent Grade Water System (San Antonio, TX, USA). When the effect of the ion-pairing regents on retention was studied, the reconstitution solution was water with the ion-pairing reagents added.

2.2. Apparatus

The HPLC system consisted of a Hewlett-Packard 1100 Series pump (Agilent, Foster City, CA, USA) and a PAL auto sampler (PAL, Columbia, MD, USA). Mobile phase A was deionized water with 0.1% formic acid or various concentrations of ion-pairing reagents. Mobile phase B was methanol with 0.1% formic acid or various concentrations of ion-pairing reagents. Gradient chromatography was performed using a Thermo Hypersil-Keystone Aquasil C18 2.1 mm × 100 mm (5 μm) packing column (Bellefonte, PA, USA). The flow rate was 0.300 mL/min. Following an isocratic period of 0.2 min at 20% B, a steep linear gradient was initiated at 0.9 min and held at 90% B for 1.6 min (0.9–2.5 min). After switching back to the initial condition (20% B for approximately 2.5 min), the program finished in 5.0 min. The sample injection volume was 35 μL.

The mass spectrometer was an Applied Biosystems API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA) with an Applied Biosystems datahanding system, which was operated in the electrospray ionization (ESI) positive-ion selected reaction monitoring (SRM) mode to observe the transitions of MNA, 2PY and phenylephrine at 137.20 > 94.10, 153.20 > 108.30 and 168.20 > 150.18, respectively. The instrumental parameters were set as follows: ion-spray voltage (IS) = 5000 V, collision energy = 18 V, NEB = 15, CUR = 10, temperature = 350 °C and dwell time = 150 ms for each compound.

The elution of ion-pairing reagents in the column was characterized using a LC-UV-quadrupole time-of-flight (QTOF) system, which comprised a Waters 2795 Separation Module with a Photodiode Array Detector (Milford, MA, USA) and a Micromass Q-Tof micro™ (Manchester, UK) mass spectrometer. The photodiode array detector was scanned from 200 to 400 nm. Ions were monitored using the negative- and positive-detection modes. The ESI conditions on the QTOF were as follows: capillary = 4.0 kV, cone = 30.0 V, extractor = 1.0 V, source temperature = 110.0 °C and desolvation temperature = 350.0 °C.

The quantitative method for methadone in human plasma was validated using a 2.1 mm × 50 mm (5 μm) C18 packing column (Advanced Chromatography Technologies, Aberdeen, Scotland) with a mobile-phase flow rate of 0.3 mL/min. Mobile phase A was deionized water and mobile phase B was acetonitrile; both also contained 0.1% formic acid. The gradient was as follows: 0 min (20% B), 0.5 min (20% B), 2.5 min (95% B), 3.0 min (95% B), 3.1 min (20% B) and finished at 4.5 min. Methadone and methadone-d9 were detected in the ESI positive-ion SRM mode, which recorded the transitions of 310.30 > 265.12 and 319.17 > 268.21, respectively. The instrumental parameters were set as follows: IS = 5000 V, NEB = 13, CUR = 10 and nebulizer temperature = 350 °C. Collision energies were 21 and 23 V for methadone and methadone-d9, respectively.

2.3. Experiments

2.3.1. Investigation of the effects of ion-pairing reagents on the retention of analytes

Analytes at a concentration of 100 ng/mL in 0.1% formic acid were used as the controls. Four perfluorinated carboxylic acids were evaluated at the same concentration (6.3 mM) in the reconstitution solution. The effect of the concentration

of the ion-paring reagents on the retention of three model compounds was further investigated using NFPA at concentrations of 0.62, 3.1, 6.2, 9.3, 12.4, 15.5, 18.6, 24.8 and 31 mM in the reconstitution solution. Gradient chromatography was performed for the experiment described above using deionized water and methanol with 0.1% formic acid as the mobile phase. Plots of retention factor *k* versus the ion-pairing reagent concentration were generated.

The mobile phase pH has a major effect on the dissociation of the secondary amino group in phenylephrine, and therefore can affect the formation of ion pairs and the retention of phenylephrine. The effect of the mobile phase pH on phenylephrine was investigated by adjusting the pH of the aqueous component of the mobile phase from 7 to 11. A 2.1 mm × 50 mm Waters Xterra® RP-18 (3.5 μm) column was used. The mobile phase consisted of methanol and 20 mM ammonium acetate with an adjusted pH (25:75, v/v). The flow rate was 0.200 mL/min. Phenylephrine (100 ng/mL) in NFBA (31 mM) was detected six times under each of the mobile-phase conditions.

2.3.2. Elution of ion-pairing reagents and its relationship with the ion suppression of plasma samples

The effects of the ion-pairing reagents on detection were compared between the reconstitution solution and the mobile phase. Drug-free human plasma was used to prepare samples with 4 μg/mL MNA and 2 μg/mL 2PY. After a 100-μl volume of the plasma sample was transferred to a borosilicate glass culture tube, the proteins were precipitated using 350 μl of 1% formic acid in acetonitrile. The sample was centrifuged at 3000 rpm for 8 min. The supernatant was then transferred to a new set of tubes and dried under dry nitrogen at 40 °C. The residue was reconstituted with 500 μL of reconstitution solution (93 mM NFPA) in reagent water. Comparisons were carried out between a mobile phase with 0.1% formic acid and a mobile phase with 0.01% NFPA.

In order to obtain a clear profile of the matrix effect of the ion-pair formation on detection, a post-column infusion experiment was carried out [22]. A syringe pump was connected with a T-piece between the analytical column and the mass spectrometer. A mixture of MNA and 2-PY (800 and 400 ng/mL, respectively) in a neat solution of 93 mM NFPA was continually infused after the analytical column. Chromatography was performed when the plasma blank extract was injected. If there were no matrix effects, the baseline would be constant; otherwise, the change in the baseline would show suppression or enhancement of the response of the analytes.

The elution profile of the ion-pairing regent NFPA was recorded using LC-UV-QTOF after 35 μL of 93 mM NFPA in reagent water was injected. As an acid, NFPA could be detected using only the negative ion-detection mode and was monitored at *m*/*z* 263. A photodiode array detector (PAD) was used as a second detector for supplementary information. A chromatogram at wavelength 212 nm was created after PAD detection.

2.3.3. Application to the determination of methadone in human plasma

Methadone stock solutions were prepared in methanol (100 μg/mL) and methadone-d9 (5 ng/mL) was used as the working internal standard solution. Calibration standard samples were freshly prepared at concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 ng/ml in human plasma. Quality controls containing 0.1, 0.25, 4.0 and 40.0 ng/ml of methadone were prepared in human plasma and divided into aliquots and stored at −20 °C.

A 50-μl volume of the plasma sample was transferred to the appropriate well of a 96-well plate and mixed with 25 μl of 5.0 ng/ml internal standard. Acetonitrile (300 μl) was added to precipitate the proteins. After the samples were vortexed for 10 min and centrifuged at 3000 rpm for 10 min, 100 μl of the supernatant was transferred to a new 96-well plate and mixed with 200 μl of reconstitution solution at 2.5:20:80 heptafluorobutanoic acid:methanol:water (v/v/v).

The quantification range, precision, accuracy, selectivity, extraction recovery and stability were evaluated for this method. Human plasma samples from six individuals were extracted and analyzed in order to evaluate the potential endogenous interferences for methadone and the internal standard. Matrix effects were evaluated for six individual human plasma lots by comparing the chromatographic peak areas of the analyte and the internal standard in solution with those of the analyte spiked into the plasma blank extracts [23]. Additional selectivity samples, which were fortified with methadone at the low-concentration quality control level (nominally 0.25 ng/mL), were prepared from six individual human plasma lots and analyzed to evaluate the potential matrix effects on the quantification.

As methadone is sensitive to light, all operations were carried out under reduced light conditions. The freeze/thaw stability after five cycles and the 24-h bench stability were studied. After the prepared samples were stored for 24 h on the auto-sampler at ambient temperature, their stability was investigated by injecting the prepared samples.

The potential for carry-over from high-concentration analyte levels was evaluated by injecting duplicate extracted matrix blanks immediately after the upper-limit of quantification calibration standards in each validation run.

3. Results and discussion

3.1. Matrix suppression using typical RP-LC separation

As a quaternary ammonium salt, MNA exhibited little or no retention (1.00 min) using the typical RP separation. It was therefore difficult to separate MNA from some endogenous polar components that might suppress the ionization of MNA when co-eluted. After extraction from a plasma sample, the sensitivity of detection for MNA decreased significantly. A 200-ng/mL sample of MNA in a plasma extract showed a much lower signal than a 20-ng/mL sample of MNA in pure

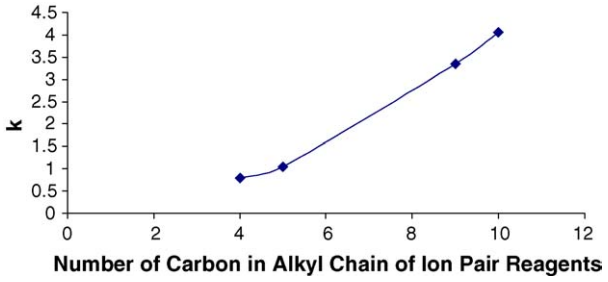

Fig. 2. Effect of different ion-pairing reagents on MNA retention.

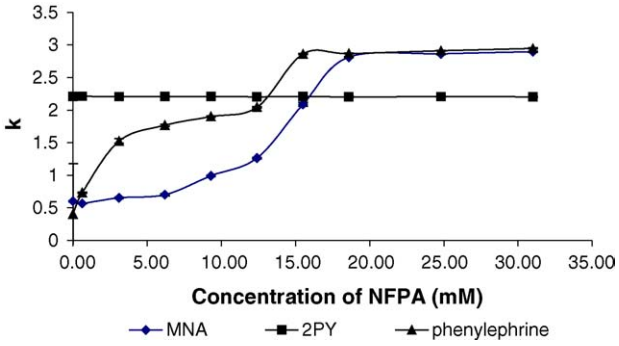

Fig. 3. Effect of the concentration of the ion-pairing reagent (PFNA) on the retention of model compounds. Experimental conditions: mobile phase A = 0.1% FA and mobile phase B = 0.1% FA in methanol ($n = 6$).

solvent. After adding 93 mM NFPA to the sample reconstitution solution, the retention time of MNA on the Aquasil C18 column was altered to 3.59 min and resulted in a 10.7-fold higher response of MNA in the plasma samples.

3.2. Comparison of different ion-pairing reagents

Fig. 2 shows that the retention time of MNA was increased with an increase in the length of the alkyl chain of the ion-pairing reagents. With no added ion-pairing reagent, MNA was almost unretained ($k = 0.47$). We added 6.3 mM HFBA and NFPA as ion-pairing reagents to the reconstitution solution, which changed the retention to 1.51 min ($k = 0.79$) and 1.81 min ($k = 1.05$), respectively. When adding the same concentration of TDFHA and PDFOA, the retention time changed to 3.83 min ($k = 3.34$) and 4.46 min ($k = 4.06$), respectively. This phenomenon is consistent with traditional ion-pair chromatography, in which ion-pairing reagents with longer alkyl chains are taken up more strongly by the column [20]. Because ion-pair chromatography involves RP separation and ion-exchange mechanisms, the uptake of the ion-pairing reagents on the column mainly determines the retention of the analytes. At a given concentration, longer chain ion-pairing reagents result in longer retention. When the concentration of NFPA was increased to 63 mM, the retention time of MNA was 3.62 min ($k = 3.10$), which was similar to the effect of TDFHA at the lower concentration (6.3 mM). However, the retention time of MNA could not be further altered due to the saturated uptake of the ion-pairing reagent on the column. This significant difference between NFPA and PDFOA reflects differences in their hydrophobicity. As a result, no concentration of the less hydrophobic reagent could afford a similar column retention [21].

Chaimbault et al., using a porous graphitic carbon column, reported that systems containing HFBA or NFPA had relatively short equilibration times, whereas systems containing TDFHA or PDFOA required longer equilibration times (35–105 min) [11]. In our present experiment, gradient HPLC program systems with HFBA or NFPA in the reconstitution solution did not require extensive equilibration times; the retention time of the analyte was reproducible after between three and six injections. However, it was difficult for systems using TDFHA or PDFOA to achieve equilibration; the retention time with these systems continued to change even after between six and ten injections. It is possible that TDFHA and PDFOA are much more hydrophobic and bind strongly to the hydrophobic stationary phase, thus making it difficult to elute out all of these reagents before each injection. TDFHA and PDFOA might have accumulated in the column such that the retention of the analyte was difficult to stabilize.

3.3. Comparison of different NFPA concentrations

Fig. 3 shows the effect of the different concentrations of NFPA on the retention of MNA, 2PY and phenylephrine. Samples in 0.1% FA were used as controls. With an increased concentration of the reagent, the MNA and phenylephrine retentions were increased; by contrast, that of 2PY was not changed, as this neutral compound could not form an ion-pair. When the concentration of NFPA was increased to 20 mM, the effect of the ion-pairing reagent on the retention of MNA and phenylephrine reached a maximum and remained constant even after increasing the concentration of the ion-pairing reagent. Snyder et al. pointed out that there might be two processes in ion-pair chromatography: RP retention and ion-exchange retention. These processes might be altered by adjustment of the amount of ion-pairing reagent that is taken up by the stationary phase [20]. When the concentration of the ion-pairing reagent was increased, more reagent was taken up by the column and the ion-exchange retention was more important. After the column was saturated with the reagent, the retention of the analyte leveled off after reaching a maximum. The results presented in Section 3.2 indicated that the ion-pairing reagent could be washed out from the column rapidly, because the equilibration of NFPA and HFBA was not required. A dynamic distribution equilibrium of the ion-pairing reagent between the column and the plug of injected mobile phase could have been established when the sample plug passed through the column. The retention of phenylephrine demonstrated two step increases (Fig. 3), which might be related to the amphoteric nature of this substance. The perfluorinated carboxylic acids are strong acids, which not only form ion pairs with positive ions but can also change the pH of the environment. When the concentration of the ion-pairing reagent was low, the pH of the environment around phenyle-

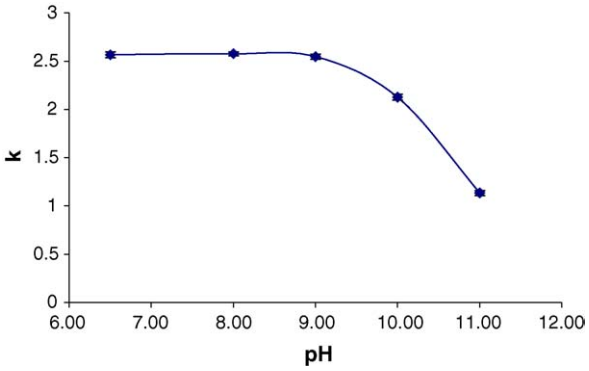

Fig. 4. Effect of the concentration of the pH of the mobile phase on the retention of phenylephrine.

phrine was close to neutral. The –OH and –NH groups could both be dissociated, and the latter formed ion pairs with perfluorinated carboxylic acids. However, with an increase in the concentration of the ion-pairing reagent, the pH of the environment around phenylephrine decreased and the dissociation of the –OH group was suppressed. The hydrophobic nature of phenylephrine was therefore increased significantly, which altered its retention to a greater extent.

3.4. Effect of pH on the retention of phenylephrine

In order to investigate the effect of pH on the retention of the analyte, the pH of the mobile phase was adjusted. A buffer of 20 mM ammonium acetate was added to provide the appropriate buffer capacity. The effect of pH on the retention of phenylephrine is illustrated in Fig. 4 according to traditional ion-pair chromatography. As the pK_a is 10.1 for the amino functionality, the dissociation of this secondary amino group was almost completely suppressed when the pH was increased to 12. Considering the stability of the column, a pH range of 7–11 was investigated. At higher pH values (such as 11), the dissociation of the secondary amino group was partially suppressed and the formation of the ion-pair decreased. Fig. 4 shows that the maximum retention occurs at a low pH where the analyte is completely ionized.

3.5. Comparison of ion-pairing reagents in the reconstitution solution versus the mobile phase

The ion-pairing reagent can change the retention time and thereby decrease the ion suppression of the matrix; nevertheless, the formation of strong ion pairs might result in depression of the electrospray signal due to the formation of a neutral ion pair. The responses of the analytes were decreased two- to four-fold after the ion-pairing reagent was added to the tuning solution (or infusion solution). In order to improve possible ion suppression, the ion-pairing reagent was added to the extraction reconstitution solution rather than the mobile phase. The signals of the analytes under these two conditions were compared. For analytes in pure solvent, the presence of ion-pairing reagents, such as HFBA and NFPA, in the mobile phase did not lead to significantly lower sensitivity than that observed with ion-pairing reagents in the reconstitution solution alone. The responses of the analytes with ion-pairing reagents in the mobile phase were 59.2–168.9% of those observed when using ion-pairing reagents in the reconstitution solution. Extracted human plasma samples containing MNA also showed no significant differences between mobile phases with and without ion-pairing reagents. However, significant ion suppression was demonstrated for 2PY in the plasma extracts; the 2PY peak in the plasma extracts could not be observed at a concentration of 2000 ng/mL (Fig. 5). These results indicated that the ion-pairing reagent suppressed ionization efficiency for the 2PY analyte in plasma extracts even though an ion pair was not formed. Post-column infusion experiments demonstrated that the ion-pairing reagent in the mobile phase caused obvious ion suppression for the 2PY plasma sample. The baseline dropped from 400 cps to almost zero at the retention time of 2PY. By contrast, the baseline remained at its original level when the ion-pairing reagent was added to the reconstitution solution. The post-column infusion experiments for MNA and phenylephrine found no significant differences between mobile phases with and without ion-pairing reagents.

3.6. Elution of ion-pairing reagents

The elution and distribution of ion-pairing reagents in the column are important factors in achieving the desired separation and preserving the detection sensitivity of the analytes. The ideal situation would be that polar compounds co-elute with ion-pairing reagents for a certain period of time and then separate after obtaining sufficient retention to avoid matrix suppression. However, the retention mechanism is complex, because the separation depends on the molecular interaction between the stationary phase, mobile phase, analyte and ion-pairing reagent. The A^+_{gp} ion is much more likely to come from the analyte in the mobile phase, rather than dissociating from the ion pair A^+IP^- in the gas phase. A^+IP^- was not observed in MS due to the formation of a neutral ion pair; moreover, the ion pair was too strong to be dissociated. In Fig. 6, the labels ‘1’ and ‘2’ denote the ion-exchange and reverse-phase separation processes [21]. The retention mechanism alternates from latter to former depending on the amount of ion-pairing reagent taken up by the stationary phase. At a low concentration of the ion-pairing reagent, RP separation is the dominant retention mechanism. The hydrophobicities of the analyte and the analyte ion pair A^+IP^- differ, therefore their retention might also vary. Due to the equilibration of the analyte and A^+IP^- between the mobile and stationary phases in Fig. 6, an asymmetrical or broad peak might be observed. In fact, two broad peaks corresponding to the analyte were recorded under certain concentrations of ion-pairing reagents (9.3 mM in the reconstitution solution). When the stationary phase takes up enough of the ion-pairing reagent, the retention of the analyte will be dominated by the ion-exchange mechanism and one sharp analyte

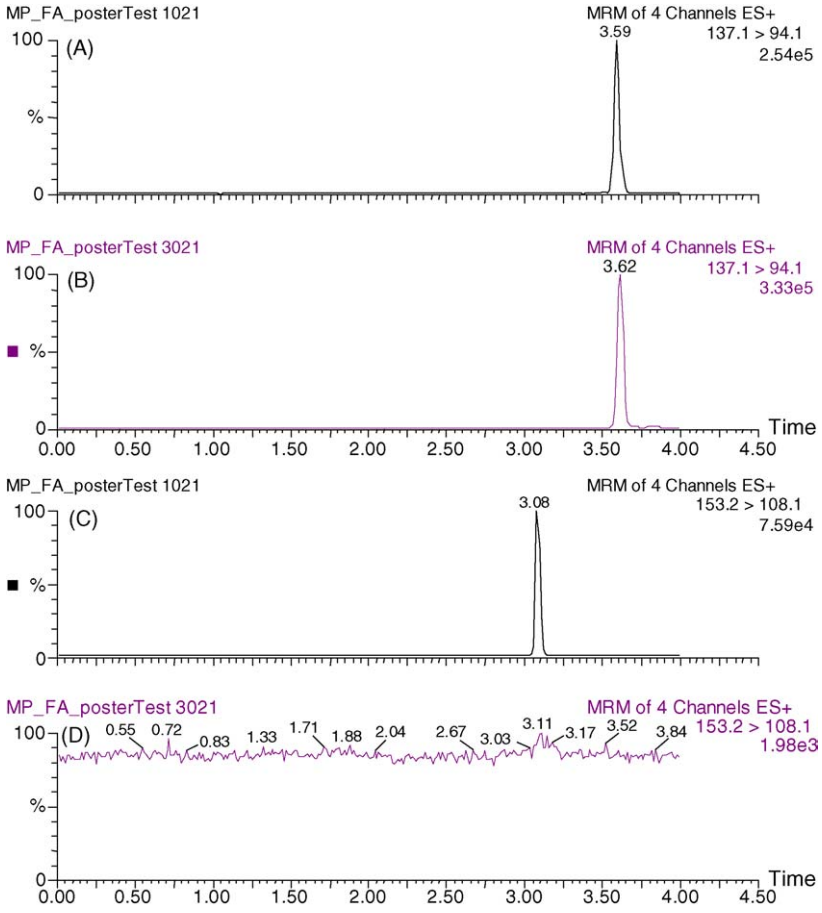

Fig. 5. Samples prepared in human plasma with MNA at 4000 ng/mL and 2PY at 2000 ng/mL. Extracted using protein precipitation. (A) Chromatogram of MNA with PFNA added to the reconstitution solution; (B) chromatogram of MNA with PFNA added to the mobile phase and the reconstitution solution; (C) chromatogram of 2PY with PFNA added to the reconstitution solution; (D) chromatogram of 2PY with PFNA added to the mobile phase and reconstitution solution.

peak will be observed (Fig. 5). After the ion-pairing reagent was injected with the reconstitution solution, the uptake of the ion-pairing reagent on the column and the ion-exchange of the analyte occurred dynamically when the plug of the injection solution passed through the column. The analyte was therefore likely to be distributed and co-eluted with the ion-pairing reagent. Although the polarities of MNA and phenylephrine differed (without an ion-pairing reagent, their retention times were 1.19 and 1.28 min, respectively), their retentions were similar after adding an ion-pairing reagent to the reconstitution solution (Fig. 3).

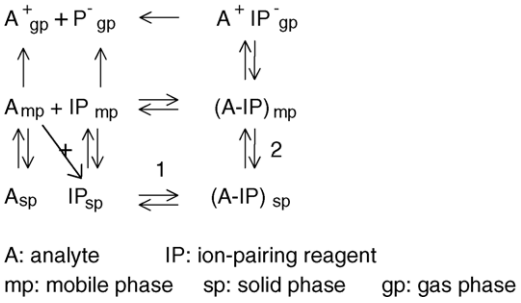

Fig. 6. Equilibrium diagram for ion-pair formation and distribution. 1 = Ion-exchange process and 2 = reverse-phase separation.

Analytes were detected using the positive-ion mode; however, this was not appropriate for the ion-pairing reagent perfluorinated carboxylic acid. Therefore, this reagent was detected using the negative-detection mode. UV absorption was used as a supplementary detector to investigate the retention of the ion-pairing reagent when it was used as a reconstitution solution additive. When 93 mM NFPA was used as the reconstitution solution, the retention time of MNA was 4.25 min and that of 2PY was 3.50 min. After 93 mM NFPA reconstitution solution was injected, a broad peak was observed at retention times of 4.00–6.00 min (MS) or 4.00–4.50 min (UV) (Fig. 7). This result supported the finding that most of the ion-pairing reagents (NFPA and HFBA at a concentration of 93 mM) could be eluted from the column. Although MNA eluted at the front end of the NFPA peak, it was not separated from the ion-pairing reagent. This explains why there was no significant difference when NFPA was used as the reconstitution solution or in the mobile phase. Even though NFPA might have resulted in ion suppression for MNA, the effect would be the same whether the ion-pairing

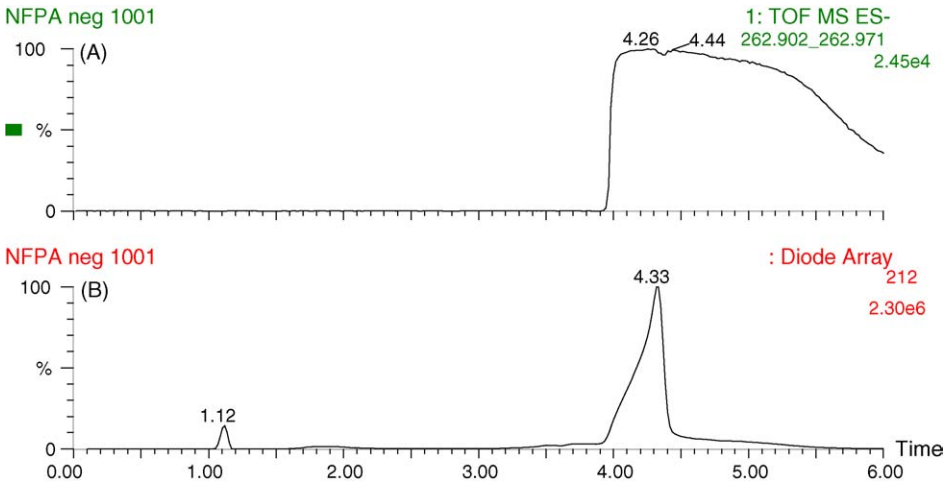

Fig. 7. Retention time of the ion-pairing reagent. (A) Chromatogram of NFPA using MS detector and (B) chromatogram of NFPA using PAD detector. Detected wavelength = 212 nm.

reagent was used in the mobile phase or the reconstitution solution.

The retention time for 2PY was 3.50 min, which was shorter than that for the ion-pairing reagent NFPA. 2PY demonstrated ion suppression when the ion-pairing reagent was used as the mobile-phase additive, but this activity was absent when the ion-pairing reagent was used as the reconstitution solution. Two mechanisms might have caused this ion suppression. Firstly, the ionization efficiency may have been decreased due to a change in the solution conditions after addition of the ion-pairing reagent (2PY can not form an ion pair with the ion-pairing reagent). The results presented in Section 3.5. show that the response of 2PY using the ion-pairing reagent in the mobile phase was about 72.7% of that using the ion-pairing reagent in the reconstitution solution. Secondly, matrix suppression was more significant when the analyte co-eluted with components of the matrix and the ion-pairing reagents, or when NFPA was added only in the reconstitution solution. Instead of co-eluting with 2PY, the ionizable components, which resulted in the matrix suppression were retained at the same retention time as NFPA. Matrix suppression was therefore decreased.

3.7. Carry-over reduction by ion-pairing reagents

Carry-over is a common chromatographic problem in LC/MS/MS bioanalytical assays. Generally, it can be corrected by adjustments of the syringe-wash or mobile-phase solutions [24,25]. The amine-containing compound methadone showed high levels of carry-over under both RP and normal-phase separations. The latter condition resulted in 20–50% lower limit of quantification (LLOQ) carry-over values. Optimizing the syringe wash, mobile-phase and reconstitution solutions did not reduce the presence of the carry-over. While a high organic mobile-phase composition is favored for carry-over reduction in the RP condition, methadone displays an extremely short retention time under these conditions. In addition, methadone (pK_a 9.1) becomes ionized in the acidic mobile phase; thus, the typical RP-HPLC methods for carry-over elimination were unfeasible. After the addition of the ion-pairing reagent HFBA in the reconstitution solution, the retention time of methadone altered from 1.07 to 2.04 min. Moreover, the carry-over levels were reduced to 10% of the LLOQ. This decrease in the carry-over peak might have been the result of both a higher organic mobile-phase composition and decreased attachment of the analyte to the LC surfaces. A high organic mobile-phase composition can decrease the deposition of the compound on the column [25]. It is well known that basic compounds can interact with the unmodified silanols of silica-based columns, resulting in band tailing [26]. The formation of ion pairs between HFBA and the amine group in methadone might reduce the interaction between methadone and the silanols of the column. In addition, this ion pairing might reduce the interaction of methadone with the surfaces of the autosampler tubing connections, which might act as chromatographic surfaces in a manner similar to the column packing [24]. Reduced interactions between methadone, the column packing and the surface of the instrument system might decrease the absorption on the instrument system directly. This method might also potentially address the carry-over problem for other polar compounds that form ion pairs.

3.8. Validation of the quantitative assay for methadone

The effects of HFBA and NFPA on the retention of analyte did not significantly differ in the experiment described above. HFBA was chosen as the ion-pairing reagent in the reconstitution solution. The calibration curve was constructed using linear least-squares regression (weighted 1/concentration^2) of the ratios of the methadone peak area to that of the internal standard plotted versus the methadone concentration. The assay was linear over the concentration range 0.1–50 ng/ml with a correlation coefficient of 0.9977. All of the concentration residuals were within 12.8%. The LOD was 10 pg/mL

Table 1
Precision and accuracy of the quality control samples

	Nominal concentration (ng/mL)			
	0.10	0.25	4.0	40.0
Intra-assay precision ($n = 6$)				
Mean	0.092	0.24	3.9	38.6
%R.S.D.	4.4	4.4	3.2	1.8
%DFN	−8.3	−5.3	−2.1	−3.4
Inter-assay precision ($n = 18$, 3×6)				
Mean	0.094	0.24	4.0	39.4
%R.S.D.	11.3	4.2	3.8	3.2
%DFN	−6.5	−3.6	−0.9	−1.6

%DFN: percentage difference from the nominal value.

based on three times the standard deviation (S.D.) of the blank. The precision and accuracy of the method were calculated as the relative S.D. (%R.S.D.) and the percentage difference from the nominal (%DFN), respectively. Table 1 summarizes the precision and accuracy of the quality control samples. The intra-assay precision (%R.S.D.) and inaccuracy were 1.8–4.4% ($n = 6$) and −8.39 to −2.1% ($n = 6$), respectively. The assay demonstrated an inter-assay precision (%R.S.D.) of between 3.2 and 11.34% ($n = 18$) and the percent inaccuracies of the inter-assay data were between −6.5 and −0.94%.

The recovery of methadone was evaluated in triplicate at three different concentrations (0.25, 4.0 and 40 ng/mL) by comparing the peak areas of the analyte spiked into extracts of the plasma blank versus the areas of the analyte controls in the same analytical run. The mean extraction recoveries for methadone were 91.7 ± 13.5, 93.7 ± 8.2 and 82.1 ± 13.9% at concentrations of 0.25, 4.0 and 40.0 ng/mL, respectively. The mean recovery of the internal standard at 5.0 ng/mL was 85.2 ± 13.3% ($n = 9$).

No significant chromatographic peaks were detected at the mass transition and retention times of the analyte or the internal standard that might interfere with quantification. Matrix effects were evaluated in six different sources ($n = 3$ per source) of plasma. No significant matrix effect was observed and the results were reproducible for all six sources; the mean matrix effect was 102.0 with a precision of 6.2%. Among the six individual samples, all six of the back-calculated values for the fortified spiked samples were less than 15% (from −4.7 to −5.1%). These results indicated that matrix suppression effects did not compromise the accuracy of the assay.

Quality controls at concentrations of 0.25 and 40.0 ng/mL were used to evaluate the stability of the analyte in the matrix. All of the quality control samples demonstrated acceptable freeze/thaw stability through five cycles, along with acceptable 24-h bench stability and 24-h prepared sample stability.

4. Conclusions

In this study, the retention of polar model compounds was successfully shifted to a suitable retention time and separated from matrix ion suppression. The volatile perfluorinated carboxylic-acid reagents provided the necessary selectivity and sensitivity improvements for the analytes. The sensitivity of detection for MNA in human plasma samples was increased 10.7-fold. The retention times of the analytes increased with the concentration of the ion-pairing reagents and the alkyl-chains of perfluorinated carboxylic acids, similar to traditional ion-pair chromatography. The effect of pH on retention was also demonstrated. Although there was no direct evidence to show that the formation of ion-pairing reagents caused significant ionization suppression in this case, adding these reagents to the reconstitution solution demonstrated several advantages, including decreasing the matrix suppression for MNA, eliminating the need for equilibration time, avoiding ion suppression for 2PY in human plasma samples, and fixing the carry-over by decreasing the possible binding on the column and instrumental systems. Although the response of ionizable polar compounds may be reduced due to the formation of ion pairs, this study suggests an alternative technique for dealing with severe matrix suppression. The successful validation of the assay for methadone in human plasma demonstrates that perfluorocarboxylic acids can provide a suitable means to establish a quantitative analytical approach for polar compounds in biological fluids using LC/MS.

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