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Optimized stationary phases for the high-performance liquid chromatography–electrospray ionization mass spectrometric analysis of basic pharmaceuticals

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Abstract

Stationary phases were investigated for HPLC coupled with electrospray ionization mass spectrometry (ESI-MS) for the analysis of basic drugs. Tricyclic antidepressants (TCAs) and β-blockers were used as model solutes. The functional groups, pentafluorophenyl (PFP), OH, CN or CH_3 were attached to the silica via a propyl chain. The effects of these stationary phases as well as C_8 and C_{18} phases on retention and peak shape of the basic drugs were studied. The CN and PFP phases adequately retained (t_R of 2 to 6 min) the basic drugs when the mobile phase was composed of 90% acetonitrile, whereas with the C_4, C_8 and C_{18} phases, less than 40% acetonitrile had to be used to provide adequate retention of the basic drugs. Because acetonitrile provides better desolvation in ESI than an aqueous solvent, it produces an increased MS signal. As an example of the HPLC–ESI-MS analysis of the β-blocker, pindolol, on a CN phase, the use of 90% acetonitrile in the mobile phase increased the ESI-MS signal by 790% when compared to a C_{18} phase which could use only 5% acetonitrile in the mobile phase for retention of the solute. In addition, the CN and PFP phases provided better peak shape than the OH phase and the hydrophobic phases (C_4, C_8 and C_{18}) and ion-pairing or ion-suppressing agents were not required. The retention behavior of the TCAs and β-blockers on each of the phases is described.

Keywords: Stationary phases, LC; Antidepressants, tricyclic; Beta-blockers

1. Introduction

For the past decade, high-performance liquid chromatography–mass spectrometry (HPLC–MS) has taken its rightful place as a routine technique in analytical laboratories, especially in the pharmaceutical and biotechnology industries [1–3]. Initially instrumental researchers focused mainly on the development and improvement of the interface between the HPLC and the MS systems [4,5]. A second focus has been to improve ion transmission in the mass spectrometer for improved sensitivity and resolution [6,7]. Other research has focused on the effects of the HPLC mobile phase conditions on electrospray ionization (ESI) MS signals [8,9]. There is, however, still a need for improving detection limits in the HPLC–MS analysis of pharmaceutical compounds in various matrices. We therefore investigated novel stationary phases in order to improve detection limits in HPLC–MS and provide good peak shape with the use of optimal ESI-MS solvents. These new station-

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ary phases were used for the analysis of drugs in support of clinical, preclinical and formulation studies of pharmaceutical compounds.

Recently, mobile phase conditions that affected the HPLC–ESI-MS analyses of nucleoside antivirals [10] and tetracycline analogs were investigated [8]. It was found that the HPLC mobile phase parameters significantly affected the results obtained from the mass spectrometer. However, little work has been reported in the literature on the role that the HPLC stationary phase plays in the HPLC–ESI-MS analyses and the determination of the best stationary phase(s) for use with HPLC–MS.

In the past, the analysis of basic pharmaceuticals by HPLC has been difficult due to secondary interactions of these drugs with the unreacted silanols [11]. Thus ion-pairing and ion-suppressing agents were added to the mobile phase to decrease these secondary interactions [12]. However, ion-pairing and ion-suppressing agents decrease the MS signal in HPLC–ESI-MS analyses [13]. Small amounts of buffer additives often facilitate ionization in ESI-MS [8], yet van Breeman et al. [14] reported that with reversed-phase columns, concentrations of 100 m*M* buffer decreased the ESI-MS signal when compared to 10 m*M* concentrations of buffer [14]. Unfortunately, buffer concentrations of 100 m*M* are often needed to decrease peak tailing for the HPLC analysis of solutes with basic groups [14]. Although ESI-MS detection is very selective, HPLC is necessary to separate solutes from endogenous compounds in complex matrices which might cause ion-suppression in the ESI interface. When the analytes are separated from the endogenous compounds the sensitivity and precision of the assay is improved. Matuszewski et al. [15] reported that retention times of 2–6 min (includes void time) for 3 cm×2.1 mm I.D. columns were necessary to reduce ionization suppression.

It has been reported that the ESI-MS response increases as the organic concentration in the liquid flow increases [16,17]. According to Kebarle and Tang [18] most organic solvents (acetonitrile, MeOH, etc.) have lower surface tension and higher volatility than aqueous solvents. The lower surface tension and higher volatility produces higher MS signals due to more efficient desolvation in ESI. Often the organic solvent is added post-column to improve the sensitivity when separations are performed with high concentrations of aqueous solvent [19]. However, this step requires additional equipment and adds complexity to the HPLC–ESI-MS process. Therefore, phases that sufficiently retain (t_R between 2 and 6 min) solutes with the use of as much acetonitrile modifier in the mobile phase as feasible would provide the best signal for ESI-MS analyses.

Our goal, therefore, was to investigate stationary phases in order to obtain the highest MS signal and good peak shape for the HPLC–ESI-MS analysis of basic pharmaceuticals. These phases must provide a balance between fast analyses and sufficient retention to separate the solutes from endogenous interferences. Thus to produce the optimum MS signal, retention times (t_R) of 2–6 min for the solutes are needed when the mobile phase contains a high concentration of acetonitrile. In addition, these phases must provide good peak shape (asymmetry factor≈1) with the use of low concentrations of buffer (<10 m*M*), and without ion-pairing or ion-suppressing agents in the mobile phase. The packings must also be stable enough to produce reproducible retention times for at least 8 h (1 working day) of analyses.

In the mid 1980s Massart et al. [20] reported that a cyanopropyl (CN) column was successful for the HPLC of basic pharmaceuticals with 90% acetonitrile in the mobile phase. In contrast, a C_{18} phase required ion-pairing agents in the mobile phase to achieve similar retention of the basic solutes. Therefore, in order to develop optimized stationary phases for HPLC–MS analyses, we investigated a stationary phase with cyano (CN), hydroxyl (OH), and pentafluorophenyl (PFP) or methyl (CH_3) groups on a propyl chain to examine their effects on retention, peak shape and MS signal. The cyanopropyl phase has been used for the HPLC of basic compounds [21], an alcohol type phase was previously used for the separation of isomers of tocopherol [22], a PFP type phase was previously used for the separation of polar solutes [23] and a C_4 phase (i.e., a methyl on the propyl chain) has been used in the reversed-phase separation of many components [24]. The PFP phases often provide better selectivity and greater retention of polar solutes than hydrophobic phases. Since C_{18} and C_8 are the most commonly used

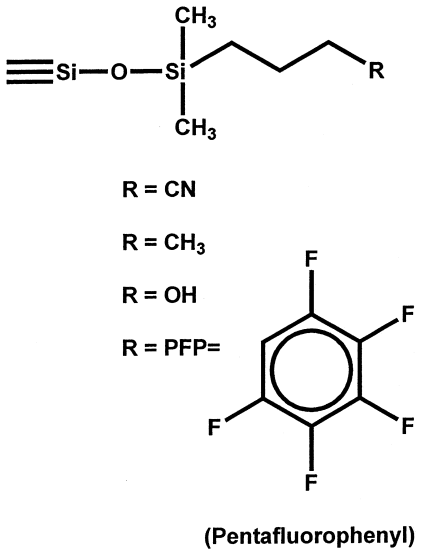

Fig. 1. Illustration of the stationary phases (C_8 and C_{18} phases not shown).

phases for HPLC analyses, these phases were also investigated for comparative purposes. Structures of the stationary phases are shown in Fig. 1.

Many procedures exist for the determination of basic drugs such as tricyclic antidepressants (TCAs) [25,26] and β-blockers by HPLC [27,28]. Many of the HPLC assays of these compounds require non-MS-compatible mobile phases or ion-pairing and ion-suppressing agents for retention and satisfactory peak shape of these solutes. Recently a silica-based hydrophobic chain phase embedded with a polar group was developed that provided good peak shape for the HPLC analysis of a β-blocker [29]. However, this phase required only 0–30% organic modifier in the mobile phase for sufficient retention of these basic pharmaceutical compounds. Atmospheric pressure ionization (API) MS has recently been used to investigate the mass spectra of the β-blocker timolol [30]. Capillary HPLC–API-MS has recently been used for the analysis of β-blockers [31]. However, in order to retain the solutes, a low concentration of acetonitrile (0–10%) was used in the mobile phase. Capillary electrophoresis (CE)–ESI-MS was also recently used for the analysis of TCAs [32]. However, due to the special ESI interface required, small injection volumes (nl) and questionable precision with some applications, CE is not a popular choice when coupled to ESI-MS. Since HPLC and HPLC–API-MS analyses have been performed with these basic drugs (pK_a values>8.0), we chose these solutes as models to investigate optimized stationary phases for the HPLC–ESI-MS analysis of basic drugs. Structures of a TCA and β-blocker are shown in Fig. 2.

2. Experimental

2.1. Reagents and standards

All compounds were obtained from Sigma (St. Louis, MO, USA). Standard stock solutions (1.0 mg/ml) were prepared by dissolving a weighed amount of the compounds in water–MeOH (90:10). The solutions were sonicated in an Ultrasonicating

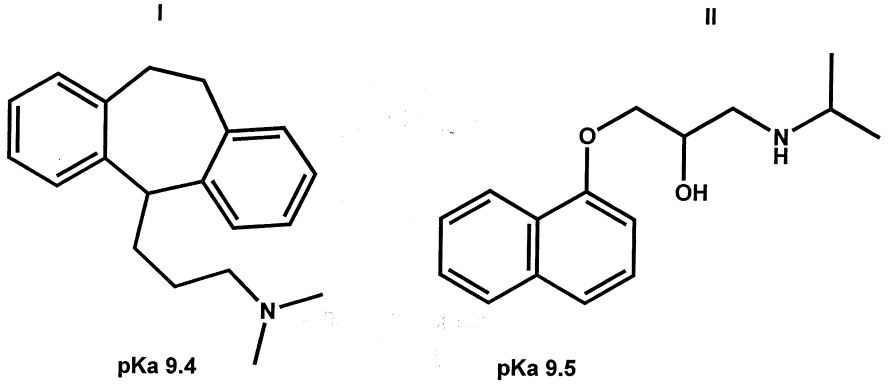

Fig. 2. Chemical structures of a TCA (I=amitriptyline) and a β-blocker solute (II=propranolol).

Bath 3200 (Bransonic, Danbury, CT, USA) for 10 min. Serial dilutions were made from the stock solutions to achieve the desired working concentrations of 1–100 μg/ml for the standards. All HPLC reagents (J.T. Baker, Phillipsburg, NJ, USA) were of HPLC-grade or better and were used without further purification. All reagents used for synthesis of the stationary phases were obtained from Silar Labs. (Scotia, NY, USA). The hydroxypropyl reagent was synthesized at Restek (Bellefonte, PA, USA). All stationary phases are commercially available from Restek. The reagents for the stationary phases were of >97% purity and used without further purification.

2.2. HPLC columns

All columns were supplied by Restek and were 3.0 cm×2.1 mm I.D. The stationary phases were monofunctional. Columns contained packings of 5 μm particles with 60 Å pores. The phases were endcapped after bonding with trimethychlorolsilane.

The HPLC column hold-up time (t_0) was estimated by monitoring the first MS signal disturbance upon an injection. The hold-up time for all the HPLC columns was approximately 0.19 min. The asymmetry factor (AF) was calculated at 10% peak height according to the following equation

$$AF = a/b \quad (1)$$

where a is the tail of the chromatographic peak and b is the front of the chromatographic peak.

2.3. HPLC conditions and apparatus

Two Jasco 980 series pumps (Tokyo, Japan) equipped with a vacuum membrane degasser delivered the mobile phase at a flow-rate of 0.4 ml/min. The mobile phase consisted of isocratic mixtures of acetonitrile and 5 m*M* ammonium acetate (aqueous) adjusted to pH 4.5 with acetic acid. A CTC LEAP Technologies HTS PAL autoinjector (Carrboro, NC, USA) injected 10-μl aliquots of the standards onto the HPLC columns.

Table 1
Molecular mass and ESI-MS parent ion information for the TCA and β-blocker solutes[a]

Drug	Molecular mass	Protonated molecular ion $(MH)^+$
β-Blockers		
Acebutolol	336.4	337.2
Alprenolol	249.4	250.3
Atenolol	266.4	267.4
Labetolol	328.4	329.3
Metoprolol	267.4	268.2
Nadolol	309.4	310.2
Oxprenolol	265.4	266.2
Pindolol	248.3	249.2
Propranolol	259.4	260.5
Sotalol	272.4	273.1
Timolol	316.4	317.6
Tricyclic antidepressants		
Amitriptyline	277.4	278.5
Clomipramine	314.8	315.4
Desipramine	266.8	267.5
Doxepin	279.4	280.5
Imipramine	280.9	281.7
Nortriptyline	263.8	264.6
Protriptyline	263.4	264.2
Trimipramine	294.4	295.2

[a] The mobile phases consisted of mixtures of acetonitrile and 5 m*M* ammonium acetate, pH 4.5 at a flow-rate of 0.4 ml/min.

2.4. Mass spectrometry

A Sciex API 3000 triple quadrupole mass spectrometer (PE-Sciex, Toronto, Canada) equipped with a turbo ionspray interface (TISP) was used for the detection of analytes. The mass spectrometer was operated at unit mass resolution. Data was acquired in the positive ion mode with an ESI probe voltage of 5000 V. Nebulizer gas and curtain gas settings were 15 and 12 p.s.i., respectively (1 p.s.i. = 6894.76 Pa). The TISP interface was operated at a temperature of 150°C and nebulizer gas settings of 7000 ml/min. Data was collected by monitoring the protonated molecular ion in the selected ion monitoring (SIM) mode with window widths of 2–4 u. Multiple analyte detection was performed by scanning Q1 from 240–340 u. Scan times were 0.5–1.0 s/scan for the MS experiments. LC2Tune version 1.4, Sample Control version 1.4 and Multiview version 1.4 were used for data collection and analysis.

3. Results and discussion

Protonated molecular ions $(MH)^+$ were observed by ESI-MS for all the solutes tested. The molecular mass and protonated molecular ion information for the solutes is listed in Table 1.

Standards of the solutes were injected into the ESI-MS system without an HPLC column to study the importance of acetonitrile concentrations in the mobile phase. Pindolol, a β-blocker and protriptyline, a TCA were used as representative solutes to demonstrate the effect the acetonitrile concentration in the mobile phase has on the ESI-MS signal. As shown in Fig. 3 for both solutes, the ESI-MS signal is a factor of 10 greater when 99% acetonitrile is

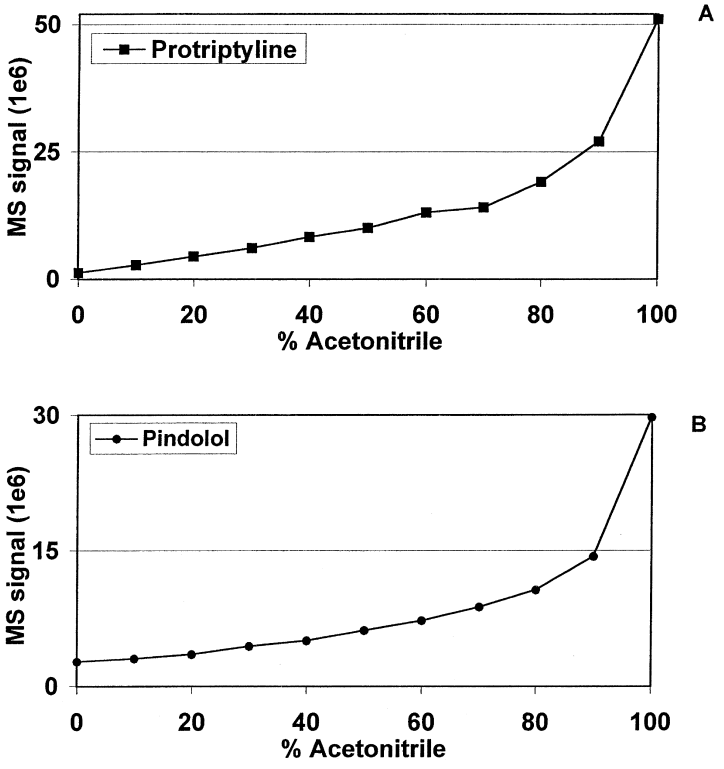

Fig. 3. MS signal enhancement with the increase of % acetonitrile in the mobile phase (acetonitrile–5 m*M* ammonium acetate, pH 4.5) for the loop injection (no HPLC column) of about 1 ng of protriptyline (A) and pindolol (B) into the ESI-MS system.

used in the mobile phase compared to when 0% acetonitrile is used in the mobile phase. These results confirm that for optimum ESI-MS signal, stationary phases are needed that give retention of solutes at high concentrations of acetonitrile.

Retention data for the solutes on the phases with 90% acetonitrile in the mobile phase are listed in Table 2. The β-blockers required <20% acetonitrile on the hydrophobic chain phases to obtain retention times greater than 2.0 min whereas the TCAs required <40% acetonitrile to achieve these results. In the current configuration, a retention time of 2.0 min corresponds to a k of ~9. The general retention trend from highest retention to the lowest retention for all the solutes tested with 90% acetonitrile is PFP, CN, OH and then C_4. Also, the TCAs showed much greater retention on the PFP phase than the β-blockers with 90% acetonitrile in the mobile phase. Typical chromatograms for the analysis of TCAs and β-blockers on a CN and PFP phase are shown in Fig. 4. Baseline resolution of solutes is not always necessary when HPLC is coupled to MS since the MS can differentiate between solutes by mass.

Table 2
Retention data of basic solutes with 90% acetonitrile in the mobile phase[a]

Drug	Retention time (min)			
	CN	OH	PFP	C_4[b]
β-Blockers				
Acebutolol	3.22	2.02	6.32	<0.5
Alprenolol	3.79	1.74	9.99	<0.5
Atenolol	3.04	2.15	4.63	<0.5
Labetolol	3.56	1.32	4.93	<0.5
Metoprolol	3.42	1.83	7.70	<0.5
Nadolol	3.37	2.15	5.58	<0.5
Oxprenolol	3.81	1.78	9.20	<0.5
Pindolol	3.64	1.81	6.60	<0.5
Propranolol	3.27	1.78	10.3	<0.5
Sotalol	3.12	1.69	5.09	<0.5
Timolol	3.37	1.74	7.53	<0.5
Tricyclic antidepressants				
Amitriptyline	3.57	1.61	12.0	<0.5
Clomipramine	3.90	1.64	13.6	<0.5
Nortriptyline	4.91	1.99	14.1	<0.5
Trimipramine	3.17	1.25	9.95	<0.5
Desipramine	3.87	2.05	13.8	<0.5
Doxepin	4.82	1.52	9.13	<0.5
Imipramine	4.94	1.76	12.4	<0.5
Protriptyline	2.92	2.07	13.4	<0.5

[a] The mobile phase consisted of ACN–5 m*M* ammonium formate, pH 4.5 (90:10) at a flow-rate of 0.4 ml/min.

[b] Similar results were obtained with C_8 and C_{18} stationary phases.

The solutes exhibited better peak shape on the CN and PFP phases compared to the OH and hydrophobic chain phases (Fig. 5). Asymmetry factors on the CN and PFP phases are 1.08 and 1.06, respectively, for the analysis of a representative solute, oxprenolol. Since oxprenolol has an AF of 1.88 on the OH phase, 1.78 on the C_{18} phases and 5.0 on the C_4 phase, these phases were not investigated further. The other solutes showed similar peak shape behavior on these phases. More efficient peak shape on the CN and PFP phases further improves detection limits in chromatographic systems.

Nortriptyline, a TCA, and pindolol, a β-blocker, were used as representative solutes to show the increase in MS signal when phases are used that retain solutes with a mobile phase of 90% acetonitrile. This mobile phase worked well for all the basic solutes tested. With the hydrophobic chain phases, it has been reported that longer chains more effectively shield the silanol groups and decrease tailing [33]. Thus due to extremely poor peak shape on the C_4 phase, a C_{18} phase was used for comparison with the CN phase. Nortriptyline and pindolol had retention times of 5.0 and 3.9 min on the CN phase with 90% acetonitrile in the mobile phase whereas the C_{18} phase only 26% and 5% acetonitrile in the mobile phase could be used to achieve retention times of 5.3 and 3.6 min. The HPLC–ESI-MS chromatograms of a TCA and a β-blocker on CN and C_{18} phases are shown in Fig. 6. Due to better desolvation with the higher acetonitrile concentrations, the CN phase gave a MS signal 3.2 and 7.9 times as large, respectively, as the signal observed with the C_{18} phase. Other phases (such as PFP) that retain solutes with 90% acetonitrile also will produce comparable increases in the ESI-MS signal when compared to hydrophobic chain phases. One hundred percent acetonitrile was used to elute the solutes to further improve the MS signal; however, irreproducible retention times were obtained, possibly due to poor solute solubility in the pure organic solvent.

For adequate throughput, retention times of ~2–6 min are important for HPLC–ESI-MS analyses. If the retention time is greater than 6 min, higher

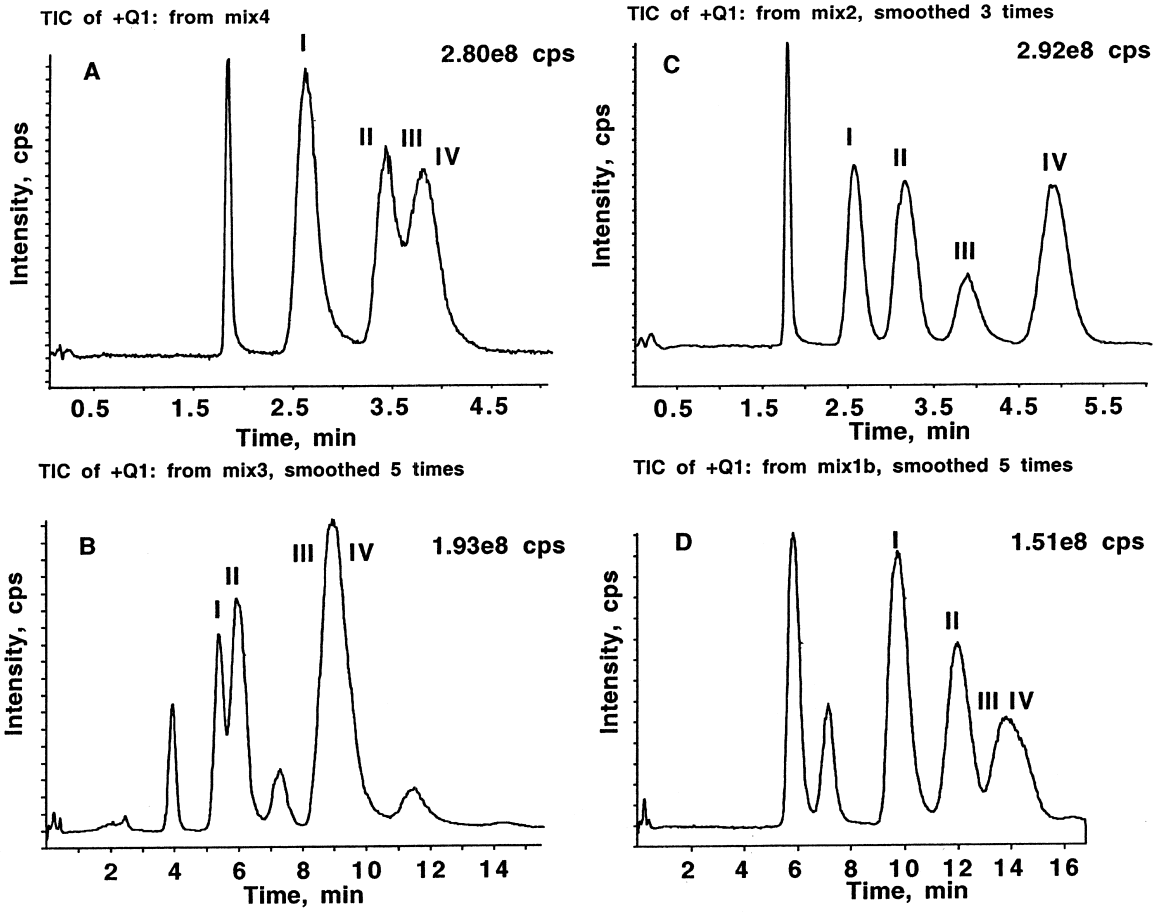

Fig. 4. Typical chromatograms for the HPLC–ESI-MS analysis of TCAs and β-blockers on a CN and PFP phase with 90% acetonitrile in the mobile phase (10% 5 m*M* ammonium acetate, pH 4.5). (A) Chromatogram for the HPLC–ESI-MS analysis of β-blockers on a CN phase; I=timolol, II=labetolol, III=pindolol, IV=oxprenolol. (B) Chromatogram for the HPLC–ESI-MS analysis of β-blockers on a PFP phase; I=atenolol, II=acebutolol, III=alprenolol, IV=propranolol. (C) Chromatogram for the HPLC–ESI-MS analysis of TCAs on a CN phase; I=protriptyline, II=desipramine, III=doxepine, IV=imipramine. (D) Chromatogram for the HPLC–ESI-MS analysis of TCAs on a PFP phase; I=amitriptyline, II=clomipramine, III=nortriptyline, IV=trimipramine.

flow-rates or shorter columns (1 or 2 cm length) can be used to decrease the analysis time. Since acetonitrile is less viscous than aqueous solvents, column backpressure decreases as more acetonitrile is present in the mobile phase. The column pressure on the CN and PFP phases was 28–30 bar when 90% acetonitrile was used in the mobile phase at a flow-rate of 0.4 ml/min. Most HPLC pumps, tubing, etc. easily handle pressures up to 275 bar. Thus flow-rates can be increased significantly to achieve faster analysis times. Chromatograms for the HPLC–ESI-MS analysis of TCAs on a PFP phase at 0.4 and 2.8 ml/min (≈400 μl/min into the source) are compared in Fig. 7. The time for the separation of four of the TCAs was decreased from 14 min per analysis to 2.5 min per analysis.

Stability of CN phases has been questioned in the past. However, retention times of the CN and PFP phases were reproducible after 2200 column volumes or about 8 h (Fig. 8).

Throughout this investigation, the triple quadrupole was employed in the Q1 mode to demonstrate the applicability of this method to single quadrupole MS. Since the ionization process is the same for MS

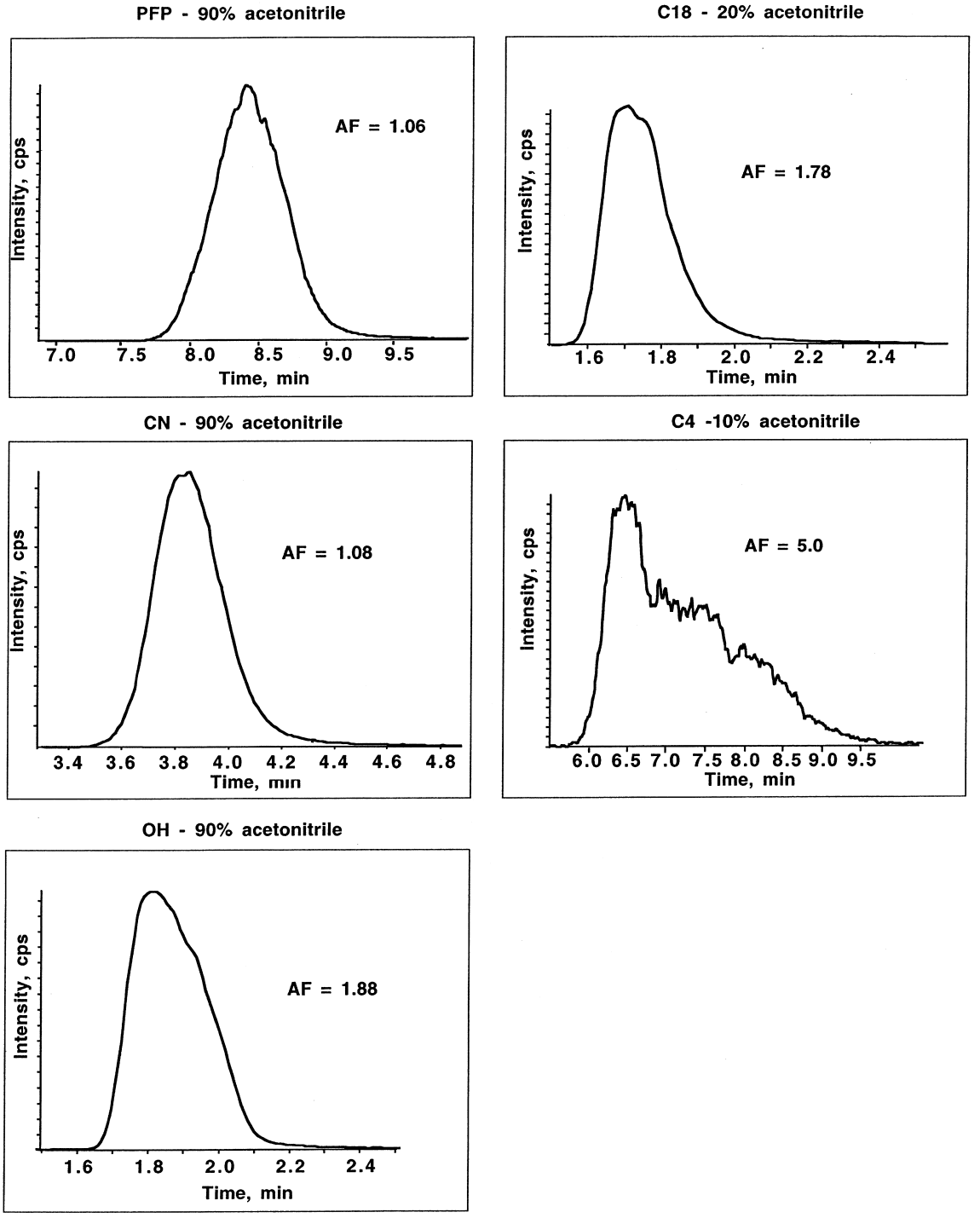

Fig. 5. Chromatograms to compare the peak shape for the HPLC–ESI-MS analysis of oxprenolol by the use of mixtures of acetonitrile–5 m*M* ammonium acetate, pH 4.5 on a C_4, C_{18}, CN, OH and PFP phase.

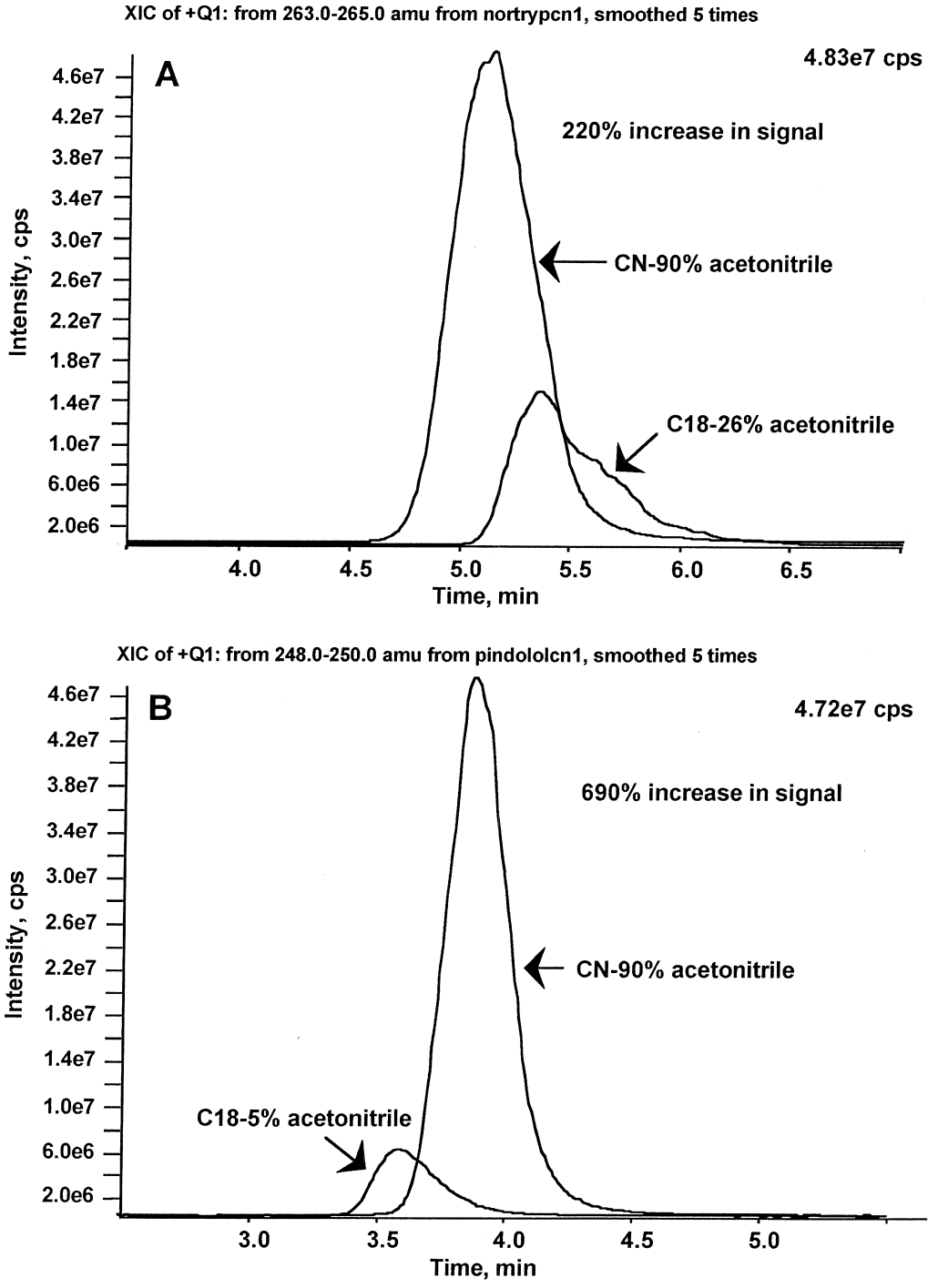

Fig. 6. Chromatograms to show the increase in the ESI-MS signal when phases are used that give retention of solutes at higher acetonitrile concentrations than hydrophobic chain phases. (A) Overlaid chromatogram to show similar retention yet increased signal on a CN phase compared to a C_{18} phase for the HPLC–ESI-MS analysis of nortriptyline (about 25 ng). (B) Overlaid chromatogram to show similar retention yet increased signal on a CN phase compared to a C_{18} phase for the HPLC–ESI-MS analysis of pindolol (about 25 ng).

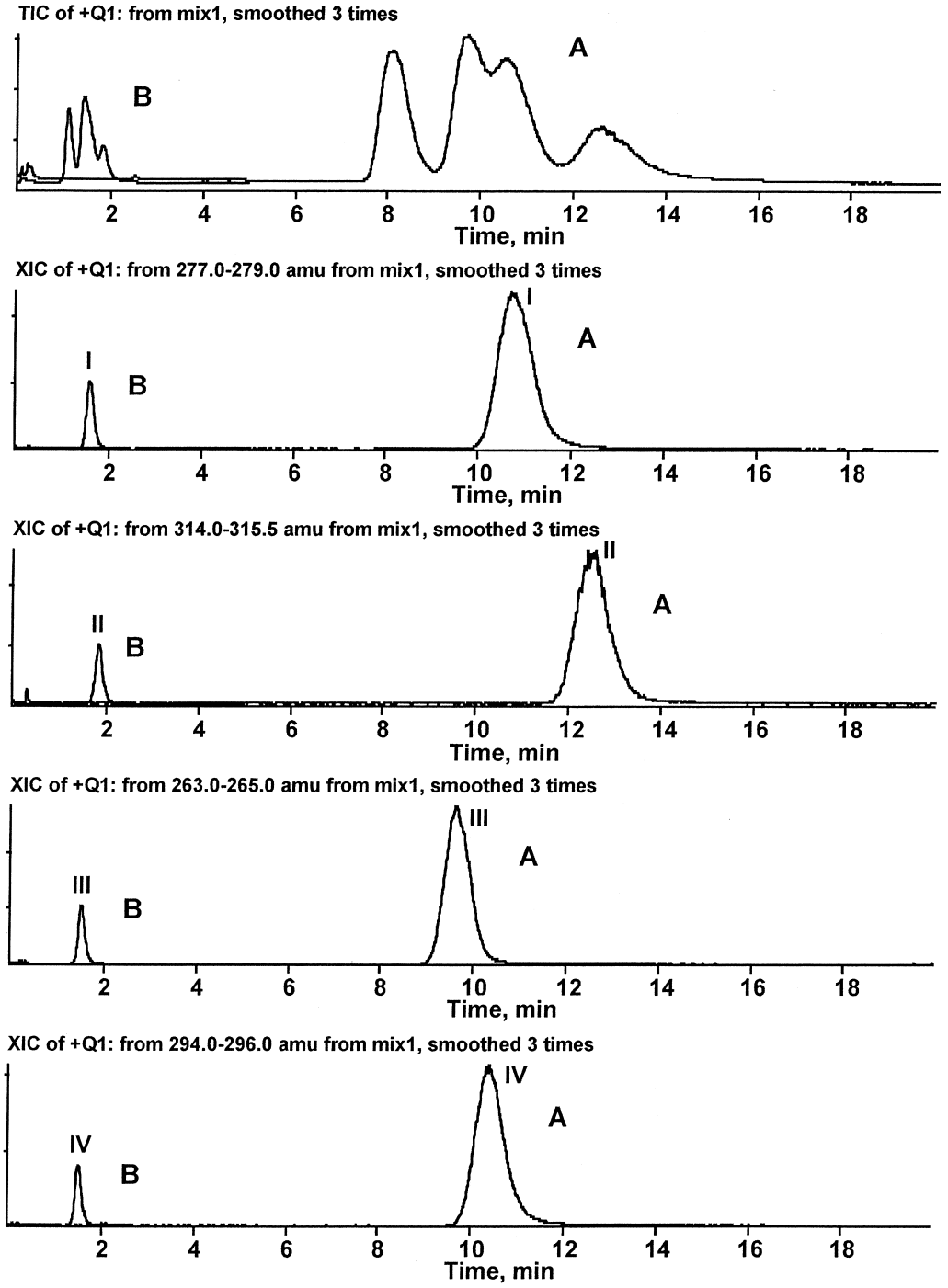

Fig. 7. Overlaid chromatograms to show the reduction of analysis times by the use of much higher flow-rates on a PFP phase that retains solutes with 90% acetonitrile (10% 5 m*M* ammonium acetate, pH 4.5) in the mobile phase. I=Nortriptyline, II=trimipramine, III=amitriptyline, IV=clomipramine. (A) 0.4 ml/min, (B) 2.8 ml/min (≈0.4 ml/min into the ESI source).

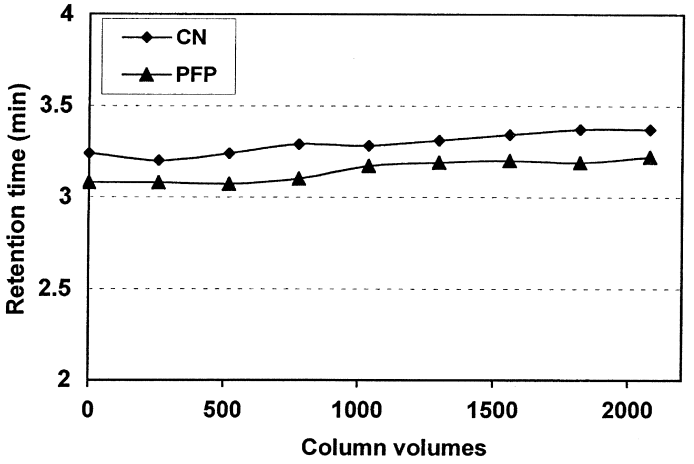

Fig. 8. Plot of the stability and reproducibility of the results for the HPLC–ESI-MS analysis of pindolol with 90% and 80% acetonitrile (10% and 20% 5 m*M* ammonium acetate, pH 4.5) in the mobile phase on the CN and PFP phases, respectively.

and MS–MS analyses, operation in the MS–MS mode would also produce signal improvements when the acetonitrile concentration in the mobile phase is increased.

4. Conclusions

Modified propyl stationary phases for HPLC were used when HPLC was coupled to ESI-MS. The phases, CN and PFP are connected by a propyl chain to a silica backbone and gave good retention (t_R of 2–6 min) of basic solutes with 90% acetonitrile in the mobile phase. These phases showed better peak shape than the popular, C_8 and C_{18} phases, and ion-pairing or ion-suppressing agents were not needed in the mobile phase. In contrast, no more than 40% acetonitrile could be used with the C_8 and C_{18} phases in analysis of the model basic solutes (TCAs and β-blockers). The use of high acetonitrile concentrations (90%), low buffer concentrations ($<$10 m*M*) and the absence of ion-pairing or ion-suppressing agents in the mobile phase provides ideal sensitivity in ESI-MS and makes the CN and PFP phases good for coupling to ESI-MS. The ESI-MS signal is increased by over 200% when 90% acetonitrile is used with the CN and PFP phases compared to the hydrophobic chain phases that require $<$40% acetonitrile for retention of solutes. The CN and PFP phases are stable and the results are reproducible. These phases have the potential of being broadly suitable for the ESI-MS analysis of other solutes with basic functionalities.

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