Transforming chiral liquid chromatography methodologies into more sensitive liquid chromatography–electrospray ionization mass spectrometry without losing enantioselectivity

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Abstract

LC–electrospray ionization (ESI) MS conditions were optimized for the individual chiral separation of 19 compounds of pharmaceutical interest using the macrocyclic glycopeptide-based chiral stationary phases in both polar organic and reversed-phase modes (RPM). The influence of mobile phase composition and MS additive type on sensitivity was investigated for all classes of compounds tested. Compounds with amine or amide groups were efficiently separated, ionized, and detected with the addition of 0.1% (w/w) ammonium trifluoroacetate to the solvent system in either the reversed-phase or polar organic mode (POM). Macrocyclic glycopeptide coupled column technology was initially used to screen all chiral compounds analyzed. Baseline resolution of enantiomers was then achieved with relatively short retention times and high efficiencies on Chirobiotic T, Chirobiotic V or Chirobiotic R narrow bore chiral stationary phases. The polar organic mode offered better limits of detection (as low as 100 pg/ml) and sensitivity over reversed-phase methods. An optimum flow-rate range of 200–400 μl/min was necessary for sensitive chiral LC–ESI-MS analysis.
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1. Introduction

Chirality has long been an important criterion for drug discovery and analysis. As a direct result of the advances made in the LC separation of enantiomers in the 1980s, the Food and Drug Administration developed a new policy for the characterization and testing of enantiomeric compounds [1]. HPLC has become the dominant technique employed for the analysis (and sometimes preparation) of chiral molecules in the pharmaceutical industry [2,3]. Consequently, a variety of chiral stationary phases (CSPs) are commercially available for the enantiomeric separation of stereogenic compounds, although only a few dominate the market. Recently, HPLC coupled to atmospheric pressure mass spectrometry (API-MS) has become a popular method for the analysis of pharmaceutical compounds due to its sensitivity, speed, and specificity. However, most existing enantiomeric separation methods were developed using UV detection and they cannot

be directly used with LC–MS due to various mobile phase and additive incompatibilities. Simply changing the mobile phase and additives of known enantioselective LC methods to ones that are API-MS-compatible often results in diminished or lost enantiomeric separations. When converting existing chiral LC methods to chiral LC–MS methods, the goal is to achieve the highest sensitivity and gain the increased information of MS without losing enantiomeric resolution and/or selectivity.

Many chiral LC methods require the use of the normal-phase mode for the enantiomeric separation. When coupled with mass spectrometric ionization sources, such as electrospray ionization (ESI) these techniques, however, are highly incompatible [4]. Normal-phase solvents such as hexane do not support the formation of ions which is well known to be critical for ESI [5]. In addition, high hexane composition introduces a possible explosion hazard in the presence of the high voltage of the electrospray needle for ESI [4]. In order to overcome these difficulties, there is no other choice but to employ extensive post-column addition of MS-compatible solvent systems [6,7], which can severely affect resolution and sensitivity. This type of massive post-column dilution

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is only acceptable when one is not sample limited and has very good separations. However, the compatibility of the reversed-phase mode (RPM) and the polar organic mode (POM) with LC–MS interfaces and detection (without the need for post-column dilution) makes them attractive direct approaches for the LC–MS of chiral compounds.

In order to achieve optimal ESI-MS sensitivity, there are restrictions not only on solvent type, but also on solvent additives. Commonly used LC additives, such as phosphate buffers, are incompatible with MS as they can contaminate ionization sources and decrease sensitivity [8]. Unfortunately when doing enantiomeric separations, simply changing the additive type to one that is MS-compatible can decrease or eliminate enantiomeric resolution and/or selectivity. Many other chromatographic parameters (such as flow-rate) also can impact MS detection [9–11].

The macrocyclic glycopeptide based chiral stationary phases, teicoplanin [12–15], vancomycin [16–18], and ristocetin A [19,20], have been used successfully in the enantiomeric separation of a variety of chiral compounds. The multi-modal capability (normal-phase, reverse phase, or polar organic modes) of these CSPs enables facile interfacing with MS ionization sources [15,21–23]. The usefulness of these macrocyclic stationary phases results from their broad selectivity and in the complementary nature of these columns, making them ideal candidates for chiral LC–MS method development [14,23].

LC chiral method development often employs the technique of directly coupling columns in series to resolve and screen a variety of chiral compounds [24–26]. Kristensen et al. used a combination of achiral and chiral columns to resolve methadone enantiomers in serum [24]. Johnson and Wainer coupled two chiral columns to improve the resolution of chiral ketones and diastereomeric alcohols [25]. More recently, Wang et al. reported the coupling of the macrocyclic glycopeptide CSPs as a fast column screening approach for HPLC [26]. All three macrocyclic glycopeptide columns, Chirobiotic R, Chirobiotic T, and Chirobiotic V were coupled together with zero dead volume fittings forming a single chiral screening column. The applicability of this technique for HPLC coupled to atmospheric pressure chemical ionization (APCI) mass spectrometry was demonstrated by Bakhtiar and co-workers [21,22].

In this study, the optimal conditions for doing chiral LC–ESI-MS were determined and the potential of adapting macrocyclic glycopeptides as a broadly applicable, LC–MS-compatible class of CSPs was considered. The glycopeptide coupled column system was used to screen a variety of compounds of pharmaceutical interest by LC–ESI-MS. The enantiomeric separations were then optimized on Chirobiotic T (teicoplanin), Chirobiotic V (vancomycin), or Chirobiotic R (ristocetin A) chiral stationary phases in either reversed-phase or polar organic phase mode. MS-compatible mobile phases were evaluated for each class of chiral compound tested. The influence of flow-rate

on MS detector sensitivity, as well as on chromatographic parameters such as resolution and selectivity were also investigated.

2. Experimental

2.1. Reagents and samples

Ammonium trifluoroacetate (NH_4TFA), ammonium acetate (NH_4OAc), and trifluoroacetic acid (TFA) were purchased from Aldrich (Milwaukee, WI, USA). All racemic compounds were obtained from Sigma (St. Louis, MO, USA), except phensuximide, coumafuryl, chloroquine, trimipramine, and metoprolol which were donated by Astec (Whippany, NJ, USA). HPLC grade methanol (MeOH) and water were acquired from Fisher (Pittsburgh, PA, USA). Formic acid and 100% pure ethanol (EtOH) were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Apper Alcohol (Shelbyville, KY, USA), respectively. All compounds were dissolved in either 100% methanol or methanol–water (50:50) and diluted to $10\,\mu g/ml$ prior to injection.

2.2. Apparatus and instrument conditions

Experiments were performed on a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with an ESI ion source. The MS system was operated in the positive ion mode using the selected ion monitoring (SIM) mode of detection at the appropriate $[M + H]^+$ for each compound. Nitrogen (Praxair, Danbury, CT, USA) was used as both sheath and auxiliary gases. Ultra-high purity helium (Linweld, Lincoln, NE, USA) was used as the dampening gas in the ion trap. Sheath and auxiliary gases ranged between 35 and 40 and 10–40 arbs (arbitrary units), respectively. MS parameters were optimized to the following: source voltage $= +4.50\,kV$, capillary voltage $= 10.0\,V$, tube lens offset $= 30.0\,V$, and capillary temperature $= 200°C$.

Separations were carried out at room temperature on $250 \times 4.6\,mm$ i.d. or $250 \times 2.0\,mm$ i.d. Chirobiotic R, Chirobiotic V, or Chirobiotic T chiral columns from Astec. All three columns, Chirobiotic R, Chirobiotic V, and Chirobiotic T ($100 \times 4.6\,mm$ i.d.) were also coupled together with zero dead volume fittings for screening of chiral compounds. The CSPs were coupled together in order of increasing polarity, ristocetin A, followed by vancomycin, followed by teicoplanin (RVT). Reversed-phase systems contained either ethanol:water or methanol:water with an MS-compatible reagent such as ammonium acetate, formic acid, TFA or NH_4TFA. Polar organic systems contained a mixture of 0.1% (w/w) NH_4TFA in methanol and 100% methanol, at varying compositions. Mobile phase flow-rates varied from 200 to $800\,\mu l/min$.

3. Results and discussion

3.1. Using MS-compatible mobile phases

Unlike the normal-phase mode, the ability of RPM and POM to seamlessly interface with MS does not place very many limitations on the assay. However, when these methods (which were developed using UV detection) are converted to ones that are MS-compatible, a number of factors, including chromatographic selectivity and efficiency, additive volatility, and ion formation or suppression, must be considered. In this study, 19 chiral compounds of pharmaceutical interest, such as β-blockers, antidepressants, and antimalarial drugs, were individually separated using volatile MS additives. Fig. 1 shows the structures and monoisotopic molecular masses for all the compounds tested.

For reversed-phase solvent systems, formic acid and TFA were used for protonation of the analytes, in addition to salts such as ammonium acetate and ammonium trifluoroacetate. The traditional mobile phase composition for the polar organic mode usually consists of methanol and/or acetonitrile and small percentages of glacial acetic acid and triethylamine (TEA). Although, acetic acid and TEA are volatile additives the combination of the acidic and basic additives can cause the neutralization of analyte ions [5]. For all the compounds tested in the polar organic mode, the use of 0.1% NH_4TFA instead of a combination of acetic acid and TEA allowed the enantiomeric separation and proper ionization of the analytes for MS detection.

In general, the chromatographic resolution and selectivity were not significantly affected by changing the nature of the LC mobile phase to MS-compatible additives described herein as long *as the optimized concentration levels of these additives were maintained*. However, the choice of volatile additive had a significant impact on signal intensity. For chloroquine enantiomers, for example, the use of ammonium trifluoroacetate provided a signal intensity that was one order of magnitude higher than that found when the same concentration (wt.%) of ammonium acetate was used. In addition, it was found that compounds with amine or amide functional groups could be effectively ionized with ammonium trifluoroacetate in both the RPM and POM. However, coumafuryl, a compound which does not contain any of those functional groups, could not be ionized at all with the addition of NH_4TFA. Ionization and separation of coumafuryl could only be achieved using a small percentage (0.001%) of TFA in the reversed-phase system.

3.2. Limits of detection for ESI-MS: reversed-phase versus polar organic

The limits of detection for reversed-phase and polar organic phase LC–ESI-MS methods were investigated. Compounds were detected by SIM at their corresponding $[M + H]^+$ values. Concentrations of 0.0001, 0.001, 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 μg/ml were injected of each compound. As can be seen in Table 1, detection limits as low as 100 pg/ml and high sensitivities (sensitivity as defined by IUPAC is the slope of the dose–response curve [27]) were achieved for many analytes such as the β-adrenergic blockers in the polar organic mode. Compounds, such as the amino acids separated in the reversed-phase mode, had the worst limits of detection and the lowest sensitivities for ESI-MS detection of all compounds tested. The differences in detection limit and sensitivity may be attributed to the significant presence of water in reversed-phase analysis. Since ESI is a desorption ionization process, the two most important considerations for MS detection are the creation of ions and the desolvation of the analyte. As it is well known that although water supports the formation of ions, its surface tension and solvation energy make it more difficult to desolvate than organic solvents such as methanol or ethanol [5], contributing greatly to the lower ionization efficiency of reversed-phase mode separations, compared to polar organic separations when using ESI-MS detection. The sensitivity of MS detection of amino acids in the reversed-phase mode, however, is increased tremendously by switching ionization sources from ESI to APCI [23].

Table 1 also presents the linearity and r^2 values of the calibration curves for selected compounds. The calibration curves were linear over two orders of magnitude. The limits of detection and linearity of both polar organic and reversed-phase methods demonstrate their applicability for mass limited sample analysis. Typical examples of mass limited analysis of chiral samples include those found in biological matrices as well as pharmacokinetic and pharmacodynamic studies.
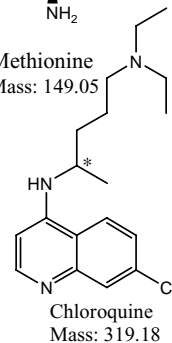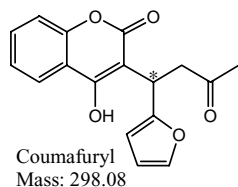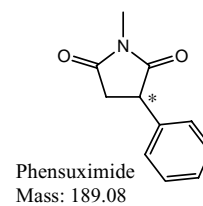
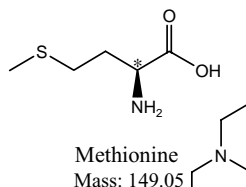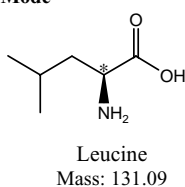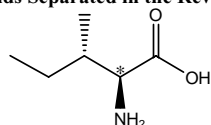## 3.3. Flow-rate and sensitivity for ESI

Mass spectrometers are generally considered mass flow-dependent detectors; that is, detector response is proportional to the total number of molecules being detected per unit of time [28]. As a result, flow-rate is an important parameter in the optimization of any chiral or non-chiral

Table 1
Limits of detection for selected compounds

Compound	SIM (*m/z*)	Column	Linearity	r^2	LOD[a]
Leucine	132	T	$y = 5E + 06x + 623361$	0.9976	50 ng/ml
Atenolol	267	T	$y = 3E + 08x + 6E + 07$	0.9985	100 pg/ml
Promethazine	284	V	$y = 9E + 07x + 2E + 07$	0.9978	1 ng/ml
Fluoxetine	310	V	$y = 4E + 07x + 5E + 06$	0.9976	1 ng/ml

[a] Based on a signal-to-noise ratio = 3; T: Chirobiotic T, V: Chirobiotic V.

Compounds Separated in the Reverse Phase Mode

Isoleucine
Mass: 131.09

Leucine
Mass: 131.09

Methionine
Mass: 149.05

Phensuximide
Mass: 189.08

Coumafuryl
Mass: 298.08

Chloroquine
Mass: 319.18

Compounds Separated in the Polar Organic Mode

Trimipramine
Mass: 294.21

Fluoxetine
Mass: 309.13

Mianserin
Mass: 264.16

Promethazine
Mass: 284.13

Clenbuterol
Mass: 276.08

Terbutaline
Mass: 225.14

Atenolol
Mass: 266.16

Pindolol
Mass: 248.15

Alprenolol
Mass: 249.17

Propranolol
Mass: 259.16

Metoprolol
Mass: 267.18

Labetolol
Mass: 328.18

Nicardipine
Mass: 465.05

Bupivacaine
Mass: 288.22
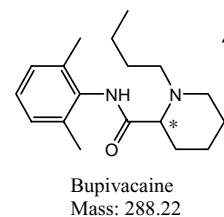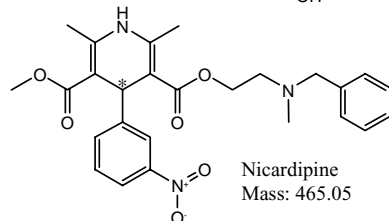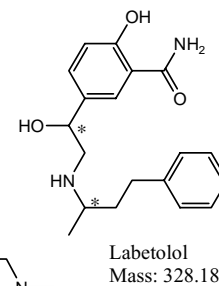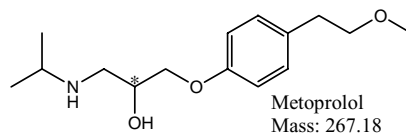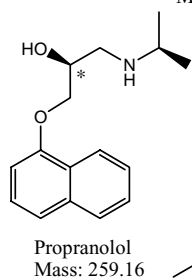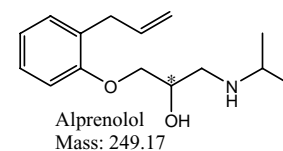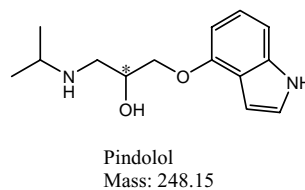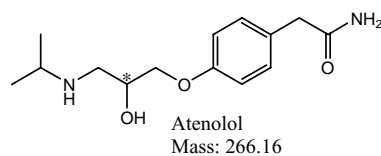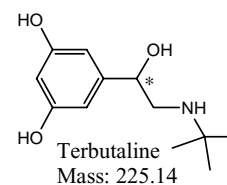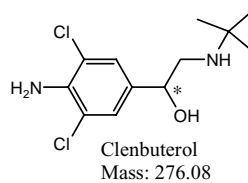
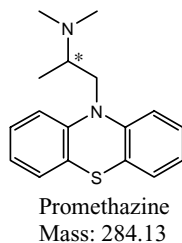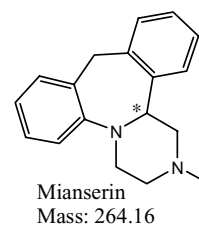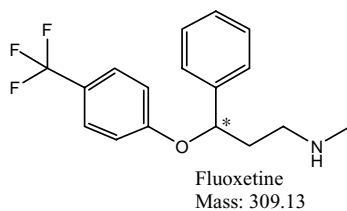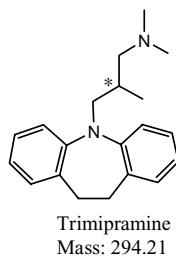Fig. 1. Structures and monoisotopic molecular masses of enantiomeric compounds separated by reversed-phase and polar organic phase modes. Chiral centers are indicated with asterisks (*).
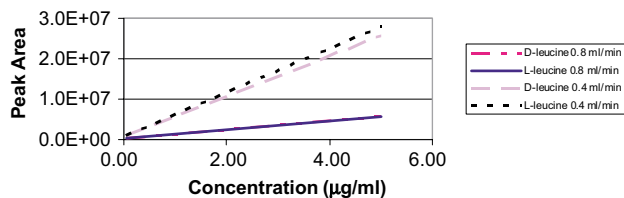
Fig. 2. Influence of flow-rate on the sensitivity of detection for LC–ESI-MS. Dose–response curves for D- and L-leucine are shown for 0.8 and 0.4 ml/min. The slopes of the calibration curves at 0.4 and 0.8 ml/min were approximately 6×10^6 and 9×10^5, respectively.

method. The effect of flow-rate on sensitivity was evaluated for leucine enantiomers. To our knowledge, specific data on exactly how much the reduction of flow-rate affects MS detection sensitivity has not been published. Using a 4.6 mm i.d. Chirobiotic T column flow-rate was varied from 400 to 800 μl/min. Fig. 2 shows the dose–response curves for D- and L-leucine at the two different flow-rates. The sensitivity for leucine at 400 μl/min was nearly an order of magnitude higher than that found at 800 μl/min. This observed behavior supports the known theory that ion sampling and gas phase ionization in ESI play a predominant role in determining sensitive detector response. Thus, ESI-MS detectors seem to be concentration-sensitive [28].

3.4. Flow-rate and chromatographic parameters

In this study, we determined that the use of narrow bore columns allowed for facile LC–ESI-MS interfacing without compromising enantioselectivity or chromatographic resolution. The optimum flow-rate using these columns was then investigated for the separation of clenbuterol enantiomers. Fig. 3 shows the separation of clenbuterol enantiomers on Chirobiotic T at flow-rates varying from 100 to 600 μl/min. Flow-rates greater than 600 μl/min could not be evaluated due to high column back pressure. At the highest flow-rates, decreased peak efficiencies were observed ($N < 2000$ plates). While resolution improved with decreasing flow-rate, selectivity remained relatively constant ($\alpha \sim 1.2$). Interestingly, a flow-rate of 300 μl/min resulted in the best overall resolution, 3.08, and peak efficiencies ($N > 5000$ plates). This observation could possibly be attributed to attaining an optimum linear velocity for the narrow bore column in conjunction with the ESI source resulting in the best chromatographic and MS response.

The smaller column diameter also resulted in an increase in detector sensitivity over conventional columns (data not shown) which can be attributed to the increased sample concentration at the detector. This supported the findings of Abian et al., which stated that samples separated with narrow bore columns were 5 times more concentrated than samples
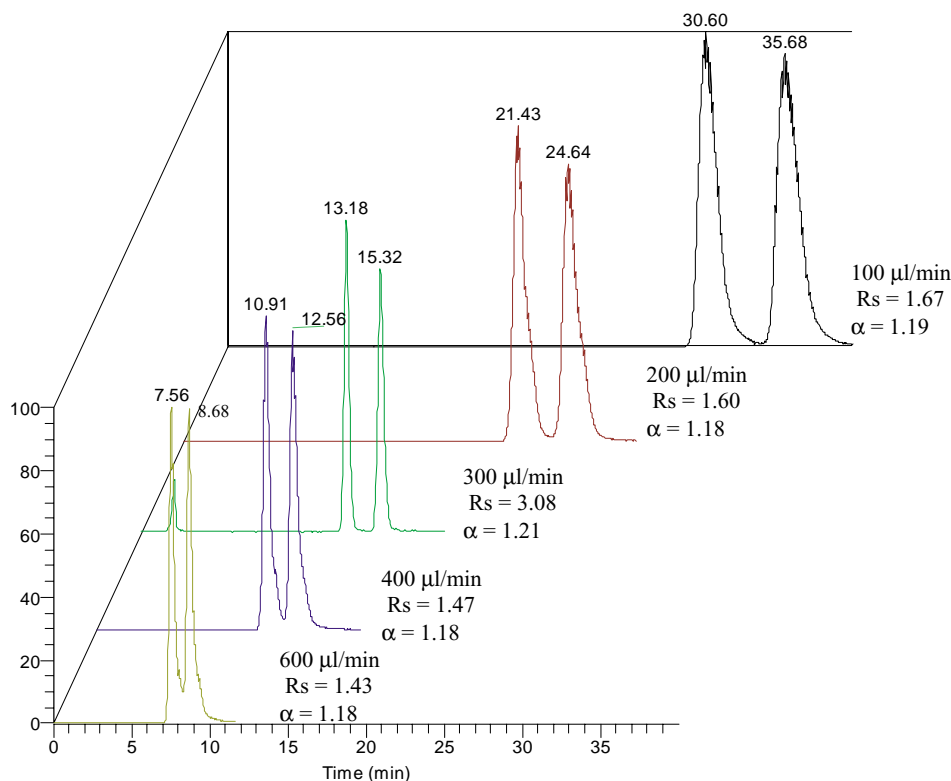

Fig. 3. Effect of flow-rate on the separation of clenbuterol enantiomers. LC–ESI-MS in SIM mode was used at *m/z* 278.0. An optimum flow-rate of 300 μl/min provided for the best resolution and enantioselectivity. The separation conditions for clenbuterol are reported in Table 2. Rs: resolution, α: selectivity.

run on conventional columns having the same length [28]. As a result of the enhanced detector response, the amount of sample necessary for detection can be decreased. The use of the narrow bore columns also allowed for a significant decrease in solvent consumption over columns run at typical 4.6 mm i.d. flow-rates.

3.5. Coupled column for chiral screening

The macrocyclic glycopeptide coupled column (RVT) was originally developed for 4.6 mm i.d. columns coupled to UV detection [26]. As previously mentioned, the applicability of the RVT technology has already been demonstrated for LC coupled to APCI-MS [21,22] with conventional columns. In our study, the RVT coupled column technology was used to screen the 19 racemic compounds using LC–ESI-MS. The separations were then optimized using narrow bore (2.0 mm i.d.) glycopeptide columns. These molecules were analyzed in either the reversed-phase mode or the polar organic mode. The results of the coupled column screening and the optimized chiral separation conditions for each compound are listed in Table 2.

According to Wang et al., if a split peak is observed on the glycopeptide coupled column, a baseline separation can be expected on at least one of the three columns, Chirobiotic R, Chirobiotic V, or Chirobiotic T [26]. Resolutions as

Table 2
Results of chiral method development using the macrocyclic coupled column system

Compound number	Compound name	Classification	Coupled column Rs	α	Optimized column (R/V/T)	Optimized mobile phase conditions	Optimized column Rs	α
Reversed-phase mode								
1	Isoleucine	Amino acid	0.40	1.11	T	50:50 (100% EtOH: 100%) 0.4 ml/min	1.73	1.26
2	Leucine	Amino acid	0.55	1.07	T	50:50 (100% EtOH: 100%) 0.4 ml/min	3.45	1.33
3	Methionine	Amino acid	0.37	1.06	T	80:20 (1.0% NH_4TFA in MeOH: 100%) 0.4 ml/min	5.24	1.57
4	Phensuximide	Anticonvulsant	0.34	1.04	R	66:34 (0.1% NH_4OAc in: 100% MeOH) 0.4 ml/min	1.74	1.12
5	Coumafuryl	Rodenticide	1.07	1.10	V	85:15 (0.001% TFA in: 100% MeOH) 0.4 ml/min	1.53	1.43
6	Chloroquine	Antimalarial	0.17	1.01	V	10:90 (0.1% NH4TFA in MeOH: 0.1% formic acid in) 0.3 ml/min	1.92	2.53
Polar organic mode								
7	Trimipramine	Antidepressant	0.12	1.01	V	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.50	1.11
8	Fluoxetine	Antidepressant	0.36	1.03	V	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.50	1.12
9	Mianserin	Antihistamine	0.67	1.08	V	30:70 (0.1% NH_4TFA in MeOH: MeOH) 0.4 ml/min	1.78	1.77
10	Promethazine	Antihistamine	0.67	1.05	V	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.2 ml/min	1.58	1.20
11	Clenbuterol	β-Adrenergic agonist	0.43	1.03	T	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	3.08	1.21
12	Terbutaline	β-Adrenergic agonist	2.52	1.18	T	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	2.67	1.36
13	Atenolol	β-Adrenergic blocker	0.44	1.03	T	50:50 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.4 ml/min	1.52	1.13
14	Pindolol	β-Adrenergic blocker	0.38	1.02	T	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.67	1.12
15	Alprenolol	β-Adrenergic blocker	1.82	1.11	T	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.71	1.13
16	Propranolol	β-Adrenergic blocker	0.55	1.03	T	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.58	1.13
17	Metoprolol	β-Adrenergic blocker	1.39	1.09	V	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.45	1.12
18	Nicardipine	Calcium channel blocker	0.71	1.07	V	10:90 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.4 ml/min	1.43	1.68
19	Bupivacaine	Local anesthetic	0.44	1.03	V	34:66 (0.1% NH_4TFA in MeOH: 100% MeOH) 0.3 ml/min	1.26	1.13

Rs $= 2(t_2 - t_1)/(w_1 + w_2)$; where t_2 and t_1 are the retention times and w_2 and w_1 are the baseline peak widths of the second and first peak, respectively. $\alpha = (t_2 - t_0)/(t_1 - t_0)$ where T: Chirobiotic T, V: Chirobiotic V, and R: Chirobiotic R.
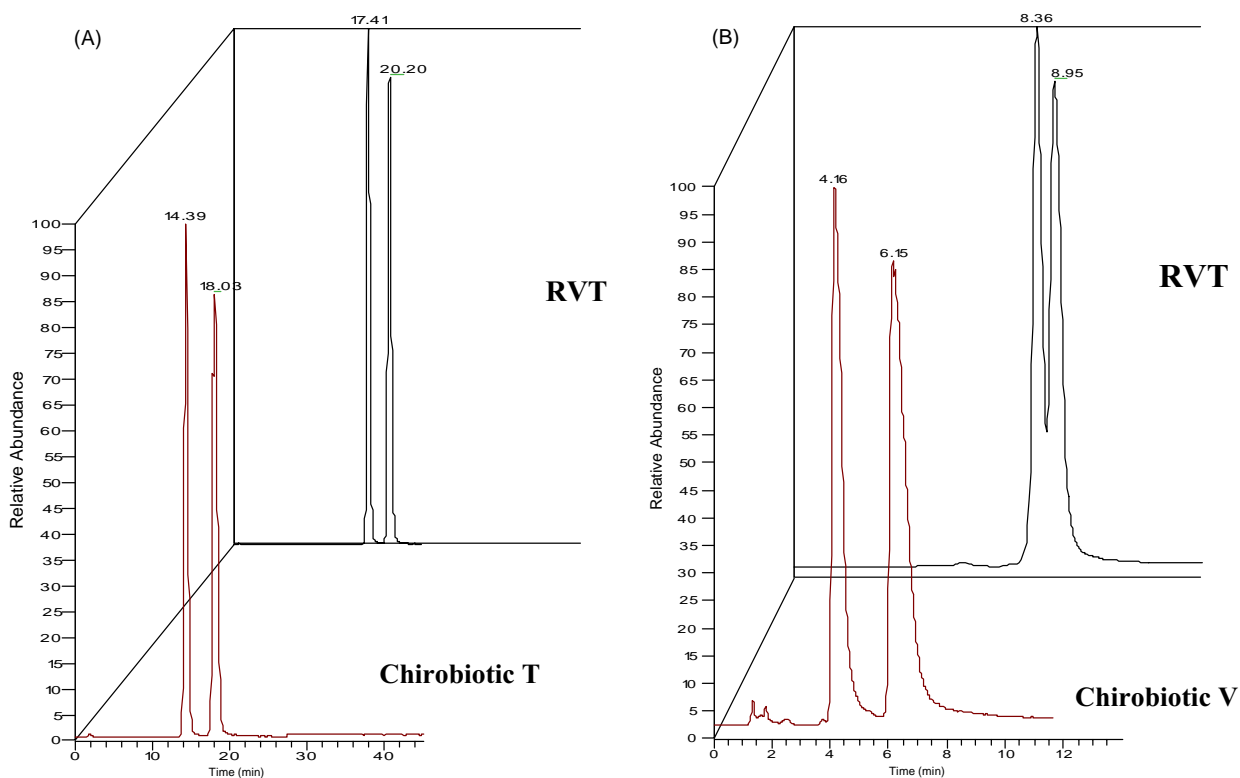
Fig. 4. Examples of chiral compounds screened on the RVT column then optimized on Chirobiotic T or Chirobiotic V in polar organic phase mode. (A) Separation of terbutaline enantiomers, SIM at *m/z* 226.0; (B) separation of mianserin enantiomers, SIM at *m/z* 265.0. Optimized conditions are reported in Table 2.
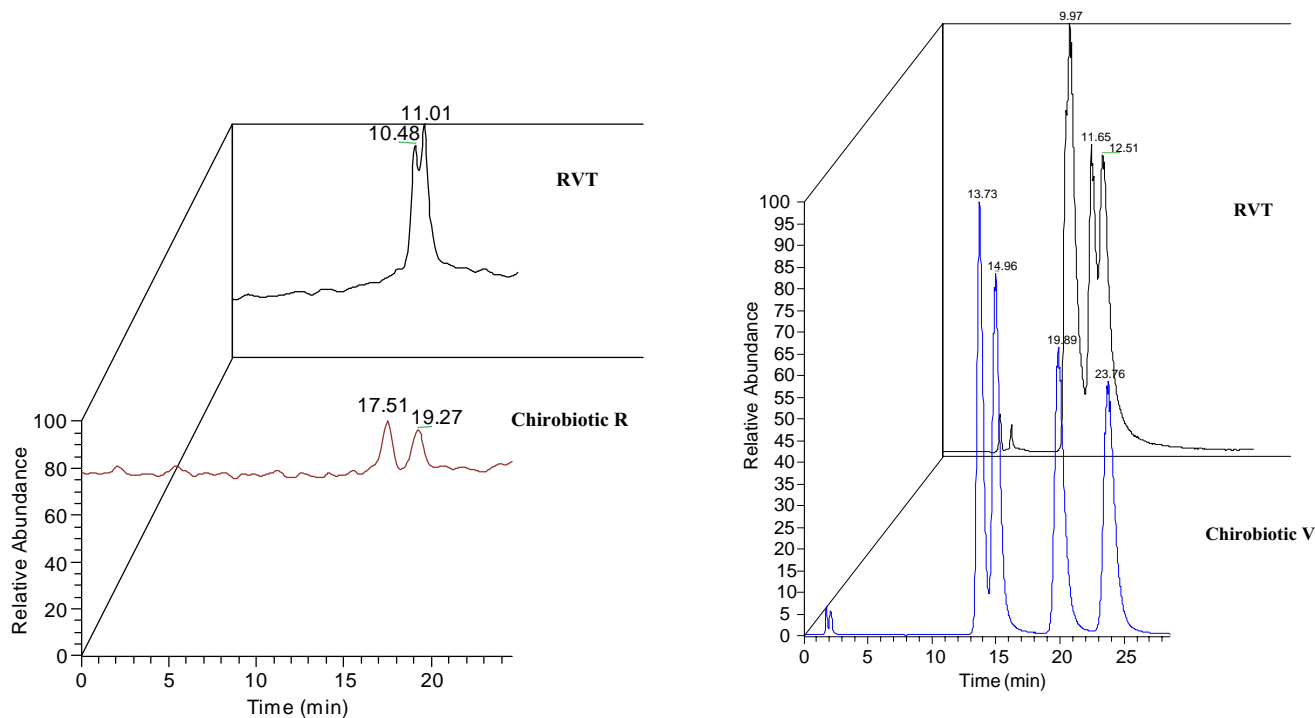

Fig. 5. Reversed-phase separation of phensuximide enantiomers screened on RVT column then optimized on Chirobiotic R. LC–ESI-MS in SIM mode was used at *m/z* 190.0. Separation conditions are reported in Table 2.

Fig. 6. Coupled column screening technique applied to labetolol, a compound with two chiral centers. Separation was optimized on Chirobiotic V under the following conditions: 34:66 (0.1% NH$_4$TFA in methanol:100% methanol); flow-rate: 0.3 ml/min, SIM at *m/z* 329.0.

low 0.12 on the RVT coupled column were able to produce baseline resolutions when conditions were optimized on at least one of the macrocyclic glycopeptide columns. All separations were optimized with run times less than 25 min on the 2.0 mm i.d. Chirobiotic columns. Fig. 4 illustrates examples of compounds screened on the RVT column then optimized on the Chirobiotic T or Chirobiotic V columns. Fig. 5 shows the RVT screen and the baseline reversed-phase separation of phensuximide enantiomers on the Chirobiotic R column. The coupled column screening technique can also be applied to compounds with more than one chiral center such as labetolol (see Fig. 6).

4. Conclusions

In this study, existing chiral LC methods were adapted to make LC–ESI-MS-compatible ones. Some general rules of thumb when converting these methods to MS amenable methodologies are as follows: (a) polar organic mobile phases are most compatible and easily adaptable to chiral LC–ESI-MS analysis. (b) Normal-phase methods are incompatible with direct LC coupling to ESI-MS. They can be used if post-column dilutions of a large excess of ESI-MS-compatible solvents is acceptable in terms of sensitivity and band broadening. (c) When possible avoid high water content reversed-phase methods when using ESI-MS detection as it tends to decrease the ionization efficiency. However, switching to APCI for reversed-phase separations produces much greater sensitivity. (d) Ammonium trufluoroacetate enhances ionization for molecules with amine or amide functionalities. (e) Optimized concentrations levels of additives should be maintained when converting existing chiral LC methods to LC–MS-compatible methodologies.

In addition, the applicability of the macrocyclic glycopeptide coupled column was demonstrated for the rapid LC–ESI-MS screening of a variety of chiral compounds of pharmaceutical interest. Slight split peaks on the RVT coupled column provided for baseline separations on at least one of the three narrow bore Chirobiotic columns. Optimum flow-rates for ESI-MS using these columns ranged between 200 and 400 μl/min. Clearly, LC–ESI-MS can be used as a valuable tool for chiral drug discovery and development.

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