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Development and validation of a GC method for quantitative determination of enantiomeric purity of a proline derivative

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ABSTRACT

Enantioselective HPLC, SFC and GC methods were evaluated for separation and quantitative determination of chiral purity of (2*R*,4*R*)-1-(1-tert-butoxyvinyl)-4-methoxypyrrolidine-2-carboxylic acid [(2*R*,4*R*)-TBMPCA], a common building block in organic synthesis. All three separation methods can provide baseline resolution of (2*R*,4*R*)-TBMPCA and its enantiomer (2*S*,4*S*)-TBMPCA; however, both enantioselective HPLC and SFC are unsuitable for quantitation of low levels of the undesired enantiomer in (2*R*,4*R*)-TBMPCA. Comparatively, the enantioselective GC method not only separates the derivatized enantioselective pair with resolution as high as 4, but also was shown to be sufficiently linear, precise, and accurate to enable quantitation of derivatized (2*S*,4*S*)-TBMPCA down to 2.4 μg/ml (0.04% of nominal concentration). The sample derivatization procedure is simple, and no sample clean-up is needed before injecting samples for enantiomeric GC analysis. Compared to the enantioselective HPLC and SFC methods, the enantioselective GC method is advantageous because of its high efficiency and high sensitivity.

1. Introduction

As part of continuing efforts to improve the safety and efficacy of drugs, both pharmaceutical companies and regulatory agencies have focused on impurity control for active pharmaceutical ingredients (API) [1]. For any drug candidate, an enantiomeric impurity can potentially produce different pharmacological, toxicological, metabolic, and pharmacokinetic properties within the chiral environment of these biological systems [2–4]. FDA Guidance infers that control of enantiomeric impurities is critical in pharmaceutical manufacturing [5].

Separation of pharmaceutical enantiomers can be achieved using different chromatographic techniques such as gas chromatography (GC) [6,7], supercritical fluid chromatography (SFC) [8,9], capillary electrophoresis (CE) [10], capillary electrochromatography (CEC) [11] and high performance liquid chromatography (HPLC) [12–14]. HPLC remains the most popular technique because it offers several practical advantages: (i) high column reproducibility, (ii) broad applicability to compounds of a wide range of polarities, and (iii) a large number of commercially available chiral stationary phases (CSPs). However, enantioselective HPLC suffers from low column efficiency [15]. In order to achieve both adequate resolution between enantiomeric pairs and other impurities, long separation times are often needed due to low peak capacity. At the same time, broad peaks require greater sample loading to detect low levels of chiral impurities (e.g., 0.1% level), which is generally limited by the compound solubility and column sample capacity.

An alternative to the standard enantioselective HPLC process is SFC, which utilizes supercritical CO_2 as a major mobile phase solvent using the same enantioselective HPLC columns with standard normal phase mode. The low viscosity and high diffusivity of supercritical CO_2 allow higher flow rates resulting in shorter run times. At the same time, higher diffusivity leads to lower mass transfer resistance resulting in better column efficiencies and sharper peaks [15]. These factors can enable SFC to provide better detection with less sample load onto the column; however, due to some practical challenges with the technique, the signal-to-noise has tended to be suboptimal, making this technique rarely used for routine analysis [9,15].

Another enantioselective separation technique is GC, which offers the advantages of high efficiency, sensitivity and speed of separation. Because of its enormous separation power, enantioselective GC makes it possible for simultaneous analysis of enantiomeric mixtures and other potential impurities or contaminants in one run, assuming one can volatilize the molecules of interest [16,17]. Flame ionization detection (FID), which is common on GC systems, provides a straightforward and universal detection format, making it a practical alternative for compounds lacking strong chromophores.

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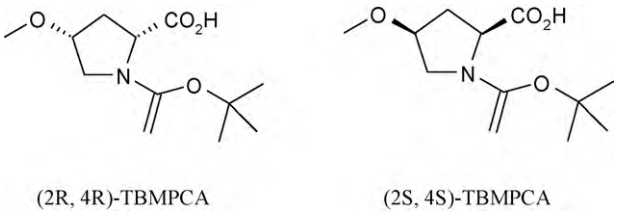

Fig. 1. Proline derivative (2*R*,4*R*)-TBMPCA and its enantiomer (2*S*,4*S*)-TBMPCA.

Enantioselective HPLC, SFC and GC all have potential advantages and disadvantages for use in practical pharmaceutical analysis. We therefore have conducted a comparison study of these techniques to assess the relative merits of each for enantioselective separation of (2*R*,4*R*)-1-(1-tert-butoxyvinyl)-4-methoxypyrrolidine-2-carboxylic acid [(2*R*,4*R*)-TBMPCA] and its (2*S*,4*S*) enantiomeric impurity (see Fig. 1). (2*R*,4*R*)-TBMPCA, a proline derivative, serves as a very important building block in pharmaceutical synthesis [9,18]. There is wide interest in a quantitative enantioselective method for this compound and its analogs; however, due to its carboxylic acid functional group and its lack of a strong UV chromophore, enantioselective separation and determination of trace level of the (2*S*,4*S*) enantiomer in (2*R*,4*R*)-TBMPCA is challenging. The relative merits of enantioselective HPLC, SFC and GC for quantitation of this model chiral pharmaceutical building block (and its enantiomer) have been evaluated.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol, isopropanol (IPA), acetonitrile (ACN), heptane, spectrophotometric grade trifluoroacetic acid (TFA) and trimethylsilyldiazomethane (2 M hexanes) were purchased from Aldrich. (2*R*,4*R*)-TBMPCA,(2*S*,4*S*)-TBMPCA, (2*R*,4*R*)-1-(1-tert-butoxyvinyl)-4-hydroxypyrrolidine-2-carboxylic acid, (2*S*,4*S*)-1-(1-tert-butoxyvinyl)-4-hydroxypyrrolidine-2-carboxylic acid and (2*S*,4*R*)-1-(1-tert-butoxyvinyl)-4-hydroxypyrrolidine-2-carboxylic acid were prepared as described in [18].

Chiralcel OD-H and OJ-H, and Chiralpak AD-H, AS-H and IC columns were purchased from Chiral Technologies (West Chester, PA). 2,6-Di-*O*-pentyl-3-trifluoroacetyl derivative of γ-cyclodextrin (G-TA) modified capillary GC column (30 m × 0.25 mm) was obtained from Astec Inc. (Whippany, NJ) and the dimethylsilyl-beta-cyclodextrin (CYCLOSILB) modified capillary GC column (30 m × 0.32 mm × 0.25 μm) was obtained from J&W Scientific (Palo Alto, CA).

2.2. Sample preparation

For the enantioselective GC method, 60 mg/ml of the proline derivative (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA stock standard and sample solutions were separately prepared in methanol. A 100 μl aliquot of each solution was transferred to a vial, followed by addition of 500 μl of methanol and 140 μl of 2 M trimethylsilyl diazomethane. The solutions were allowed to stand at room temperature for 30 min with the vial cap loose since nitrogen is formed during the reaction. After 30 min, the reaction solution was diluted to 1.0 ml with methanol. The nominal concentrations for the proline derivatives (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA were 6 mg/ml.

Since trimethylsilyldiazomethane is volatile, and does not interfere with the separation of derivatized the (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA using the GC method in this study, it was not necessary to destroy the excess of derivatization reagent; therefore, the reaction solution was injected directly [19]. In addition, the formation of the esters using trimethylsilyldiazomethane was confirmed using enantioselective GC/MS.

The derivatized sample stability was studied by injecting the sample for the enantioselective GC analysis every 30 min. It was found that there is no significant changes of the peak areas (±3.0%) of derivatized (2*R*,4*R*)-TBMPCA spiked with 6.0 μg/ml of (2*S*,4*S*)-TBMPCA for reaction times from 30 min to 14 h. After 14 h, some extraneous peaks were observed in the chromatograms, and these unknown peaks may interfere with the detection of derivatized (2*S*,4*S*)-TBMPCA; therefore, the derivatized samples were analyzed between 30 min to 14 h following preparation.

For enantioselective HPLC and SFC separation, (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA were dissolved in ACN to 10 mg/ml for initial method development. UV detection at 210 nm was employed since the compounds lack strong chromophores.

2.3. Equipment

SFC experiments used a SFC Fusion™ A5 analytical instrument (Aurora SFC Systems, Inc. CA) connected to an Agilent 1100 HPLC system. A high pressure flow cell (Palo Alto, CA) was coupled to the HPLC system. Agilent ChemStation version B.04.01 was used for the instrument control and the data collection. An Agilent 1100 HPLC and Agilent 6890 gas chromatograph (Palo Alto, CA) were used for enantioselective HPLC and GC separations, respectively.

3. Results and discussion

3.1. Enantioselective HPLC and SFC methods

AD-H, OD-H, AS-H and OJ-H CSPs and several mobile phase modifiers including ethanol and IPA without any additives, and with TFA as an acid additive were screened for both enantioselective HPLC and SFC separation of (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA to identify the best CSP and mobile phase/modifier. Among the CSPs resolving the enantiomeric pairs, the AS-H column gives the best resolution and an acidic additive helps sharpen the peak due to suppression of ionization of the acidic analytes.

The screening methods were used as a starting point and further method optimization was performed to improve separation by fine tuning the mobile phase/modifier composition and flow rate for both enantioselective HPLC and SFC and adjusting the back pressure for enantioselective SFC. By mainly changing the mobile phase composition, a resolution of 3.5 in 25 min was achieved using the optimized HPLC method as shown in Fig. 2. In both SFC and HPLC, decreasing the proportion of alcohol modifier helps retain the compounds. For example, when the methanol modifier was decreased from 3% to 1% in the SFC method, the sample retention increased significantly, yet there was no improvement in the resolution because of poor peak shapes. Additionally, baseline noise as high as 3 mAU, likely attributable to the CO_2 pump in the SFC system, was observed. Increasing methanol modifier in SFC improves the peak shape as well as decreases the baseline noise slightly, but sacrifices the resolution. The decreased resolution prevented quantitative determination of the minor enantiomeric impurity because of the peak overlap. The back pressure in SFC system also affects the retention and peak shape too. Higher back pressure results in less retention and better peak shape whereas lower back pressure provides more retention and broader peak shape. After fine tuning the back pressure, methanol modifier, and acid additive proportions, it was found that a resolution of 2.1 could be achieved in 5 min with the reasonably good peak shapes using 3% methanol with 0.2% TFA at back pressure of 120 bar (see Fig. 3). It is noted that significant baseline noise (approximately 1 mAU) is still observed

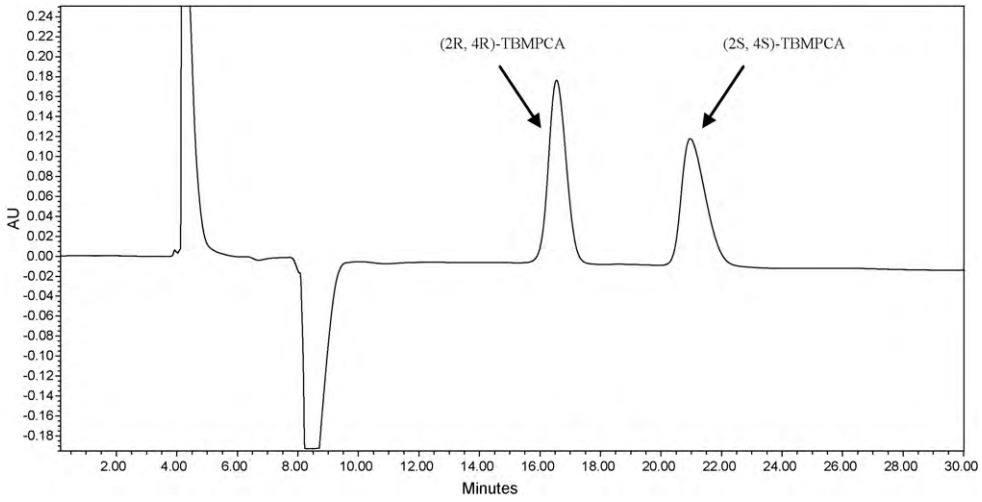

Fig. 2. Typical enantioselective HPLC chromatogram of (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA mixture. Conditions: AS-H column, 4.6 mm × 250 mm; mobile phase, heptane:ethanol:TFA (98:2:0.25, v/v/v); flow rate = 0.8 ml/min; ambient condition; 10 mg/ml (2*R*,4*R*)-TBMPCA,(2*S*,4*S*)-TBMPCA in acetonitrile; UV detection at 210 nm.

with SFC when 3% methanol was used as shown in the expanded chromatogram inserted in Fig. 3.

3.2. Enantioselective GC method

Due to its superior column efficiency, enantioselective GC is a powerful alternative to enantioselective HPLC and SFC. Generally, separation of free carboxylic acids by GC is difficult because of their low volatility, strong adsorption on solid supports and/or dimerization side reactions [20]. Chemical derivatization of carboxylic acids is often performed to increase volatility and enable GC analysis [6,7,19,20]. The ester derivatives are of moderate polarity and are more volatile than their carboxylic acid precursors. Among existing derivatization methods, trimethylsilyldiazomethane is most readily utilized to use to convert carboxylic acids to their methyl esters using methanol as the solvent [6,7,19,20]. The enantioselective GC method can separate the derivatization reagent from derivatized (2*R*,4*R*)-TBMPCA and its enantiomer, therefore, no sample clean-up is needed prior to GC analysis.

3.2.1. Column selection

CYCLOSILB and G-TA columns were investigated for separation of trimethylsilyldiazomethane derivatized proline derivative (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA. As shown in Fig. 4A and B, baseline separation can be achieved using both columns. The derivatized (2*S*,4*S*)-TBMPCA elutes earlier than the derivatized (2*R*,4*R*)-TBMPCA on the CYCLOSILB column, while the elution order is reversed on the G-TA column. The results indicate that the separation mechanism could be different for these two columns. Inclusion could be dominant on CYCLOSILB column, while dipole–dipole interaction is the main mechanism associated with the G-TA column [14].Using the G-TA column, derivatized (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA are separated in 20 min with resolution of approximately 4, while it takes at least 40 min to achieve the baseline separation on the CYCLOSILB column (see Fig. 4A and B). Since the resolution and separation times for the G-TA column are superior to the CYCLOSILB column, the G-TA column was selected for further method development in this study.

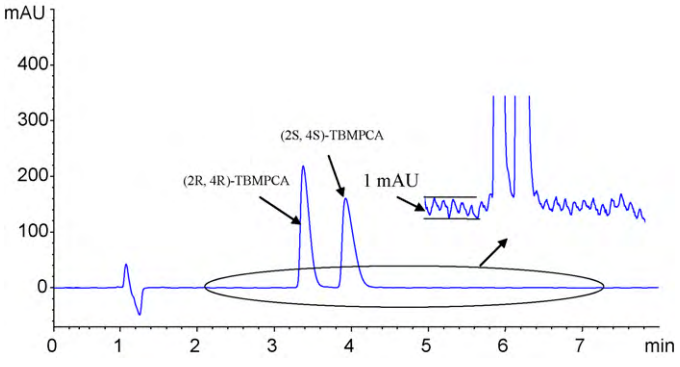

Fig. 3. Typical enantioselective SFC chromatogram of (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA mixture. Conditions: AS-H column, 4.6 mm × 250 mm; mobile phase, CO_2:methanol:TFA (97:3:0.2, v/v/v); flow rate = 3 ml/min; 40 °C; 10 mg/ml (2*R*,4*R*)-TBMPCA,(2*S*,4*S*)-TBMPCA in acetonitrile; UV detection at 210 nm.

3.2.2. Effect of split ratio

Slight tailing in the major peak observed using the G-TA column (see Fig. 4B) could potentially be due to overloading the column with sample. The injection split ratio was optimized using 6.0 mg/ml of derivatized (2*R*,4*R*)-TBMPCA standard, spiked with 6.0 μg/ml of (2*S*,4*S*)-TBMPCA. Both the major peak of derivatized (2*R*,4*R*)-TBMPCA and the minor peak of derivatized (2*S*,4*S*)-TBMPCA increase in area as the split ratio decreases, consequently, the peak tailing increased, indicating that the column was overloaded. On the other hand, as the split ratio increases, both peak area and signal-to-noise (S/N) decrease. For example, as the split ratio increases from 60 to 80, S/N for 6.0 μg/ml of (2*S*,4*S*)-TBMPCA decreases from 24 to less than 10. With a split ratio of 60, peak tailing is not an issue and S/N is reasonable; therefore, this split ratio was used for further experiments.

3.3. Enantioselective HPLC vs. SFC vs. GC

Using the optimized method conditions tabulated in Table 1, separation parameters for the enantioselective HPLC, SFC and GC methods were calculated and are listed in Table 2. All three techniques provided adequate resolution in a reasonable separation time. Compared to HPLC and GC, the enantioselective SFC method exhibits lower separation power and limited sensitivity. The relatively poor signal-to-noise in SFC/UV only allows quantitation down to 0.3 area% of the undesired enantiomer, which is much higher than 0.05% requirement for limit of quantitation (LOQ) for this case. Consideration was given to quantitation of the low-level enantiomer versus an external standard. The SFC/UV method would require a sample concentration as high as 300 mg/ml to achieve a 0.1% quantitation limit; however, at these high con-

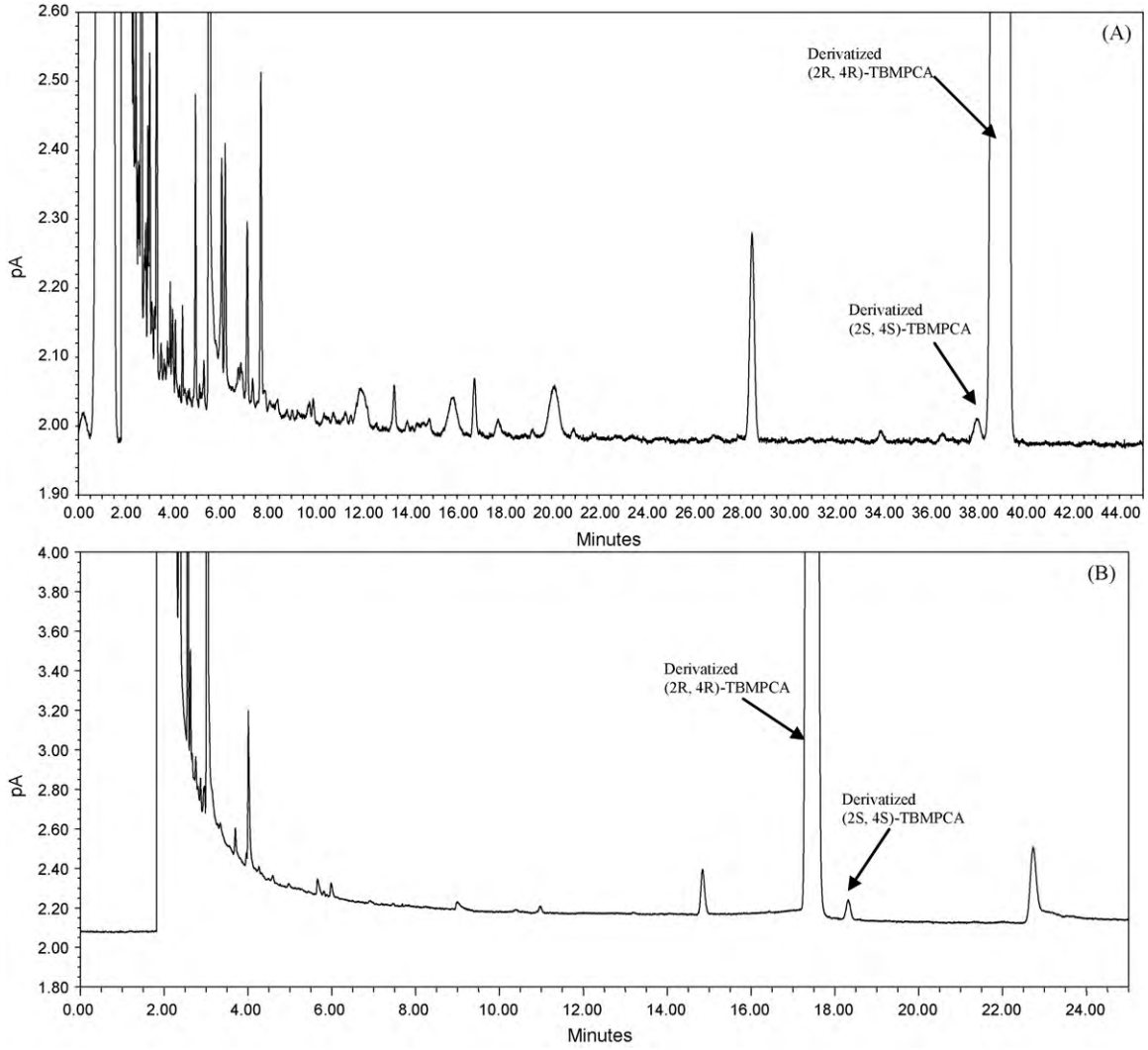

Fig. 4. Typical enantioselective GC chromatograms of derivatized (2*R*,4*R*)-TBMPCA, spiked with 0.1% (2*S*,4*S*)-TBMPCA. (A) CYCLOSILB column, 30 m × 0.32 mm, 0.25 μm thickness; 140 °C isothermal; inlet pressure = 10 psi; split ratio = 30. (B) G-TA column, 30 m × 0.25 mm, 0.10 μm thickness; 140 °C isothermal; inlet pressure = 19 psi; split ratio = 50.

centrations, the main band peak is very broad due to column overloading (the tailing factor (TF) as high as 3.4, in Table 2) and accurate determination of the low-level enantiomeric impurity that elutes on the tail is not possible. Finally, the solubilities of (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA in ACN are just below 300 mg/ml so an alternate dissolving solvent would need to be identified.

In this case, enantioselective HPLC provides better resolution than enantioselective SFC. However, even with resolution as high as 3.5, the enantioselective HPLC separation still exhibits major peak tailing with TF as high as 3.1 (see Table 2) at the high concentrations necessary to achieve accurate quantitation at the 0.1% level. As evident by the poor accuracy and system precision data in Table 2, this HPLC/UV method is suitable only as a limit test for the low-

Table 1
Chromatographic conditions for enantioselective HPLC, SFC and GC methods.

Chromatograph parameters	Enantioselective HPLC	Enantioselective SFC	Enantioselective GC
Column	AS-H column 4.6 × 250 mm	AS-H column 4.6 × 250 mm	G-TA modified capillary GC column 30 m × 0.25 mm × 1.0 μm
Detector	UV at 210 nm	UV at 210 nm	FID
Detector temperature	N.A.	N.A.	250 °C
Injector temperature	N.A.	N.A.	200 °C
Carrier gas	Heptane:ethanol:TFA	CO_2:MeOH:TFA	Helium
Mobile phase	(98:2:0.25, v/v/v)	(95:3:0.2%, v/v/v)	
Flow rate	0.8 ml/min	3.0 ml/min, at 120 bar back pressure	N/A—at constant pressure of 19 psi
Injections	5 μl	5 μl	1.0 μl
Split ratio	N.A.	N.A.	60
Oven temperature	Ambient	40 °C, 8 min	140 °C isothermal
Sample concentration[a]	200 mg/ml in ACN	~300 mg/ml	6 mg/ml Dissolved in methanol, refer to sample preparation

[a] Necessary to achieve at least 0.10 area % quantitation limit.

Table 2
Chromatographic separation parameters for enantioselective HPLC, SFC and GC methods.

Separation parameters	Enantioselective HPLC	Enantioselective SFC	Enantioselective GC
Resolution[a]	3.5	2.1	4.0
Column efficiency[b] (plate numbers)	3,500	3,600	120,000
Selectivity[c]	1.6	1.3	1.0
Tailing factor (TF)[d]	3.1	3.4	1.2
Quantitation limit[e] (relative to nominal conc.)	0.2 mg/ml (0.1% of nominal conc.)	0.3 mg/ml (0.1% of nominal conc.)	3 μg/ml (0.05% of nominal conc.)
Nominal conc.	200 mg/ml	300 mg/ml (not soluble)	6 mg/ml
Accuracy[f]	72.8–251.6%	Not assessed	85.8–103.1%
System precision (%RSD)	38.3% at 0.2 mg/ml (n = 5, spiked)	5.6% at 0.3 mg/ml (n = 5)	3.2% at 6 μg/ml (n = 7, spiked)

[a] Resolution = $\frac{1.18(t_2-t_1)}{W_{0.5,1}+W_{0.5,2}}$, where t_1 and t_2 are the retention times for the less and more retained peaks, while $W_{0.5,1}$ and $W_{0.5,2}$ are the half peak width of the less and more retained peaks, respectively.
[b] Average of plate numbers for the peaks of the enantiomeric pairs.
[c] Selectivity = $\frac{t_2-t_0}{t_1-t_0}$, where t_0 is the void time.
[d] TF = $\frac{AB}{2AC}$, where AB is the peak width and AC is the left half peak width at 5% of the peak height; TF was measured for the peak at nominal conc.
[e] Quantitation limit = conc. level, where S/N ≥ 10.
[f] Spiking recovery experiments using 3 replicates at 3 levels for the enantioselective HPLC and 5 levels for the enantioselective GC.

level undesired enantiomer in (2*R*,4*R*)-TBMPCA. Refractive index (RI) detection in conjunction with enantioselective HPLC was evaluated, but did not demonstrate improved sensitivity. Alternative detectors in conjunction with HPLC, such as evaporative light scattering detector (ELSD) [21] and charged aerosol detector (CAD) [22], may provide sufficient sensitivity to allow more accurate low-level quantitation of (2*R*,4*R*)-TBMPCA. Finally pre-column derivatization techniques to enhance UV sensitivity could be considered.

Compared to enantioselective HPLC and SFC, the GC method provides the best resolution due to high column efficiency (as high as 120,000 plates, in Table 2) although it has the lowest selectivity. Using the conditions listed in Table 1, the enantioselective GC/FID demonstrated good sensitivity for these non-UV sensitive enantiomeric model compounds. An LOQ as low as 2.4 μg/ml of (2*S*,4*S*)-TBMPCA, approximately 100 times lower than the HPLC/UV and SFC/UV methods, was achieved. With the best resolution, efficiency and sensitivity among three enantioselective methods, the GC method was successfully validated.

The potential impurities in the (2*R*,4*R*)-TBMPCA sample were similarly derivatized with trimethylsilyldiazomethane and none interfered with detection of either (2*R*,4*R*)-TBMPCA or (2*S*,4*S*)-TBMPCA derivatives as summarized in Table 3. Good linearity was achieved at both nominal assay and low-level concentration ranges. Within the concentration range from 2.4 to 12 μg/ml (n = 5), the linear equation $y = 330.41x - 0.101$ ($R^2 = 0.997$) was obtained for (2*S*,4*S*)-TBMPCA, while within the concentration range from 4.8 to 7.2 mg/ml (n = 6), the linear equation $y = 279.29x - 133.88$ ($R^2 = 0.999$) was achieved for the (2*R*,4*R*)-TBMPCA.

The RSD for derivatized (2*R*,4*R*)-TBMPCA at 6 mg/ml is 3.8% (n = 7), while the RSD for the derivatized (2*S*,4*S*)-TBMPCA at 6 μg/ml is 3.2% (n = 7). The recoveries for (2*S*,4*S*)-TBMPCA in (2*R*,4*R*)-TBMPCA at five concentration levels ranged from 85.8% to 103.1%. Most RSDs are less than 5.0% (n = 3 at each level), except at the concentration of 3.0 μg/ml, an RSD of 7.9% was observed. The overall RSD for recovery accuracy is 8.4% (n = 15). The limit of quantitation and the limit of detection are estimated to be 2.4 μg/ml (0.04% of nominal concentration) and 0.8 μg/ml (0.01% of nominal concentration), respectively. At the limit of quantitation level (0.04%), the signal-to-noise is more than 10 and the method precision (RSD) is less than 5%.

Table 3
Specificity table.

Compounds	Retention time (min)
(2*R*,4*R*)-TBMPCA	18.57
(2*S*,4*S*)-TBMPCA	17.69
(2*R*,4*R*)-TBHPCA[a]	23.08
(2*S*,4*S*)-TBHPCA[b]	23.05
(2*S*,4*R*)-TBHPCA[c]	38.05

[a] (2*R*,4*R*)-TBHPCA = (2*R*,4*R*)-1-(1-tert-butoxyvinyl)-4-hydroxypyrrolidine-2-carboxylic acid.
[b] (2*S*,4*S*)-TBHPCA = (2*S*,4*S*)-1-(1-tert-butoxyvinyl)-4-hydroxypyrrolidine-2-carboxylic acid.
[c] (2*S*,4*R*)-TBHPCA = (2*S*,4*R*)-1-(1-tert-butoxyvinyl)-4-hydroxypyrrolidine-2-carboxylic acid.

4. Conclusion

The enantioselective HPLC, SFC and GC methods developed in this study provide baseline separation of enantiomeric pair (2*R*,4*R*)-TBMPCA and (2*S*,4*S*)-TBMPCA. The limited resolving power and low sensitivity of the enantioselective SFC/UV method precludes its application for low-level quantitation. Comparatively, HPLC and GC methods provided much better resolution in a reasonable separation time. The peak tailing due to sample overloading in enantioselective HPLC, however, interfered with low-level quantitative determination of the enantiomeric impurity eluting after the major enantiomer. Thus the enantioselective HPLC/UV is not suitable for quantitative determination of low-levels of (2*S*,4*S*)-TBMPCA in (2*R*,4*R*)-TBMPCA. Although sample derivatization is needed to provide sufficient volatility for GC analysis, the sample treatment process is simple, and no sample clean-up is needed for this procedure since the derivatization reagent is well resolved from the peaks of interest. The enantioselective GC method exhibits high efficiency and high sensitivity and is the better method for quantitation of the enantiomeric pair (2*S*,4*S*)-TBMPCA and (2*R*,4*R*)-TBMPCA.

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