Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1602–1608
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Chiral purity assay for Flindokalner using tandem mass spectrometry: Method development, validation, and benchmarking

Brandy L. Young [a], R.G. Cooks [a,*], Michelle C. Madden [b], Michael Bair [c], Jingpin Jia [b], Anne-Françoise Aubry [b], Scott A. Miller [b]

[a] *Department of Chemistry, Purdue University, West Lafayette, IN 47907, United States*
[b] *Bristol-Myers Squibb Company, One Squibb Drive, New Brunswick, NJ 08903, United States*
[c] *Department of Chemistry, Florida State University, Tallahassee, FL 32306, United States*

Received 19 October 2006; received in revised form 22 December 2006; accepted 28 December 2006
Available online 8 January 2007

Abstract

The present work demonstrates the application and validation of a mass spectrometry method for quantitative chiral purity determination. The particular compound analyzed is Flindokalner, a Bristol-Myers Squibb drug candidate for post-stroke neuroprotection. Chiral quantification of Flindokalner was achieved using tandem mass spectrometry (MS/MS) and the kinetic method, a gas phase method used for thermochemical and chiral determinations. The MS/MS method was validated and benchmarked against two separate chromatographic techniques, chiral high performance liquid chromatography with ultra-violet detection (LC/UV) and achiral high performance liquid chromatography with circular dichroism detection (LC/CD). The chiral purity determination of Flindokalner using MS/MS proved to be rapid (3 min run time for each sample) and to have accuracy and precision comparable to the chiral LC/UV and achiral LC/CD methods. This method represents an alternative to commonly used chromatographic techniques as a means of chiral purity determination and is particularly useful in rapid screening experiments.

Keywords: Kinetic method; Tandem mass spectrometry; Trimeric cluster ion; Chiral chromatography; Circular dichroism

1. Introduction

Chiral therapeutics represent approximately one-third of the drug market and compared to racemics, enantiomerically pure chiral drugs often have fewer adverse toxicological effects [1,2]. As a result, analytical techniques used to determine chiral purity are gaining interest as seen from the diversity of technologies that include nuclear magnetic resonance spectroscopy (NMR) [3], high performance liquid chromatography (HPLC) [4,5], capillary electrophoresis (CE) [6,7], circular dichroism (CD) [8], and many more [9]. Despite the array of chiral analysis techniques available, the pharmaceutical industry standard in terms of the quantitative determination of chiral purity remains chromatographic in nature, particularly chiral HPLC, often with ultraviolet or visible (UV–vis) detection [10].

Achiral chromatographic techniques are generally rugged and selective. This combination of features provides the analyst with a reliable and routine separation technique. In spite of the advantages of chromatography, significant problems persist when developing chiral procedures. For example, chiral columns can be delicate, expensive, and analysis time is often long. In instances where multiple batch analyses are necessary, there is a need for chiral methods with higher throughput. Methods which can quickly and accurately assess the chiral purity of a sample have the potential to reduce the research and development time for chiral therapeutics.

While mass spectrometry (MS) has been perceived as a "chirally blind" technique as enantiomers have identical mass/charge (m/z) ratios, successful procedures that use MS for chiral separation and quantification have been developed [11–13]. They are based on ionic reactions with chiral reference compounds. Mass spectrometry is an attractive analytical technique for chiral purity determination due to its speed, high sensitivity, molecular specificity, tolerance to impurities, and ability to probe the analyte in a solvent free environment.

* Corresponding author. Tel.: +1 765 494 9421; fax: +1 765 494 9421.
E-mail address: cooks@purdue.edu (R.G. Cooks).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.12.027

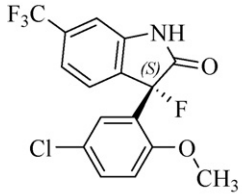
Procedures that use MS for chiral analysis include (1) the kinetic method, which is used to recognize and quantify mixtures of chiral molecules by evaluating the dissociation kinetics of metal-coordinated cluster ions [14], (2) ion/molecule reactions to study gas phase reactions between enantiomers and inclusion complexes [15,16], (3) the generation of labeled host-guest diastereomeric adducts that are used for the purpose of studying the degree of complexation [13,17], and (4) solution-phase kinetic resolution, which is a hybrid method in which reactions of chiral analytes with a mass-tag reagent produce a diastereomer, followed by mass spectrometric measurements on the resulting products [18–21]. The first two methods use tandem mass spectrometry (MS/MS), a highly specific experiment that lends additional advantages when analyzing complex mixtures. This paper focuses on work that uses the kinetic method formalism to achieve chiral identification and quantification.

Chiral quantification of Flindokalner, a potassium channel opener for post-stroke neuroprotection [22–26], through the use of MS/MS and the kinetic method is reported here. The motivation of this work was to validate and benchmark a newly developed chiral MS/MS method for Flindokalner against existing chiral LC/UV and achiral LC/CD methods, by comparing the linearity, accuracy, precision, and analysis time of the methods.

2. Experimental

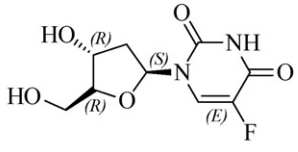
2.1. Materials

Flindokalner (Fig. 1 (**1a**)), 3-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indol-2-one, and its enantiomer (Fig. 1 (**1b**)) were both synthesized at Bristol-Myers Squibb Company (New Brunswick, NJ) [27]. Lithium chloride, ammonium acetate, glacial acetic acid, methanol, acetonitrile, and (+)-5-fluorodeoxyuridine (Fig. 1 (**2**)), were all purchased from Sigma–Aldrich. Water was generated using a Milli-Q® UV Plus water purifying system (Millipore, Billerica, MA). Methanol and acetonitrile were analytical grade.



Flindokalner

$C_{16}H_{10}ClF_4NO_2$
Exact Mass: 359.0336
Mol. Wt.: 359.7027



(+)-5-Fluorodeoxyuridine

$C_9H_{11}FN_2O_5$
Exact Mass: 246.0652
Mol. Wt.: 246.1924

1a = S-enantiomer
1b = R-enantiomer

Fig. 1. Chemical structures of Flindokalner (S-enantiomer, **1a**), its enantiomer (**1b**), and (+)-5-fluorodeoxyuridine (**2**).

2.2. Standards, quality control samples, and sample preparation procedure for chiral LC/UV and achiral LC/CD

Stock solutions of the two enantiomers, Flindokalner and **1b**, were made by dissolving the compounds separately in a methanol:water (60:40) solvent mixture at a concentration of 300 μM. Linearity standards were prepared by volumetrically mixing the two stock solutions to give chiral purity percentages for Flindokalner of 0, 24.9, 49.9, 64.9, 74.9, 84.9, 89.9, 94.8, and 99.8%. Quality control (QC) samples were prepared in a similar fashion with chiral purity percentages for Flindokalner of 92.8, 96.8, 98.8, and 99.3%. Flindokalner samples of unknown chiral purity were prepared by dissolving the sample in a methanol:water (60:40) solution. All solutions were analyzed by chiral LC/UV and achiral LC/CD methods.

2.3. Standards, QC samples, and sample preparation procedure for chiral MS/MS analysis

A stock solution of LiCl and (+)-5-fluorodeoxyuridine was prepared at a concentration of 180 μM each in methanol:water (60:40). The standards, QC samples, and unknown Flindokalner samples that were prepared for the chiral LC/UV and achiral LC/CD analysis were further diluted with the stock solution of LiCl and (+)-5-fluorodeoxyuridine to a final concentration of 100 μM each (i.e. a 1:1:1 molar ratio of analyte:(+)-5-fluorodeoxyuridine:LiCl).

2.4. Chiral chromatography instrumental conditions

The chiral LC/UV experiments were performed using an Alliance® 2695 Separation Module with a 996 Photodiode Array Detector (Waters Corp., Milford, MA). An isocratic, chiral, chromatographic separation was performed using an analytical scale CHIRALCEL OD-R column (Chiral Technologies Inc., Exton, PA) with an internal diameter of 4.6 mm, length of 250 mm, and a particle size of 10 μm. The column was maintained at ambient temperature and the mobile phase consisted of a premixed solution of methanol:water (85:15). Instrumental parameters used to conduct this analysis included a flow rate of 0.75 ml min^{-1}, 20 μl injection volume, and a run time of 20 min. A photodiode array detector was used to collect data from 200 to 400 nm and extracted UV chromatograms at 220 nm were used for all data analysis. Data acquisition and analysis for LC/UV were performed using Millennium™ Version 4.00 software (Waters Corp., Milford, MA). All data calculations were made using Microsoft Excel 2002 service pack 3.

2.5. Achiral chromatography instrumental conditions

The achiral LC/CD experiments were performed using an 1100 Series pump (Agilent Technologies, Palo Alto, CA) and a CD-2095+ circular dichroism detector (Jasco Inc., Easton, MD). An isocratic, achiral, chromatographic separation was performed using a Zorbax SB-C18 column (Agilent Technologies Inc., Palo Alto, CA) with an internal diameter of 4.6 mm, a length of 250 mm, and a particle size of 5 μm. The column

was maintained at ambient temperature and the mobile phase consisted of a premixed solution of acetonitrile and 20 mM ammonium acetate in water (68:32). The pH of the mobile phase was adjusted with acetic acid to a final value of 5.0. Instrumental parameters used to conduct this analysis included a flow rate of 1.0 ml min^{-1}, 20 μl injection volume, and a run time of 9 min. UV detection was performed at 220 nm. Data acquisition and analysis for LC/CD were performed using Millennium™ Version 4.00 software (Waters Corp., Milford, MA). All data calculations were made using Microsoft Excel 2002 service pack 3.

2.6. Mass spectrometry instrumental conditions

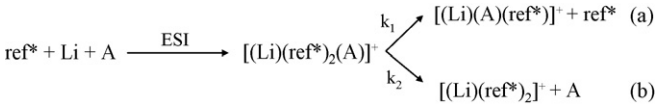
The MS/MS analysis was performed using a Micromass Quattro micro API™ triple quadrupole mass spectrometer (Waters Corp., Milford, MA) with direct loop injection sample introduction using an Alliance® 2695 Separation Module. All MS experiments were performed via loop injection using a 10 μl injection volume. The HPLC pump was set at a flow rate of 0.05 ml min^{-1} using a premixed mobile phase of methanol:water (75:25). Positive ion electrospray ionization (ESI) was used with a capillary voltage of 3.5 kV, a cone voltage of 20 V, a source temperature of 100 °C, a desolvation temperature of 150 °C, a cone gas flow of 60 l h^{-1}, and a desolvation gas flow of 300 l h^{-1}. For the collision-induced dissociation used to record the MS/MS data, argon was used as the collision gas with a collision gas pressure of 0.6 mTorr and collision energy of 5 eV, unless stated otherwise. Quantitative MS/MS data were collected using multiple reaction monitoring (MRM) with precursor ion to product ion transitions of *m/z* 858 to *m/z* 612 and *m/z* 858 to *m/z* 499. The dwell time of the MRM channels was 0.2 s with an interchannel delay of 0.05 s. The total run time was 3 min. All MS/MS data were acquired and analyzed using MassLynx™ Version 3.5 software (Waters Corp., Milford, MA).

2.7. Chiral purity determination of Flindokalner by chiral LC/UV and achiral LC/CD

Chiral purity determinations by LC/UV were made by evaluating the UV area percent (AP) of the individually separated enantiomers, Flindokalner and **1b**. The chiral purity was determined by dividing the integrated UV area for Flindokalner ($AP_{Flindokalner}$) by the total integrated UV area for both enantiomers ($AP_{Flindokalner} + AP_{\mathbf{1b}}$). For achiral LC/CD, the chiral purity of Flindokalner was determined by generating a response curve of the g-factor (a spectrum showing the differential absorbance of left- and right-circularly polarized light, divided by the UV absorbance spectrum at a set wavelength) [28] versus a set of linearity standards of known chiral purity. The chiral purity of a sample was determined using the least squares fit of the response curve.

2.8. Chiral purity determination of Flindokalner by chiral MS/MS

For the MS/MS analysis, a singly charged trimeric cluster ion $[(Li)(A)(ref^*)_2]^+$ (A represents Flindokalner, **1b**, or a mixture of the two enantiomers and ref* represents (+)-5-fluorodeoxyuridine) was observed after the ESI process. The trimeric cluster ion was mass selected and dissociated using low energy collision induced dissociation (CID) to competitively form the dimeric product ions, $[(Li)(A)(ref^*)]^+$ and $[(Li)(ref^*)_2]^+$, by loss of A or ref* from the trimeric cluster ion. This is illustrated in Scheme 1. From the kinetic method formalism [29,30], the ratio of fragmentation rates, k_1 and k_2 (rate constants for the competitive loss of the reference and analyte), are logarithmically related to the chiral purity of A. These rates can be determined from the relative ion abundance ratio, *R*, of the product ions in the MS/MS analysis, Eq. (1).



Scheme 1. Competitive dissociation of the singly charged trimeric cluster ion $[(Li)(A)(ref^*)_2]^+$, where fragmentation rates k_1 and k_2 represent the respective losses of (a) the neutral reference or (b) the neutral analyte.

$$R = \frac{[(Li)(A)(ref^*)]^+}{[(Li)(ref^*)_2]^+} \quad (1)$$

A linear relationship is expected between ln(*R*) and chiral purity of the analyte, Flindokalner. This relationship is used to construct a response curve and the obtained non-weighted linear least squares fit equation is used to determine chiral purity of unknown samples.

3. Results

3.1. Chiral MS method optimization

Fig. 2 shows the mass spectrum of a solution containing LiCl, Flindokalner, and (+)-5-fluorodeoxyuridine. This figure illustrates how efficiently the trimeric cluster ion of interest at *m/z* 858 ($[(Li)(A)(ref^*)_2]^+$) is generated. Ion source conditions and flow rates were adjusted to maximize the formation of *m/z* 858.

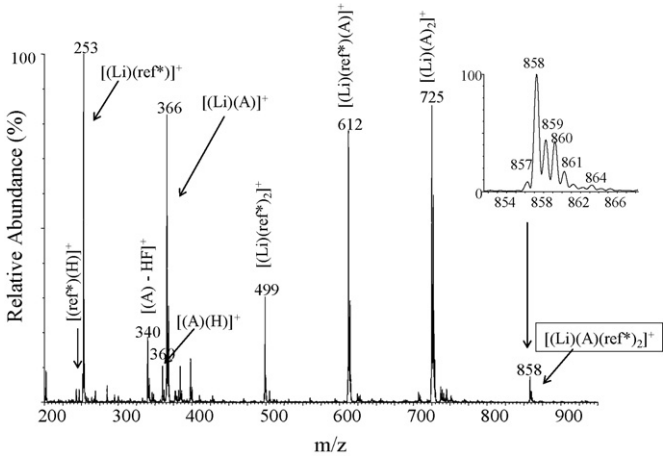


Fig. 2. Full scan MS of a loop injected mixture of Flindokalner (*A*), LiCl, and (+)-5-fluorodeoxyuridine (ref*) at 100 μM. The trimer of interest is *m/z* 858 $[(Li)(A)(ref^*)_2]^+$ as shown in the inset with resolved ^{13}C, ^{37}Cl, and ^{6}Li isotopes.

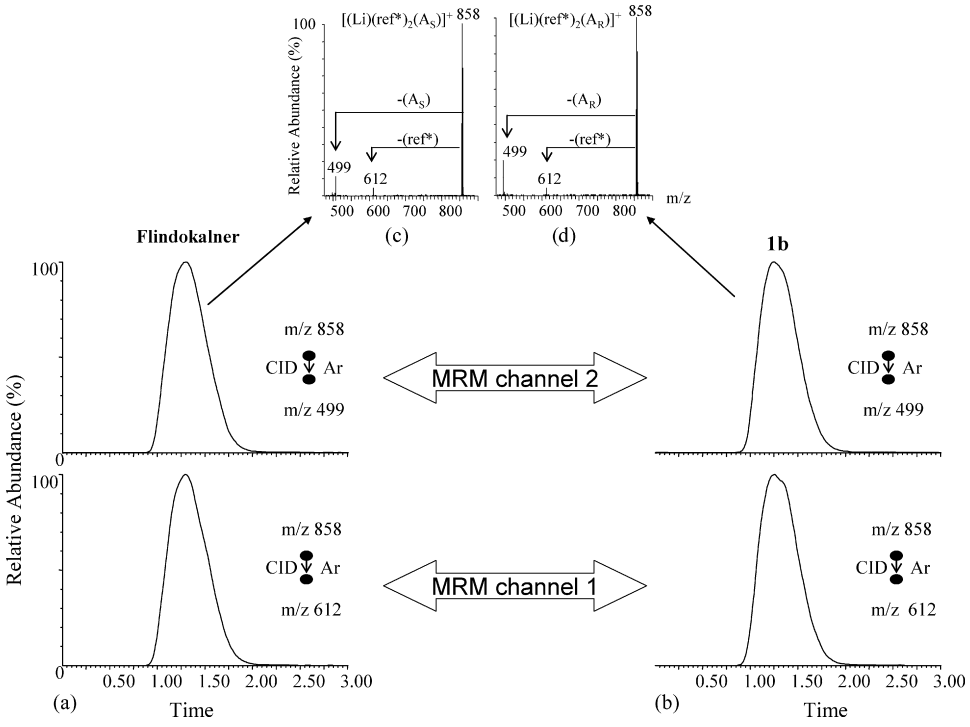


Fig. 3. MRM responses for the reactions *m/z* 858 to *m/z* 499 and *m/z* 858 to *m/z* 612 for (a) a solution containing Flindokalner, LiCl, and (+)-5-fluorodeoxyuridine (ref*) and (b) a solution containing **1b**, LiCl, and (+)-5-fluorodeoxyuridine. The ratio for the pure enantiomer Flindokalner and **1b** were calculated using the respective areas from the MRM transition providing an R_{chiral} of 1.88 using a collision energy of 5 eV and a collision gas pressure of 0.6 mTorr. The inset shows the changes in the peak heights using full scan MS/MS for (c) Flindokalner (A_S) and (d) **1b** (A_R).

Fig. 3 illustrates the MS/MS data for the trimeric cluster ion (*m/z* 858) showing the formation of the dimeric product ions of interest, *m/z* 499 ($[Li(ref^*)_2]^+$) and *m/z* 612 ($[Li(A)(ref^*)]^+$) using a collision energy of 5 eV and a collision gas pressure of 0.6 mTorr. Fig. 3a shows the MRM responses for the reactions *m/z* 858 to *m/z* 499 and *m/z* 858 to *m/z* 612 generated from a solution of Flindokalner, LiCl, and (+)-5-fluorodeoxyuridine upon loop injection. Fig. 3b depicts the same MRM transitions for a solution containing **1b**, LiCl, and (+)-5-fluorodeoxyuridine. The ratios, R_S and R_R (for Flindokalner and **1b**, respectively), were determined by integrating the areas of the respective MRM transitions, Eq. (1). R_{chiral} was then determined from the following expression, Eq. (2).

$$R_{chiral} = \frac{R_R}{R_S} = \frac{[(Li)(A_{\mathbf{1b}})(ref^*)]^+ / [(Li)(ref^*)_2]^+}{[(Li)(A_{Flindokalner})(ref^*)]^+ / [(Li)(ref^*)_2]^+} \quad (2)$$

R_{chiral} is a numerical indication of how sensitive the chosen system (Li, ref*, and A at some specific operating conditions) is at facilitating chiral discrimination. An R_{chiral} of unity means that the chosen system fails to create a stereochemically dependent interaction. The further R_{chiral} is from unity the more stereoselective the interaction [31–33].

The degree of chiral recognition achieved varies with collision energy and collision gas pressure. R_{chiral} for this system was optimized by varying the collision energy (3–10 eV) and collision gas pressure (0.2–1.0 mTorr) as illustrated in Fig. 4. R_{chiral} versus collision energy (collision gas pressure set at 0.6 mTorr)

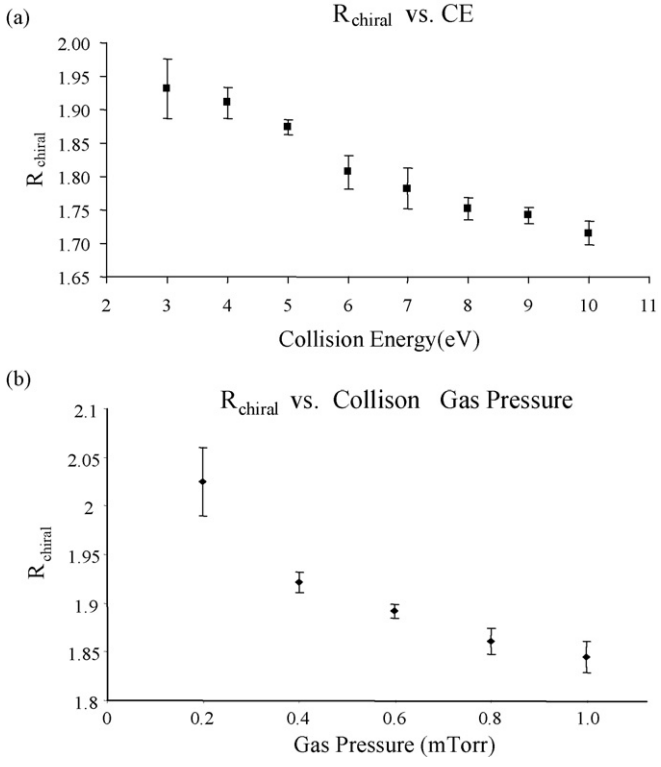


Fig. 4. Effect of operation conditions on chiral discrimination. (a) R_{chiral} vs. collision energy at a fixed collision gas pressure of 0.6 mTorr and (b) R_{chiral} vs. collision gas pressure at a fixed collision energy of 5 Ev.

shows a maximum R_{chiral} of 1.93 at a collision energy of 3 eV. A collision gas pressure of 0.2 mTorr gives a maximum R_{chiral} of 2.03 at 5 eV collision energy. In addition to a high R_{chiral}, the method also requires high precision in the measurement of this ratio, which can be judged from the standard deviation (S.D.) of the R_{chiral} measurement (shown as error bars in Fig. 4). Thus, a collision energy of 5 eV and collision gas pressure of 0.6 mTorr was preferred despite the lower R_{chiral} value of 1.88.

Additionally, R_{chiral} can vary significantly. Other systems show selectivity values that range from 2 to 2000 [34]. As a result, other reference analogues and metals (alkali and first row transition metals) were screened to get the largest R_{chiral} value. The reference/metal combinations were excluded for one of two reasons. The combinations, when combined with Flindokalner did not form the necessary trimeric cluster ion or the obtained R_{chiral} was unity. When the reference, (+)-5-fluorodeoxyuridine, was combined with Li^+ and Flindokalner both criteria were satisfied, trimeric cluster ion formation and an R_{chiral} greater than unity. The R_{chiral} value obtained is comparable with previous reports [12].

3.2. Chiral MS/MS method validation procedure for Flindokalner

The linearity of the system was observed over a range of 0–99.8% chiral purity using linearity standards of 0, 24.9, 49.9, 74.9, and 99.8% Flindokalner. The ln(*R*) versus chiral purity data were plotted (data not shown) and a non-weighted linear least squares fit was generated that produced a slope of 0.0063, *y*-intercept of −0.2671, and a correlation coefficient, r^2, of 0.9995. Since a linear relationship was demonstrated between ln(*R*) and chiral purity using conditions that gave an R_{chiral} value of 1.88, it was determined that this system and these experimental conditions could be used to quantify the chiral purity of Flindokalner. Therefore, a 3-day validation procedure was developed to test the linearity, accuracy, and reproducibility of the chiral MS/MS method.

3.3. Linearity

Linearity was assessed each day using freshly prepared standards and the slope, *y*-intercept, and correlation coefficient were determined. The linearity of this system was demonstrated using chiral purity values of Flindokalner at 49.9, 64.9, 74.9, 84.9, 89.9, 94.8, and 99.8%, as the unknown samples of interest would have chiral purity values in the range of approximately 95–100%. Table 1 summarizes the linearity results for the 3-day validation. Correlation coefficients, r^2, were obtained each day with an average of 0.9963. The slope and *y*-intercept reproducibility had a standard deviation of ± 0.0001 and ± 0.0216, respectively.

Table 1
Linearity data summary for the 3-day validation using chiral MS/MS

Day	Slope	*y*-Intercept	Correlation coefficient (r^2)
1	0.0063	−0.2880	0.9983
2	0.0062	−0.3297	0.9982
3	0.0061	−0.2993	0.9923
Average	0.0062	−0.3057	0.9963
S.D.	0.0001	0.0216	0.0034

Table 2
Intra-day accuracy and precision

Day 1 (n = 3)[a]				
Expected chiral purity	92.8	96.8	98.8	99.3
Injection 1	89.8	95.5	94.0	99.0
Injection 2	93.1	98.1	98.4	99.8
Injection 3	92.8	96.9	95.6	100.8
Mean	91.9	96.8	96.0	99.9
% R.S.D.	2.0	1.4	2.3	0.9
% Bias[b]	−1.0	0.0	−2.9	0.6

[a] *n* represents the number of measurements obtained.
[b] The root-mean-square of the % bias for the four QC samples was calculated to be 1.6%.

3.4. Accuracy

Intra-day accuracy was assessed by comparing the measured chiral purity of all QC samples on day 1 to their expected chiral purity. Inter-day accuracy was determined by comparing the measured chiral purity of the 99.3% QC sample, prepared daily in triplicate, to the expected chiral purity. Intra- and inter-day accuracies were calculated using% bias ((measured chiral purity − nominal chiral purity)/nominal chiral purity × 100) and are shown in Tables 2 and 3. The intra-day accuracy had % bias values that ranged from −2.9 to 0.9% with a root-mean-square of 1.6%. Inter-day accuracies for the 99.3% QC sample ranged in % bias from −2.4 to 0.9% with a root-mean-square of 1.6% over the validation period.

3.5. Reproducibility

Intra-day precision was determined by calculating the percent relative standard deviation (% R.S.D.) for each QC measurement on day 1. Inter-day precision was assessed by comparing the% R.S.D. of the measured chiral purity of the three freshly prepared 99.3% QC samples on each day over the 3-day validation period. The intra- and inter-day reproducibility values are shown in Tables 2 and 3. The intra-day precision ranged from 1.4 to 2.3% R.S.D. with an average of 1.2% for all QC samples on day 1. The inter-day precision ranged from 1.0 to

Table 3
Inter-day accuracy and precision

99.3% QC				
	Day 1 (n = 9)[a]	Day 2 (n = 9) [a]	Day 3 (n = 9) [a]	Avg. (n = 27) [a]
Mean	100.2	98.5	96.9	98.5
% R.S.D.	1.2	1.0	1.3	1.2
%Bias	0.9	−0.8	−2.4	1.6[b]

[a] *n* represents the number of measurements obtained.
[b] The % bias average was determined using the root-mean-square.

1.3% R.S.D. with an average precision of 1.2% for the validation study.

4. Discussion

The same set of standards and QC samples used on day 1 of the chiral MS/MS validation study were also analyzed using the chiral LC/UV and achiral LC/CD methods. In addition, three separate samples of Flindokalner with unknown chiral purity were analyzed by all three chiral methods. A comparison of the data obtained from the three methods including % bias and % R.S.D. is shown in Table 4. The performance of the MS/MS method was similar to the achiral LC/CD method in both accuracy and precision but neither method performed as well as the chiral LC/UV method for the pure QC samples.

In addition to accuracy and precision, the time for method development as well as the time for analysis is also of interest with respect to benchmarking the chiral MS/MS method against the chiral LC/UV and achiral LC/CD methods [35]. Table 5 gives the time it took to develop the chiral methods discussed here, the number of injections required to determine the chiral purity of a sample for the given method, and the sample-to-sample analysis time. The MS/MS method took less than a week to develop. The number of injections required to determine the chiral purity of a single sample for the three methods differs in that only single injections are required for the chiral LC/UV method while triplicate injections are suggested for the achiral LC/CD and chiral MS/MS methods due to their lower inherent precision. The chiral MS/MS method, however, is still the most rapid, in spite of the number of injections needed, since the analysis time is only 3 min. This is even further improved when using higher flow rates in the chiral MS/MS method which can reduce run times to < 1 min (data not shown). The achiral LC/CD method has the largest disadvantage in overall analysis time required, since the analysis time of a single sample requires ∼225 min per sample compared to 60 and 75 min for chiral LC/UV and chiral MS/MS, respectively. The achiral LC/CD analysis time could be improved with faster chromatography or with fewer injections, however, the selectivity could be compromised as a result of optically active impurities overlapping and the precision of the analysis would suffer with fewer injections.

The fast analysis time along with reasonably accurate and precise chiral purity determinations, over the range of 1–99%, makes chiral MS/MS a promising technique. In addition to speed and accuracy, the added complexity of using a chromatographic chiral column is not needed to perform the chiral measurement. The use of mass spectrometry as a means of detection eliminates the requirement of the analyte to have a chromophore or to subject your analyte to a derivatization procedure to afford a chromophore. This method is also a trace analysis procedure, which is advantageous in instances where there is a limited amount of sample available. Similar MS/MS kinetic methods for chiral analysis have been used for complex mixtures [36] and extended to ternary chiral systems [37], indicative of the versatility of the method. Conversely, when using complex matrices for a chiral HPLC analysis, baseline resolution of the enantiomers and related impurities or matrix interferences can be difficult to achieve; however, this is avoided by the chiral MS/MS procedure which has superior specificity as a result of mass-filtering for the analyte of interest and its product ions while excluding all unwanted non-isobaric ions.

Limitations associated with this method involve the limited knowledge of reference–analyte interactions, a safe route being to use a reference that is structurally similar to the analyte [38].

Table 5
Comparison of method development time, number of injections required to determine the chiral purity of a single sample, and analysis time for the chiral LC/UV, achiral LC/CD, and chiral MS/MS methods

	Chiral LC/UV	Achiral LC/CD	Chiral MS/MS
Method development time	1 week	1 week	1 week
Number of injections[a]	3[b]	25[c]	25[d]
Sample-to-sample analysis time (min)	20	9	3[e]
Total analysis time (min) for 1 sample	60	225	75
Total analysis time (min) for 6 samples	160	360	120
Total analysis time (min) for 25 samples	540	873	291

[a] Number of injections required to determine the chiral purity of a single sample for the given method.
[b] Single injection of blank, QC injection for system suitability, and sample.
[c] Single injection of blank with triplicate injection of seven standards and sample.
[d] Single injection of blank with triplicate injection of seven standards and sample.
[e] Analysis time could be reduced to 1 min with higher flow rates.

Table 4
Comparison of results obtained from chiral MS/MS, chiral LC/UV, and achiral LC/CD methods

Sample	Expected chiral purity	Measured chiral purity MS (UV) [CD]	% Bias MS (UV) [CD]	% R.S.D. MS (UV) [CD]
QC 1	92.9	91.9 (92.9) [93.6]	−1.1 (0.0) [0.8]	2.0 (0.1) [0.7]
QC 2	96.8	96.8 (96.8) [96.6]	0.0 (0.0) [−0.2]	1.4 (0.1) [1.5]
QC 3	98.8	96.0 (98.9) [98.3]	−2.9 (0.1) [−0.5]	2.3 (0.0) [0.6]
QC 4	99.3	100.2 (99.4) [98.5]	0.9 (0.1) [−0.8]	1.2 (0.1) [0.2]
Sample 1	Unknown	99.5 (99.9) [99.2]	N/A	1.3 (0.0) [0.2]
Sample 2	Unknown	99.8 (99.9) [99.3]	N/A	0.6 (0.0) [0.6]
Sample 3	Unknown	100.7 (99.9) [99.9]	N/A	1.7 (0.0) [1.2]

The chosen reference molecule helps facilitate chiral recognition due to changes in stereospecificity. The cluster formation (trimeric complex ion or dimeric complex ion [34]) is necessary for this procedure. Choosing a reference compound and forming a cluster affect the performance of the MS/MS method since the trimeric cluster ion is often a minor component generated in the ion source of the mass spectrometer and thus often approaches a low-level quantitation scenario (as noted in Fig. 2). The choice of MS/MS instrument and experimental conditions are critical in order to reproducibly measure the fragment ion abundances. In addition, successful chiral distinction can only be achieved if R_{chiral} is greater than or less than unity. The combination of reference, metal ion and analyte must be selected such that the analyte–reference interactions are maximized and easily measured from the relative ion abundances in the MS/MS experiment. Lastly, obtaining an appropriate R_{chiral} value can require many experimental trials which could potentially affect the method development time.

Mass spectrometry provides a rapid and sensitive method for chiral analysis. In a gas-phase environment, enantiodiscrimination methods are relatively easy to develop and the experimental conditions are readily optimized so that chiral recognition can be obtained; although, it remains difficult to predict the arrangement of ligands about the metal center which provides the stereoselective interaction. The use of the kinetic method and MS/MS for chiral purity determination has been shown here to be a rapid means of obtaining chiral purity information and represents a good alternative to chiral LC in early pharmaceutical development, especially when multiple samples must be analyzed. The validated chiral MS/MS method for Flindokalner produced results that have accuracies and precision comparable to the chiral LC/UV and achiral LC/CD methods and are compatible with current standards in pharmaceutical development [39].

5. Acknowledgments

This work was supported by the National Science Foundation, 0412782-CHE, the U.S. Department of Energy, Office of Energy Research, and Bristol-Myers Squibb Pharmaceutical Research Institute. B. L. Young is grateful to BMS for the opportunity to collaborate and conduct research at their facility and to the colleagues at BMS who offered support, encouragement, and thoughtful comments.

References

[1] P. Chen, Angew. Chem. Int. Ed. 42 (2003) 2832–2847.
[2] S.C. Stinson, Chem. Eng. News 78 (2000) 55–79.
[3] J.P. Morken, M.A. Evans, J.P. Morken, J.P. Morken, J. Am. Chem. Soc. 124 (2002) 9020–9021.
[4] T.E. Beesley, R.P.W. Scott, Chiral Chromatography, John Wiley & Sons Inc., New York, 1998.
[5] Y. Tang, J. Zukowski, D.W.I. Armstrong, J. Chromatogr A 743 (1996) 261–271.
[6] X. Zhu, B. Lin, A. Jakob, S. Wuerthner, B. Koppenhoefer, Electrophoresis 20 (1999) 1878–1889.
[7] G. Blaschke, B. Chankvetadze, J. Chromatogr. A 875 (2000) 3–25.
[8] T. Hattori, Y. Minato, S. Yao, M.G. Finn, S. Miyano, Tetrahedron Lett. 42 (2001) 8015–8018.
[9] T.J. Ward, D.M. Hamburg, Anal. Chem. 76 (2004) 4635–4644.
[10] FDA, Guidance for Industry, August 2000.
[11] M.G. Finn, Chirality 14 (2002) 534–540.
[12] B.L. Young, L. Wu, R.G. Cooks, in: K.W. Busch, M.A. Busch (Eds.), Mass Spectral Methods of Chiral Analysis, Elsevier B.V., Oxford, 2006, pp. 593–658.
[13] M. Sawada, Mass Spectrom. Rev. 16 (1997) 73–90.
[14] W.A. Tao, R.G. Cooks, Anal. Chem. 75 (2003) 25A–31A.
[15] C.B. Lebrilla, Acc. Chem. Res. 34 (2001) 653–661.
[16] G. Grigorean, J. Ramirez, S.H. Ahn, C.B. Lebrilla, Anal. Chem. 72 (2000) 4275–4281.
[17] M. Sawada, in: N.M.M. Nibbering (Ed.), Chiral Compounds, Elsevier, 2004.
[18] J. Guo, J. Wu, G. Siuzdak, M.G. Finn, Angew. Chem. Int. Ed. 38 (1999) 1755–1758.
[19] D.D. Diaz, S. Yao, M.G. Finn, Tetrahedron Lett. 42 (2001) 2617–2619.
[20] S. Yao, J. Meng, G. Siuzdak, M.G. Finn, J. Org. Chem. 68 (2003) 2540–2546.
[21] B.N. Brewer, C. Zu, M.E. Koscho, Chirality 17 (2005) 456–463.
[22] D. Zhang, R. Krishna, L. Wang, J. Zeng, J. Mitroka, R. Dai, N. Narasimhan, R.A. Reeves, N.R. Srinivas, L.J. Klunk, Drug Metab. Dispos. 33 (2005) 83–93.
[23] P. Hewawasam, V.K. Gribkoff, Y. Pendri, S.I. Dworetzky, N.A. Meanwell, E. Martinez, C.G. Boissard, D.J. Post-Munson, J.T. Trojnacki, K. Yeleswaram, L.M. Pajor, J. Knipe, Q. Gao, R. Perrone, J.E. Starrett, Bioorg. Med. Chem. Lett. 12 (2002) 1023–1026.
[24] B.S. Jensen, CNS Drug Rev. 8 (2003) 353–360.
[25] V.K. Gribkoff, J.E. Starrett Jr., S.I. Dworetzky, P. Hewawasam, C.G. Boissard, D.A. Cook, S.W. Frantz, K. Heman, J.R. Hibbard, K. Huston, G. Johnson, B.S. Krishnan, G.G. Kinney, L.A. Lombardo, N.A. Meanwell, P.B. Molinoff, R.A. Myers, S.L. Moon, A. Ortiz, L. Pajor, R.L. Pieschl, D.J. Post-Munson, L.J. Signor, N. Srinivas, M.T. Taber, G. Thalody, J.T. Trojnacki, H. Wiener, K. Yeleswaram, S.W. Yeola, Nat. Med. 7 (2001) 471–477.
[26] P. Hewawasam, M. Erway, S.L. Moon, J. Knipe, H. Weiner, C.G. Boissard, D.J. Post-Munson, Q. Gao, S. Huang, V.K. Gribkoff, N.A. Meanwell, J. Med. Chem. 45 (2002) 1487–1499.
[27] D.D. Dischino, V.K. Gribkoff, J.E. Starrett, J. Rinehart, T. Spears, P. Hewawasam, G. Luke, J. Label. Compd. Radiopharm. 46 (2003) 139–149.
[28] B.R. Baker, R.L. Garrell, Faraday Discuss. 126 (2004) 209–222.
[29] R.G. Cooks, J.S. Patrick, T. Kotiaho, S.A. McLuckey, Mass Spectrom. Rev. 13 (1994) 287–339.
[30] R.G. Cooks, P.S.H. Wong, Acc. Chem. Res. 31 (1998) 379–386.
[31] V.R. Meyers, M. Rais, Chirality 1 (1989) 167–169.
[32] V.A. Davankov, V. Meyer, M. Rais, Chirality 2 (1990) 208–210.
[33] V.A. Davankov, Chirality 9 (1997) 99–102.
[34] L. Wu, E.C. Meurer, B. Young, P. Yang, M.N. Eberlin, R.G. Cooks, Int. J. Mass Spectrom. 231 (2004) 103–111.
[35] K.A. Schug, W. Lindner, J. Sep. Sci. 28 (2005) 1932–1955.
[36] L. Wu, A.W. Tao, R.G. Cooks, J. Mass Spectrom. 38 (2003) 386–393.
[37] L. Wu, R.L. Clark, R.G. Cooks, Chem. Commun. 137 (2003) 136–137.
[38] X. Zheng, R.G. Cooks, J. Phys. Chem. A 106 (2002) 9939–9946.
[39] FDA, Innovation Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products, U.S. Department of Health and Human Services Food and Drug Administration, 2005.