Published in final edited form as:

J Liq Chromatogr Relat Technol. 2017 ; 2017: 1–8. doi:10.1080/10826076.2017.1333962.

Determination of the enantiomeric purity of epinephrine by HPLC with circular dichroism detection

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Abstract

Several hundred drug substances approved by the U.S. Food and Drug Administration are chiral molecules. For the enantiomeric purity assessment, current practice is to develop separation techniques using chiral columns or mobile phase modifiers to separate enantiomers before detection. An alternative approach is to use currently accepted HPLC assay methods and use chiral-specific detectors to confirm whether the correct enantiomer is present. In this paper, adding a circular dichroism (CD) detector to an achiral HPLC method from the US Pharmacopeia (USP) is shown to be amenable for the determination of the enantiomeric purity of epinephrine, a substance used to treat anaphylaxis. This HPLC-UV-CD approach was able to detect the inactive D-(+) enantiomer at 1% of the total epinephrine composition. The linearity, accuracy, and precision of HPLC-UV-CD were evaluated and compared to analyses using a chiral HPLC method. Additionally, an epinephrine drug product was analyzed for assay (concentration) and enantiomeric purity. The results from achiral and chiral methods were identical within the experimental error. Overall, achiral chromatography performed using a USP method with CD detection may serve as a general means of determining chiral drug enantiomer purity and avoids the need for the development of additional chiral-specific methods for each individual drug.

Graphical abstract

Keywords

Adrenaline; chiral; circular dichroism; enantiomer; epinephrine; HPLC

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Introduction

One estimate calculates that more than half of all active drug ingredients, representing several hundred compounds, have chiral structures.^[1] Currently, the most common approach of determining enantiomer purity is to use chiral columns or chiral mobile phase modifiers to separate enantiomers prior to detection. Implementing a monitoring process to ensure that each chiral product contains the appropriate enantiomer purity requires the development, validation, and acceptance of several hundred chiral methods. In addition, specific validation would be needed for each chiral product to which the method applies. An alternative approach is to use currently validated HPLC assay methods to separate the substance of interest from other components and apply chiral-specific detectors such as circular dichroism (CD) or optical rotation to verify that the correct enantiomer is present and meets the minimum enantiomeric purity criteria.^[2] A series of articles have explored the potential for this approach,^[3–16] but these studies have not focused on compatibility with methods validated by the US Pharmacopeia (USP).

Circular dichroism spectroscopy takes advantage of the differential absorbance (ΔA) between left- and right-handed polarized light for chiral molecules. This value is proportional to the ellipticity (θ), which relates to the change in the angle of a light vector after passing through the sample. Ellipticity is typically expressed in units of milli-degrees and is dependent upon the enantiomeric composition of a sample as well as the concentration. CD signals can be normalized by the sample concentration, so that the response is solely related to the enantiomeric composition. This value is called the g-factor ($g = \frac{\Delta A}{A}$) and can be used to determine the enantiomeric purity of a chiral substance.^[2]

Epinephrine is an enantiopure drug which has been found to be an effective treatment for anaphylaxis in emergency situations.^[17–20] Epinephrine is biologically active in the L-(–) configuration, while the D-(+) form is considered an impurity. L-(–) epinephrine is known to undergo racemization and degradation during storage, which results in a partial loss of effectiveness.^[21–22] Given the wide use of this drug, the ability to assay the drug and confirm that the product meets specifications is critical for patient safety. In a previous study, expired EpiPens, an injectable epinephrine drug product, were tested for total available epinephrine^[23] and found that the total amount of epinephrine degraded over time, with the content of some samples reaching approximately 50% of the label claim 5 years after the expiration date. In a separate study, samples of epinephrine-containing drug products were found to contain 1.3–5.7% of the inactive D-(+) enantiomer.^[24]

Several methods for the determination of epinephrine enantiomeric purity have been demonstrated, including HPLC following derivation with chiral reagents,^[25,26] using chiral chromatographic columns,^[27] and by capillary electrophoresis using a chiral mobile phase.^[28] Here, the feasibility of simplifying the method development process for enantiomer purity analysis was explored by adding CD detection to an achiral HPLC method (i.e., the current achiral USP HPLC method) to determine the amount of the D-(+) enantiomer in a mixture of L-(–) and D-(+) epinephrine. The linearity, accuracy, and precision were evaluated, and the limits of detection (LOD) and quantitation (LOQ) were determined. Finally, an epinephrine drug product was analyzed to determine the enantiomer

composition, and results are compared with the analysis using a traditional chiral HPLC method.

Materials and methods

Chemicals, samples, and reagents

All chemicals were used as received unless otherwise stated. Mobile phase buffers were prepared with >18 M Ω water and filtered through a HVLP 0.45 μ m filter (Millipore, Billerica, MA) prior to use. HPLC-grade methanol and acetonitrile (Fisher Scientific, Pittsburgh, PA) were added after filtration. Epinephrine standard stock solutions (Toronto Research Chemicals, Toronto, Canada) were prepared individually from separate enantiomers in the achiral HPLC mobile phase. Standard enantiomeric composition solutions were prepared volumetrically from stock solutions and were mixed well prior to analysis. The epinephrine-injectable drug product (Adrenaline[®], 1.0 mg/mL L-(–) epinephrine, JHP Pharmaceuticals, Rochester, MI) (Expired on Jan. 2016) was diluted 10-fold with water prior to use. Analysis was performed 13 months after the expiration date.

Liquid chromatography with circular dichroism detection

Liquid chromatographic analysis of epinephrine standard solutions was performed using the current USP HPLC method for epinephrine injection solutions.^[29] The mobile phase buffer consisted of 50 mM sodium phosphate (Fisher Scientific), 2.4 mM sodium 1-octanesulfonate (Sigma-Aldrich, St Louis, MO), and 0.15 mM ethylenediaminetetraacetic acid (Sigma-Aldrich). The pH was adjusted to 3.8 using phosphoric acid (EMD, Darmstadt, Germany) and the buffer was mixed with methanol to form an 85/15 (v/v) solution of buffer/methanol. LC instrumentation consisted of a Jasco LC-4000 Series (Jasco Inc., Maryland, USA) equipped with a 4.6 $\times$ 150 mm X-Bridge C8 column (Waters, Milford, MA) with 3.5 μ m particles. An isocratic elution method was run at a flow rate of 2 mL/min, using a column temperature of 40°C. Twenty microliter injections of 100 μ g/mL epinephrine samples were performed, and 280 nm UV detection was facilitated by a photodiode array detector (MD-4010, Jasco Inc.). CD detection (CD-4095, Jasco Inc.) was also used to record the ellipticity and g-factor responses at a response time of 2 s. The CD detector wavelength was set to 230 nm utilizing a 20 nm bandwidth and was allowed to equilibrate for at least 6 hr prior to analysis. All instrumentation was controlled using Chrom-NAV software (Jasco Inc.), which also performed data collection and analysis. The g-factor was recorded as the magnitude of signal at the retention time of the epinephrine ellipticity peak. Reported data represent an average of three separate injections.

Chiral chromatography

Chiral separation of epinephrine enantiomers was performed by a slight alteration of a method provided by Shodex, a manufacturer of chiral HPLC columns. The mobile phase was prepared by making a 200 mM sodium chloride (Fisher Scientific) aqueous solution consisting of 0.05% glacial acetic acid (EMD), which was combined to form a 95/5% (v/v) aqueous/acetonitrile mixture. Instrumentation consisted of an Agilent 1290 HPLC (Agilent Technologies, Santa Clara, CA) with separation of epinephrine enantiomers facilitated by an ORpak CDBS-453 4.6 $\times$ 150 mm column (Shodex, New York, NY). A flow rate of 0.5

mL/min was used in isocratic mode and the column was cooled to 10°C. The injection volume was set to 20 µL, and epinephrine solutions were prepared at 100 µg/mL. UV detection was monitored at 280 nm, and an additional CD detector (CD-4095, Jasco Inc.) was incorporated through an Agilent 35900E multichannel interface (Agilent Technologies). CD detection parameters were identical to those previously described. OpenLAB Software (Agilent Technologies) was used to control HPLC instrumentation and to record and analyze data. Epinephrine concentrations were determined by integrating UV peak areas and comparing responses with those of known standards. Triplicate injections were performed for all solutions.

Circular dichroism spectroscopy

CD spectra of epinephrine solutions were recorded using a Jasco J-815 CD Spectrometer (Jasco Inc.). Epinephrine standard solutions were prepared in the phosphate/methanol HPLC mobile phase described above. All spectra were recorded at room temperature using a 1-cm path length quartz cuvette. Epinephrine solution spectra were background subtracted with that of the mobile phase. Scans were conducted between 200 and 300 nm at 100 nm/min, with a resolution of 0.5 nm, and were averaged over 20 accumulations. Spectra Manager software (Jasco Inc.) was used to control instrumentation and record measurements.

Confidence intervals

Confidence intervals (CI) for the slope and *y*-intercept of regression lines were calculated using the equation below with regression parameters determined by MS Excel. Here, *t* is the corresponding value for *n* – 2 degrees of freedom in the two-tailed student's *t*-test, and *s* represents the standard error of the slope (*m*) or intercept (*b*).

$$CI=m \text{ or } b \pm (t \times s)$$

All reported intervals were calculated using a *t*-value corresponding to a confidence level of 95%.

Results and discussion

Circular dichroism detection of epinephrine

To determine the optimal wavelength for CD detection of epinephrine, spectra of L-(–) and D-(+) epinephrine enantiomers were collected using a CD spectrometer. Figure 1 shows the spectra for each enantiomer, which were prepared in the LC mobile phase to account for the effects of pH and solvent composition. The most intense signals were observed around 230 nm, indicating that the differential absorbance due to the chiral center was most sensitive around this wavelength. This was consistent with other reported epinephrine CD spectra^[30] and thus, 230 nm was selected as the CD detection wavelength.

Next, epinephrine solutions were injected onto a HPLC system equipped with both UV and CD detectors, with analysis conditions consistent with the current achiral USP monograph method for epinephrine injection solutions. Chromatograms for an injection of a 10/90% D-

(+)/L-(−) epinephrine mixture are displayed in Figure 2a. The UV peak (upper) confirmed the retention time of epinephrine and demonstrated the concurrent elution of both enantiomers. Negative ellipticity and g-factor signals (middle and lower, respectively) at the epinephrine retention time indicated the excess of the L-(−) enantiomer. Injection of a racemic epinephrine mixture resulted in an identical UV peak compared to the 10/90% mixture, but the ellipticity and g-factor responses were noticeably reduced (Figure 2b).

Determination of the linearity and LOQ/LOD for achiral HPLC-UV-CD

Standard 100 µg/mL epinephrine solutions with different enantiomer compositions were analyzed using the USP achiral method with CD detection. For each injection, the g-factor magnitude was recorded at the retention time of the epinephrine ellipticity peak. As shown in Figure 3, the g-factor was found to be linear ($r^2 = 0.9999$) over the complete range of possible D-(+) enantiomer compositions. Notably, this included solutions with a low percentage of D-(+) epinephrine (*inset*, $r^2 = 0.9945$), which is important for monitoring racemization of the active L-(−) enantiomer to the inactive D-(+) configuration. LOD and LOQ, respectively, were determined using the standard error (s) and slope of the regression line (m) as shown in below equations.

$$\text{LOD} = \frac{3.3 \times s}{m}$$
$$\text{LOQ} = \frac{10 \times s}{m}$$

Using this method, the LOD for the percentage of D-(+) epinephrine in 100 µg/mL samples was determined to be 1.0%, and the LOQ was calculated at 3.1% (Table 1). The current USP monograph for epinephrine injection solutions does not specify a D-(+) enantiomer limit.^[29]

Determination of the chiral HPLC detection limit

To provide a comparison for the detection limit of the achiral method, a similar analysis was performed using a chiral separation. Thus, a chiral HPLC column was used to separate epinephrine enantiomers prior to detection. Figure 4 displays the UV (upper) and ellipticity responses (lower) to injections of (a) a racemic and (b) a 10/90% D-(+)/L-(−) epinephrine solution. These chromatograms show a baseline resolved separation of the L-(−) and D-(+) enantiomers before detection. To compare detection and quantitation limits with the achiral HPLC-UV-CD method, injections of 100 µg/mL epinephrine solutions consisting of 0 to 10% of the D-(+) enantiomer were performed, and the UV peak areas were used to construct a calibration curve for the percentage of D-(+) epinephrine in each solution (not shown). Using the slope and standard error with the last two equations, the LOD for D-(+) epinephrine was determined to be 0.5 µg/mL, and the LOQ was found to be 1.5 µg/mL. These concentrations correspond to 0.5 and 1.5% of the total epinephrine content in 100 µg/mL solutions, respectively (Table 1), approximately half that of the HPLC-UV-CD method.

Comparing the accuracy and precision of achiral and chiral methods

Next, the accuracy of each HPLC method was determined by calculating the average recovery for triplicate injections of 2–10% D-(+) epinephrine standard preparations. The

recovery, reported as a percentage, is defined as the measured amount of the D-(+) enantiomer divided by the prepared quantity. Due to the stated intent of monitoring racemization of pure L-(−) drug products, only recoveries for solutions with low amounts of the D-(+) enantiomer (2–10%) were examined. As shown in Table 2, both methods produced nearly 100% recovery for solutions with D-(+) enantiomer compositions of 8 and 10%. However, as the D-(+) enantiomer composition decreased from this point, the accuracy of both methods largely declined. In fitting with the higher LOQ for the CD detector compared to UV detector with chiral separation, recoveries for solutions with lower D-(+) enantiomer concentrations were less accurate for the achiral method compared to the chiral method. For these solutions, achiral analysis resulted in concentration estimates within ~10% of the true value, whereas this error was reduced to ~5% for the chiral method.

The precision of each HPLC method was evaluated using the relative standard deviation (RSD) for the D-(+) epinephrine recovery from the injections described above. A complete comparison for the precision analysis is provided in Table 2. Similar to the accuracy data, both methods were more precise for solutions consisting of 8–10% of the D-(+) enantiomer, as these RSD values were under 5%. However, at lower D-(+) compositions, there was more variability. Although the achiral method showed respectable precision for 4% D-(+) solutions (5.5%), this value increased dramatically to 30.7% when the D-(+) composition was lowered to 2%. This is probably due to the fact that 2% D-(+) is below the LOQ for the achiral method, so measurements in this range will have a larger variation. Alternatively, the LOQ for the chiral method was below 2%, and hence this method showed less variation at lower D-(+) concentrations.

Linear regression analysis of the achiral method

An additional evaluation for the accuracy and precision of the achiral HPLC-UV-CD method was performed using linear regression analysis. Thus, a linear equation was fit to the values determined by the CD detector for the D-(+) composition of epinephrine standard solutions when plotted against the actual values (Figure 5a). For accurate methods, this analysis should produce a regression line with a slope equal to 1 and a *y*-intercept of 0.^[31] To evaluate the achiral method's accuracy, 95% confidence intervals for the slope and the *y*-intercept of the fitted line were calculated as described in "Materials and Methods" section. These were 1.00 ± 0.10 and 0.3 ± 0.7 , respectively (Table 3). Both intervals contained their ideal values, suggesting a good agreement between the measured and actual compositions. Additionally, the coefficient of determination was close to 1 ($r^2 = 0.9722$), indicating good repeatability.

The same procedure was performed using the average D-(+) composition determined from the achiral and chiral analyses, which provided a direct comparison for results obtained from each method (Figure 5b). The 95% confidence intervals for the slope and *y*-intercept of the regression equation were 0.97 ± 0.22 and 0.3 ± 1.4 , respectively (Table 3). Once again, both of these intervals contained their ideal values, and data were strongly correlated between methods ($r^2 = 0.9851$). These data suggested that the achiral method could perform similarly to the chiral analysis for determining epinephrine enantiomer purity.

Assessing the use of CD detection for determining enantiomer composition

Current USP testing criteria require epinephrine-injectable drug products to contain a quantity between 90 and 115% of the labeled amount.^[29] With an LOQ of 3.1% for the D-(+) enantiomer, HPLC-UV-CD would be sufficient in most cases to verify the required concentration of the active L-(−) enantiomer. Unlike other drug products where the presence of the wrong enantiomer may be harmful, D-(+) epinephrine is not considered toxic, and it is regularly found in epinephrine-containing drug products at a level of around 5%.^[25,32] Thus, the ability to accurately and precisely determine trace levels of the D-(+) enantiomer is not critical to drug safety but can impact drug potency. Here, the achiral HPLC-UV-CD method was found to be suitable for the purpose of monitoring the D-(+) enantiomer composition in epinephrine drug products to ensure quality. Incorporating a CD detector with existing instrumentation is an easy way to simultaneously confirm the enantiomer composition while determining the assay value using a currently validated achiral HPLC method. This avoids the need to develop and perform an additional chiral separation method and decreases cost and analysis time.

Determination of the chiral purity of an epinephrine drug product

Adrenaline[®], an injectable epinephrine solution with a label claim of 1 mg/mL of the L-(−) enantiomer, was analyzed to determine its enantiomeric purity. Using the achiral HPLC method with CD detection, the total epinephrine concentration was determined to be 1.06 mg/mL, and the D-(+) enantiomer composition was calculated to be 0.5% (Table 4). Next, the same product was analyzed using the chiral HPLC method. Similar to the achiral analysis, the total epinephrine concentration was determined to be 1.07 mg/mL with 0.6% in the D-(+) configuration. For each analysis, the amount of D-(+)-epinephrine measured was below the LOQ for their respective method, indicating the sample containing a high purity for the L-(−) enantiomer.

Conclusions

Circular dichroism spectroscopy was evaluated as a means of determining epinephrine enantiomer purity following achiral HPLC. Using the HPLC-UV-CD method, the enantiomeric compositions of epinephrine solutions could be accurately and precisely determined at suitable levels to meet USP testing criteria. A commercially available epinephrine-injectable sample was analyzed, and results agreed with analysis performed using a traditional chiral separation. The incorporation of a CD detector with achiral chromatography permits the simultaneous analysis of epinephrine purity, concentration, and enantiomeric composition using the assay method from the USP monograph for epinephrine injection. The use of a CD detection strategy may be generally applicable to other chiral drug products and may avoid the need for development of additional chiral-specific methods for each chiral drug.

Acknowledgments

Funding

Funding was obtained through the Research Participation Program at the Center for Drug Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

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Figure 1. Circular dichroism spectra of 750 $\mu\text{g/mL}$ L-(-) and D-(+) epinephrine (red and blue, respectively) in 85/15% 50 mM sodium phosphate/methanol, pH 3.8.

Figure 2.

Chromatograms for epinephrine solutions following HPLC using an achiral stationary phase. UV (upper), ellipticity (middle), and g-factor (lower) responses were recorded for injections of 100 $\mu\text{g/mL}$ epinephrine solutions. Standard solutions consisted of (a) 90/10% L-(-)/D-(+) epinephrine and (b) a racemic mixture.

Figure 3. g-Factor versus % D-(+) epinephrine for achiral HPLC analysis of 100 $\mu\text{g/mL}$ epinephrine standards. The inset displays the same data in the 0–10% D-(+) epinephrine range.

Figure 4. Chromatograms for injections of epinephrine following HPLC with chiral methods. 100 $\mu\text{g/mL}$ standards of (a) a racemic mixture and (b) a 90/10% L-(-)/D-(+) epinephrine solution were detected using UV (upper) and ellipticity (lower).

Figure 5.

Composition of D-(+) epinephrine in standard solutions determined from achiral HPLC analysis versus (a) the actual value and (b) the average amount determined from chiral analysis for triplicate injections.

Table 1

Limits of detection (LOD) and quantification (LOQ) for the percent of the D-(+) enantiomer in 100 µg/mL epinephrine solutions for achiral and chiral analysis methods.

Analysis method	LOD(%)	LOQ(%)
Achiral HPLC	1.0	3.1
Chiral HPLC	0.5	1.5

LOD, limits of detection; LOQ, limits of quantitation.

Table 2

Comparison of recovery metrics of D-(+) epinephrine using achiral and chiral methods. The recovery was determined as the percentage of the calculated versus the actual D-(+) epinephrine composition. Reported values represent an average of three determinations.

Analysis method	Achiral HPLC		Chiral HPLC		
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	
% D-(+) epinephrine	2	105.9	30.7	95.3	0.4
	4	110.8	5.5	99.5	7.2
	6	93.5	12.3	103.4	0.1
	8	100.6	4.6	98.8	3.6
	10	100.4	2.4	99.7	2.8

RSD, relative standard deviation.

Table 3

Regression statistics for best-fit lines in Figure 5a (actual values) and 5b (chiral HPLC). Intervals represent a confidence level of 95%.

Comparison method	Slope	Intercept (%)	r^2
Actual values	1.00 ± 0.10	0.3 ± 0.7	0.9722
Chiral HPLC	0.97 ± 0.22	0.3 ± 1.4	0.9851

Table 4

Determination of the concentration and enantiomeric composition of an epinephrine drug product.

Analysis method	[Epinephrine] (mg/mL)	D-(+) (%)
Achiral HPLC	1.06	0.5
Chiral HPLC	1.07	0.6