Research Article

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Chiral and Achiral Enantiomeric Separation of (±)-Alprenolol

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Abstract: The chiral separation of enantiomers is crucial for pharmacovigilance within drug discovery. Although a large number of prescribed medications are marketed as pure enantiomers, this is not always the case and many are in fact racemic mixtures. Drug scandals, such as that of Thalidomide in 1961, provide a clear example of the social and economic repercussions that can be caused by negligence of these chiral compounds. Two high performance liquid chromatography (HPLC) methods are presented to determine, separate and quantitate a commonly prescribed chiral beta blocker, (-)-Alprenolol. The first method utilises a chiral column to physically separate the two enantiomers of Alprenolol in 25 minutes, before quantitating with two detectors. Fluorimetry gave the better limit of detection of 0.16-0.41ng and a correlation coefficient of 0.999. The second method used an achiral column coupled with polarimetry to quantitate (-)-Alprenolol without the need for physical separation in 10 minutes. The limit of detection achieved was 27-37μg and demonstrated a correlation coefficient of -0.999.

Keywords: Separation; Enantiomer; Chiral; Achiral; HPLC.

1 Introduction

Beta blockers impede the effects of epinephrine and norepinephrine within cardiac and smooth muscle resulting in a decrease in heart rate and blood pressure [1]. Alprenolol is a non-polar beta blocker commonly prescribed for the treatment of cardiovascular presentations such as hypertension, angina and cardiac arrythmia [2,3,4]. Numerous substances found in nature, many of them pharmaceutical medicines such as Alprenolol, are chiral [5]. Typically, only one enantiomer is therapeutically active, and the other is either inactive or toxic. This was highlighted in the media by the 1961 Thalidomide drug scandal, which demonstrated a further need for research into the separation of these two enantiomeric forms [6,7]. It has been verified that for beta blockers, including Alprenolol, the pharmacokinetics differ greatly between the two enantiomers [8]. For a class of drug that is marketed as a "racemic dose", only the (-)-Alprenolol accounts for the majority of the beta blocking activity [9]. A large number of pharmaceutical products are sold under the ruse of being pure enantiomers but are still in fact racemic mixtures [10]. As a result, changes to pharmaceutical manufacturing guidelines aim to increase safety and subsequently avoid the reoccurrence of similar scandals [11]. This has led to a greater need for robust, reliable and high throughput analytical techniques to be developed for enantiomeric separation and detection.

Attempts at the resolution of enantiomeric forms of Alprenolol and other beta blockers has been done previously using capillary electrophoresis (CE) [12,13], micellar electrokinetic chromatography (MEKC) [14,15], capillary isotachophoresis coupled with nuclear magnetic resonance (cITP/NMR) [16], nano-LC [11] and HPLC with an acid glycoprotein (AGP) column [3,17-19]. These previous citations, however, have demonstrated various limitations such as poor sensitivity, inability to identify low purity chiral samples and lengthy method preparations [3,16-19]. The ability of chromatographic methods to simultaneously separate, analyse and quantitate a multitude of substances within a mixture demonstrates their superiority over other analytical techniques [20]. While HPLC is an analytical technique that is rarely surpassed in achieving separation, one of the most difficult challenges it has faced is perfecting the resolution of enantiomers [21]. As a result of the rise in demand to characterise enantiomers and determine their purities, the development of new methods for the separation of a wide range of enantiomers has progressed rapidly [22-24].

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There are two routes that can be taken with chiral separation by HPLC. Firstly, the use of a chiral column with standard detection and secondly the use of an achiral column with a chiral detector. Both methodologies will be investigated in this study. The advantages of a chiral HPLC method are that the high sensitivity of standard HPLC detectors, such as UV-Vis and Fluorescence, can be applied to chiral mixtures [3]. For the chiral opportunity, cyclodextrins (CDs) were employed as the chiral selector for the racemic separation, as compared to other selection options they offer many advantages [11]. The geometric arrangement of the molecule on the inner surface of the column creates a hydrophobic cavity to allow for chiral selectivity and the formation of inclusion complexes [25]. The size, polarity and structure of the guest analyte within the CDs surface all influence the formation of this inclusion complex, however if the molecular fit of the analyte is efficient then the chiral recognition will be more selective [26,27]. It has previously been reported that the presence of aromatic rings, such as that found within the structure of Alprenolol, increases the likelihood of inclusion complexes to develop [28]. Due to their opposite three-dimensional configurations the enantiomers of racemic mixtures associate differently with the cyclodextrin molecules and form inclusion complex diastereomers. It is expected in this case that there will be hydrophilic interactions between the C2 and C3 of the hydroxyl groups on the CD and the Alprenolol structure [29]. This type of column is characteristic for its enantioselectivity and is known for its elite separation of enantiomers and different pharmaceutical ingredients [30].

For the achiral method development, in-line polarimetry is chosen to distinguish the enantiomers as well as fluorescence and photometry for comparison. Although known for its low sensitivity up against the more commonly chosen detectors, such as a UV-Visible (UV-Vis) and Diode Array (DAD), polarimetry offers chiroptic data to explicitly separate and determine the identity of the enantiomers without the need for prior separation [31,32]. To the best of the authors knowledge the present study is the first to report two HPLC methods, one chiral and one achiral, capable of fully resolving racemic Alprenolol.

2 Experimental

2.1 Chemicals and Reagents

Racemic Alprenolol and (-)-Alprenolol Hydrochloride (1-(o-Allylphenoxy)-3-(isopropylamino)-2-propanol hydrochloride) >98% were purchased from Sigma Aldrich, UK. Analytical grade Acetonitrile (ACN), Ethanol (EtOH), Glacial Acetic Acid, Trifluoroacetic Acid (TFA) and Triethylamine (TEA) were supplied by Merck, UK. LiChrosolv® Ultrapure Grade Methanol (MeOH) was supplied by Merck, UK. LC grade water (H_2O) was prepared by passing de-mineralised water through a Milli-Q filtration system (Millipore, Bedford, MA).

2.2 Instrumentation

The chiral LiChroCART® 250-4 ChiraDex® (5µm) (Merck, UK) and the achiral Supelco LiChrospher® RP-8 125-4 (5µm) (Sigma, UK) chromatographic columns were tested. The flow rates used were in the ranges recommended by the manufacturers; 0.7mL/min for the chiral LiChroCART® column, and 1.0mL/min for the achiral LiChrospher®.

All measurements were achieved using a Hitachi-Merck (Darmstadt, Germany) high performance liquid chromatograph (HPLC) consisting of the following modules; a L6200 pump, an AS-4000 autosampler and a D-6000 interface. While the chiral stationary phase was in use, the following modules were coupled together; an L-4250 UV-visible detector (UV-Vis) and F-1080 fluorescence detector. The injection volume was set at 20µL. When the achiral stationary phase was applied, the F-1080 fluorescence detector and L-4250 UV-visible detector (UV-Vis) are employed alongside a ChiraMonitor® 2000 polarimeter. All integrations and instrument parameters were controlled with Hitachi-Merck HM software. Analog optical rotation data acquired from the ChiraMonitor® 2000 was transformed to digital by the Pico ADC-100 (Picotechnology Ltd., Cambridge, UK). The fluorescence detector was set at 230nm (absorption), 278nm (excitation) and 400nm (emission). The polarimeter laser was set, as described by Lloyd [33], providing 30mW of light at 830nm. The injection volume was set at 12µL. All injections were done in triplicate.

2.3 Chromatographic Conditions

The optimised reverse-phase chiral chromatographic separation was achieved with a total run time of 25 minutes. Experimentation used an isocratic gradient, with a mobile phase comprised of ACN:MeOH:Triethylamine:Acetic Acid (98:2:0.2:0.4, v/v). Flow rate was set at 1mL/min. The (+) and (-)-Alprenolol were identified based on standard retention times.

The optimised reverse-phase achiral chromatographic separation was achieved with a total run time of 10 minutes. Again, an isocratic gradient was used, with a mobile phase consisting of Methanol:Water:TFA (50:50:0.4, v/v). Flow rate was set at 0.7mL/min. The (+)- and (-)-Alprenolol were identified based on their optical rotation as identified by polarimetric data.

All solvents were filtered through a 0.2-micron filter and degassed in an ultrasonic bath prior to analysis. The column temperature was controlled at room temperature in both instances.

2.4 Mobile Phase and Sample Preparation

Standard stock solutions of the (-)-Alprenolol and Racemic (±)-Alprenolol were prepared respectively at a concentration of 15mg/mL in methanol. The racemic (±)-Alprenolol was diluted appropriately with methanol to create five calibration standards utilised for the chiral method, based on the known sensitivity of the detectors. Calibration standards for the achiral method were made by mixing the two stock solutions to contain 50, 55, 65, 75, 85 and 100% (-)-Alprenolol. All solutions were prepared weekly and stored in the fridge (4°C).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 Determination of Alprenolol Enantiomers using a Chiral Column

3.1.1 Optimisation of the Mobile Phase

Chiral separations are very sensitive to changes in temperature, pH, ionic strength, polarity and composition of the mobile phase [34]. A small change in these parameters can cause drastic changes in the retention and resolution of compounds within a mixture. Careful control of the experimental conditions, therefore, is necessary to obtain reproducible results. Optimising the mobile phase, in particular, is a key parameter required to be controlled as it has such a large influence on the signal received by the detectors [35]. Changing the constituents of the mobile phase can have a vast impact on resolution of structurally related compounds when using reversed-phase HPLC [36,37]. In this case, however, the high basicity of Alprenolol leads to complex mobile phase manipulation.

Initial injections of the racemic mixture utilised ACN as the mobile phase, however both enantiomers were observed to have a high affinity for the column and eluted slowly. From these initial trial runs, although lengthy, the time of the injection peak was eluded (~3 minutes). Development from this point forward aimed to fully resolve the enantiomers, but with elution as close to the injection peak as possible to ensure a quick run time. The literature expresses enantioresolution as an advantage of mobile phase modifiers, for which primary alcohols such as MeOH are seen as far superior to branched alcohols such as isopropanol (IPA) [38,39,40]. MeOH, therefore, was added to the mobile phase in small incremental amounts over a series of injections. This demonstrated a decrease in the retention of the Alprenolol analytes as the ratio of MeOH to ACN increased. Unfortunately, with this, the resolution of the enantiomers decreased. The optimum ratio of ACN to MeOH was found to be 98:2, finding a compromise between retention on the stationary phase and resolution. Other modifiers, such as acid buffer, were also trialled as previous studies have demonstrated how enantioselectivity is dependent on pH [39,40]. Acetic acid was chosen as the buffer of choice, as it also improved peak tailing, with the best output in results seen at 0.4% [41]. The literature also discusses the advantages of mobile phase additives, explaining how the type and ratio selected can impact enantioselectivity and resolution [38,42]. TFA is known to have a dominant effect on chiral separations, however for more basic drugs TEA has been proven to demonstrate a higher success rate [38]. In light of this, 0.2% TEA was added to the mobile phase.

Retention times for the (-) and (+)-Alprenolol enantiomers were achieved at approximately 19 and 21 minutes (Figures 1 and 2). Due to broad peak shapes, complete baseline separation between the two enantiomers was not achieved (<0.8 USP Resolution, Peak Tailing >1.5) [43]. Regardless of further changes made to the mobile phase, including trial of a gradient and other method parameters, this resolution and peak shape could not be improved. Mechanisms for enantiomer separation are not clearly defined in the literature, however Pirkle & Pochapsky detail a three-point rule that requires at least three instantaneous stereochemical interactions between the chiral stationary phase and the analyte [44]. A lack of these published interactions between the stationary phase and the enantiomers could explain the subpar baseline resolution. Despite this, it was deemed that the method was acceptable for use, and development moved onto determination of enantiomeric purity.

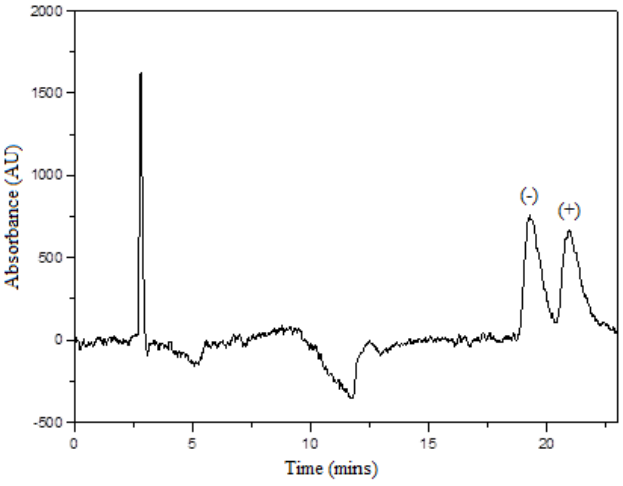

Figure 1: Final chiral method parameters for the separation of Alprenolol racemic mixture using photometric detection. Elution of (-)-Alprenolol and (+)-Alprenolol were seen at ~19 and 21 minutes respectively.

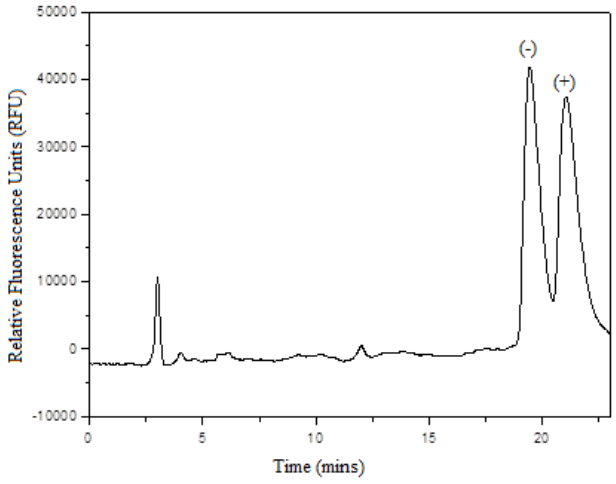

Figure 2: Final chiral method parameters for the separation of Alprenolol racemic mixture using fluorescence detection. Elution of (-)-Alprenolol and (+)-Alprenolol were seen at ~19 and 21 minutes respectively.

3.1.2 Quantitation of Alprenolol Enantiomers

Early injections of the racemic mixture demonstrate that the peak heights/areas of the Alprenolol enantiomers are not identical. This is an immediate indication that the racemic mixture is not entirely racemic and does not contain exactly 50% of each enantiomer. Using the analytical signals received from the photometric and fluorometric detectors, alongside the method previously published by Sanchez et al., the ratio of each enantiomer in the sample can be determined [45]. Here, the peak height, molar absorption coefficient and the concentrations of each enantiomer in the racemic mixture are used to calculate the enantiomeric purity as 52.5% (-)-Alprenolol and 47.5% (+)-Alprenolol. These values assigned to each enantiomer within the racemic mixture were used to calculate the total µg of (±)-Alprenolol contained in each calibration standard. This transpires to be 49-75µg across the calibration range. Linearity was assessed at five levels between these two values.

Least squares regression has been discussed in the literature as the most commonly used multivariate regression method, particularly for building calibration models [46]. When compared with other multivariate regression models, the least squares method demonstrates a higher separation power as its algorithms are designed in such a way as to counteract the presence of predictor variables when there are relatively few samples [47]. The linear calibration model is most easily achieved by performing a linear least squares regression of the instrument response versus the mass of the analyte.

Calibration plots were generated to investigate the linearity of the method, by plotting the total peak area (or peak height) for both enantiomers against the total (±)-Alprenolol present in each calibration standard (µg). The tested standards were prepared according to the sensitivity of the detectors. Peak area calibration plots for photometric detection gave a correlation coefficient of 0.993, and fluorometric detection gave a correlation coefficient of 0.991 (Figures 3 and 4). Peak height calibration plots for photometric detection gave a correlation coefficient of 0.991, and fluorometric detection gave a correlation coefficient of 0.990.

It was also determined exactly how much of each enantiomer (µg) was present in the calibration standards, as a result of knowing the initial ratio in the racemic mixture. Calibration plots were therefore constructed using the individual enantiomer peak heights/areas instead of the combined values at each chosen calibration level. The regression equations, correlation coefficients and limits of detection generated for both enantiomers using the peak heights and areas with photometric and fluorometric detection are shown in Table 1.

Results demonstrate that fluorometric detection, for both peak area and peak height, produce the best results. Limits of detection (LoD) were considered to be the calibration standards which produced signal-to-noise ratios of 3, as per ICH guidelines [43]. The LoD determined for the fluorescence signals were between 0.16-0.41ng, whereas those for the photometric signals were much higher with great variation between 26-630ng. Interestingly, although peak area typically gives better

Table 1: Calibration data for L (-) Alprenolol and D (+) Alprenolol (N = 3).

	Photometric			Fluorometric		
	Regression Equation (y=m x +c)	Correlation Coefficient (R^2)	LoD (ng)	Regression Equation (y=m x +c)	Correlation Coefficient (R^2)	LoD (ng)
L(-)Alprenolol Area	y =12.35x + 313.18	0.991	340	y = 1275.96 x + 1808.28	0.999	0.41
L(-)Alprenolol Height	y = 22.14x + 200.08	0.996	26	y = 1872.97x + 6353.84	0.996	0.19
D(+)Alprenolol Area	y = 15.95x + 373.45	0.941	630	y = 1640 x + 2236.63	0.998	0.25
D(+)Alprenolol Height	y = 20.05x + 238.33	0.994	36	y = 1765.67x + 3114.94	0.999	0.16

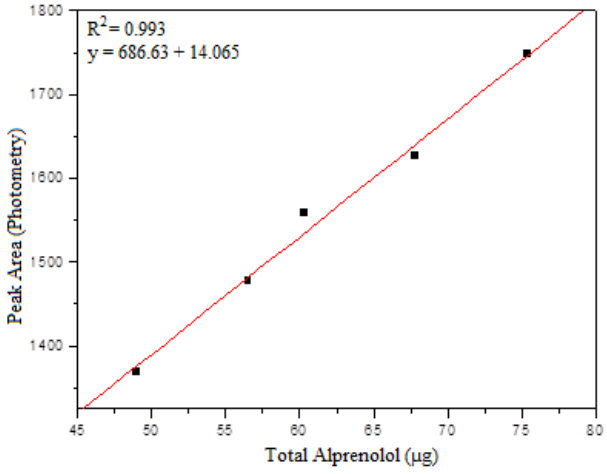

Figure 3: Photometry calibration plot using peak area as a function of the total (±)-Alprenolol in each calibration standard (µg).

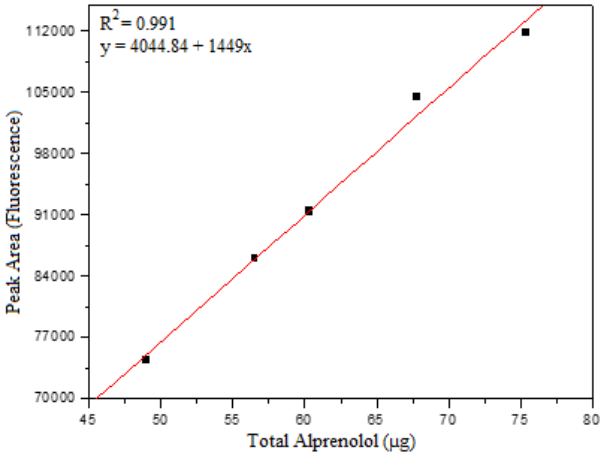

Figure 4: Fluorescence calibration plot using peak area as a function of the total (±)-Alprenolol in each calibration standard (µg).

results with chromatographic methods than peak height in terms of calibrations, the better limit of detection for the photometric results are those of the peak height [48]. This can be seen across both data sets, as the results derived from the peak heights deduct higher sensitivity than those of peak areas. The literature does suggest, however, that peak height can be more reliable when dealing with the lower end of a calibration set i.e. determination of limit of detection [49]. The correlation coefficients for both fluorescence and photometric detection are close to 1, but the data from the fluorescence detector is again superior.

3.2 Determination of Alprenolol Enantiomers using an Achiral Column

3.2.1 Optimisation of the Mobile Phase

Aside from the alternate column, another difference from the previous method is the detectors used. Polarimetry has a high dependence on many variables, including the composition of the mobile phase, the temperature, oscillations of the flow and the impurities of the solvents [50]. The selection of the mobile phase, specifically, is crucial since it intervenes in the optical rotation of the enantiomers in the polarimetric detector [51-54]. Biot's law states that this rotational power produced by an enantiomer will be directly proportional to the quantity of each enantiomer present and therefore the output signal given. This, in turn, has a direct effect on the sensitivity of the technique [54]. In a racemic mixture, however, the rotation being produced in the plane of polarised light by the positive enantiomer is counteracted by the rotation being produced by the negative enantiomer, which gives a final optical rotation of zero [52]. From this it could be inferred that the optical rotation produced by a sample of a chiral substance will be proportional to the difference in quantities of the two enantiomers and, therefore, their concentrations.

The mobile phase composition of the achiral method was optimised, similarly to the chiral method reported, by altering the buffers and mobile phase modifiers. Initial

injections of the racemic mixture utilised MeOH as the mobile phase to analyse the retention of the enantiomers. MeOH was chosen over ACN as it is commercially cheaper, however the literature suggests that the RP column interactions are stronger than those of ACN, which leads to increased separation power of compounds [55]. Alprenolol was poorly retained by the column, however, and eluted prior to the injection peak (~2 minutes). The addition of water was investigated with the aim to increase the retention of the analytes on the column, therefore separating the injection peak and the Alprenolol. After various ratios of MeOH:H_2O were trialled it was observed that there was a linear relationship between the ratio of water added to the mobile phase and the retention of the analyte on the column. This is a common relationship that has been observed in the literature since the 1990's [56]. The optimal composition of mobile phase here was deemed to be MeOH:H_2O 50:50. TFA was utilised as a mobile phase additive (0.4%) as it has been previously demonstrated to have a dominant effect on chiral separations for both acidic and basic chiral drugs [38].

It was identified that under these conditions the polarimetric detector was sufficiently sensitive, and two signals are obtained for the enantiomers at 7.08 minutes (425 secs) and 7.92 mins (475 secs). For the photometric and fluorescence detectors, a broad single peak for Alprenolol eluted at ~7 minutes indicating coelution of the two enantiomers, lending these detectors somewhat obsolete. This lack of physical separation of the enantiomers was expected due to the achiral nature of the RP-8 column, however the signals received from these detectors can still be used to determine the total amount of the enantiomers in a sample.

Unfortunately, as the polarimeter required a high sample concentration in order to get a signal observable over the high level of noise, the resulting peak of interest when detected using photometry and fluorescence has prominent tailing. The peak broadening is also increased further as a result of the columns inability to resolve the enantiomers. This will in turn cause deviations in the linearity of the response from these detectors, as they are close to their capability limits, and will therefore not be used for any calculations.

3.2.2 Determination of Alprenolol Enantiomeric Purity using an Achiral Column

Calibration standards, produced as detailed previously, were run on the now optimised achiral method. The calibrations plot the difference in polarimetric peak area or peak height of the two enantiomers as a function of the percentage of (-)-Alprenolol in the mixture. The calibrations for difference in peak height and peak area showed peak area to be the superior measurement, with a correlation coefficient of 0.996. The correlation coefficient for difference in peak height was 0.980. Both calibrations display a strong, robust correlation to the percentage of (-)-Alprenolol present in the sample, however the linearity of the method still requires improvement.

The calibration equations generated for the data sets shown were used to calculate the percentage of (-)-Alprenolol in the prepared racemic mixture at 51.8% (which would therefore put the (+)-enantiomer at 48.2%). Comparing these results to those obtained using the chiral column it is seen that they are very close; 52.5% with the chiral column and 51.8% with the achiral column. This initially suggests that these two methods are comparable with each other, however it cannot be said which is superior as the true value of the (-) and (+)-Alprenolol in the mixture is unknown.

3.2.3 Correcting Linearity Deviations from the Polarimetric Signal

Deviations of a polarimetric signal from ideal linearity can be demonstrated through an exploration of refractive index artefacts (RIA) and chemical law [54]. In order to eliminate these phenomena of measurements, corrections must be made to calibration equations [52,54]. As previously discussed, Biot's law explains that the response from positive and negative enantiomers either counteract each other or give a response that indicates a higher presence of one enantiomer [54]. It would be logical to assume that this value is obtained only due to the effect of optical rotation, however, this is not entirely accurate [57]. Signals obtained from pure enantiomer injections give only positive values for (+)-enantiomers and only negative values for (-)-enantiomers, and as a result the effect of RIA is low compared to the analyte signal. The closer the ratio of the enantiomers in a mixture is to 50%, however, the greater the impact of these other factors [54]. Literature suggests that the main factors that influence RIA are the refractive index of the mobile phase, the analyte concentration and the flow of the mobile phase, all of which can be corrected [5]. RIA corrections were applied to each of the calibration points, and calibration equations updated. Excellent correlation coefficients of 0.999 for both peak area and peak height are achieved after reprocessing, further demonstrating the importance of applying RIA corrections to data obtained by polarimetric

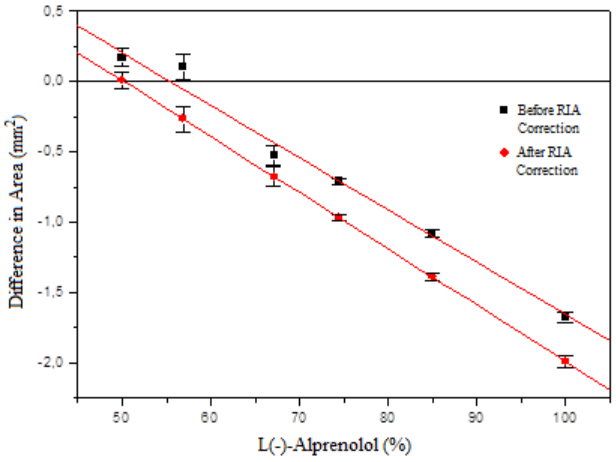

Figure 5: Achiral calibration plots before and after RIA corrections, plotting the difference in polarimetric signal peak area as a function of L(-)-Alprenolol percentage.

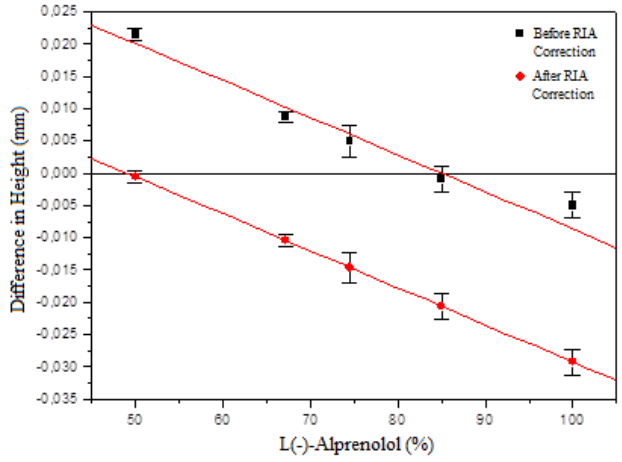

Figure 6: Achiral calibration plots before and after RIA corrections, plotting the difference in polarimetric signal peak height as a function of L(-)-Alprenolol percentage.

detection. The negative correlation coefficients represent the negative correlation seen throughout the calibrations, with LoD seen between 27-37µg. The degree of RIA corrections can be seen in Figures 5 and 6.

The corrections eliminated any deviations in linearity due to the aforementioned effects and, as expected, the racemic mixture gives a signal difference of zero for both data sets. Using the corrected calibration lines, the percentage of (-)-Alprenolol in the racemic mixture is calculated. Based on the difference in area of the polarimetric signals 52.7% is obtained. The difference made when applying RIA corrections can be seen by comparing pre and post RIA results with that of the chiral method.

4 Conclusions

Previous attempts at the resolution of Alprenolol enantiomers, as well as other beta blockers, has been attempted but have demonstrated various limitations. Here, two high performance liquid chromatography methods are presented which separate and quantitate (±)-Alprenolol using chiral and achiral routes.

Mobile phase parameters such as mobile phase modifiers, acids and additives were investigated to achieve optimised chromatographic conditions. The (±)-Alprenolol analytes had a high affinity for the column under initial chiral conditions and eluted slowly. Development, leading to a final mobile phase of ACN:MeOH:TEA:Acetic Acid (98:2:0.2:0.4 v/v), allowed enantioresolution of the two compounds in 25 minutes.. The mobile phase manipulation for the achiral method was crucial due to the nature of the polarimetric detector. This method utilised Methanol:Water:TFA (50:50:0.4, v/v) to separate the (±)-Alprenolol enantiomers in just 10 minutes.

Using the developed chiral method, it was determined that the racemic (±)-Alprenolol standard did not contain equal quantities of both enantiomers. Peak height, molar absorption coefficient and the concentrations of each enantiomer were used to calculate enantiomeric purity at 52.5% (-)-Alprenolol. Least squares regression algorithms were used to investigate linearity of the method for each individual enantiomer. The analysis was carried out for both detectors using peak area/height versus mass of analyte. Fluorescence was deemed the far superior detector, achieving correlation coefficients between 0.996 and 0.999, and limits of detection between 0.16-0.14ng.

Calibrations standards were run on the optimised achiral method parameters to assess linearity of the method. Peak area was found to be the statistically superior variable over peak height in this instance with a correlation coefficient of 0.996, showing that the linearity of the method still required improvement. Linearity deviations from the polarimetric signal were corrected in order to eliminate refractive index artefacts, which produced updated correlation coefficients of 0.999, and limits of detection between 27-37ug. Enantiomeric purity, here, was determined to be 52.7% (-)-Alprenolol.

The calibration models for both methods demonstrated above acceptable limits, indicating their strength and utility. They present two ways to separate, detect and quantitate (±)-Alprenolol enantiomers although each with their limitations. The chiral method produces enantioresolution in 25 minutes with good limits of detection, although the unique columns can be expensive. The achiral method allows for determination of both

enantiomers in 10 minutes, using relatively inexpensive instrumentation, although limits of detection suffer as a result. The shorter run time lends the advantage, however, of being more environmentally viable with less solvent waste.

Conflict of interest: Authors declare no conflict of interest.

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