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Chiral separation and detection enhancement of propranolol using automated pre-column derivatization

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Abstract: In the liquid chromatgraphic analysis of pharmaceuticals, two challenges are often encountered: detectivity and chiral separations. Propranolol, a beta adrenergic blocker, is a pharmaceutical compound that faces both of these limitations. In this study, both limitations are overcome simultaneously using derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC), a highly fluorescent and chiral reagent. The derivatization is automated using an autosampler with an AutoMix™ microrobotic feature, which greatly contributes to the efficiency and reproducibility of the method when manipulating microlitre volumes of sample and reagents. The method yields excellent separation of the diasteriomers, has a detection limit of 1 picomol, good reproducibility and linearity in the 50–400 pmol range (on column). In addition, this method is simple, requires no elevated temperature, no chiral stationary or mobile phases and can be easily automated.

Keywords: *Propranolol; FLEC; chiral; racemic mixtures; diastereomers; automated sample preparation.*

Introduction

Poor detectivity due to the native characteristics of the analyte group is a common limitation to chromatographic methods. In the case of HPLC analysis of amino acids, detectivity problems have been solved through both pre- and post-column derivatization schemes with detection limits extended to sub-picomolar amounts (on column) [1].

Since pharmaceutical enantiomers can have different therapeutic characteristics and effectiveness, the determination of enantiomeric purity has become increasingly important in the pharmaceutical industry. Due to their identical physical and chemical properties, enantiomers can be separated by HPLC only when a chiral reaction or interaction is involved. Currently there are three different HPLC methods for chiral separations: Converting enantiomers to diastereomers by pre-column derivatization with optically active reagents; using chiral mobile phase; using chiral stationary phase. The last two methods offer advantages in easy sample preparation and preservation of analyte structure but are costly and not very flexible in the selection of stationary and/or mobile phase. The first method is just the opposite. It is more cost effective and very flexible in the selection of HPLC parameters. Also, the derivatization may be chosen to enhance detection. On the other hand, the method is more time-consuming as the derivatization process is an additional sample preparation step. However, an LC autosampler with derivatization capabilities can easily automate the process. Further comparison of the three methods has been well documented by Szepesi [2].

Propranolol [1-isopropylamino-3-(1-naphthoxy)-2-propanol], a chiral beta adrenergic blocker, exists in the form of *R*-(+) and *S*-(−) isomers with different pharmacological properties while only the *S*-(−) isomer has blocking activity [3]. Propranolol has been derivatized with *N*-trifluoroacetyl-*S*-(−)-prolyl chloride [4] and also with 1-phenylethyl isocyanate [5]. The detection limits were at the nanogram [4] to microgram [5] level and neither method was automated. In this study an alternative automated method is developed for chiral separation as well as detection enhancement of propranolol.

It has been shown that in the analysis of amino acids, the amino group reacts with the chloroformate of 9-fluorenylmethyl chloroformate (FMOC) to form a fluorescent derivative [6–8]. This reaction is fast even at room

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temperature and forms a highly fluorescent derivative for sensitive detection.

When the methyl group in FMOC is replaced by an ethyl group, the reagent is (±)-1-(9-fluorenyl)ethyl chloroformate (FLEC) which is chiral and is available in its pure enantiomeric (+) form. It has been used in the chiral separation of amino acid enantiomers and chiral amines [9, 10]. Similar to FMOC, it is highly fluorescent and the derivatives are quite stable. In this study, propranolol is derivatized with FLEC (Fig. 1) to show the chiral separation and detection enhancement in the HPLC analysis.

All the derivatizations were performed with an LC autosampler that has an AutoMix™ microrobotic feature which permits all sample preparation steps, i.e. reagent addition, mixing and extraction, to be carried out automatically [11].

Experimental

Instrumentation

The HPLC system consisted of a 9010 pump, 9095 AutoSampler, Fluorichrom II fluorescence detector, 9050 UV–vis detector, LC Star Workstation and MicroPak SP C_8 4 mm × 15 cm column from Varian Chromatography Systems (Walnut Creek, CA, USA).

Materials

DL-Propranolol hydrochloride was purchased from Sigma Chemical (St Louis, MO, USA). FLEC reagent was obtained from EKA Nobel AB, Surte, Sweden. Sodium bicarbonate (reagent grade) was from J.T. Baker (Phillipsburg, NJ, USA). Boric acid (analytical reagent) was purchased from Mallinckrodt (St Louis, MO, USA). Sodium acetate (HPLC grade) and sodium hydroxide (50% solution) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Glacial acetic acid, acetonitrile and acetone (both HPLC grade) were products of Burdick & Jackson (Muskegon, MI, USA).

Methods

Propranolol stock standard (1.6 μmol ml^{-1}) was prepared in water and diluted with water to 400, 100, 25, 5, 2.5 and 1 nmol ml^{-1}.

A 20 μl volume of (native) propranolol in the range 1.6–1 nmol ml^{-1} was injected to determine the detection limit using UV absorbance (254 nm) and a signal-to-noise level of 5.

Another propranolol stock standard (80 nmol ml^{-1}), was prepared in diluent (boric acid–sodium bicarbonate buffer) (0.10 M, pH 8.5 with sodium hydroxide) and diluted by serial dilutions with diluent to 40, 20, and 10 nmol ml^{-1}. The 40 nmol ml^{-1} standard was used for studying FLEC derivatization and the reproducibility of the method. The 20 nmol ml^{-1} standard was used for comparing the results between UV and fluorescence detection of the FLEC derivative. All four standards were used in the linearity study.

FLEC reagent (4 mM in dry acetone) was prepared.

The 9100 AutoSampler was programmed to AutoMix 20 μl of propranolol standard and 20 μl of FLEC reagent, stand for the reaction

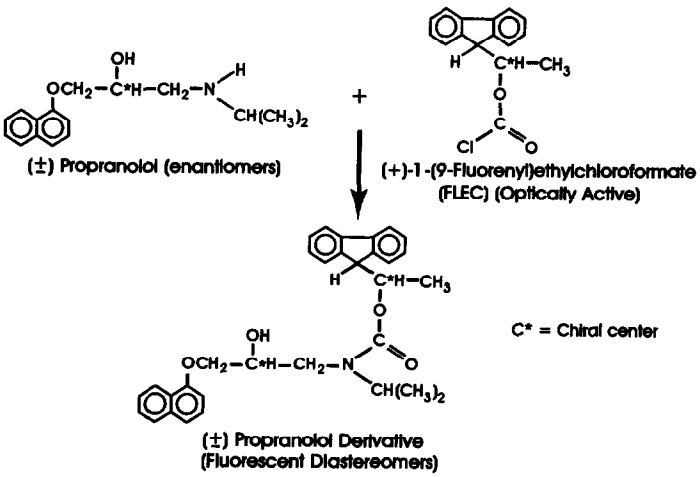


Figure 1
Analysis of (±) propranolol using pre-column derivatization (FLEC).

time of 10 min, and finally inject 10 μl onto the HPLC.

For the reproducibility study, six aliquots of 40 nmol ml^{-1} standards were derivatized separately and injected (immediately following each derivatization), all on the same day.

For the linearity study, the 80, 40, 20 and 10 nmol ml^{-1} standards were derivatized and injected (immediately following each derivatization), all on the same day.

A blank without propranol was included.

HPLC conditions were as follows. Column: MicroPak SP C8 (4 mm × 15 cm) at ambient temperature; mobile phase: sodium acetate (pH 4.0; 0.02 M)–acetonitrile (30:70, v/v) at 2 ml min^{-1}; detection: fluorescence using excitation 265 nm and emission 345 nm or UV absorbance at 254 nm.

Results

Using UV absorbance at 254 nm as the detection method, the detection limit of propranolol was found to be 100 pmol on column. FLEC-derivatized propranolol produced resolved twin peaks at 6.7 and 7.2 min, which correspond to the diastereoisomeric derivatives. Excess FLEC elutes at 11–13 min and there is no interference from the blank (Fig. 2). The derivatives were stable up to one day.

The difference between detection by UV (254 nm) and detection by fluorescence (excitation 265 nm, emission 345 nm) for FLEC-derivatized propranolol are shown in Fig. 3. With an amount as low as 100 pmoles on column, UV detection gave a small poorly-shaped peak for derivative 1 and a much larger peak for derivative 2. The disproportionate size of the second peak was found to be due to interference from the blank. Whereas, fluorescence detection yields two sharp peaks of equal size, as expected with racemic mixtures. The detection limit for the derivatives was found to be 10 pmol (on column) for UV (derivative 1 only) and 1 pmol for fluorescence.

Six replicate derivatizations and injections had good reproducibility with relative standard deviations of 1.1 and 1.8% for the two derivatives. Table 1 shows standards from 50 to 400 pmol (on column) were linear with correlation coefficients of 0.99960 and 0.99998 for the two derivatives.

Discussion

Chiral separation and detection enhancement of propranolol are achieved by means of derivatization using a chiral and fluorescent reagent. When UV absorbance is used for detection of the derivative, the detection limit is improved 10-fold over UV detection of native propranolol. When fluorescence is used for detection, the detection limit is improved

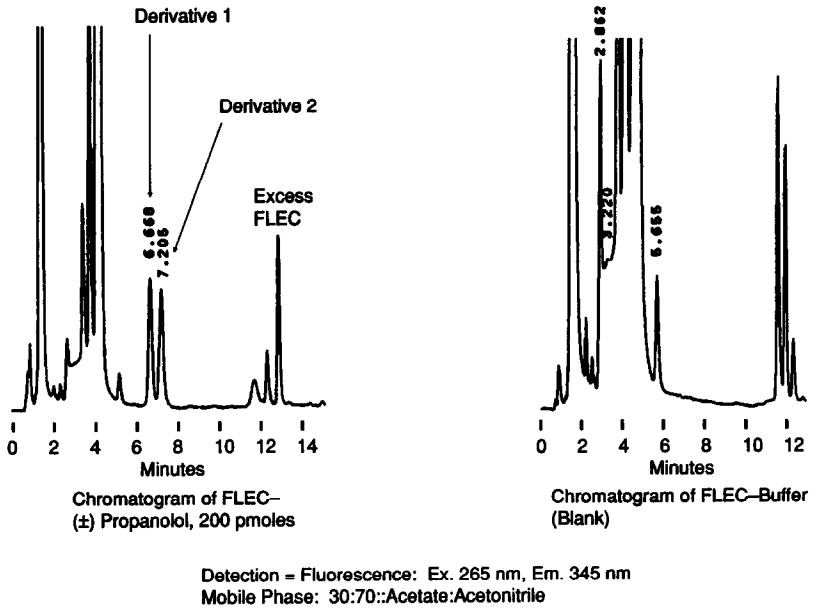
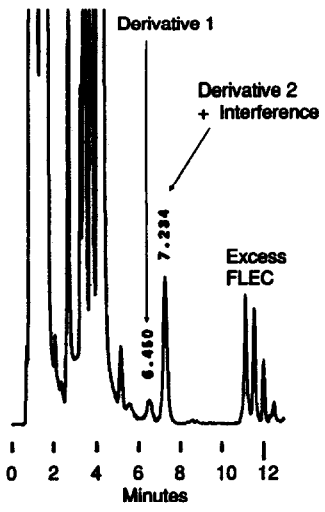


Figure 2
FLEC derivatization of (±) propranolol.



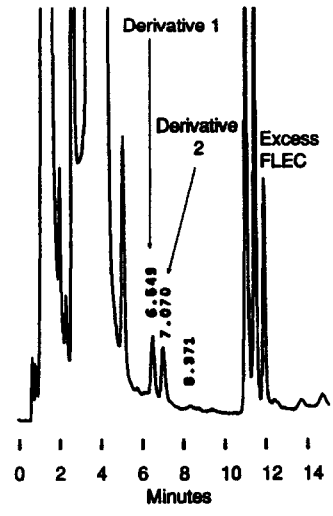


Figure 3
Detection enhancement using fluorescence versus UV.

Table 1
Linearity of FLEC-propranolol

FLEC-propranolol (pmol)	Area count	
	Derivative 1	Derivative 2
0	0	0
50	109743	123638
100	233833	253163
200	473936	494519
400	998174	990321

100-fold. This method is particularly useful when low sample concentrations or limited sample volume is being handled.

Chiral separation is excellent using FLEC as the derivatization reagent with no interference from the blank. Fluorescence detection in this case is not only 10 times more sensitive than UV detection, but it also eliminates the interference detected by UV absorbance. Excess FLEC elutes late in the chromatogram so that there is no interference problem and back extraction to eliminate the excess is unnecessary.

Conclusions

The method reported here is a very simple method for the chiral separation and detection enhancement of propranolol. No special chiral stationary phase or mobile phase is used. The use of a pure enantiomeric derivatization reagent is very cost effective. The current cost of FLEC is approximately $0.075 per assay according to this method. The derivatization completes within 10 min at room temperature and can be fully automated. The efficiency and precision of routine micro analyses can be greatly enhanced by using an LC AutoSampler as mentioned above. The results show good separation, linearity and reproducibility. The detection limit is as low as 1 pmol.

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