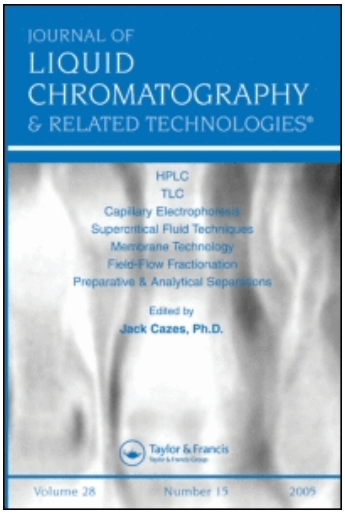

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OPTIMIZED ENANTIOSELECTIVE SEPARATION OF CLENBUTEROL ON MACROCYCLIC ANTIBIOTIC TEICOPLANIN CHIRAL STATIONARY PHASE

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ABSTRACT

An isocratic and simple high performance liquid chromatographic method was developed for the direct resolution of clenbuterol enantiomers. The method involved the use of the macrocyclic antibiotic teicoplanin chiral stationary phase column known as Chirobiotic T. Several mobile phase compositions were studied to optimize the resolution of racemic clenbuterol. The role of using different solvents, e.g. methanol, ethanol, and acetonitrile as organic modifiers and the effect of triethylamine and acetic acid on the enantioseparation of clenbuterol were investigated.

The best base-line separation was achieved using mobile phase consisting of ethanol containing 0.3% acetic acid and 0.2% triethylamine where the stereochemical separation factor (α) obtained was 1.58 and the stereochemical resolution factor (R_s) was 1.48. Under these chromatographic conditions, the (-)-R-clenbuterol eluted first with a capacity factor $k`_1$ of 1.89 followed by the (+)-S-enantiomer with a capacity factor of $k`_2$ of 2.99.

INTRODUCTION

Clenbuterol is a potent, orally active β_2-adrenoceptor agonist with powerful bronchodilator properties. It is used therapeutically to relieve respiratory disorders in humans and animals.[1] Clenbuterol is also used in veterinary medicine as a uterine relaxant.[2] When clenbuterol and salbutamol are administered in multiples of the therapeutic dose, these compounds improve nitrogen retention causing an increase in muscle growth and a reduction of body fat.[3] Clenbuterol has been used in beef production to promote this metabolic effect. Clenbuterol is one of the drugs abused by athletes and especially body-builders and there is an increasing misuse of the drug in muscle building establishments. History has made it abundantly clear that although drug abuse is not new to sport, these specific drugs of abuse are continuously changing, and thus the physician must be ever mindful.[4]

Clenbuterol is known to inhibit skeletal muscle atrophy in rodents secondary to disuse,[5] injury,[6] denervation,[7] cachexia, and endotoxiemia.[8,9] However, it was observed from various animal models that aging did not attenuate the anabolic or accentuate the catabolic effect of the drug.[10] Clinically, human aging is associated with declining muscle mass and functional reserve.[11,12] This age related decline is often accelerated by diseases of old age and malnutrition.[13] As a result muscle strength declines below the threshold required for independent performance of daily activities. Therefore, clenbuterol may be useful in preventing or reducing age-related decline in muscle mass and strength.

Cardiac hypertrophy is an effect of clenbuterol in rat.[14,15] Therefore, clenbuterol may be a hope for treatment of cardiomyopathy, a well known clinical condition caused by several heart diseases.

Clenbuterol is chemically known as 4-amino-3,5-dichloro-α-[(1,1 dimethylethyl)amino]-methyl-benzenemethanol, or 1-(4-amino-3,5 dichloro-phenyl)-2-*tert*-butylaminoethanol. It is marketed as a racemate mixture of 1:1 ratio of the two enantiomers R and S (Figure 1).

Clenbuterol racemate has been resolved on chiral α_1-acid glycoprotein.[16] Recently, Abou-Basha and Aboul-Enein[17] described a direct isocratic enantioselective separation of clenbuterol by chiral HPLC using a urea type chiral stationary phase made of S-indolone-2-carboxylic acid and R-1-(α-naphenyl ethylamine) known as Chirex 3022 column under normal phase conditions.

The aim of this study is to design an optimized high performance liquid chromatographic method for the enantioseparation of clenbuterol to be applied for (a) semi-preparative scale for the separation of few milligrams of clenbuterol

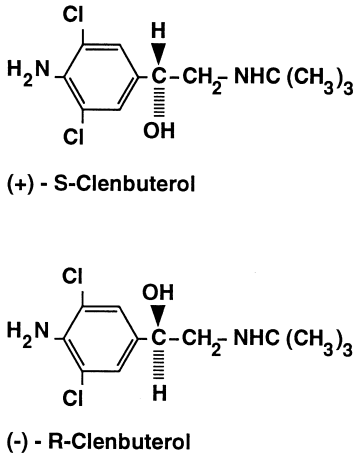

Figure 1. The absolute configuration of clenbuterol.

enantiomers to be pharmacologically evaluated for their inhibitory effect on skeletal muscle atrophy and (b) enantioselective analysis of clenbuterol enantiomers in plasma. Macrocyclic antibiotic teicoplanin was used as a chiral selector in this study, the chemical structure of which is shown in Figure 2.

EXPERIMENTAL

Chemicals

Absolute ethanol (EtOH) was obtained from E. Merck (Darmstadt, Germany). HPLC grade methanol (MeOH), acetonitrile (ACN), and triethylamine (TEA) were purchased from Fisher Scientific (Springfield, NJ, USA). Glacial acetic acid (AcOH) was bought from BDH chemicals (Poole, England). Clenbuterol-hydrochloride (batch #843031) was a generous gift supplied by the Dr. Karl Thomae Company Ltd. (Biberach, Germany).

Methods

A stock solution of clenbuterol-hydrochloride was prepared in its respective mobile phase (see below) or solvent components of which 20 μL, corresponding to 4.5 nmoles, was injected onto a Waters Liquid Chromatograph (Milford, MA, USA) consisting of 717 WISP autosampler, a 600E multisolvent delivery pump, a 5200 printer/plotter, a 990+ photodiode array detector with data acquisition, and analysis software loaded into a NEC Powermate 2

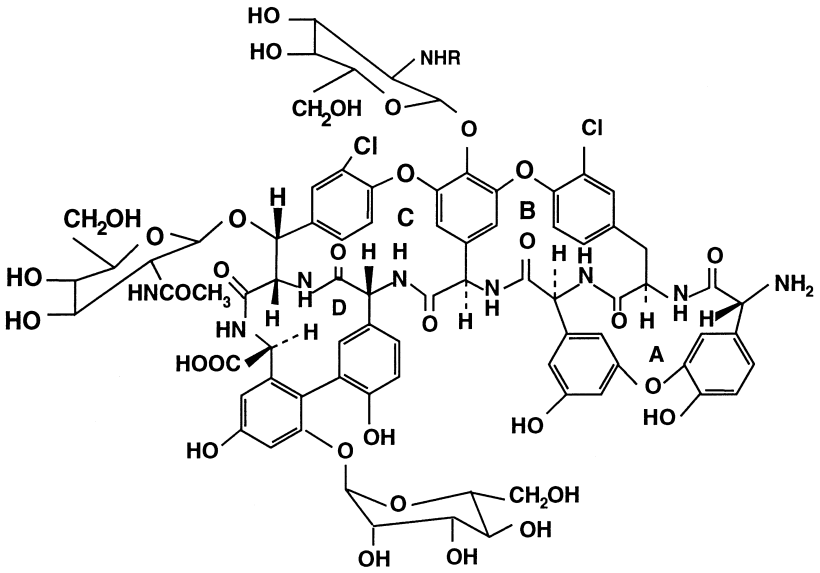

Figure 2. The proposed structure of the macrocyclic glycopeptide teicoplanin. This is covalently bonded to a silica gel and used as the chiral stationary phase commercially known as Chirobiotic T (R = CH_3 - decanoic acid).

computer. The chiral stationary phase used in this study was a macrolide type antibiotic, teicoplanin known as Chirobiotic T (serial # 8614) (25 cm x 4.6 mm id, with 5 μ spherical shape particle size silica) and an accompany guard column (2 cm x 4mm id). The set was purchased from Advanced Separation Technologies (Whippany, NJ, USA).

The mobile phases were the following. All other chromatographic conditions were as described in the Figure 3 legend:

1. MeOH, 0.2% TEA;

2. MeOH, 0.3% AcOH, 0.2% TEA;

3. EtOH, 0.3% AcOH, 0.2% TEA;

4. MeOH/EtOH (60/40, v/v), 0.3% AcOH, 0.2% TEA;

5. MeOH/ACN (45/55, v/v), 0.3% AcOH, 0.2% TEA; and

6. MeOH/ACN (70/30, v/v), 0.3% AcOH, 0.2% TEA.

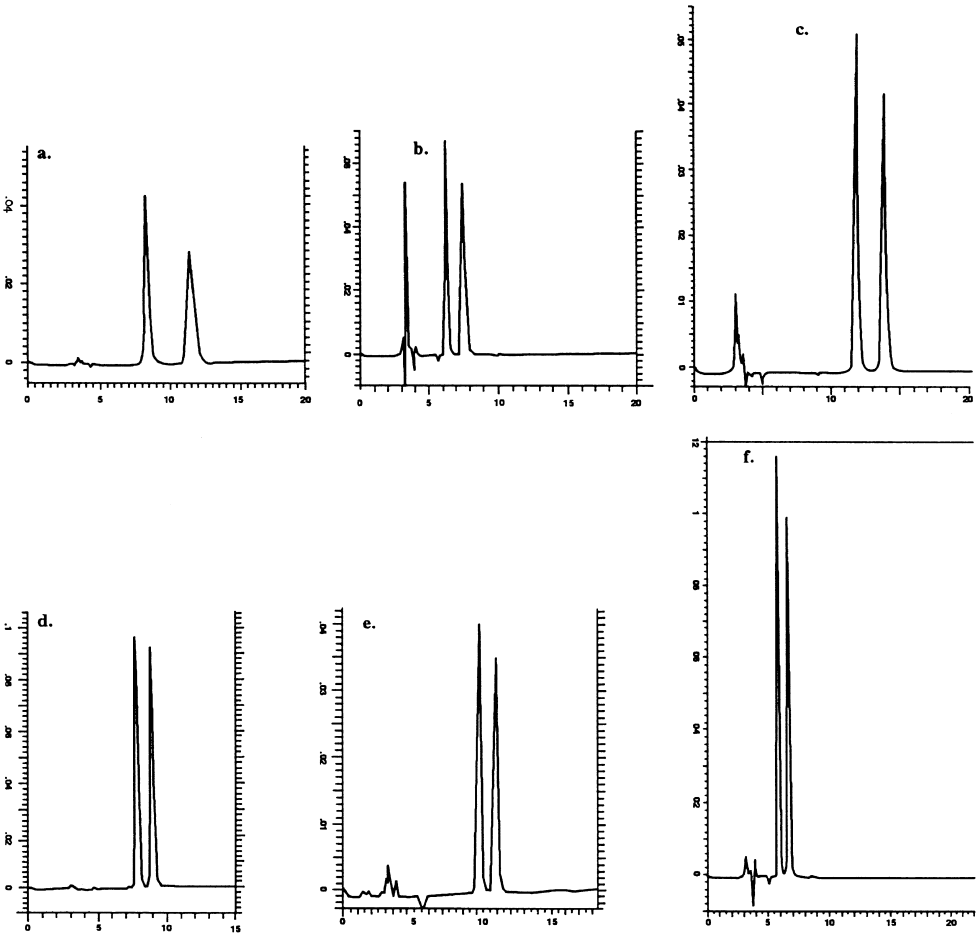

Figure 3. Chromatograms obtained for the enantiomeric resolution of clenbuterol, using the following mobile phases: a) EtOH, 0.3% AcOH, 0.2% TEA, b) MeOH/EtOH (60/40,v/v), 0.3% AcOH, 0.2% TEA, c) MeOH/ACN (45/55, v/v), 0.3% AcOH, 0.2% TEA, d) MeOH/ACN (70/30, v/v), 0.3% AcOH, 0.2% TEA, e) MeOH, 0.2% TEA, f) MeOH, 0.3% AcOH, 0.2% TEA. Chromatographic conditions: flow rate = 1.0 mL/min. pressure = 400 - 1060 psi, temp. = 23°C, chart speed = 0.5 cm/min., wavelength detection = 246 nm, sample quantity = 4.5 nmoles.

Chromatographic Parameters

Capacity factors (k`) were calculated using the equation $k`=(V_r-V_o)/V_o$ where V_r is the elution volume and V_o is the void volume. The separation factor (α) was calculated using the equation of $k`_2/k`_1$ where $k`_1$ and $k`_2$ are the capacity factors of the first and second eluted peaks. The stereochemical resolution

factor (R_s) was calculated according to the formula $R_s=2(tR_2-tR_1)/w_1+w_2$ X chart speed (cm/min) where w_1, tR_1 and w_2, tR_2 are the peak widths and retention time of the first and second eluted peaks, respectively.

RESULTS AND DISCUSSION

The purpose of this study was to select a suitable mobile phase that gave satisfactory retention times for the chromatographic peaks of interest, such that the chromatographic conditions could be adapted to (a) semi-preparative chromatography to separate a few milligrams of each individual enantiomer for their pharmacological evaluation; and (b) a plasma analysis for the clenbuterol enantiomers. The mobile phase of choice would possess the best limit of detection among those mobile phases tested, producing sharp symmetrical peaks with satisfactory peak heights.

Mobile phase compositions containing methanol, ethanol, and triethylamine (TEA) resolved the clenbuterol enantiomers (Figure 3). Without TEA, only one peak with distorted shape was detected. Replacing the alcohol by acetonitrile as the organic solvent produced poor results. The addition of acetic acid did not play a major role in chiral discrimination. However, peak sharpness and symmetry was improved when acetic was added to mobile phases containing alcohol and TEA (Figures 3c and 3f, respectively).

Ethanol presence in the mobile phase produced broader chromatographic peaks (Figure 3a) and favorable R_s value (see Table 1). Its short-coming was the low absorbance values (peak heights). A mixture of methanol/ethanol (Figure 3b) improved the peak heights indicating that methanol is the preferred alcohol. This is evident when methanol is the sole organic solvent in the mobile phase (Figure 3f). Although, the absorbance values obtained are favorable, the retention times are not suitable for a plasma analysis. A compromise was achieved when methanol was mixed with acetonitrile (Figure 3d). The chromatographic results showed good resolution with sharp symmetric peaks and retention times that would be distinguishable from those generated by plasma components. Hence, the methanol/acetonitrile mixture was the best mobile phase from those tried in this study.

Utilizing the same chromatographic conditions as those in Figure 3d, by substituting the photodiode array detector with the Shodex OR-1 optical rotation detector (JM Sciences, NY, USA), the optical rotation sign was determined for each chromatographic peak; they were identified as (-)-R-clenbuterol and (+)-S-clenbuterol, respectively (Figure 1).

The column (Chirobiotic T) used in this study belongs to a class of chiral selectors that are known as macrocyclic antibiotics. The chemical structure of teicoplanin (Figure 2) contains twenty chiral centers, creating a complete chiral environment. There are peptide, carbohydrate and ionizable groups, as well as

Table 1

Chromatographic Parameters, Capacity Factor (k'), Separation Factor (α), and Resolution Factor (R_S) for the Resolution of Clenbuterol

Mobile Phase*	k_1'	k_1'	α	R_S
1. EtOH, 0.3% AcOH, 0.2% TEA	1.89	2.99	1.58	1.48
2. MeOH/EtOH(60/40, v/v), 0.3% AcOH, 0.2% TEA	1.16	1.59	1.37	0.95
3. MeOH/ACN (45/55, v/v), 0.3% AcOH, 0.2% TEA	3.09	3.78	1.22	1.00
4. MeOH/ACN (70/30, v/v), 0.3% AcOH, 0.2% TEA	1.72	2.11	1.17	0.93
5. MeOH, 0.2% TEA	2.38	2.79	1.17	0.79
6. MeOH, 0.3% AcOH, 0.2% TEA	1.02	1.31	1.28	0.82

* TEA = triethylamine; AcOH = acetic acid; ACN = acetonitrile.

fused rings (A, B, C, and D, respectively). This paper will not include a discussion on the enantioselective mechanisms that are responsible for the chiral separation of the clenbuterol enantiomers. However, the interactions that can occur between this chiral stationary phase (CSP) and the analyte include hydrogen bonding, inclusion complexes, π-π bonding, steric interactions, and dipole stacking. These macrolide type CSPs have great potential for separating different classes of chiral compounds.

The plasma analysis for the clenbuterol enantiomers is currently being developed in our laboratory. It is worth mentioning that after injecting 260 reconstructed racemic clenbuterol residues extracted from pooled human plasma there was no apparent deterioration in the column performance. Although there has been a general limitation for commercially available chiral columns to be used for biological assay, Chirdiotic T proves to be an exception.

In summary, this study showed that the use of alcohols namely methanol and ethanol in the mobile phase results in better enantioselective separation of clenbuterol. However, replacement of methanol and/or ethanol by acetonitrile led to a significant decrease in enantioselectivity and resolution factor.

It is of interest to mention also that resolution was achieved in presence of triethylamine alone in the mobile phase. However, the presence triethylammonium acetate formed by addition of acetic acid and triethylamine to the mobile phase resulted in higher enantioselectivity and better resolution factor.

This indicates that resolution of clenbuterol enantiomers are proceeded through ion pairing mechanism. The results of this investigation are summarized in Table 1 and visualized in Figure 3.

CONCLUSION

Direct separation of clenbuterol enantiomers was achieved on teicoplanin chiral stationary phase column known as Chirobotic T. The method is isocratic, simple and fast. The method was optimized to be suitable for separation of a few milligrams of each individual enantiomer on a semi-preparative scale and also for analysis of clenbuterol in plasma which is currently being investigated.

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