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# ENANTIOMERIC RESOLUTION AND DETERMINATION OF CLENBUTEROL BY HPLC TECHNIQUE IN PLASMA AND PHARMACEUTICAL FORMULATIONS USING POLYSACCHARIDE CHIRAL STATIONARY PHASE AND UV DETECTION

MOHAMED M. HEFNAWY, MONA AL-SHEHRI, MAHA SULTAN, GAMAL A.E.MOSTAFA*
*College of Pharmacy, Department of Pharmaceutical Chemistry, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia*

Clenbuterol enantiomers were directly separated and determined in plasma and pharmaceutical formulation by a selective HPLC method using cellulose-based polysaccharide chiral stationary phase (CSP) known as OJ-RH. Enantiomeric resolution was achieved with a mobile phase consists of acetonitrile: 0.3M sodium perchlorate (16 %: 84 %), (v/v), a flow rate of 0.9 ml/min and a UV detection set at 247 nm. The method validated for its linearity, accuracy, and precision and robustness. The standard calibration curves were linear over the range of 0.5-50 µg/ml for each enantiomer with detection limit of 0.1 µg/ml. There was no significant difference between inter- and intra-day studies for each enantiomer which confirmed the reproducibility of the assay method. The method is highly specific where the co-formulated compounds did not interfere. The stability of clenbuterol enantiomers under high temperature was studied. The results showed that the drug is stable for at least 7 days at 80$^{o}$C. The mean extraction efficiency for R-(-)- and S-(+)- clenbuterol from plasma was in the ranges 93-102 % at 7.5 - 40 µg/ml level for each enantiomers. The overall recoveries of clenbuterol enantiomers from pharmaceutical formulations were in the ranges 97 - 103 % with %RSD ranged from 1.81-2.35 %. The assay method proved to be chiral quality control for clenbuterol formulations by HPLC and to therapeutic drug monitoring.

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*Keywords:* Clenbuterol enantiomers, Polysaccharide-based CSP, Solid-phase Extraction, Plasma and Pharmaceutical formulations

## 1. Introduction

Molecular chirality plays a central role in receptor–ligand interactions and functional biologic effects. Therefore the control and determination of the enantiomeric composition of chiral drug substances is a key issue for both the pharmaceutical industry and regulatory agencies [1]. Over the past three decades, high performance liquid chromatography (HPLC) has been the most productive tool for resolving enantiomers for both analytical and preparative purposes, and its separation power has had a tremendous impact on the pharmaceutical industry [2].

Chiral separations can be achieved with HPLC through the following approaches: (a) direct separation of racemates to their corresponding enantiomers using chiral stationary phases (CSPs)[3-5]; or (b) indirect separation of diastereoisomers, formed by the reaction of the enantiomers with a chiral derivatizing agent, using achiral stationary phases [ 6,7 ]; or (c) separation of chiral derivatives, formed by the reaction with non-chiral derivatizating agents, using CSPs[8]. Direct methods based on CSPs are the preferred separation approaches, since they are simple and rapid to apply at both analytical and preparative scales.

Several chiral stationary phases (CSPs) have been developed and used for the chiral resolution of a variety of racemates. A large number of CSPs have been developed in the last 25

*Corresponding author: gamal_most@yahoo.com

years including brush, ligand-exchange, cyclodextrin, protein, and polysaccharide types [9-17]. Among the commercially available polysaccharide-based CSPs, polysaccharide phenylcarbamates and esters/benzoates represent an important class of CSPs, which have shown excellent enantioselectivity for a wide range of race mates possessing a variety of chemical functionalities [18]. They are not only effective under normal-phase conditions, but also under reversed-phase conditions [19].

Clenbuterol is belonging to the family of β-adronists. It is a sympathomimetic drug with potent $\beta_2$-adrenoceptor stimulating properties and used for the treatment of pulmonary diseases. Its bronchodilatory effect on smooth muscle is the main pharmacological action responsible for alleviation of asthmatic attacks, as well as treating other respiratory disorders [20-22]. Besides the smooth muscle relaxation effect, clenbuterol is used to increase the muscle growth [23]. It is also one of the drugs abused by athletes as a performance-enhancing agent [24]. It was reported that clenbuterol might be used to treat cardiomyopathy, a clinical condition caused by several heart diseases [25].

Clenbuterol is chemically known as 4-amino-3,5-dichloro-α-[(1,1-dimethylethyl)amino]-methyl-benzenemethanol [Fig.1]. There is one chiral center in its molecule. It was reported that the $\beta_2$-agonistic as well as the $\beta_1$-antagonistic effect of clenbuterol resides in the R-(−)-isomer and the S-(+)-isomer does not seem to contribute to the pharmacological effects [26]. Therefore, enantioseparation of clenbuterol is very important for preparative and analytical purposes.

The separation of clenbuterol enantiomers was first attempted using Chirex 3022 [27], reversed phase C18 [28] and imide-type chiral (Chirex 3005) [29, 30]. Enantiomeric resolution of cleanbuterol was reported [22, 31] using macrocyclic antibiotic CSP. Both methods are based on using chirobiotic T [22] and vancomycin [31] to separate and determine clenbuterol enantiomers in plasma.

A search in the literature indicated that the resolution and quantitation of optically active clenbuterol using polysaccharide-based CSP has not yet been reported.

The purpose of this work was to evaluate the possibility of the enantioseparation of clenbuterol on polysaccharide-based CSP and to develop direct and validated method to determine the drug in human plasma and in pharmaceutical formulations. Also the stability of clenbuterol enantiomers under different temperature degrees was studied.

## 2. Materials and methods

### Apparatus

The HPLC instrument (Jasco, Japan) is equipped with a pump (model PU-980), a UV/VIS detector (model UV-975) and injection valve with 20-µL sample loop. Signal acquisition and data handling were performed with LG computer connected to the instrument. The CSP used in this study was the ester derivative of cellulose-based CSP, known as OJ-RH (15 × 0.46 cm i.d.) purchased from Daicel Chemical Industries, LTD. (Illkirch Cedex, France).

### Materials and reagents

R-(-)- and S-(+)- clenbuterol and bunolol were obtained from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from BDH Chemicals (Poole, UK). Analytical grade sodium perchlorate, potassium acetate, and disodium hydrogen phosphate were purchased from BDH Chemicals (Poole, UK). Deionized water was purified using a cartridge system (Picotech water system, RTP, NC, USA). Oasis HLB and Sep-Pak C18 cartridges (1ml) were obtained from Waters Corp. (Milford, MA, USA). Human plasma was obtained from King Khalid University hospital (Riyadh, KSA), and was kept frozen until use.

### Chromatographic conditions

The mobile phase was acetonitrile: 0.3 M sodium perchlorate (16: 84) (v/v). The mobile phase was filtered through a Millipore membrane filter (0.2 µm) from Nihon, Millipore (Yonezawa, Japan) and degassed before use. The flow rate was 0.9 ml/min and the detection wavelength (UV) was set at 247 nm.

### Preparation of stock and standard solutions

Stock solutions of individual R-(-)- and S-(+)- clenbuterol were prepared in methanol to

give a concentration of 1000 μg/ml. Working standards solution (100 μg/ml) were prepared by dilution of individual aliquot of stock solution with the same solvent. The internal standard bunolol was prepared in methanol to give a concentration of 100 μg /ml. Appropriate dilutions of the individual stock solutions of clenbuterol were made and used for constructing the calibration curves and for spiking the plasma.

**Preparation of tablet solution**

0.5 g of the tablet powder contains: 0.005g of Magnesium stearate, 0.01g of calcium hydrogen phosphate, 0.0025 g of silicon dioxide, 0.15 starch and 0.3325 g of Cellulose. 10 mg of the clenbuterol was added to the tablet powder, finely powdered, and transferred into a 100-ml calibrated flask, and then 40 ml of distilled water was added. The content of the flask were swirled and sonicated for 15 minutes. The contents were filtered, and the first portion of the filtrate was rejected. Part of the remaining filtrate was diluted quantitatively with methanol to obtain the required concentrations for HPLC analysis. Accurately measured aliquots of the supernatant were transferred to 5 ml volumetric flasks containing 750 μl of the internal standard and diluted to 5 ml with methanol to give final concentration of 2.5, 20 and 40 μg/ml of clenbuterol.

**Preparation of spiked plasma**

Human plasma sample (0.5 ml) was placed into individual Eppendrof tube and accurately measured aliquots of R-(-)- and S-(+)- clenbuterol were added. Then 0.5 ml of the internal standard solution was added to each tube and diluted with methanol to 2 ml and mixed well to give final concentrations of 7.5, 25 and 40 μg/ml of each clenbuterol enantiomers. The mixture was sonicated for 5 min. Blank human plasma samples were processed in the same manner using methanol instead of clenbuterol enantiomers.

**Assay method**

Oasis HLB and Sep-Pak C18 and elution solvent consisted of absolute methanol were used. Cartridges were attached to a vacuum manifold (VacElute, Harbor City, CA, USA) and conditioned with two column volumes of methanol and two column volumes of deionized water before applying the plasma samples. Care was taken that the cartridges did not run dry. Blank and spiked plasma samples were transferred into the cartridges and vacuum was applied to obtain a flow of 0.5 ml/min. After the entire plasma samples had been aspirated through the cartridges, the cartridges were washed with 2 ×500 μl deionized water. The cartridges were then dried under vacuum for 3 min. All cartridges were eluted with 2 × 1 ml of elution solvents and 20 μl was injected into the HPLC system.

The absolute recoveries of each enantiomer from plasma and pharmaceutical preparations were calculated by comparing drug peak area of the spiked analyte samples to unextracted analyte of stock solution that has been injected directly into an HPLC system.

Calibration plots for the R-(-)- and S-(+)- clenbuterol in standard solution were prepared by diluting stock solutions to yield seven concentrations over the range of 0.5–50 μg/mL for each enantiomer, respectively. Also, calibration plots for the R-(-)- and S-(+)- clenbuterol in plasma were prepared by diluting stock solutions with pooled human plasma over the range of 2.5 - 50 μg/mL for each enantiomer, respectively. Calibration standards at each concentration were extracted and analyzed in triplicate. Calibration curves of clenbuterol enantiomers were constructed using the observed D/IS peak area ratio versus nominal concentrations of the analytes. Least squares linear regression analysis of the data gave slope, intercept and correlation coefficient data. From this data, a first order polymonial model was selected for each analyte.

The within-run and between-run accuracy and precision of the assays in plasma were determined by assaying three QC samples in triplicate over a period of 3 days. Precision was reported as (% R S D) = (S D/mean) x 100. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Percent accuracy was reported as % relative error = [(measured concentration - nominal concentration)/ nominal concentration] ×100. The LOD and the LOQ were determined as 3 and 10 times the baseline noise, respectively, following the United States Pharmacopoeia [27].

The selectivity of the assay was checked by analyzing six independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analytes. Moreover, the selectivity of the assay was checked by analyzing ten placebo tablets samples. The

chromatograms of these placebo tablets samples were compared with chromatograms obtained by analyzing prepared tablets containing the drug.

The stability of sample solution was tested by the proposed HPLC method over a period of 7 days. The freshly prepared solutions at room temperature and the 7 day-stored samples in thermostatic oven at 80$^{o}$C were analyzed by the optimized proposed HPLC method.

## 3. Results and discussion

### Optimization of the chromatographic conditions

The chemical structure of clenbuterol enantiomers and bunolol (IS) are shown in Fig. 1. The separation of clenbuterol enantiomers was first attempted on a cellulose carbamate CSP (Chiralcel OD-R column). The mobile phases used consist of acetonitrile with different buffers (sodium perchlorate, potassium acetate, and disodium hydrogen phosphate in different concentrations) in different ratios (40:60 to 10: 90). In all experimental conditions no separation was observed.

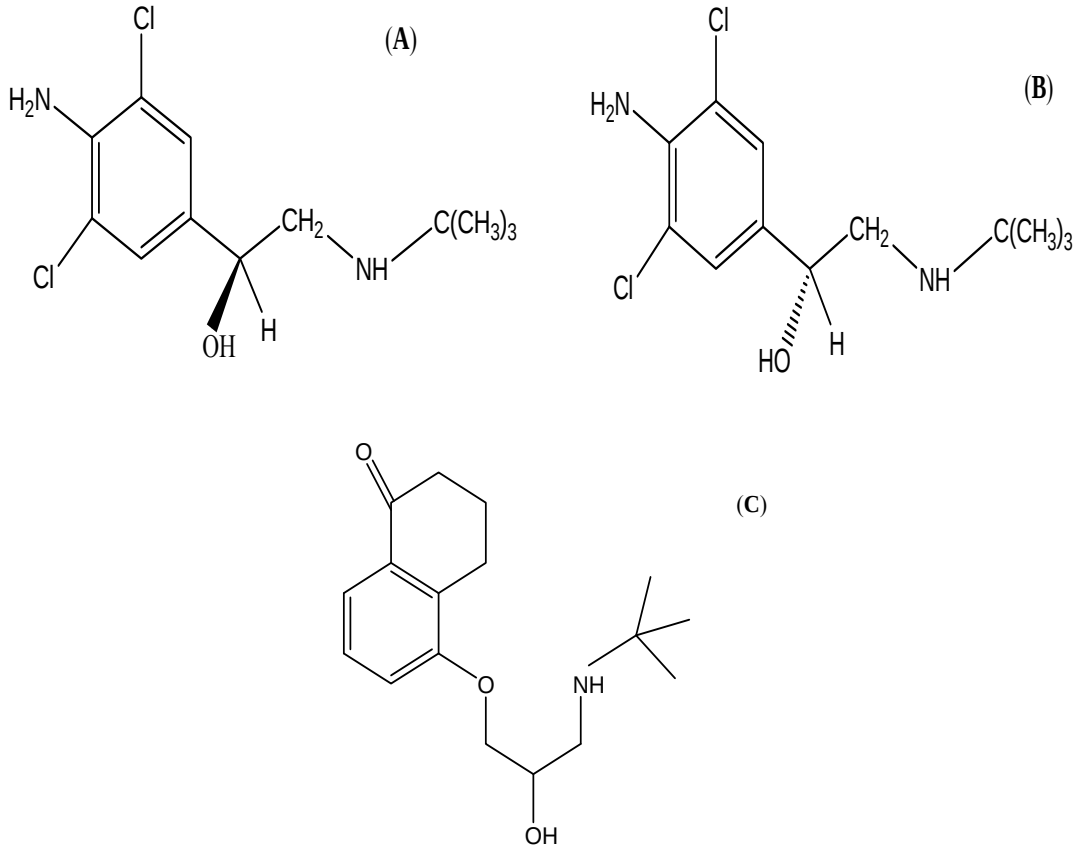


*Fig. 1. Chemical Structures of (A) R-(-)-clenbuterol, (B) S-(+)-clenbuterol , and (C) (±) bunolol (IS).*

The column has been changed to Cellulose ester CSP (Chiralcel OJ-RH column) and again all the above mentioned mobile phases that have been tried on Chiralcel OD-R column. There was no separation when using the potassium acetate buffer and partial separation with disodium hydrogen phosphate buffer. The best resolution was obtained with sodium perchlorate buffer (0.3 M).

The mobile phase consisted of acetonitrile: 0.3 M sodium perchlorate (20 :80) resulted in about 50 % resolution of the two enantiomers. We changed the ratio of acetonitrile. A complete resolution was achieved with acetonitrile: 0.3 M sodium perchlorate (16 : 84) (Fig.2).

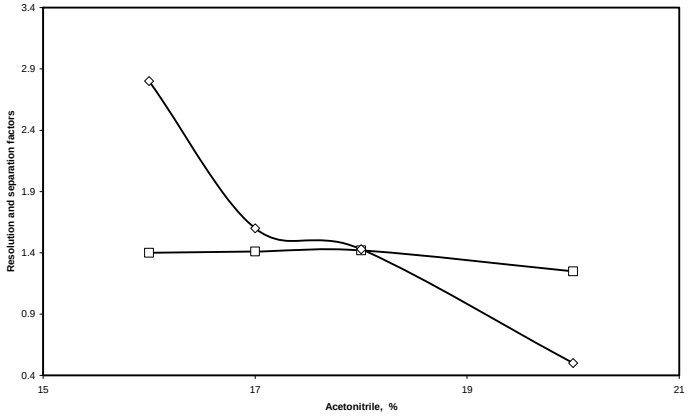


*Fig. 2. Effects of acetonitrile content in the mobile phase on the resolution of clenbuterol enantiomers (Rs ◊, and α □).*

Sodium perchlorate 0.15, 0.25, 0.3, 0.4 and 0.5 M were used as a buffer in the mobile phase with acetonitrile (84: 16) (v/v). Decreasing the strength of sodium perchlorate < 0.3 M, resulted in decreasing the resolution factor. Using 0.15 M sodium perchlorate completely diminished the resolution. Sodium perchlorate 0.3, 0.4 and 0.5 M nearly gave the same resolution. We have chosen 0.3M sodium perchlorate as an optimum value.

Although the retention times decreased when increasing the content of acetonitrile, the resolution factor (*Rs*) decreased. Separation factor (*α)* and resolution factor gave maximum values of 1.53 and 3.78, respectively, when 16 % (v/v) of acetonitrile was used, and the capacity factors (*k)* were 9.12 and 12.55 for S-(+)- and R-(-)-enantiomer, respectively.

We found that changing the pH value of the mobile phase acetonitrile (16%): sodium perchlorate (0.3M) (16: 84 %) did not affect the resolution of the two enantiomers. The values of pH used ranged from 3 to 6.5. The value 6.5 was used as optimum pH value. At the last, the effect of mobile phase composition on the resolution of clenbuterol enantiomers using OJ-RH column was summarized in Table 1.

*Table 1. Chromatographic parameters for the separation of clenbuterol enantiomers and the internal standard.*

| Analyte | $R_S$ [a] | $\alpha$ [b] | $k$ [c, d] | $T_R$ [d,e] |
|---|---|---|---|---|
| R-(-)-clenbuterol | 3.78 | 1.53 | 9.12 ± 0.06 | 18.64 ± 0.10 |
| S-(+)-clenbuterol | 2.65 | 1.37 | 12.55 ± 0.07 | 24.94 ± 0.12 |
| Bunolol | [f] | [f] | 5.94 ± 0.02 | 12.79 ± 0.05 |

[a] Resolution factor, calculated as $R_s = (t_2-t_1)/0.5(w_1+w_2)$. Where $t_2$ and $t_1$ are the retention of the second and first peaks, $w_1$ and $w_2$ are the peak width of the first and second peaks.
[b] Separation factor, calculated as $k_2/k_1$
[c] Capacity factor, calculated as $T_R - T_o / T_o$
[d] Mean ± SD, n = 3
[e] Retention time
[f] Not calculated

**Applications to plasma samples**

In the course of developing a solid phase extraction (SPE) procedure for plasma sample clean up, several types of cartridges investigated (Water Oasis HLB and Sep-Pak C18, C8 and cyanopropyl). The cyanopropyl cartridge and an octyl (C8) SPE column also found to be unacceptable due to low recoveries (< 62%) for clenbuterol enantiomers and internal standard. Oasis HLB cartridge showed recoveries in excess 70% whereas an octadecyl (C18) SPE column gave high recoveries for clenbuterol enantiomers and internal standard (more than 90%) while at the same time removing endogenous interference. Fig. 3A and B show chromatograms of a blank plasma sample and a calibration sample, respectively. The retention times for R-(-)- and S-(+)-clenbuterol were 18.56 ± 0.10 and 24.94 ± 0.12 min, respectively.

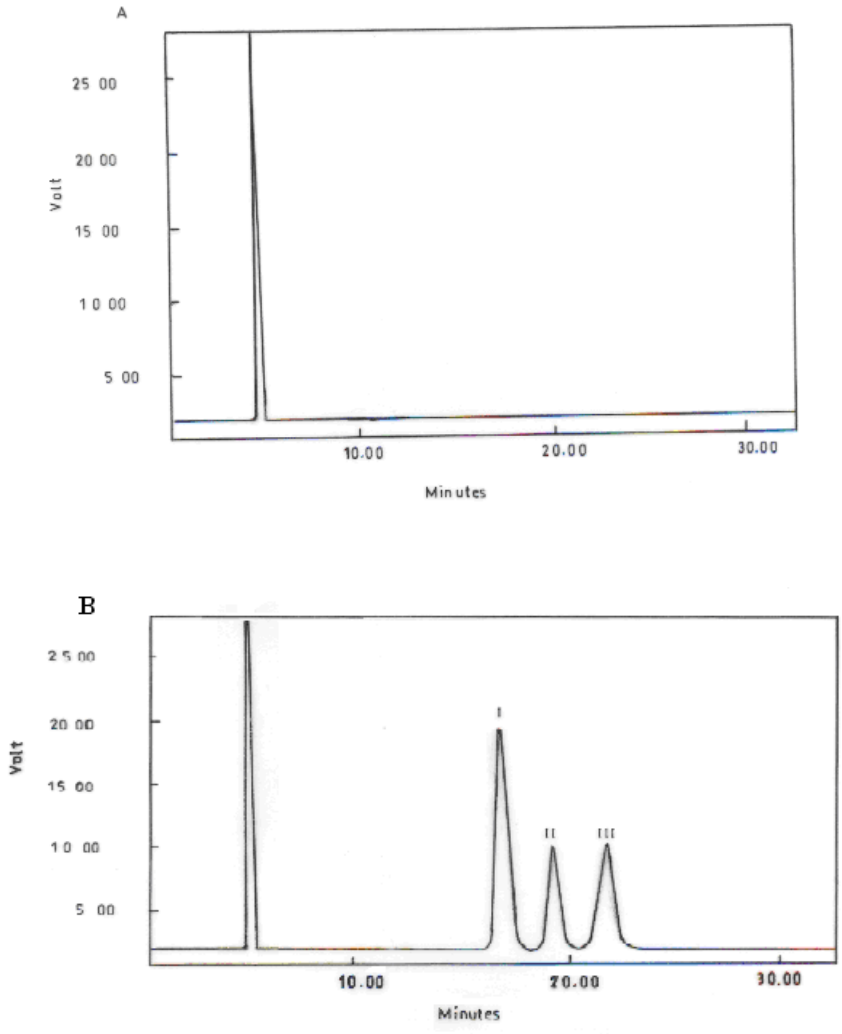


*Fig. 3. Chromatograms of (A) blank human plasma and (B) spiked with 12.5 µg/ml of R-(-)-clenbuterol (II), S-(+)-clenbuterol (III) and 15 µg/ml (± ) bunolol (I) using OJ-RH column (mobile phase: acetonitrile: sodium perchlorate 0.3 M (16: 84, v/v), flow rate: 0.9 ml/min, UV detection: 247 nm).*

**Validation of the proposed method**

***Linearity, precision and accuracy***

The method is extensively validated as the United States Food and Drug Administration (FDA) guidelines [32, 33] and is rugged and adequately sensitive for routine subject sample of analysis .The linear regression analysis of R-(-)- and S-(+)- clenbuterol was constructed by

plotting the peak area ratio of each enantiomer to the internal standard (*y*) versus analyte concentration (*x*). The standard calibration curves were linear over the range of 0.5 - 50 µg/ml for each enantiomer with detection limit of 0.1 µg/ml. The calibration curves in plasma samples were linear in the range of 2.5 - 50 µg/ml for R-(-)- and S-(+)-clenbuterol with correlation coefficient ($r^2$) of more than 0.998 [34].

Table 2 , shows the results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the least squares treatment of the results along with standard deviation of the slope ($S_b$) and intercept ($S_a$) on the ordinate and the standard deviation of the residuals ($S_{y/x}$).

*Table 2. Validation parameters for the determination of clenbuterol enantiomers using the proposed method in spiked plasma samples.*

| Parameters | R-(-) clenbuterol | S-(+) clenbuterol |
|---|---|---|
| Concentration range µg/ml | 2.5-50 | 2.5-50 |
| Intercept (a) | - 0.0300 | 0.0850 |
| Slope (b) | 0.0306 | 0.0290 |
| Correlation coefficient ($r^2$) | 0.9984 | 0.9988 |
| $S_{y/x}$ | 0.0774 | 0.0418 |
| $S_a$ | 0.0948 | 0.0512 |
| $S_b$ | 0.0013 | 0.0007 |
| LOQ (µg/ml)[a] | 2.5 | 2.5 |
| LOD (µg/ml)[b] | 0.5 | 0.5 |

[a] Average of six determinations.
[b] Average of five determinations.

A summary of the accuracy and precision results is given in Table 3. The acceptance criteria (within-run and between-run % RSD of < 15 % and accuracy between 85 and 115%) were met in all cases. The precision and accuracy of the method were determined by using plasma samples spiked at three levels (Table 3). The data indicate that within-run precision and accuracy (n= 6) as expressed by % RSD and % error were 1.59 - 2.88 % and 0.20 - 3.06 %, respectively for R-(-)- clenbuterol and 2.13 - 3.15 % and 1.48 - 4.66 % for S-(+)- clenbuterol, respectively. The between-run precision and accuracy (n=6) expressed by % RSD and % error were 2.31- 3.40 % and 0.36 - 2.27 % for (-)-R clenbuterol and 2.41 - 4.50 % and 1.05 - 6.13 % for (+)-S clenbuterol, respectively. The detailed analytical data are shown in Table 3.

*Table 3. Accuracy and precision data for clenbuterol enantiomers in spiked plasma samples.*

| Analyte | Actual concentration (μg/ml) | Experimental concentration (μg/ml) | Recovery (%) | RSD (%)[c] | Relative, Error (%)[d] |
|---|---|---|---|---|---|
| Within-day [a] | 7.5 | 7.27 ± 0.21 | 96.93 | 2.88 | -3.06 |
| R-(-)- | 25 | 25.05 ± 0.55 | 100.20 | 2.19 | 0.20 |
| clenbuterol | 40 | 40.27 ± 0.64 | 100.67 | 1.59 | 0.67 |
| S-(+)- | 7.5 | 7.15 ± 0.17 | 95.33 | 2.37 | -4.66 |
| clenbuterol | 25 | 25.37 ± 0.80 | 101.48 | 3.15 | 1.48 |
| | 40 | 40.77 ± 0.87 | 101.92 | 2.13 | 1.92 |
| Between-day [b] | 7.5 | 7.33 ± 0.20 | 97.73 | 2.31 | -2.26 |
| R-(-)- | 25 | 25.09 ± 0.85 | 100.36 | 3.38 | 0.36 |
| clenbuterol | 40 | 39.09 ± 1.33 | 97.72 | 3.40 | -2.27 |
| S-(+)- | 7.5 | 7.04 ± 0.17 | 93.86 | 2.41 | -6.13 |
| clenbuterol | 25 | 25.51±1.15 | 102.04 | 4.50 | 2.04 |
| | 40 | 39.58 ± 1.49 | 98.95 | 3.76 | -1.05 |

[a] Mean ± SD based on average of six determinations.
[b] Mean ± SD based on average of six determinations
[c] Expressed as % RSD : (S.D./ mean) × 100
[d] Calculated as (experimental concentration - actual concentration / actual concentration) × 100

***Limit of detection and limit of quantitation***

The LOD as defined in the experimental section were 0.5 μg/ml for R-(-)- and S-(+)-clenbuterol (Table 2). The LOQ of each calibration graph was 2.5 μg/ml for each enantiomer. The good linearity of the calibration graphs and the negligible scatter of experimental points are evident by the values of the correlation coefficient and standard deviation [27].

***Application to pharmaceutical formulations***

The validity of the method developed here was applied to various concentrations taken from the pharmaceutical formulations for determining their content of clenbuterol enantiomers. The values of the overall drug percentage recoveries and the %RSD value of R-(-)- and S-(+)-enantiomers are presented in

Table 4, indicating that these values are acceptable and the method is accurate and precise.

*Table 4. Determination of clenbuterol enantiomers in pharmaceutical formulations by the proposed method.*

| Pharmaceutical preparation | Enantiomer | Nominal conc. (μg/ml) | Measured conc. (μg/ml) | Recovery (%) |
|---|---|---|---|---|
| Clenbuterol tablet[a] | R-(-)- | 2.5 | 2.57 | 102.80 |
| | | 20 | 19.45 | 97.25 |
| | | 40 | 40.63 | 101.57 |
| Overall recovery (± SD) | | | | 100.54 ± 2.37 |
| RSD (%) | | | | 2.35 |
| | S-(+)- | 2.5 | 2.56 | 102.40 |
| | | 20 | 19.59 | 97.95 |
| | | 40 | 39.94 | 99.85 |
| Overall recovery (± SD) | | | | 100.06 ± 1.82 |
| RSD (%) | | | | 1.81 |

[a] Prepared tablets in our lab.

***Selectivity***

The analytical figures of merit for this method are shown in Table 1. R-(-)- and S-(+)-clenbuterol enantiomers were well separated under the HPLC conditions applied. Retention times were 18.64 and 24.94 for R-(-)- and S-(+)- enantiomers, respectively. No interference was observed in drug free human plasma samples (Figures 3A and B). Otherwise, there is no peak detected at the retention time of individual clenbuterol enantiomer and of internal standard at the level of LOQ or less. Excipients commonly coformulated with the studied drug such as magnesium stearate, cellulose, starch, calcium hydrogen phosphate, colloidal silicon dioxide and coloring agents, also did not interfere with the determination of clenbuterol enantiomers, indicating the high selectivity of the method (Fig. 4).

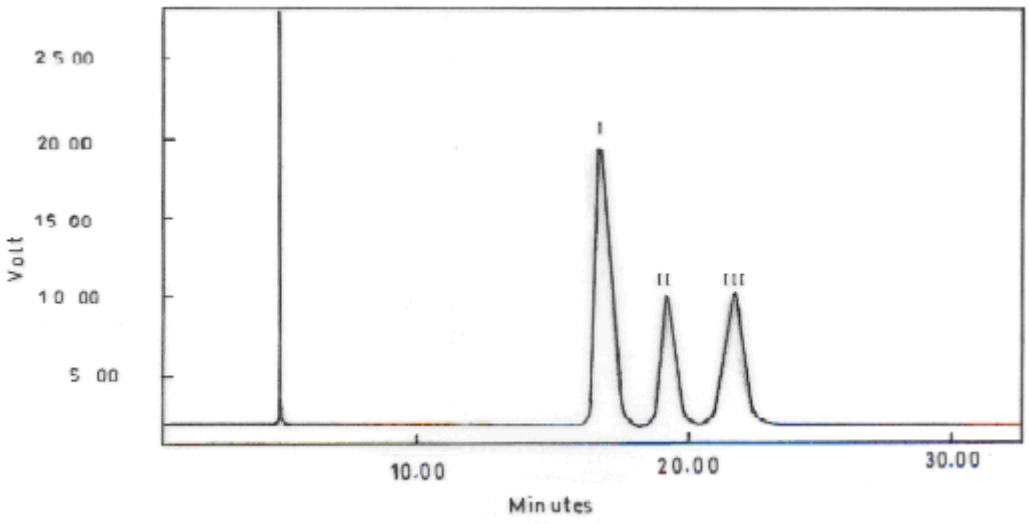


*Fig. 4. Chromatogram of 10 µg/ml of R-(-)-clenbuterol (II), S-(+)-clenbuterol (III) recovered from clenbuterol prepared tablets and 15 µg/ml (± )bunolol (I) using OJ-RH column (mobile phase: acetonitrile: sodium perchlorate 0.3 M (16:84, v/v), flow rate: 0.9 ml/min, UV detection: 247 nm).*

***Stability of sample solutions***

The stability of standard solutions under different temperature degrees was tested by the proposed HPLC method over a period of 7 days. The freshly prepared solutions at room temperature and the 7-day-sored samples at 50, 60, 70 and 80°C were analyzed by the proposed HPLC method. The concentrations of clenbuterol enantiomers in the stored samples were calculated and compared to that present in the freshly prepared samples. From the results, we can conclude that there are no degradation products at elevated temperature and the drug is stable at 80°C without degradation.

## 4. Conclusion

An enantioselective HPLC method that enabled direct separation and determination of clenbuterol enantiomers in plasma and in pharmaceutical formulations using OJ-RH column was developed. The method used an efficient and simple SPE procedure for sample clean-up of plasma. The standard calibration curves were linear over the range of 0.5 - 50 µg/ml for each enantiomer with a detection limit of 0.1 µg/ml. The method was selective where the coformulated compounds did not interfere. The drug was stable for at least 7 days at 80$^{o}$C. The method has a satisfactory sensitivity, precision, accuracy, and reproducibility.

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