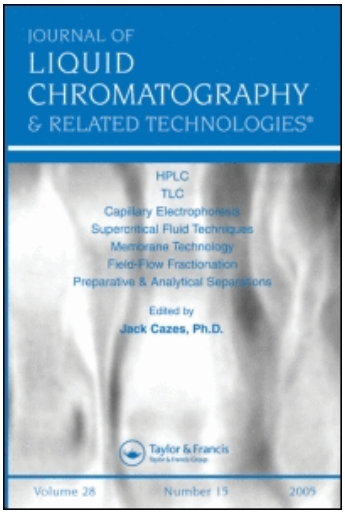

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DETERMINATION OF CLENBUTEROL HCl IN HUMAN SERUM, PHARMACEUTICALS, AND IN DRUG DISSOLUTION STUDIES BY RP-HPLC

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ABSTRACT

A simple, reliable, and rapid RP-HPLC method has been developed for the determination of clenbuterol HCl in human serum and pharmaceuticals, in order to carry out drug dissolution studies for clenbuterol tablets. This compound is well separated on a C_{18} column by using the mobile phase consisting of a mixture of acetonitrile and ion-pair buffer (32:68; v/v) at a flow rate of 1.5 mL/min.

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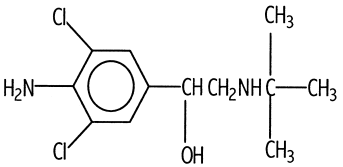
Detection was carried out using a UV detector at 244 nm. ephedrine HCl was used as an internal standard. Minimum detection limit obtained was 3.78 ng/mL. This method was applied, without any interferences from the excipients, for the determination of the drug in tablet formulation, human serum, and drug dissolution studies.

INTRODUCTION

Clenbuterol hydrochloride is a direct-acting, sympatho-mimetic (β_2-agonist) agent. It is used as a bronchodilator in the management of asthma and obstructive lung diseases and reduction of stress symptoms. The reduction of stress symptoms has led to the abuse of clenbuterol, causing it to be added to the International Olympic Committee's list of doping substances.[1,2].



Structural formula of clenbuterol

The determination of trace amounts of analytes in biological samples and pharmaceuticals is a well-known problem. So, in order to reduce the effect of interfering components and to enrich the analytes of interest, sample pretreatment is necessary in most cases.

Some methods have been reported for the analysis of clenbuterol. Most of them involve the determination in biological matrices, such as brain tissue,[3] liver,[4-6] urine, [6-9] hair,[10,11] and biological fluids and matrices.[12-15] These methods are highly sensitive but quite complicated, require a long analysis time (>10 min), and time-consuming separation procedure.

The main purpose of an oral solid dosage form is to make available a certain and defined amount of active substance to human body through the gastrointestinal system.[16] Drug dissolution testing is an integral part of pharmaceutical development and routine quality-control monitoring of drug release characteristics. The in vitro dissolution profiles obtained from dissolution rate studies have also been used in an attempt to characterize the in vivo behaviour of drugs with success.[17-19]

It is of interest to mention, that the dissolution rate studies of clenbuterol and determination from tablet dosage forms of this substance have not yet been

reported by HPLC. The main objective of this work is to develop a simple, sensitive, reliable, and fast HPLC method which uses UV detection for the determination of clenbuterol in human serum and pharmaceuticals, and to carry out drug dissolution studies of this drug from its tablets dosage formulation.

The developed HPLC method was applied to the in vitro dissolution rate studies of clenbuterol tablets used by the standard addition technique. This method was also succesfully applied to the analysis of clenbuterol in human serum and pharmaceuticals without any interferences from the excipients.

EXPERIMENTAL

Instrumentation

A liquid chromatographic system consisted of a Waters Isocratic LC pump 510 (Waters, Milford MA, USA), with an autosampler (Model 717 plus), equipped with a model 481 Waters UV detector. The chromatograms were analysed with a chromatographic workstation (Baseline 810).

Dissolution rate and quantitative determination studies were realised at 244 nm wavelength. The dissolution rates of clenbuterol from tablets was performed on Caleva 7ST dissolution apparatus (G.B. Caleva Inc., England).

Chemicals and Reagents

Clenbuterol hydrochloride and clenbuterol tablets (Spiropent®, claimed labeled amount 20 μg clenbuterol/tablet) was kindly supplied by K.Thomae Pharmaceuticals Inc. (Biberach, Germany). The internal standard, ephedrine hydrochloride, was received from Fako Pharmaceuticals Inc. (Istanbul, Turkey). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). All other chemicals (analytical grade) were obtained from Sigma (St. Louis, MO, USA) or Merck. Doubly distilled water was used.

For dissolution studies, working solutions of 0.1 N HCl (pH 1.2), which is adequate to physiological conditions in gastric fluids, were used.

Chromatographic Conditions

Clenbuterol is well separated on a Waters C_{18} (150 x 4.6 mm, particle size 5 μm) column by using the mobile phase consisting of a mixture of acetonitrile and ion-pair buffer (32:68; v/v) at a flow rate of 1.5 mL/min. The ion pair buffer contained 0.02 M octane sulfonic acid sodium salt and 0.02 M acetic acid. The pH

of the mobile phase was adjusted to pH 3.8 with 0.1 M NaOH. The mobile phase was prepared daily, filtered, and sonicated before use.

Standard Stock Solution

Internal standard solution was prepared by dissolving 10 mg ephedrine HCl in 10 mL mobile phase. A stock solution of clenbuterol HCl was prepared by dissolving 10 mg clenbuterol HCl in 10 mL mobile phase. Standard solutions for HPLC were prepared with mobile phase by varying the concentration range of clenbuterol in the range of 40-50000 ng/mL and maintaining the concentration of ephedrine (internal standard) at a constant level of 20 μg/mL.

The calibration curve for HPLC analysis was constructed by plotting the ratio of the peak area of the drug to that of internal standard against clenbuterol concentration.

Determination of Clenbuterol HCl from Tablets

Not less than ten tablets were weighed, crushed, and powdered. An appropriate amount of the powdered tablet was accurately weighed to correspond to 100 μg/mL of clenbuterol, transferred in a 10 mL volumetric flask, diluted with mobile phase, sonicated for 10 minutes, and then completed to the volume with the same solution. The above solution was filtered through 0.45 micron Whatman filter paper.

After filtration, appropriate solutions were prepared by taking suitable aliquots of clear filtrate and addition of the appropriate internal standard, and diluting them with the mobile phase in order to obtain a final solution. 50 μL of this solution was injected and chromatograms were recorded. The amount of clenbuterol per tablet was calculated from a linear regression equation.

Recovery Studies

To study the accuracy, reproducibility, precision, and to check the interference from excipients used in the formulation of the above method, a recovery experiment was carried out. In order to know whether the excipients show any interference with the analysis, known amounts of pure drug were added to the different pre-analysed formulations of clenbuterol, including a constant concentration of internal standard, and the mixtures were analysed by the proposed method. After three repeated experiments, the recoveries were calculated.

Recovery Studies in Human Serum

Serum sample, obtained from healthy individuals (after obtaining their written consent), were stored frozen until assay.

After gentle thawing, 2 mL aliquots of serum were spiked with 100 μg/mL of clenbuterol solution (dissolved in mobile phase), 200 μL acetonitrile (for participitation of proteins), maintaining the concentration of ephedrine at a constant level. The tubes were vortexed for 2 min and then centrifuged for 10 min at 4000 g. The supernatant was taken carefully. Serum samples including various concentrations of clenbuterol and a constant amount of internal standard were injected into the HPLC column.

In Vitro Dissolution Studies for Tablets

Drug release was carried out according to the USP dissolution procedures for the single entity products with the use of a USP paddle-stirrer type of apparatus in 500 mL of 0.1 N HCl (pH 1.2, gastric medium), at a stirring rate of 75 rpm. The temperature of the cell was maintained at 37.5°C by use of a thermostatic bath. At each sample interval, an exact volume of a sample was withdrawn from each flask and replaced immediately with an identical volume of fresh medium. A correction factor was included in the calculations to account for the drug lost in the samples. At predetermined time intervals (2,4,6,8,10,15,20,30,45,60,75,90 min), the concentration of clenbuterol in dissolution medium were determined by the standard addition method using the proposed HPLC technique. The dissolution test data were obtained from the average of six parallel studies.

RESULTS AND DISCUSSION

The mobile phase was chosen after several trials with acetonitrile-ion pair buffer and methanol-ion pair buffer in various proportions, and at different pH values. The mobile phase composition that is described in the experimental section was found suitable to carry out this study.

Figure 1 shows a typical chromatogram obtained for the analysis of a standard clenbuterol solution using the proposed method. At a flow rate of 1.5 mL/min, the retention times for ephedrine (IS) and clenbuterol were 3.75, 7.95 min, respectively. For both compounds, sharp and symmetrical single peaks were obtained with good baseline resolution. Linearity was obtained for clenbuterol in the concentration range 40-50,000 ng/mL. Table 1 represents calibration characteristics for the peak area ratio of varying amounts of clenbuterol to a constant level of ephedrine (20 μg/mL). The injection volume was 50 μL.

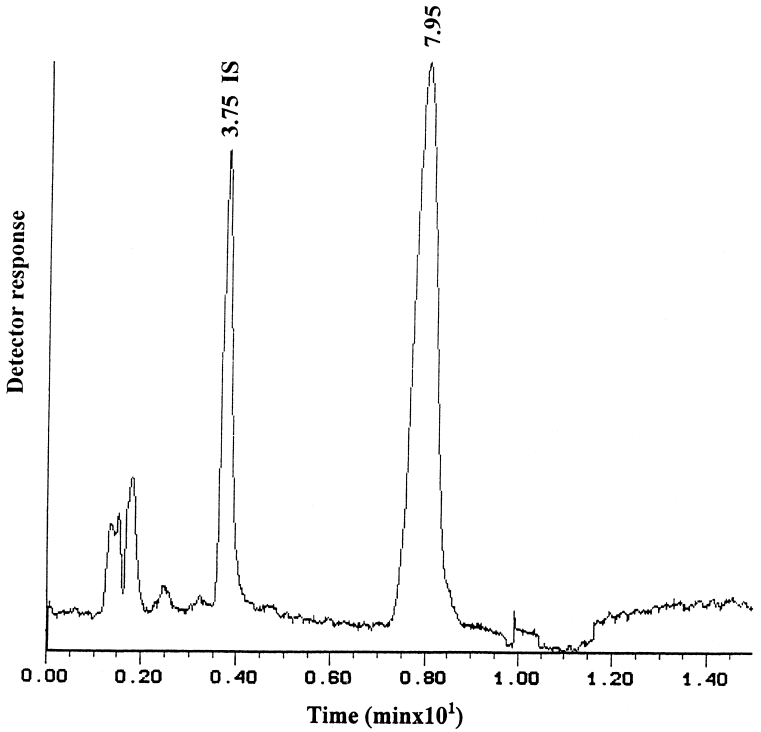


Figure 1. Typical chromatogram of a standard solution of clenbuterol (1000 ng/mL) and ephedrine (IS) (20 μg/mL) in the mobile phase.

Table 1. Analytical Parameters for the Determination of Clenbuterol

Linearity Range	40 ng/ml-50000 ng/ml
Slope of Calibration Graph	3.3×10^{-3}
Intercept	5.03×10^{-3}
Correlation Coefficient (r)	0.9999
RSD % of Slope	1.09
RSD % of Intercept	0.953
Detection Limit	3.78 ng/ml
Quantitation Limit	12.6 ng/ml

The limit of detection (LOD) and quantitation (LOQ) of the procedure were shown in Table 1, which was calculated on the peak area using the following equations: LOD: 3 s/m and LOQ: 10 s/m, whereas, the noise estimate is the standard deviation of the peak areas (five injections) of the drug and m is the slope of the corresponding calibration curve.

To study the accuracy, precision, and reproducibility of the proposed method, intra and inter-day variability studies were carried out at two different concentrations, as shown in Table 2. It was concluded that there was no significant difference for the assay tested within day and between day.

Quantitative Determination

The proposed method was used for analysis in clenbuterol in pharmaceutical dosage form and in human serum samples. The results of the analysis of clenbuterol tablets indicate that the proposed method can be used for quantitation and routine quality control analysis of clenbuterol in commercial samples (Table 3). Figure 2 shows a typical chromatogram obtained for the analysis of clenbuterol in tablet dosage form.

In order to check the accuracy and precision of the developed method, we have also carried out a recovery experiment by adding known amounts of standards to the preanalysed formulation, keeping the internal standard concentration at a constant level. The results of the recovery analysis are presented in Table 3. High percentage of recovery shows that the method is free from the interferences of the excipients used in the formulations. In order to check the applicability of the method to biological materials, the recovery studies performed for clenbuterol quantitation in human serum was done by means of the calibration graph method, achieving for each analysis the specified percent recoveries.

Table 2. Within-Day and Between-Day Precision of Clenbuterol Standards

Theoretical Concentration (ng/mL)	Within-Day Measured Concentration (ng/mL)*		Between-Day Measured Concentration (ng/mL)**	
	Mean	RSD %	Mean	RSD %
45	44.82	0.44	44.76	0.38
500	499.76	0.58	497.45	0.41

*Mean values represent five different clenbuterol standards for each concentration.
**Between-day reproducibility was determined from five different runs over a 2-week period.

Table 3. Results of the Determination and the Recovery Analysis of Clenbuterol in Tablet Dosage Forms

Labelled Claim (μg per tablets)	20
Mean of Amount Found (μg)*	19.87
RSD % of Amount Found	0.57
Added (μg)	10
Recovered (μg)**	9.94
Recovery %	99.42
RSD % of Recovery	0.18

*Each value is the mean of five experiments.
**Each value is the mean of three experiments.

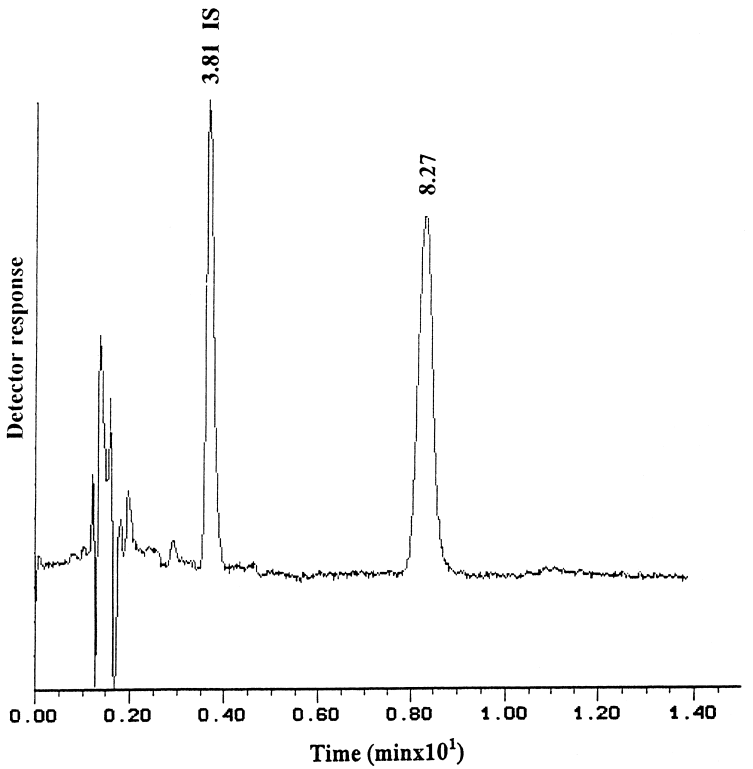


Figure 2. Chromatogram obtained from a pharmaceutical dosage form containing 500 ng/mL clenbuterol and 20 μg/mL ephedrine (IS).

Typical chromatograms of a blank human serum and a serum sample spiked with clenbuterol are shown in Figure 3a and 3b, respectively. The internal standard and clenbuterol gave well-separated, sharp symmetrical peaks with retention times of 3.92 and 8.37 min, respectively. There are no extraneous peaks in chromatograms obtained for serum samples. Blank serum samples were spiked with clenbuterol to achieve final concentrations of 100 ng/mL, 500 ng/mL, and 1000 ng/mL. The results were given in Table 4. The proposed method gives reproducible results, easy to perform, and is sensitive enough for the determination of clenbuterol in human serum (Table 4).

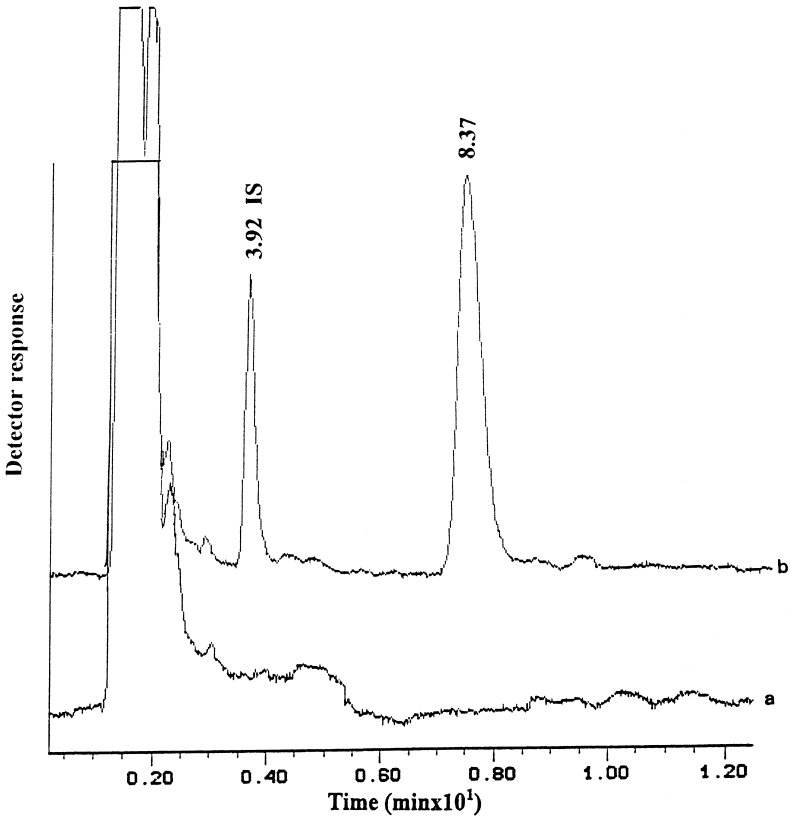


Figure 3. Chromatogram of blank serum (a) and serum spiked with 1000 ng/mL of clenbuterol and 20 μg/mL ephedrine (IS) (b).

Table 4. Results Obtained for Clenbuterol Analysis from Human Serum

Clenbuterol Added (ng/ml)	100	500	1000
n	3	3	3
Clenbuterol Found (ng/ml)	99.07	489.4	987.4
RSD %	1.35	0.61	0.47
Average Recovery %	99.1	97.9	98.7

In Vitro Dissolution Studies for Tablets

The proposed method was also applied to the quantitation of clenbuterol in dissolution rate studies of the samples obtained from the tablets using the standard addition method. Clenbuterol tablet formulation, which includes 20 μg active compound, was investigated with the paddle dissolution method. The release rate profile was drawn as the percentage drug dissolved from the tablet versus time (Figure 4).

The release data were evaluated according to the different kinetics, namely, zero order, first order, Hixson-Crowell, and Weibull Distribution[17,20,21] (RRSBW). All studied kinetics, and their related rate constants and determination coefficients and other parameters, are summarized in Table 5.

For releasing profile, the best compliance according to the highest determination coefficient and lowest sum of weighed squared deviation values for clenbuterol dosage form was found to be the Weibull distribution.[20] The release of active material from tablet was attained to 63.2 %, at the end of 9.94 min. When the shape factor (β) value, which is one of the parameters of Weibull distribution, is larger than 1, it is characteristic for a slower initial rate followed by an accelerated approach to the

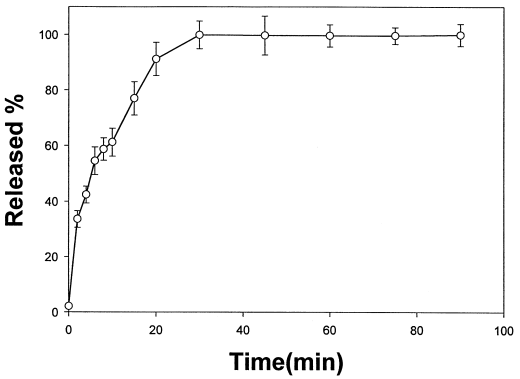


Figure 4. In vitro dissolution profiles of clenbuterol tablets by proposed HPLC method.

Table 5. Kinetic Assesment of Release Data

Zero Order	kr°	9.63×10^{23}
	r^2	0.577
	SWSD	0.290
First Order	kr	4.760
	r^2	0.764
	SWSD	0.360
Hixson-Crowell	k	0.136
	r^2	0.754
	SWSD	0.255
Weibull Distribution	$T_{(min)}$	9.94
(RRSBW)	β	1.08
	r^2	0.878
	SWSD	0.219

kr: Release rate constant of first order kinetic; kr^{o}: Release rate constant of zero order kinetic; k: Release rate constant of Hixson-Crowell kinetic; r^2: Determination coefficient; SWSD: Sum of weighed squared deviations; β: Shape factor; $T_{(min)}$: Value stands for the time for 63.2% release of the drug.

final plateau, i.e., an initial upward curvature and sigmoid overall appearance.[20] The value obtained was 1.08 which is larger than 1. The release of clenbuterol from the tablets tested were completed within 30 min in the proposed method.

CONCLUSION

The proposed method gives good resolution between clenbuterol and internal standard within a short analysis time (< 10 min). The method is simple, rapid, sensitive, reproducible, and nowhere involves use of complicated sample treatment. High percentage of recovery shows that the method is free from the interferences of the excipients used in the formulation. Also there are no extraneous peaks in chromatograms obtained during analysis of the drug in serum samples.

The need of dissolution control in drug production is indispensible to ensure drug quality. The method described, is shown to be useful in routine quality control analysis and dissolution studies of clenbuterol tablet formulations.

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