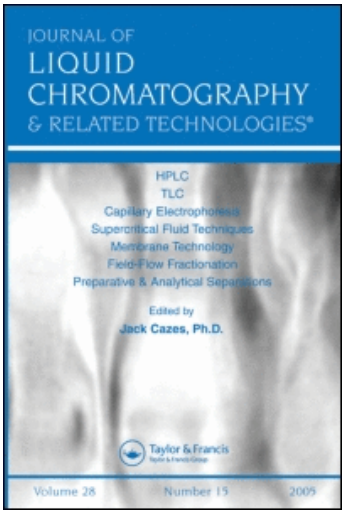

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A High Performance Liquid Chromatographic Method for the Determination of Albuterol Enantiomers in Human Serum Using Solid Phase Extraction and a Sumichiral-OA Chiral Stationary Phase

Amanda G. Adams[a]; James T. Stewart[b]
[a] Department of Chemistry, North Georgia College, Dahlonega, Georgia [b] Department of Medicinal, Chemistry College of Pharmacy The University of Georgia, Athens, Georgia

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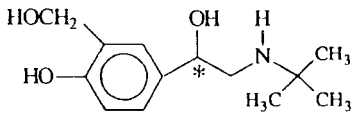
A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ALBUTEROL ENANTIOMERS IN HUMAN SERUM USING SOLID PHASE EXTRACTION AND A SUMICHIRAL-OA CHIRAL STATIONARY PHASE

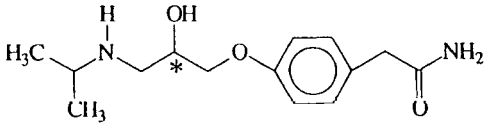
AMANDA G. ADAMS[1] AND JAMES T. STEWART[2]
[1]*Department of Chemistry*
North Georgia College
Dahlonega, Georgia
[2]*Department of Medicinal Chemistry*
College of Pharmacy
The University of Georgia
Athens, Georgia 30602-2352

ABSTRACT

A chiral high performance liquid chromatographic method was developed for the simultaneous assay of S(+) and R(-) albuterol in human serum. The assay utilizes solid-phase extraction on a silica column as a sample clean-up step. The chiral separation was accomplished under isocratic conditions using a Sumichiral OA 4700 column and a mobile phase consisting of 350:410:40:2 v/v/v/v hexane/methylene chloride/absolute methanol/trifluoroacetic acid at a flow rate of 1.0 mL/min. The enantiomers were measured using fluorescence detection set at 228 nm excitation and an emission filter of >280nm. Racemic atenolol was used as internal standard. Drug to internal standard peak height ratios were linear over a 2-20 ng/mL range for each enantiomer. The limit of detection of each analyte was 2.0 ng/mL (S/N=3). The lowest quantifiable level of each enantiomer was 3 ng/mL.



Albuterol



Atenolol

Figure 1 - Chemical structures of albuterol and atenolol (internal standard).

INTRODUCTION

Albuterol, also known as salbutamol, 2-(tert-butylamino)-1-(4-hydroxy-3-hydroxymethylphenyl) ethanol (Fig. 1), is a β-adrenergic stimulant. It has been used to relieve bronchospasm in patients with reversible obstructive airway diseases (1,2). The half-life of albuterol is reported to be 6h and plasma concentrations in man are reported to be in the 0.5-26 ng/mL range (3).

Methods of analysis reported for albuterol have been reviewed (4). The earliest reported procedures for racemic albuterol involved gas chromatography - mass spectrometry (GC-MS)(5,6). A

radioimmunoassay (RIA) method has been reported for racemic albuterol by Loo et al (7). HPLC procedures for albuterol in plasma have become popular. Bland et al reported a procedure which separates racemic albuterol from human plasma using solid phase extraction followed by normal phase chromatography on a silica column (8). Linearity of the method was in the 1-16 ng/mL range using fluorescence detection. Wu et al presented a procedure in which racemic albuterol was separated from plasma using a back extraction approach followed by chromatography on a silica column using an aqueous-organic mobile phase and fluorescence detection (9). Sensitivity of the procedure was reported to be 0.5 ng/mL. A method by Ong et al used an immunoaffinity chromatography clean-up of human plasma prior to chromatographic separation and fluorescence detection of racemic albuterol(10). Recently, an HPLC method for racemic abuterol using amperometric detection was reported by Tamisier-Karolak et al (11). The limit of detection was stated to be 0.5 ng/mL with 5-10% precision. Another electrochemical method for racemic albuterol in human plasma has been reported by Sagar et al (12). Tan and Soldin report a procedure in which albuterol enantiomers are separated from a biological matrix (urine) and chromatographed on an acid glycoprotein column.(AGP) (13), The column temperature was maintained at 0°C and the enantiomers were detected using electrochemical detection (glassy carbon at + 750

mV vs Ag/AgCl). Sensitivity of the method was reported to be around 250 ng/mL. Complete separation of the two enantiomers was not achieved (Rs = 1.06). We evaluated Tan's procedure in our laboratory and it was found that the difficulty of maintaining a 0°C column termperature coupled with the initial high cost and frequent replacement of the chiral AGP column were distinct disadvantages of the method. This laboratory has recently reported an HPLC determination of R(-) and S(+) albuterol enantiomers in human serum using solid phase extraction and chemical derivatization with 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl isothiocyanate to form the diasteromeric thioureas of albuterol (14). The method employed reverse-phase chromatography on an octadecylsilane column with an acetonitrile/water mobile phase. The procedure was found to be sensitive in the low ng/mL range and provided good accuracy and precision.

It was of interest to develop a chiral separation for R(-) and S(+) albuterol without the need for a pre-column chemical derivatization step. It had been suggested in the technical literature from the Sumika Chemical Analysis Service that albuterol enantiomers could be separated on a Sumichiral OA urea-type chiral column using 240:140:20:1 v/v/v/v hexane/1,2-dichloroethane/methanol/trifluoroacetic acid. No application of this separation was made for albuterol enantiomers in human serum. This paper describes a chiral HPLC determination of S(+) and R(-) albuterol enantiomers in human serum using a silica solid phase

extraction column clean-up and direct injection of the eluate onto a Sumichiral OA column using a hexane/methylene chloride based mobile phase. The method is sensitive and precise. The separation is achieved within 30 min with a limit of detection of 2 ng/mL (S/N = 3).

EXPERIMENTAL

Apparatus - The HPLC system consisted of a Varian Model 2510 pump (Walnut Creek, CA, USA), a Rheodyne Model 7125 injector equipped with a 100 μL loop (Cotati, CA USA), a Kratos Model 980 fluorescence detector (Ramsey, NJ USA) set at an excitation wavelength of 228 nm with an emission filter, >280 nm (WG 280, Schott). The PMT setting was 0.055, range 0.010 and a 1 s rise time. A Houston Instrument strip-chart recorder (Series 4500 Microscribe, Houston, TX) set at 5 mVfs and a chart speed of 15 cm/hr was used to trace the chromatograms. The Sumichiral 0A4700 chiral column (YMC, Wilmington, NC, 250 mm x 4.0 mm, 5μm), was used at ambient temperature (22 ± 1°C).

Reagents and Chemicals - Racemic atenolol (internal standard, see Fig. 1) was obtained from Sigma Chemical Co. (St. Lousi, MO, USA), R(-) albuterol acetate and S(+) albuterol acetate were gifts from Glaxo, Inc. (Research Triangle Park, NC,USA). Trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) HPLC grade Methylene chloride, hexane and absolute methanol, were obtained from J.T. Baker (Phillipsburg, NJ, USA). Silica Bond-Elut columns (1 mL, Cat. No. 1210-

2010) were obtained from Analytichem International (Harbor City, CA, USA). Drug free human serum was purchased from Biological Specialty Corporation (Lansdale, PA, USA).

Preparation of Mobile Phase - The mobile phase consisted of a mixture of 350:410:40:2 v/v/v/v hexane/methylene chloride/absolute methanol/trifluoroacetic acid and delivered at a flow rate of 1.0 mL/min. The solution was filered through a 0.45 μm nylon 66 membrane filter (Magna, Honeoye Fall, NY, USA) and sonicated prior to use.

Preparation of Standard Solutions - Stock solutions (2 and 0.2 μg/mL) of S(+) albuterol acetate and R(-) albuterol acetate calculated as albuterol base were prepared in HPLC water and stored at 4°C. These solutions were used to prepare spiked albuterol in human serum. A racemic atenolol stock solution (10 μg/mL) used as the internal standard was also prepared in water and stored at 4°C until use.

Preparation of Spiked Human Serum Samples - Accurately measured aliquots (10, 25 and 50 μL of 0.2 μg/mL and 10 μL of 2 μg/mL) of the S(+) and R(-) albuterol stock solutions were each added to 1 mL volumetric flasks followed by the addition of 15 μL of atenolol stock solution. Blank human serum was added to volume to give standard solutions containing 2, 5, 10, and 20 ng/mL of each albuterol enantiomer.

Assay Method - A 1 mL silica solid phase extraction column was conditioned with one column volume each of acetonitrile and water. (Note-do not allow sorbent to dry). Human serum (1.0 mL) containing

albuterol was applied to the column and aspirated at a slow speed followed by increased vacuum (10 mm) for 2 min. When all of the serum sample was aspirated through the column, the column was washed with 1 mL of water followed by 1 mL of acetonitrile. The column was then dried under full vacuum (50 mm) for 2 min. The albuterol and atenolol enantiomers were eluted with 4-500 μL aliquots of absolute methanol.

The methanol eluent was evaporated to dryness using a nitrogen stream at 50°C and the residue was reconstituted in 200 μL of mobile phase using a vortex mixer for 5 min. Duplicate 100 μL aliquots of the reconstituted solution were injected into the liquid chromatograph.

Calculations - The peak heights of each albuterol enantiomer and the first atenolol peak (T_1) were manually measured for each sample. Four concentrations ranging from 2-20 ng/mL of S(+) and R(-) albuterol were used to construct the calibration curves. Least-square linear regression analysis of drug to internal standard peak-height ratios (D/IS) versus albuterol concentrations was performed. The concentrations of each albuterol enantiomer in a given sample were then calculated using the slope and intercept data in the equation: D/IS = slope (Albuterol concn.) + intercept.

RESULTS AND DISCUSSION

Chromatographic separation of S(+)and R(-) albuterol on the Sumichiral OA column was initially investigated using a mobile phase of

hexane/dichloroethane/methanol/trifluoroacetic acid, which had been described in Sumichiral technical literature. Retention times of the enantiomers were in the 18-25 min range. In efforts to shorten retention and achieve sensitivity in the desired low nanogram level for both enantiomers, the mobile phase was modified using various proportions of hexane/methylene chloride/methanol/trifluoroacetic acid. The final result of this study was creation of a mobile phase that gave retention times in the 9-12 min range with sharp and symmetrical peaks for each enantiomer.

System suitability for the albuterol enantiomers was based on the following chromatographic parameters and the performance of the method throughout validation. The retention times of S(+) and R(-) albuterol were 9.94 ± 0.15 and 11.60 ± 0.20 min., respectively (n = 7). Capacity factors (k') for the S(+) and R(-) enantiomers were 3.97 ± 0.08 and 4.80 ± 0.10, respectively (n = 7). The respective number of theoretical plates for the S(+) and R(-) enantiomers were 9888 ± 296 and 13459 ± 460 per 25 cm column (n = 7). Relative retention of S(+) and R(-) albuterol was expressed by the separation factor α, calculated to be 1.21 ±0.01 (n = 7) from the ratio of the capacity factors. These data indicated that the method should be suitable for analysis of albuterol enantiomers in human serum.

The retention times of atenolol enantiomers (internal standard) were 15.89 ± 0.20 and 17.37 ± 0.21 min, respectively (n = 7). The

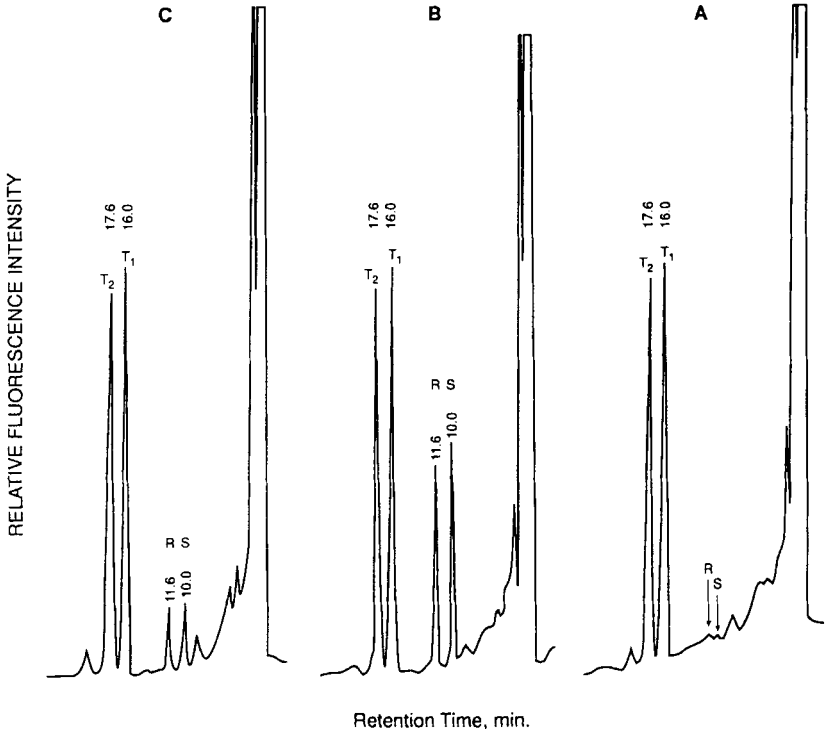


Figure 2 - Typical chromatograms of (A) blank human serum and (B) and (C) spiked human serum containing 20 and 5 ng/mL each of S(+) and R(-) albuterol, respectively. T_1 and T_2 are assigned to the enantiomers of atenolol (0.15 μg/mL) with the shorter and longer elution times, respectively.

order of elution of these enantiomers was not determined. Therefore, the peaks were designated T_1 and T_2 based on the shortest and longest elution time, respectively. The quantitation of each albuterol enantiomer was based on the peak height of the T_1 atenolol peak.

No interferences were observed in blank human serum at the retention times of S(+) and R(-) albuterol. Figure 2 shows typical

chromatograms for blank human serum, and serum spiked with 20 and 5 ng/mL of the S(+) and R(-) enantiomers

The absolute recoveries of S (+) and R(-) albuterol from human serum by the solid-phase extraction method were 84.5 ± 10.6% and 70.4 ± 7.2, respectively, at the 20 ng/mL levels (n = 8). The absolute recovery of atenolol from serum calculated as the racemate was 76.7 ± 6.1% at the 0.15 μg/mL level (n = 5). Comparison of peak heights of extracted to unextracted analyte or internal standard gave absolute recovery data.

System reproducibility was determined by replicate injections of each albuterol enantiomer at 3 and 15 ng/mL. Percent relative standard deviation of D/IS ratios of peak height data for the repeat injections at these concentrations were 14.5 and 10.9%, respectively, for the S(+) albuterol and 9.3 and 11.7%, respectively, for the R(-) albuterol (n = 10).

Standard curves were fitted to a first degree polynomial, $y = a x + b$, where y is the ratio of drug/internal standard peak heights, a and b are constants, and x is albuterol concentration. Precision and accuracy of the method was ascertained using spiked human serum samples in the 2-20 ng/mL range. The data shown in Table 1 indicates that precision was in the 3-9% range and accuracy in the 0.1-10% range for both albuterol enantiomers.

The minimum detectable concentration of each enantiomer was determined to be 2.0 ng/mL (S/N = 3). The lowest quantifiable level of

Table 1 - Analysis of Spiked Albuterol Enantiomers in Human Serum

Amount Added ng/mL	Amound Found*, ng/mL	RSD%	Percent Error
S(+) Albuterol			
2.89	2.68 ± 0.09	3.5	7.1
15.40	14.49 ± 0.46	3.2	5.9
R(-) Albuterol			
2.89	2.61 ± 0.24	9.1	9.7
15.43	15.43 ± 0.75	4.9	0.05

* Based on n = 4.

albuterol with reasonable precision and error was approximately 3 ng/mL of each enantiomer (S(+) 0.4% RSD, 4.2% error; (R(-), 4.77% RSD, 15.2% error).

In conclusion, an HPLC method has been developed and validated for the analysis of S(+) and R(-) albuterol enantiomers in human serum. The method is suitable for the separation and quantification of each enantiomer over a 2-20 ng/mL range.

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