British Journal of Pharmaceutical Research
2(4): 228-237, 2012

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High Performance Liquid Chromatographic Method Development and Its Validation for Salbutamol

Selvadurai Muralidharan[1*] and Jayaraj Kumar[1]

[1]Faculty of Pharmacy, AIMST University, Semeling, Bedong, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. SM designed the study, performed the analysis, Experimental part, wrote the protocol, and wrote the first draft of the manuscript. JK managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

Received 7th March 2012
Accepted 21st June 2012
Published 9th November 2012

ABSTRACT

Aims: A simple and sensitive high performance liquid chromatographic (HPLC) method was developed for quantification of salbutamol in rat plasma. Terbutaline was used as an internal standard (IS).
Study Design: Validation study.
Methodology: The present method used solid phase extraction of salbutamol from rat plasma. Chromatographic separation achieved isocratically on reversed-phase c_{18} column (250 × 4.6 mm, 5µ) and the column effluent was monitored by uv detector at 276 nm. The mobile phase used was acetonitrile: 50mm ammonium acetate (ph 7.0), (80: 20 % v/v) at a flow rate of 1.0 ml/min.
Results and Discussion: This method was linear over the range of 50.0 – 1000.0 ng/ml with regression coefficient greater than 0.99.
Conclusion: The method was found to be precise, accurate and specific during the study. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC–MS/MS or GC–MS/MS that are complicated, costly and time consuming rather than a simple HPLC–UV method. The method was successfully applied for pharmacokinetic study of salbutamol in rats.

**Corresponding author: murali23pharm@hotmail.com;*

Keywords: Simple Method; high performance liquid chromatography; validation; salbutamol; rat plasma; pharmacokinetic study.

1. INTRODUCTION

Salbutamol is chemically 2-(hydroxymethyl)-4-[1-hydroxy- 2-(tert-butylamino) ethyl] phenol. It is a short-acting β_2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and COPD. It is usually given by the inhaled route for direct effect on bronchial smooth muscle. This is usually achieved through a metered dose inhaler, Nebulizer or other proprietary delivery devices. In these forms of delivery, the effect of Salbutamol can take place within 5 to 20 minutes of dosing. It can also be given orally or intravenously. Despite the fact that SS is well absorbed, its systemic bioavailability is only 50% due to extensive presystemic metabolism in the gastrointestinal tract and liver [1]. Salbutamol Sulphate in pharmaceuticals has been assayed using visible spectrophotometric methods based on reactions such as redox, reducing and then chelating, oxidative coupling, diazotization and coupling, nitrosation, nitration, nitration followed by Meisenheiner complex formation and charge-transfer complex formation [2-12].

However, any of these procedures suffer from some disadvantage, such as poor sensitivity, heating or extraction step, critical working conditions or the use of organic solvents and are hence unsatisfactory for routine analysis [13]. A number of analytical methods exist for the determination of Salbutamol in biological fluids, including reversed phase high- performance liquid chromatography [14-17] equipped with ultraviolet [18,19], Fluorescent Detection [20] electrophoresis [21-23] amperometric [24,25] thin layer chromatography [26] cation exchange [27] direct conductivity [28] gamma radiation [29] and liquid chromatography mass spectrometric detection [30]. The low plasma concentration and long resident time after topical application of the drug justify the necessity of developing a sensitive (HPLC) method. There were no simple, rapid and reproducible methods so far reported for the estimation of salbutamol in plasma. The objective of the present investigation was to develop a new, rapid and sensitive RP-HPLC method for the estimation of salbutamol in rat plasma. In the present study, a more sensitive, and precise (HPLC) method was to be developed and applied to the pharmacokinetic study.

2. EXPERIMENTAL

2.1 Materials and Reagents

Salbutamol (98.35%) and Terbutaline (99.56%) (Figs. 1a and 1b) reference standards were procured from Cipla Pharmaceutical Pvt. Ltd. (Mumbai, India) and Alembic Pharmaceuticals Ltd. (Baroda, Gujarat, India) respectively. Acetonitrile (HPLC grade) and methanol (HPLC grade), Merck KGaA, Germany. Ammonium acetate (analytical grade) and orthophosphoric acid were purchased from Systerm, Malaysia. Milli-Q water purification system supplied by Millipore was used for the preparation of the aqueous mobile phase.

2.2 Equipment

HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50µL loop volume. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan). Thermo C_{18} (250 x 4.6 mm i.d., 5µ) was used for the present analysis.

2.3 Preparation of the Calibration Standards and Quality Control (QC) Samples

The stock solutions of salbutamol and Terbutaline were prepared in water and acetonitrile mixture 1:1 at a concentration of 1.0 mg/mL each. The working solutions of 100.0 and 40.0 µg/mL were prepared by appropriately diluting the stock solutions of salbutamol and Terbutaline. Salbutamol working solution was used to prepare the spiking stock solutions for construction of six-point calibration curve (50.0 -1000.0 ng/mL) and QC samples at three different levels (100.0, 750.0, 1000.0 ng/mL). All the stock solutions were refrigerated (2-8ºC) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 25.0 µL of respective spiking stock solutions to 475.0 µL of control rat plasma and then aliquoted. These were stored at -70ºC until analysis.

2.4 Sample Preparation for Analysis

At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. Samprep SPE Columns C_{18}- (50µm, 70 A) 100mg/mL solid phase extraction cartridge was conditioned with methanol, water sequentially. To this 20.0 µL aliquot of the plasma containing salbutamol were pipetted into micro tubes and 5.0 µL of internal standard (50.0 µg/mL terbutaline) was loaded. The cartridge was washed with 2.0mL of water. The drug and internal standard was eluted from the cartridge using water and acetonitrile mixture 1:1. The Resulting Solution was evaporated to dryness. The evaporated residue was reconstituted with 250 µL of reconstitution solution (mobile phase ratio). 20.0 µL of the reconstituted samples was injected to the HPLC system for analysis. All the procedures were performed at room temperature.

2.5 Chromatographic Conditions

The samples were chromatographed on a Thermo C_{18} (250 x 4.6 mm i.d., 5µ) column with a flow rate of 1.0 mL/min. The mobile phase used was acetonitrile – ammonium acetate (80:20 % v/v). Ammonium acetate used was 50mM solution in water with pH being adjusted to 7.0 with orthophosphoric acid solution. The injection volume was 20.0 µL. The UV-visible detector was set at 276 nm.

3. VALIDATION

The method has been validated [31] for selectivity, sensitivity, recovery, linearity, precision, accuracy and stability.

3.1 Selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing salbutamol and the internal standard with those obtained from blank samples.

3.2 Sensitivity

Sensitivity was determined in terms of LLOQ (Lower Limit of Quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte.

3.3 Linearity

For linearity, different concentrations of standard solutions were prepared to contain 50.0 ng/mL to 1000.0 ng/mL of salbutamol containing 5.0 µL of internal standard (50.0 µg/mL terbutaline). These solutions were analysed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor vs concentration of the standard solutions. Standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit.

3.4 Precision and Accuracy

The precision of the method was determined by intraday precision and interday precision. The intra-assay precision and accuracy was calculated for five replicates at each Lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The results are given in Table 2 as part of the method validation.

Table 2. Intraday and Interday accuracy and precision of Salbutamol in rat plasma

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD %	R.E. %
Inter-day (n=3)			
50	44.21	6.92	-11.58
250	235.38	3.63	-5.40
750	739.35	0.91	-1.42
1000	959.19	3.00	-4.08
Intra-day (n=3)			
50	43.09	6.41	-13.82
250	238.10	1.98	-4.76
750	736.31	1.70	-1.82
1000	980.92	1.06	-1.90

3.5 Stability Studies

Stability study was carried out. Room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and long term stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 hours by injecting four replicates of prepared stock dilutions of salbutamol equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of salbutamol and internal standard at 3 and 8 hours was carried out against the zero hour value. Refrigerated stock solution stability was determined at 7, 14 and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. The stability studies of plasma samples spiked with salbutamol were subjected to three freeze - thaw cycles, short term stability at room temperature for 3 h and long term stability at – 70ºC over four weeks. In addition, stability of standard solutions was performed at room temperature over 6 h and after freezing for four weeks. The stability of triplicate spiked

animal plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with salbutamol was investigated at room temperature for 1.00 to 3.00 h before extraction. The plasma samples for long term stability were stored in the freezer at -70º C until the time of analysis.

4. RESULTS AND DISCUSSION

Liquid-liquid extraction was attempted using various organic solvents like diethyl ether, chloroform, ethyl acetate, dichloromethane, petroleum ether. As salbutamol is hydrophobic in nature, also combination of these solvents at different ratios had been tried with different precipitating agents. Since the drug was poorly soluble in the above said organic solvents, it resulted in poor extraction efficiency. Protein precipitating agents like acids and alkalis were used at different pH ranges to extract the drug efficiently.

Solid phase extraction can be carried out to achieve the higher extraction efficiency. Hence, the present study employed solid phase extraction of the drug from rat plasma.

4.1 Selectivity

No interfering endogenous compound peak was observed at the retention time of analyte. Under chromatographic conditions, the retention times of salbutamol and terbutaline were 9.12 min and 14.50 min respectively. Representative chromatograms of Lower Limit of Quantitation (LLOQ) and one study sample containing salbutamol are shown in Figs. 1 and 2 respectively.

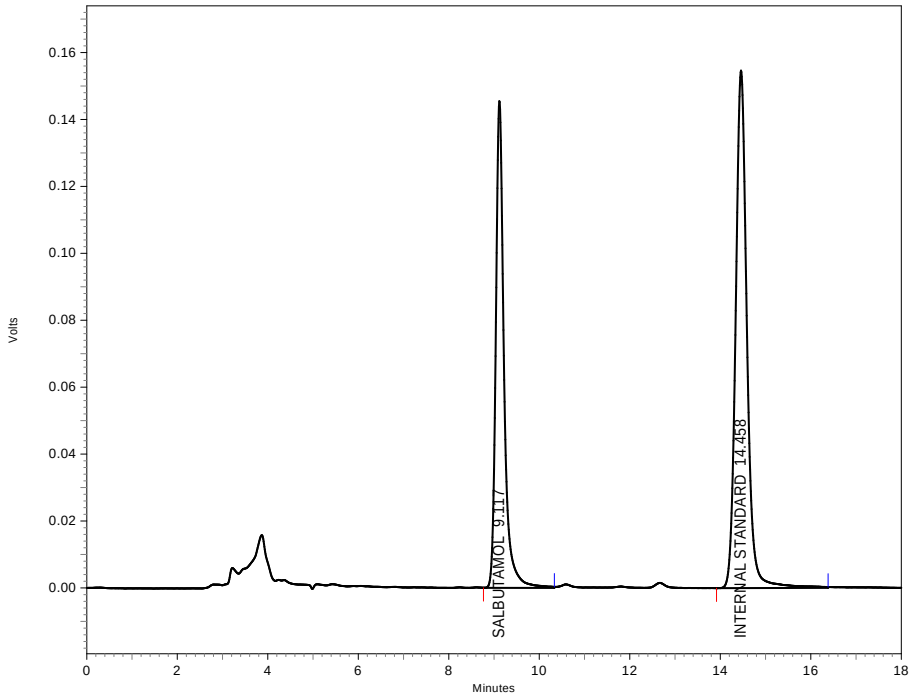

Fig. 1. Typical Chromatogram of Standard Solution

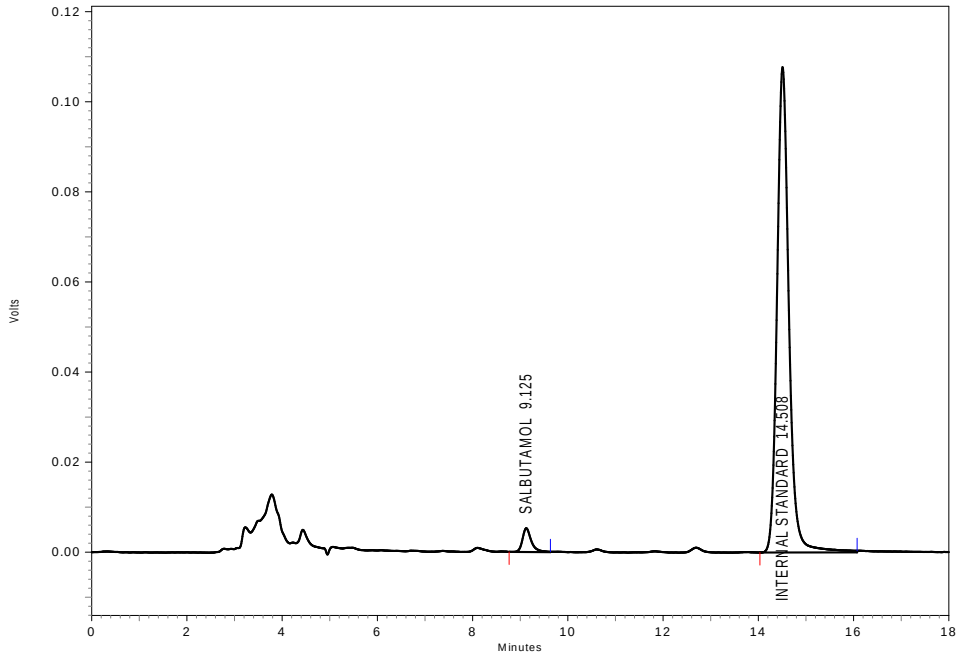

Fig. 2. Typical Chromatogram of Sample Solution

4.2 Sensitivity (Lower Limit of Quantitation)

The sensitivity of the experiment was carried out at LLOQ level. The average percentage deviation from the nominal concentration was less than 12.0 % and the precision was within 1.4 % relative standard deviation (R.S.D.).

4.3 Linearity

The calibration curves were linear over the range of 50.0-1000.0 ng/mL. The correlation coefficient was > 0.9996**.** Calibration curve data of salbutamol result are shown in Table 1.

4.4 Precision and Accuracy

Both intra-day and inter-day accuracy and precision of the method were determined by Analysis of the control rat plasma spiked with salbutamol at LLOQ, LQC, MQC and HQC. All QCs concentration was calculated using the calibration curve. The accuracy and precision of the method were described as a percentage bias and the percentage relative standard deviation, respectively.

Table 1. Inter-run accuracy and precision of plasma calibration standards for Salbutamol

Standard concentration (ng/mL)	Average calculated Concentration (ng/mL)	SD	R.E. %
50	44.60	7.88	-10.79
150	143.99	2.66	-4.00
250	236.03	3.33	-5.58
500	477.93	1.76	-4.41
750	738.75	1.03	-1.50
1000	950.39	3.64	-4.96

RSD=Relative standard deviation; R.E.=Relative error

4.5 Stability

Analysis of the stock solution was performed at 1000.0 ng/mL. After storage for 15 days at 2-8ºC and at room temperature for 6h, more than 98% of salbutamol remained unchanged, based on peak areas in comparison with freshly prepared solution of salbutamol (1000.0 ng/mL) This suggests that the salbutamol in standard solution is stable for at least 15 days when stored at 2-8ºC and for 6h at room temperature.

Bench top stability of salbutamol in plasma was investigated at LQC and HQC levels. This revealed that the salbutamol in plasma was stable for at least 6 h at room temperature. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with salbutamol at LQC and HQC level did not affect the stability of salbutamol Long term stability of the salbutamol in plasma at -70ºC was also performed after 30 days of storage at LQC, HQC levels. The results of the stability studies are shown in Table 3. The average long term stability was 93.75%. The above results indicated that the salbutamol was stable in the studied conditions.

Table 3. Stability Study of Salbutamol

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD	R.E. %
Bench top (n=5)			
50	23538	3.63	-5.40
1000	959.19	3.00	-4.08
Freeze thaw Stability (n=5)			
50	238.10	1.98	-4.76
1000	980.92	1.06	-1.90
Long term Stability (n=5)			
50	238.10	1.98	-4.76
1000	980.92	1.06	-1.90

5. CONCLUSION

A simple and sensitive method for the determination of salbutamol in rat plasma by HPLC was developed and validated. The method consisted of sample preparation by Solid Phase Extraction, followed by chromatographic separation and UV detection. No interfering peaks were observed at the elution times of salbutamol and IS. Adequate specificity, precision and

accuracy of the proposed method were demonstrated over the concentration range of 50.0-1000.0 ng/mL. The method was accurate, reproducible, specific and applicable to the evaluation of pharmacokinetic profiles of salbutamol in rats.

COMPETING INTERESTS

Authors have declared no competing interests exist.

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