Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 1, 2012

Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LEVALBUTEROL A β_2-ADRENERGIC AGONIST BY RP-HPLC METHOD

A. S. GHEMUD[1], B. SANTHAKUMARI[2*], A. B. PHARNE[3], M. M. JADHAV[4], K. S. JAIN[1], M. J. KULKARNI[2]

[1.]Department of Pharmaceutical Chemistry, Sinhgad College of Pharmacy, Vadagaon (BK) Pune, 411041, Maharashtra, India, [2.]Department of Center for Materials Characterisation, National Chemical Laboratory, Pune 411008, Maharashtra, India, [3.]Department of Pharmaceutical Chemistry, CYMET's Siddhant College of Pharmacy, Pune 412109, Maharashtra, India, [4.]Dr. D.Y. Patil College of Pharmacy, Pimpri, Pune 411018, Maharashtra, India. Email: b.santhakumari@ncl.res.in

Received: 7 Oct 2011, Revised and Accepted: 27 Nov 2011

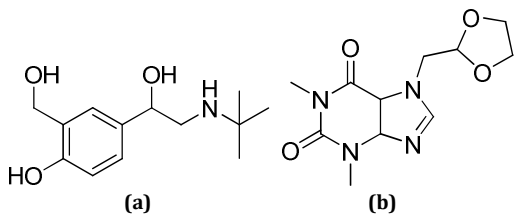
ABSTRACT

A simple, selective, rapid, precise and economical Reverse-Phase HPLC method has been developed and validated for quantitative determination of Levalbuterol in rat plasma. Levalbuterol is β_2-adrenergic agonist commonly used as a bronchodilator for the treatment of patients with asthma. Doxophylline is used as an internal standard. The method was carried out with UV spectrophotometric detection using a Perkin Elmer Series 200 HPLC system equipped with X-Bridge Shield C18 column (3.5 µm, 4.6×150mm) and a guard column of the same type, at a flow rate of 0.8mL/min. Detection was carried out at 276 nm. The mobile phase consisted of 50mM ammonium bicarbonate (pH 7.8) and acetonitrile. The retention times of Levalbuterol and Doxophylline were 9.6 and 12 min respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification and stability. The proposed method uses less biological material and is MS compatible. This Method can be applicable for pharmacokinetic studies using HPLC or LC-MS.

Keywords: RP-HPLC, Levalbuterol, Doxophylline, Gradient

INTRODUCTION

Methods of measuring drugs in biological media are becoming increasingly important for the study of bioavailability & bioequivalence studies, quantitative evaluation of drugs and their metabolites, new drug development, clinical pharmacokinetics, research in basic biomedical and pharmaceutical sciences and therapeutic drug monitoring. Levalbuterol fig.1 (a), chemically 2-(Hydroxymethyl)-4-[(1*S*) 1-hydroxy-2-(tert-butylamino) ethyl] phenol is a single isomer beta2-agonist that differs from racemic albuterol by elimination of (S)-Albuterol. Levalbuterol leads to activation of beta2-adrenergic receptors on airways smooth muscle leading to the activation of adenylate cyclase, which increases the intracellular concentration of cAMP. The increase in cAMP is associated with the activation of protein kinase A, which in turn, inhibits the phosphorylation of myosin and lowers intracellular ionic calcium concentrations, resulting in muscle relaxation and bronchodilation. Levalbuterol relaxes the smooth muscles of all airways, from the trachea to the terminal bronchioles. Increased cAMP concentrations are also associated with the inhibition of the release of mediators from mast cells in the airways[1-4]. Doxophylline fig1 (b), chemically 7-(1, 3-dioxolan-2-ylmethyl)-1, 3-dimethylpurine-2, 6-dione, is a xanthine bronchodilator. In this study it is used as an internal standard (IS).



(a) (b)

Fig. 1: Chemical structure of (a) Levalbuterol and (b) Doxophylline

Literature survey revealed that validated HPLC method for the quantification of Levalbuterol in plasma is not reported [5-9]. For the estimation of the drugs present in the biological fluid, HPLC method is considered to be more suitable since this is a powerful and rugged method. It is also extremely specific, linear, precise, accurate, sensitive and rapid. In this study we have developed a mass spectrometry compatible HPLC method with a protein precipitation extraction and improved sensitivity for the determination of Levalbuterol in plasma and the developed method is validated as per regulatory requirements.

MATERIALS AND METHODS

Chemicals

Levalbuterol and Doxophylline(IS) was gifted by FDC Ltd. Mumbai. HPLC grade solvents (Acetonitrile, Methanol) were obtained from Merck and milli-Q water was from SG Series Compact Pretreatment Module. AR Grade ammonium bicarbonate was purchased from Sigma Aldrich.

Instrument

The Perkin Elmer Series 200 LC system equipped with a UV detector and an autosampler was used. Chromatographic separations were performed using the X-Bridge Shield C18 (3.5 µm, 4.6×150mm) column and analyzed by LC software Turbochrome work station.

Preparation of solutions

50mM ammonium bicarbonate buffer was prepared by dissolving approximately 1.96 gm of ammonium bicarbonate in 500 ml of water and the pH was adjusted to 7.8 with acetic acid.

Preparation of standard

Levalbuterol and Doxophylline stock solutions were prepared with a concentration of 1 mg/ml by dissolving in methanol and the stock solutions were stored in the refrigerator. Spiking solutions of Levalbuterol for the preparation of calibration standards and quality control samples were prepared in methanol and spiked in to the plasma at the ratio of 1:8. The calibration curve was generated using seven calibration standards with the concentrations of 2.5µg/ml (STD 1), 5 µg/ml (STD 2), 10 µg/ml (STD 3), 15 µg/ml (STD 4), 20 µg/ml (STD 5), 25 µg/ml (STD 6) and 30 µg/ml (STD 7). The Quality Control samples were prepared with the concentrations of 5 µg/ml (LQC), 15 µg/ml (MQC) and 25 µg/ml (HQC). The bulk spiked calibration

standards and quality control samples were stored in the freezer. Internal standard dilution was prepared.

Sample preparation and extraction

Levalbuterol from the plasma was extracted by using protein precipitation extraction technique. Blood samples were collected in heparinised tubes and immediately placed on ice and taken to the lab where they were centrifuged at 5000rpm for 5 min at room temperature. The resulting plasma samples were stored at -75°C until analysis. Aliquot 160µl of plasma into eppindorf tubes and added 20µl of internal standard dilution and vortexed to mix the contents. 20µl of above sample is added and Levalbuterol is extracted by using methanol as a precipitating solvent and vortexed for 30sec. Then the extract was centrifuged at 4°c, 7000rpm for 10 min. The supernatant was taken and transferred to HPLC vials.

HPLC method

The mobile phase used was 50 mM ammonium bicarbonate (pH 7.8) (phase A) and 100% acetonitrile (Phase B). Before analysis, the mobile phase was filtered through 0.45 µm filter paper and then degassed ultrasonically for 15 min. A gradient method was developed table 1. For analysis the mobile phase was initially composed of 95% solvent-A and held for 1 min. The mobile phase composition was then linearly programmed to 95% solvent-B in 20 min and held for 1 min. The mobile phase condition was returned to the starting solvent mixture in 1 min. The system was allowed to equilibrate for 10 min before the next injection. The analysis was conducted at a flow rate of 0.8 ml/min. The eluent was monitored at a wavelength of 276nm. The total run time was 20 min and injection volume was 20µl.

Table1: The chromatographic conditions optimized for analysis of Levalbuterol by RP-HPLC

Drug	Mobile Phase			Flow Rate (ml/min)	Detection Wavelength (nm)	Injection Volume (µl)	Retention Time (min.)
	Time (min)	Buffer %A	ACN %B				
Levalbuterol	0	95	5	0.8	276	20	9.6
	5	80	20				
	10	50	50				
	15	20	80				
	17	5	95				
	19	95	5				
	20	95	5				

Method validation

The method performance was evaluated for selectivity, accuracy, precision, linearity, and robustness, stability during various stress conditions including bench top stability, freeze thaw stability, autosampler stability, stability of stock solutions etc. and recovery [10, 11]

Linearity

Calibration curves were constructed using linear regression (with weighting of $1/x^2$) within the range of 2.5-30µg/ml of Levalbuterol.

Recovery

Recovery of analyte was evaluated by comparing response of Levalbuterol in three quality control samples (LQC, MQC and HQC) with the response of Levalbuterol in equivalent aqueous solutions.

Precision and Accuracy

For precision and accuracy studies, samples were prepared at three concentration levels, low (LQC), medium (MQC) and high (HQC) quality controls. Corresponding to 5, 15 and 25µg/ml respectively with six replicates each. Precision was evaluated at inter and intra batch.

Ruggedness

The ruggedness of the method was studied by changing the experimental conditions such as,

Different HPLC instruments (Waters HPLC, Shimadzu HPLC)

Different operators in the same laboratory

Stability studies

The stability of Levalbuterol in solutions and plasma samples was evaluated during method validation. Levalbuterol stability was evaluated using two concentration levels (low and high quality control, corresponding to 5 and 25µg/ml respectively). The stability of Levalbuterol was also evaluated in post extracted samples kept in the autosampler at 4°C, 24 hours, as well as in plasma samples kept at freezer and after being stressed to 3 freeze-thawing cycles (24 hours each cycle). All samples described above were compared to freshly prepared Levalbuterol samples at the same concentration level.

RESULTS AND DISCUSSION

Chromatographic Optimization

RP-HPLC method was developed for Levalbuterol, which can be conveniently employed for routine analysis in biological fluids. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for drug was selected based on its polarity. Different trials were taken and the final working mobile phases are listed in table 1. The retention times of Levalbuterol and Doxophylline were 9.6min and 12min, respectively. The chromatograms have been shown in fig.2 and fig.3. The method is validated as per regulatory guidelines.

Selectivity

The described method used reversed-phase HPLC for separation of Levalbuterol from Doxophylline (IS) and was shown to be selective for the analyte and its IS (retention times for Levalbuterol and Doxophylline were 9.6min and 12min respectively). No interfering peaks were observed with the same retention time of the analyte when different plasma samples were analysed. Fig.4 and fig.5 represents the chromatogram of blank plasma sample and plasma sample spiked with drugs respectively.

Linearity

Linearity was demonstrated from 2.5-30 µg/ml. fig.6 shows calibration curve of Levalbuterol. The calibration curve includes 7 calibration standards which are distributed throughout the calibration range. The average correlation coefficient was found to be 0.998 with goodness of fit.

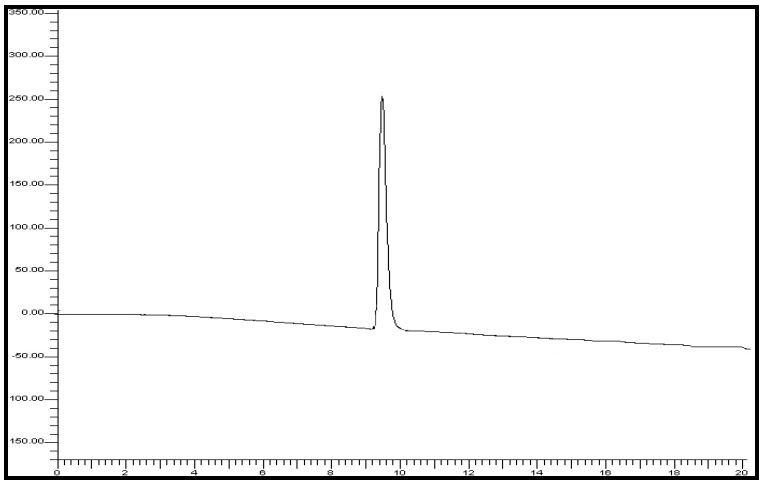


Fig. 2: Typical chromatogram of Levalbuterol (RT-9.6min)

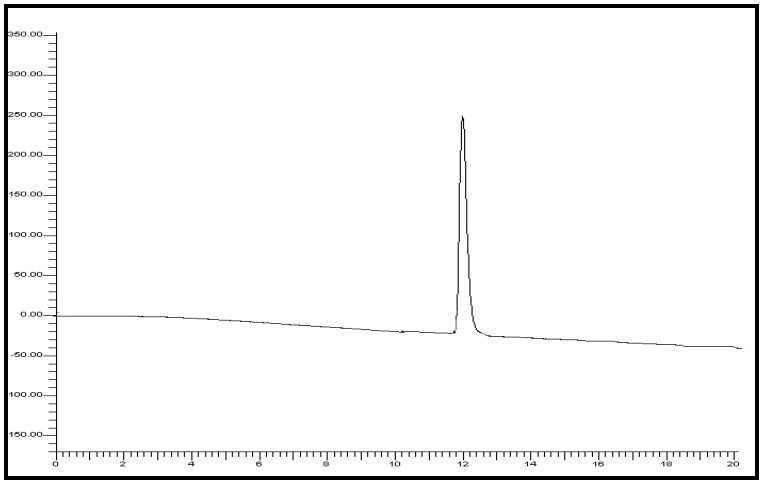


Fig. 3: Typical chromatogram of Doxophylline (RT-12min)

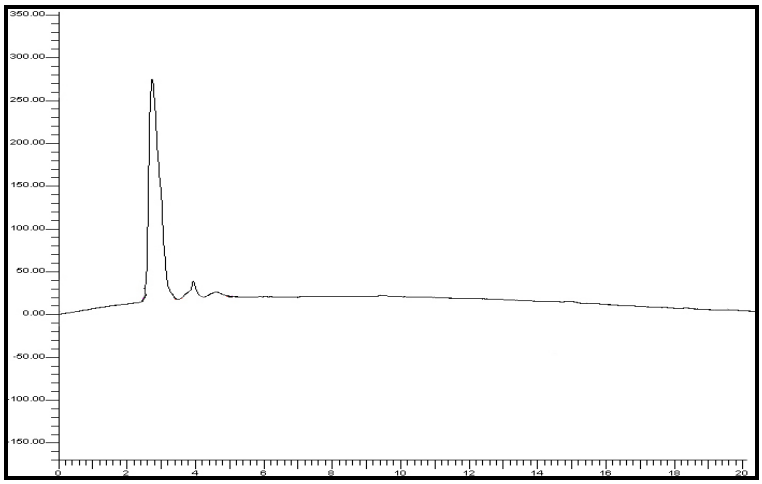


Fig. 4: Typical chromatogram of blank plasma sample

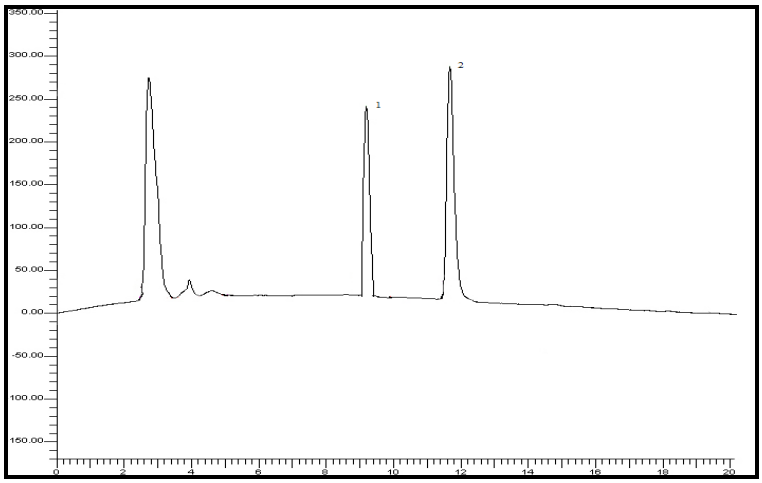


Fig. 5: Chromatogram of blank plasma spiked with (1) Levalbuterol and (2) Doxophylline(IS)

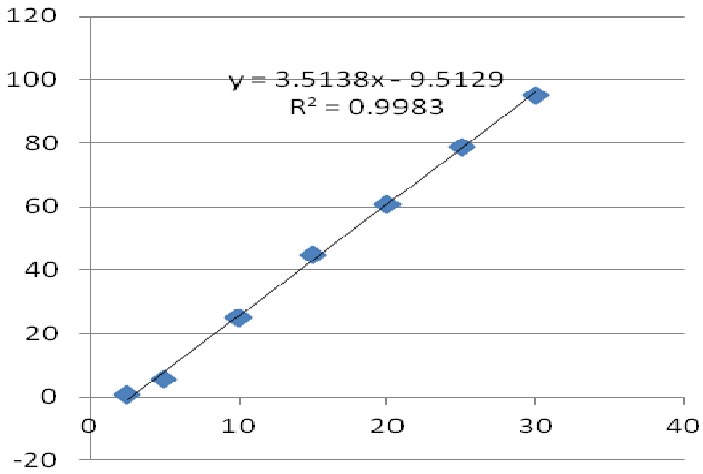


Fig. 6: Calibration Curve of Levalbuterol

Accuracy and Precision

Accuracy and precision was evaluated by analysing 3 batches. Each batch consisted of six replicates of LQC, MQC and HQC. Precision was evaluated both interday and intraday batches. The interday and intraday precision and accuracy of the method for each Levalbuterol concentration levels (5, 15 and 25 µg/ml) are represented in table 2. The mean accuracy for each concentration level ranged from 93.2 to 98.24 and the mean precision for each concentration level ranged from 0.79 to 5.22.

Table 2: Intraday and Interday Precision and Accuracy of Levalbuterol

Concentration (µg /ml)	Accuracy (% nominal) (n=6)	Precision(%CV) (n=6)	
		Interday	Intraday
5	93.2	1.97	5.22
15	96.86	0.79	1.01
25	98.24	1.21	1.03

Recovery

The recovery was evaluated by comparing response of extracted and unextracted sample. Extracted samples include six replicates of extracted LQC, MQC and HQC samples. Unextracted samples included the aqueous solutions equivalent to extracted samples. The average recovery for Levalbuterol in plasma was ranged from 89.2 to 96.87% for the low, medium and high quality control samples with an average of 92.6% table 3.

Table 3: Recovery result of Levalbuterol from plasma

Concentration (µg /ml)	Mean Relative Recovery (%) (n=6)
5	89.27
15	91.69
25	96.87

Stability Studies

Stability studies were performed to evaluate the stability of Levalbuterol both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include stock solution stability in stock solution, bench top stability in plasma, freeze thaw stability in plasma, long term storage stability in plasma and auto sampler stability of processed samples. All Stability evaluations were performed as per international regulatory guidelines. Levalbuterol stock solution (1mg/ml) remained stable when stored at refrigerator

conditions for 30 days including the storage at room temperature for 12 hours. Levalbuterol was stable in plasma samples when stored at room temperature for 5 hours 30 min at -20^{0}C. Levalbuterol was found to be stable for three freeze and thaw cycles. Levalbuterol in the processed samples was stable for 24 hours when stored in the autosampler.

Ruggedness

The ruggedness of the method was carried out by changing the instrument and by different analyst in the same lab. The percentage CV of the HQC and LQC were found to be 5.5 and 3.3% respectively.

Table 4: Validation Parameters of Levalbuterol by HPLC method

Sr. No.	Parameters	Results
1.	Selectivity	Pass
2.	System suitability	Pass
3.	Accuracy and precision	Pass
4.	Linearity	R^2 = 0.998
5.	Recovery	Pass
6.	Short term stock stability	12 Hrs.
7.	Long term stock stability	30days
8.	Bench top stability	5 Hrs 30min
9.	Freeze thaw stability	Pass (3cycles)
10.	Auto sampler Stability	24 Hrs
11.	Ruggedness	Pass

CONCLUSION

The current validated HPLC method for Levalbuterol offers good accuracy and significant advantages in terms of sensitivity, selectivity and sample preparation. It can be used for the estimation of Levalbuterol in bio-fluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of Levalbuterol without any interference. The validation parameters are shown in table 4. From the results of all the validation parameters here we conclude the developed method can be applied in bioequivalence, pharmacokinetic and toxicokinetic studies with desired precision and accuracy along with high-throughput.

ACKNOWLEDGMENT

Authors are thankful to FDC Ltd. Mumbai for providing drugs as gift samples.

REFERENCES

1. U.S. Department of Health and Human Services Food and Drug administration Centre for Drug Evaluation and Research (CDER) Centre for Veterinary Medicine (CVM), Guidance for Industry Bioanalytical Method Validation, May 2001 BP.
2. McIntosh CH, Demuth HU, Pospisilik JA, Pederson R., Dipeptidyl peptidase IV inhibitors. How do they work as new antidiabetic agents? Regul Pept. 2005, 159–165.
3. Weber AE. Dipeptidyl peptidase IV inhibitors for the treatment of diabetes. J Med Chem. 47, 2004, 4135–4141.
4. Lauster CD, McKaveney TP, Muench SV. Levalbuterol: A novel oral therapy for type 2 diabetes mellitus. Am J Health Syst Pharm. 64, 2007, 1265–1273.
5. Handan He, Phi Tran, Hequn Yin, Harold Smith, Yannick Batard, Lai Wang, Heidi Einolf, Helen Gu, James B. Mangold, Volker Fischer and Dan Howard. Absorption, Metabolism, and Excretion of [^{14}C] Levalbuterol, a Novel Dipeptidyl Peptidase 4 Inhibitor, in Humans, Drug Metabolism and Disposition**,** 37, 2009, 536-544.
6. Cornelius Hess & Frank Musshoff & Burkhard Madea, Simultaneous identification and validated quantification of 11 oral hypoglycemic drugs in plasma by electrospray ionisation liquid chromatography–mass spectrometry, Analytical and bioanalytical chemistry, 400, 2011, 33-41.
7. G. Hendriks, Review Theoretical models in LC based bioanalytical method development, Journal of Pharmaceutical and Biomedical Analysis, 49, 2009, 1-10.
8. Subhash V. Deshmane, Madhuri A. Channawar, Anil V. Chandewar, Unmesh M. Joshi, Kailash R. Biyani, Chitosan based sustained release mucoadhesive buccal patches containing verapamil Hcl. International Journal of Pharmacy and Pharmaceutical Sciences, 1, (2009) 216-229.
9. Aarifkhan M. Pathan*, Madhuri A. Channawar, Bharti. V. Bakde, Dr. Anil V. Chandewar, Development and in vitro evaluation of salbutamol sulphate mucoadhesive buccal patches. International Journal of Pharmacy and Pharmaceutical Sciences, 3, (2010)
10. International Conference on Harmonization (ICH), Validation of analytical methods definitions and terminology, ICH Q2 A, 1994.
11. International Conference on Harmonization (ICH), Validation of analytical methods: methodology, ICH Q2 B, 1996.