Journal of Applied Pharmaceutical Science Vol. 10(08), pp 131-138, August, 2020
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2020.10815
ISSN 2231-3354

Simultaneous detection and quantification of bronchodilators in pure form and from *in-vitro* drug release of a novel combinational formulation

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ARTICLE INFO

Received on: 09/03/2020
Accepted on: 04/06/2020
Available online: 05/08/2020

Key words:
RP-HPLC, salbutamol, ipratropium, method development, validation, ICH guidelines.

ABSTRACT

The analytical method was developed and validated for the quantification of salbutamol sulfate (SS) and ipratropium bromide (IPB) in accordance with the International Council for Harmonization guidelines in its pure form. The chromatographic partition was completed utilizing a blend of acetonitrile:phosphate buffer (30:70 v/v) with the pH scale adjusted to 3.0 using o-phosphoric acid at a flow rate of 1 ml/minute in Luna C-18(2)(150 × 4.6 mm i.d., 5 µm) column. The wavelength for detection was fixed at 212 nm. The SS and IPB showed a standard linearity curve in the range of 2–12 µg/ml, with retention time at 2.4 and 3.8 minutes, respectively. The developed method was reported to be specific, linear ($r^2 \geq 0.999$), precise at intraday and interday levels (% relative standard deviation < 2.0%), accurate (% recovery: 96.02%–103.62%), and robust. The limit of detection and limit of quantification for SS was found to be 0.42 and 1.26 µg/ml, while that of IPB was 0.44 and 1.34 µg/ml, respectively. Additionally, the developed method was effectively applied in quantifying SS and IPB from its pure, commercial, and in-house prepared transdermal system to understanding the *in-vitro* drug release pattern from patches.

INTRODUCTION

Salbutamol sulfate (SS), chemically known as (RS)-1-(4-hydroxy-3-hydroxy-methyl phenyl)-2-(tert-butylamino)ethanol sulfate, is formulated as a 1:1 sulfate salt. It is the most extensively used sympathomimetic, indicating prominent bronchodilator and less cardiovascular impacts. It mitigates the congestion related to asthma, chronic obstructive pulmonary disease (COPD), and other forms of allergic airways disease (The Merk Index, 2006). The medication is additionally utilized for the anticipation of premature labor and exercise-evoked bronchospasm. Pharmacological action is a result of adenylyl cyclase stimulation, thus increasing the intracellular cyclic-adenosine monophosphate levels, promoting relaxation of the smooth muscles of bronchi and the uterus (Springhouse, 2008).

The drug is official in European Pharmacopoeia, British Pharmacopoeia, and Indian Pharmacopoeia. Different methods of detection of SS have been accounted for, either alone or in combination with other drugs, using chromatographic analytical techniques, like high-performance liquid chromatography (Walode *et al*., 2013), liquid chromatography–tandem mass spectrometry (Zhou *et al*., 2015), gas chromatography (Srichana *et al*., 1998), and by other nonchromatographic procedures (Basavaiah *et al*., 2006; Basavaiah *et al*., 2007). Various other principles for its detection are also reported (Muralidharan and Kumar, 2012)

Ipratropium bromide (IPB) is a congener of atropine obtained by treating atropine with isopropyl bromide, chemically described as 8-azoniabicyclo [3.2.1] octane, 3-(3-hydroxy-1-oxo-2-phenylpropoxy)-8-methyl 8-(1-methyl ethyl) bromide (Abdine *et al*., 2003a). It was designed as an orally active, exceptionally polar drug which inadequately infiltrates the blood–brain barrier, thus preventing central side effects, while the sole significant side effect is dry mouth, at dosages higher than the therapeutic dose. IPB is a white to off-white crystalline substance freely soluble in

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water and methanol, sparingly soluble in ethanol, and insoluble in lipophilic solvents (Abdine *et al*., 2003b). The drug is used by inhalation, in the treatment of COPD and allergic rhinitis. It blocks all subtypes of muscarinic receptors, resulting in a decrease in the formation of cyclic guanosine monophosphate (c-GMP) that effectively averts the increase in intracellular concentration of calcium, thereby improving the contractility of smooth muscles and restraining mucus secretion (Mario *et al*., 2012).

IPB is devoid of structural conjugation (Abdine *et al*., 2003c); therefore, no analytical methods have been reported by the conventional UV method. The official methods for estimating IPB were the titrimetric method with silver nitrate for the assay of pure substance; BP (1998) used an high performance liquid chromatography (HPLC) method for the assay in pressurized inhalation, while the European, Japanese and American pharmacopoeia described other official methods. Hassan (2000) extended the application of derivative spectroscopy for its estimation. HPLC techniques were reported to quantify IPB in aerosol, liquid for nebulization (Nayak *et al*., 1992), and its related substances (Simms *et al*., 1998). Other methods reported are a radioreceptor assay method (Ensing *et al*., 1988) and a nonaqueous titration method. The detailed spectrophotometric methods depended on the oxidation of IPB with alkaline $KMnO_4$ and complexation with iodine and bromocresol green (Hassan, 2000).

Not many methods have been accounted for in the simultaneous estimation of SS and IPB by HPLC. Most of the reported methods for their quantification depicted limitations (Jyothi *et al*., 2012; Kasawar and Farooqui, 2010; Nagaraju and Appaji, 2014; Vankudoth *et al*., 2013).

These bronchodilators are usually available in the market, either as pressured metered-dose inhalers, dry powder inhalers, soft mist inhalers, or as nebulization solutions. Challenges experienced by incorrect inhaler device usage bringing about befuddled COPD patients prompts suboptimal drug administration, thus rendering poor adherence to medical care (Braido *et al*., 2016). The alarming rise in mortality and morbidity is caused by the progressive nonreversible disease COPD and the potential drawbacks of the inhaler therapy used in the management of COPD; the economic burden experienced by patients due to hospitalization and exacerbation (Kallaru *et al*., 2015) happening due to nonadherence of therapy can be resolved by application of the novel drug delivery systems, such as the transdermal drug delivery system which offers numerous advantages, such as increased bioavailability, decreased frequency of dosing, capacity to entrap both hydrophilic and lipophilic compounds, reduced toxicity, and easy termination of medication by removal of the formulation (Ali *et al*., 2015).

Precise evaluation of the drugs trapped in the matrix is vital, since it is the integral factor that can be related to its therapeutic effectiveness. Hence, we felt the requirement to develop a validated analytical method for the estimation of SS and IPB and to quantify the *in-vitro* drug release from the in-house manufactured transdermal patch.

EXPERIMENTAL STUDY

Materials

HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Mumbai, India), IPB (CAS No-18559-94-9) was acquired from Vamsi Laboratories, Sholapur, India, and SS (CAS No-60205-81-4) was gifted by Elegant Drugs, India, and was used as supplied. Evonik Degussa India Pvt. Ltd., Mumbai, was benevolent enough to gift samples of Eudragit Polymer Eudragit RL 100 (RLPO) and Eudragit RSPO. The plasticizer triethyl citrate and mercury were bought from S.D. Fine Chemicals Ltd., Mumbai. Different reagents used were of analytical quality.

METHODS

Instrumentation

The chromatographic framework comprised the Shimadzu Prominence HPLC system (Kyoto, Japan) equipped with a pump (LC-20AD), photo diode array detector (SPD-M20A), an online degasser (DGU-20A5), an autosampler (SIL-20AC HT), a rheodyne injection valve with a 20-µl loop and a column oven (CTO-10ASVP). Data acquisition was rendered using the Shimadzu LC software solution (version 1.25).

Chromatographic conditions

A Luna C18(2) column (150 × 4.6 mm i.d., 5-µm particle size; Phenomenex, CA, USA) fitted with a security guard column (C18 ODS; 4 × 3.0 mm i.d.; Phenomenex, CA, USA) was utilized for the chromatographic separation of SS and IPB. The mobile phase injected at a flow rate of 1 ml/minute involved a blend of acetonitrile:phosphate buffer (pH adjusted to 3.0 ± 0.5 with o-phosphoric acid) in 30:70 v/v ratio. The column temperature was 30°C and the detection wavelength was 212 nm. The mobile phase was filtered and degassed prior to use. The chromatographic run injecting 10-µl volume was stopped at the end of 6 minutes. Before the injection of standards and samples, the column was equilibrized with the mobile phase for 60 minutes. Stock solutions of 1,000 µg/ml of SS and IPB were prepared using the mobile phase and necessary dilutions were made to acquire concentrations of 2–12 µg/ml.

Validation studies

The developed HPLC method was validated in accordance with the guidelines of the International Council for Harmonization (ICH) (International Conference On Harmonization, 2005) for various parameters, such as the system suitability, precision, linearity, accuracy, specificity, robustness, limit of quantification (LOQ) and detection (LOD). System suitability tests were carried out by injecting six replicates of standard SS and IPB solutions and determining the tailing factor, number of theoretical plates, and percentage of relative standard deviation (%RSD) of retention time and peak area. Linearity was measured by using the least squares regression model to assess the slope, *y*-intercept, and correlation coefficient. The calibration curves were obtained from various SS and IPB concentrations ranging from 2 to 12 µg/ml and were tested in triplicates. Precision was evaluated by determining the repeatability of three different standard solutions for six times on the same day (intraday) and examining the same three standard arrangements three times on three successive days (interday). Accuracy was tested at three diverse concentration levels by spiking the pre-analyzed commercially available samples with known concentrations of SS and IPB and later deciding the %RSD. Specificity was studied by

checking the blank patches chromatograms amid *in-vitro* release to check for potential interference by the excipients added. The LOD and LOQ values were decided numerically based on the standard deviation of the response and the slope. They were expressed as LOQ=*3.3* $\sigma \div S$ and LOQ=*10*$\sigma \div S$. The robustness of the developed reverse phase high performance liquid chromatography (RP-HPLC) method was evaluated by deliberate changes to the optimized chromatographic conditions based on the %RSD values of the system suitability parameters, such as change of ± 0.2 ml/minutes, ± 2%, ±5°C, and ± 2 nm in the flow rate, mobile phase composition, column temperature, and wavelength, respectively.

Fabrication of patches

Four films coded F1, F2, F3, and F4 were prepared according to the composition stated in Table 6, by dissolving the drugs to the previously prepared methanolic polymeric solution comprising plasticizer, sonicated to remove entrapped air bubbles, and the final mixture was made to 10 ml. Drug-loaded polymer solutions were casted onto a flat-bottomed Anumbra petri dish using mercury as a substrate and dried overnight at room temperature (Parhi and Suresh, 2016).

Formulation considerations

The total dose (Eriksen and Robinson, 2006) for once-daily controlled-release formulation of SS and IPB was calculated using the available pharmacokinetic data as per Robinson Eriksen's equation. The loading dose for incorporation into the formulation was calculated considering the radius 4.4 cm of the Anumbra molds (Parhi and Suresh, 2016).

In-mano evaluation

The prepared films were graded either as transparent or translucent based on the opacity of the film, their texture was given two grades (smooth/rough), and finally, the nature of the film was noted as either flexible or brittle. The patches which could withstand *in-mano* stress to maintain its structural integrity and the film which could be rolled and folded were termed as tensile and pliable, respectively (Ham *et al*., 2013). A study of tack properties enabled us to understand the adhesive nature of the film. It was studied a week after the preparation of the transdermal patches, by pressing the thumb lightly on a patch for about 5 seconds and then quickly withdrawing. The folding endurance was measured manually by subjecting a small strip of the film (2 cm × 2 cm) to repeated folds in the same place. The number of times the film was folded without developing visible cracks or breaking was noted (Shailesh *et al*., 2011).

Surface pH

The surface pH of the patches was determined by Bottenberg et al.'s method and reported in Table 6. The surface pH of a 1-cm^2 patch, previously soaked in 0.5 ml double-distilled water for 1 hour, was noted by bringing a glass electrode close to the surface of the patch and permitting it to equilibrate for 1 minute (Bharkatiya *et al*., 2010).

Drug content

Patches of 1-cm^2 area, placed in tubes containing 10-ml mobile phase, were shaken with the help of a rotary flask shaker for 24 hours at room temperature, till the complete dissolution of the drugs. After prior filtration, 0.5 ml drug aliquots was pipetted out into vials containing 1.5 ml of mobile phase, and each film formulation was tested in triplicate and the results are expressed as the mean and standard deviation in Table 6 (Vijaya *et al*., 2012).

In-vitro drug release studies

A Franz diffusion system having six vertical 12.5-ml capacity receptor cells with an effective diffusion area of 1.77 cm^2 and a PermeGear diffusion system (Perme Gear, Inc. Hellertown, PA) filled with phosphate buffer pH 7.4 were used as the diffusion medium. A magnetic stirrer bar of 10 × 2.5 mm served for the continuously stirring the receptor solution maintained at 37°C ± 0.5°C. The dialysis membrane of pore size 0.45 μ, soaked in phosphate buffer overnight, was mounted between the donor and receptor compartments so that it just touched the receptor fluid's surface. The prepared transdermal film was placed on the dialysis membrane and covered with aluminum foil, 0.5 ml sample aliquots diluted to 2 ml was withdrawn every 2 hours (for 24 hours), and the released drug was quantified by detecting using the HPLC at 212 nm. The drug release rate is estimated and shown in Figure 3 and 4.

Analysis of commercial formulations

The application of the method in estimating drug content from marketed formulation was tested by appropriate dilution of capsules and the results are shown in Table 4. The typical chromatograms of a pure, commercial formulation and *in-vitro* release pattern are shown in Figure 2.

RESULTS AND DISCUSSION

Analytical method development

Absence of conventional method

The concern in evaluating the stated drugs simultaneously by the simple analytical technique UV–visible spectrophotometry was confirmed by the trials of the simultaneous equation method and the derivative spectroscopic method. The low absorbing nature of IPB and almost similar zero-crossing points made their simultaneous estimation tedious. Subsequently, our aim was to build another method of analysis for the estimation of these drugs.

Optimization of chromatographic conditions

The RP-HPLC method was optimized by changing different parameters, such as mobile phase composition, flow rate, detection wavelength, and column oven temperature, with the true objective to acquire sharp and symmetrical peaks with excellent baseline separation. The Luna C18(2) column (150 × 4.6 mm i.d., 5 µm) was used as the stationary phase and the detection wavelength was fixed at 212 nm due to enhanced sensitivity. No drug elution was observed during the trials comprising methanol–water, methanol–acetonitrile, methanol–potassium and dihydrogen phosphate buffer in various proportions, but when methanol is replaced by acetonitrile, peaks with shorter retention time were eluted. Therefore, further trials were carried out by altering the proportions of acetonitrile and buffer. The ACN:PBS (30:70 v/v) ratio was finalized and gave well-resolved peaks with no significant improvement in peak shape by additional

trials. Mobile phase was also considered as a diluent to avoid disturbances in the baseline. The temperature was set at 30°C and the flow rate at 1.0 ml/minute, thus resulting in a well-resolved peak with better shape, shorter retention time, and lesser tailing factor.

METHOD VALIDATION

Analytical method validation is carried out to guarantee that the developed method is reliable and adequate for its intended purpose. The proposed method was validated in accordance with the ICH guidelines by reviewing parameters of system suitability, specificity, linearity, accuracy, precision, LOD, LOQ, and robustness.

System suitability

System suitability test guarantees that the set chromatographic conditions ensure proper separation, and the parameters are abridged and presented in Table 1. The high number of theoretical plates (n > 2,000) exhibited good column efficiency and the tailing factor values (T<2) indicated good peak symmetry. The %RSD of the retention time and peak area was within 1%, indicating the chromatographic setup's reproducibility and repeatability (%RSD<1). The resolution (Rs) of 4.8 between SS and IPB indicated a high degree of peak separation (Rs>2.0). All tested parameters for further validation and sample analysis were within acceptable limits, suggesting the suitability of the chromatographic arrangement.

Specificity

The retention times of SS and IPB were 2.4 and 3.8 minutes, respectively. No peaks were eluted when the mobile phase alone or blank patches were exposed to similar experimental conditions as that of drug-loaded films, while the drug-loaded patches and the commercial formulations showed distinct peaks as that of pure drugs, thus indicating no interference from the formulation ingredients. Chromatograms depicting specificity is shown in Figure 1.

Linearity, detection, and quantification limits

The calibration curves of SS and IPB were linear over the tested concentration range of 2–12 µg/ml and the coefficient of correlation for both bronchodilators was higher than 0.999, proposing an excellent correlation and good linearity for the method developed is presented in Table 1. The LOD values were found to be 0.42 µg/ml for SS and 0.44 µg/ml for IPB, while the LOQ values were found to be 1.26 µg/ml for SS and 1.34 µg/ml for IPB, indicating a sufficiently sensitive method to determine the drug even from low permeable matrix systems.

Precision

Intraday and interday precision results are expressed in %RSD in Table 2 and in both cases were found to be below 2%, demonstrating that the developed method is precise.

Accuracy

Table 3 summarizes the mean %recoveries, indicating the low variability and closeness between experimental and true values.

Robustness

Table 5 depicts the robustness data with %RSD values (<1) and system suitability parameters (theoretical plate, n > 2,000; tailing factor, T<2). The parameter tested was found to be satisfactory, and hence confirms that the developed method is robust.

Characterization of transdermal systems and applicability of HPLC method

Consideration of formulation parameters

SS of 5 mg and IPB of 1 mg per patch were found as the optimum dose. Our aim was to prepare an 8-cm^2 patch while the area of the anumbra plate was found to be 60.7904 cm^2. Hence, the amount of the drug required was approximately 40 mg of SS and 8 mg of IPB.

Table 1. System suitability parameters of SS and ipratropium bromide (8 µg/ml).

Parameters	SS	Ipratropium Bromide	Acceptance criteria
Retention time (t_R, minute)	2.4	3.8	–
%RSD of retention Time	0.12	0.13	< 1 for $n \geq 5$
Peak area	124,264	63,606	–
%RSD of peak area	0.26	0.53	< 1 for $n \geq 5$
Theoretical plates (N)	4,264.48	9,569.40	> 2,000
Tailing factor (T)	1.39	1.27	≤ 2.0
Resolution (Rs)	–	4.837	NLT 2.5
Regression equation	y = 15,770.75x−1,158.12	y = 8,029.595x−921.333	–
Limit of detection µg/ml	0.42	0.44	–
Limit of quantitation µg/ml	1.26	1.34	–
Calibration range µg/ml	02–12	02–12	–
Correlation coefficient (r^2)	0.999	0.999	–

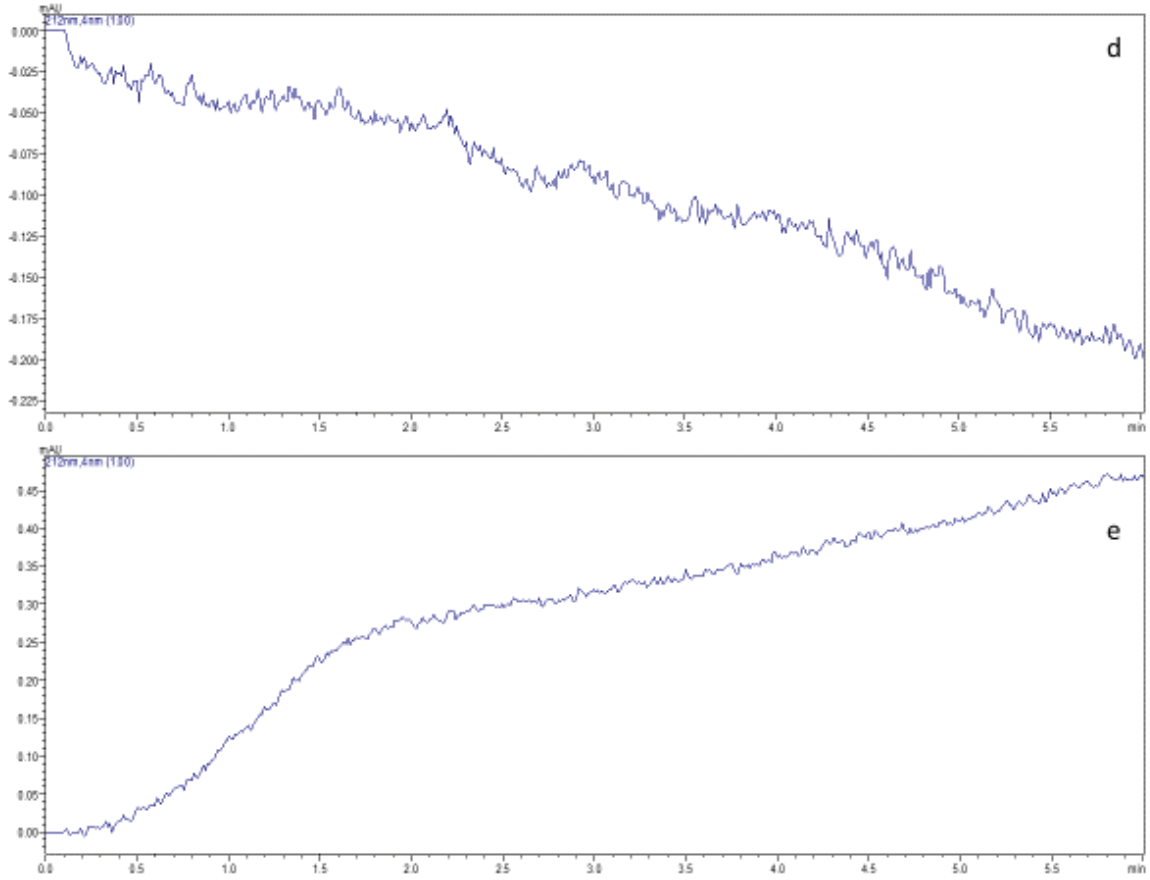

Figure 1. Typical chromatogram showing blank mobile phase (d) and release from the blank patch (e).

Table 2. Precision data of SS and ipratropium bromide.

Analyte			Intraday(n = 6)		Interday (n = 3)					
					Day 1		Day 2		Day 3	
			PA	%RSD	PA	%RSD	PA	%RSD	PA	%RSD
SS	Conc. (µg/ml)	2	30,968	0.255955	31,604	0.218396	31,245.67	1.829866	31,009.67	0.319765
		6	94,398	0.554327	93,926	0.420062	94,117	0.718306	94,220.67	0.745611
		12	19,1275.3	0.267783	19,1470.7	0.564672	19,2074.7	0.712153	19,1177.7	0.225127
Ipratropium bromide	Conc. (µg/ml)	2	14,700.67	0.141298	14,748	1.870703	14,735.33	0.955133	14,697.33	0.184628
		6	47,156	0.187892	46,126	0.147613	46,045.67	0.235777	47,164.33	0.226473
		12	96,716.33	0.317762	97,859.67	1.844633	96,199.67	0.603476	96,586.33	0.445445

RSD = relative standard deviation; n = number of replicates.

Table 3. Recovery studies of SS and ipratropium bromide.

Recovery study		Compound contents (µg/ml)	Quantity added (µg)	Theoretical amount (µg)	Recovered amount (µg)	[a]Recovery (%)
SS	50%	5	2.5	7.5	7.85	103.62 ± 1.74
	100%	5	5	10	10.53	100.41 ± 1.42
	150%	5	7.5	12.5	12.52	99.3 ± 1.31
Ipratropium Bromide	50%	2	1	3	3.14	96.02 ± 1.05
	100%	2	2	4	4.10	99.70 ± 1.34
	150%	2	3	5	5.25	99.13 ± 1.42

[a]n = 3.

In-mano evaluation

All formulations appeared to be as transparent, flexible, tensile, pliable, and have a smooth texture. All the stated characteristics were more vivid with the increasing content of RL. Hence, the developed films are said to possess the prerequisites for an optimized patch. The tackiness of the films was in the following order F1>F2>F3>F4. This pattern can be correlated with the direct proportionality between tackiness and RS content. The effect of the plasticizer, polymer, and solvent is ruled out, since these factors were unaltered in these formulations. The films exhibited no signs of folds even after 100 folds, specifying the ability of the films to be unaffected when it experiences the natural fold of the skin.

Table 4. Assay of commercial formulation containing SS and ipratropium bromide.

Commercial formulation	% Purity[a]
SS	102.25 ± 0.17
IPB	104.2 ± 1.56

[a]*n* = 3.

Surface pH

The surface pH of all the formulations is presented in Table 6, and the results indicate the compatibility of the prepared patch with the skin.

Drug content

Drug content analysis was carried out for all the prepared transdermal systems and the results displayed in Table 6 show good uniformity and a higher degree of reproducibility with a low standard deviation. The drug content from the marketed formulation is presented in Table 4.

In-vitro drug release studies

The developed RP-HPLC method was also extended to quantify the drug release pattern of the *in-house* manufactured patches and the *in-vitro* release profile of SS and IPB from formulations is graphically shown in Figures 3 and 4, respectively.

Table 5. Robustness data of salbutamol sulphate and ipratropium bromide.

Parameters	Modification	Observations							
		%RSD of retention time		%RSD of peak area		Theoretical plate(*N*)		Tailing factor (*T*)	
		SB	IPB	SB	IPB	SB	IPB	SB	IPB
Flow rate ml/minute	0.8	0.153744	0.533008	0.10548	0.13263	3,885.746	11,489.77	1.105667	1.239333
	1.0 optimized	0.116279	0.127666	0.257016	0.530828	3,996.13	9,569.404	1.3905	1.266833
	1.2	0.225168	0.11759	0.178951	0.112505	3,239.593	8,379.211	1.3	1.27675
Column temperature °C	35	0.073887	0.156074	0.138592	0.107405	3,610.267	10,095.05	1.284	1.26625
	30 optimized	0.116279	0.127666	0.257016	0.530828	3,996.13	9,569.404	1.3905	1.266833
	25	0.11972	0.065159	0.082622	0.104448	4,026.188	9,645.283	1.44025	1.2575
Mobile phase composition (ACN:Buffer)	28:72	0.10427	0.114792	0.134354	0.104293	3,633.512	9,947.064	1.15525	1.231
	30:70 optimized	0.116279	0.127666	0.257016	0.530828	3,996.13	9,569.404	1.3905	1.266833
	32:68	0.053491	0.027318	0.286341	0.352809	4,130.206	9,604.47	1.4385	1.27225
Wavelength nm	210	0.297142	0.314428	0.62525	0.890202	3,771.245	8,705.013	1.245667	1.282333
	212 optimized	0.116279	0.127666	0.257016	0.530828	3,996.13	9,569.404	1.3905	1.266833
	214	0.246143	0.325508	0.703474	0.28485	4,128.632	8,647.875	1.255667	1.277333

Table 6. Evaluation of the films incorporating SS and ipratropium bromide

F.Code	SS	IPB	RL	RS	Plasticizer	Solvent	Surface pH	Drug content	
								SS	IPB
F1	40	8	1	4	TEC	Methanol	6.58 ± 0.18	11.88 ± 0.42	2.18 ± 0.13
F2	″	″	2	3	″	″	6.80 ± 0.14	11.78 ± 0.48	2.23 ± 0.17
F3	″	″	3	2	″	″	6.50 ± 0.20	11.65 ± 0.17	2.26 ± 0.20
F4	″	″	4	1	″	″	6.67 ± 0.15	11.95 ± 0.40	2.26 ± 0.17

*Salbutamol Sulfate (SS) and Ipratropium Bromide (IPB) in mg; Eudragit RLPO (RL); Eudragit RSPO (RS); Triethyl citrate (TEC).

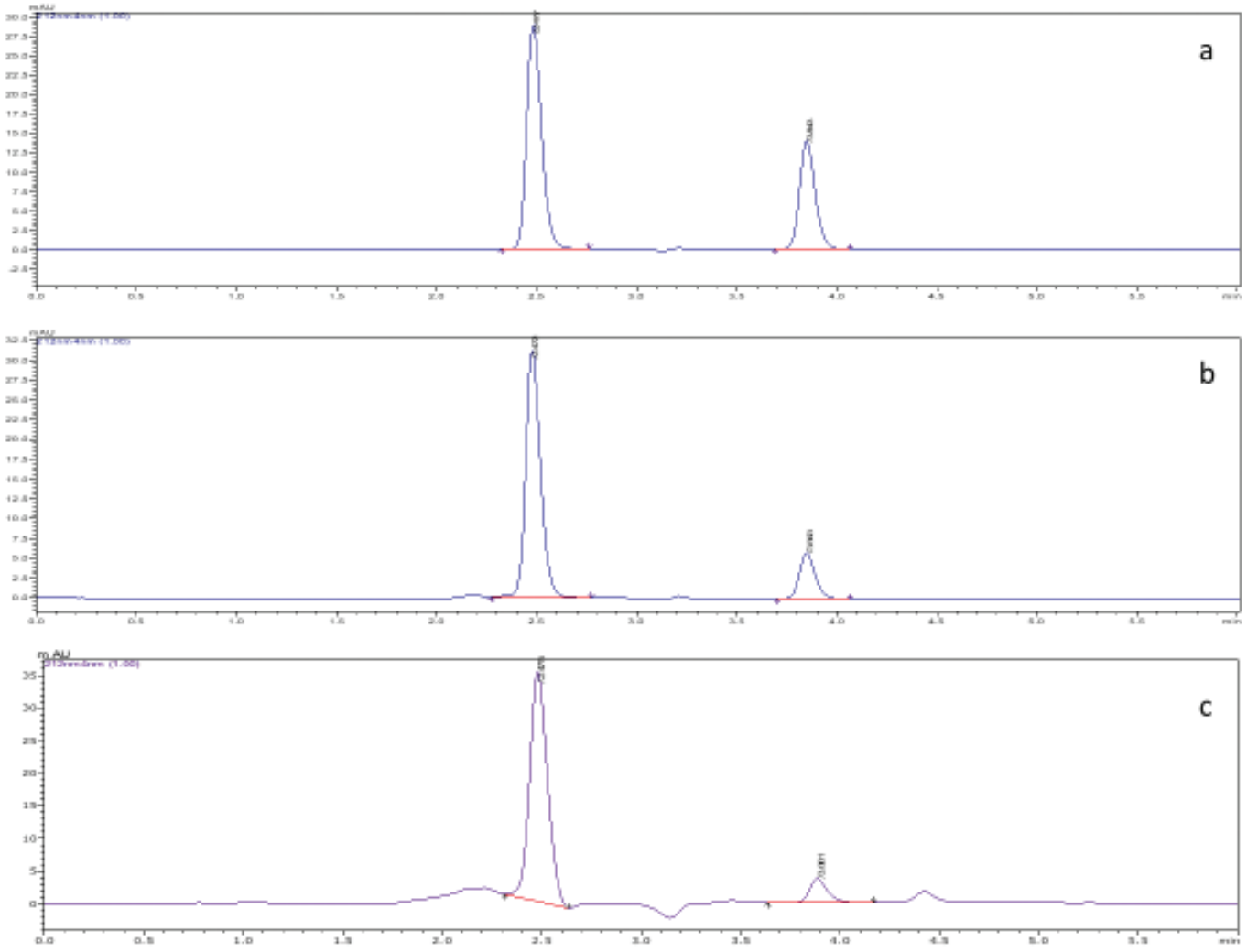

Figure 2. Typical chromatogram showing the peaks of salbuatmol sulfate and ipratropium bromide. (a: pure drugs; b: commercial samples c: release from patch).

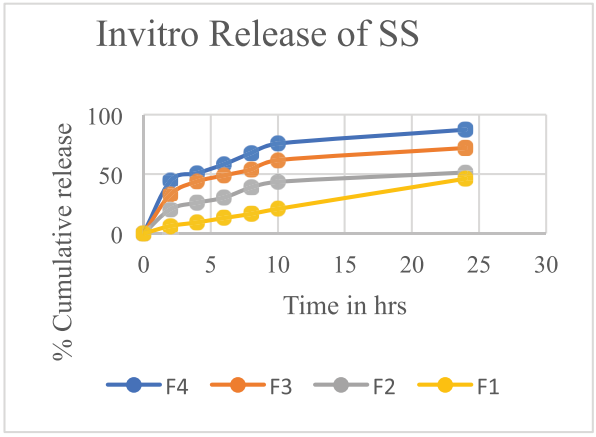

Figure 3. Graph depicting the *in vitro* release studies of salbutamol sulphate from formulations and F1–F4 transdermal patches using a dialysis membrane.

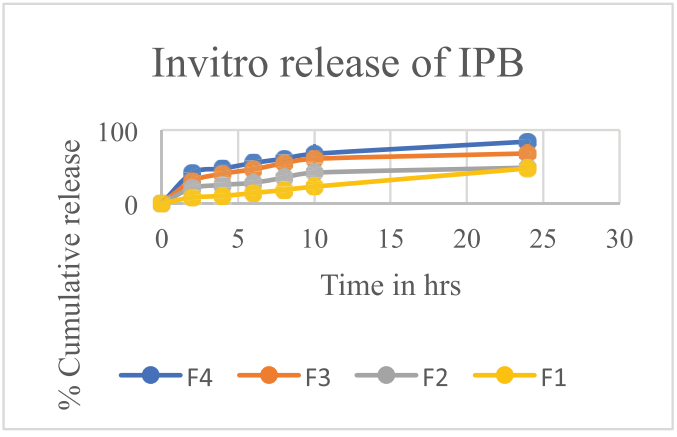

Figure 4. Graph depicting the *in-vitro* release studies of ipratropium bromide from formulations and F1–F4 transdermal patches using a dialysis membrane.

CONCLUSION

In conclusion, the strategically developed, ICH-validated RP-HPLC method for successful quantification of SS and IPB content in pure and marketed formulation is hereby stated. The validation parameters are in accordance with the ICH guidelines. This method adequately helps to quantify drugs released from in-house manufactured transdermal patches at regular intervals and was found to be more economical, namely factors showing decreased elution time (6 minutes), flow rate (1 ml/minute), and consumption of costly solvents.

ACKNOWLEDGMENTS

The authors acknowledge KAHER, Belagavi, for funding the project and providing all research facilities. The authors appreciatively acknowledge Elegant Drugs, Evonik Degussa, India, for providing the gift samples of SS and polymers RLPO and RSPO, respectively. Support extended by Dr. Malleswara Rao Peram and Mr. Satveer Jagwani is also acknowledged.

CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

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How to cite this article:
Raj SM, Jamakandi VG, Jalalpure SS, Ronad PM. Simultaneous detection and quantification of bronchodilators in pure form and from *in-vitro* drug release of a novel combinational formulation. J Appl Pharm Sci, 2020; 10(08):131–138.