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Development and validation of RP HPLC PDA method for the simultaneous estimation of salbutamol sulphate and ambroxol hydrochloride in pharmaceutical dosage forms

V. SriKalyani, D. Meena Bharathi, M. Anusha, B. Chandra Priyanka and Buchi N. Nalluri*

Department of Pharmaceutical Analysis, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, AP, India

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ABSTRACT

A simple, specific and accurate reverse phase liquid chromatographic method was developed for the simultaneous estimation of Salbutamol sulphate (SAL) and Ambroxol hydrochloride (AMB) in bulk and pharmaceutical dosage forms. A Phenomenex C_{18} column (250 x 4.6mm; 5 µm) with mobile phase containing 15mM ammonium acetate: acetonitrile (16:84% v/v) was used at isocratic mode and eluents were monitored at 227nm. The retention times of SAL and AMB were 3.1 min and 4.5 min respectively and showed a good linearity in the concentration range of 4-20µg/mL for SAL and 30-150µg/mL for AMB with a corrɛlation coefficient (R) of 0.999 and 0.999. The percentage assays were found to be 99.36 and 100.81 respectively for SAL and AMB. The proposed method was validated as per ICH guidelines and successfully applied for the simultaneous estimation of SAL and AMB in bulk and dosage forms.

Keywords: Salbutamol Suphate, Ambroxol hydrochloride, Simultaneous estimation, Phenomenex C_{18} column, PDA detection, Validation.

INTRODUCTION

Salbutamol sulphate (SAL) is chemically bis [(1RS)-2-[(1, 1-dimethylethyl) amino]-1-[4-hydroxy-3-(hydroxymethyl) phenyl] ethanol] sulphate, is a β2-adrenergic receptor agonist used for the relief of broncho-spasm in conditions such as asthma and chronic obstructive pulmonary disease [1-5]. Ambroxol hydrochloride (AMB) is chemically 1 ({[2– Amino – 3, 5 dibromo phenyl] –methyl} amino) cyclohexanol monohydrochloride and it is a mucolytic agent. It stimulates mucociliary action and liquefies the mucous and clears the air passages in the respiratory tract [6-9]. Combination of SAL and AMB is available in India and is used for the treatment of asthma and bronchitis.

Literature survey reveals that SAL and AMB alone or in combination with other drugs were analysed by RP-HPLC [10-16], LC-MS [17], UV spectrophotometric [18-20] and TLC [21] methods. Only two RP-HPLC methods were reported for the simultaneous estimation of SAL and AMB in combination using potassium phosphate buffer, which is not LC-MS compatible. Hence, the main objective of the present investigation was aimed at developing a validated sensitive and rapid RP-HPLC-PDA method for the simultaneous estimation of SAL and AMB in bulk and dosage forms with a mobile phase that is compatible with LC-MS analysis.

EXPERIMENTAL SECTION

Reagents and Chemicals

SAL and AMB were gift samples from Darwin Laboratories, India. Acetonitrile, water and formic acid were purchased from E. Merck, Mumbai, India. All the solvents and reagents were of HPLC grade. SALMUCOLITE®, (Manufactured by Cheminnova Remedies Pvt Ltd, Hyderabad) a tablet containing SAL (2mg), and AMB (30mg) was commercially purchased.

Equipment

A Shimadzu Prominence HPLC system provided with DGU-20A3 degasser, LC-20AD binary pumps, SIL-20AHT auto sampler, and SPD-M20A PDA detector. Data acquisition was carried out using LC solutions software. The chromatographic analysis was performed on Phenomenex C_{18} RP column (250 × 4.6mm; 5µm).

Chromatographic Conditions

Mobile phase consisting of 15mM ammonium acetate: acetonitrile (16:84% v/v) was used in isocratic mode and the mobile phase was filtered through nylon disc filter of 0.45µm (Millipore) and sonicated for 3 min before use. The flow rate was maintained at 1 mL/min with an injection volume of 20µL. Eluents were monitored at 227 nm and the separation was achieved at ambient temperature.

Preparation of Stock and Standard Solutions

The stock solutions of SAL and AMB (1mg/mL) were prepared by dissolving 10 mg of each drug separately in a 10mL volumetric flask using methanol as diluent. The working standard solutions of concentration ranging from 4-20 µg/mL of SAL and 30-150 µg/mL of AMB were prepared by appropriately diluting the stock solutions with acetonitrile as diluent.

METHOD VALIDATION

The method was validated according to the ICH guidelines [22].

Linearity

A linear relationship was evaluated across the range of the analytical procedure with a minimum of five concentrations. A series of standard solutions of SAL and AMB were prepared over a concentration range of 4-20µg/mL (4, 8, 12, 16, 20 µg/mL) and 30-150µg/mL (30, 60, 90, 120& 150µg/mL) respectively from stock solutions and injected in triplicate. Linearity was evaluated by a plot of peak areas as a function of analyte concentration, and the test results were evaluated by appropriate statistical methods where by slope, intercept, and regression (R^2) correlation coefficients (R) were calculated.

Precision

Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Precision was measured in terms of repeatability of application. Repeatability of standard application was assessed by using a minimum of six determinations at 100% of the test concentration (4µg/mL of SAL and 60 µg/mL of AMB). The standard deviation and relative standard deviation (RSD) were reported for precision. Less than 2% RSD for peak areas indicates the developed method was precised and the data was presented in Table-1.

Accuracy

Accuracy was established across the specified range of the analytical procedure. Accuracy (recovery) of the method was tested by spiking 80, 100 and 120% of SAL (4µg/mL) and AMB (60µg/mL) standard concentrations. The accuracy of the analytical method was analysed in triplicate. The % recovery and the % RSD were calculated at each level of addition.

Robustness

Robustness of the method was determined by altering the experimental conditions such as flow rate and wavelength intentionally. The chromatographic parameters viz., capacity factor, tailing factor, theoretical plate number and % assay were recorded. The flow rate was maintained at 1.0mL/min. To study the effect of flow rate, the flow rate was changed by ±20% and the effect of wavelength was studied by changing wavelength by ±1nm.

Specificity

Specificity is a measure of the degree of interference in the analysis of the complex sample mixtures such as analyte mixed with the formulation excipients or the known impurities. Specificity of the method was carried out by

comparing chromatogram of the placebo (in house made) with that of the sample for checking any interference peaks.

Limit of Detection (LOD) and Limit of Quantification (LOQ)
LOD and LOQ were determined by calibration curve method. Standard solutions of SAL and AMB were prepared in the concentration range of 4-20µg/mL for SAL and 30-150µg/mL for AMB and injected (20µL) in triplicate. Average peak area of SAL and AMB was plotted against concentration. LOD and LOQ were calculated by using following equations: LOD = (3.3 ×σ)/m; LOQ= (10.0×σ)/m (where, σ is the standard deviation of the responses and m is mean of the slopes of the calibration curves).

Assay
Twenty tablets were weighed individually and finely powdered and the powder blend equivalent to 2mg of SAL and 30mg of AMB was accurately weighed and transferred to a 10 mL volumetric flask. 5 mL of methanol was added to solubilize and was sonicated for 5 min . Volume was made up to the mark with diluent. The above solution was filtered using Nylon disposable Syringe Filter (13 mm, 0.45 µm). Aliquots of the filtrate were diluted using acetonitrile and analysed in triplicate. The amount present in the each tablet was calculated by comparing the area of standard SAL and AMB with that of the tablet sample.

RESULTS AND DISCUSSION

The present investigation was carried out with a view to develop a RP HPLC PDA method for the simultaneous estimation of SAL and AMB in bulk and dosage forms. Initial trials were carried out on Phenomenex C_{18} column (250×4.6 mm;5µm) using 15mM ammonium acetate (pH 6.5) and acetonitrile (40:60% v/v) at a flow rate of 1.0mL/min as mobile phase and acetonitrile as the diluent. The quantification was carried out at 227nm. Under these conditions SAL was eluted at 2.79 min and AMB at 13.72 min. The SAL was almost eluted with the solvent front.
In the other trial, mobile phase ratio was changed to 30:70% v/v of 15mM ammonium acetate (pH 6.5) and acetonitrile at a flow rate of 1.0mL/min and under these conditions, SAL was eluted at 2.64 min and AMB at 5.40 min. However, there is no proper resolution between the solvent front and the SAL peak. In further trials, the organic phase was increased to 85% v/v and under these conditions the SAL was eluted at 2.89 min and AMB at a 4.77 min respectively.

Finally, the mobile phase was maintained at a ratio of 16:84% v/v of 15mM ammonium acetate and acetonitrile at a flow rate of 1.0mL/min in order to achieve proper resolution of both the SAL and AMB peaks respectively. Under these conditions both the SAL and AMB peaks were eluted at 3.14 min and 4.55 min respectively. Peaks were symmetrical and tailing factor was within the limits. For quantitative analytical purpose, wavelength was set at 227 nm which provides better reproducibility without interference. The peak purity indices were also found to be greater than 0.9999 and this indicates peak purity of the both the drugs SAL and AMB used in the analysis. A sample chromatogram of SAL and AMB and peak purity profiles were given in Figure 1 along with UV spectra.

METHOD VALIDATION
The method has been validated as per ICH-Guidelines for following parameters:

Linearity
A linear relationship was evaluated across a concentration range of 4-20µg/mL for SAL and 30-150mg/mL for AMB in triplicate. The concentration range was selected based on 80-120% of the test concentration. Peak area and concentrations were subjected to least square regression analysis. The regression coefficient (R^2) was found to be 0.997 and 0.999 and shows good linearity in the concentration ranges selected. The data of the calibration curve was given in Table 1 and chromatograms were shown in Figure 2.

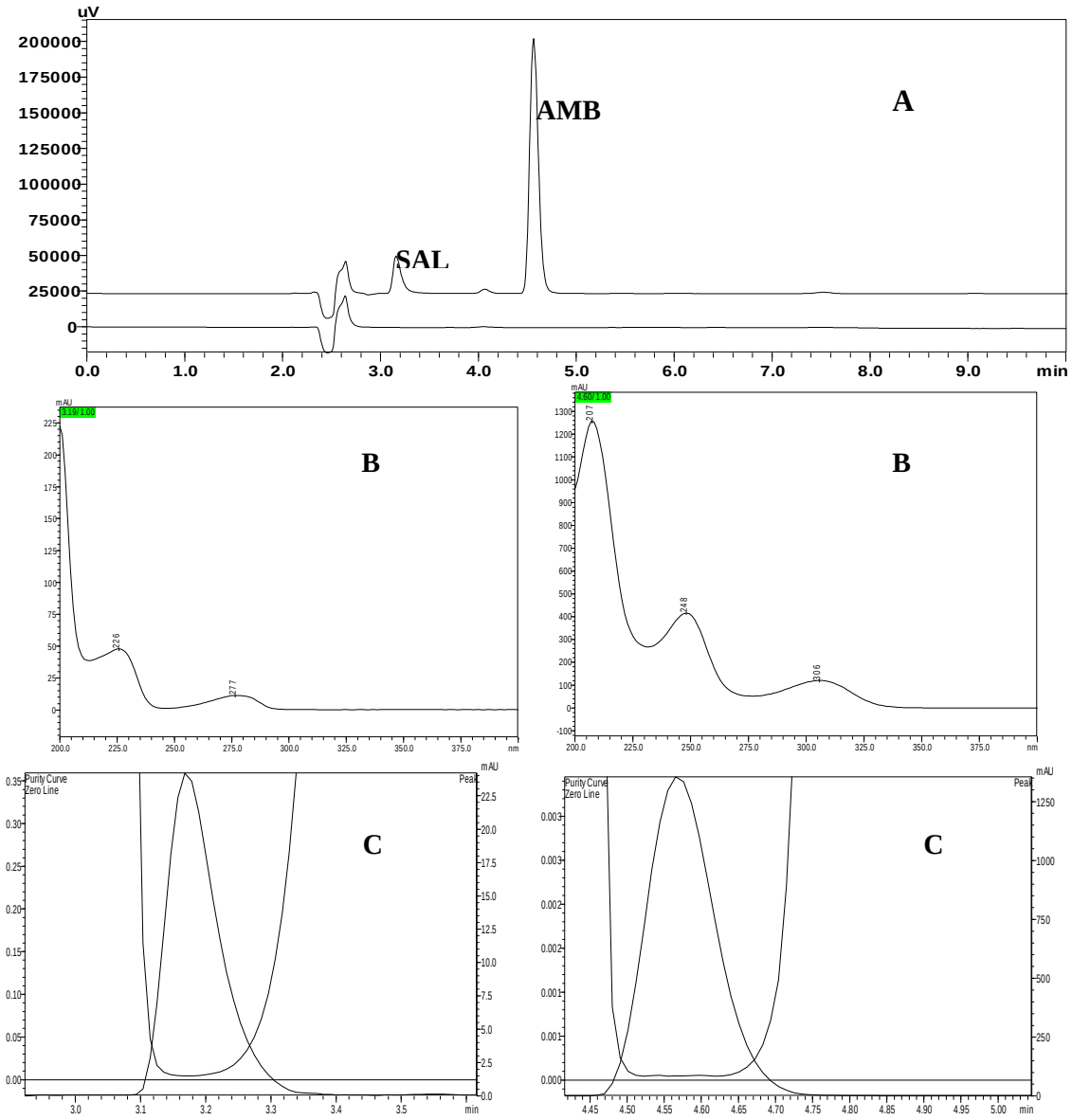

Fig. 1 Standard Chromatogram of SAL (4µg/mL) and AMB (30µg/mL) mixture (A); Peak purity curves of SAL and AMB (B) and (C) - UV spectra

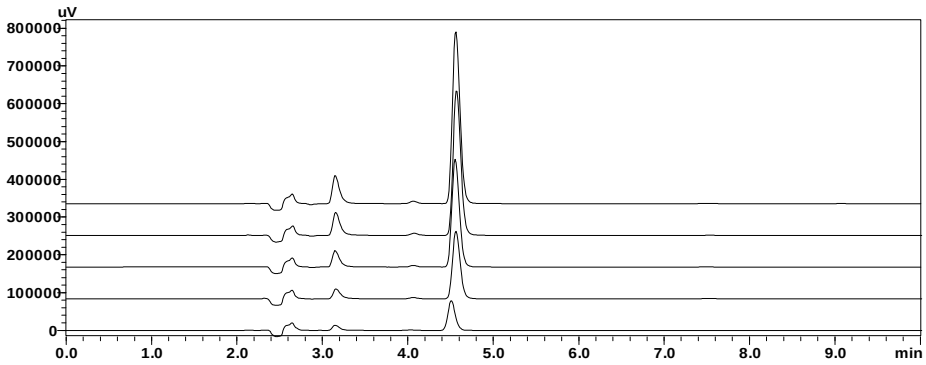

Fig. 2 Overlay of SAL (4-20µg/mL) and AMB (30-150µg/mL) standard chromatogram

System Precision

Precision studies were carried out in terms of repeatability. Six replicates of standard concentration (4µg/mL of SAL and 60µg/mL of AMB) was evaluated and the data given in Table 1. The % RSD was found to be below 2.

Method Precision

Repeatability was carried using six replicates of sample preparations from the homogenous blend of marketed formulation at a concentration of 4µg/mL of SAL and 60µg/mL of AMB. The data was given in Table 1. The % RSD was found to be below 2.

Accuracy

Accuracy of the method was examined by performing recovery studies using standard addition method by spiking the known quantities at 80, 100 & 120% of the standard concentrations. The analyte peak is evaluated by 3D plot of the chromatogram in order to confirm the existence of components at 3.15 min, and 4.54 min elution time of SAL and AMB in Figure 3. The percent recoveries were found to be 99.92-100.8 and 99.22-101.97 respectively for SAL and AMB. These results indicate a good accuracy of the method to that of the labelled claim. The obtained recovery results were given in Table 1.

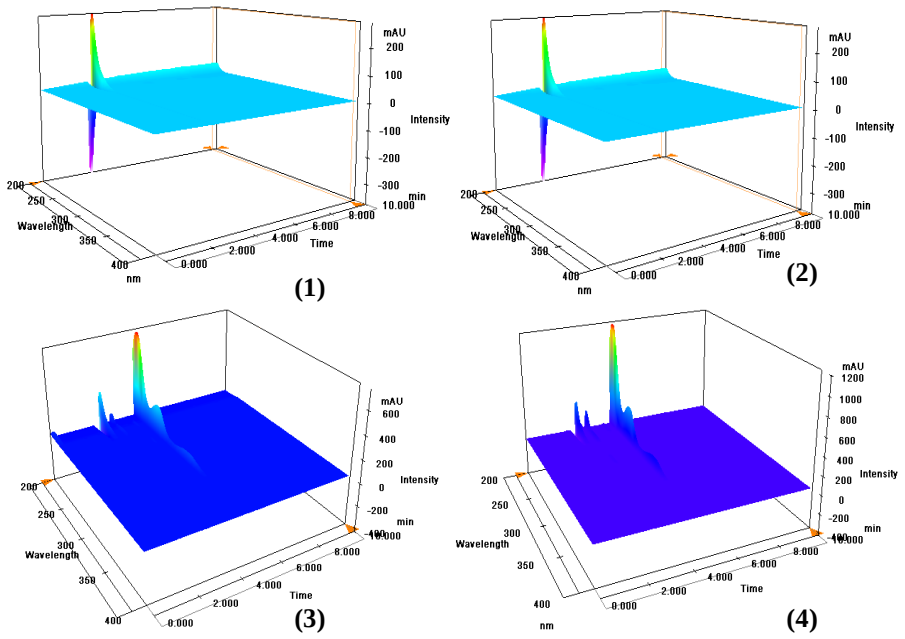

Fig 3. 3 D plots of Blank (1), Placebo (2), Sample (3), Standard (4) chromatograms

Table 1. Linearity, Accuracy and Precision data

Validation data of SAL and AMB			
	PARAMETERS	**SAL**	**AMB**
Linearity (n=3)	**Concentration**	4-20 µg/mL	30-150 µg/mL
	Regression equation	y=26574x-23132	y=21599x-17288
	Regression Coefficient(R^2)	0.997	0.998
	Correlation coefficient (R)	0.999	0.999
Accuracy (n=3)	**% Level of Addition**	Mean Percent Recovery(% RSD)	Mean Percent Recovery(%RSD)
	80	99.62 (0.33)	100.09 (0.68)
	100	100.25 (0.28)	100.33 (0.12)
	120	100.86 (0.07)	101.39 (0.29)
Precision (n=6)		**SAL**	**AMB**
System Precision	**Average peak area of the standard sample (%RSD)**	132389.8 (0.19)	1072544 (0.08)
Method Precision	**Average peak area of the assay sample (%RSD)**	88609 (0.13)	1161031 (0.22)

Specificity

The specificity of the method was established by injecting the solutions of diluent, placebo, standard and test sample (formulation) individually to examine any interference. From the overlay chromatogram shown in Figure 4, it can be inferred that there were no co-eluting peaks at the retention time SAL and AMB. These results show that peak of analyte was pure and the excipients in the formulation did not interfere with the analysis.

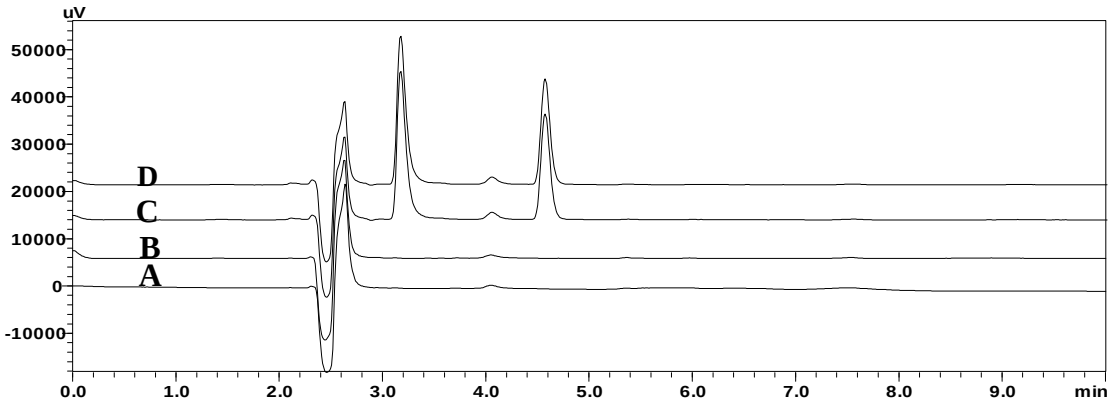

Fig 4. Overlay chromatogram of (A) Placebo, (B) Blank, (C) Sample, (D) Standard chromatograms

Robustness

As part of the robustness, deliberate changes in the flow rate and wavelength, were made to evaluate the impact on the method. Retention times were significantly changed with flow rate but no change was found due to change in wavelength, however % assay values, tailing factor, capacity factor and theoretical plate number were within limits and these results indicated minor changes in the flow rate and wavelength didn't affect the assay results. The data was given in Table 2.

Table 2. Robustness data

Robustness data relating to change in flow rate					
Drug	**Flow rate (mL/min)**	**Retention time (min)**	**Theoretical Plates**	**Tailing factor**	**% Assay**
SAL	0.8	3.93	6049.45	1.82	99.51
	1.0	3.16	5637.66	1.76	99.36
	1.2	2.62	5125.29	1.85	99.82
AMB	0.8	5.66	10939.82	1.31	100.62
	1.0	4.57	9864.03	1.27	100.81
	1.2	3.79	9049.91	1.33	100.98
Robustness data relating to wavelength change (nm)					
SAL	226	3.16	5636.48	1.76	99.32
	227	3.16	5637.66	1.76	99.36
	228	3.16	5644.36	1.75	99.39
AMB	226	4.57	9862.17	1.23	100.80
	227	4.57	9864.03	1.27	100.81
	228	4.57	9862.24	1.27	100.83

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated from the average slope and standard deviation from the calibration curve. LOD for SAL and AMB was found to be 0.021µg/mL and 0.034µg/mL respectively. LOQ for SAL and AMB was found to be 0.06µg/mL and 0.10µg/mL respectively. These results indicate that the method is sensitive enough to carry out the routine analysis for the simultaneous estimation of SAL and AMB in bulk and dosage forms.

System Suitability

System suitability studies were carried out by injecting 8µg/mL of SAL & 60µg/mL of AMB at injection volumes ranging from 10µL-50µL. The data was given in Table 3. With increment of injection volumes, the % RSD for tailing factor and theoretical plate number were calculated and was less than 2 and is satisfactory.

Table 3. System suitability parameters

Parameters	**SAL**(% RSD)	**AMB** (% RSD)
Retention Time (min)	3.15 (0.36)	4.54 (0.19)
Tailing Factor	1.7	1.20
Theoretical Plates (#)	5530.83 (0.74)	8703.35 (0.71)
Capacity factor (k)	2.184 (0.52)	3.708 (0.64)

Assay

The percentage assay values of SAL and AMB in the tablet was found to be 99.36 and 100.81 respectively. The results were found to be within the limits and the developed LC conditions can be used for the assay of SAL and AMB in different dosage forms.

Stability of the Stock Solution

The stability of the stock solution was determined by analyzing the samples under refrigeration (8±1°C) at different time intervals up to 48hrs. The % variation in assay values at different time intervals were found 0.31 for SAL and 0.064 for AMB from the initial zero time interval solution, thus indicating that the solutions were stable for a period of 48hrs when stored at 8±1°C.

CONCLUSION

The proposed RP-HPLC-PDA method was validated as per International Conference on Harmonisation (ICH) Guidelines, and found to be applicable for routine quality control analysis for the simultaneous estimation of SAL and AMB using isocratic mode of elution. The results of linearity, precision, accuracy and specificity, proved to be within the limits. The method provides selective and simultaneous quantification of SAL and AMB without interference from diluent and placebo. Overall, the proposed method is highly sensitive, reproducible, reliable, rapid and specific and also has the unique advantage of LC conditions being compatible with MS detection and hence can be successfully employed in the routine analysis for the simultaneous estimation of SAL and AMB in bulk and dosage forms.

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