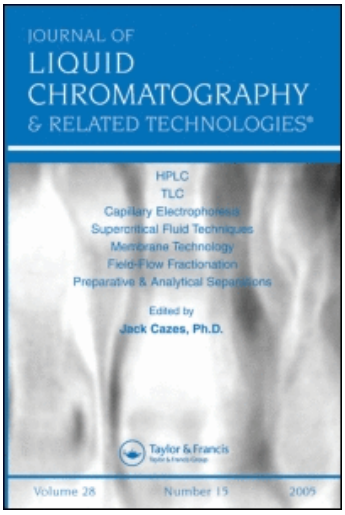

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SIMULTANEOUS HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ACETAMINOPHEN, SALICYLAMIDE, PHENYLTOLOXAMINE, AND RELATED PRODUCTS

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ABSTRACT

A stability indicating high performance liquid chromatography method has been developed for simultaneous determination of acetaminophen, salicylamide and phenyltoloxamine. The reversed-phase method utilizes UV detection at 220 nm and a C8 column. This paper presents the data to support linearity, precision, specificity, and robustness of the method. The known potential degradation products of acetaminophen, p-aminophenol, p-nitrophenol, precursor impurity p-hydroxyacetophenone, the potential degradation product of salicylamide, salicylic acid, and precursor impurity ethylsalicylate were separated for quantitation simultaneous with parent compounds. Quantification was achieved by peak area and external standard method. This method can be employed in determining stability, assay, content uniformity, and dissolution of the combination in pharmaceutical dosage forms.

INTRODUCTION

Antihistamine and analgesic combinations are used to relieve sneezing, runny nose, sinus, and nasal congestion (stuffy nose). Also, these combinations are used to treat fever, headache, and aches and pain associated with colds, influenza, and hay fever arthritis (rheumatism).[1] Several of these combination active ingredients are available on the market. One such product is the combination of acetaminophen, salicylamide, and phenyltoloxamine in the form of tablets and capsules.

Acetaminophen is widely used for its nonsalicylate, analgesic, and antipyretic actions. Salicylamide is used as an analgesic and antipyretic in several combination products. Several studies have indicated that the combination of acetaminophen and phenyltoloxamine have significant additive analgesic activities in several kinds of pains. These kinds of analgesic adjuvant effects have been confirmed by clinical studies.[2-4] Other studies have indicated that several antihistaminic agents, but not all, have direct analgesic effects.[5,6]

High performance liquid chromatographic (HPLC) methods for acetaminophen and salicylamide either as individual components or combination formulations have been reported.[7-16] Similarly, analysis of phenyltoloxamine has been reported individually and in combination with other pain relievers and antihistamines.[17] A method to analyze the combination of these three has not been reported to the best of our knowledge. A reliable, stability, and impurity indicating simultaneous HPLC method would be of significance to manufacturers and quality control departments. Stability and impurity indicating HPLC analysis is important in order to ensure the efficacy and safety of a drug substance or a dosage form. Drugs and dosage forms are inevitably contaminated by low concentrations of impurities. Data supporting the linearity, specificity, and limits of detection and quantification for acetaminophen, salicylamide, phenyltoloxamine citrate, and their potential degradation products and precursor impurities is presented in this study.

EXPERIMENTAL

Materials

All the reagents and solvents used in this study were of HPLC grade. Monobasic potassium phosphate, phosphoric acid, salicylic acid, ethyl salicylate, p-nitrophenol, p-aminophenol, and p-hydroxyacetophenone were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Acetaminophen, salicylamide, and phenyltoloxamine citrate were gifts from Seatrace Pharmaceuticals (Gadsden, AL). Deionized water was prepared using Fistreem II glass still (Barnstead, UK).

Equipment

Chromatographic analysis was performed using Varian LC instrument (Varian Associates, Walnut Creek, CA) equipped with Varian pump (Model 9010), autosampler (Model 9095) with a 100 µL loop, UV absorbance detector (Model 9050), and Dinamax MacIntegrator I (Rainin Instrument Co., Woburn, MA) data acquisition system. The detection AUFS was set at 0.01. DU series 60 Spectrophotometer (Beckman Instruments, Fullerton, CA) was used in specificity studies with control and degraded samples.

Method Development

Two different gradients (Method I & Method II) were developed. First one for assay and quantification of degradation products and the second one for assay. The appropriate method can be chosen, should the need arise. Mobile phase consisted of a mixture of three components, mobile phase A was 0.1 M phosphate buffer, adjusted to pH 2.7 with phosphoric acid. Mobile phase B was methanol and mobile phase C was acetonitrile. The mobile phases were filtered, degassed and pumped at 1 mL/min. Injection volume was 50 µL. The column used was a C8, 5µ (150 X 4.6 mm) prodigy column from Phenomenex (Torrance, CA).

Method I was developed for assay, content uniformity, and quantification of degradation products. In this method, the three components were maintained at a proportion of 81:15:4 during 0-12 min elution. The composition was programmed to reach a proportion of 72:12:16 and 43:14:43 by the end of 19 and 30 min respectively. The total run time was 35 min and equilibration time was 10 min.

Method II was developed for assay and content uniformity. In this method, a gradient set at 95:0:5 and was changed to 55:0:45 in 17 min and the equilibration time was 10 min. In the initial stages of developing method II, a change from 100 % buffer to 50 % buffer in 15 min was attempted. With this approach, the peak shape of acetaminophen was not good and the retention time of the peaks, particularly acetaminophen was not consistent. This problem was not seen when the starting mobile phase had 5 % acetonitrile. Use of 100 % water or buffer is not recommended as starting mobile phase because reverse-phase columns equilibrate poorly in these conditions.[18] The final mobile phase in the gradient was set at 45 % acetonitrile because at higher concentrations (>50 % acetonitrile) of organic phase, phosphate was precipitated out of the solution when they were mixed outside.

The mobile phase reservoir and the column were maintained at ambient temperature. These phases were either mixed online or premixed and used. When the solvents were premixed, the pH of the phosphate buffer was adjusted

to pH 2.7 before mixing other solvents. It is always recommended that the pH of the mobile phase not be adjusted after mixing the solvents. The problems associated with such adjustment are discussed elsewhere.[19] Premixing the mobile phases had some advantages over online mixing. When online mixing was used, there was a severe problem of phantom peaks. This problem totally disappeared when the same solvent systems were premixed and used. Premixing the mobile phase may also reduce the peak spikes associated with the dissolved air in pure solvents. Usually the dissolved air will be more in pure solvents than in the solvent mixtures. Also, premixing reduces the mechanical problems associated with mixing the solvents online.[20]

Robustness

Robustness is defined as the capacity of a method to remain unaffected by deliberate variations in method parameters.[21] The robustness of the methods was evaluated at different pH values (2.3, 2.5, 2.7, and 3.0) and different potassium phosphate monobasic salt concentrations in the mobile phase. The concentration of potassium phosphate monobasic was varied by ± 0.1 g/L. Peak characteristics and retention times were compared in evaluating robustness of the methods.

Stability Studies

Initially, typical stability protocols were attempted.[22] But because the drug substances tested were found to be extremely stable, more stressful conditions were selected to yield degradation products. Pure drug solutions of concentration 0.02 mg/mL were prepared in 1N hydrochloride, 1N sodium hydroxide, and 10 % hydrogen peroxide. Acetaminophen and salicylamide were dissolved in minimum amounts of methanol before the addition of acid, base, and peroxide. Aliquots of each solution were stored at 60°C separately for specified intervals. These aliquots were diluted with acid, base, or distilled water to yield a pH of 3 before injecting onto the column.

Sensitivity Factors, Detection and Quantitation Limits

The precision of the chromatographic system was determined using the relative standard deviation of the response factors for the different peaks in the injections of the standard solutions. Response factor was calculated as RF = DR/C where DR is the detector response (peak area) and C is the concentration of the analyte. The sensitivity factor was calculated by dividing the response factor of the drug with the response factor of respective degradation product and impurity. The detection limit for all these components was evaluated till response/noise ratio was 3. The limit of quantification for all the components was evaluated till response/noise ratio was 10. Response considered in these

calculations is the peak area of the chemical and the noise is the area associated with short-term noise (also known as high-frequency noise).

RESULTS AND DISCUSSION

Retention Times and Linearity

Method I is useful in quantification of acetaminophen, salicylamide, and phenyltoloxamine along with precursor impurities and potential degradation products. The total run time was 35 min followed by 10 min equilibration time.

The known impurity and degradation products have different retention times and separated adequately from the major components, suggesting this method is stability indicating. A representative chromatogram is shown in Figure 1.

Method II was developed for the assay of the three active components. The total run time of method II was 17 min followed by 10 min equilibration time. The retention times with both methods were lying within 100 ± 5 % range (Figure 2). The linearity parameters and ranges for the components are shown in Tables 1 & 2. Linearity of ethylsalicylate was not determined because of its poor aqueous solubility and high volatility. The correlation coefficient for all other components in the range of linearity was 0.999 or better.

Method Development

In the initial method development studies, an isocratic HPLC method was attempted to separate active ingredients and potential degradation products and impurities. The detection wavelength was chosen after scanning the individual components using a UV spectrophotometer. The UV spectrum of acetaminophen in water indicated a major band at 242 nm and a minor band at 280 nm. The ultraviolet spectrum of salicylamide in water indicated maxima at about 235 nm and 300 nm and that of phenyltoloxamine indicated a considerable increase in absorbance from 240 nm to 200 nm and a minor band at about 270 nm.

A wavelength of 220 nm was selected for the HPLC method to optimize the detection of phenyltoloxamine and thus avoid the adjustments in either wavelength or detector sensitivity required in order to accurately and precisely quantitate all the components. If methanol were to be used in the method, it would be preferable not to use λ_{max} less than 220 nm, as this could result in drifts in absorbance due to large variability in the absorbances of methanol and water at lower wavelengths.[23]

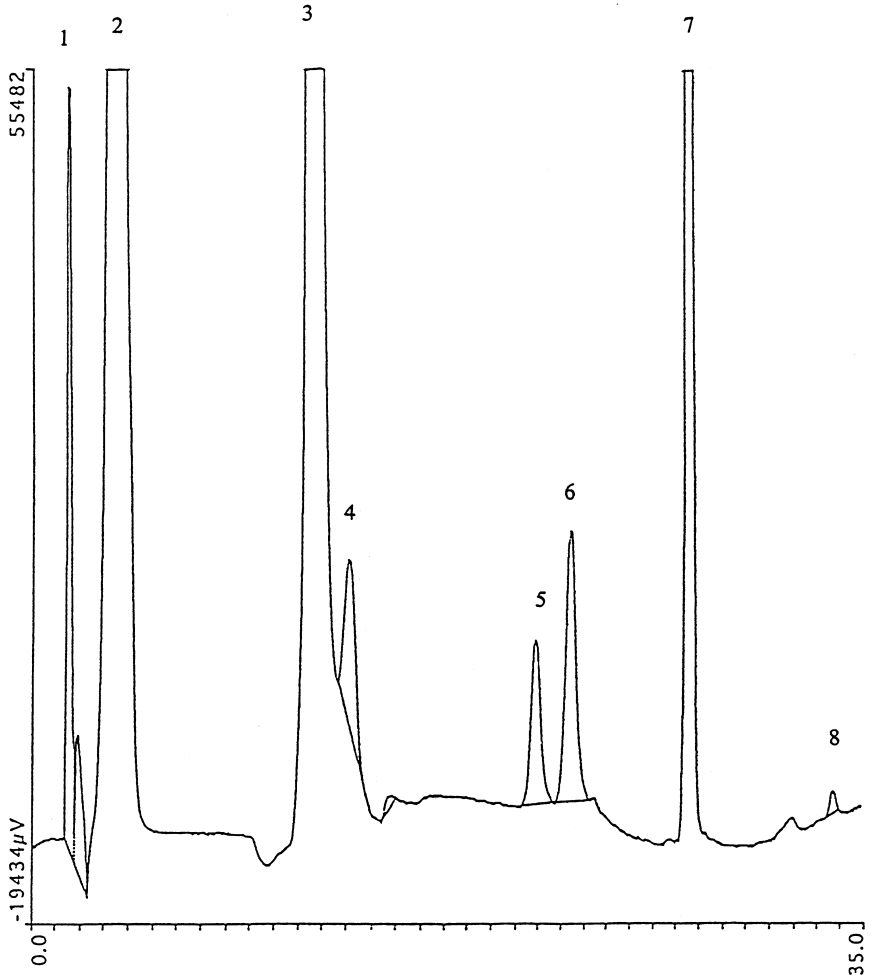
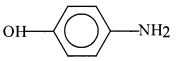
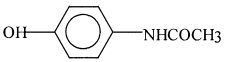
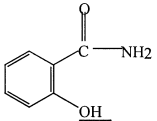
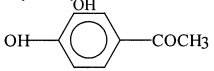
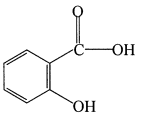
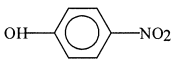
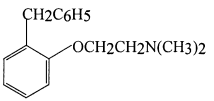
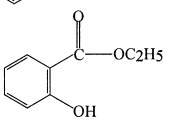


Figure 1. Chromatogram of Acetaminophen, Salicylamide and Phenyltoloxamine spiked with their potential related substances. Peak identities: (1) p-aminophenol, (2) acetaminophen, (3) salicylamide, (4) p-hydroxyacetophenone, (5) p-nitrophenol, (6) salicylic acid, (7) phenyltoloxamine, (8) ethyl salicylate.

This separation had become difficult because of wide range of polarities. For some pairs of the compounds, the polarities were too close to get good separation. Subsequently, a gradient method containing acetonitrile-buffer combination as mobile phase was attempted. Though the resolution was overall good, satisfactory resolution for nitrophenol-salicylic acid and p-hydroxyacetophe-

Structure	Name	Retention times I	Retention times II	Sensitivity factors
	p-aminophenol	1.5	*	1.33
	acetaminophen	3.3	5.7	1.00
	salicylamide	11.7	10.9	1.00
	p-hydroxyacet -ophenone	13.1	*	2.13
	salicylic acid	20.9	*	0.58
	p-Nitrophenol	22.4	*	1.18
	phenyltoloxamine	27.2	15.9	1.00
	ethylsalicylate	33.8	*	ND

* Method I is not defined ND Not determined

Figure 2. Chemical structures; retention times and sensitivity factors of components, degradation products, and impurities.

none-salicylamide pairs was not obtained (Figure 3). The resolution between these pairs was achieved by using acetonitrile-methanol-buffer combination. This ternary mixture of methanol, acetonitrile, and phosphate buffer yielded good resolution and reduced the overall run time when compared with acetonitrile-phosphate buffer mobile phase system.

The pH of the buffer used in these studies was 2.7. The peak shapes were good and the retention times were quite consistent. Also, the above gradient

Table 1

Linearity Data for Individual Components, Impurities and Degradation Products in Method 1

Compound	Concentration (mg/mL)	Slope (μVs mL/mg)	(μVs)	Intercept R^2
p-Aminophenol	0.02-0.00008	3.9 X 10^7	470	0.999
Acetaminophen	0.3-0.00006	3.13 X 10^7	23491	0.999
Salicylamide	0.2-0.00004	4.74 X 10^7	12601	0.999
p-Hydroxyaceophenone	0.02-0.00008	6.85X10^7	105	0.999
Salicylic acid	0.02-0.00008	2.90 X 10^7	-2364	0.999
4-Nitrophenol	0.02-0.00002	4.44 X 10^7	29	0.999
Phenyltoloxamine	0.22-0.00004	2.52 X 10^7	818	0.999
Ethylsalicylate	ND	ND	ND	ND

Peak areas are in μVs. ND = not determined.

method was tested with mobile phases containing an ion-pairing agent (heptane sulfonic acid, 1 g/L). Though the peak shapes were quite excellent, the retention times were quite unstable with time. All the peaks were moving towards t_0 with repeated injections. Thus, a gradient method with optimum isocratic conditions for the first 12 min was developed and chosen for further studies.

Table 2

Linearity Data for Individual Components, Impurities and Degradation Products in Method 2

Compound	Concentration (mg/mL)	Slope (μVs mL/mg)	(μVs)	Intercept R^2
Acetaminophen	0.3-0.00006	3.17 X 10^7	23832	0.999
Salicylamide	0.2-0.00004	4.89 X 10^7	1492	0.999
Phenyltoloxamine	0.2-0.00004	2.50 X 10^7	13036	0.999

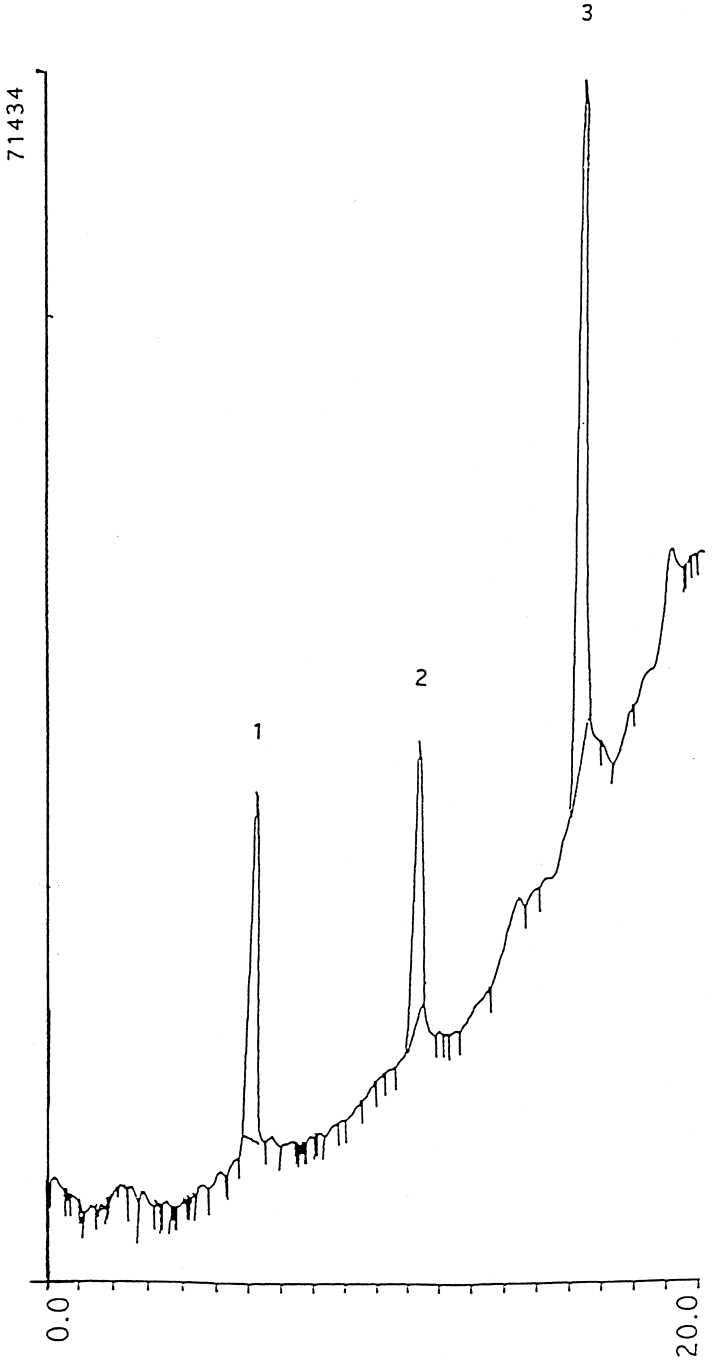


Figure 3. Chromatogram of (1) Acetaminophen, (2) Salicylamide and (3) Phenyltoloxamine.

Precision and Linearity

The relative standard deviation of the response factors of individual components in the injections of the standards in the two methods was typically less than 2.0 %.

External standard method was used in quantification of the drugs, degradation products, and impurities. This method is suitable when 1) the sample preparation procedures are simple, 2) when the run times are extended as in the case of detection of potential extraneous peaks (degradation products and impurities) and 3) when the analysis is for specific concentration point and narrow range (ex: dosage form acceptance and release studies).[21] Also, it will not complicate the method by introducing one more peak to be separated as in the case of internal standard method.[24]

Well dried acetaminophen, salicylamide, phenyltoloxamine, and other components were used to prepare the standards. Standards of acetaminophen were prepared in the range of 0.06-300 μg/mL, standards of salicylamide in the range of 0.04-200 μg/mL, and that of phenyltoloxamine in the range of 0.04-200 μg/mL. Standards for the degradation products and the impurities were prepared in the range of 0.08-20 μg/mL. All these solutions except ethylsalicylate were prepared in a solvent system containing methanol, acetonitrile, and distilled water in the ratio of 15:4:81 to avoid problems associated with injecting a weaker or strong solvent than the mobile phase. Guidelines for injection solvent selection are described by Dolan.[25]

Standards of ethylsalicylate were prepared in pure methanol because of its poor water solubility. All the standards were prepared by serial dilutions in triplicates. At least 5 different concentration standards were injected for each component. Injection volume was always 50 μL. Linearity of acetaminophen, salicylamide, phenyltoloxamine, and other components was evaluated in the above concentrations. Plot of residuals was used in the determination of linearity. In this method, a plot of residuals vs. measured analyte concentration was plotted. The residual at each data point is the difference between the measured area at a given concentration and the calculated area using the slope and intercept determined by a fit of all data. Linearity was assumed in the concentration range with the residuals distributed above and below the zero residual line, with no obvious outliers. Also, linearity was confirmed by determining the response factor at each measured concentration and plotting this response factor vs. logarithm of the analyte concentration. Linearity was assumed in the range where the response factors at their respective concentrations were in the range of 5% of their average value.

Limits of Detection and Quantification

The limits of detection for acetaminophen, salicylamide, phenyltoloxamine, p-nitrophenol, p-aminophenol, salicylic acid, p-hydroxyacetaminophen were 3 X 10^{-3}, 2 X 10^{-3}, 2 X 10^{-3}, 1 X 10^{-3}, 1 X 10^{-3}, 1 X 10^{-3}, and 2 X 10^{-3} μg respectively.

The limits of quantification for these compounds were 0.15 μg/mL, 0.08 μg/mL, 0.10 μg/mL, 0.10 μg/mL, 0.05μg/mL, 0.05 μg/mL, and 0.10 μg/mL respectively.

Solution Stability

Method I was employed to study solution stability. It has been found that during the stressed conditions, acetaminophen degraded significantly whereas salicylamide and phenyltoloxamine were quite stable. The stress conditions do not exactly represent the environment these dosage forms would be exposed. These tests were performed to yield degradation products. Acetaminophen degraded rapidly in the acid medium and yielded p-aminophenol. Salicylamide degraded to yield salicylic acid. This reaction was faster in acidic medium compared to basic medium. Phenyltoloxamine yielded unknown degradation products in acidic and basic media and the retention times of these peaks were 24.2, 28.8, and 30.3 mins respectively. All these were well separated from the parent compound and other components of interest.

Specificity

The eluents of acetaminophen, salicylamide, and phenyltoloxamine were collected after injecting standards and stress samples on to the column. All these were obtained using method I. The normalized UV scans associated with the peaks of acetaminophen, salicylamide, and phenyltoloxamine in the samples indicated that the peaks of both the standards and degraded samples were similar, demonstrating the specificity of this HPLC method in quantitative analysis of the major components.

Sensitivity Factors

The sensitivity factors for p-aminophenol, p-nitrophenol, and p-hydroxyacetophenone were calculated relative to acetaminophen and the sensitivity factor for salicylic acid was calculated relative to salicylamide. The sensitivity factors for these components are 1.33, 1.18, 2.12, 0.58, respectively. These sensitivity factors can be used to quantify degradation products or precursor impurities relative to their parent compounds.

Robustness

The robustness of the method was evaluated by determining the qualitative (tailing, resolution, stability in retention times) and quantitative changes in the chromatographic results of the same sample relative to the mobile phase pH and concentration of phosphate. At pH 2.3, there was poor resolution for salicylamide-p-hydroxyacetophenone and p-nitrophenol-salicylic acid pairs. The retention times reduced drastically for all the peaks with repeated injections. Also, the acetaminophen and salicylamide peaks were broader.

The resolution and peak shapes were quite good at pH 2.5 but there was a profound shift in the retention times. The retention times of all the peaks excepting for phenyltoloxamine decreased with time. At pH 3.0, the results were quite similar to that of pH 2.3. There was poor resolution with the same pairs and peaks of p-nitrophenol and salicylamide were broader and merging with repeated injections. The resolution was excellent and the retention times were quite consistent at pH 2.7.

Thus, it is recommended that the mobile phase be used at pH 2.7. There was no significant change in the chromatographic results with alteration in phosphate concentrations in the mobile phase.

CONCLUSIONS

Two different methods have been developed. The first method is to identify and quantify acetaminophen, salicylamide, and phenyltoloxamine along with its degradation products and precursor impurities and the second method can be used in the assay of formulations containing the above three drugs. These methods have been shown to be linear, robust, reproducible, sensitive, and stability indicating.

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