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# Simultaneous assay of ephedrine hydrochloride, theophylline, papaverine hydrochloride and hydroxyzine hydrochloride in tablets using RP-LC

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**Abstract**

The analytical problem was to control the quality of imported antiasthmatic tablets containing ephedrine hydrochloride, theophylline, papaverine hydrochloride and hydroxyzine hydrochloride. The aim of the analytical method for the assay was to separate, identify and quantify all compounds, at the same time. A gradient capable RP-LC system was used, using a commercially packed Nucleosil C18 column connected to a dual channel variable, programmable wavelength detector. The analysis was performed in the gradient program of increasing concentration of acetonitrile in water. The influence of pH of the mobile phase was established. The proposed method is reliable, reproducible, easy to perform and satisfies the aim. 

*Keywords:* Ephedrine hydrochloride; Theophylline; Papaverine hydrochloride; Hydroxyzine hydrochloride; RP-LC; Simultaneous assay; Antiasthmatic tablets

## 1. Introduction

Multicomponent antiasthmatic preparations, like the one that contains ephedrine hydrochloride, theophylline, papaverine hydrochloride and hydroxyzine hydrochloride, are often an analytical problem, since combined substances may have different chemical structure but very similar physical-chemical properties (solubility, polarity, collygative properties, etc.). Neither pharmacopoeial monographs [1–5] nor available reported methods [6–14] have demonstrated sufficient specificity for the simultaneous separation, identification and quantification of all the four active substances mentioned above.

The Institute of Pharmacy is the national official laboratory for quality control of medicines. Therefore, in order to systematically control the quality of some imported medicines for the ther-

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apy of asthma we had to set up an analytical strategy for the complete evaluation of their quality. For the assay, we decided to design an RP-HPLC method with acceptable separating power, which had also to be reliable, reproducible, easy to perform and low cost. The method was validated in order to satisfy its further use for the routine quality control of tablets containing the mentioned antiasthmatic active substances. It was not the aim of the study to set up stability indicating power of the method and there are no data presented with such intention.

This paper describes the development of the method that meets the proposed aim—a reliable, reproducible, easy to perform and low cost routine RP-HPLC method for the simultaneous assay of ephedrine hydrochloride, theophylline, papaverine hydrochloride and hydroxyzine hydrochloride in tablets.

## 2. Experimental

### 2.1. Materials

#### 2.1.1. Test samples

Test samples were imported pharmaceuticals of the oral dosage form of tablets containing 0.02 g ephedrine hydrochloride, 0.02 g papaverine hydrochloride, 0.1 g theophylline and 0.01 g hydroxyzine hydrochloride as active substances. Other substances in the formulation were Avicel PH 101, maize starch, talc and magnesium stearate.

#### 2.1.2. Chemicals

Acetonitrile, manufactured by Merck (Darmstadt, Germany) was HPLC gradient grade. The same company manufactured orthophosphoric acid p.a. and purified water used was of HPLC quality.

### 2.2. Methods

#### 2.2.1. Apparatus

The HPLC system used during development of the method consisted of the two LKB 2150 HPLC pumps (LKB Bromma, Sweden) controlled by Gradient Controller LKB LC 2152 and connected by Rheodyne high pressure gradient mixer (Rheodyne, Redwood, CA) and were equipped with a Rheodyne 7125 manual injector with a 10-μl sample loop. The two detectors were connected in series. The first was UV diode-array Rapid Spectral Detector LKB 2140 (LKB, Bromma, Sweden), and the other was a UV/Vis variable programmable wavelength LC 1210 Detector (GBC Scientific Equipment, Dandenong, Victoria, Australia). Chromatographic data monitored by the diode-array detector were acquired, stored and processed by an IBM PC i386 compatible computer using Wavescan EG (LKB Bromma, Sweden) and Nelson Analytical 3000 Series (CDS, CA) software. The variable wavelength detector was connected to the HP 3396 Series II Integrator (Hewlett-Packard, USA). Later, for the validation of the method and routine work, the system was simplified by exclusion of the diode-array detector and computer system. A column heating compartment was not used, but environmental temperature conditions in Instrumental Laboratory were controlled and continually maintained at $20 \pm 2$°C.

#### 2.2.2. Detector program

The BC LC 1210 detector was programmed to change detection wavelengths from 220 nm at start time, to 240 nm at 3.70 min and again to 220 nm at 10.70 min. By default, the signal was zeroed at wavelength change and rise-time for $\lambda$ change was set up at 0.01 min. The sensitivity of the detector was set up for a range of 1.0 AUFS for both channels.

#### 2.2.3. Integrator program

By default it was set up that minimum peak width for detection would be 0.05. The integration method was by peak area, and the method for reintegration and recalibration was on the basis of average peak areas. Calibration table updating was automatic, and ephedrine hydrochloride peak was used as reference peak. Peak detection time window was 0.2 min. Timed events program was as described in Table 1.

Table 1
Timed events program for HP 3396 Series II Integrator

| Time (min) | Event | Value |
|---|---|---|
| 0.00 | Signal zero | 0 |
| 0.00 | Threshold | 2 |
| 2.00 | Attenuation | 4 |
| 4.50 | Attenuation | 7 |
| 4.50 | Threshold | 8 |
| 9.00 | Attenuation | 9 |
| 11.70 | Attenuation | 5 |
| 13.70 | Signal zero | 0 |
| 15.00 | Program events time-table | Stop |

### 2.2.4. Stationary phase

The stationary phase was a commercial Nucleosil® C18 pre-packed HPLC column, pore diameter of 100 Å, 150 mm × 4.6 mm and particle size 5 μm (Sigma-Aldrich, Cat. No. Z226173, Sigma, St Louis, MO). Between injector and column, the pre-column was mounted with 0.22-μm pore size replaceable sintered glass filter (Rheodyne, CA).

### 2.2.5. Mobile phase

Depending on gradient conditions, there were two solvents used in the composition of mobile phase. For pump A, the solvent was a mixture of acetonitrile and water (5:95 v/v), pH $2.40 \pm 0.1$ adjusted with *o*-phosphoric acid. For pump B, the solvent was a mixture of acetonitrile and water (40:60 v/v), pH $2.40 \pm 0.1$ adjusted with *o*-phosphoric acid. During the chromatographic runs, the flow of mobile phase was maintained at 1.50 ml $min^{-1}$. The composition of mobile phase was changed from 2% of pump B to 100% of pump B, according to the gradient program (Fig. 1).

### 2.2.6. Gradient program

The chromatographic run was started under isocratic conditions of 2% of the solvent from

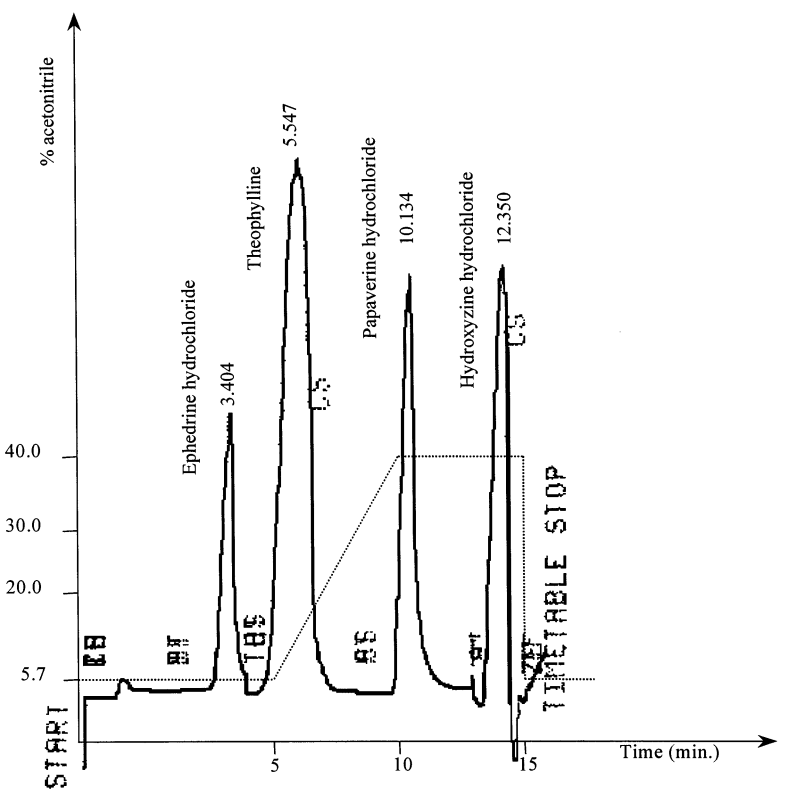


Fig. 1. Graphical presentation of gradient program with typical chromatogram.

pump B in the mobile phase composition during the first 5.1 min. At that time point, gradient was started with a linear increase of the volume of the solvent from pump B, from 2 to 100% in 4.9 min. After 10 min of chromatographic run the HPLC system was maintained for next 5 min under isocratic conditions of 100% of the solvent from pump B. The gradient program was finished at 15.1 min with the return of mobile phase composition to start conditions of 2% solvent from pump B. The HPLC system was equilibrated for 5 min under those conditions between two chromatographic runs.

*2.2.7. Reference standard stock solution*

Appropriate quantities of reference standard substances of active components were weighed and dissolved in the solvent to obtain the following concentrations: ephedrine hydrochloride $9.92 \times 10^{-3}$ mol $l^{-1}$ (2.0 mg $ml^{-1}$); theophylline $5.04 \times 10^{-2}$ mol $l^{-1}$ (10.0 mg $ml^{-1}$); papaverine hydrochloride $5.32 \times 10^{-3}$ mol $l^{-1}$ (2.0 mg $ml^{-1}$) and hydroxyzine hydrochloride $2.23 \times 10^{-3}$ mol $l^{-1}$ (1.0 mg $ml^{-1}$). As solvent, a mixture of acetonitrile and water (20:80 v/v), pH $2.00 \pm 0.1$ adjusted with *o*-phosphoric acid, was used.

*2.2.8. Reference standard working solution*

Appropriate quantities of reference standard substances of active components were weighed and dissolved in the solvent to obtain the following concentrations: ephedrine hydrochloride $9.92 \times 10^{-4}$ mol $l^{-1}$ (0.2 mg $ml^{-1}$); theophylline $5.04 \times 10^{-3}$ mol $l^{-1}$ (1.0 mg $ml^{-1}$); papaverine hydrochloride $5.32 \times 10^{-4}$ mol $l^{-1}$ (0.2 mg $ml^{-1}$) and hydroxyzine hydrochloride $2.23 \times 10^{-4}$ mol $l^{-1}$ (0.1 mg $ml^{-1}$). As solvent, a mixture of acetonitrile and water (20:80 v/v), pH $2.00 \pm 0.1$ adjusted with *o*-phosphoric acid, was used. Injections were made with the aid of a 100-μl glass syringe (Cat. No. 80665, Hamilton, Reno, NV).

*2.2.9. Reference standard solutions for calibration*

Appropriate volumes of standard stock solution of active components were transferred and diluted by the solvent to obtain the following range of concentrations: ephedrine hydrochloride $4.96–1.49 \times 10^{-4}$ mol $l^{-1}$ (0.1–0.3 mg $ml^{-1}$); theophylline $2.52–7.57 \times 10^{-3}$ mol $l^{-1}$ (0.5–1.5 mg $ml^{-1}$); papaverine hydrochloride $2.66–7.98 \times 10^{-4}$ mol $l^{-1}$ (0.1–0.3 mg $ml^{-1}$) and hydroxyzine hydrochloride $1.12–3.35 \times 10^{-4}$ mol $l^{-1}$ (0.05–0.15 mg $ml^{-1}$). As solvent, a mixture of acetonitrile and water (20:80 v/v), pH $2.0 \pm 0.1$ adjusted with *o*-phosphoric acid, was used.

*2.2.10. Sample solution*

Twenty tablets were pulverized in a mortar and a quantity corresponding to average tablet mass was weighed and extracted by the solvent with the aid of ultrasonication, to obtain appropriate concentrations of active compounds with respect to reference standard working solution. Before injection all solutions were filtered through a 0.22-μm PTFE syringe filter.

## 3. Results and discussion

During a development stage of the presented HPLC method, a wide range of commercial prepacked RP-HPLC columns with different stationary phases (C18, C8, ODS-2, CN, $NH_2$, etc.) were screened. The best separating properties were obtained for C18 packaging, especially for those with end-capped, spherical particle-columns like Nucleosil® and Partisil®. The chromatographic behavior of analyzed active compounds was evaluated by varying the qualitative and quantitative composition and pH of the mobile phase using a Nucleosil® C18 $150 \times 4.6$ mm column.

### *3.1. The influence of organic modifier in mobile phase composition*

When methanol was used as the organic modifier of water mobile phase, baseline resolution of papaverine hydrochloride and hydroxyzine hydrochloride could be achieved only with a high difference between initial and final methanol concentration in the gradient program. The absorbance due to mobile phase change had been significantly increased during such gradient elution and has resulted in gradient baseline drift at the lower wavelengths, where it was only possible to detect and quantify ephedrine hydrochloride

and hydroxyzine hydrochloride. In fact, the gradient baseline was overlapping the hydroxyzine hydrochloride peak.

The retention behavior of the analyzed drugs under the influence of different concentrations of acetonitrile in water was investigated. The acetonitrile concentration range between 0 and 40% was examined by increasing the concentration in 2.5% increments while flow rate was maintained at 1.50 ml $min^{-1}$ and the pH of the mobile phase was $2.4 \pm 0.1$.

The least values for peak tailing factor and the highest response factor for ephedrine hydrochloride and theophylline peaks were achieved with concentrations of 3 and 5% of acetonitrile, respectively. Papaverine hydrochloride started to elute with 17.5% of acetonitrile and hydroxyzine hydrochloride could not elute until the acetonitrile concentration in the mobile phase had reached 22.5%. Good retention parameters for hydroxyzine hydrochloride were obtained with 32.5% acetonitrile. Theophylline, papaverine hydrochloride and hydroxyzine hydrochloride were overlapping if final gradient concentrations were set above 40%. The obtained experimental results have defined the optimal gradient profile.

The segmented gradient started in the first 5 min with isocratic elution with 5.8% of acetonitrile, followed by the gradient acetonitrile concentration change of 6.98% per min until 40% had been reached at 10 min. From 10 min, up to the end of the chromatographic run, isocratic conditions of 40% acetonitrile concentration were taking place.

### 3.2. The influence of pH of mobile phase

In the course of setting up the adequate chromatographic conditions, the influence of pH of the mobile phase was investigated. The method selectivity and retention behavior of analytes has been evaluated in the pH range from 2.0 to 7.5 in 0.5 pH unit increments.

Results have shown that pH of the eluent greater than 4.0 induced a significant increase of the retention parameters and the effect of peak tailing for all active compounds. At pH greater than 6.0, papaverine hydrochloride and hydroxyzine hydrochloride did not elute during chromatographic runs.

For pH in the range between 3.0 and 4.0, better chromatographic behavior of analytes was observed. Acceptable values for retention parameters were obtained for the pH in the range of 2.0–3.0. The influence of mobile phase having pH 2.2, 2.4, 2.6, 2.8 and 3.0 on retention of compounds of interest was investigated. The experimental results obtained for this pH range demonstrated that optimal values for retention parameters were achieved with pH of mobile phase adjusted to $2.40 \pm 0.1$ which was later confirmed by validation of the proposed method.

### 3.3. The influence of column sample load and detection wavelength

In order to impose the optimal column sample load, experiments were designed to cover 50-, 20- and 10-μl injection volumes for the Rheodyne injector. It was concluded that good retention characteristic, sensitivity and adequate column loading of analytes are achieved if they are introduced into the system with a 10-μl injection loop.

The high differences in the label amounts of active compounds (20 mg ephedrine hydrochloride, 10 mg hydroxyzine hydrochloride, 10 mg papaverine hydrochloride and 100 mg of theophylline per tablet) and in their molar absorption coefficients determined the need to vary detection wavelengths during chromatographic runs. For the purposes of method development and validation and with the aim to use it for routine laboratory work, technical performances of dual channel, programmable wavelength detector were fully used.

Ephedrine hydrochloride and hydroxyzine hydrochloride as active compounds with the lowest declared content in investigated antiasthmatic tablets demonstrate the highest sensitivity and UV absorbance between 206 and 210 nm. Since there is significant baseline drift within that range due to increasing acetonitrile concentration in the eluent, the detection wavelengths for these analytes had been shifted to 220 nm. That choice has contributed to good sensitivity, precision and accuracy of the method for ephedrine hydrochloride and hydroxyzine hydrochloride assay.

At the same wavelength, theophylline, due to high molar absorption coefficient as well as the highest concentration in the sample solution, had shown maximal response factor which caused undesirable effects on method precision and accuracy. This fact was the major reason for selecting 240 nm as the detection wavelength for theophylline, where its UV spectra exhibit the minimal absorbance, so that this compound had lower but yet more adequate detector response. In this manner, further dilution of sample solution to be submitted to chromatography and/or additional chromatographic run was avoided, which has contributed to simplicity, speed and cost effectiveness of the method.

Under the same detection conditions, papaverine hydrochloride, analyte, whose concentration in the sample solution was five times lower than that for theophylline, exhibited maximal absorbance, better sensitivity and precision compared to results obtained at 220 nm.

The method precision was evaluated for all four analytes for different wavelengths. The C.V.% values for ephedrine hydrochloride, theophylline, papaverine hydrochloride and hydroxyzine hydrochloride at 220 nm were 2.53, 11.23, 3.77 and 2.55% respectively while at 240 nm, the values were 5.67, 2.35, 1.22 and 7.30%. On the basis of these results, a detector wavelength change program was proposed.

So, for the separation and simultaneous identification and assay of ephedrine hydrochloride, hydroxyzine hydrochloride, papaverine hydrochloride and theophylline the RP-HPLC method was developed and suggested.

### 3.4. Validation of the method

#### 3.4.1. Performance parameters

The established chromatographic conditions had demonstrated good baseline resolution for all compounds of interest that had enabled reliable and reproductive integration based on the peak areas.

Calculated average retention times of the tested active substances were as follows (min): ephedrine hydrochloride 3.476, theophylline 5.588, papaverine hydrochloride 10.152 and hydroxyzine hydrochloride 12.392, and average R.S.D. was 3.08%.

On the basis of the retention times and peak widths, the characteristic performance parameters were determined.

The retention time of mobile phase (0.827 min) was used as hold-up time for the capacity factor calculations. The capacity factors ($k'$) were: ephedrine hydrochloride 3.20, theophylline 5.76, papaverine hydrochloride 11.28 and hydroxyzine hydrochloride 13.98. The presented chromatographic conditions ensured adequate retention of all active compounds, since $k'$ values obtained satisfied the condition $k' \geq 1.0$.

The method has enabled good resolution of analytes since values of resolution factors of adjacent peaks, calculated according to the Ph. Eur. equation, were greater than 1.0 (1.475 for theophylline/ephedrine hydrochloride, 2.655 for papaverine hydrochloride/theophylline and 3.54 for hydroxyzine hydrochloride/papaverine hydrochloride).

#### 3.4.2. Identification

The acceptance criteria for validation of identification and assay method were established. The specificity of identification test was demonstrated by the absence of any peak from placebo solution corresponding to retention time of active compounds. The presence of ephedrine hydrochloride, theophylline, papaverine hydrochloride and hydroxyzine hydrochloride was confirmed by matching their retention times and UV/Vis spectra obtained in the apex of appropriate peaks on chromatograms of sample and standard solutions.

The assay method was submitted to validation evaluation of precision, accuracy, linearity, detection and quantification limits for all compounds of interest.

#### 3.4.3. Linearity

A calibration plot for ephedrine hydrochloride was made using the least squares method of mean values of peak areas for five data points and has demonstrated linearity over the range of concentration $4.96 \times 10^{-4}$–$1.96 \times 10^{-3}$ mol $1^{-1}$. The slope of the calibration plot ($b$) was $6.1476 \times 10^{6}$, $y$-axis intercept ($a$) 7981.84 and correlation coeffi-

Table 2
Summary data of method linearity, accuracy and precision validation

| Validation parameters | Criteria and acceptance limits | Ephedrine hydrochloride | Theophylline | Papaverine hydrochloride | Hydroxyzine hydrochloride |
|---|---|---|---|---|---|
| Linearity | Correlation coefficient (NLT 0.99) | 0.9984 | 0.9927 | 0.9972 | 0.9959 |
| | Significance of intercept, $t$-test ($t_{tab} = 3.180$) | 0.263 | 0.095 | 0.305 | 0.467 |
| | Significance of intercept (%), ratio of intercept vs. working concentration peak area (max. 2.0%) | 0.64 | 0.495 | 0.981 | 1.81 |
| Accuracy | % Recovery ($n = 7$) | 1.25 | 1.93 | 1.27 | 1.92 |
| | $t_{\alpha,k}$ ($t_{tab} = 2.4469$) | 1.270 | 2.1424 | 1.2345 | 1.2345 |
| Precision | R.S.D. (%) ($n = 10$) | 1.05 | 2.46 | 0.70 | 2.15 |

cient 0.9984. The $y$-axis intercept significance was challenged, zero hypothesis tested applying Student's $t$-test. Since a value was less than the tabular one it was concluded that the intercept is not significantly different from zero. The intercept and peak area ephedrine hydrochloride ratio for working concentration ($9.92 \times 10^{-4}$) was 0.64% which was within acceptance limits ($\pm 2\%$), the criteria that was set for linearity of the method. R.S.D. of the detector response factor for ephedrine hydrochloride was 2.06%.

On the basis of experimental results obtained for theophylline, a linearity calibration plot was constructed as a function of peak area vs. five different concentrations in the range $2.52–7.57 \times 10^{-3}$ mol $l^{-1}$. The regression equation $y = 2.3237 \times 10^{7}x + 115\,602$ was determined with correlation coefficient of 0.9927. The intercept on the $y$-axis ($a$) was not statistically significant, since obtained experimental $t_a$ value (0.095) was less than the tabular value (3.18) for the same probability and number of degrees of freedom. The linearity challenge within the suggested range was completed by demonstrating the ratio of theophylline linearity plot intercept and peak area for working concentration was 0.495%.

The linearity of the assay method for papaverine hydrochloride was defined by the regression equation $y = 1.5355 \times 10^{8}x + 304\,388$ for peak areas and five corresponding concentrations in the range of $2.66–7.98 \times 10^{-4}$ mol $l^{-1}$. Linearity was evaluated and confirmed, since the correlation coefficient had been 0.9972, and the intercept was not statistically significantly different from zero ($t_a = 0.305$; $t_{tab} = 3.180$), the ratio of plot intercept and the papaverine hydrochloride peak area of working concentration (0.495%) was within specification limits. The value of detector response factor R.S.D. was 2.19%.

The obtained peak areas for hydroxyzine hydrochloride in the concentration range $1.12–3.35 \times 10^{-4}$ mol $l^{-1}$ were shown to follow a linear function and were represented by the equation $y = 2.617 \times 10^{7}x + 48\,204$. The correlation coefficient was 0.9959 and intercept ($a$) was not statistically significant ($t_a = 0.467$; $t_{tab} = 3.180$). A linear relationship was also demonstrated since the value of ratio of plot intercept and peak area at working concentration is within acceptance limits. A brief summary of linearity, accuracy and precision validation data for all compounds is presented in Table 2.

### 3.4.4. Accuracy and precision

In the course of evaluation of ephedrine hydrochloride assay method accuracy, it has been found that the experimental value of the confidence coefficient ($t_{exp}$) was 1.27, and that it was less than the tabular value (for $P = 0.05$ and $k = n - 1$), so that it could be concluded that the proposed method is accurate. The R.S.D. of the assay method for ephedrine hydrochloride was 1.05%.

Confidence coefficient of the theophylline assay ($t_{exp} = 2.142$) was less than the tabular value,

which qualified the proposed method as accurate. Good repeatability of results for theophylline was established since R.S.D. was 2.47%.

The suggested HPLC method was found to be accurate for assay of papaverine hydrochloride because the experimental value of the confidence coefficient (1.2345) was less than the tabular value. Good precision of the method was confirmed since the R.S.D. was 0.7%.

The hydroxyzine hydrochloride assay confidence coefficient was determined in the course of accuracy evaluation ($t_{exp} = 1.238$) and when compared to the tabular value, it was concluded that the method for this compound was accurate and precise with R.S.D. of 2.19%.

The presented method could be considered accurate and precise although some R.S.D. values were greater then 2.0%, which was mainly influenced by the fact that simultaneous assay of four components was taking place. A summary of results obtained for validation of the method accuracy and precision are presented in Table 2. Data for accuracy are obtained for the seven spiked placebo samples and for precision by estimation of results for 10 samples.

*3.4.5. Detection and quantification limits*

The signal-to-noise ratio, S/N = 3 was used as the criteria for detection limits and they were established to be 50 ng for ephedrine hydrochloride, 250 ng for theophylline, 50 ng for papaverine hydrochloride and 25 ng for hydroxyzine hydrochloride. The detection limit of the proposed RP-HPLC method for the simultaneous assay of these four active compounds was determined by the minimal detectable amount of ephedrine hydrochloride, the analyte which under the suggested chromatographic conditions demonstrated the lowest detector response.

The method quantification limit (LOQ) at S/N = 10 was 0.2 μg for ephedrine hydrochloride, 1 μg for theophylline, 0.2 μg for papaverine hydrochloride and 1 μg for hydroxyzine hydrochloride. This has been influenced by the fact that the proposed conditions have imposed the greatest threshold value for theophylline integration.

During method development and validation, the two imported batches of antiasthmatic tablets were assayed. All of the obtained results had been within specified acceptance limits for the finished product. A typical chromatogram is presented in Fig. 1. The R.S.D. of the assay of ephedrine hydrochloride was 1.74%, 1.93% for hydroxyzine hydrochloride, 1.01% for papaverine hydrochloride and 2.78% for theophylline.

## 4. Conclusion

The proposed HPLC method is specific, selective, accurate, precise and sensitive as well as quick for simultaneous identification and assay of ephedrine hydrochloride, hydroxyzine hydrochloride, papaverine hydrochloride and theophylline in tablets.

Bearing this in mind, the method could be used as a Standard Operating Procedure for the identification and assay of any combination of these active substances or as single compounds in solid oral pharmaceutical dosage forms. For such purpose, it seems that only optimization of sample solution preparation could be required.

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