TLC-spectrodensitometric and microemulsion RP-HPLC chromatographic methods for determination of orphenadrine and paracetamol

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Received: 25th March, 2010; Accepted: 4th April, 2010

ABSTRACT

Two chromatographic methods were developed for determination of paracetamol and orphenadrine citrate in presence of p-aminophenol. In the first method, paracetamol, orphenadrine citrate and p-aminophenol were separated on TLC silica gel 60F254 plate using ethyl acetate: acetone: methanol: glacial acetic acid (5: 4: 1: 0.1, by volume) as mobile phase. The obtained bands were then scanned at 220nm. The second method is a RP-HPLC method in which paracetamol, orphenadrine citrate and p-aminophenol were separated on a reversed-phase C18 column using micro-emulsion as mobile phase at a flow rate of 1mL.min−1 and UV detection at 225nm. The proposed methods were successfully applied for determination of paracetamol and orphenadrine citrate in pure form and in their pharmaceutical formulation.

INTRODUCTION

Paracetamol (PAR), 4-acetamidophenol, is an effective analgesic and antipyretic for treatment of minor, non-inflammatory conditions in patients who are prone to gastric symptoms[1]. Orphenadrine citrate (ORP), (RS)-(dimethyl-2-(2-methylbenz hydroxy) ethyl) amine citrate, is used as skeletal muscle relaxant[1]. Thus, tablets containing PAR and ORP show combined analgesic and skeletal muscle relaxant effects. This combination is widely prescribed for patients with gastric problems who cannot tolerate ordinary non-steroidal anti-inflammatory drugs.

There are many reports for the determination of PAR and ORP separately[2-5] or in combination with other drugs[6-9]. Few methods have been reported for determination of PAR and ORP in binary mixture[10-13]. None of these methods has been reported for simultaneous determination of PAR and ORP in presence of p-aminophenol (PAP); a potential impurity of PAR reported in BP and USP and considered the major degradation product of paracetamol[2,14].

Therefore, the objective of this work is to develop sensitive, selective and reproducible methods for simultaneous determination of PAR and ORP in presence of PAP. Chromatographic methods are well known for providing high selectivity and sensitivity when used for determination of pharmaceutical drugs. In this paper, two chromatographic methods, namely, TLC-spectrodensitometry and reversed phase high performance liquid chromatography (RP-HPLC) have been proposed for simultaneous determination of PAR and
ORP in presence of PAP that can be used for routine quality control analysis of these drugs either in bulk powder or in pharmaceutical formulation.

**EXPERIMENTAL**

**Apparatus**

1. UV lamp with short wavelength 254 nm (USA).
2. TLC scanner 3 densitometer (Carnage, Muttenz, Switzerland). The following requirements are taken into consideration:
   - Slit dimensions: 6.00×0.45
   - Micrometer, Scanning speed = 20mm/s
   - Data resolution = 100μm / step
3. Sample applicator for TLC Linomat IV with 100μl syringe (Carnage, Muttenz, Switzerland).
4. TLC plates (20×20 cm) coated with silica gel 60 F 254 (Merck KgaA, Darmstad, Germany).
5. Shimadzu Class - LC 10 AD Liquid Chromatograph supplied with Shimadzu SPD-10 A UV-VIS Detector (Shimadzu Corporation, Japan). Phenomenex C$_{18}$ (25cm×4.6mm i.d, 5μm particle size) column was used as a stationary phase for HPLC determinations (USA).
6. Sonix TV ss-series ultrasonicator (USA).

**Materials**

**Pure samples**

1. Paracetamol and Orphenadrine were kindly supplied by the tenth of Ramadan Co. for pharmaceutical industries and diagnostic reagents (RAMEDA), 10$^{th}$ of Ramadan city, Egypt. Their purity were found to be 100.01±0.837 and 99.21±0.602, respectively, according to BP methods.$^{[14]}$
2. P-amino phenol (99 %) was obtained from Riedel-dehaen-AG-Germany.

**Market samples**

Orphamol® tablet (Batch No 06524) labeled to contain 450mg of paracetamol and 35 mg of Orphenadrine citrate, is from the tenth of Ramadan Co. for pharmaceutical industries and diagnostic reagents (RAMEDA), 10$^{th}$ of Ramadan city, Egypt.

**Reagents**

All reagents and chemicals used were of analytical grade and were used without further purification.

1. Ethyl acetate (Merck, Darmstadt, Germany).
2. Acetone (Merck, Darmstadt, Germany).
3. Triethyl amine (Merck, Darmstadt, Germany).
4. Methanol HPLC grade (Sigma Aldrich, Germany).
5. Acetonitrile HPLC grade (Sigma Aldrich, Germany).
6. Deionized water (SEDICO pharmaceutical Co., 6$^{th}$ October City, Egypt).
8. Butanol (Merck, Darmstadt, Germany).
9. Potassium dihydrogen phosphate (Sigma Aldrich, Germany).
10. Berj 35 (Merck, Darmstadt, Germany).

**Preparation of standard solutions**

1. Stock standard solutions (1mg.mL$^{-1}$) : weigh accurately 0.1gm of PAR, ORP and PAP into three separate 100mL volumetric flasks, add 50mL methanol to each flask, shake to dissolve then complete the volume to the mark with methanol.
2. Working standard solutions (100μg.mL$^{-1}$): transfer accurately 10mL of each of PAR, ORP and PAP stock standard solutions (1mg.mL$^{-1}$) into three 100mL volumetric flasks, then complete the volume to the mark with methanol.

**Preparation of micro-emulsion mobile phase**

Micro-emulsion mobile phase was prepared by mixing 15gm of Berj 35, 5mL ethyl acetate and 30mL butanol in a beaker with stirring and gentle heating then complete the volume to one liter with phosphate buffer (pH = 3).

**Procedures**

**TLC- spectrodensitometric method**

**Linearity and construction of calibration curves**

Apply accurate aliquots equivalent to (0.2-1.4μg.mL$^{-1}$) of PAR, (0.3-3μg.mL$^{-1}$) of ORP and (0.2-1.4μg.mL$^{-1}$) of PAP from their corresponding working solution (100μg.mL$^{-1}$) to thin layer chromatographic plates (20×20cm) as band using the Camage TLC sampler. Leave a space of 1cm between each band and 1.5cm from the bottom edge of the plate. Develop the plate in a chromatographic tank previously saturated.
for an hour with the developing mobile phase, ethyl acetate: acetone: glacial acetic acid (5: 4: 1:0.1, by volume) by ascending chromatography at room temperature.

Detect the bands under UV-lamp and scan at 220nm under the specified experimental conditions. Construct the calibration curves for each compound by plotting the peak area/ 100 versus the corresponding concentration and then compute the regression equations.

**Analysis of laboratory prepared mixtures**

Prepare mixtures containing PAR, ORP and PAP in different ratios. Proceed as mentioned under linearity and construction of calibration curves. Calculate the concentrations of the three compounds from their corresponding regression equations.

**RP-HPLC method**

**Linearity and construction of calibration curves**

Transfer accurate aliquots equivalent to (50-2000)μg of PAR, (50-2500)μg of ORP and (50-2200)μg of PAP from their corresponding working solutions (100μg.mL⁻¹) into three separate sets of a series of 10mL volumetric flasks. Complete the volume with methanol. Make triplicate 20μL injections for each concentration. Record the chromatograms at ambient temperature maintaining the flow rate at 1mL.min⁻¹ and detect the effluent at 225nm. The separation was done on a C₁₈ column using prepared micro-emulsion as a mobile phase. Construct the calibration curves for each compound by plotting the peak area/ 10⁴ versus the corresponding concentration and then compute the regression equations.

**Analysis of laboratory prepared mixtures**

Prepare mixtures containing PAR, ORP and PAP in different ratios. Proceed as mentioned under linearity and construction of calibration curves. Calculate the concentrations of the three compounds from their corresponding regression equations.

**Application of the proposed methods to pharmaceutical formulations**

**For the TLC-spectrodensitometric method**

Thoroughly powder ten tablets of Orphamol® and mix, then weigh accurately an amount of the powder equivalent to 450mg of PAR and 35mg of ORP in 250ml beaker. Add 70mL of methanol; stir magnetically for 30 minutes then filter through filter paper into a 100mL volumetric flask. Wash the beaker and the funnel then complete the volume with methanol to get a concentration of 4.5 and 0.35mg.mL⁻¹ for PAR and ORP respectively.

Appropriate dilutions were made to bring up a concentration of 4.50 and 0.35μg.mL⁻¹ for PAR and ORP, respectively and a concentration of 0.45 and 0.035μg.mL⁻¹ PAR and ORP, respectively. The proposed spectrodensitometric method was applied for the analysis and calculation of PAR, ORP and PAP concentrations.

**For the RP-HPLC method**

The above procedure was repeated but appropriate dilution was made to bring up a concentration of 90.00 and 7.00μg.mL⁻¹ for PAR and ORP, respectively. The proposed RP-HPLC method was applied for the analysis and calculation of PAR, ORP and PAP concentrations.

**RESULTS AND DISCUSSION**

**Method development**

**TLC- spectrodensitometric method**

This technique offers a simple way to quantify directly on TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing the peak height of the unknown band to a standard curve from reference materials chromatographed simultaneously under the same condition[18].

The method is based on the difference in R\textsubscript{f} values of PAR ( R\textsubscript{f} = 0.80 ), PAP ( R\textsubscript{f} = 0.69) and ORP ( R\textsubscript{f} = 0.12 ) as shown in figure 1.

A linear correlation was obtained between the peak area /100 and concentration of each compound in the range of 0.2-1.4μg.band⁻¹ for PAR, 0.3-3μg.band⁻¹ for ORP and 0.2-1.4μg.band⁻¹ for PAP. The regression equations were calculated and found to be:

\[ Y_1 = 1.1904C_1 + 0.1151 r_1 = 0.9992 \]
\[ Y_2 = 0.0942C_2 + 0.0373 r_2 = 0.9991 \]
Y_3 = 0.478 C_3 + 0.3121 \quad r_3 = 0.9992

where Y_1, Y_2 and Y_3 are the peak area/100, C_1, C_2 and C_3 are PAR, ORP and PAP concentration in µg. mL^{-1} respectively and r_1, r_2 and r_3 are the correlation coefficients.

**RP-HPLC method**

A validated isocratic RP-HPLC method with UV detection was developed for simultaneous determination of PAR, ORP in presence of PAP.

It depends on the chromatographic separation of PAR, ORP and PAP using C18 (25cm × 4.6mm i.d. 5µm particle size) column and micro-emulsion (formulated by mixing 15gm of Berj35 with 5mL ethyl acetate and 30mL butanol and completing the volume to 1 liter with phosphate buffer (PH = 3)) as a mobile phase with UV detection at 225nm, figure 2.

A linear correlation was obtained between the peak area/10^4 and concentration of each compound in the range of 5-200µg.mL^{-1} for PAR, 5-250µg.mL^{-1} for ORP and 5-220µg.mL^{-1} for PAP. The regression equations were calculated and found to be:

Y_1 = 0.7455 C_1 + 34.179 \quad r_1 = 0.9991
Y_2 = 1.0516 C_2 + 0.936 \quad r_2 = 0.9991
Y_3 = 0.5413 C_3 + 2.1183 \quad r_3 = 0.9992

where Y_1, Y_2 and Y_3 are the peak area/100, C_1, C_2 and C_3 are PAR, ORP and PAP concentration in µg. mL^{-1} respectively and r_1, r_2 and r_3 are the correlation coefficients.

**Method optimization**

**TLC- spectrodensitometric method**

Studying of the optimum parameters for maximum separation was carried out by trying different developing systems with different ratios, but complete separation of PAR, ORP and PAP was achieved by using ethyl acetate: acetone: methanol: glacial acetic acid (5:4:1:0.1, by volume) as developing mobile phase. Also different scanning wavelengths were tested, but the best sensitivity obtained when 220nm was chosen as scanning wavelength.

**RP-HPLC method**

To optimize the proposed HPLC method, all of the experimental conditions were investigated. The stationary phase choice was selected to be the reversed-phase over the normal phase separation due to the drawbacks of the normal phase, e.g. hydration of silica with water that can cause peak tailing. C_{18} column is preferred for mixture separation than C_8 column, as it gives higher resolution.

Different mobile phase systems of different composition and proportions were tried e.g. (methanol: water), (acetonitrile: water) (0.01 M KH_2PO_4-methanol-acetonitrile-isopropyl alcohol (420: 20: 30: 30, by volume) pH was adjusted to 7.9. However, complete separation of the two drugs in presence of PAP was achieved by using micro-emulsion prepared as men-
Method validation

Linearity

The linearity of the proposed methods was evaluated by analyzing eight concentrations ranging between 0.2-1.4 µg.mL⁻¹ for PAR, 0.3-3 µg.mL⁻¹ for ORP and 0.2-1.4 µg.mL⁻¹ for PAP in case of TLC-spectrodensitometric method and 5-200 µg.mL⁻¹ for PAR, 5-250 µg.mL⁻¹ for ORP and 5-2200 µg.mL⁻¹ for PAP in case of RP-HPLC method. Each concentration was repeated three times. The assay was performed according to the experimental conditions previously mentioned. The linearity of the calibration graphs were validated by the high value of the correlation coefficient and the low intercept value, (TABLE 1).

Precision

Repeatability

Repeatability of the results for concentrations of 0.3, 0.6, 0.9 µg.mL⁻¹ for each in case of TLC-spectrodensitometric method and 20.00, 40.00 and 60.00 µg.mL⁻¹ for each drug in case of RP-HPLC method were performed by three replicate determinations to estimate intra-day variation (TABLE 1). 

TABLE 1 : Results of assay validation parameters of the proposed methods for the determination of PAR, PAP and ORP in their mixture

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TLC-spectrodensitometric method</th>
<th>RP-HPLC method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAR</td>
<td>PAP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.2-1.4(µg.band⁻¹)</td>
<td>0.2-1.4(µg.band⁻¹)</td>
</tr>
<tr>
<td>Slope</td>
<td>1.1904</td>
<td>0.478</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.115</td>
<td>0.312</td>
</tr>
<tr>
<td>Correlation coefficient(r)</td>
<td>0.9992</td>
<td>0.9992</td>
</tr>
<tr>
<td>Accuracy (mean ± SD)</td>
<td>100.14±1.807</td>
<td>100.21±1.537</td>
</tr>
<tr>
<td>(RSD%)³</td>
<td>1.264</td>
<td>1.201</td>
</tr>
<tr>
<td>(RSD%)³</td>
<td>1.622</td>
<td>1.324</td>
</tr>
<tr>
<td>LOD</td>
<td>0.05(µg.band⁻¹)</td>
<td>0.05(µg.band⁻¹)</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.16(µg.band⁻¹)</td>
<td>0.155(µg.band⁻¹)</td>
</tr>
</tbody>
</table>

³Average of three determinations

In addition, the effect of pH was studied by using buffers of different pH values. It was found that pH = 3 was suitable for proper resolution and peak shape.

Finally, a satisfactory separation was obtained by using micro-emulsion as a mobile phase, maintaining the flow rate of 1 mL min⁻¹ with UV detection at 225 nm.

Table 2a: Results of accuracy for determination of pure PAR, PAP and ORP by the proposed spectrodensitometric method

<table>
<thead>
<tr>
<th>Taken(µg.band⁻¹)</th>
<th>Found(µg.band⁻¹)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR</td>
<td>PAP</td>
<td>ORP</td>
</tr>
<tr>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1.20</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>1.40</td>
<td>1.40</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Mean ±SD 100.14± 100.21± 98.81±
1.807 1.537 0.833

Average of three determinations

Table 2b: Results of accuracy for determination of pure PAR, PAP and ORP by the proposed RP-HPLC method

<table>
<thead>
<tr>
<th>Taken(µg.mL⁻¹)</th>
<th>Found(µg.mL⁻¹)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR</td>
<td>PAP</td>
<td>ORP</td>
</tr>
<tr>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>30.00</td>
<td>40.00</td>
<td>40.00</td>
</tr>
<tr>
<td>50.00</td>
<td>60.00</td>
<td>70.00</td>
</tr>
<tr>
<td>100.00</td>
<td>120.00</td>
<td>100.00</td>
</tr>
<tr>
<td>150.00</td>
<td>150.00</td>
<td>150.00</td>
</tr>
<tr>
<td>200.00</td>
<td>200.00</td>
<td>200.00</td>
</tr>
</tbody>
</table>

Mean ±SD 99.93 ± 99.89 ± 99.96 ±
0.431 0.365 0.439

Average of three determinations

Acknowledgment

This work was supported by the University Grants Commission, New Delhi.
TABLE 3: Application of standard addition technique to analysis of PAR and ORP in Orphamol® by the proposed methods

<table>
<thead>
<tr>
<th>Orphamol® tablet, Batch No.06524</th>
<th>TLC-spectrodensitometric method</th>
<th>RP-HPLC method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Taken (µg.mL⁻¹)</td>
<td>Found a (µg.mL⁻¹)</td>
</tr>
<tr>
<td>Par</td>
<td>0.200</td>
<td>0.198</td>
</tr>
<tr>
<td></td>
<td>0.400</td>
<td>0.402</td>
</tr>
<tr>
<td></td>
<td>0.600</td>
<td>0.600</td>
</tr>
<tr>
<td></td>
<td>0.800</td>
<td>0.809</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>0.200 ±0.896</td>
<td>100.16 ±0.896</td>
</tr>
<tr>
<td>ORP</td>
<td>0.350</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>0.800</td>
<td>0.804</td>
</tr>
<tr>
<td></td>
<td>1.000</td>
<td>1.006</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>0.350 ±0.892</td>
<td>100.55 ±0.892</td>
</tr>
</tbody>
</table>

aAverage of six determinations

TABLE 4: Determination of PAR and ORP in laboratory prepared mixtures by the proposed methods

<table>
<thead>
<tr>
<th>Mix. no. Ratio (PAR:ORP)</th>
<th>TLC-spectrodensitometric method</th>
<th>RP-HPLC method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAR</td>
<td>ORP</td>
</tr>
<tr>
<td></td>
<td>Taken (µg.mL⁻¹)</td>
<td>Found a (µg.mL⁻¹)</td>
</tr>
<tr>
<td>1 195: 15</td>
<td>195.00</td>
<td>194.94</td>
</tr>
<tr>
<td>2 130: 10</td>
<td>130.00</td>
<td>130.08</td>
</tr>
<tr>
<td>3 65: 5</td>
<td>65.00</td>
<td>65.10</td>
</tr>
</tbody>
</table>

bAverage of three determinations

Intermediate precision

Nine replicate determinations in different 3 days were used to estimate inter-day variation. The coefficient of variation at these concentration levels was calculated (TABLE 1).

Range

The calibration range was established through considerations of the practical range necessary according to adherence to linearity and the concentration of PAR, ORP and PAP to give accurate, precise and linear results (TABLE 1).

Detection and quantitation limits

According to the ICH recommendations, the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits, LOD = 3.3S.D./Slope, LOQ = 10×S.D./Slope (TABLE 1).

Accuracy

The accuracy of the results was checked by applying the proposed method for determination of different blind samples of PAR, ORP and PAP. The concentrations were calculated from the corresponding regression equations. The results obtained as shown in TABLE 2a & 2b.

Accuracy of the methods was assured by the use of the standard addition technique. The results obtained for PAR, ORP and PAP were compared with the expected results. The good recoveries of standard addition technique as shown in TABLE 3 suggested good accuracy of the proposed methods.

Specificity

Specificity of the proposed methods was achieved by the analysis of different laboratory prepared mixtures of PAR, ORP and PAP within the linearity range. Satisfactory results were shown in TABLE 4.

The proposed TLC-spectrodensitometric and RP-HPLC methods were successfully applied for the determination of PAR and ORP in their pharmaceutical formulation (Orphamol® tablets). Results obtained are...
TABLE 5: Statistical analysis of parameters required for system suitability testing of HPLC method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Obtained value</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Rs)</td>
<td>5.71</td>
<td>7.99 &gt;0.8</td>
</tr>
<tr>
<td>Capacity factor (K')</td>
<td>5.33</td>
<td>18.67 1-10 acceptable</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1.20</td>
<td>1.05 &lt; 1.52</td>
</tr>
<tr>
<td>Selectivity factor (α)</td>
<td>3.50</td>
<td>&gt; 1</td>
</tr>
<tr>
<td>Number of theoretical plate (N)</td>
<td>1444</td>
<td>2227 Increases with separation efficiency</td>
</tr>
<tr>
<td>HETP (cm.plate⁻¹)</td>
<td>0.017</td>
<td>0.011 The smaller the value, the higher the efficiency</td>
</tr>
</tbody>
</table>

shown in TABLE 3 which reveals that there is no interference by excipients and additives.

Robustness

The effect of different factors in case of TLC-spectrodetisitometric and RP-HPLC methods was studied to obtain the optimum parameters for complete separation.

System suitability testing for HPLC

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as whole. System suitability is used to ensure system performance before or during the analysis of the drugs. System suitability was checked by calculating the capacity factor (K'), tailing factor (T), column efficiency (N), the selectivity factor (γ) and resolution (Rs), where the system was found to be suitable as shown in TABLE 5.

CONCLUSION

The proposed methods are efficient for providing sensitive, accurate and reproducible quantitative analysis for the determination of PAR, ORP in bulk powder and in pharmaceutical formulation, without any interference from excipients or PAP.

TLC- spectrodensitometric method has the advantages that several samples can be run simultaneously using a small quantity of mobile phase and provides high sensitivity and selectivity. RP- HPLC method using micro-emulsion as mobile phase provides rapid, short analysis time, simple and selective method. Micro-emulsion has advantage over normal mobile phase in separation of compounds with close similar structure.

Statistical analysis was performed by comparing the results of the suggested procedures with those of manufacturer method. No significant difference was observed regarding accuracy and precision, as shown in TABLE 6. However, the proposed methods have the advantage of determining PAR and ORP in presence of PAP using single mobile phase instead of double mobile phase as the case with the manufacturer method.

The suggested methods provide selective, accurate and sensitive analytical procedures for the determination of PAR and ORP in presence of PAP. They are suitable for routine analysis and quality control of PAR and ORP in its pharmaceutical formulation.

REFERENCES