DEVELOPMENT AND VALIDATION OF FIRST ORDER DERivative SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF PREGABALIN, METHYLCOBAMIN, AND ALPHA LIPOIC ACID IN MULTICOMPONENT DOSAGE FORM

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S. J. Thakkar Pharmacy College, Rajkot, Gujarat, India

ABSTRACT: Objective: To develop and validate a novel and easy first order derivative spectroscopic method for the simultaneous determination of multicomponent dosage form which contains Methylcobalmine, Alpha lipoic acid and Pregabalin. Method: Measurement was achieved by selecting different Zero crossing points i.e ZCP for Methylcobalmine and Alpha lipoic acid at 436.24 nm, ZCP for Alpha lipoic acid and Pregabalin at 338.0 nm and ZCP for Pregabalin and Methylcobalmin at 307.03 nm. The method was validated according ICH guideline. The proposed method was applied for quantification of all three drugs in the marketed formulations. Results: The method was found linear in the range of 100-140µg/ml for Pregabalin, 1.14µg/ml for Methylcobalmine and 130-170µg/ml for Alpha lipoic acid respectively. The co-efficient co-relation was found to be 99.5 % for Pregabalin, 99.56 for Methylcobalmine and 99.61 for Alpha lipoic acid. The accuracy and precision were within acceptable limits. Conclusion: The method is simple, accurate, precise, rapid and cheap. The proposed method can be used for analysis of multicomponent marketed formulation.

INTRODUCTION: Pregabalin (PRG), (S)-3-((aminomethyl)-5-methylhexanoic acid (Fig.1) is an antiepileptic used in the treatment of peripheral neuropathy. Its molecular weight is 159.2 g/mol with empirical formula C₇H₁₇NO₂. Methylcobalamin (MCA), (1R,2R,4S,7S)-7-((2S)-3-hydroxy-2-phenylpropanol)oxy)-9,9-dimethyl-3-oxa-9-azioniatricyclo[3.3.1.0²,4]nonane (Fig.2) is vitamin supplement used in inadequacy of Vitamin-B₁₂. Its molecular weight is 1344.38 g/mol with empirical formula C₆₅H₇₁CoN₁₃O₁₄P₄. Alpha lipoic acid (ALA), (R)-5-(1,2-dithiolan-3-yl)pentanoic acid (Fig. 3) ⁵. It is an universal reducing agent which prevents oxidative damage of brain cells ⁶. Its molecular weight is 1344.38 g/mol with empirical formula C₆₅H₇₁CoN₁₃O₁₄P⁵.

Combination of PRG, MCA and ALA treats the problems related to all types of neuropathy and epilepsy ⁷. PRG is manifested to be very efficacious and well permitted in the treatment of neurogenic dysaesthesia. MCA increases myelin sheath formation thereby reforms brain cells ⁶. ALA is also used as universal antioxidant which disallows oxidative damage of brain cells ⁷. The marketed formulation is NERVUP PG which contains pregabalin 75 mg, methylcobalamin 750...
mcg and alpha lipoic acid 100 mg. Literature survey revealed that only few HPLC methods for the analysis of simultaneous estimation of Pregabalin, Methylcobalamin and Alpha lipoic acid have been reported.

In the present work we are focused to accomplish the optimum chromatographic conditions for the simultaneous determination of PRG, MCA and ALA in the combined dosage form. The developed method can be implemented successfully as quality control tool. The method was validated as per ICH guideline to access the replicability and extensive practicability of the developed method.

**Material Procurement:**
Pregabalin, Methylcobalamin and Alpha lipoic acid was procured as a gift sample from Alembic Pharmaceuticals, Vadodara, Influx Pharmaceuticals, Gandhinagar, Sunvij drugs ltd., Vadodara. NERVUP PG was purchased from local pharmacy. Analytical Grade solvents were procured from Merck, Mumbai.

**Instruments and Equipment:**

**UV Spectrophotometer:**
- Make and Model: Shimadzu UV 1800
- Type: Double Beam Spectrophotometer
- Scanning Speed: Fast, Medium, Slow
- Cuvettes: Matched Quartz cuvettes pair with 1 cm path length

**Electronic Weighing Balance:**
- Make and Model: Shimadzu, Japan; AUX220
- Capacity: MAX.: 220 gm; Min: 10 mg
- Readability (Deviation): 0.1 mg

**First Order Derivative Spectrophotometric Method:**

**Selection of Suitable Wavelengths for Analysis:**
Solutions of PRG (10 μg/ml), MCA (10 μg/ml) and ALA (10 μg/ml) were derivatized using 200 μg/ml Bromocresol green reagent in water. Solutions containing appropriate concentration of PRG, MCA and ALA in water were scanned using UV spectrophotometer in “Spectrum mode” in the range of 800 - 200 nm and their spectra were stored in computer.

Spectra were converted to first order derivative spectra (Δλ = 10 and scaling factor = 15) using UV Probe software (Ver.2.33). First order derivative spectra of drugs were overlaid. From overlaid spectra ZCP of both the drugs were selected as analytical wavelengths for detection.
Preparation of Standard Solutions:

Preparation of Derivatizing agent (BCG solution): 200μg/ml of bromocresol green was prepared by accurately weighing 200 mg and dissolving in 100 ml water.

Preparation of PRG standard stock solution: Accurately weighed 10 mg of PRG was transferred to 10 ml volumetric flask, diluted up to the mark with water to give a stock solution having strength of 1000 μg/ml.

Preparation of MCA standard stock solution: Accurately weighed 10 mg of MCA was transferred to 10 ml volumetric flask, diluted up to mark with water to obtain final concentration of 1000 μg/ml MCA.

Preparation of ALA standard stock solution: Accurately weighed 10 mg of ALA was transferred to 10 ml volumetric flask, diluted up to the mark with water to give a stock solution having strength of 1000 μg/ml.

Preparation of Calibration Curves:
Spectra of prepared tertiary mixture containing concentration 100 + 1 + 120, 110 + 1.1 + 130, 120 + 1.2 + 140, 130 + 1.3 + 150 and 140 + 1.4 + 160 μg/ml PEG + MCA + ALA were recorded in the range of 200 to 800 nm using BCG solution prepared in water as blank. Spectra were converted to first order derivative spectra (Δλ = 10 and scaling factor = 15) using UV Probe software (Ver.2.33). Amplitude (dA/dλ) of both the drugs was measured at selected wavelengths. PRG was measured at 436.24 nm (ZCP of ALA and MCA), ALA was measured at 307.03 nm (ZCP of PRG and MCA) and MCA was measured at 338 nm (ZCP of PRG and ALA). Standard calibration curves of dA/dλ against concentration were plotted.

Preparation of Sample Solution:
Test solution of 7500 μg/ml of PRG, 750 μg/ml of MCA, 1000 μg/ml of ALA were taken for analysis of PRG, MCA and ALA.

RESULTS AND DISCUSSION:

Validation of Proposed Method:
Validation of developed method was carried out according to ICH guideline for validation of Analytical Procedure Q2(R1):

Preparation of Calibration Curves:
Spectra of prepared tertiary mixture containing concentration 100 + 1 + 120, 110 + 1.1 + 130, 120 + 1.2 + 140, 130 + 1.3 + 150 and 140 + 1.4 + 160 μg/ml PEG + MCA + ALA were recorded in the range of 200 to 800 nm using BCG solution prepared in water as blank. Spectra were converted to first order derivative spectra (Δλ = 10 and scaling factor = 15) using UV Probe software (Ver.2.33). Amplitude (dA/dλ) of both the drugs was measured at selected wavelengths. PRG was measured at 436.24 nm (ZCP of ALA and MCA), ALA was measured at 307.03 nm (ZCP of PRG and MCA) and MCA was measured at 338 nm (ZCP of PRG and ALA). Standard calibration curves of dA/dλ against concentration were plotted.

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Preparation of Sample Solution:
Test solution of 7500 μg/ml of PRG, 750 μg/ml of MCA, 1000 μg/ml of ALA were taken for analysis of PRG, MCA and ALA.
Limit of detection (LOD) and limit of quantitation (LOQ):
LOD and LOQ were calculated from the data obtained from the linearity studies. For each of the six replicate determinations, slope and y-intercept of the plot was determined. Average of slope (s) and standard deviation of the y intercept (σ) were computed. From these values, the parameter LOD and LOQ were determined using following equations (On the basis of response and slope of the regression equation):

\[
LOD = \frac{3.3 \times SD}{Slope}
\]

\[
LOQ = \frac{10 \times SD}{Slope}
\]

Where; \( \sigma \) = Standard deviation of response
\( S \) = Slope of calibration curve

TABLE 3: RESULT OF LOD, LOQ BY FIRST ORDER DERIVATIVE METHOD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PRG</th>
<th>MCA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>5.0915 µg/ml</td>
<td>0.01893 µg/ml</td>
<td>5.4640 µg/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>15.4290 µg/ml</td>
<td>0.05737 µg/ml</td>
<td>16.5576 µg/ml</td>
</tr>
</tbody>
</table>

Accuracy:
Accuracy was calculated by addition of standard drug to preanalyzed sample at 3 different concentration level and computing percentage recoveries. Accurately weighed content equivalent to 750 mg PRG, 0.75 mg MCA and 100 mg ALA were taken according to amount given in Table 5. To that amount of standard added according to Table 5, 0.15 ml was taken from above solution to have 80%, 100% and 120% level and AUC were determined. Percentage recovery were found and it was obtained between 98 – 102 %. Accuracy was assessed using 9 determinations over 3 concentration level covering the specific range (e.g., 3 concentration and 3 replicates each of the total analytical procedure).

Sample solutions for accuracy were prepared as follows:

TABLE 4: ACCURACY STUDY OF FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

<table>
<thead>
<tr>
<th>Level</th>
<th>Amount of sample taken in (mg)</th>
<th>Amount of standard added</th>
<th>Amount of sample taken in (mg)</th>
<th>Amount of standard added</th>
<th>Amount of sample taken in (mg)</th>
<th>Amount of standard added</th>
<th>PRG</th>
<th>MCA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>45</td>
<td>15</td>
<td>0.45</td>
<td>0.15</td>
<td>60</td>
<td>20</td>
<td>60</td>
<td>0.54</td>
<td>80</td>
</tr>
<tr>
<td>100%</td>
<td>45</td>
<td>30</td>
<td>0.45</td>
<td>0.30</td>
<td>60</td>
<td>40</td>
<td>75</td>
<td>0.68</td>
<td>100</td>
</tr>
<tr>
<td>120%</td>
<td>45</td>
<td>45</td>
<td>0.45</td>
<td>0.45</td>
<td>60</td>
<td>60</td>
<td>90</td>
<td>0.82</td>
<td>120</td>
</tr>
</tbody>
</table>

Standard solution of PRG (1000 µg/ml): Weigh accurately 10mg of PRG transferred in to 10ml volumetric flask, made up to mark with methanol.

Standard solution of PCM (1000 µg/ml): Weigh accurately 10mg of MCA transferred in to 10ml volumetric flask, made up to mark with methanol.

Standard solution of ALA (1000 µg/ml): Weigh accurately 10mg of ALA transferred in to 10ml volumetric flask, made up to mark with methanol.

Precision:
Precision of method was computed by two means: Repeatability and Intermediate precision.

Repeatability: System Precision and Method Precision:

System Precision:
Solution containing mixture of 120 µg/ml of PRG, 1.2 µg/ml of MCA and 140 µg/ml of ALA (100% Test concentration) were prepared from their respective stock solution. Prepared solution were analysed six times as per the proposed method. The mean amplitude (dA/dλ) with its standard deviation were computed for both the drugs.

Method Precision:
Six replicate solution containing mixture of 120µg/ml PRG, 1.2 µg/ml MCA and 140 µg/ml ALA were prepared from their respective stock solution. Prepared solution were analysed as per the proposed method. The mean % labelled claim with its standard deviation and % relative standard deviation were computed for both the drugs.
Intermediate Precision: Intraday and interday precision was determined in terms of % RSD. Intraday precision was determined by analysing PRG, MCA and ALA in combined solution at three independent concentration range of their respective calibration range for three days.

Procedure for Intraday Precision: Combined solution containing the mixture of PRG, MCA and ALA as 120, 1.2 and 140 µg/ml (100% Test concentration) were prepared as described in section 5.2.1.5 were analysed on 3 times on the same day and %RSD were calculated.

Preparation for Interday Precision: Combined solution containing the mixture of PRG, MCA and ALA 120, 1.2 and 140 µg/ml (100% Test concentration) were prepared as described in section 5.2.1.5 were analysed 3 times on 3 different days and %RSD were calculated.

Robustness:
Solution containing mixture of 120 µg/ml PRG, 1.2 µg/ml MCA and 140 µg/ml ALA was prepared from their respective sample solution prepared as per described in section 5.2.1.5. Prepared solution was analyzed as per proposed method with small but

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### TABLE 5: RESULT OF REPEATABILITY BY FIRST ORDER DERIVATIVE METHOD

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Concentration (µg/ml)</th>
<th>PRG</th>
<th>MCA</th>
<th>ALA</th>
<th>PRG</th>
<th>MCA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120</td>
<td>1.2</td>
<td>150</td>
<td>-0.0521</td>
<td>-0.0914</td>
<td>0.0254</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>120</td>
<td>1.2</td>
<td>150</td>
<td>-0.0529</td>
<td>-0.0936</td>
<td>0.0251</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>1.2</td>
<td>150</td>
<td>-0.0514</td>
<td>-0.0926</td>
<td>0.0250</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>1.2</td>
<td>150</td>
<td>-0.0526</td>
<td>-0.0921</td>
<td>0.0252</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>1.2</td>
<td>150</td>
<td>-0.0531</td>
<td>-0.0918</td>
<td>0.0259</td>
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</tr>
<tr>
<td>6</td>
<td>120</td>
<td>1.2</td>
<td>150</td>
<td>-0.0517</td>
<td>-0.0949</td>
<td>0.0253</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 6: RESULT OF INTRADAY PRECISION FOR FIRST ORDER DERIVATIVE METHOD

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Mean dA/dλ ± S.D (n= 3)</th>
<th>% RSD</th>
<th>Conc. (µg/ml)</th>
<th>Mean dA/dλ ± S.D (n= 3)</th>
<th>% RSD</th>
<th>Conc. (µg/ml)</th>
<th>Mean dA/dλ ± S.D (n= 3)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>-0.0378 ± 0.0006</td>
<td>1.7388</td>
<td>1</td>
<td>-0.0775 ± 0.0012</td>
<td>1.6473</td>
<td>130</td>
<td>0.0071 ± 0.0001</td>
<td>1.7129</td>
</tr>
<tr>
<td>120</td>
<td>-0.0513 ± 0.0006</td>
<td>1.2968</td>
<td>1.2</td>
<td>-0.0925 ± 0.0011</td>
<td>1.1904</td>
<td>150</td>
<td>0.0253 ± 0.0003</td>
<td>1.2594</td>
</tr>
<tr>
<td>140</td>
<td>-0.0560 ± 0.0007</td>
<td>1.3410</td>
<td>1.4</td>
<td>-0.1048 ± 0.0014</td>
<td>1.3393</td>
<td>170</td>
<td>0.0205 ± 0.0002</td>
<td>1.4137</td>
</tr>
<tr>
<td>Average % RSD</td>
<td>1.3138</td>
<td></td>
<td>Average % RSD</td>
<td>1.3923</td>
<td></td>
<td>Average % RSD</td>
<td>1.4620</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 7: RESULT OF INTER DAY PRECISION FOR FIRST ORDER DERIVATIVE METHOD

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Mean dA/dλ ± S.D (n= 3)</th>
<th>% RSD</th>
<th>Conc. (µg/ml)</th>
<th>Mean dA/dλ ± S.D (n= 3)</th>
<th>% RSD</th>
<th>Conc. (µg/ml)</th>
<th>Mean dA/dλ ± S.D (n= 3)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>-0.0378 ± 0.0006</td>
<td>1.7388</td>
<td>10</td>
<td>-0.0771 ± 0.0013</td>
<td>1.7953</td>
<td>120</td>
<td>0.0071 ± 0.0001</td>
<td>1.4478</td>
</tr>
<tr>
<td>120</td>
<td>-0.0523 ± 0.0006</td>
<td>1.2961</td>
<td>12</td>
<td>-0.0927 ± 0.0013</td>
<td>1.4077</td>
<td>150</td>
<td>0.0253 ± 0.0003</td>
<td>1.3965</td>
</tr>
<tr>
<td>140</td>
<td>-0.0616 ± 0.0011</td>
<td>1.8856</td>
<td>14</td>
<td>-0.1051 ± 0.0017</td>
<td>1.6447</td>
<td>160</td>
<td>0.0443 ± 0.0006</td>
<td>1.4733</td>
</tr>
</tbody>
</table>

Average % RSD | 1.6401 | Average % RSD | 1.6159 | Average % RSD | 1.4992 |
deliberate change in spectroscopic conditions as listed below:

i. Scanning speed: Fast, Medium and Slow

ii. Methanol from different manufacturers. Methanol GR Grade: Merck ltd., India, Qualigens Fine Chemicals Pvt. Ltd., India

The mean % labeled claim with its standard deviation and % relative standard deviation was computed at each level.

<table>
<thead>
<tr>
<th>Robustness</th>
<th>PRG % RSD</th>
<th>MCA % RSD</th>
<th>ALA % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Scanning speed</td>
<td>1.5297</td>
<td>1.4156</td>
<td>1.5109</td>
</tr>
<tr>
<td>Change in BCG brand</td>
<td>1.4560</td>
<td>1.3867</td>
<td>1.4289</td>
</tr>
</tbody>
</table>

Analysis of Pharmaceutical Dosage Form:
Twenty capsules were weighed accurately and their average weight was determined. The capsule content equivalent to 75 mg PRG, 0.75 mg MCA and 100 mg of ALA were weighed and transferred to 10 ml volumetric flask. To this flask, 5 ml of water was added and the flask was sonicated for 5 min. The volume was adjusted up to the mark with water. The solution was then filtered through Whatman filter paper no. 41μm. Filtrate contained mixture of 120μg/ml PRG, 1.2μg/ml MCA and 140μg/ml ALA. An aliquots of 0.12 ml of the filtrate solution was suitably diluted to 10 ml with water to get a final concentration of 120μg/ml of PRG, 1.2μg/ml of MCA and 130 μg/ml of ALA. Spectrum of prepared solution was recorded in spectroscopic condition.

Spectrum was converted to first order derivative spectrum (Δλ = 10, scaling factor = 15) using UV Probe software (Ver.2.33). Amplitudes (dA/dλ) of PRG, MCA and ALA were measured at 252.58 nm and 369.84 nm respectively. Concentrations of PRG, MCA and ALA were computed by putting value of their amplitudes in respective standard regression equation obtained from calibration curve.

The analysis procedure was repeated six times with tablet dosage form.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Labelled Claim (mg)</th>
<th>Amount obtained (mg)</th>
<th>% Labelled claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRG</td>
<td>MCA</td>
<td>ALA</td>
<td>PRG</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>0.75</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>0.75</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>0.75</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>0.75</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>0.75</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>0.75</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Label claim</th>
<th>% Labelled claim</th>
<th>SD (n=6)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRG</td>
<td>75 mg</td>
<td>99.6266</td>
<td>1.0833</td>
<td>1.4531</td>
</tr>
<tr>
<td>MCA</td>
<td>0.75 mg</td>
<td>99.5983</td>
<td>1.9357</td>
<td>1.9357</td>
</tr>
<tr>
<td>ALA</td>
<td>100 mg</td>
<td>100.2083</td>
<td>1.5053</td>
<td>1.5147</td>
</tr>
</tbody>
</table>

FIG.4: FIRST ORDER SPECTRUM OF CAPSULE FORMULATION BY FIRST ORDER DERIVATIVE METHOD
CONCLUSION: The developed method was established to be precise accurate, linear, robust and specific for determination of PRG, MCA and ALA. The method was developed and validated as per ICH guideline and all parameters tested were found to be within limits.

ACKNOWLEDGEMENT: I am thankful to Alembic Pharmaceuticals, Vadodara and Influx Pharmaceuticals, Gandhinagar, Sunvij drugs ltd., Vadodara for providing gift samples of Pregabalin, Methylcobalamin and Alpha lipoic acid for research.

REFERENCES:


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