A pragmatic approach to the analysis of a combination formulation

Noshin Mubtasim a, Eva Rahman Kabir a,*, Ashis Kumar Podder a, Subrata Bhadra b

a Department of Pharmacy, BRAC University, Dhaka, Bangladesh
b Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka, Bangladesh

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a Department of Pharmacy, BRAC University, Dhaka, Bangladesh
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KEYWORDS
Rosuvastatin calcium; Amlodipine besylate; Compatible; Combination formulation; Method validation

Abstract The aim of the paper was to formulate a combined oral dosage form of rosuvastatin calcium and amlodipine besylate and to develop and validate an analytical method to be adopted for both routine quality control assay and in vitro dissolution studies of the formulation.

The proposed combination formulation has shown compatibility with the chosen excipients, verified through FT-IR study. A novel gradient RP-HPLC method was developed and validated according to the ICH guideline which was found to be suitable for the simultaneous estimation of rosuvastatin calcium and amlodipine besylate from the formulation. The retention time of 2.7 and 6.08 min allows the analysis of large amount of samples with less mobile phase which makes the method economic. The dissolution profiles of both the drugs in different dissolution medium were encouraging which makes the combination formulation of rosuvastatin calcium and amlodipine besylate superior and effective in achieving patient compliance.

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Abbreviations: RP-HPLC, reverse phase high performance liquid chromatography; THF, tetrahydrofuran; CVD, cardiovascular disease; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme-A; Ca2+, calcium; PDA, photo diode array; LC, liquid chromatography; FT-IR, Fourier Transform Infrared spectroscopy; IR, infrared; µg, microgram; ml, milliliter; FDA, Food and Drug Administration; USP, United States Pharmacopeia; µl, microliter; % RSD, percentage relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation; BP, British Pharmacopeia; ICH, International Conference on Harmonization

* Corresponding author at: Flat 503, Concord Nessa, House 16, Road 59, Gulshan, Dhaka, Bangladesh. Tel.: +880 1714073632.
E-mail address: eva.kabir@gmail.com (E.R. Kabir).
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1. Introduction

Cardiovascular diseases such as coronary heart disease, cerebrovascular disease, atherothrombosis, ischemic heart disease, and peripheral arterial disease are found to be prevalent among different age groups of people especially among the young generation. According to a report by Saquib et al., the death rate from cardiovascular diseases (CVD) would be 4 times higher in 2010 and 21 times higher in 2025 compared to its corresponding rate in 2003 (Saquib et al., 2012). Hypertension and dyslipidemia are important, modifiable cardiovascular (CV) risk factors that frequently coexist, and together have an effect on CV risk that may be greater than expected from the simple addition of the risk associated with each condition (Blank et al., 2005).

2. Need of combination therapy

Novel drug delivery systems are constantly being developed for various purposes such as the expansion of markets and indications, the extension of product life cycles, or the generation of opportunities. Even after advancement in the management of cardiovascular diseases (CVD) during the last several years, they are still the main cause for morbidity and mortality (Gowda et al., 2012). Many hypertensive symptoms of hyperlipidemic patients may be reduced using the combination formulation of antihyperlipidemic and antihypertensive agents. Combined dosage form of two or more drugs has been proven useful in multiple therapies as they offer better patient compliance than a single drug. It is well recognized that a single drug, even when used in maximal recommended dosage will control no more than 50% of a hypertensive population (Shaikh et al., 2010). On the other hand, the skillful use of two or more agents in combination can improve hypertension control rates to well above 80% (Shaikh et al., 2010). Therefore, the rational for combination therapy is to encourage the use of lower doses of drug to reduce patient’s blood pressure with the goal to minimize dose dependent side effects and adverse reactions (Atram et al., 2009). The fixed-dose combination containing the antihypertensive agent amlodipine and the cholesterol lowering agent atorvastatin is the first combination of its kind designed to treat two risk factors for cardiovascular disease (Bashir et al., 2011). Atorvastatin has rapid access to non-hepatic tissues due to the hydrophobicity which results in some undesirable side effects. These unwanted side effects associated with combined dosage of atorvastatin and amlodipine may be reduced when rosuvastatin is used in place of atorvastatin. An assortment of techniques has been described for the quantification of rosuvastatin alone or in combination with other products (Gowda et al., 2012). The reverse phase-high performance liquid chromatography (RP-HPLC) methods described for simultaneous determination of rosuvastatin and amlodipine in pharmaceutical preparations (Banerjee and Vasava, 2013; Tajane et al., 2012) however, is not developed for in vitro dissolution profile of rosuvastatin calcium and amlodipine besylate. Therefore, a simple, accurate, efficient and reproducible reverse phase HPLC method has been developed and validated for the simultaneous determination of rosuvastatin calcium and amlodipine besylate at 240 nm in combined tablet dosage form and has been applied successfully for in vitro dissolution studies.

Rosuvastatin, chemically described as bis [(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-methyl (methyl-sulphonyl) amino] pyrimidin-5-yl] (3R, 5S) -3, 5-dihydroxyhept-6-enolic acid] (Fig. 1), is another member of the drug class statin. It is hydrophilic and this makes it hepatoselective. This drug may thus be considered as a substitute of atorvastatin to formulate a new combination of drug for dose-related reduction in systolic blood pressure, diastolic blood pressure and low density lipoprotein cholesterol in patients with co-morbid hypertension and dyslipidemia. It competitively inhibits HMG-CoA reductase enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early rate-limiting step in cholesterol biosynthesis (Reddy et al., 2011).

Amlodipine besylate, chemically described as 3-ethyl-5-methyl(±)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5 pyridinedicarboxylate, monobenzenesulphonate (Fig. 2), is a long-acting dihydropyridine class of calcium channel blocker, approved for treating hypertension and both vasospastic and chronic, stable angina (Blank et al., 2005). It selectively inhibits the transmembrane influx of Ca2+ ion across L-type calcium channels, without changing serum calcium concentration. Thus it relaxes the muscles lining the arteries and lowers blood pressure. It also expands coronary arterioles which increases the flow of blood to the heart and prevents heart pain (angina) resulting from reduced flow of blood to the heart that is caused by coronary artery spasm (contraction). It is more vasoselective with lower negative inotropic effects and reflex tachycardia is less prominent since fluctuations in plasma levels are less pronounced with these agents (Drug information reference, 2003).

3. Materials and methods

The present research started with the development of a proposed combination formulation of a statin with a calcium channel blocker. Excipients, used for the preparation of the combined formulation tablets of rosuvastatin and amlodipine, were initially chosen on the basis of the existing formulation of atorvastatin and amlodipine and their compatibility with the
active ingredients verified using FTIR study. When the results came positive, the proposed formulation of the statin with the calcium channel blocker was adopted as the combination formulation and this was further studied. An assay based method was then developed and validated for the simultaneous estimation of rosuvastatin and amlodipine from the combination formulation which has been used further to characterize the in vitro dissolution profile of rosuvastatin calcium and amlodipine besylate.

3.1. Chemical and pharmaceutical preparation

Reference standard of rosuvastatin calcium and amlodipine besylate was donated by the two of the leading local pharmaceutical companies of Bangladesh, Square Pharmaceuticals Limited, Bangladesh and Eskayef Bangladesh Limited, which are certified to be 96.36% and 99.33% pure respectively. The test tablets of the combination formulation of 10 mg rosuvastatin calcium and 5 mg amlodipine besylate used were manufactured in-house. The excipients needed to make the tablets were gifted from Eskayef Bangladesh Limited and ACI Limited. The commercially available preparation of rosuvastatin (label claim rosuvastatin calcium INN equivalent to rosuvastatin 10 mg) and amlodipine (label claim 5 mg amlodipine) used in the analysis, were collected from the local market. The water used for the preparation of buffer was purified by distillation. All the solvents used for the study were of HPLC grade.

3.2. Instrumentation

The HPLC system consisted of a high pressure binary gradient pump (LC-20AT; Shimadzu), SIL-20AHT auto sampler, CTO-10ASvp column temperature oven, SPD-M20A PDA detector has been used for doing all the experiments including the development and its subsequent validation. All the components of the system are controlled by using CBM-20 Alite system controller. Data acquisition was done using lab solution LC workstation multi PDA software. The dissolution test was carried out using Universal Dissolution Tester (model: UDT 804-B).

3.3. Compatibility studies

The drug-excipient compatibility studies were done to select the excipients that are physically and chemically compatible with the API, using Fourier Transform Infrared spectroscopy. This was done by separately mixing each drug entity with the individual excipient in the ratio of 1:1. A separate FT-IR study of the standard sample of rosuvastatin calcium and amlodipine besylate was also done. The IR spectrum exhibiting the transmittance of different functional groups of the pure sample of rosuvastatin and amlodipine within 4000–400 cm⁻¹ region was checked, studied and recorded (Figs. 3 and 4) and their comparison with the IR spectrum exhibiting transmittance of those same functional groups was done in presence of each of the excipients individually (Tables 1 and 2).

3.4. HPLC method

A reversed phase HPLC system was used to analyze both compounds with a sufficient separation and a fine peak shape owing to the relatively nonpolar properties of rosuvastatin calcium and amlodipine besylate. Therefore, all the experiments were carried out on a Luna 5μ C18 column (250 mm × 4.60 mm) at ambient temperature using different conditions of various mobile phases systematically. The mobile phase systems that were initially fixed after extensive literature
review, focusing on the gradient elution of rosuvastatin and amlodipine, are as follows:

(i) Phosphate buffer (pH 2.5): acetonitrile in the ratio 55:45% v/v (Banerjee and Vasava, 2013).
(ii) Acetonitrile: THF: water at pH 3 in the ratio 68:12:20% v/v (Tajane et al., 2012).

The suitable wavelength for detection of rosuvastatin calcium and amlodipine besylate was selected from the overlain spectrum of rosuvastatin and amlodipine.

3.5. Preparation of solutions for assay

3.5.1. Standard preparation

Standard stock solution of rosuvastatin and amlodipine was prepared by dissolving 25 mg rosuvastatin and 12.5 mg amlodipine respectively with a small quantity of mobile phase into a clean dry 100 ml volumetric flask. It was then sonicated for 20 min and the final volume of the solution was then made up to 100 ml with the mobile phase. 4 ml solution was taken into 100 ml volumetric flask to obtain a concentration of 10 l g/ml rosuvastatin and 5 l g/ml amlodipine.

3.5.2. Sample preparation

A total of 20 tablets were accurately weighed and powdered in a clean dry mortar. An amount equivalent to 10 mg of rosuvastatin and 5 mg of amlodipine was taken in a conical flask and dissolved in small quantity of mobile phase with the aid of ultrasonication for 15 min. The resultant solution was then filtered, through Whatman filter paper, into a clean, dry 100 ml volumetric flask and the final volume was made up to 100 ml with the mobile phase. From the solution, 1 ml was taken out into 10 ml volumetric flask and dilution was done with the mobile phase.

### Table 1 FT-IR study of rosuvastatin calcium (standard) and its comparison with the mixed sample of rosuvastatin calcium and individual excipient.

|                         | O—H stretching | Dual response | S═O stretching |
|-------------------------|----------------|---------------|---------------|
| ALCOHOL                 | 3420.87        | 2969.55       | 2928.04       |
| Broad & strong          | 3550–3200      | 2968.55       | 2931.90       |
|                         |                | 2966.62       | 2930.93       |
| Rosuvastatin calcium    |                | 2968.55       | 2930.93       |
| RSV + pregelatinized modified starch | 3440.16 | 2934.79 | 2916.47 |
| RSV + microcrystalline cellulose | 3433.41 | 2969.51 | 2915.50 |
| RSV + sodium starch glycolate | 3420.87 | 2952.15 | 2915.50 |
| RSV + colloidal SiO₂    | 3421.83        | 2956.97       | 1156.36       |
| RSV + butylated hydroxyanisole | 3428.76 | 2956.97 | 1156.36 |

Figure 4 FT-IR study of amlodipine besylate standard.
phase to get a concentration of 10 \mu g/ml rosuvastatin and 5 \mu g/ml amlodipine. From this solution further dilutions were done and injected into the system to get the chromatogram.

### 3.6. Method validation

The suggested RP-HPLC method was validated with respect to the corresponding parameters such as linearity, accuracy, precision, sensitivity, ruggedness, and robustness according to USP and ICH guidelines.

### 3.7. In-vitro dissolution study

The *in vitro* dissolution study of the combined formulation of rosuvastatin calcium and amlodipine besylate, was carried out using USP-type II dissolution test apparatus. The drug release study was conducted using two different dissolution media to ascertain their percentage of release according to the respective dissolution profile mentioned in FDA reports. For the study of dissolution profile of rosuvastatin, 900 ml 0.05 M sodium citrate buffer of pH 6.6 was used as the dissolution medium where agitation speed of 50 rpm was maintained at (37 ± 0.5) °C for 60 min; and for amlodipine 500 ml 0.01 N HCl was used as dissolution medium with agitation speed of 75 rpm, maintained also at temperature (37 ± 0.5) °C for 60 min. Aliquots of about 10 ml had been withdrawn after 10, 20, 30, 45 and 60 min and filtered. The filtrates were then finally filtered through 0.2 \mu disk filter and prepared vials were analyzed with the validated RP-HPLC method for assay. The dissolution profile of the

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**Table 2** FT-IR study of amlodipine besylate (standard) and its comparison with the mixed sample of amlodipine besylate and individual excipients.

|                      | N–H stretching Medium | N–H stretching Primary amine | C–H stretching Secondary amine | C–O stretching Strong α, β-unsaturated ester | S–O stretching Strong Sulfone |
|----------------------|-----------------------|------------------------------|---------------------------------|---------------------------------------------|-----------------------------|
| Amlodipine besylate  | 3300.31               | 3157.58                      | 3069.81                         | 1696.45                                    | 1125.5                      |
| AMD besylate + pregelatinized modified starch | 3285.85               | 3155.65                      | 3066.92                         | 1696.45                                    | 1125.5                      |
| AMD besylate + microcrystalline cellulose | 3420.91               | 3169.15                      | 3066.92                         | 1696.45                                    | 1125.5                      |
| AMD besylate + sodium starch glycolate | 3291.63               | 3155.65                      | 3083.31                         | 1696.45                                    | 1125.30                     |
| AMD besylate + colloidal SiO2 | 3290.76               | 3155.67                      | 3085.61                         | 1696.45                                    | 1125.5                      |
| AMD besylate + butylated hydroxyanisole | 3329.25               | 3154.68                      | 3068.85                         | 1696.45                                    | 1125.5                      |
| AMD besylate + Mg stearate | 3292.60               | 3164.33                      | 3066.92                         | 1696.45                                    | 1125.5                      |

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**Figure 5** Chromatogram of rosuvastatin calcium and amlodipine besylate reference standard.

**Table 3** System suitability study of rosuvastatin calcium.

| Rosuvastatin calcium | Tailing factor | Theoretical plate | Peak area | Retention time |
|----------------------|----------------|-------------------|-----------|----------------|
| Average              | 1.153          | 6359              | 140,766   | 6.187          |
| STD                  | 0.017          | 36.73             | 33.13     | 0.006          |
| RSD (%)              | 1.45           | 0.578             | 0.024     | 0.089          |

**Table 4** System suitability study of amlodipine besylate.

| Amlodipine besylate | Tailing factor | Theoretical plate | Peak area | Retention time |
|---------------------|----------------|-------------------|-----------|----------------|
| Average             | 1.035          | 10,737            | 160,458   | 2.594          |
| STD                 | 0.003          | 18.97             | 313.42    | 0.002          |
| RSD (%)             | 0.28           | 0.177             | 0.195     | 0.082          |
combination formulation tablets of rosuvastatin and amlodipine was compared with that of separate commercial preparations of amlodipine and rosuvastatin alone.

4. Result & discussion

4.1. IR spectral analysis

The compatibility study of rosuvastatin calcium and amlodipine besylate with the selected excipients came out positive which enabled us to adopt the formula to formulate the combination dosage form.

4.2. Chromatographic conditions

The mobile phase composition of phosphate buffer (pH 2.5) and acetonitrile in the ratio 55:45% v/v that was set at a flow rate of 1.5 ml/min was chosen because it was found optimal to resolve the peak at 240 nm with retention time 2.7 min and 6.08 min for amlodipine and rosuvastatin respectively (Fig. 5). 10 µl samples were injected at each run.

4.3. Method validation

4.3.1. System suitability test

Freshly prepared samples of rosuvastatin calcium and amlodipine besylate were injected six times into the chromatographic system under the optimized chromatographic conditions to check all the important parameters such as column efficiency (theoretical plates), peak tailing, retention factor, and resolution. The % RSD for the peak area (Tables 3 and 4) response was found to be less than 2%.

4.3.2. Linearity

The linearity of calibration curves was established by plotting a graph between concentrations versus corresponding peak area of the sample (Figs. 6 and 7) and co-relation coefficient, slope and y-intercept were determined. Five different concentrations of sample solutions were prepared in the concentration range of 80%, 90%, 100%, 110%, and 120% from the standard stock solution of rosuvastatin calcium and amlodipine besylate and injected into the HPLC system. The detector response was found to be linear with 8 µg/ml to 1.2 µg/ml concentration of rosuvastatin calcium and the 4 µg/ml to 6 µg/ml concentration of amlodipine besylate. The co-relation coefficient was found to be 0.992 for rosuvastatin and 0.995 for amlodipine (Table 5).

4.3.3. Accuracy

The accuracy of the assay method was evaluated with the recovery of the standards from excipients (Tajane et al., 2012). Accuracy was carried out at three concentrations i.e. 80%, 100% and 120% of the target concentration of both the drugs. The concentration of solutions were prepared and injected six times. The mean percentage of recovery (Table 5) for both the drugs was found to range from 98% to 102% for both rosuvastatin and amlodipine which suggests the accuracy of the method for their simultaneous estimation.

4.3.4. Precision

The intraday and interday precisions were assessed by multiple sampling of homogenous sample of 10 µg/ml rosuvastatin calcium and of 5 µg/ml amlodipine besylate. The percentage

| Validation parameters | Rosuvastatin calcium | Amlodipine besylate |
|-----------------------|---------------------|---------------------|
| **Linearity**         | Linear equation     | $y = 16237620x - 3055.68$ |
|                       | Correlation coefficient ($R^2$) | $0.992949109$       |
| **Accuracy**          | % of recovery       | 80%: 102.88%        |
|                       |                     | 100%: 101.97%       |
|                       |                     | 120%: 98.67%        |
| **Precision**         | Interday precision peak area (%RSD) | 0.099 | 0.222 |
|                       | Intraday precision peak area (%RSD) | 0.099 | 0.149 |
| **Ruggedness**        | Peak area (%RSD)    | Analyst 1: 1.187    |
|                       |                     | Analyst 2: 1.20     |
| **LOD**               | Concentration (µg/ml) | 0.06 | 0.018 |
| **LOQ**               | Concentration (µg/ml) | 0.22 | 0.095 |

Figure 6  Calibration curve of rosuvastatin calcium.

Figure 7  Calibration curve of amlodipine besylate.
relative standard deviation (Table 5) was found to be less than 2% for both interday and intra-day precision (Sagar et al., 2012).

### 4.3.5. Ruggedness

Ruggedness was determined by verifying the percentage relative standard deviation of the measurement of the two analysts in the same laboratory. For this purpose, six replicate samples were analyzed. The percentage relative standard deviation (%RSD) was found to be less than 2% for both the drugs (Table 5).

### 4.3.6. Sensitivity

Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from the signal-to-noise ratio (Hosseini, 2011). Limit of detection is defined as the lowest concentration of analyte resulting in a peak area three times that of the baseline noise (Hosseini, 2011). On the other hand, the limit of quantitation is defined as lowest concentration of analyte that provide a peak area that of ten times the baseline noise (Hosseini, 2011). The LOD value for rosuvastatin calcium and amlodipine besylate was found to be 0.06 μg/ml and 0.018 μg/ml and the LOQ value for rosuvastatin calcium and amlodipine besylate was found to be 0.095 μg/ml and 0.22 μg/ml respectively (Table 5).

### 4.3.7. Robustness

The robustness of an analytical procedure was assessed by measuring its capacity to remain unaffected by small but deliberate variations in method parameters which provides an indication of its reliability for routine analysis (Sagar et al., 2012). To determine robustness of the proposed method, test samples were prepared and analyzed by varying analytical parameters while keeping the other parameters unchanged such as the composition of mobile phase (±5%), flow rate (±2%), column temperature (±5 °C), and wavelength (±5). None of the alteration caused a significant change in peak area, percentage of relative standard deviation, tailing factor and retention time (Sagar et al., 2012). The results are recorded in Table 6.

### 4.3.8. In vitro dissolution study

A typical acceptance criterion for dissolution release of drugs from immediate release tablet is about 80% of label amount in 45 min (Ummapathi et al., 2011). Both preparations (market and the combination formulation) were found to release an average of 95% rosuvastatin and 93% of amlodipine within 45 min (Tables 7 and 8), without showing any hindrance to the release pattern of other drug (Fig. 8). The dissolution pattern complies with the BP Guidance standards as well as with the in-house specifications (rosuvastatin calcium is an INN

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| Conditions | Retention time | Peak area | Tailing factor Average %RSD |
|------------|---------------|-----------|-----------------------------|
| Rosuvastatin calcium | ACN:Buffer (48:52) | 8.285 | 158,392 | 0.224 | 1.060 |
| | ACN:Buffer (42:58) | 5.166 | 155,370 | 0.076 | 1.023 |
| | Flow rate (1.3 ml/min) | 6.356 | 72,352 | 0.04 | 1.045 |
| | Flow rate (1.7 ml/min) | 5.654 | 139,265 | 0.10 | 1.040 |
| | Column temperature (20 °C) | 6.495 | 157,389 | 0.075 | 1.042 |
| | Column temperature (30 °C) | 6.234 | 157,247 | 0.096 | 1.052 |
| | Wavelength (235 nm) | 6.315 | 157,443 | 0.151 | 1.045 |
| | Wavelength (245 nm) | 6.345 | 157,622 | 0.178 | 1.047 |
| Amlodipine besylate | ACN:Buffer (48:52) | 2.405 | 62,771 | 0.213 | 1.133 |
| | ACN:Buffer (42:58) | 3.411 | 62,786 | 0.098 | 1.203 |
| | Flow rate (1.3 ml/min) | 3.130 | 72,348 | 0.036 | 1.187 |
| | Flow rate (1.7 ml/min) | 2.431 | 55,437 | 0.084 | 1.169 |
| | Column temperature (20 °C) | 2.673 | 63,159 | 0.737 | 1.185 |
| | Column temperature (30 °C) | 2.799 | 63,432 | 0.124 | 1.189 |
| | Wavelength (235 nm) | 2.765 | 63,175 | 0.161 | 1.184 |
| | Wavelength (245 nm) | 2.759 | 63290.4 | 0.246 | 1.181 |

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| Table 6 Robustness study of rosuvastatin calcium and amlodipine besylate. |
|-----------------------------|-----------------------------|-----------------------------|
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| | Wavelength (245 nm) | 2.759 | 63290.4 | 0.246 | 1.181 |

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| Table 7 Dissolution profile of rosuvastatin calcium. |
|-----------------------------|-----------------------------|-----------------------------|
| Time interval | Dissolution media | % of drug release Formulated combination preparation Market preparation |
| Rosuvastatin calcium | After 10 min | 0.05 M sodium | 88.03 | 83.89 |
| | After 20 min | citrate buffer of pH 6.6 | 91.65 | 90.86 |
| | After 30 min | pH 6.6 | 94.06 | 92.7 |
| | After 45 min | | 96.99 | 94.07 |
| | After 60 min | | 98.5 | 98 |

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Figure 8 Comparative drug release pattern of rosuvastatin calcium and amlodipine besylate.
drug), indicating suitability of the proposed method for the dissolution study of the two drugs (see Fig. 9).

5. Conclusion

The proposed combination formulation of rosuvastatin calcium and amlodipine besylate has shown compatibility with the chosen excipients, verified through FT-IR study. A novel gradient RP-HPLC method was developed and validated according to the ICH guideline which was found to be suitable for the simultaneous estimation rosuvastatin calcium and amlodipine besylate from the combination formulation. The retention time of 2.7 and 6.08 min allows the analysis of large amount of samples with less mobile phase which makes the method economic. The dissolution profiles of both the drugs in different dissolution medium were encouraging which makes the combination formulation of rosuvastatin calcium and amlodipine besylate superior and effective in achieving patient compliance. The complete design of the study done can be depicted in a flowchart as shown in Fig. 9.

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Table 8  Dissolution profile of amlodipine besylate.

| Time interval | Dissolution media | % of drug release |
|---------------|-------------------|-------------------|
|               | Formulated        | Market preparation|
|               | combination       | preparation        |
|               | preparation       |                   |
| Amlodipine besylate | 0.01 N HCl | 58.69 | 90.08 |
| After 10 min | 71.56 | 92.16 |
| After 30 min | 83.62 | 98 |
| After 45 min | 92.56 | 102 |
| After 60 min | 99.65 | 105 |

Figure 9  Flowchart of the study design.
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