

Assay method for quality control and stability studies of a new anti-diabetic and anti-dyslipidemic flavone (S002-853)*

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ABSTRACT

Background: Flavonoid-rich extract of the plant is long known for its anti-diabetic activities in traditional medicine. S002-853, a new flavone derivative synthesized by Central Drug Research Institute (CDRI) has been used for the present study. **Objectives:** The present study aimed at development of an assay method for quality control (QC) and stability studies of a new anti-diabetic and anti-dyslipidemic agent CDRI compound S002-853. **Materials and Methods:** A validated high-performance liquid chromatography analysis method for S002-853 was developed for in process QC and stability studies. The separation was achieved on a RP-C18 (25 cm × 0.4 cm, 5 μm, Phenomenex) at 240 nm with flow rate of 1.0 ml/min. This method was applied successfully in establishing forced degradation and drug-excipient testing protocols as per International Conference on Harmonization guidelines. **Results:** The result of estimation and stress testing studies indicated a high degree of selectivity of this method. S002-853 was most stable at pH 7 and under photolytic conditions. The temperature degradation pattern of S002-853 was found to follow the zero order degradation. **Conclusion:** The method described is easy and simple hence can be easily reproduced. This method can be very useful for bulk manufacture QC, and drug development process.

Key words: Anti-diabetic, anti-dyslipidemic, flavone derivative, high performance liquid chromatography, in process quality control

INTRODUCTION

Diabetes mellitus is one of the most common endocrine metabolic disorders and had become a global epidemic with significant impact on society and economy. There are 382 million people living with diabetes and may reach 471 million by 2035. The rapid increase in type-2 diabetes mellitus (T2DM) among people aged between 30 and 39 years, and in children including adolescents, is of particular concern.^[1] The prevalence increases with age and varies widely between different populations and ethnic groups. T2DM (non-insulin-dependent) accounts for approximately 90% of patients with diabetes mellitus, with type-1 (insulin-dependent) accounting for the remainder.^[2]

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One of the main contributing factors to this burden is the changed sedentary life style, which promotes obesity as a result incidence of diabetes.^[3] Patients with T2DM have increased cardiovascular mortality due to recurrent thrombotic events.^[4,5] Several epidemiology studies have revealed that patients with T2DM are at increased risk for developing memory impairment, dementia and Alzheimer's disease.^[6] Characteristic factors of this heterogeneous disorder include insulin resistance, obesity, hypertension and a common form of dyslipidemia, low high-density lipoprotein cholesterol, in insulin sensitivity and impaired insulin secretion by pancreatic β cells.^[7] Although the primary cause of type-2 diabetes remains unclear, the inability of β cells to compensate for reduced insulin sensitivity in peripheral tissues eventually leads to β-cell failure and deterioration in glucose homeostasis, ultimately leading to overt T2DM. T2DM is a complex disease and both genetic, and environmental factors appear to contribute to its development.^[8-10]

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Medicinal plants play an important role in the management of diabetes mellitus especially in developing countries where resources are meager. Flavonoids and chalcones are among the most ubiquitous groups of polyphenolic compounds in foods of plant origin. As integral constituents of the diet, they may exert a wide range of beneficial effects on human health. Flavonoid-rich extract of the plant is long known for its anti-diabetic activities in traditional medicines.^[11-13] Flavonoids and chalcones produce such biological effects through their free radical scavenging anti-oxidant activities and metal ion chelating abilities.^[14,15] Within the secondary metabolite of the flavonoid class, flavones define one of the largest subgroups. They express various biological activities such as anti-bacterial, anti-cancer, anti-tumor, anti-protozoal, anti-oxidant, anti-convulsant.^[16,18]

In the quest to develop compound with efficacy, low toxicity, and more ever affordable for treatment we utilized flavones for the synthesis of hybrid molecule as anti-diabetic and anti-dyslipidemic agents by substitution with thermogenic as well as insulin sensitizing pharmacophores. Central Drug Research Institute (CDRI), Lucknow while shouldering the responsibility of this project, accepted the challenge to develop new anti-diabetic and anti-dyslipidemic as well as to test their activity. A few compounds have had been launched in the market, and several are still in the preclinical phase from this institute. S002-853 [Figure 1, C₃₆H₃₇NO₆] is such derivative, demonstrated to exhibit promising anti-diabetic and anti-dyslipidemic activity.^[19-22] Compound hence was taken up for further studies pertaining to the development of pharmaceutical dosage form.

MATERIALS AND METHODS

Standard S002-853 was prepared in the Medicinal and Process Chemistry Division. Milli-Q pure water was obtained from a Millipore Elix water purification system (Millipore India Pvt. Ltd. [New Delhi, India]). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile (ACN) were purchased

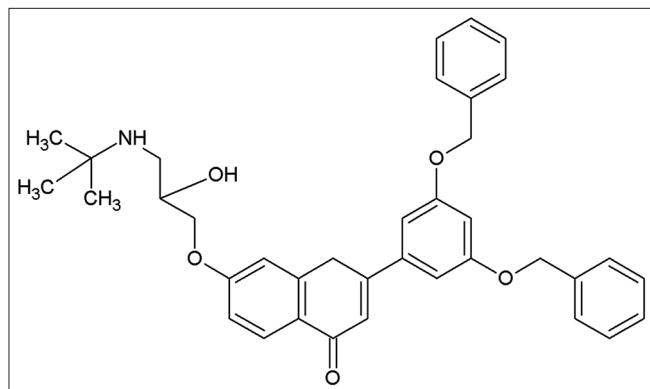


Figure 1: Chemical structure of S002-853

from Merck, Ltd. (Mumbai, India). Sodium hydroxide, hydrochloric acid, hydrogen peroxide, acetic acid, sodium bicarbonate, sodium metabisulphite, sodium chloride, sodium nitrate, potassium chloride of certified grades were purchased from SD Fine Chem Ltd, Mumbai, India. Other reagents used were of analytical grade. Excipients namely hydroxypropyl methyl cellulose (HPMC), methyl cellulose, ethyl cellulose, lactose, polyvinyl pyrrolidone (PVP), micro crystalline cellulose, magnesium stearate was purchased from SD Fine Chem Ltd, Mumbai, India and Sigma Aldrich.

Apparatus and chromatographic conditions

The HPLC workstation used was from Shimadzu, Japan; equipped with SCL-10A VP system controller, LC-10AT VP twin pump, SPD-10A VP UV-VIS detector, photo diode array detector (SPD M10A), Rheodyne injector with 20 μ l injection loop. The separation was achieved on a reverse phase analytical column (RP) C-18 (25 cm \times 0.4 cm, 5 μ m, Phenomenex). The mobile phase consisted of a mixture of ACN: 0.05% glacial acetic acid in water (70:30) system. Both the solvents were filtered and degassed before use. Chromatography was performed at 27°C \pm 3°C at a flow rate of 1.0 ml/min. The monitoring wavelength for the column effluent was taken at 240 nm. The data acquisition and processing were done using Shimadzu HPLC software Class-VP (V 5.03). Humidity chambers and photostability chambers (Thermolab, Mumbai, India) used were equipped with UV and visual light tubes.

Preparation of calibration standards and quality control samples

Stock standard solution of compound S002-853 was prepared by weighing \sim 10 mg of the compound and making the volume to 10 ml with ACN. Working standard solutions were prepared by serial dilution in ACN in the range of 1–50 μ g/ml. The quality control (QC) samples were prepared at three different concentration levels (1 μ g/mL [low QC], 6.25 μ g/mL [medium QC] and 25.0 μ g/mL [high QC]) for S002-853.

Preparation of sample solution

The sample of the compound S002-853 was prepared by weighing 5 mg of different batches and dissolving in 25 ml of ACN, and then 1 ml of that was further diluted in 10 ml. Similarly, the samples from various studies were weighed and diluted.

Method validation

The method was validated with respect to parameters including selectivity, limit of quantitation (LOQ), limit of detection (LOD), linearity, precision, accuracy, ruggedness, robustness and recovery according to International Conference on Harmonization (ICH) guidelines.

Specificity and selectivity

The chromatographic interferences were assessed by comparing chromatograms of blank ACN with that of samples spiked with S002-853 in ACN. Specificity was established by determination of peak purity index of the drug peak using a photodiode array (PDA) detector.

Linearity

The linearity of standard solutions was evaluated by analyzing a set of standards ranging from 1 to 50 µg/mL. Plots of peak area (response) against analyte concentration were used. The slope, intercept and correlation coefficient of each calibration curve were determined using linear regression (LINREG) analysis. In the regression equation $y = mx + c$, where m corresponds to the slope of line, x is the concentration of standard solution in µg, y is the peak area and c is the intercept of the straight line on y axis.

Limits of detection and quantitation

The LOD and LOQ were measured according to the ICH guidelines (ICH 2003). LOD and LOQ were estimated with the signal to noise ratio of 3 and 10, respectively.

Intra-day and inter-day accuracy and precision

In order to evaluate the intra- and inter-day precision and accuracy of the assay, QC samples at low, medium and high concentrations were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV) for the analysis of QC samples in three replicates and inter-day precision was determined by the analysis of three replicates QC samples on three different days. The concentrations of S002-853 were quantitated using the LINREG line of the calibration standards. The percent deviation of the calculated concentrations of the standard from the actual concentrations (% deviation from the actual concentration, [% DFA]) was used to calculate the intraday and inter-day accuracy. The % relative standard deviation (RSD) was used to calculate inter-day precision.

Robustness

The variable factors used to determine the robustness of the analytical method were percentage of ACN, flow rate of mobile phase and age of the column (new and old column).

Ruggedness

The ruggedness of the method was tested by using two HPLC systems of Class-VP, Shimadzu Corporation, Kyoto, Japan consisted of binary pump having UV detector and HPLC system described above. The parameters were checked within replicates in intraday and inter-day accuracy and precision by variations.

Recovery

Known amount of S002-853 was added to the mixed contents of the samples, and the quantity of S002-853 was determined by interpolation on the corresponding calibration graphs. The recovery was calculated by using the following equation: Recovery (%) = (Amount detected) × 100 / (Amount spiked) %.

Physico-chemical parameters**Purity**

The purity of the compound was checked, and its identity was established by HPLC and nuclear magnetic resonance/fast atom bombardment-mass, respectively.

Solubility

The solubility of S002-853 was checked in various solvents such as methanol, ethanol, chloroform, acetone, ACN, ethyl acetate, and dichloromethane. This was done by taking 1 ml of each solvent and adding an excess amount of the compound to it. The amount dissolved in the solvent was determined by HPLC. All the experiments were performed in triplicate.

Partition coefficient (log P)

Log P for S002-853 was calculated by shaking the compound with the octanol/buffer (0.05M phosphate buffer, pH = 7.4) and octanol/water layer mixture for about 4 h, then centrifugation for 20 min at 4000 rpm. Then, after the layers were separated, each was analyzed by HPLC after proper dilution and concentration were calculated.

Dissociation constant

The dissociation constant determination (pKa) value of any compound can be determined using different methods such as the potentiometry, spectrophotometry and capillary electrophoresis. Spectrophotometric determination of dissociation constant (pKa) for S002-853 using Albert equation was carried out. In this method, absorbance of S002-853 in different pH (ranging from acidic pH 2, 3, 4, 5, 6 and basic pH 8, 9, 10, 11 and 12 solutions) using spectrophotometric method was observed.

$$pK^1 = pH + \log \frac{A_i - A}{A - A_M}$$

Where, A_i and A_M represent the absorbance of the basic and the acidic form, respectively, of S002-853, and A denotes the absorbance at the given pH and wavelength.

Stability studies**Effect of pH**

Acid-base degradation studies were carried out to obtain the pH for maximum stability. This information

is required for the preparation of the final formulation. Effect of pH on this compound was checked by taking 1 ml of stock solution (each) of S002-853 in 10 ml volumetric flasks and the volumes were made up with buffers of pH 2–10. Samples were withdrawn at different time intervals, diluted appropriately, and 20 µl were injected on to the HPLC column to analyze as described above. The reaction rate constants were calculated by LINREG program.

Effect of temperature

For conducting accelerated temperature stability studies, the samples of S002-853 (50 mg) were kept at 37°C, 50°C and 60°C for 2 months in different ovens. The samples so stored were examined for caking, liquefaction, discoloration, odor and gas formation. The sample (10 mg) was withdrawn from vials at every 10 days up to 2 months and dissolved in ACN and diluted suitably for estimation of products by HPLC method.

Effect of temperature and moisture as per international conference on harmonization guidelines

In the presence of moisture, many drug substances hydrolyze, react with other excipients or oxidize. These reactions can be accelerated by exposing the solid drug to different relative humidity conditions. Three vials of S002-853 were kept at 4°C (in refrigerator), 30°C ± 2°C with 65% ± 5% RH and 40°C ± 2°C with 75% ± 5% RH (in Thermolab humidity cum photo stability chambers). The stored samples were examined for discoloration and odor or gas formation. The sample (2 mg) was withdrawn from each vial after regular intervals (0, 3, 6, 9, 12, 18 and 24 months). They were dissolved, diluted suitably in ACN and analyzed by HPLC.

Photostability studies

The photolytic stability of S002-853 was performed by exposing S002-853 to light from UV lamp in a photolytic chamber (Thermolab) at 1.2 million/h Luxes. The drug (20 mg) was placed in Petri dishes and exposed to UV light for a period of 57 h. The samples were withdrawn after 5, 24, and 57 h and analyzed by HPLC. The drug stored under the same conditions but protected from light was used as control for comparison.

Forced degradation studies

S002-853 was subjected to various forced degradation conditions as per ICH guidelines to effect partial degradation preferably in 20–30% range. S002-853 was refluxed (40°C) with three different reagents, that is, alkali (0.1 N NaOH), acid (0.1 N HCl) and peroxide (3% H₂O₂) for 1 h, followed by neutralization with acetic acid, sodium bicarbonate and sodium metabisulfite for alkali,

acid and peroxide, respectively, and final volume was made up to 10 ml with methanol. This was further diluted five times with ACN and injected (20 µl) in triplicate on HPLC. The % remaining concentration of S002-853 was calculated with the help of the reference standard.

Drug excipient studies

Interaction between the active constituent and excipients can alter stability and bioavailability of drugs, thereby, affecting its safety and/or efficacy. A number of experimental techniques (i.e. differential scanning calorimetry, Fourier transform infrared spectroscopy, X-ray powder diffraction, scanning electron microscopy, HPLC etc.) have been used to investigate the interaction between drug and excipients.^[23,24] Equimolar mixtures (100 mg each) of S002-853 and the chosen excipients were maintained at 40°C ± 2°C for a period of 7 days in glass vials. A control sample containing only the extract was also kept under similar conditions. After 7 days, all samples were withdrawn, and 5 ml methanol-water (1:1) solution was added to each vial. These samples were then individually vortexed to ensure mixing and then filtered. The subsequent filtrate achieved of concentration 20 mg/ml was further diluted to achieve the final test samples of 1 mg/ml each. These were analyzed by HPLC for estimation of S002-853 content and to observe any changes in the fingerprint pattern or additional peaks.

RESULTS

Final separation was achieved in an isocratic mobile phase consisting of a mixture of ACN: 0.05% glacial acetic acid (70:30) system at a flow rate of 1 mL/min. This method was capable in resolving S002-853 at a retention time of about 7.9 min with quality peak shape [Figure 2]. The PDA studies indicated that the method was sufficiently specific. The calibration curves ($n = 3$) showed a linear relationship between peak area and concentration over the range of 1–50 µg/mL with the correlation coefficient (r^2) of 0.998. Based on the signal-to-noise ratio of 3 and 10, the LOD came out to be 0.284 µg/ml. However, the LOQ was 2.84 µg/ml. The recoveries of the analyte samples were in the range of 98–101% and the % CV and % DFA were not more than 3% [Table 1].

The compound S002-853 was found 98.85% pure by HPLC. The solubility data are given in Table 2. The partition coefficient of S002-853 in octanol and water is 2.77, and that in octanol and phosphate buffer pH 7.4 is 3.00. Spectrophotometric determinations of the pKa and pKb values of S002-853 were found to be 4.2 and 9.8, respectively.

It was found that the drug is most stable at pH 7 [Table 3]. The temperature degradation rate constant at 25°C was

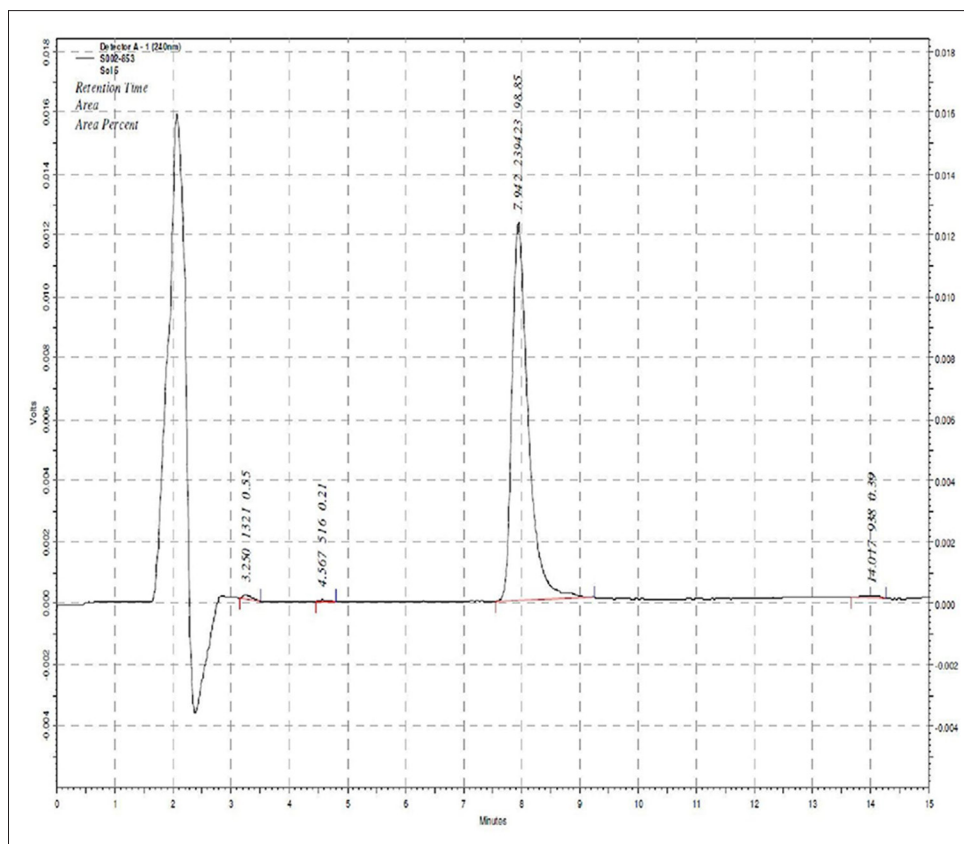


Figure 2: High-performance liquid chromatography of S002-853

Table 1: Validation parameters of S002-853

Parameters	S002-853
Linearity range ($\mu\text{g/ml}$)	1-50
Detection limit ($\mu\text{g/ml}$)	0.284
Quantitation limit ($\mu\text{g/ml}$)	2.84
Slope	40077.67
Intercept	-11843.45
Correlation coefficient	0.998
Intra assay variation % CV	<2
Intra assay variation % DFA	<3
Inter assay variation % CV	<2

CV: Coefficients of variation; DFA: Deviation from actual

Table 2: Solubility data of S002-853

Solvent	Solubility (mg/ml)
Acetone	3.5
Acetonitrile	1.3
Chloroform	20.0
Dichloromethane	16
Ethanol	5.0
Ethyl acetate	3.4
Hexane	Practically insoluble
Methanol	2.7
Water	Practically insoluble

calculated to be $K = 0.17457/\text{week}$, $t_{1/2} = 5.5$ years, $t_{10} = 57.28$ weeks and $E_a = 59.73$ kJ/mole [Table 4]. At

controlled humidity environments and upon exposure to UV lamp (negligible decomposition) drug is quite stable. In forced degradation studies, stress conditions utilizing alkali medium afforded maximum degradation, followed by acidic and consecutively peroxide. The drug was not found compatible with PVP [Figure 3].

DISCUSSION

Method development

The purpose of the study was to develop a validated HPLC method for in process QC and stability studies of CDRI compound S002-853. The chromatographic conditions were optimized to obtain chromatograms with better peak shape within a short time. Out of the many permutations and combinations employed the most suitable and optimized solvent system was chosen. Initially, we tried the separation experiments with columns like C-18 endcapped (250 mm, 4 mm, 5 μm , Merck) or C₈ (250 mm, 4 mm, 5 μm , Merck) or CN (250 mm, 4 mm, 5 μm , Merck). Several solvent systems comprising of different percentage of methanol-water or ACN-water were used, but the resolution among the peaks of the main compound not found satisfactory. The best separation was achieved on a RP C-18 (25 cm \times 0.4 cm, 5 μm , Phenomenex).

Method validation

The PDA studies indicated that the method was sufficiently specific. The purity angle value was less than the threshold angle, indicating that the drug peak was pure by nature. The result of estimation and stress testing studies indicated a high degree of selectivity of this method. No interfering peaks were observed at the retention time of the analyte. The method provided adequate sensitivity for the determination of S002-853 in bulk drug substance and dosage forms. Precision of the assay was investigated with respect to both repeatability and reproducibility. The percentage

RSD of assay S002-853 during assay method precision and in intermediate precision study were within acceptable limits (ICH 2003) confirming good precision of the method. The accuracy was also found to be within acceptable limits at all calibration points. The analytical method was found to be rugged with instrumental and environmental variation and also sufficiently robust within the range of tested conditions.

Physico-chemical parameters and stability studies

The solubility data indicate that the compound S002-853 is lipophilic in nature, and it is slightly soluble in water.

The samples of pH studies were collected at different days and analyzed by HPLC. The % remaining concentration was calculated from the calibration curves. The temperature degradation pattern of S002-853 was found to follow the zero order degradation, which indicated that the degradation of the compound is concentration independent. The rate constant (K), half-life ($t_{1/2}$), shelf-life (t_{10}) and energy of activation (E_a) at 25°C were calculated by LINREG. The % concentration remaining of S002-853 was found to be 23.87% in alkaline medium (0.1 N NaOH), 50.43% in acidic medium (0.1 N HCl), and 65.81% in 3% H₂O₂. In the drug excipients interaction, it was found that micro crystalline cellulose; lactose and magnesium stearate has no effect on S002-853. HPMC, EC and MC retard the release of the drug from the matrix [Figure 3].

Table 3: Effect of pH on the compound S002-853

pH	R (correlation coefficient)	K (rate constant)	T ₅₀ (days)	T ₉₀ (days)
4	0.9960	3.066×10 ⁻²	40.078	8.016
5	0.9945	2.153×10 ⁻²	44.71	8.941
6	0.99997	2.0188×10 ⁻²	51.170	10.234
7	0.9408	1.060×10 ⁻²	76.79	15.36
8	0.96885	1.522×10 ⁻²	59.09	11.818
9	0.9898	1.868×10 ⁻²	50.524	10.105
10	0.9982	2.958×10 ⁻²	38.80	7.76

Table 4: Temperature degradation of compound S002-853

Temperature (°C)	Reaction rate constant (K)	Half-life (weeks)	Shelf-life (weeks)
4*	0.0281	1779.35	355.87
25*	0.17456	286.43	57.28
37	0.3965	128.12	25.62
50	1.4715	34.73	6.946
60	1.8774	26.48	5.296

*Calculated

CONCLUSION

The developed and validated HPLC method is reproducible and can be routinely used for QC of S002-853 in bulk

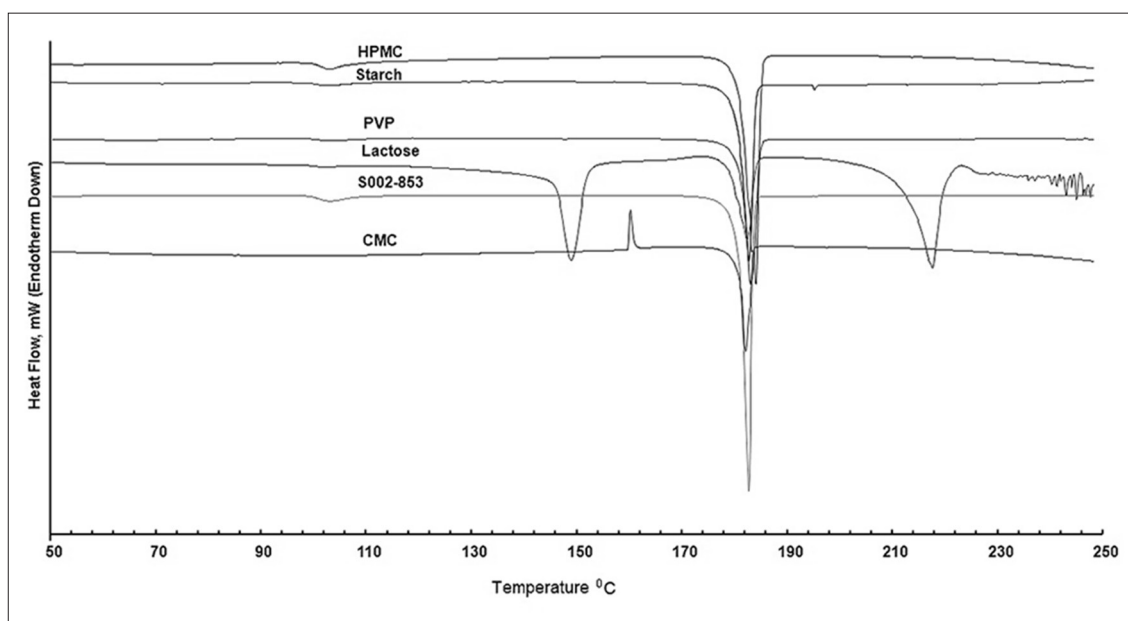


Figure 3: Differential scanning calorimetry of S002-853 with various excipients

manufacture, with ongoing stability studies for drug development process. Analytical studies were attempted on CDRI compound S002-853 to generate data beneficial for formulation development, further studies and Investigational New Drug application on the same. It provides base line separation of the compound of interest S002-853. The bulk samples of compound S002-853 from the Medicinal and Process Chemistry were checked by the above method. The results of stress testing undertaken according to the ICH guide lines reveal that the method is also selective and stability-indicating for S002-853. The solubility data indicate that the compound S002-853 is lipophilic in nature, and it is slightly soluble in water. The drug candidate is most sensitive towards alkaline condition as compared to acidic and oxidative conditions. The reported method can be very useful for further studies on bulk manufacture QC and in drug development process.

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