Enhancement of Bioavailability of Fenofibrate with Alpha Tocopherol and Phospholipids as Solubilizers

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Abstract

The purpose of this study was to investigate the effect of different solubilizers namely alpha tocopherol, soy phosphatidylcholine 70, Phospholipon 80H, and Phospholipon 90H on the bioavailability of sustained release fenofibrate pellets using fluid bed coating by applying Taguchi design to optimize the type and concentration of solubilizer at four levels namely 0.5%, 1%, 1.5%, and 2%. The pellets were prepared by loading the fenofibrate blended with other excipients onto the core sugar pellets with the aid of the binder solution. Taguchi experimental runs with alpha tocopherol 1% and Phospholipon 90H 2% (test) showed significant differences in in vitro dissolution behavior of drug compared to the pure drug. The pharmacokinetics of pure drug and test was evaluated in healthy male Wistar rats and found that t1/2 was reduced significantly (4.36 and 4.02 hours) while AUC0-t (32.14 ± 6.38 μg h/ml, 36.94 ± 6.2 μg h/ml), Cmax (6.7 ± 2.31 μg/ml, 9.8 ± 2.2 μg/ml) were improved markedly compared to the pure drug with t1/2 (7.339±4.1 3.1 hours), AUC0-t (11.89 ± 8.13 μg h/ml), and Cmax (5.137 ± 3.37 μg/ml). The extent of the mean plasma exposure of fenofibrate was 2.7 and 3.1 fold higher in animals treated with test. The ANOVA results revealed that type and concentration of solubilizer are crucial for enhancement of in vitro dissolution profile. Hence use of solubilizers may be the promising way to improve the oral bioavailability of fenofibrate.

Introduction

Oral drug delivery continued to be the preferred route of drug administration especially in instances of multiple frequency of dosing. However, the effective drug delivery through oral route still remains a major challenge for highly lipophilic drugs in the pharmaceutical field owing to their poor bioavailability [1]. Prerequisite for effective oral drug delivery is the dissolution of pharmaceutical drug formulation in the gastro-intestinal lumen. It has been estimated that about 40 to 70 percent of New Chemical Entities (NCE’s) entering drug development process possess inadequate aqueous solubility resulting in poor absorption thereby resulting in poor bioavailability [2].

Several attempts had been made in literature to enhance the oral bioavailability of poorly soluble and highly lipophilic drugs. More recently, micronized fenofibrate showed significantly improved dissolution and enhanced oral bioavailability [3]. The present study aims at enhancement of oral bioavailability of fenofibrate. Fenofibrate is a lipid lowering highly lipophilic drug with low water solubility and high membrane permeability included in class II of biopharmaceutical drug classification system. It has a lipid-water distribution coefficient of 5.24 and aqueous solubility of less than 0.5 mg/liter with half life of 20 hours. Due to its virtual aqueous insolubility it is poorly absorbed from digestive tract leading to consequences of incomplete, irregular oral bioavailability [4]. The therapeutic dose required to be administered must thus be increased to obviate the disadvantage.

To decrease the daily dose of drug and frequency of administration, fenofibrate was formulated into sustained release pellets by incorporating HPMC K4M as sustained release matrix forming polymer. The drug and excipient blend was loaded on to the core pellets with the aid of the binder solution and various process parameters were optimized using fluid bed coater that is more automated and advanced technique compared to conventional pan coating. The potential use of vitamin E preferably alpha tocopherol as antioxidant to protect plasma low density lipoproteins [5] and the use of alpha tocopherol as solubilizer for poorly soluble and highly lipophilic drug griseofulvin is well reported [6]. Owing to the dual advantages Alpha Tocopherol (AT) was selected as solubilizer for fenofibrate. Presence of food increased absorption of fenofibrate by nearly 35% compared with fasting conditions [7]. Therefore different phospholipids namely Soy Phosphatidyl Choline 70 (SPC 70), Phospho Lipon 80H (PL 80H) and Phospho Lipon 90H (PL 90H) were incorporated as solubilizers owing to advantages that they occur naturally in biological membranes, freely available in nature, relatively effective at low concentrations. Phospholipids are physiological lipids present in all living species. Among all phospholipids, phosphatidylcholine (PC) is the most abundant biomolecule and the only one available in pure form on industrial scale. Phosphatidylcholine is non-toxic and no limits have been established for maximum daily intake. However, their potential usage as oral drug delivery carriers for poorly water-soluble fenofibrate has not been investigated. In this study, fenofibrate-loaded alpha tocopherol and phospholipids sustained release pellets were prepared and evaluated both in vitro and in vivo.

Materials and Methods

Materials

Fenofibrate (unmicronized) was obtained from Alembic Private Limited (Gujarat, India), Povidone K30 (Plasdone K 29/32) was

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obtained from ISP (New Jersey, USA), HPMC-E5 (Pharmacoat 606) was obtained from SHIN-ETSU (Tokyo, Japan), HPMC K4M (Methocel K4M) was obtained from Dow chemicals (Asia Pacific). Alpha tocopherol was obtained from (BASF, Norway), soy phosphatidyl choline 70 (containing phosphatidyl choline 70%) was obtained from Sonic Biochem Extractions (Madhya Pradesh, India). Phospholipon 80H (containing 70% of phosphatidyl choline, 6% of lysophosphatidyl choline) and Phospholipon 90H (containing 90% of phosphatidyl choline, 4% of lysophosphatidyl choline) were gift samples from Lipoid GmbH (Newark, USA), sugar spheres and sugar was obtained from RA Chem Pharma Limited (Hyderabad, India). Lipin Retard® marketed by Solvay Pharmaceuticals, Inc. All other reagents used were of analytical grade.

Methods

Drug loading: Drug was loaded onto sugar spheres using fluidized bed processor (Model Glatt FBE-250). The sugar spheres were loaded into the fluidized bed processor and warmed for about 10-15 minutes. Binder solution was prepared by dissolving Povidone K30 and sugar in purified water under constant stirring. Unmicronized fenofibrate which was pre blended and pulverized (0.5 mm screen pulverizer) with other excipients was incorporated intermittently into the solution under constant stirring. The obtained solution was loaded onto the circulating sugar spheres (800 μm). The wet spheres were dried simultaneously during the process of circulation in wurster chamber. The process was continued till the complete solution was consumed. The various processing variables namely inlet air temperature (40-45°C), outlet air temperature (30-33°C), product temperature (36-40°C), chamber humidity (58-60%), air flow (85 m³/hr), compressed air pressure (1.5-3.0 kg/cm²)The peristaltic pump rpm (10-35) and spray rate (2 ml/min) were optimized in the preliminary trials.

Design of experiment to optimize drug loaded fenofibrate pellets: Taguchi L16 design of experiment was used to study the effect of two variables namely, type of solubilizer and concentration of solubilizer at four different levels as given in Table 1 and Table 2. All the experimental runs were analyzed using Minitab statistical software package (version 15).

Characterization methods

Infrared Spectroscopy analysis of drug and excipients: FT-IR spectra were obtained using FT-IR spectrometer (Model Nicolet 5700) by the conventional KBr pellet method. The samples were grounded gently with anhydrous KBr and compressed to form pellet. Scanning range was 400-4000 cm⁻¹ and the resolution was 4 cm⁻¹ (Table 3).

Sieve analysis and usable yield or % theoretical yield of pellets: Auchi experimental runs were evaluated for particle size distribution using standard set of sieves and were shaken using RoTap sieve shaker (Model RX-29) for about 20 minutes. The formulations for which not less than 90% of pellets passed through #16 (1180 μm) and not less than 90% of pellets retained on #20 (850 μm) were selected for further studies and were weighed after drug loading. The theoretical yield was calculated respectively.

Mechanical Crushing force: At least 20 pellets from the usable yield fraction of each formulation were evaluated for their diametrical crushing force using a tablet strength tester (Electrolab EH 01) [8].

Abrasion Resistance: A pre-weighed sample (approximately 10 g) taken from the usable yield fraction was placed in an abrasion drum (Vankel Industries Inc. USA) that was configured to raise and drop the pellets from 200 mm. The stress levels on pellets were enhanced by adding 1mm glass beads. After 100 revolutions at 25 rpm, the mass retained on the sieve (1190 μm) was weighed and the abrasion resistance was calculated as the percentage loss of mass between initial and final weights of each pellet batch. Each batch was analyzed in triplicate.

Residual moisture: The residual water content present in the pellets after drying was determined by United States Pharmacopoeia (USP) method using Karl Fischer titrator. The equipment was pre-calibrated and standardized with disodium tartarate dihydrate. Pellets, approximately 250 mg were accurately weighed and immediately placed in the moisture analyzer for titration with Karl Fischer reagent. Each batch was analyzed in triplicate.

Flow Properties: Bulk and tapped density of pellets was determined using USP density apparatus. The bulk density, tapped densities were determined initially from which Hausner’s ratio was calculated. The angle of repose was determined using fixed funnel method [9].

Assay of the prepared pellets

High performance liquid Chromatography (HPLC) method of analysis: The HPLC system consisted LC-10AT pump system, an auto injector SIL-10A (Shimadzu) at a flow rate of 1.0 ml/min of mobile phase, peak detection by UV detector (SPD-10AV) at wavelength 286 nm. A reverse phase column Hypersil BDS C18, 150 mm X 4.6 mm kept at 25°C by a column oven with 5 μm particle size was used as stationary phase. A mixture of 30% of orthophosphoric acid buffer in 70% of acetonitrile (30:70 v/v) was used as mobile phase. The injection volume was 20 μl. The samples were detected at 286 nm and integrated using the RF 10A (version 1.1) LC Solutions software program. Samples were diluted with mobile phase before injection and run for 12 minutes.

In vitro dissolution study of prepared pellets: Accurately weighed amount of pellets equivalent quantity of 250 mg fenofibrate were filled into size ‘1’ capsules and the dissolution was performed in 1000 ml of 0.1M Sodium lauryl sulfate in water as biorelevant dissolution medium, using USP Type II (paddle) apparatus (Electrolab) at temperature of 37 ± 0.5°C for 8 hours. Aliquots of 5 ml were withdrawn for every one hour and were replaced with fresh medium. The samples were analyzed spectrophotometrically (UV 2450) at maximum wavelength (λmax) of drug at 291.0 nm against the medium as the blank. For each dissolution run, a mean of six determinations was recorded.

Pharmacokinetic study in rats

The pharmacokinetics of drug and test (AT 1% and PL 90H 2%) was evaluated following oral administration. The study was conducted at G. Pulla Reddy College of Pharmacy, Hyderabad, India as per the regulations specified by the Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments.
on Animals (CPSEA). In total 18 (6 per group) male Wistar rats (6-7 weeks old) weighing between 180-230 g were used for the study. All rats had free access to tap water and pelleted diet (Ssniff rats pellet food, Ssniff Spezialdiaten, Germany). The rats were housed in a cage and maintained on a 12 h light/dark at room temperature (21°C to 24°C) and relative humidity of 50 to 70% and acclimatized to study area conditions for at least 5 days before dosing. General and environmental conditions were strictly monitored. The room underwent 10 fresh air change cycles per hour. Rats were implanted with canula in the jugular vein for blood sampling. The surgery was performed two days before dosing under anesthesia. The animals were fasted at least 10 h prior to dose administration and for 4 hours post dose with free access to water. Individual oral doses of the test and reference samples were prepared (10 mg/kg free base) and accurately weighed drug material was carefully transferred into the dosing syringe containing aliquot of gelatin gel. Transfer the sample into the syringe barrel was accomplished either using a butter-paper funnel/with a spatula; the funnel was weighed before and after transferring drug to account for any loss by sticking to
funnel. Separate funnels were used to prepare each dose. After transfer of the drug material into the syringe, an aliquot of gelatin was placed on top of the drug powder, thus effectively sandwiching it between 2 layers of gelatin. The sample was attached to an oral feeding needle and administered into the stomach. After dosing, syringe was rinsed with 1mL of water and dosed again. Serial blood samples (50 µL) were withdrawn from the cannulated jugular vein at: Pre dose, 1, 2, 4, 6, 8, 9 and 24 h post-dosing and collected in labeled tubes containing 20 µL of EDTA dipotassium dehydrate solution (200mM) per ml of blood as anticoagulant. Blood samples were held on ice until centrifuged at 10000 rpm; 4°C for 10min. Plasma was transferred to individual Eppendorf tubes and stored below -70°C until bioanalysis.

Bioanalysis

The samples were analyzed by High performance liquid chromatography (HPLC Model no: API LC-1018, Shimadzu, Japan) by using reverse phase column (Hypersil BDS C18 150 mm × 4.6 mm) and UV detector at 286nm using 30:70 orthophosphoric acid buffer and acetonitrile. Clofibric acid stock solution was used as the internal standard. Under these conditions, no interference was observed for both samples and pure drug. The standard curve was linear from 1ng/ml to 2000 mg/ml.

Pharmacokinetic data analysis

The area under the drug concentration-time curve from zero to 24 h (AUC_{0→24h}) and Mean Residence Time (MRT) were calculated using noncompartmental analysis (WinNonlin 2.1, Pharsight Corp., Mountain View, CA). The maximal plasma concentration of drug (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were directly obtained from plasma data and area under the curve (AUC), elimination rate constant (K_{e}) and half life (t_{1/2}), were also computed.

Results and Discussion

Infrared Spectroscopy analysis of drug and excipient mixture

The principal peaks of pure fenofibrate were observed at 1729.4, 1651.0, 1598.9 and 925.1 cm⁻¹. The characteristic peaks for drug and excipients mixture were found at 1729.6, 1650.1, and 1599.1 and 925.5 cm⁻¹. No peaks were found at these wave numbers for excipients indicating no possible interaction between drug and the polymer therein (Figure 1).

Characterization of taguchi experimental runs

The characterized properties for Taguchi runs given in Table 4 suggests that the two factors under consideration namely type and concentration of solubilizers have insignificant effect on the properties of % theoretical yield, mechanical crushing force, abrasion resistance, residual moisture, assay and flow properties.

In vitro release studies

The in vitro dissolution profile of Taguchi experimental runs indicates that for Run 1 to Run 2 with 0.5%, 1% alpha tocopherol and Run 5 to Run 16 with 0.5%, 1%, 1.5%, 2% of soy phosphatidyl choline 70, Phospholipon 80H and Phospholipon 90H the release was acceptable with 10-30% of release in first hour, 50-75% drug in fourth hour and greater than 75% of release at the end of seventh hour. While for Run 3 and Run 4 containing 1.5% and 2% alpha tocopherol, the release was greater than 30% in first hour and greater than 90% of drug was released at the end of seventh hour (Table 5).

The statistical analysis (Figure 2a) suggests that factor A, type of solubilizer has pronounced and significant effect on dissolution behavior, since the obtained P value is 0.020 (P < 0.05). The effect can be indicated in descending order as
Phospholipon 90H > alpha tocopherol > soy phosphatidyl choline 70 > Phospholipon 80H

While concentration of solubilizer (Figure 2b) suggests no pronounced effect on dissolution behavior of drug in lipid phase, since obtained P value is 0.756, (P > 0.05) increase in concentration suggested enhanced dissolution behavior of drug.

Comparative dissolution profiles for Taguchi optimized runs with that of marketed are elucidated (Figure 3).

Pharmacokinetic parameters

The comparative average serum concentration-time profiles of fenofibrate following oral administration of 10 mg/kg in the rat (Figure 4).

Statistical Analysis of data

ANOVA results were obtained using the Minitab statistical software package (version 15). The response factor or dependent factor studied was dissolution behavior of drug. The effect of two parameters effect of solubilizer and concentration of solubilizer on the in vitro dissolution of drug were studied. The F, P values obtained was 4.81, 0.020 when the response was compared with factor A, namely type of solubilizer, the obtained F, P values when the response was compared with factor B, namely concentration of solubilizer were 0.40 and 0.756 respectively. The tabulated F value with three degrees of freedom in the numerator and denominator is 15.4. Therefore the variances cane be considered significantly indifferent and the P < 0.05 when the response was studied against factor A stating that factor A has insignificant effect on the response variable. While P > 0.05 when the response was studied against factor B stating that factor B has significant effect on the response variable.

Model independent methods

Model independent methods were used to compute the Mean Dissolution Time (MDT) and also to compare Taguchi experimental runs.
runs to the marketed formulation by calculating similarity ($f_s$) and difference factor ($f_d$) that signifies the Taguchi experimental runs for acceptance or rejection for further studies. The computed values are given in Table 6.

**Test for Comparison of means of reference and optimized Taguchi experimental runs**

Two formulations of Taguchi experimental runs were optimized based on characterized properties, ANOVA, release kinetics, mean dissolution time, similarity and difference factor. Of all the Taguchi experimental runs, Run 2, with 1% alpha-tocopherol and Run 16, with 2% Phospholipon 90H were proved to be the optimized runs with respective to highest similarity factor and highest drug release. Therefore the two formulations were compared with reference for statistical significance. For this, a two-sided Paired-Sample $t$'s test was applied. The obtained values were compared with standard tables for significance at confidence intervals of 95% [10]. The summary of test is briefed out in Table 7.

The resultant $t$'s was found to be 5.200626976 for Run 2, and was found to be 4.28116546 for Run 16. For a two sided test at 5% level, a $t$ value of 2.36 is needed for significance with seven degrees of freedom (Since n=8). Therefore the difference is significant at 5% level, stating that the optimized formulations have better bioavailability compared to marketed, which has already been evidenced from (Figure 3), (Table 6). The summary of test is given in Table 7.

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![Figure 3: Comparative release profiles of optimized Taguchi runs with marketed formulation.](image)

![Figure 4: Comparative average serum concentration-time profiles of Taguchi optimized runs with marketed formulation and pure drug.](image)

<table>
<thead>
<tr>
<th>Experimental Runs</th>
<th>Calculation of MDT, $f_s$, and $f_d$ for the dissolution data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDT</td>
</tr>
<tr>
<td>Run $1^*_1$</td>
<td>0.001953</td>
</tr>
<tr>
<td>Run $2^*_2$</td>
<td>0.00172479</td>
</tr>
<tr>
<td>Run $3^*_3$</td>
<td>0.757755</td>
</tr>
<tr>
<td>Run $4^*_4$</td>
<td>0.674866</td>
</tr>
<tr>
<td>Run $5^*_5$</td>
<td>0.00105709</td>
</tr>
<tr>
<td>Run $6^*_6$</td>
<td>0.00218616</td>
</tr>
<tr>
<td>Run $7^*_7$</td>
<td>0.001483015</td>
</tr>
<tr>
<td>Run $8^*_8$</td>
<td>0.00202604</td>
</tr>
<tr>
<td>Run $9^*_9$</td>
<td>0.014877</td>
</tr>
<tr>
<td>Run $10^*_2$</td>
<td>0.0123761</td>
</tr>
<tr>
<td>Run $11^*_2$</td>
<td>0.0100919</td>
</tr>
<tr>
<td>Run $12^*_2$</td>
<td>0.002579166</td>
</tr>
<tr>
<td>Run $13^*_2$</td>
<td>0.00565444</td>
</tr>
<tr>
<td>Run $14^*_2$</td>
<td>0.003653</td>
</tr>
<tr>
<td>Run $15^*_2$</td>
<td>0.00267744</td>
</tr>
<tr>
<td>Run $16^*_2$</td>
<td>0.00726911</td>
</tr>
<tr>
<td>Marketed</td>
<td>0.011559507</td>
</tr>
</tbody>
</table>

**Table 6: Descriptive statistical model independent methods for calculation of MDT, $f_s$ and $f_d$ for the dissolution data (n=6).**

Model independent methods suggests that the mean dissolution time for all Taguchi experimental runs were constant and almost the cumulative percent of drug release throughout the test time was predicted to release the drug for in sustained manner for eight hours given in Table 6. While the MDT for Run 3 and Run 4, of 0.757755 and 0.674866 suggests rapid drug release in first intervals of test period indicating substantial increase in concentration of alpha tocopherol. Alpha tocopherol is a lipophilic potential antioxidant that might interact with membrane phospholipids and modulates the membrane fluidity and hence lead to the rapid solubilization of the drug and it may be anticipated that the critical micelle concentration of alpha tocopherol may be greater than 1%. Therefore the enhanced concentrations of alpha tocopherol above 1% lead to the rapid in vitro release of drug in initial hours and had no effect on in vitro dissolution behavior of drug. The $f_s$ and $f_d$ factors suggests that twelve out of sixteen runs were similar to reference with $f_s$ value between 50-100 and Run 2, Run 16, are more closer to the reference with $f_d$ of 61.19 and 80.48. The two side Paired-Sample $t$ test suggested significant difference between reference and test runs and indicated Run 2 to be more bioavailable and may result in enhanced absorption from the gastrointestinal tract as evidenced by use of D-α-tocopheryl PEG 1000 succinate (TPGS), a derivative of vitamin E that has a relatively low critical micelle concentration and has been found to increase the oral absorption of drugs including vancomycin through micelle formation [11]. For hydrophobic drugs, increased wettability upon exposure to biological fluids may lead to improved dissolution thereby enhanced bioavailability that was in turn evidenced from the pharmacokinetic parameters where there was marked improvement in bioavailability.
rate and extent compared to the pure drug. Significant differences were found in AUC_{0-24} as well as in C_{max} and t_{1/2} between the test and reference formulations as shown in the Table 6. The hypothesized mechanism is the interaction and fluidization of membrane phospholipids thereby assisting in drug transport by enhancing the lipid solubility [12] as in the case of L-deaminophenylalanine where the transport of loseroxaphidyl choline treatment increased the apparent permeability coefficient, P_{eff} even under circumstances where the cell monolayer integrity was only slightly altered and also evidenced by 8 fold increase in concentration of miconazole indicating that the enhancement effect involved high partition of miconazole into the skin by the use of phospholipid and dodecyl 2-(N,N-dimethyl amino) propionate [13,14]. Therefore phosphatidylcholine embedded micellar systems are responsible for enhanced permeability. The difference in three types of phospholipids may be owed to the difference in percentage of phosphatidyl choline (PC) as evidenced by the solubility of miconazole that increased in proportion to the hydrogenated phosphatidylcholine concentration [14]. Run with Phospholipon 90H containing 90% PC and 4% of lyso phosphatidyl choline, the byproduct of PC resulted in better in vitro dissolution and higher drug release. These results are similar to that of bile salts containing soybean phosphatidylcholine and sodiumdeoxycholate which were used as absorption enhancers for bioavailability of fenofibrate that showed 1.57 fold in bioavailability compared to liposomes containing soybean phosphatidylcholine and cholesterol [15]. Hence use of alpha tocopherol and phospholipids at lower concentrations and may be potential and safe solubilizers for improving dissolution thereby absorption of poorly water soluble drugs similar to dendrimers [16]. These may also have potential effects on other poorly water soluble drugs like griseofulvin, cetoxatefime sodium, cetazidime pentahydrate, cyclosporin A and lovastatin that made use of alpha tocopherol and medium fatty acids (sodium caprylate, caprate), cyclodextrins (β-cyclodextrin, hydroxypropyl β-cyclodextrin) and bile salts (sodium cholate, deoxycholate) for solubilization of drug and absorption enhancement [17]. There may be effect on absorption kinetics similar to norfloxacain where the absorption kinetics was improved by the use of phospholipid and bile salts (sodium cholate, deoxycholate) for solubilization of highly lipophilic drugs like fenofibrate and the like.

**Acknowledgements**

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**References**


**Table 7: Test for Comparison of means of reference and optimized Taguchi experimental runs using a two side Paired-Sample T test (n=6).**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Marketed formulation</th>
<th>Run 2</th>
<th>Run 16</th>
<th>Δ = Ref - Run 2</th>
<th>Δ = Ref - Run 16</th>
<th>R = Reference/ Run 2</th>
<th>R = Reference/ Run 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.6±0.21</td>
<td>27±0.007</td>
<td>21.2±0.08</td>
<td>9.4±0.03</td>
<td>3.6±0.14</td>
<td>0.65±0.02</td>
<td>0.83±0.21</td>
</tr>
<tr>
<td>2</td>
<td>35.8±0.15</td>
<td>44.3±0.15</td>
<td>36.2±0.1</td>
<td>8.4±0.17</td>
<td>0.41±0.07</td>
<td>0.89±0.05</td>
<td>0.98±0.28</td>
</tr>
<tr>
<td>3</td>
<td>51.6±0.21</td>
<td>57.8±0.17</td>
<td>52.3±0.12</td>
<td>6.4±0.09</td>
<td>0.53±0.28</td>
<td>0.895±0.14</td>
<td>0.98±0.28</td>
</tr>
<tr>
<td>4</td>
<td>63.5±0.01</td>
<td>69.6±0.07</td>
<td>64.5±0.19</td>
<td>6.1±0.02</td>
<td>1.03±0.04</td>
<td>0.91±0.05</td>
<td>0.98±0.02</td>
</tr>
<tr>
<td>5</td>
<td>74.4±0.28</td>
<td>78.6±0.06</td>
<td>75.3±0.28</td>
<td>4.2±0.01</td>
<td>0.92±0.7</td>
<td>0.946±0.32</td>
<td>0.98±0.05</td>
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<td>6</td>
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<td>86.7±0.03</td>
<td>84.7±0.07</td>
<td>4.1±0.02</td>
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<td>7</td>
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<td>2.9±0.08</td>
<td>0.987±0.10</td>
<td>0.97±0.007</td>
</tr>
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</table>

* Indicates the significant difference at 5% level


