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VIRTUAL SCREENING OF COFORMERS FOR ATORVASTATIN Co-CRYSTALLIZATION AND THE CHARACTERIZATIONS OF THE Co-CRYSTALS

D. Gozali [1], S. Megantara [1], J. Levita [*1], H.H. Bahti [2], S.N. Soewandhi [3] and M. Abdassah[1]

Faculty of Pharmacy [1], Universitas Padjadjaran, Jl. Raya Bandung-Sumedang km. 21 Jatinangor Sumedang, Indonesia
Department of Chemistry [2], Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran Jl. Raya Bandung-Sumedang km.21 Jatinangor Sumedang, Indonesi
School of Pharmacy [3], Bandung Institute of Technology, Jl. Ganesha 10 Bandung

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Correspondence to Author:
Jutti Levita

Department of Chemistry , Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jl. Raya Bandung-Sumedang km.21 Jatinangor Sumedang, Indonesia

E-mail: jutti.levita@unpad.ac.id

ABSTRACT: Atorvastatin calcium (ATC) is very slightly soluble in water and it is classified under BCS class II drugs. A widely used method to enhance the solubility of drugs is co-crystallization. In this work, we screened six co-formers for ATC by employing molecular docking method. The work was continued by co-crystallization process using slurry method, solubility assay of the mixtures using HPLC, and characterization of the co-crystal by PXRD, DSC and SEM. Based on molecular docking, the best co-former is aspartame (Ei = -4.70 kcal/mol). The docking result fits the solubility assay of the ATC-aspartame co-crystal (136.77% increasing of solubility compared to ATC). ATC-aspartame co-crystal shows better dissolution profile (91.62 % in 60 minutes) than ATC (73.54 % in 60 minutes). The characteristic peaks of ATC and aspartame were gone, whilst new peaks appeared after slurry process. The ATC-aspartame characterization by PXRD, DSC and SEM positively confirmed that the co-crystallization of ATC-aspartame using slurry method was successful.

INTRODUCTION: Atorvastatin (PubChem CID 60823), or its IUPAC name is (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5 dihydroxy heptanoic acid (**Fig.1**), is in a group of drugs called HMG-CoA reductase inhibitors. Atorvastatin calcium (ATC), a hemi-calcium salt, is very slightly soluble in water, phosphate buffers at pH 7.4, and acetonitrile, freely soluble in methanol.

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According to the Biopharmaceutical Classification System (BCS), ATC is classified under BCS class II drugs that exhibit poor aqueous solubility and high permeability. The intestinal permeability of atorvastatin is high at the physiologically intestinal pH (6–6.5). However, it is reported that the absolute bioavailability of atorvastatin is 12% after a 40 mg oral dose [1-3].

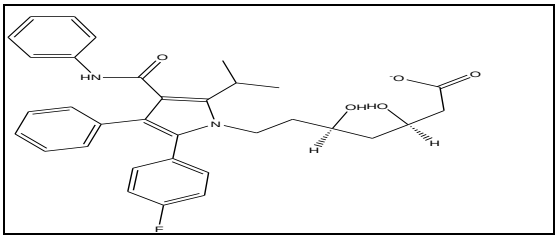


FIG.1 2D STRUCTURE OF ATORVASTATIN CHEMSPIDER ID 54809
(downloaded as *mol* format from www.chemspider.com)

A widely used method to enhance the solubility of poorly water soluble drugs is co-crystallization. A co-crystal is a multi-component crystal which involves non-covalent interactions between API and its co-formers. There are several approaches or tools to predicting the formation of co-crystal between API and co-crystal former molecule (co-former), such as solubility-based approach, Hansen solubility parameter tool [1], or computational calculation by employing molecular docking method [4]. Previous studies showed that co-crystal of calcium atorvastatin was successfully formed using isonicotinamide [5] and natural or synthetic polymers [2, 6, 7] as co-formers.

In this work, we developed virtual screening of co-formers for ATC by employing molecular docking method as proposed by Siswandi [4]. AutoDock (The Scripps Research Institute) was used for docking. Parameters observed were type and energy (Ei) of interaction. The work was continued by co-crystallization process characterized by PXRD, DSC, FTIR and SEM. The solubility and dissolution rate assay of the mixtures were performed using HPLC.

MATERIALS AND METHODS:

Hardware and programs:

Personal computer equipped with Linux Ubuntu 14.04 LTS, Intel Core i5 2.30 GHz processor DRAM 4 GB was used in this work. Programs were SPORES, Open Babel GUI 2.2.3, PLANTS1.2, and MGLTools1.5.6 shell script for intial preparation of the ligands and Auto Dock 4.2.3 for docking process.

Molecular modelling:

2D structures of atorvastatin (Chem Spider ID: **54809**) and its co-formers in *.mol* format were downloaded from www.chemspider.com. All molecules were geometry optimized using molecular mechanics method in Portable_Hyperchem_8.0. and calculated their QSAR properties using the same program. All *.mol* files of the molecules were converted into *.pdb* files by employing Open Babel GUI 2.2.3. The files then were opened in Auto Dock 4.2.3 and converted into *.pdbq* files by adding polar hydrogen and Kollman charges. The *.pdbq* files were converted into *.pdbqt* by calculating their torsion angles and were ready to be used for docking. The dimension of grid box was set to 40x40x40 $Å^3$ and other parameters were set to default. Docking was repeated 10 times for each co-former. Parameters observed were type and energy (Ei) of interactions.

Preparation of ATC-the best conformer co-crystal by slurry method

ATC commercial material with purity of >99% was obtained from PT. Kimia Farma, tbk, Indonesia. Benzoic acid, fumaric acid, citric acid, aspartame, tartaric acid, and succinic acid were obtained from Merck Chemicals. Methanol, acetonitrile, and other reagents were purchased from Merck Chemicals without any purification.

Atorvastatin calcium 6.05 g and the best conformer according to *in silico* result, were carefully weighed and mixed homogeneously in a mortar. 15 mL of methanol was added to the mixture until it became slurry. The product was dried at 40^0 C for 48 hours. The solid product was stored in a desiccator under vacuum.

Characterization of the product:

Powder X-ray Diffraction (PXRD):

X-ray diffraction patterns were traced on atorvastatin, ATC- cocrystal, and aspartame by employing an X-ray diffractometer (X'pert pro pan analytical, Netherland). The samples were analyzed using Ni-filtered CuKa radiation (l¼ 1.5418 ˚A) under the following condition: voltage 40 kV, current 40 mA, receiving slit 0.2 inches, 2y range of 5–75 1C, scan rate 0.0401/s.

Differential Scanning Calorimetry (DSC):

Thermal analysis was performed using differential scanning calorimeter (Linseis, USA). Under nitrogen flow of 20 ml/min, approximately 4-4.5 mg of sample was placed in a sealed aluminium pan and heated at a scanning rate of 10°C/min from 30°C to 300°C. An empty aluminium pan was used as reference.

Scanning electron microscope (SEM):

The analysis was carried out using scanning electron microscope (JSM 6100, Jeol, Japan). Atorvastatin and co-crystal of ATC-aspartame were mounted onto the stubs using double-sided adhesive tape and then coated with a thin layer of

gold palladium alloy (150–200A°). The microscope was operated at an acceleration voltage of 20 KV, working distance (12–14 mm). The magnification was set ×500.

Solubility Studies:

Solubility studies were performed according to the method described by Higuchi and Connors. The saturation solubility of pure atorvastatin calcium, ATC-aspartame, and aspartame in water was determined by adding an excess of the drugs to 50 ml distilled water in conical flask and were rotated in a orbital shaking incubator for 48 h at 37^0C ± 0.5^0C. The saturated solutions were filtered through a 0.45 μm membrane filter, suitably diluted with water and analyzed by HPLC.

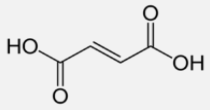
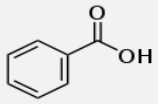
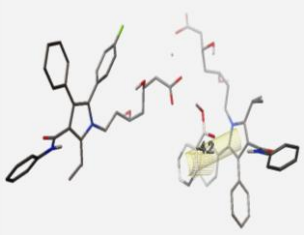
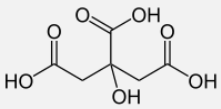
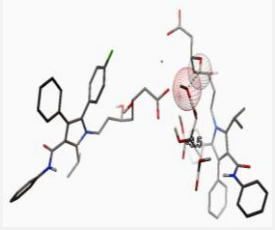
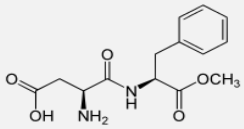
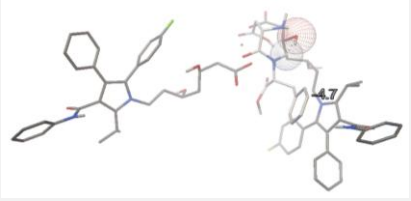
In vitro Dissolution Studies:

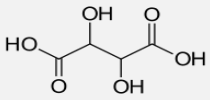
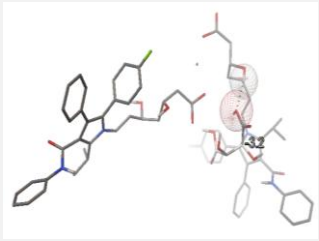
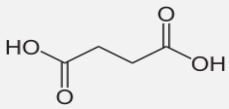
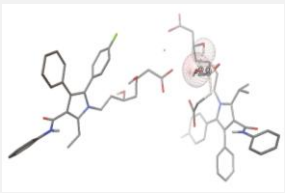
In vitro dissolution studies of pure ATC trihydrate and ATC-aspartame and physical mixture were conducted with the USP type II apparatus (paddle type). The dissolution studies were performed using phosphate of pH 6.8 buffer as dissolution medium at 37±0.5^0C with 75 rpm speed. Samples of each preparation equivalent to 80mg of drug were added into the dissolution medium. The sample of 1ml aliquots were withdrawn periodically (5, 10, 15, 30, 45 and 60 min) and were quantified by HPLC. Detection was set at 246 nm.

RESULTS AND DISCUSSION: Atorvastatin molecule (**Fig.1**) contains four aromatic rings, 4 HBDs (hydrogen bond donor) and 6 HBAs (hydrogen bond acceptor), hence it is possible to form co-crystals with certain co-formers.

Molecular docking proved that all co-formers only interacted with one ATC molecule, and the highest affinity to ATC was showed by aspartame (interaction energy = -4.70 kcal/mol). Of the six co-formers, only benzoic acid undergoes hydrophobic interaction as indicated by two π-π interactions.

TABLE 1: VIRTUAL SCREENING OF co-FORMERS FOR ATC

Co-former	2 D structure	Interaction	Ei (kcal/mol)
Fumaric acid			-3.20 1 Hydrogen bond
Benzoic acid			-4.20 π-π interaction
Citric acid			-3.50 1 Hydrogen bond
Aspartame			-4.70 1 Hydrogen bond

Tartaric acid			-3.20 1 Hydrogen bond
Succinic acid			-2.90 1 Hydrogen bond

Mixtures of ATC and the co-formers were tested for their solubilities and compared with ATC itself. Based on solubility assay (**Table 2**) carried out using Higuchi and Connor method, ATC is slightly soluble in water (22.08µg/mL). By mixing it with citric acid, the solubility increases 119.11% (26.30 µg/mL), whereas with benzoic acid and aspartame the increasing of solubility are 130.93% (28.91 µg/mL) and 136.77% (30.20 µg/mL), respectively.

TABLE 2: SOLUBILITY ASSAY

Compound	Solubility in water (µg/mL)
ATC	22.08
ATC- citric acid	26.30
ATC- succinic acid	14.32
ATC- fumaric acid	23.73
ATC- benzoic acid	28.91
ATC- tartaric acid	19.70
ATC- aspartame	30.20

The selection of solvent used in the slurry method is very important, where the solvent should not be able to dissolve at least small portion of the parent's components. Methanol was used as liquid phase in preparation of ATC-aspartame co-crystal because both compounds are soluble in it. During slurry process, the mixtures changed to amorphous phase and recrystallized rapidly. X-ray powder diffraction was used to monitor the co-crystallization. The presence of methanol as a liquid phase increases the rate of co-crystal formation due to enhancement of molecular diffusion.

TABLE 3: DISSOLUTION TEST

Time (minute)	% of ATC-aspartame	% of ATC
0	0	0
5	41.22	25.92
10	48.24	41.00
15	65.39	53.56
30	80.83	57.74
45	86.00	69.62
60	91.62	73.54

Dissolution test is showed in **Table 3**. ATC-aspartame co-crystal shows better dissolution profile (91.62 % in 60 minutes) than ATC (73.54 % in 60 minutes). This data proved that co-crystallization of ATC with aspartame co-former enhances the solubility of ATC.

The formation of co-crystals is primarily characterized by PXRD. If the resulting PXRD pattern of the solid product is different from the starting materials, it can be concluded that a new solid phase was formed.

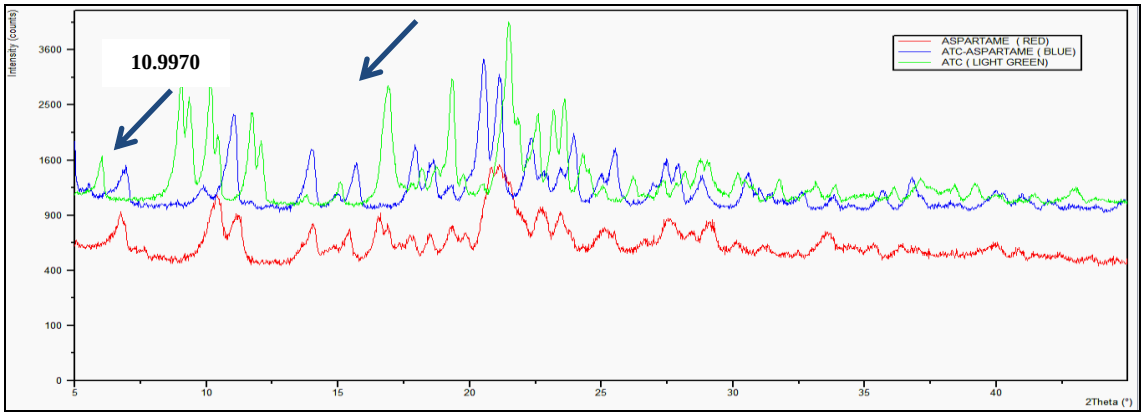


FIG.2: PXRD PATTERN OF ATC (GREEN), ASPARTAME (RED) AND ATC-ASPARTAME (BLUE)

The PXRD pattern ATC-aspartame co-crystal (blue) is showed in **Fig. 2**, which proves that the characteristic peaks of ATC (green) and aspartame (red) were gone, whilst new peaks (indicated by blue arrows in **Fig.2**) appeared after slurry process. The changes in the position of the peaks after co-crystallization process indicated that there was a formation of ATC-aspartame co-crystal in a 1:2 molar ratio. Detailed information of the PXRD peaks is showed in **Table 4.**

TABLE 4: 2-THETA (^{0}A) PEAK POSITION

ATC	Aspartame	ATC- Aspartame
6.0471	6.7452	5.5077
9.0297	10.3941	6.9335
10.1720	11.1557	9.8547
11.7268	14.0431	10.9970*
16.8671	15.4392	13.9996
19.3103	16.6132	15.6931
21.4680	20.8333	17.9142
22.6102	21.1189	21.1507*

*indicates the 2-theta position of the new peaks

Melting point is a fundamental physical property, which is determined by the temperature at which the solid phase is at equilibrium with the liquid phase. DSC is the most preferred technique for obtaining comprehensive melting point data.

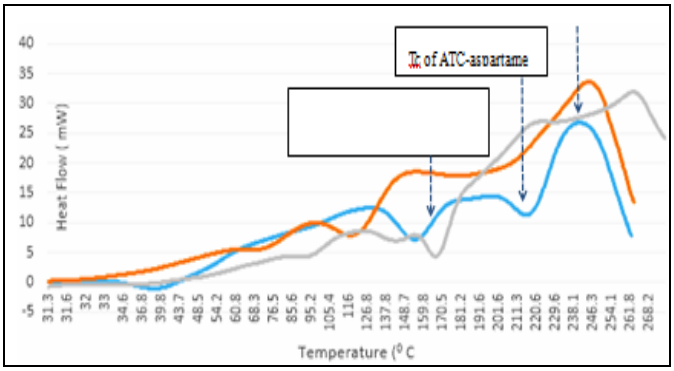


FIG.3 DSC THERMOGRAM OF ATC (GREY), ASPARTAME (ORANGE), AND ATC-ASPARTAME (BLUE)

Fig.3 showed positively the presence of ATC-aspartame co-crystal, as proved by the dip (minimum) on the blue thermogram. This minimum or Tc (crystallization temperature) indicates the heat-off when crystals are formed. The continuous heating past its Tc will form another thermal transition that is Tm or melting temperature (**Fig.3**). ATC-aspartame melted at 168.30°C while ATC Tm was detected at 179.90°C. Further analysis using SEM was performed to differentiate and characterize the crystal of ATC-aspartame (**Fig.4**). **Fig.4** shows the difference between aspartame, ATC, and ATC-aspartame.

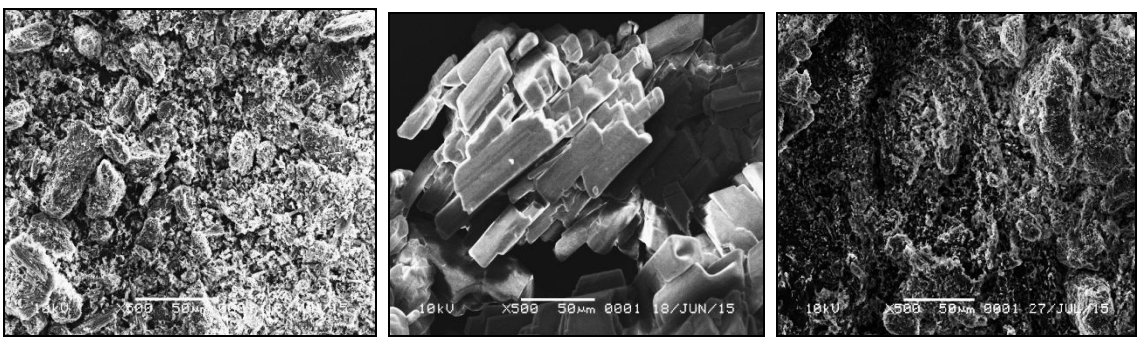


FIG.4: CRYSTALS OF ASPARTAME (LEFT), ATC (MIDDLE), AND ATC-ASPARTAME (RIGHT) AT 500x MAGNITUDE

CONCLUSION: The best co-former is aspartame (Ei = -4.70 kcal/mol). The docking result fits the solubility assay of the ATC-aspartame co-crystal (136.77% increasing of solubility compared to ATC). ATC-aspartame co-crystal shows better dissolution profile (91.62 % in 60 minutes) than ATC (73.54 % in 60 minutes). The ATC-aspartame characterization by PXRD, DSC and SEM positively confirmed that the co-crystallization of ATC-aspartame using slurry method was successful.

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