COMMUNICATION

Crystal Engineering of Nutraceutical Phytosterols: New Cocrystal Solid-Solutions

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A cocrystal screening conducted with a solid solution of three phytosterols (β-sitosterol, campesterol and stigmasterol) and a set of coformers with strong hydrogen bond donors reveals that multicomponent solid-solutions are preferentially formed instead of pure cocrystals and much enriched with β-sitosterol with respect to stigmasterol, a natural product with cytotoxicity concerns.

Phytosterols are plant steroids with a structure similar to cholesterol. Their structures vary only in the carbon side chains and/or the presence or absence of a double bond (e.g. sitosterol and sitostanol). Phytosterols are widely distributed in the plant kingdom and specially found in vegetable oil, nuts, seeds and avocados. However, they are not synthesized by the human body and therefore their presence in the body is the result of their consumption as part of the diet. Particularly, the β-Sitosterol is the most common dietary phytosterol.[1] Several clinical studies have demonstrated that phytosterols reduce serum cholesterol levels by inhibiting cholesterol absorption in the intestinal lumen.[2] Particularly, beta-sitosterol is useful for the reduction of serum total and LDL-cholesterol levels because the beta-sitosterol competes with cholesterol for up taking the cholesterol by the cells or by interfering with the esterification of cholesterol. In fact, beta-sitosterol has been approved by the FDA (Food and drug Administration) for that indication.[3] Moreover, Phytosterol-induced treatment sensitivity has been studied with multidrug resistance in cancer therapy. Recently β-sitosterol, has shown to have an effect in drug-resistant colorectal cancer cells by inhibiting breast cancer resistance protein expression.[4] It is known that different solid forms of an active ingredient can have different properties, and offer certain advantages with regard to solubility or bioavailability. Thus, the discovery of new solid forms allows for improving the pharmacokinetic and/or pharmacologic or other

physicochemical properties of the active ingredients. In recent years cocrystal formation has emerged as a viable strategy towards improving the pharmacokinetic data of active ingredients.[5] One of the main issues is to select the appropriated pharmaceutical acceptable coformers which can interact satisfactorily with the active ingredient and, at the same time, provide to the new entity with advantageous physicochemical properties, like bioavailability or stability .[6]

Impurities in the raw material may significantly impact crystallization process giving rise a new crystal forms, either stables or metastables[7] or can reduce the nucleation rate.[8] A particular case is the study of a multicomponent crystal of variable stoichiometry in which the impurity is a compound whose structure and size is similar to the main compound in the crystal lattice. This kind of compounds are called nonstoichiometric substitutional mixed crystals (or crystalline solid solutions), (CSS).[9]

CSS have attracted interest in the past decade among crystallographers,[10-11] mainly because they have the potential to design tunable materials for pharmaceutical applications, which makes solid solutions relevant in the scope of crystal engineering.[12-13] However, these solid forms are not well understood yet and are difficult to obtain.[14] Since the solid state of CSS is scarcely studied with respect to pharmaceutical and nutraceutical compounds not much is known about how the formation of CSS can impact the physicochemical properties of the crystal form. For instance, the difficulty to identify the number of molecules in the asymmetric unit, especially in chiral compounds, as a racemic solid solution of enantiomers[15-17] or a mixture of two diastereomers.[18] In this sense, a good approach to identify potential CSS formers is by taking advantage of the isostructurality and isomorphicity concepts[19-21] but at present it is difficult to determine whether two or more molecules will be completely miscible in the crystal structure as well as their random distribution in the crystal lattice.

In this work, we aimed to extend the solid-state knowledge of the important nutraceutical β-sitosterol by engineering new cocrystals. And for the first time new multicomponent forms of β-sitosterol containing other phytosterols in the crystal lattice are reported. The new cocrystals, in form of CSS, have been discovered through an experimental cocrystal screening by using a broad set of thermodynamic and kinetic experimental conditions.

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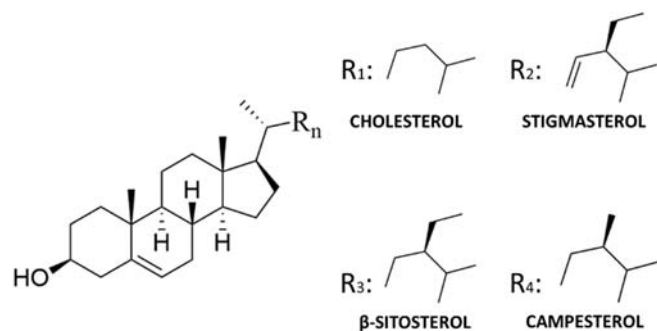
Figure 1. Molecular structures of sterols. Aliphatic chain differences are present from C_{19} (R_n schematic representation)

Since commercial sources of β-sitosterol at affordable prices are usually mixtures of phytosterols in the form of solid solutions,[22] we decided to conduct the study with such mixture, in the form of a ternary solid solution containing campesterol and stigmasterol as the starting material. Thus, we have performed a comprehensive cocrystal screen with a set of coformers, which includes carboxylic acids and phenol-containing compounds from a variety of 30 organic solvents, which produced 370 crystalline solids (see ESI, for experimental and characterization details).

Nine multicomponent forms of β-sitosterol (SIT) with different proportion of phytosterols (SIT CSS) (one solvate and eight cocrystals) have been obtained through a cocrystal screening with five out of the twenty-seven coformers tested. One solvate form with benzyl alcohol (BzOH) in a 4:1 molar ratio (as a tetartohydrate form, SIT-BzOH-H$_2$O). Eight SIT CSS have been obtained with five coformers: three forms with propionic acid (ProA) in two different stoichiometries: one in a 2:1 molar ratio (as a hemihydrate form, SIT-ProA-H$_2$O I) and two in a 4:1 molar ratio (as an tetartohydrate form, SIT-ProA-H$_2$O II and as an acetonitrile tetartosolvate form SIT-ProA-ACN); one form with zymonic acid (ZA) in a 2:1 stoichiometry (as an hemihydrate form, SIL-ZA-H$_2$O); one form with gallic acid (GA) in a 4:1 stoichiometry (as an anhydrous form, SIL-GA); two with 4-hydroxybenzoic acid (4-HBA) in a 1:1 stoichiometry (one as an anhydrous form, SIT-4-HBA I and one as a hemihydrate form, SIT-4-HBA-H$_2$O II) and finally one

with 3,4-dihydroxybenzoic acid (3,4-DHBA) in a 1:2 stoichiometry (as a monohydrate, SIT-3,4-DHBA-H$_2$O). Since our raw material is a CSS of three phytosterols, accurate determination of each phytosterol content is essential for the intellectual property implications and the full characterization of the new forms. In this sense, the phytosterols present in all new CSS forms were quantified and the content of each phytosterol determined using GC and HPLC techniques. The details about GC and HPLC methods are provided in ESI. Quantification shows that in average β-sitosterol (~ 86%) is in higher proportion followed by campesterol (~ 9%) and stigmasterol (~ 5%), respectively. (Table 1)

Experiments conducted with propionic acid as the coformer produced three different cocrystal forms with two different stoichiometries, according to NMR and SCXRD measurements. In particular, experiments conducted generally through kinetic control conditions produced a 2:1 cocrystal solid solution (for instance, use of water as an antisolvent in the precipitation of a solution of the phytosterols solid solution in propionic acid), while thermodynamic control experiments, produced a 4:1 cocrystal solid solution (for instance, slurry experiment for one day in propionic acid-acetone suspension). While NMR experiments could not confirm the presence of water Single X-ray Diffraction measurements allowed to not only confirm the stoichiometry but also determine the presence of a molecule of water in the crystal lattice, being new forms SIT-ProA-H$_2$O I and SIT-ProA-H$_2$O II 2:1:1 and 4:1:1 SIT CSS:propionic acid:water Cocrystal Solid Solution hydrates, respectively. The last crystal form is an ACN solvate with 4:1:1 stoichiometry confirmed by SCXRD and it was obtained by ACN atmosphere diffusion through SIT CSS-propionic acid acetone solution (SIT-ProA-ACN).

Unfortunately, the low proportion of two phytosterols (stigmasterol and campesterol) together with the low quality of the single crystals and the presence of substitutional disorder (deduced from the large size of the ellipsoids associated to the carbon atoms at the end of the aliphatic chains) due to the presence of the three phytosterols make difficult to determine the crystal structures with precision. Thus, we have refined them considering that all the molecules present in the lattice are β-sitosterol. However, these data are still valuable for the purposes of characterization of the new cocrystals.

Table 1. HPLC quantification of the different phytosterols in the standard sample and new CSS

Crystal form	Stigmasterol		Campesterol		β-Sitosterol	
	RT[a] (min)	Area[b] (%)	RT (min)	Area (%)	RT (min)	Area (%)
β-sitosterol standard (72.5%)	6.443	10.75	7.933	19.6	9.267	70.19
CSS SIT-ProA-H$_2$O I	6.256	7.09	7.777	9.76	9.167	83.14
CSS SIT-ProA-H$_2$O II	6.267	3.70	7.800	9.55	9.200	86.75
CSS SIT-ZA-H$_2$O	6.133	7.33	7.700	9.35	9.067	84.32
CSS SIT-GA	6.367	5.51	7.822	9.38	9.222	85.11
CSS SIT-4-HBA I	6.252	3.59	7.733	9.06	9.100	87.35
CSS SIT-4-HBA-H$_2$O II	6.250	6.03	7.767	9.16	9.167	84.82
CSS SIT-3,4-DHBA-H$_2$O	6.284	5.45	7.767	9.27	9.167	85.27
Standard deviation (SD)[c]	6.262±0.064	5.22±1.64	7.760±0.041	9.27±0.34	9.147±0.056	85.64±1.71

a: "RT" means retention time; b: The area is expressed in percentage (W/W); c: Average of all the cocrystals

Net grinding experiments conducted with pyruvic acid as the coformer and SIT CSS, produced a new cocrystal with a different coformer in the crystal lattice as a consequence of an *in situ* chemical reaction in solution which transformed pyruvic acid in zymonic acid. This transformation has been previously reported in literature.[23] Moreover, experiments conducted with pyruvic acid containing 7% of zymonic acid (determined by NMR) as the coformer (solvent-mediated transformation in cyclohexane at 25 °C for four days) produced a 2:1:1 zymonic acid cocrystal solid solution. No evidences of pyruvic acid cocrystal solid solution has been observed. In a similar way as with propionic acid cocrystals, low quality crystal structure determination confirmed the presence of water with a 2:1:1 stoichiometry. Interestingly, comparative cell parameters suggest that it corresponds to a zymonic acid solid solution different from the bulk powder.

Solvent-mediated transformation experiments between SIT CSS and gallic acid in AcOEt at 25 °C for one day produced an anhydrous 4:1 gallic acid cocrystal solid solution (SIT-GA). As previously described, low quality crystal structure determination confirmed a 4:1 stoichiometry.

Experiments conducted with 4-hydroxybenzoic acid as a coformer produced two different cocrystal forms, one anhydrous and one hemihydrate with the same stoichiometry, according to NMR and TGA measurements. In particular, solvent-mediated transformation experiments between SIT CSS and 4-hydroxybenzoic acid (in a molar ratio 1:1.1) in AcOEt at 25 °C for two days produced an anhydrous 1:1 4-hydroxybenzoic acid cocrystal solid solution (SIT-4-HBA I). On the other hand, reaction crystallization experiments with the coformer saturated in AcOEt at 25 °C for one day produced a hemihydrate 2:2:1 4-hydroxybenzoic acid cocrystal solid solution (SIT-4-HBA-H$_2$O II).

Reaction crystallization experiments conducted with 3,4-dihydroxybenzoic acid as the coformer saturated in AcOEt at

dihydroxybenzoic acid cocrystal solid solution (SIT-3,4-DHBA-H$_2$O).

As said before, although great efforts were devoted to produce good quality single crystals of all the new CSS forms, in all cases thin needles were obtained. Five crystal structures have been determined but, unfortunately, as a consequence of problems derived from poor data, the refinement can be used mainly to estimate their stoichiometry. On the other hand, high-resolution PXRD diffractograms of the new forms were indexed and the lattice parameters were refined by means of LeBail fits by using Dicvol04 software.[24] Indexed cell parameters were compared with the single crystal analysis to confirm that the single crystals and the bulk powders correspond to the same solid forms. Comparative crystallographic data and refinement details of CSS form are shown in Table 2.

In absence of good quality SCXRD data it is not possible to analyze with precision the structural features of the new multicomponent solid forms and, in fact it is not the subject of this paper although another future research is intended to focus on that particular issue. We think that for the purposes of this paper it is more important to discuss on the proportion of phytosterols in the new cocrystals. Thus, the most important common feature of all the cocrystals is that the coformer, as expected, is inserted forming layers between the alcohol moieties of each sterol solid solution. Interestingly, the resulting CSS are enriched with β-sitosterol and with a variable and low proportion of stigmasterol and campesterol. The lower proportion of stigmasterol with respect to the other phytosterols in the solid solution is probably due to the different flexibility of the aliphatic chain. Stigmasterol, in contrast to β-sitosterol and campesterol has a double bond in carbon 19, which increases its conformational rigidity at the end of the chain and probably fits worst with the other phytosterols in the crystal lattice. However, this proportion is variable depending on the

Table 2. Crystallographic data and refinement details of β-sitosterol crystalline solid solution: R-factor for SCXRD and R$_{wp}$ for PXRD

Crystal form CSS		a (Å)	b (Å)	c (Å)	α (°)	β (°)	γ (°)	V (Å^3)	Z	T (K)	Space group	R(%)
SIT-BzOH-H$_2$O	PXRD	38.21(3)	9.935(2)	7.640(2)	88.48(1)	93.38(3)	96.34(4)	2877(2)	1	298	P1	9.62
	SCXRD	37.763(14)	9.730(4)	7.597(3)	84.846(9)	86.089(8)	88.219(9)	2772.8(19)	1	100	P1	4.61
SIT-ProA-H$_2$O I	PXRD	40.0(2)	7.617(2)	9.631(1)	90	97.03(3)	90	2914(2)	2	298	P2$_1$	6.88
	SCXRD	39.635(8)	7.5391(16)	9.439(2)	90	95.216(6)	90	2808.8(10)	2	293	P2$_1$	4.46
SIT-ProA-H$_2$O II	PXRD	28.16(1)	7.568(2)	26.235(9)	90	92.09(2)	90	5587(3)	2	298	P2$_1$	11.5
	SCXRD	27.183(3)	7.4971(7)	26.373(3)	90	92.569(6)	90	5369.2(10)	2	100	P2$_1$	10.1
SIT-ProA-ACN	SCXRD	36.973(9)	9.702(3)	7.581(2)	82.962(5)	86.112(5)	89.740(5)	2692.7(13)	1	100	P1	10.6
SIT-ZA-H$_2$O	PXRD	39.51(2)	6.982(3)	20.126(7)	90	95.18(4)	90	5528(4)	4	298	P2$_1$	10.1
	SCXRD	77.42(2)	7.6086(18)	9.924(3)	90	90.948(7)	90	5845(3)	4	293	C2	6.02
SIT-GA	PXRD	38.54(5)	13.812(4)	10.882(3)	90	92.33(5)	90	5788(8)	2	298	P2$_1$	11.1
	SCXRD	38.1228(19)	13.6989(6)	10.7439(5)	90	93.002(2)	90	5603.2(5)	2	100	P2$_1$	8.97
SIT-4-HBA I	PXRD	38.16(5)	14.196(6)	10.549(4)	90	92.0(1)	90	5711(8)	8	298	P2$_1$	21.3
SIT-4-HBA-H$_2$O II	PXRD	42.87(3)	7.083(5)	8.361(5)	107.3 (1)	108.8(1)	89.06(3)	2285(3)	3	298	P-1	7.21
SIT-3,4-HBA-H$_2$O	PXRD	38.91(4)	14.017(8)	10.701(5)	90	92.41(9)	90	5832(8)	6	298	P2$_1$	22.6

25 °C for one day produced a monohydrate 1:2:1 3,4-

coformer present in the CSS and the experimental conditions of

production. For instance, depending on the conditions 4-HBA produces two different CSS with different distribution of phytosterols. Moreover, form I is the one with the lowest amount of stigmasterol (only 3.59%) and the highest amount of β-sitosterol. For comparison reasons we determined the content of the three phytosterols present in a commercial analytical standard, which contains 10.75% of stigmasterol. These results show that in principle it is possible to modify the content of a particular phytosterol in the solid by changing the experimental conditions.

Although relevant progress has been achieved in the production of some phytosterols in pure form, the extraction and purification techniques are still complicated and time-consuming, particularly with high costs when large amounts of pure sterols are required.[25,26] Furthermore, the origin of the natural sources affects to the final compositions of phytosterols. But in general terms the simplest isolation approach based on crystallizations can produce β-sitosterol with purity in the 70% range. Further purification to >90% purity can be achieved with expensive chromatographic techniques.[27] In this sense, our results can be particularly advantageous from an industrial point of view since it has been recently reported that stigmasterol accumulation can cause cardiac injury and promote mortality.[28] As said before, current clinical strategies are designed to reduce the levels of cholesterol by consuming diets rich in phytosterols. However, in the form of a nutraceutical formulation a mixture of phytosterols can have a significant percentage of stigmasterol (as in our starting material), which can represent a potential risk factor for heart disease by inducing cardiac fibrosis. Particularly, individuals suffering of the rare illness called sitosterolemia (defects in their phytosterol absorption process) are more prone to be affected by high levels of stigmasterol.[29] Cocrystal Solid Solutions with a reduced level of stigmasterol can represent a new research line to develop efficient phytosterol formulations richer in β-sitosterol and depleted in stigmasterol suitable for clinical cardiovascular applications.

Conclusions

Multicomponent molecular crystals of more than three components are a challenge, which Desiraju has approached successfully by crystal engineering with up to six components.[30,31] Here, we have conducted a cocrystal screening between a phytosterol solid solution containing β-sitosterol, campesterol and stigmasterol and a series of coformers, which include carboxylic acids and phenolic compounds. 8 new cocrystal solid solutions containing four different compounds have been discovered and characterized. The discovered CSS are a new example of this poorly studied family of solid forms and show a low and variable proportion of stigmasterol with respect to the other two phytosterols, which can provide a valid strategy to formulate β-sitosterol in the form of a multicomponent solid solution with reduced amounts of stigmasterol, a product with toxicity concerns. Further studies are being conducted to study the physicochemical properties and the effects of our new solid forms *in vivo*.

Conflicts of interest

There are no conflicts to declare.

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