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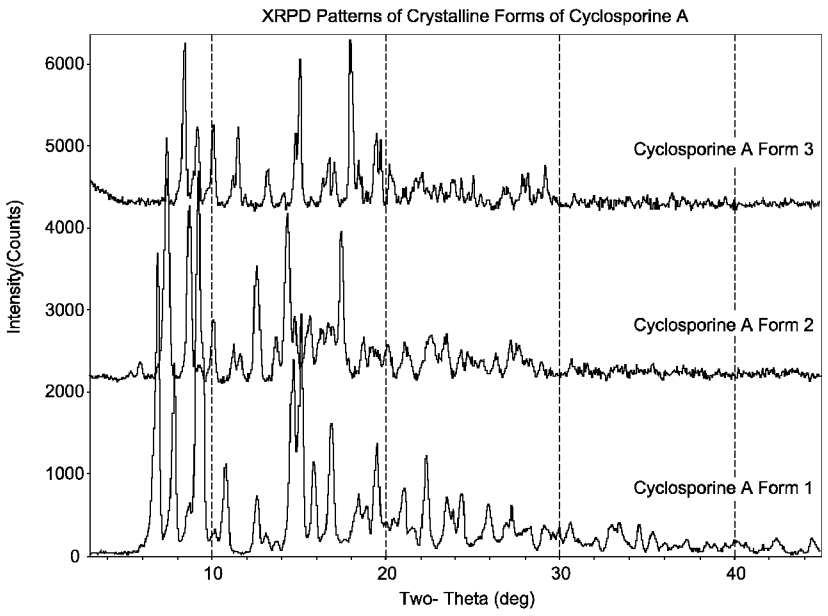

FIG. 1

(57) Abstract: Disclosed herein are methods of making cyclosporin A Form 2.

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CYCLOSPORIN A FORM 2 AND METHOD OF MAKING SAME

By Inventors Ke Wu and Scott W. Smith

CROSS-REFERENCE TO RELATED APPLICATION

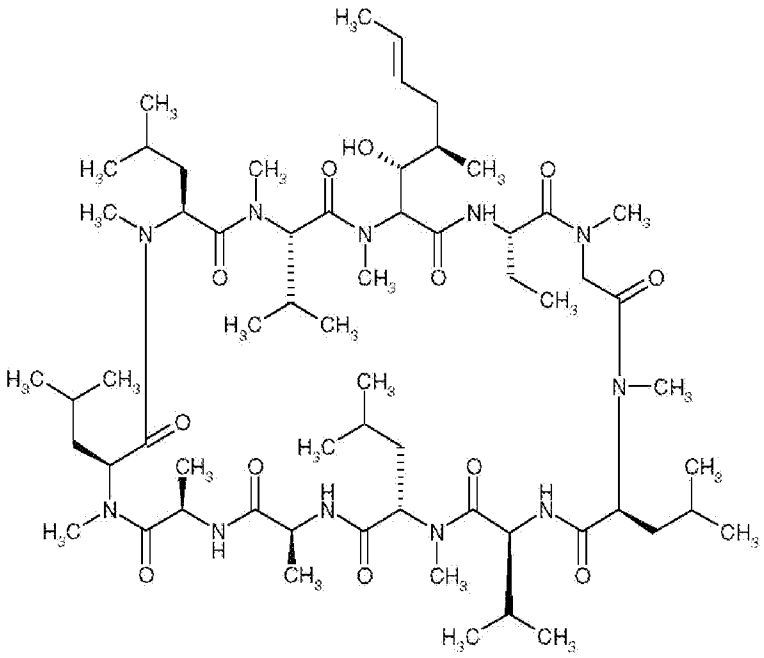
This patent application claims priority to U.S. Provisional Patent Application No. 61/559,830, filed November 15, 2011, the entire contents of which are hereby incorporated by reference.

BACKGROUND

Disclosed herein is a method for making a new crystalline form of cyclosporin A.

Cyclosporin A

Cyclosporin A (CsA) is a cyclic peptide having the following chemical structure:



Its chemical name is cyclo[[(*E*)-(2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-*N*-methylglycyl-*N*-methyl-Lleucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*methyl-L-valyl]. It is also known by the names cyclosporine, cyclosporine A, ciclosporin, and ciclosporin A. It is the active ingredient in Restasis® (Allergan, Inc., Irvine,

California), an emulsion comprising 0.05% (w/v) cyclosporin. Restasis® is approved in the United States to increase tear production in patients whose tear production is presumed to be suppressed due to ocular inflammation associated with keratoconjunctivitis sicca.

Cyclosporin A is known to exist in an amorphous form, liquid crystal form, tetragonal crystalline form (Form 1), and an orthorhombic form (Form 3). The inventors describe here a method of making a new crystalline form, cyclosporin A Form 2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts characteristic X-ray powder diffraction (XRPD) patterns of CsA in a new crystalline form (designated as Form 2 herein), tetragonal form (designated as Form 1 herein), and orthorhombic form (designated as Form 3 herein).

Figure 2 depicts the XRPD diffractogram of CsA crystalline Form 2.

Figure 3 depicts the water sorption/desorption profile of CsA Form 2.

Figure 4 depicts MDSC analysis of CsA Form 2 recovered from 0.04% formulation with 1% PS80.

Figure 5 depicts a cycle of heating and cooling used to generate the CsA Form 2 generated by a method according to the invention.

Figure 6 shows the x-ray diffraction pattern (XRPD) of CsA Form 2 obtained according to a method of the invention, using amorphous cyclosporin A as the starting material.

Figure 7 shows the XRPD of CsA Form 2 obtained according to a method of the invention, using tetragonal cyclosporin A as the starting material.

Figure 8 shows the XRPD of CsA Form 2 obtained according to a method of the invention, using orthorhombic cyclosporin A as the starting material.

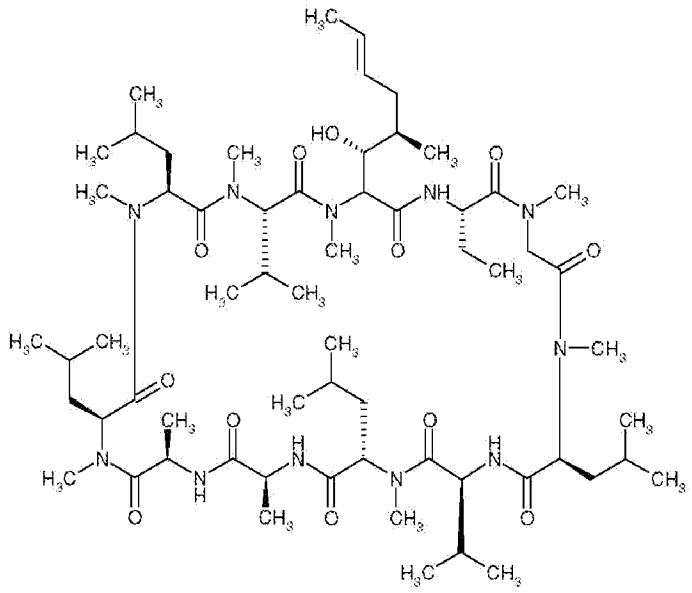
Figure 9 shows the simulated XRPD pattern of cyclosporine A forms.

DETAILED DESCRIPTION

The XRPD pattern of CsA Form 2 differs significantly from the tetragonal form and orthorhombic form (FIG. 1). The major crystalline peaks for CsA form 2 appear at (2θ) when scanned by an X-ray diffractometer with X-ray source as Cu Kα radiation, λ = 1.54 Å, at 30 kV /15 mA: 7.5, 8.8, 10.2, 11.3, 12.7, 13.8, 14.5,

15.6 and 17.5 (d-spacing in crystal lattice at about 11.8, 10.0, 8.7, 7.8, 7.0, 6.4, 6.1, 5.6 and 5.1Å, respectively, Fig. 2). These major peaks are defined as those being unique to Form 2 relative to the orthorhombic or tetragonal forms; as well as, peaks having an intensity greater than 5 times the background.

In one embodiment, the new crystalline form (Form 2) of CsA is a nonstoichiometric hydrate of Cyclosporin A. In another embodiment, the crystalline Form 2 is represented by the formula:



$X\ H_2O$,

wherein X is the number of molecules of water and varies from 0 to 3. In one embodiment, X in the above formula is 2.

Form 2 appears to be a kinetically stable form of CsA in aqueous suspensions. Suspensions containing Form 2 show no conversion to other known polymorphic or pseudomorphic forms upon storage. It has been found that Form 1 and the amorphous form convert to Form 2 in the presence of water.

The single crystal structure of the hydrate form of CsA Form 2 has been determined and the crystal structure parameters are listed in Table 2. These results indicate that Form 2 is unique compared to other known crystalline forms of cyclosporine A.

Table 1: Crystal data and data collection parameters of crystal structure solution of CsA Form 2.

formula	$C_{62}H_{115}N_{11}O_{14}$
formula weight	1238.67
space group	P 2_1 2_1 2_1 (No. 19)
a (Å)	12.6390(5)
b (Å)	19.7582(6)
c (Å)	29.568(2)
volume (Å^3)	7383.8(7)
Z	4
d_{calc} (g cm^{-3})	1.114
crystal dimensions (mm)	0.27 x 0.18 x 0.12
temperature (K)	150
radiation (wavelength in Å)	Cu K_α (1.54184)
monochromator	confocal optics
linear abs coef (mm^{-1})	0.640
absorption correction applied	empirical[a]
transmission factors (min, max)	0.80, 0.93
diffractometer	Rigaku RAPID-II
h, *k*, *l* range	-13 to 13 -21 to 21 -32 to 21
2θ range (deg)	5.38–115.00
mosaicity (deg)	1.31
programs used	SHELXTL
F_{000}	2704.0
weighting	$1/[\sigma^2(Fo^2)+(0.0845P)^2+0.0000P]$ where $P=(Fo^2+2Fc^2)/3$
data collected	37360
unique data	9964
R_{int}	0.077
data used in refinement	9964
cutoff used in R-factor calculations	$F_o^2>2.0s(F_o^2)$
data with I>2.0s(I)	6597
number of variables	834
largest shift/esd in final cycle	0.00
$R(F_o)$	0.061
$R_w(F_o^2)$	0.145
goodness of fit	1.037
absolute structure determination	Flack parameter[b] (0.0(3))

The asymmetric unit of this CsA Form 2 contains one cyclosporine A molecule and two water molecules. It is possible that any small molecule that can hydrogen bond to water could play the role of space filler, which would give a range of potential structures running from the orthorhombic dihydrate to distorted monoclinic dihydrate. The XRPD pattern calculated from the single-crystal structure is shown in Figure 9 and it matches the experimental pattern shown in Figure 2. These matching patterns further corroborate that Form 2 is a unique and pure crystalline form of cyclosporine A.

Without wishing to be bound by theory, thermogravimetric analysis combined with KF titration and vapor sorption desorption analysis (VSA) suggest that CsA Form 2 is a non-stoichiometric hydrate of CsA. The vapor sorption

analysis of Cyclosporine Form 2 indicates that water content in the new crystal form reversibly varies with relative humidity as shown in Fig. 3. Similar to the tetragonal form, the new CsA form undergoes a phase transition to a liquid crystal or amorphous form at 124.4 ^{o}C prior to melting as indicated by the modulated differential calorimetric (MDSC) analysis (Figure 4).

Further details regarding CsA Form 2 may be found in U.S. Patent Application No. 13/480,710, the entire contents of which are incorporated by reference herein.

Methods of Obtaining cyclosporin A Form 2

By precipitation from Polysorbate 80

Cyclosporin A Form 2 may be obtained by suspending amorphous cyclosporin A in water containing Polysorbate 80 (polyoxyethylene sorbitan-mono-olleate), followed by heating the solution in to a temperature of between about 55 °C and about 75 °C, and storing it at that temperature for at least between about 18 and about 48 hours, after which one removes the precipitate, cyclosporin A Form 2.

One can use in this method cyclosporin A at a concentration of between about 0.001% and about 10%. As used here, the term “about,” when used in connection with a value, means a value that is reasonably close to the stated value.

One can therefore use in this method cyclosporin A at a concentration of about 0.001% (w/v), about 0.005% (w/v), about 0.01% (w/v), about 0.02% (w/v), about 0.03% (w/v), about 0.04% (w/v), about 0.05% (w/v), about 0.06% (w/v), about 0.07% (w/v), about 0.08% (w/v), about 0.09% (w/v), about 0.1% (w/v), about 0.2% (w/v), about 0.3% (w/v), about 0.4% (w/v), about 0.5% (w/v), about 0.6% (w/v), about 0.7% (w/v), about 0.8% (w/v), about 0.9% (w/v), about 1% (w/v), about 2% (w/v), about 3% (w/v), about 4% (w/v), about 5% (w/v), about 6% (w/v), about 7% (w/v), about 8% (w/v), about 9% (w/v), or about 10% (w/v) cyclosporin A.

One can use in this method Polysorbate 80 at a concentration of between about 0.1% and 10%, such as about 0.1% (w/v), about 0.2% (w/v), about 0.3% (w/v), about 0.4% (w/v), about 0.5% (w/v), about 0.6% (w/v), about 0.7% (w/v), about 0.8% (w/v), about 0.9% (w/v), about 1% (w/v), about 2% (w/v), about 3%

(w/v), about 4% (w/v), about 5% (w/v), about 6% (w/v), about 7% (w/v), about 8% (w/v), about 9% (w/v), or about 10% (w/v) Polysorbate 80.

After suspending the cyclosporin A in the Polysorbate 80, one can heat the solution to a temperature of between about 55 °C and about 75 °C, such as about 55 °C, about 56 °C, about 57 °C, about 58 °C, about 59 ° C, about 60 °C, about 61 °C, about 62 ° C, about 63 °C, about 64 °C, about 65 ° C, about 66 °C, about 66 °C, about 67 ° C, about 68 °C, about 69 °C, about 70 °C, about 71 °C, about 72 °C, about 73 °C, about 74 °C, or about 75 °C.

One can store the heated solution at one of the foregoing temperatures for a length of time of between about 18 and about 48 hours, such as about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 25 hours, about 26 hours, about 27 hours, about 28 hours, about 29 hours, about 30 hours, about 31 hours, about 32 hours, about 33 hours, about 34 hours, about 35 hours, about 36 hours, about 37 hours, about 38 hours, about 39 hours, about 40 hours, about 41 hours, about 42 hours, about 43 hours, about 44 hours, about 45 hours, about 46 hours, about 47 hours, or about 48 hours.

After preparing and heating the solution, and maintaining it at the desired temperature, the resulting precipitated solid may be recovered by any standard method, such as by vacuum filtration. Following the recovery, the precipitate may then be washed and dried. For example, it may be washed with water and then dried under vacuum at an elevated temperature (for example, about 40 °C), then at room temperature. Other washing and drying techniques may also be used.

Using cyclosporin A Form 2 as a seed crystal

Cyclosporin A Form 2 may also be formed using cyclosporin A Form 2 as a seed crystal. In this method, one can suspend amorphous cyclosporin A in an aqueous solution of Polysorbate 80 and heat the solution as described above. One can then seed the solution with cyclosporin A Form 2, then maintain the solution at the temperatures and for the durations described above, constantly stirring the solution while doing so. At the conclusion of this process, one can then recover the precipitate as described above.

One can use between about 0.01 to about 1 g seed crystal / L water in this process. For example, one can use about 0.01 g/L, about 0.02 g / L, about 0.03 g

/ L, about 0.04 g/L, about 0.05 g/L, about 0.06 g/L, about 0.07 g/L, about 0.08 g/L, about 0.09 g/L, about 0.1 g/L, about 0.2 g / L, about 0.3 g / L, about 0.4 g/L, about 0.5 g/L, about 0.6 g/L, about 0.7 g/L, about 0.8 g/L, about 0.9 g/L, or about 1 g of seed crystal per liter of water.

One can heat the suspension of amorphous cyclosporin A seed crystal of cyclosporin A Form 2 to a temperature of between about 45 °C to about 65 °C before adding it to the solution, or one can leave the seed crystal at room temperature before adding it. For example, one can heat the seed crystal of cyclosporin A Form 2 to about 45 °C, about 46 °C, about 47 °C, about 48 °C about, about 49 °C, about 50 °C, about 51 °C, about 52 ° C, about 53 °C, about 54 °C, about 55 ° C, about 56 °C, about 57 °C, about 58 °C, about 59 °C, about 60 °C, about 61 °C, about 62 °C, about 63 °C, about 64 °C, or about 65 °C before adding it to the solution.

For example, one can suspend about 30 g cyclosporin A in a solution of 900 ml water containing 1% (w/v) Polysorbate 80. One can heat the solution to 65 °C, and then seed it with 0.2 g of cyclosporin A Form 2 at a temperature of 52 °C. The solution is stirred for about 22 hours at a temperature of between about 61 °C and 65 °C. The resulting precipitate may be recovered as described above.

By heating and cooling in certain solvent systems

In very general terms, cyclosporin A Form 2 may be obtained by 1) suspending cyclosporin A in either water, or in a solution of water and acetonitrile, 1,4-dioxane, or ethanol; 2) heating the suspension at a certain rate; 3) cooling the suspension at a certain rate; 4) repeating the cycle of heating and cooling; 5) and recovering the precipitate that results. The choice of solvent is critical: the inventors were able to find no structural feature or other property that predicted whether a solvent would or would not cause CsA Form 2 to form.

One can use in this embodiment cyclosporin A of the liquid crystal, tetragonal, or orthorhombic form, or one can use an amorphous form. The choice of starting material yields CsA Form 2 having very slightly different characteristics (see Figures X and Y), but the important point is that one can use different starting material and still obtain CsA Form 2.

In the first step of this method, one prepares a solution by suspending the desired starting material (that is, liquid crystal, tetragonal, orthorhombic form, or

amorphous cyclosporin A) in water, or by suspending the starting material in acetonitrile, in 1,4-dioxane, or in ethanol, each dissolved in water. One can use in this step between about 0.01 to about 1 g starting material / L water. For example, one can use about 0.01 g/L, about 0.02 g / L, about 0.03 g / L, about 0.04 g/L, about 0.05 g/L, about 0.06 g/L, about 0.07 g/L, about 0.08 g/L, about 0.09 g/L, about 0.1 g/L, about 0.2 g / L, about 0.3 g / L, about 0.4 g/L, about 0.5 g/L, about 0.6 g/L, about 0.7 g/L, about 0.8 g/L, about 0.9 g/L, or about 1 g of starting material per liter of water. The desired solvent (acetonitrile, 1,4-dioxane, or ethanol) is added in that amount which results in the solution having a mole fraction of water of between about 0.75 and 1. For example, the solvent is added in an amount resulting in the solution having a mole fraction of about 0.75, about 0.76, about 0.77, about 0.78, about 0.79, about 0.80, about 0.81, about 0.82, about 0.83, about 0.84, about 0.85, about 0.86, about 0.87, about 0.88, about 0.89, about 0.90, about 0.91, about 0.92, about 0.93, about 0.94, about 0.95, about 0.96, about 0.97, about 0.98, about 0.99, and about 1.

In the second step of this method, one then heats the solution to a temperature of about 5 °C to about 50 °C at the rate of about 0.01 °C per minute to about 1 °C per minute. In one embodiment, one can heat the solution to a temperature of about 5 °C to about 10 °C, about 10 °C to about 15 °C, about 15 °C to about 20 °C, about 20 °C to about 25 °C, about 25 °C to about 30 °C, about 30 °C to about 35 °C, about 35 °C to about 40 °C, about 40 °C to about 45 °C, or about 45 °C to about 50 °C. In another embodiment, one can heat the solution to a temperature of about 5 °C to about 15 °C, about 15 °C to about 25 °C, about 25 °C to about 35 °C, about 35 °C to about 45 °C, or about 40 °C to about 50 °C. For example, one can heat the solution to a temperature of about 1 °C, about 2 °C, about 3 °C, about 4 °C, about 5 °C, about 6 °C, about 7 °C, about 8 °C, about 9 °C, about 10 °C, about 11 °C, about 12 °C, about 13 °C, about 14 °C, about 15 °C, about 16 °C, about 17 °C, about 18 °C, about 19 °C, about 20 °C, about 21 °C, about 22 °C, about 23 °C, about 24 °C, about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, about 37 °C, about 38 °C, about 39 °C, about 40 °C, 41 °C, about 42 °C, about 43 °C, about 44 °C, about 45 °C, about 46 °C, about 47 °C, about 48 °C, about 49 °C, or about 50 °C.

In one embodiment, one heats the solution at a rate of about 0.01 °C / min to about 0.05 °C / min, about 0.05 °C / min – 0.1 °C per minute, about 0.1 °C / min to about 0.2 °C / min, about 0.2 °C / min to about 0.3 °C / min, about 0.3 °C / min to about 0.4 °C / min, about 0.4 °C / min to about 0.5 °C / min, about 0.5 °C / min to about 0.6 °C / min, about 0.6 °C / min to about 0.7 °C / min, about 0.7 °C / min to about 0.8 °C / min, about 0.8 °C / min to about 0.9 °C / min, or about 0.9 °C / min to about 1 °C / min. For example, one can heat the solution at the rate of about 0.01 °C / min, about 0.02 °C / min, about 0.03 °C / min, about 0.04 °C / min, about 0.05 °C / min, about 0.06 °C / min, about 0.07 °C / min, about 0.08 °C / min, about 0.09 °C / min, about 0.1 °C / min, about 0.2 °C / min, about 0.3 °C / min, about 0.4 °C / min, about 0.5 °C / min, about 0.6 °C / min, about 0.7 °C / min, about 0.8 °C / min, about 0.9 °C / min, or about 1 °C / min.

In the third step of this method, one then cools the solution to a temperature of between about 1 °C to about 22 °C. In one embodiment, one can cool the solution to a temperature of about 1 °C to about 5 °C, about 5 °C to about 10 °C, about 10 °C to about 15 °C, about 15 °C to about 20 °C, or about 17 °C to about 22 °C. In another embodiment, one can cool the solution to a temperature of about 1 °C to about 10 °C, about 5 °C to about 15 °C, about 10 °C to about 20 °C, or about 15 °C to about 22 °C. For example, one can cool the solution to a temperature of about 1 °C, about 2 °C, about 3 °C, about 4 °C, about 5 °C, about 6 °C, about 7 °C, about 8 °C, about 9 °C, about 10 °C, about 11 °C, about 12 °C, about 13 °C, about 14 °C, about 15 °C, about 16 °C, about 17 °C, about 18 °C, about 19 °C, about 20 °C, about 21 °C, or about 22 °C.

The solution may be cooled at the same or different rate at which it is heated. In one embodiment, one cools the solution at a rate of about 0.01 °C / min to about 0.05 °C / min, about 0.05 °C / min to 0.1 °C per minute, about 0.1 °C / min to about 0.2 °C / min, about 0.2 °C / min to about 0.3 °C / min, about 0.3 °C / min to about 0.4 °C / min, about 0.4 °C / min to about 0.5 °C / min, about 0.5 °C / min to about 0.6 °C / min, about 0.6 °C / min to about 0.7 °C / min, about 0.7 °C / min to about 0.8 °C / min, about 0.8 °C / min to about 0.9 °C / min, or about 0.9 °C / min to about 1 °C / min. For example, one can cool the solution at the rate of about 0.01 °C / min, about 0.02 °C / min, about 0.03 °C / min, about 0.04 °C / min, about 0.05 °C / min, about 0.06 °C / min, about 0.07 °C / min, about 0.08 °C / min, about 0.09 °C / min, about 0.1 °C / min, about 0.2 °C / min, about 0.3 °C / min,

about 0.4 °C / min, about 0.5 °C / min, about 0.6 °C / min, about 0.7 °C / min, about 0.8 °C / min, about 0.9 °C / min, or about 1 °C / min.

One may then proceed to recover any precipitate that has formed, using the methods described above, or one may repeat the steps of heating and cooling, using the same or different temperatures and the same or different rates of heating and cooling. In one embodiment, one repeats the steps of heating and cooling once, that is, one first heats then cools the solution, then heats and cools the solution again. In another embodiment, one repeats the steps of heating and cooling two times, that is, one first heats then cools the solution, then heats and cools the solution a second time, then heats and cooling the solution a third time. In another embodiment, one repeats the steps of heating and cooling three times, that is, one first heats then cools the solution, then heats and cools the solution a second time, then heats and cooling the solution a third time, and then heats and cooling the solution a fourth time. During each of the heating steps, one can heat the solution to the same or different temperature, and at the same or different rate; likewise, during each of the cooling steps, one can heat the solution to the same or different temperature, and at the same or different rate.

In one embodiment, one begins to cool the solution immediately after heating the solution to the desired temperature. In another embodiment, one maintains the solution at the heated temperature for between about 0 and about 25 hours before beginning to cool it. For example, one can maintain the solution at the heated temperature for about 0 to about 5 hours, about 5 to about 10 hours, about 10 to about 15 hours, about 15 to about 20 hours, or about 20 to 25 hours; in another embodiment, one can maintain the solution at the heated temperature for about 0 to about 10 hours, about 5 to about 15 hours, about 10 to about 20 hours, or about 15 to about 25 hours. For example, one can maintain the solution at the heated temperature for about 0.5 hours, about 1 hours, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, or about 25 hours.

If repeating a heating-cooling cycle, one can immediately heat the solution after it has cooled to the desired temperature, or one can maintain the solution at

the cooled temperature for between about 0 and about 24 hours before beginning to heat it again. For example, one can maintain the solution for about 0 to about 5 hours, about 5 to about 10 hours, about 10 to about 15 hours, about 15 to about 20 hours, or about 20 to 25 hours; in another embodiment, one can maintain the solution at the cooled temperature for about 0 to about 10 hours, about 5 to about 15 hours, about 10 to about 20 hours, or about 15 to about 25 hours. For example, one can maintain the solution at the cooled temperature for about 0.5 hours, about 1 hours, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, or about 25 hours

At the conclusion of the heating and cooling cycle, one can hold the solution at the final, cooled temperature for about 0 to about 24 hours before recovering the precipitate as described above.

EXAMPLES

The invention is further illustrated by way of the following examples.

Example 1

Cyclosporin A (CsA), either of the amorphous, tetragonal (F1), or orthorhombic form, was suspended in water, acetonitrile, dioxane, or ethanol, as described in Table 1, below:

Table 1: Cyclosporin suspension used to make cyclosporin form 2

CsA Form	Solvent		Mole fraction of water
	1	2	
amorphous	Water	acetonitrile	0.87
F1	Water	acetonitrile	0.87
F3	Water	acetonitrile	0.87
F1	water	none	1.00
F1	water	dioxane	0.90
F1	Water	Ethanol	0.89

Each of the above solutions was heated to 50 °C at a rate of 0.1 °C per minute, and maintained at that temperature for 600 min; the solution was then cooled to 20 °C at the same rate, and held at that temperature for 300 min; this cycle of heating and cooling was repeated twice more, as illustrated in Figure 5 and summarized at Table 2:

Table 2: Thermocycling profile

Step	Temp (°C)	Heating Rate (°C/min)	Duration (min)	Total time
Hold	20	---	30.00	00:30:00
Ramp	50	0.1000	300.00	05:30:00
Hold	50	---	600.00	15:30:00
Ramp	20	- 0.1000	300.00	20:30:00
Hold	20	---	600.00	1.06:30:00
Ramp	50	0.1000	300.00	1.11:30:00
Hold	50	---	600.00	1.21:30:00
Ramp	20	- 0.1000	300.00	2.02:30:00
Hold	20	---	600.00	2.12:30:00
Ramp	50	0.1000	300.00	2.17:30:00
Hold	50	---	600.00	3.03:30:00
Ramp	20	- 0.1000	300.00	3.06:30:00
Hold	20	---	300.00	3.13:30:00

The x-ray powder diffraction pattern (XRPD) of cyclosporin A form 2 thus obtained, using amorphous cyclosporin A as the starting material, is illustrated at Figure 6; the XRPD of cyclosporin A form 2 obtained using tetragonal cyclosporin A as the starting material is illustrated at Figure 7; the XRPD of cyclosporin A form 2 obtained using orthorhombic cyclosporin A as the starting material is illustrated

at Figure 8. XRPD patterns of CsA forms were obtained using a Rigaku MiniFlex X-ray diffractometer (Cu K_α radiation, λ = 1.54 Å, at 30 kV and 15 mA). The instrument was calibrated with a silicon standard with a reference peak at 28.44° (2-theta). The X-ray diffraction experiments were performed from 3° to 45° (2-theta) at a scan rate of 0.5° or 1° (2-theta) per minute and a step width of 0.05° (2-theta).

Experimental conditions that did not produce cyclosporin A Form 2 are shown below in Table 3:

Table 3 - Experimental conditions that did not produce cyclosporin A Form 2

#	CsA Form	CsA Weight (mg)	Solvent		Volume (µL)		Final Form	Mole fraction of water
			1	2	1	2		
1	Amorphous	53.77	water	acetone	100	42	F1+F2	0.91
2	Amorphous	61.70	water	ethylene glycol	100	42	amorphous	0.88
4	Amorphous	41.17	water	n/a	100	n/a	amorphous	1.00
5	F1	66.74	water	acetone	100	42	F1+F2	0.91
6	F1	74.51	water	ethylene glycol	100	42	F1	0.88
8	F1	68.59	water	n/a	100	n/a	amorphous	1.00
9	F3	49.70	water	acetone	100	42	F3	0.91
10	F3	69.45	water	ethylene glycol	100	42	F3	0.88
12	F3	70.87	water	n/a	100	n/a	F3	1.00
16	F1	43.07	water	methanol	100	42	amorphous + F2	0.84

Methods of treatment

CsA Form 2 obtained by the methods of the invention may be used to treat any condition of the eye which is known to be amenable to topical treatment with cyclosporin A (such as with Restasis®). For example, compositions of the invention may be used to treat patients suffering from dry eye, to treat blepharitis and meibomian gland disease, to restore corneal sensitivity that has been impaired due to refractive surgery on the eye, to treat allergic conjunctivitis and atopic and vernal keratoconjunctivitis, and to treat ptyregia, conjunctival and corneal inflammation, keratoconjuntivitis, graft versus host disease, post-transplant glaucoma, corneal transplants, mycotic keratitis, Thygeson's superficial punctate keratitis, uveitis, and Theodore's superior limbic keratoconjunctivitis, among other conditions.

The International Dry Eye Workshop (DEWS) defines dry eye as “a multifactorial disease of the tears and ocular surface that results in symptoms of

discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface, accompanied by increased osmolarity of the tear film and inflammation of the ocular surface." It includes those conditions, such as keratoconjunctivitis sicca, that are caused by tear deficiency or excessive evaporation of tears.

Blepharitis is a chronic disorder producing inflammation of the anterior and posterior lid margin, with involvement of skin and its related structures (hairs and sebaceous glands), the mucocutaneous junction, and the meibomian glands. It can also affect the conjunctiva, tear film, and the corneal surface in advanced stages and may be associated with dry eye. Blepharitis is commonly classified into anterior or posterior blepharitis, with anterior affecting the lash bearing region of the lids, and posterior primarily affecting the meibomian gland orifices.

Meibomian gland disease most often occurs as one of three forms: primary meibomitis, secondary meibomitis, and meibomian seborrhea. Meibomian seborrhea is characterized by excessive meibomian secretion in the absence of inflammation (hypersecretory meibomian gland disease). Primary meibomitis, by contrast, is distinguished by stagnant and inspissated meibomian secretions (obstructive hypersecretory meibomian gland disease). Secondary meibomitis represents a localized inflammatory response in which the meibomian glands are secondarily inflamed in a spotty fashion from an anterior lid margin blepharitis.

Impaired corneal sensitivity often occurs after refractive surgery, such as photorefractive keratectomy, laser assisted sub-epithelium keratomileusis (LASEK), EPI-LASEK, customized transepithelial non-contact ablation, or other procedures in which the corneal nerves are severed. Impaired corneal sensitivity may also occur after viral infection, such as by HSV-1, HSV-2, and VZV viruses. Patients with impaired corneal sensitivity often complain that their eyes feel dry, even though tear production and evaporation may be normal, suggesting that "dryness" in such patients is actually a form of corneal neuropathy that results when corneal nerves are severed by surgery or inflamed after viral infection.

Allergic conjunctivitis is an inflammation of the conjunctiva resulting from hypersensitivity to one or more allergens. It may be acute, intermittent, or chronic. It occurs seasonally, that is, at only certain time of the year, or it occurs perennially, that is, chronically throughout the year. Symptoms of seasonal and perennial allergic conjunctivitis include, in addition to inflammation of the

conjunctiva, lacrimation, tearing, conjunctival vascular dilation, itching, papillary hyperlasia, chemosis, eyelid edema, and discharge from the eye. The discharge may form a crust over the eyes after a night's sleep.

Atopic keratoconjunctivitis is a chronic, severe form of allergic conjunctivitis that often leads to visual impairment. Symptoms include itching, burning, pain, redness, foreign body sensation, light sensitivity and blurry vision. There is often a discharge, especially on awakening from a night's sleep; the discharge may be stringy, ropy, and mucoid. The lower conjunctiva is often more prominently affected than the upper conjunctiva. The conjunctiva may range from pale, edematous, and featureless to having the characteristics of advanced disease, including papillary hypertrophy, subepithelial fibrosis, formix foreshortening, trichiasis, entropion, and madurosis. In some patients the disease progresses to punctate epithelial erosions, corneal neovascularization, and other features of keratopathy which may impair vision. There is typically goblet cell proliferation in the conjunctiva, epithelial pseudotubular formation, and an increased number of degranulating eosinophils and mast cells in the epithelium. CD25+T lymphocytes, macrophages, and dendritic cells (HLA-DR.sup.+, HLA-CD1+) are significantly elevated in the substantia propria.

Like atopic keratoconjunctivitis, vernal keratoconjunctivitis is a severe form of allergic conjunctivitis, but it tends to affect the upper conjunctiva more prominently than the lower. It occurs in two forms. In the palpebral form, square, hard, flattened, closely packed papillae are present; in the bulbar (limbal) form, the circumcorneal conjunctiva becomes hypertrophied and grayish. Both forms are often accompanied by a mucoid discharge. Corneal epithelium loss may occur, accompanied by pain and photophobia, as may central corneal plaques and Trantas' dots.

What is claimed is:

1. A method for making cyclosporin A form 2, the method comprising the steps of:

a) preparing a suspension of cyclosporin A in a solvent comprising water and an mmmingredient selected from the group consisting of acetonitrile, 1,4-dioxane, and ethanol;

b) a first heating-cooling cycle comprising heating the suspension to a temperature of between about 5 °C and about 50 °C, followed by cooling the suspension to a temperature of about 1 °C to about 35 °C;

c) a second heating-cooling cycle comprising heating the suspension to a temperature of between about 5 °C and about 50 °C, followed by cooling the suspension to a temperature of about 1 °C to about 35 °C; and

d) a third heating-cooling cycle comprising heating the suspension to a temperature of between about 5 °C and about 50 °C, followed by cooling the suspension to a temperature of about 1 °C to about 35 °C.

2. The method of claim 1, wherein the solvent comprises water and acetonitrile.

3. The method of claim 2, wherein the molar fraction of water in the solvent is between about 0.8 and about 1.0.

4. The method of claim 3, wherein the molar fraction of water in the solvent is about 0.87.

5. The method of claim 1, wherein the solvent comprises water and 1,4-dioxane.

6. The method of claim 5, wherein the molar fraction of water in the solvent is between about 0.8 and about 1.0.

7. The method of claim 6, wherein the molar fraction of water in the solvent is about 0.90.

8. The method of claim 1, wherein the solvent comprises water and ethanol.

9. The method of claim 8, wherein the molar fraction of water in the solvent is between about 0.8 and about 1.0.

10. The method of claim 9, wherein the molar fraction of water in the solvent is about 0.89.

11. The method of any of claims 1-10, wherein the first, second, or third heating-cooling cycle comprises heating the suspension to a temperature of between about 5 °C and about 50 °C at the rate of about 0.05 °C to about 2 °C per minute, followed by cooling the suspension to a temperature of about 1 °C to about 35 °C at the rate of about 0.01 °C to about 1 °C per minute.

12. The method of any of claims 1-10, wherein at least two of the heating-cooling cycles comprise heating the suspension to a temperature of between about 5 °C and about 50 °C at the rate of about 0.05 °C to about 2 °C per minute, followed by cooling the suspension to a temperature of about 1 °C to about 35 °C at the rate of about 0.01 °C to about 1 °C per minute.

13. The method of any of claims 1-10, wherein the first, second, and third heating-cooling cycles comprise heating the suspension to a temperature of between about 5 °C and about 70 °C at the rate of about 0.05 °C to about 2 °C per minute, followed by cooling the suspension to a temperature of about 1 °C to about 35 °C at the rate of about 0.05 °C to about 2 °C per minute.

14. The method of claim 13, wherein the first, second, and third heating-cooling cycles comprise heating the suspension to a temperature of between about 5 °C and about 50 °C at the rate of about 0.1 °C per minute, followed by

cooling the suspension to a temperature of about 5 °C at the rate of about 1 °C per minute.

15. A method for making cyclosporin A form 2, the method comprising the steps of:

a) preparing a suspension of cyclosporin A in a solvent;

b) heating the suspension;

c) adding cyclosporin A form 2 to the suspension;

d) stirring the suspension; and

e) isolating cyclosporin A form 2 from the suspension.

16. The method of claim 15, wherein the step of heating the suspension comprises heating the suspension at a temperature of between about 40 °C and 70 °C.

17. The method of claim 16, wherein the step of heating the suspension comprises heating the suspension at a temperature of between about 60 °C and 65 °C.

18. The method of any of claims 15-17, wherein the step of stirring the suspension comprises stirring the suspension at a temperature of between about 20 °C and 70 °C.

19. The method of claim 18, wherein the step of stirring the suspension comprises stirring the suspension at a temperature of between about 60 °C and 65 °C.

20. The method of any of claims 15-19, wherein the step of stirring the suspension comprises stirring the suspension for between about 24 and about 72 hours.

21. The method of any of claims 15-19, wherein the step of stirring the suspension comprises stirring the suspension for between about 22 and about 23 hours.

22. Cyclosporin A form 2, produced by the method of any of claims 1-21.

23. A method of treating an ocular condition selected from dry eye, blepharitis, meibomian gland disease, impaired corneal sensitivity, allergic conjunctivitis, atopic keratoconjunctivitis, vernal keratoconjunctivitis, and ptyregia, the method comprising the step of administering to a patient the cyclosporin A form 2 obtained by the method of claim 22.

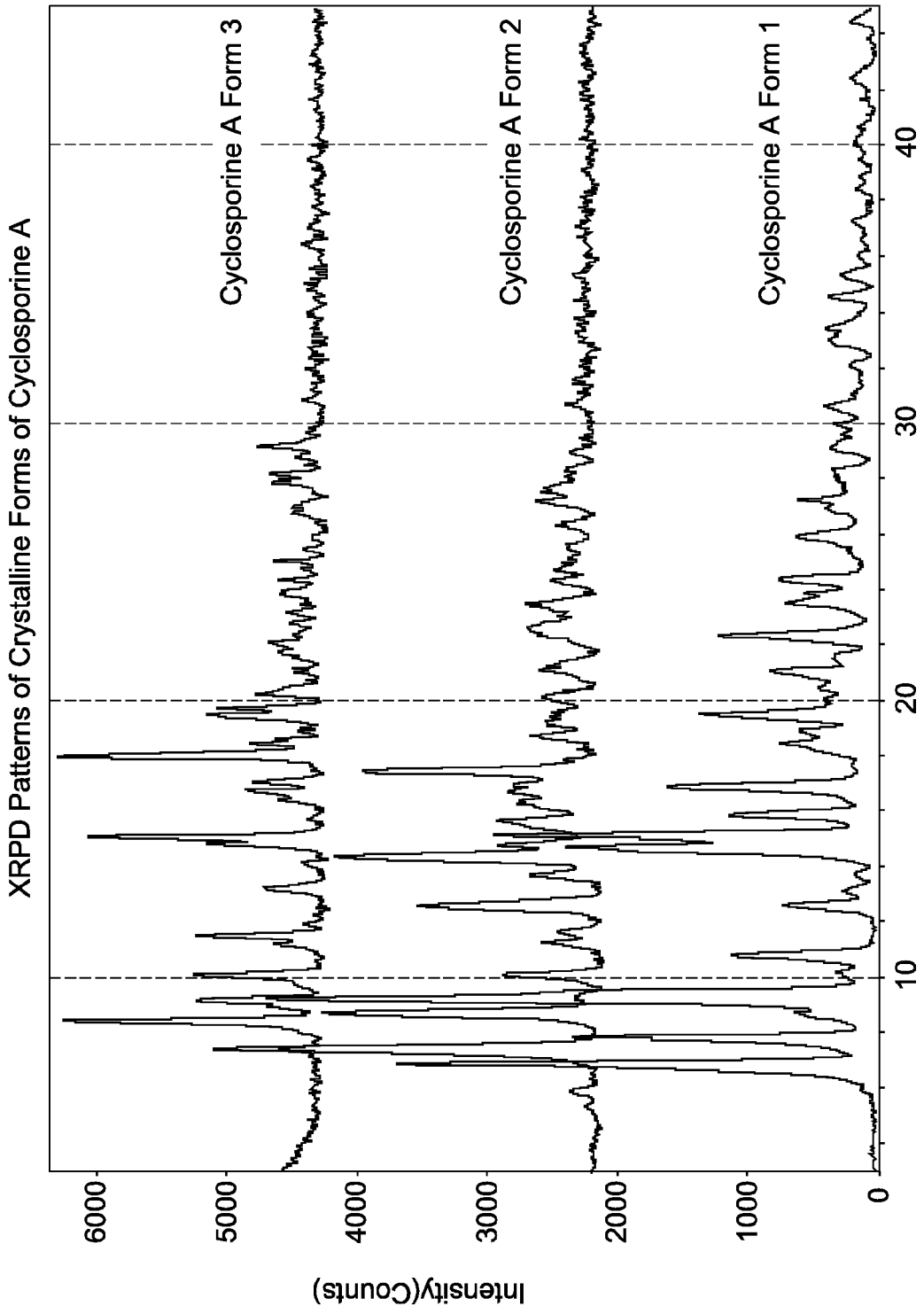

XRPD Patterns of Crystalline Forms of Cyclosporine A
Cyclosporine A Form 3
Cyclosporine A Form 2
Cyclosporine A Form 1
Intensity(Counts)
0
1000
2000
3000
4000
5000
6000
10
20
30
40
Two- Theta (deg)

FIG. 1

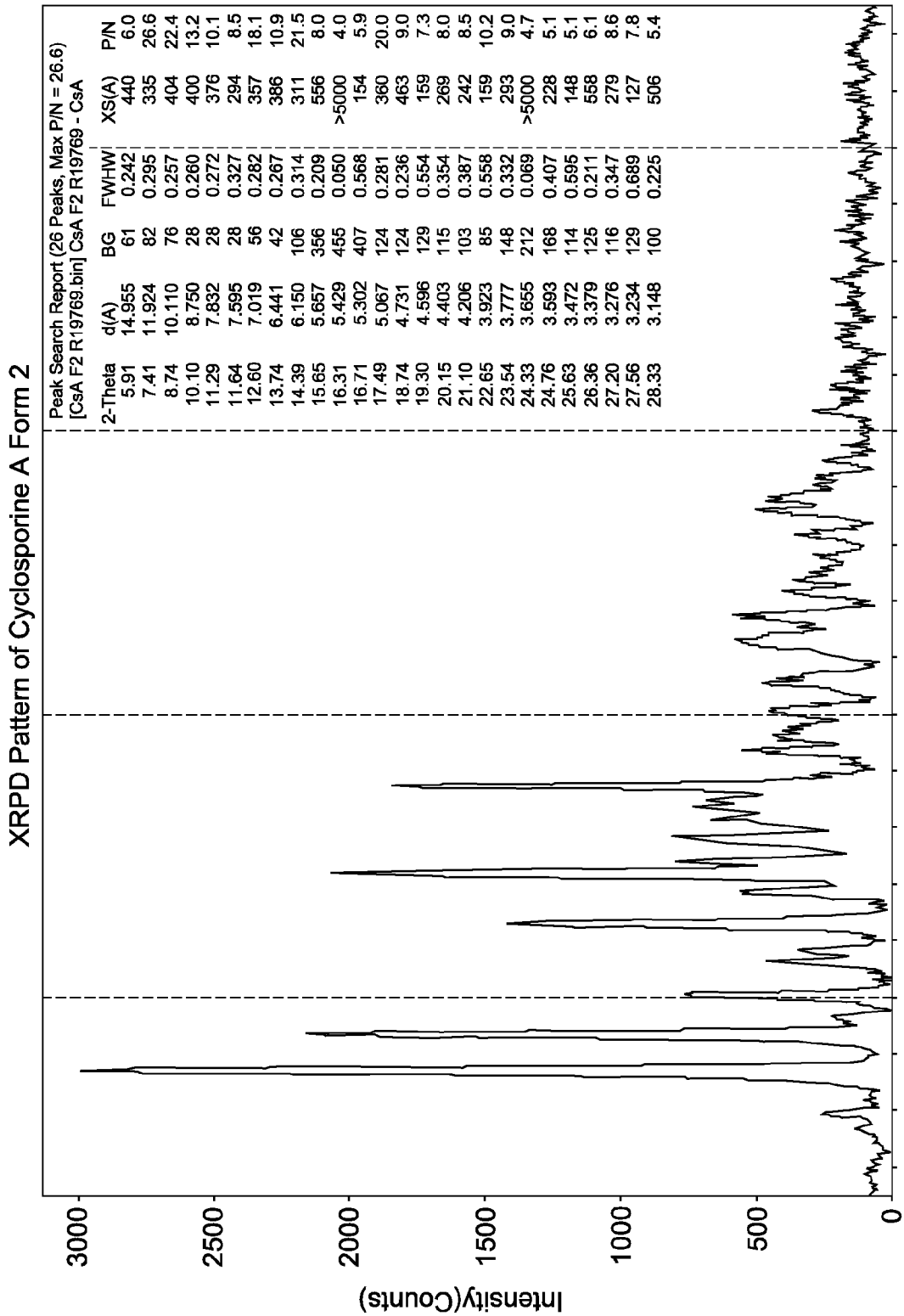

XRPD Pattern of Cyclosporine A Form 2
Peak Search Report (26 Peaks, Max P/N = 26.6)
[CsA F2 R19769.bin] CsA F2 R19769 - CsA
2-Theta d(A) BG FWHW XS(A) P/N
5.91 14.955 61 0.242 440 6.0
7.41 11.924 82 0.295 335 26.6
8.74 10.110 76 0.257 404 22.4
10.10 8.750 28 0.260 400 13.2
11.29 7.832 28 0.272 376 10.1
11.64 7.595 28 0.327 294 8.5
12.60 7.019 56 0.282 357 18.1
13.74 6.441 42 0.267 386 10.9
14.39 6.150 106 0.314 311 21.5
15.65 5.657 356 0.209 556 8.0
16.31 5.429 455 0.050 >5000 4.0
16.71 5.302 407 0.568 154 5.9
17.49 5.067 124 0.281 360 20.0
18.74 4.731 124 0.236 463 9.0
19.30 4.596 129 0.554 159 7.3
20.15 4.403 115 0.354 269 8.0
21.10 4.206 103 0.387 242 8.5
22.65 3.923 85 0.558 159 10.2
23.54 3.777 148 0.332 293 9.0
24.33 3.655 212 0.069 >5000 4.7
24.76 3.593 168 0.407 228 5.1
25.63 3.472 114 0.595 148 5.1
26.36 3.379 125 0.211 558 6.1
27.20 3.276 116 0.347 279 8.6
27.56 3.234 129 0.689 127 7.8
28.33 3.148 100 0.225 506 5.4
Intensity(Counts)
3000
2500
2000
1500
1000
500
0
10
20
30
40
Two-Theta (deg)

FIG. 2

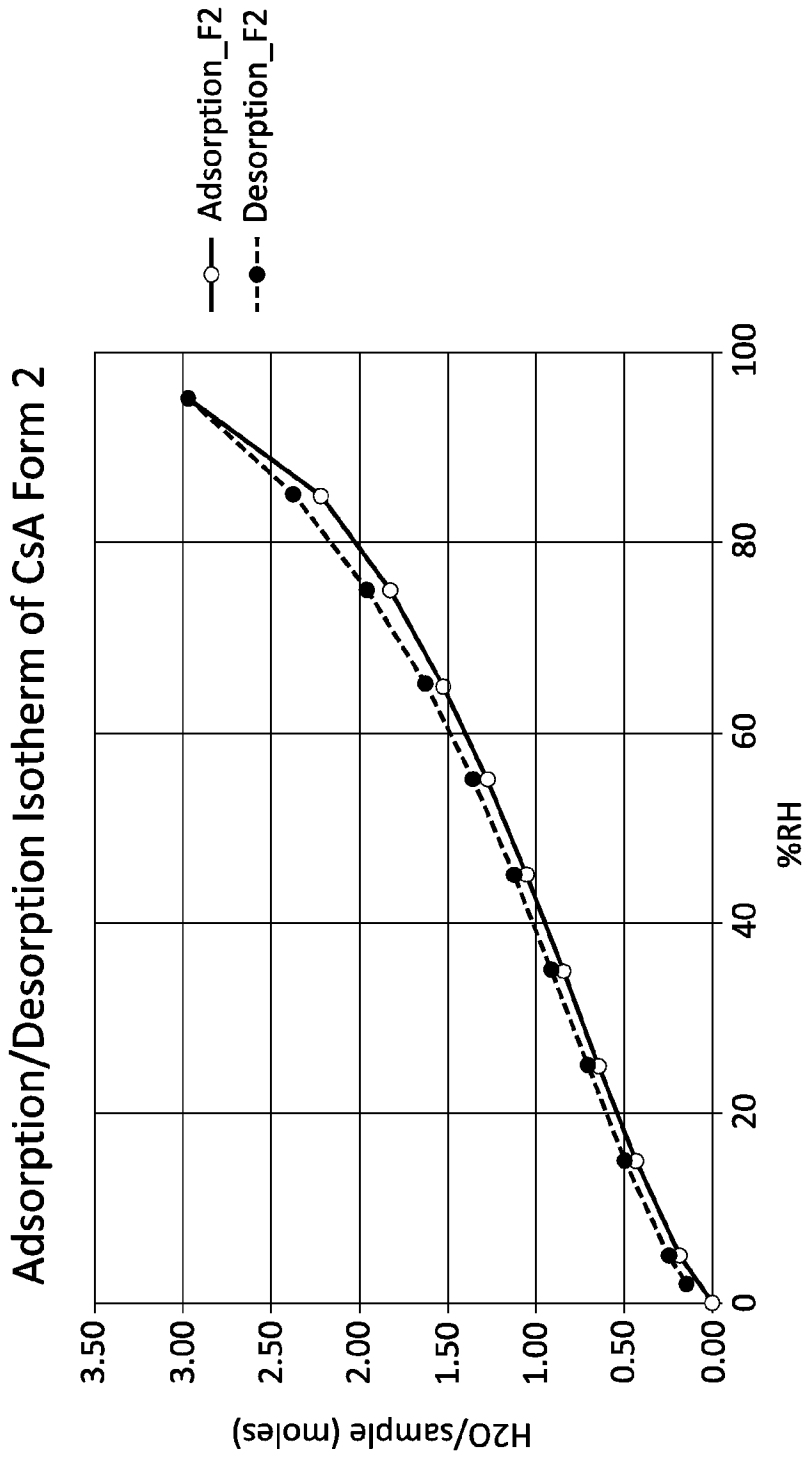

Adsorption_F2
Desorption_F2
Adsorption/Desorption Isotherm of CsA Form 2
H2O/sample (moles)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
20
40
60
80
100
%RH

FIG. 3

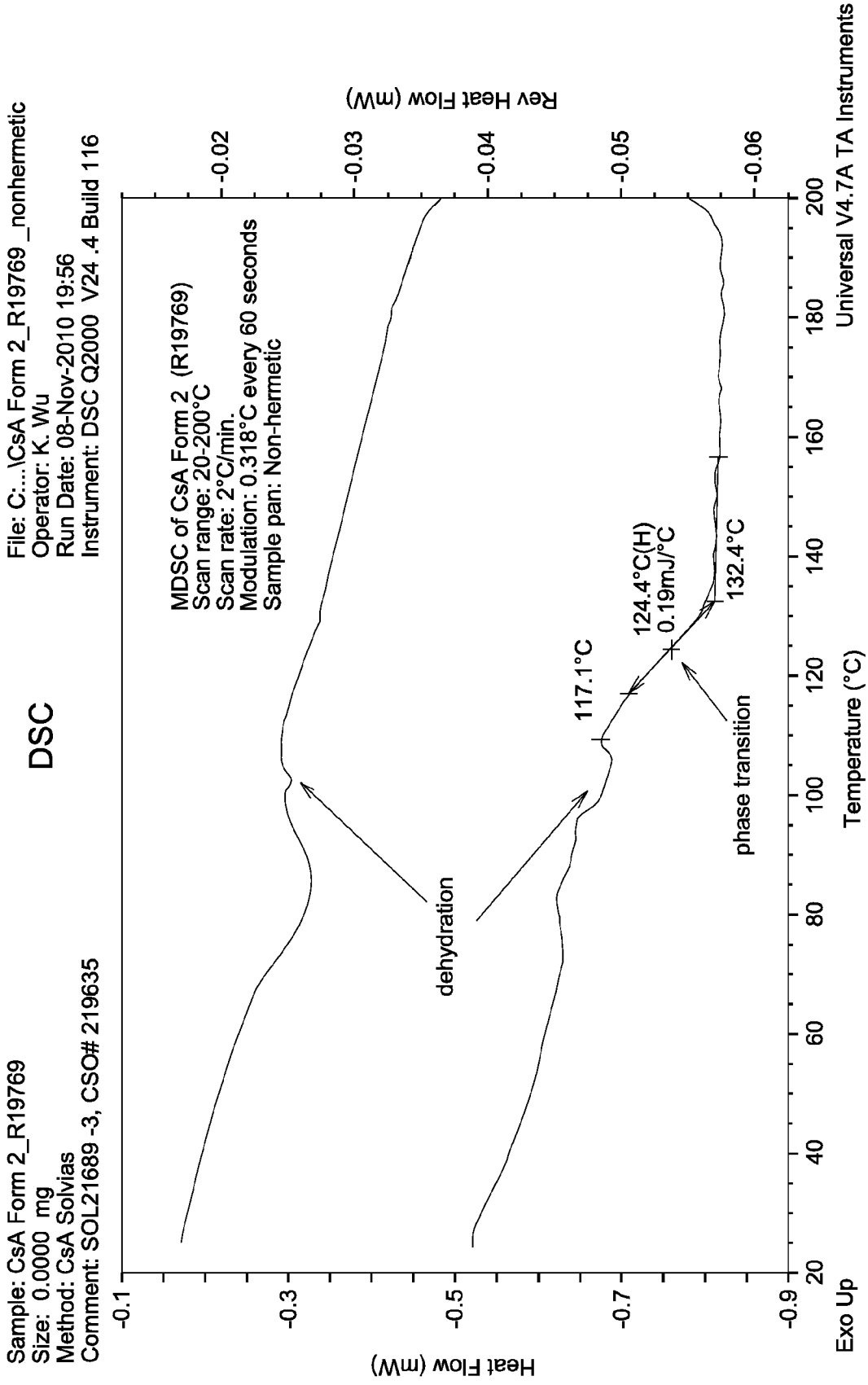
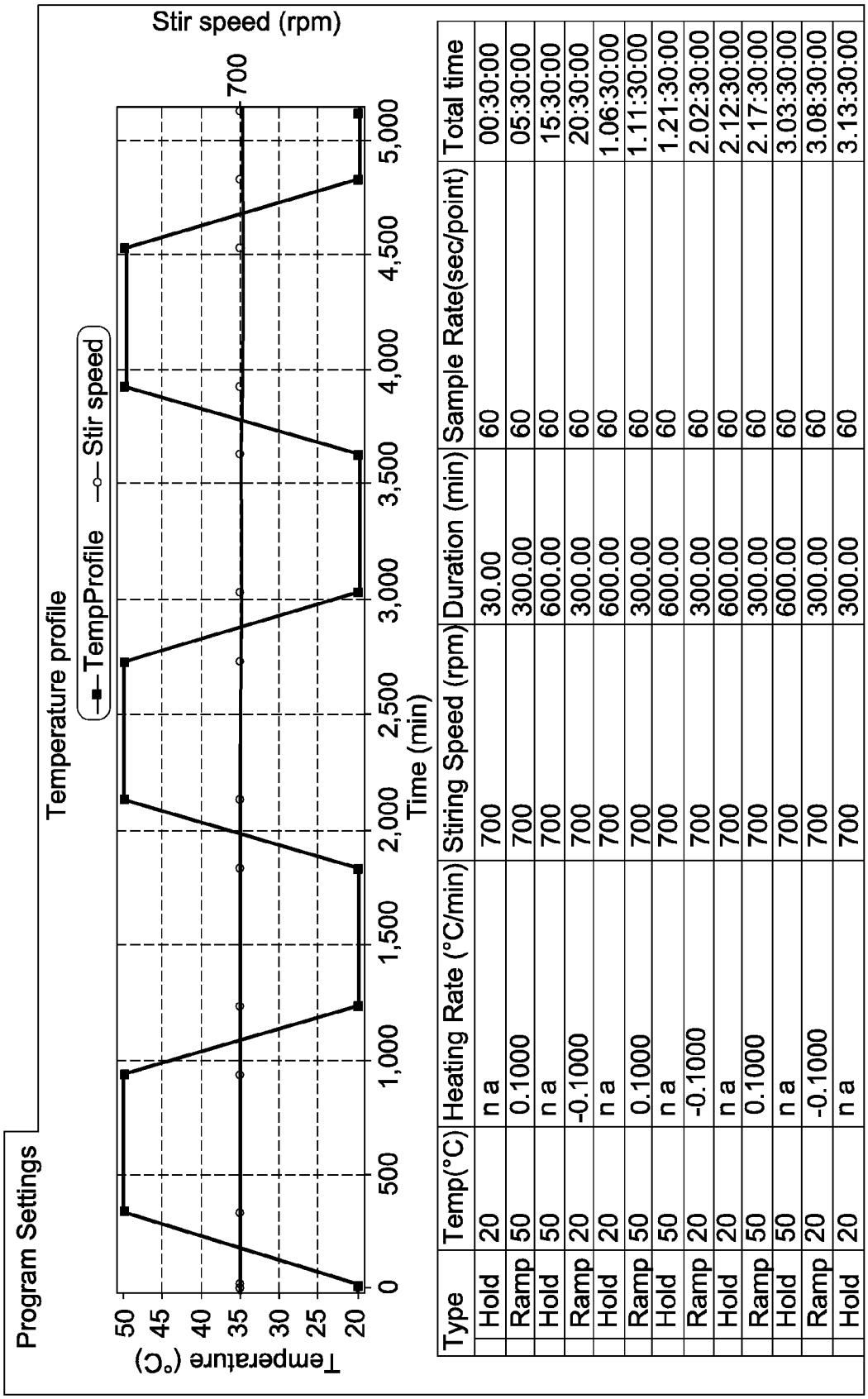

Sample: CsA Form 2_R19769
Size: 0.0000 mg
Method: CsA Solvias
Comment: SOL21689 -3, CSO# 219635
DSC
File: C:...\CsA Form 2_R19769 _nonhermetic
Operator: K. Wu
Run Date: 08-Nov-2010 19:56
Instrument: DSC Q2000 V24 .4 Build 116
MDSC of CsA Form 2 (R19769)
Scan range: 20-200°C
Scan rate: 2°C/min.
Modulation: 0.318°C every 60 seconds
Sample pan: Non-hermetic
dehydration
117.1°C
124.4°C(H)
0.19mJ/°C
132.4°C
phase transition
Heat Flow (mW)
Rev Heat Flow (mW)
Temperature (°C)
Exo Up
Universal V4.7A TA Instruments

FIG. 4



Type	Temp(°C)	Heating Rate (°C/min)	Stiring Speed (rpm)	Duration (min)	Sample Rate(sec/point)	Total time
Hold	20	n a	700	30.00	60	00:30:00
Ramp	50	0.1000	700	300.00	60	05:30:00
Hold	50	n a	700	600.00	60	15:30:00
Ramp	20	-0.1000	700	300.00	60	20:30:00
Hold	20	n a	700	600.00	60	1.06:30:00
Ramp	50	0.1000	700	300.00	60	1.11:30:00
Hold	50	n a	700	600.00	60	1.21:30:00
Ramp	20	-0.1000	700	300.00	60	2.02:30:00
Hold	20	n a	700	600.00	60	2.12:30:00
Ramp	50	0.1000	700	300.00	60	2.17:30:00
Hold	50	n a	700	600.00	60	3.03:30:00
Ramp	20	-0.1000	700	300.00	60	3.08:30:00
Hold	20	n a	700	300.00	60	3.13:30:00

FIG. 5

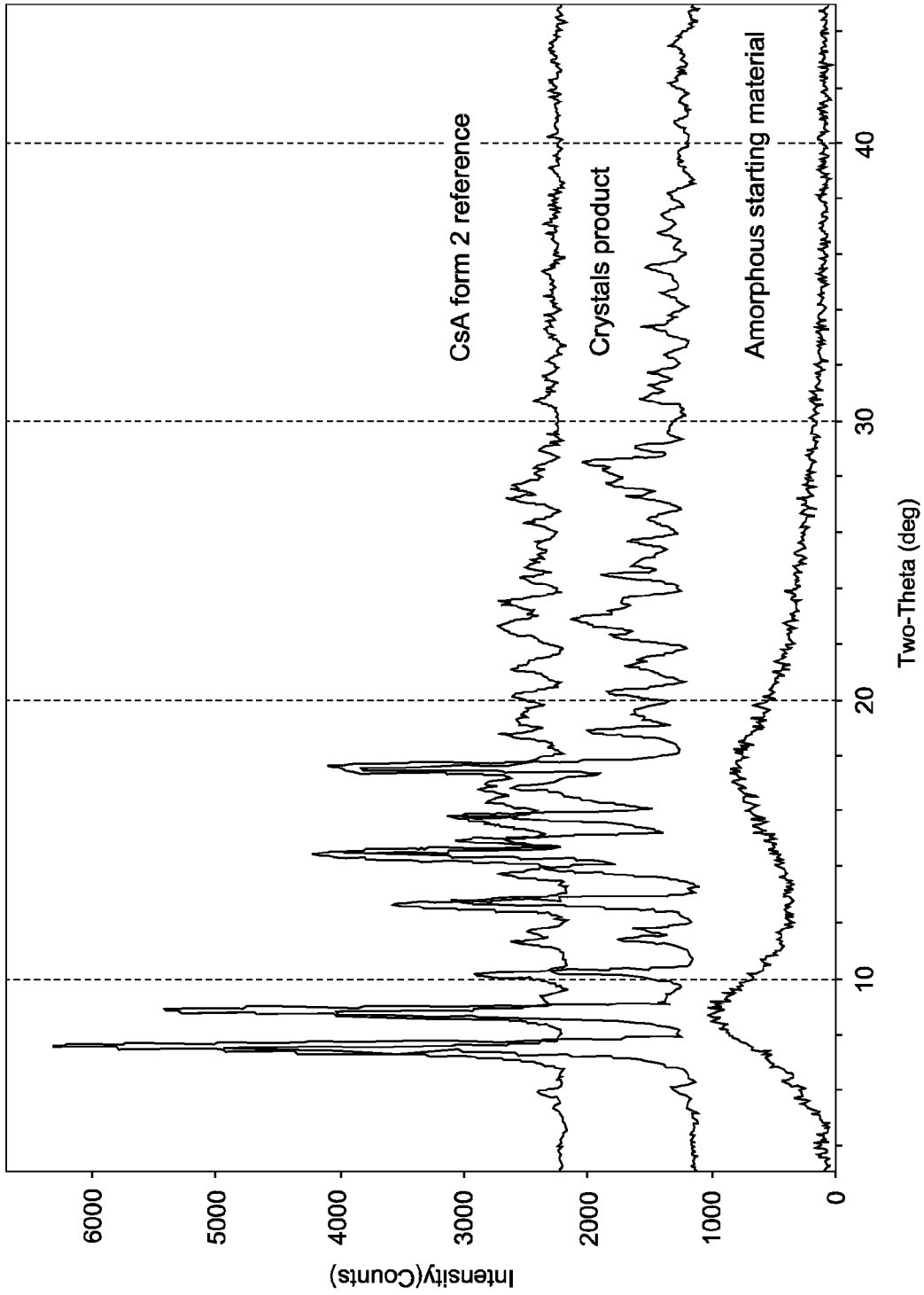

CsA form 2 reference
Crystals product
Amorphous starting material
Intensity(Counts)
Two-Theta (deg)
0
1000
2000
3000
4000
5000
6000
10
20
30
40

FIG. 6

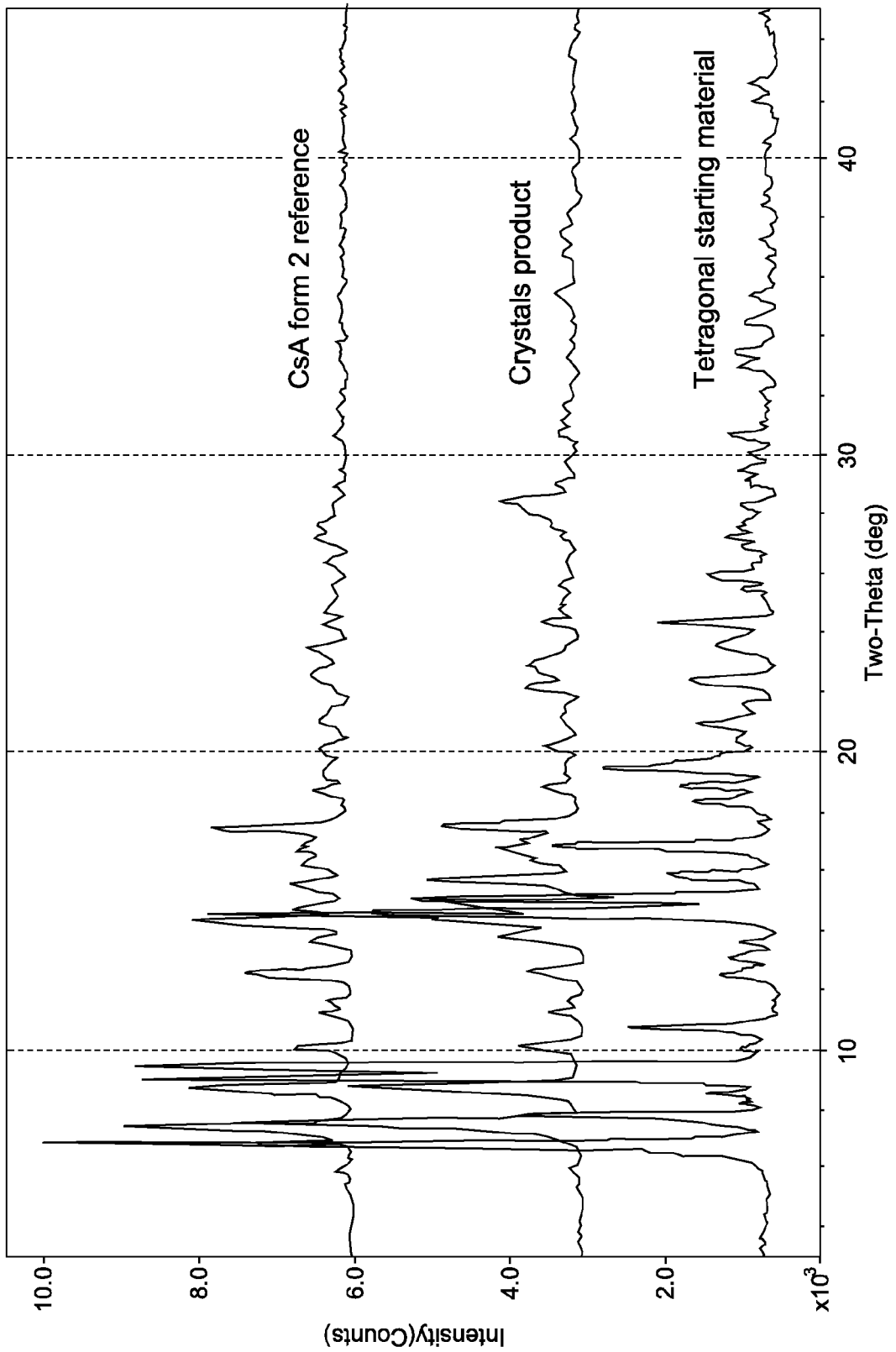

CsA form 2 reference
Crystals product
Tetragonal starting material
Intensity(Counts)
10.0
8.0
6.0
4.0
2.0
x10^3
10
20
30
40
Two-Theta (deg)

FIG. 7

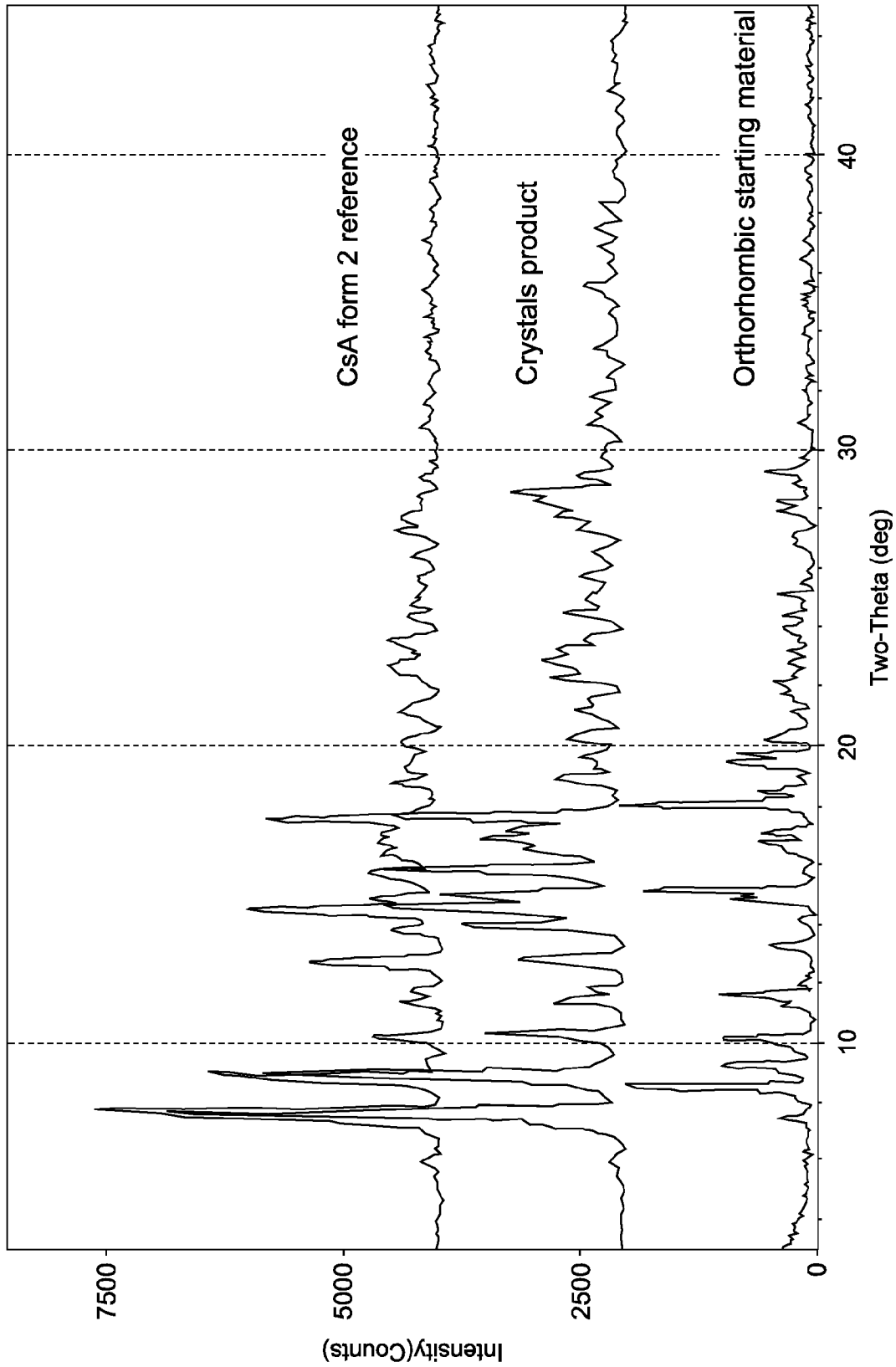

CsA form 2 reference
Crystals product
Orthorhombic starting material
Intensity(Counts)
7500
5000
2500
0
10
20
30
40
Two-Theta (deg)

FIG. 8

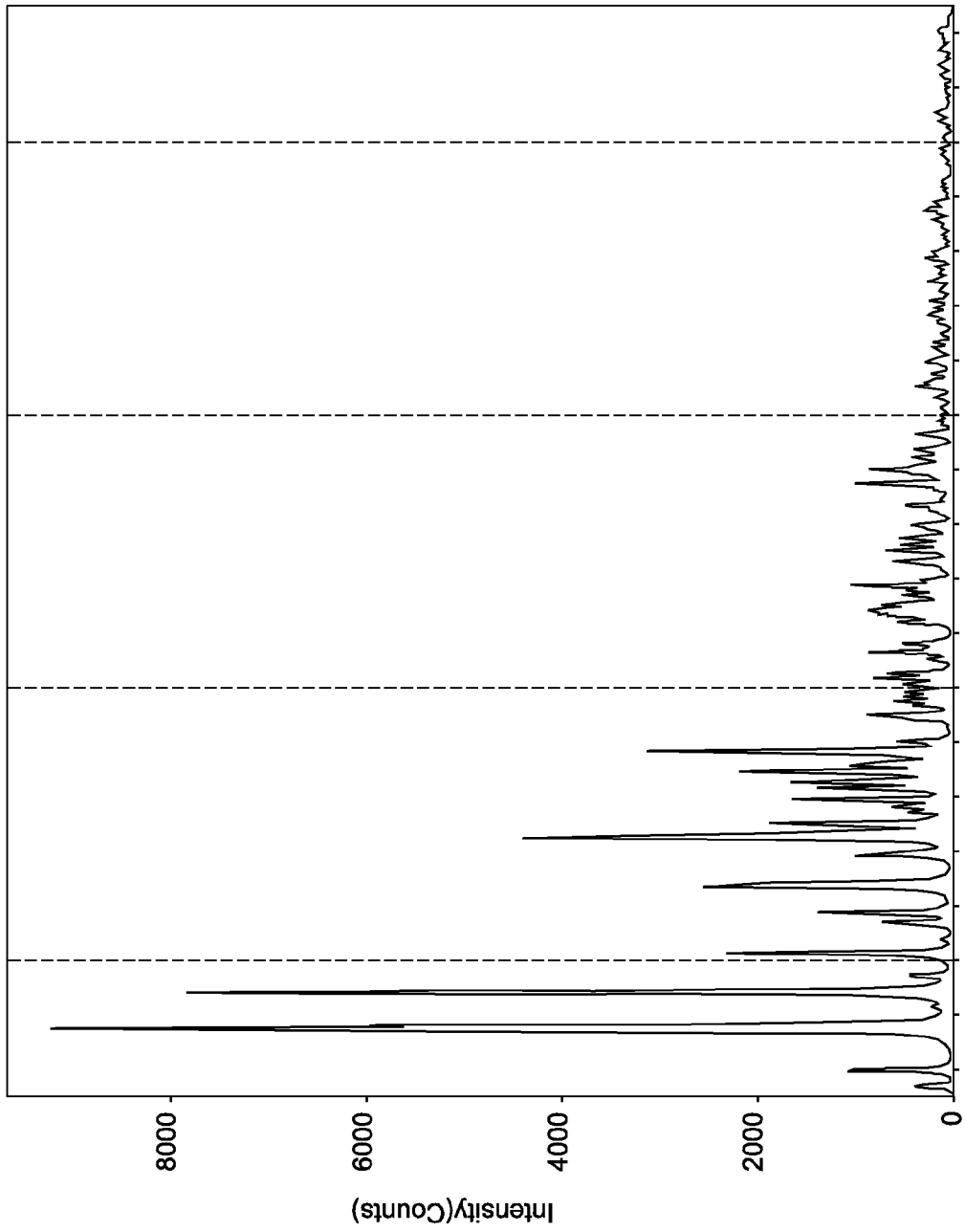

Intensity(Counts)
0
2000
4000
6000
8000
10
20
30
40
Two-Theta (deg)

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/064985

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/64 A61K38/13 A61P27/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 211 848 A (SANDOZ LTD.) 12 July 1989 (1989-07-12) the whole document -----	22,23
E	WO 2012/166610 A1 (ALLERGAN INC.) 6 December 2012 (2012-12-06) cited in the application the whole document -----	22,23

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 2013	21/02/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Masturzo, Pietro

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/064985

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2211848 A	12-07-1989	AT 403163 B	25-11-1997
		AU 2705588 A	22-06-1989
		BE 1002665 A4	30-04-1991
		CA 1341396 C	05-11-2002
		CH 677926 A5	15-07-1991
		CY 1799 A	17-02-1995
		DE 3843054 A1	27-07-1989
		DK 705488 A	22-06-1989
		ES 2012580 A6	01-04-1990
		FI 885869 A	22-06-1989
		FR 2624863 A1	23-06-1989
		GB 2211848 A	12-07-1989
		GR 88100850 A	31-03-1994
		HK 102894 A	30-09-1994
		IE 62006 B1	14-12-1994
		IT 1235356 B	30-06-1992
		JP 1211598 A	24-08-1989
		JP 7091316 B	04-10-1995
		LU 87409 A1	07-07-1989
		NL 8803125 A	17-07-1989
		NO 885625 A	22-06-1989
		NZ 227384 A	25-09-1991
		SE 503174 C2	15-04-1996
		SE 8804570 A	22-06-1989
WO 2012166610 A1	06-12-2012	US 2013023482 A1	24-01-2013
		WO 2012166610 A1	06-12-2012