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Characterization of mesophases of a spontaneously gel-forming compound

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The phases formed by 10, 20, 30, 40 and 50 mg ml^{-1} solutions of a compound, named RWJ-47428, in water have been studied with optical polarizing microscopy and X-ray diffraction. All solutions were found to exhibit the nematic liquid crystalline phase over a wide temperature range. Changes in optical textures showed a transition to the isotropic phase above 130°C in the most concentrated solution. The dimensions (length and width) of the orienting unit were determined to be 22 Å and 4.9 Å. Higher concentration samples exhibit both the nematic and the lamellar phases.

1. Introduction

Gonadotropin releasing hormone (GnRH) is an endogenous decapeptide that can induce the production of both luteinizing hormone and follicle releasing hormone. Because of its functions in the regulation of both female and male hormones, GnRH agonists and antagonists have potential medical use in the treatment of various gynaecological disorders. In particular, numerous pharmaceutical companies have evaluated the efficacy of various GnRH antagonists in the treatment of uterine fibroids, endometriosis and breast cancers. However, most GnRH antagonists exhibit an undesired physicochemical property, which is aggregation or gelation as solution concentrations are increased above the critical micellar concentration. This physicochemical property has created many challenges for fomulation scientists.

A material synthesized at R. W. Johnson Pharmaceutical Research Institute, and named RWJ-47428, is a potent GnRH antagonist in the animal model demonstrating very weak immunological or allergic reactions when the compound was administered by subcutaneous injections [1]. Some of the more common side effects, such as histamine release, were not as profound as with other GnRH antagonists. However, it still behaves like other GnRH antagonist analogues which self-associate to form a liquid crystalline phase [2, 3].

Analytical techniques applied to characterize the phase or liquid crystalline structure include light scattering [4], optical microscopy [5], solid state NMR [6], spin-label electron spin resonance spectroscopy (ESR) [7], circular dichroism (CD) [8], FTIR [9], Raman spectroscopy [10] and thermal analysis [11]. For example, optical birefringence was observed in the peptide liquid crystals of Deterelix [12], a GnRH antagonist developed by Syntex. In the present study, we examined the gelation property of RWJ-47428 in various vehicles. Also, both polarizing optical microscopy (POM) and low angle X-ray diffraction (XRD) were utilized to investigate the liquid crystal properties of aqueous solutions of RWJ-47428. The ultimate goal of this investigation was to identify the conditions for gelation and the liquid crystalline phase of RWJ-47428 in order to assist the formulators in developing a clinical dosage.

2. Experimental

2.1. *Materials*

RWJ-47428, obtained from Chemical Development, Global Chemical, Pharmaceutical and Preclinical Division of R. W. Johnson Pharmaceutical Research Institute exists as an acetate salt. The synthesis and purification methods and structural details of this compound have been previously reported [13, 14]. RWJ-4728 dissolves readily in an aqueous environment. The solution foams upon shaking at ambient temperatures indicating that the compound is surface active and acts like a surfactant in forming micelles above the critical micellar concentration (CMC) of 1.4 mg ml^{-1}. At high concentrations, the solution becomes viscous and lightly turbid. All chemicals

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utilized in the preparation of vehicles to solubilize RWJ-47428 were reagent grades and were used without further purification. In, both the optical POM and XRD experiments, water was the vehicle of choice. Formulations were prepared at concentrations of 5, 10, 20, 30, 40 and 50 mg ml^{-1}.

2.2. *Gelation concentrations in different vehicles*

RWJ-47428 solutions were prepared at various concentrations using different solubilizing agents and buffers. In an attempt to determine the concentrations at which gelation would occur *in vivo*, an aliquot of 100 µl of each formulation was transferred into a 2 ml glass vial containing 0.5 ml of rat plasma. Instantaneous gel formations were observed and the gelation concentrations were recorded for each vehicle. To maximize the drug concentration before which gelation would not occur, common solubilizing and complexing agents such as hydroxypropyl-beta-cyclodextri n were included in some of the tested vehicles.

2.3. *Polarizing optical microscopy*

It is well established that optically anisotropic materials such as liquid crystals form characteristic textures [15] when observed under a polarizing microscope with polarizer and analyser crossed. Each liquid crystalline phase has texture that is unique and a trained eye can correctly identify the phase from its appearance.

A sample looks black under POM in the non-liquid crystalline isotropic phase, or if the optic axis of the liquid crystal nematic phase is oriented parallel to the axis of the microscope, i.e. perpendicular to the glass slide. Under such conditions, a slight pressure on the slide causes the material to flow and reorient its optic axis. If the material is birefringent, as are most liquid crystalline phases, the molecular reorientation gives rise to visible patterns of different colours. In an unaligned liquid crystalline sample, the optic axis takes trajectories determined by the surface conditions and elastic properties of the phase. Liquid crystalline phases thus exhibit characteristic 'textures' which are used to identify them.

Two types of microscope slide were prepared with RWJ-47428 solutions. Simple microscope slides were prepared using a glass plate and a thin cover plate. These were good for observing the effect of flow on the orientation of the optic axis caused by slight pressure or shear force applied to the cover slip. However, since the slide edges were open to air, the solvent (water) tended to evaporate rapidly and observations were unreliable, especially at elevated temperatures. The second type of slide was prepared with 0.1 mm gap flat capillaries. After filling, the capillaries were sealed by carefully fusing their ends with a Bunsen burner. The slides were placed in a Mettler FP-90 hot stage with FP-82HT attachment so that the temperature could be regulated and changed during the evaluation. We typically refrained from approaching 100°C for fear of sample decomposition and breaking of capillary slides due to increase in internal pressure. On occasion, especially for the most concentrated (50 mg ml^{-1}) sample, the temperature was raised well above boiling point of water to look for any changes in optical texture, which would indicate a phase transition.

2.4. *X-ray diffraction*

Almost all XRD measurements were conducted in 1.0 or 1.5 mm thick Lindman glass capillaries. Their ends were fused to make an airtight seal. The capillary samples were placed in an oven with a temperature control and stability of ± 2 K. A pair of permanent rare-earth magnets were placed inside the oven on opposite sides of the sample to produce a magnetic field ~ 2 kG at the sample. This was done to determine if the sample oriented in response to the magnetic field at room temperature. The oven was mounted on the triple-axis goniometer of a Seimens polymer XRD system. This spectrometer uses a sealed copper target, a graphite monochromator, and an area detector model X-1000.

Sample temperature was varied up to 90°C and diffraction patterns recorded for long periods (5–30 min) of time due to extremely weak scattering from these samples. Weak scattering was attributed to the small amount of active material in the solution. Consequently, X-ray scattering experiments were very difficult to perform and even more difficult to interpret, especially in dilute solutions because of the scattering from water, glass, and the oven's Mylar windows. Attempts to perform diffraction measurements on a high resolution spectrometer that used an 18 kW rotating X-ray generator were unsuccessful. The increase in the resolution by approximately a factor of 1000 resulted in a decrease in the diffracted intensity in the same proportion, making the signal to background ratio too small for high resolution measurements.

3. Results and discussion

3.1. *Gelation concentrations in different vehicles*

In this study, most of the vehicles tested formed an *in vitro* gel spontaneously. The gelation concentrations for each vehicle are summarized in the table. [It should be pointed out that the words *gel* and *gelation* are used for the viscous phase obtained for the sake of consistency with previous publications related to such materials, and should not be taken to mean the formation of a crosslinked 'gel' phase.] Based on these data, investigational formulations were prepared. Propylene glycol and hydroxypropyl-beta-cyclodextri n were found to

Table. Effect of various vehicles on the gelation potentials of RWJ-47428 in rat plasma.

Vehicle	Gelation conc./ mg ml^{-1}
Acetic acid 0.2%	<2
Acidified normal saline (pH 1)	>1
Citric acid 0.5%	<2
Dextrose 5%	<2
Glycine 5%	<2
Hydroxypropyl-beta-cyclodextrin 10%	>10
Mannitol 5%	<2
Methanesulfonic acid 0.5%	<2
Normal saline	<0.5
Propylene glycol	>2

inhibit the gelation or liquid crystal phase formation of RWJ-47428. *In vitro* aggregation in 10 mg ml^{-1} solution was prevented by hydroxypropyl-beta-cyclodextrin. RWJ-47428 is less susceptible to gelation in an acidic pH environment compared with vehicles at neutral or basic pH. Acidified normal saline vehicle adjusted to pH 1 prevented self-association at concentrations of up to 1 mg ml^{-1}.

3.2. *Polarizing optical microscopy*

Samples of RWJ-47428 appeared to have bright texture when viewed under a polarizing microscope, confirming the optical birefringence in these samples. The overall brightness of the textures increased with the concentration as would be expected because the birefringence is expected to increase with the concentration. Different areas of the cell appeared to have roughly the same direction of the optic axis, which appeared to be associated with the direction of flow during filling. This phenomenon, termed as *flow alignment*, is a well known characteristic of nematic liquid crystals.

Changes in the brightness of the texture were observed as the cell was rotated between the polarizers. The cell completed a cycle of varying light intensity (i.e. bright–less bright) every 90° of rotation. This indicated that the sample was birefringent and that the optic axis was oriented along the filling direction, generally perpendicularly to the microscope axis. Small pressure on the cover plate deformed the sample and caused changes in the texture. In dilute solutions, bright flashes were observed when deformed, demonstrating the optical anisotropy and deformability, as is the case with most liquid crystalline phases. Some of the areas retained features indicative of the flow caused by the applied pressure.

Slides prepared with flat capillaries were used to make texture observations over a wide temperature range for extended periods of time. Bright and elongated regions appeared to be uniformly aligned along the direction of flow during filling. Rotation of the capillaries under the polarizing microscope showed changes from bright to dark. At intermediate angles, the orientational defects became more visible. For a 10 mg ml^{-1} sample, as shown in figure 1 (*a*), dark bands were clearly visible. These dark lines, characteristics of a nematic liquid crystal, always form closed loops.

Similar results were obtained for the 5, 20, and 30 mg ml^{-1} samples. For a 40 mg ml^{-1} sample, figure 1 (*b*) shows the nematic texture in the centre of the capillary as well as near the edge. However, figure 2 (*a*) depicts both nematic texture as well as focal-conics starting at the straight dark line in the second quadrant of the picture. The latter is a characteristic texture of the lamellar (smectic A-like) phase. Since this region was near the edge of the cell and in the proximity of an air bubble, the concentration in this region appears to have increased significantly to form the lamellar phase. Consequently, this sample was in the nematic phase and appeared to have some regions of higher concentration with the lamellar phase.

In the case of the 50 mg ml^{-1} sample, textures supported the conclusion that this mixture also formed the nematic phase. Representative textures are shown in figure 2 (*b*) for areas in the middle and near the edge of the cell. The textures remained relatively unchanged even when the temperature of the cell was raised above 100°C. At approximately 130°C, a significant change was

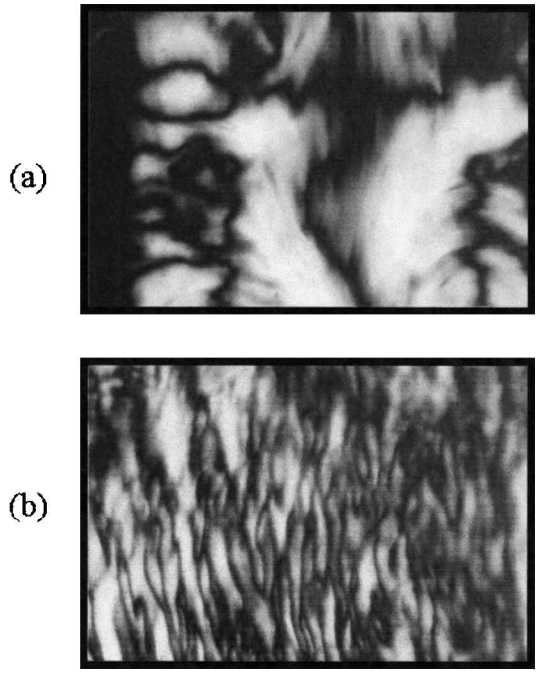

Figure 1. Characteristic nematic textures in (*a*) 10 mg ml^{-1} and (*b*) 40 mg ml^{-1} solutions of RWJ-47428.

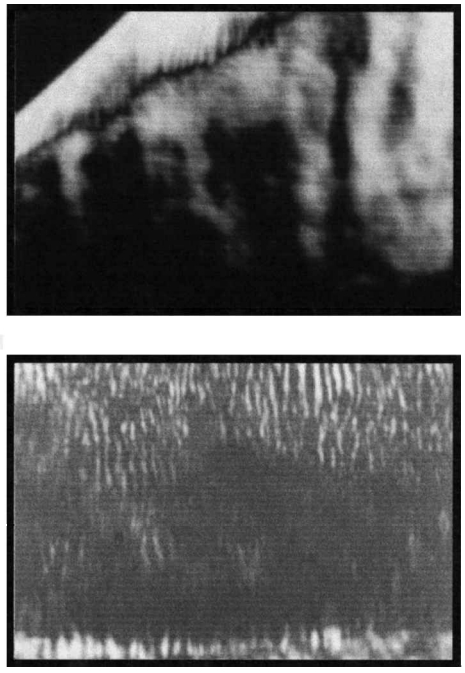

Figure 2. (*a*) Focal-conic texture characteristic of the lamellar phase is visible near the dark line in the 2nd quadrant of the picture along with the nematic texture, in the 40 mg ml^{-1} solution. (*b*) Nematic and lamellar textures are visible in the middle and near the bottom edge of the capillary, respectively.

observed; the cell became uniformly dark and had undergone a transition to the isotropic phase, although some bright specks were still visible in dehydrated parts of the sample. Upon cooling, the nematic texture reappeared, illustrating that the transition was thermodynamically reversible.

3.3. *X-ray diffraction*

The X-ray scattering from these solutions was rather weak mainly because the amount of solute in water was (5–50 parts in 1000) very small. Increasing the amount of sample in the X-ray beam beyond 1.5 mm resulted in diminished X-ray intensities due to absorption by the solvent, water. The samples were filled in capillaries of 1 and 1.5 mm diameter. Since water was the dominant material in the path of the X-rays, scattering from a capillary filled with pure water was recorded to determine background contributions from water that included a band of scattering from 18° to 22°. This band appeared, as expected, in diffraction patterns of all samples and it made the observation of mostly weak diffraction peaks from dilute (5, 10, and 20 mg ml^{-1}) samples difficult.

X-ray scattering from a fresh 30 mg ml^{-1} sample showed no easily identifiable peaks up to an elevated temperature of 90°C. However, when the sample was slowly cooled in the presence of the magnetic field from 90°C to room temperature over a period of several hours and then left in the field for several days, two pairs of reflections in the shape of arcs became clearly visible. These are marked by small arrows in figure 3, and in figure 4 on an expanded scale with readjusted contrast.

The corresponding diffraction angle for the pair of reflections at large angle is 18°. It should be noted that the line joining these two reflections was at 90° to the direction of the applied magnetic field. The second pair of reflections, at small angles, was oriented along the direction parallel to the field. The pair was masked by background scattering near the beam stop and is more visible only on an expanded scale in figure 4. The average angle for this scattering was estimated to be at approximately 4°. The appearance of these reflections was due to the alignment of elongated microscopic entities (micelles or segments of the molecule), possessing a small but positive diamagnetic susceptibility anisotropy, in the direction of the applied field. We estimate the two dimensions, length and width (or diameter) of these entities, to be approximately 22 and 4.9 Å, respectively. These reflections prove the existence of orientational order in this nematic liquid crystalline phase with very small diamagnetic susceptibility anisotropy. The broad reflection, originating from water, was estimated to be centred at 27.5° corresponding to a spacing of ~3.3 Å, representing the average intermolecular distance of water.

A room temperature diffraction pattern showed the two arc-like small reflections at the upper left and lower right hand corners. When the temperature of this sample

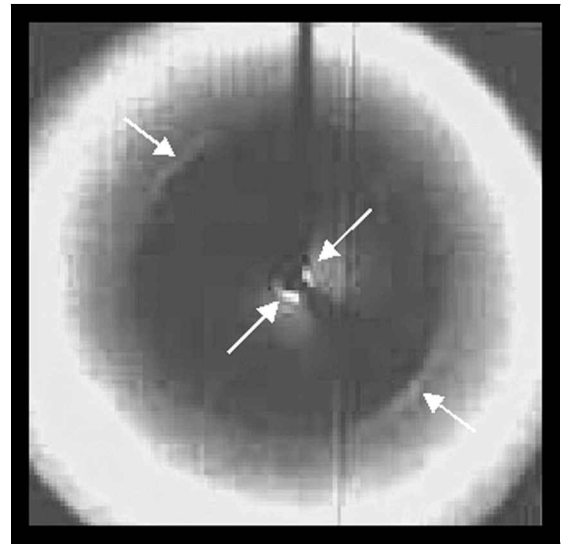

Figure 3. X-ray diffraction pattern of an aligned 30 mg ml^{-1} sample. Two sets of arc-like reflections marked with small arrows are visible. The small angle pair of reflections is parallel to the field while the large angle reflections are perpendicular.

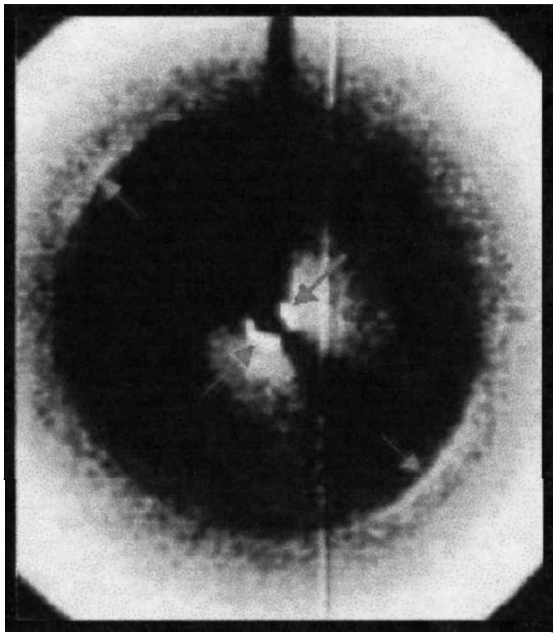

Figure 4. X-ray diffraction pattern of figure 3 on an expanded scale and with readjusted contrast to show the four reflections (marked with arrows) clearly. The magnetic field is parallel to the arrows pointing to the small angle reflections.

was raised to 80°C, the intensity of these reflections diminished significantly suggesting a disruption of field-induced alignment accompanied by a lowering of the nematic order parameter. Such is often the case for nematic liquid crystals.

For a 40 mg ml^{-1} sample, a diffraction ring, indicative of an unaligned sample, was observed at room temperature. The fact that the reflections were easily visible even without the director alignment, is because its sample had a higher concentration of RWJ-47428. Attempts to align this sample were unsuccessful because of increased viscosity and resulting ineffectiveness of the magnetic field. The dimensions estimated from the large and small angle reflections of this sample are essentially the same as given above for the 30 mg ml^{-1} sample.

The 50 mg ml^{-1} sample also yielded essentially the same diffraction patterns but with one difference. In this case, the large angle diffraction arcs were observed in the direction parallel to the field (they were perpendicular to the field for the field-aligned 30 mg ml^{-1} sample), caused by flow alignment during the filling process. This behaviour was also observed in thin cells prepared for optical observation. Although optical examinations showed characteristic focal-conic textures of the lamellar phase in small regions, the lamellar phase would have given rise to bright and multiple reflections in X-ray experiments. The absence of such reflections leads to the conclusion that, at the concentration studied, the samples did not form a lamellar phase.

4. Summary

Formulations of RWJ-47428 in commonly used injectable vehicles, such as normal saline and 5% dextrose, have the tendency to spontaneously form a viscous phase. Using both polarizing optical microscopy and X-ray diffraction techniques, RWJ-47428 was found to exhibit the nematic liquid crystalline phase. Macroscopic alignment of the sample's optic axis was clearly visible in the nematic phase of high concentration solutions. Magnetic alignment and its loss at higher temperatures are tell-tale signs of the nematic phase. The two dimensions of the entities undergoing orientational order to form the nematic phase in this system were determined with XRD. At this time, we do not have enough information to infer the nature and exact shape of these entities. It should be possible, in future, to determine if these dimensions are consistent with the size of the RWJ-47428 molecule or a sub-molecular unit.

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