Quality by Design-Guided Silymarin-Phospholipid Phytosomal Complex for Enhanced Oral Delivery and Hepatoprotection: A Short Review

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Abstract*—Silymarin, the standardised flavonolignan extract of the fruits and seeds of milk thistle (Silybum marianum), is among the most widely used hepatoprotective phytopharmaceuticals, and its antioxidant, membrane-stabilising, anti-inflammatory and regenerative actions in the liver are well documented. Its oral utility is nevertheless limited by unfavourable biopharmaceutics. Silymarin is a poorly water-soluble, Biopharmaceutics Classification System Class II material whose principal constituent, silybin, undergoes extensive first-pass glucuronidation and sulfation, so that its absolute oral bioavailability is very low and conventional products must be given at a high dose several times a day. Complexation with phospholipid, the technology commonly termed phytosome, addresses this mismatch by converting a polar phenolic into an amphiphilic complex through hydrogen bonding with the head group of phosphatidylcholines, and silybin-phosphatidylcholine complexes are already marketed. Published silymarin phytosome studies, however, have largely relied on one-factor-at-a-time or simple factorial optimisation. This short review examines the case for a silymarin-phospholipid phytosome developed within a Quality by Design framework, using a three-factor Box-Behnken design with the phospholipid-to-drug molar ratio, reaction temperature and reaction time as variables. The hepatic pharmacology and biopharmaceutical limitations of silymarin, the chemistry and preparation of phytosomes, the formulation variables, the precedent in the literature, the optimisation design, the characterisation, dissolution, solid-dosage and stability programme, and a framework for relating in vitro improvement to in vivo exposure and hepatoprotection in a carbon tetrachloride model are discussed in turn. The review concludes that a design-space-optimised silymarin phytosome is a rational, experimentally tractable route to improved oral delivery, and it identifies the scientific and translational challenges that remain.

***Index Terms*—Silymarin; silybin; phytosome; phospholipid complex; oral bioavailability; Quality by Design; Box-Behnken design; hepatoprotection; carbon tetrachloride**

I. INTRODUCTION

Chronic liver disease spans alcohol-related liver disease, non-alcoholic fatty liver disease and its progressive form, steatohepatitis, viral hepatitis, drug-induced liver injury and cirrhosis, and together these conditions account for a very large global burden of illness and death, estimated at about two million deaths a year [1]. The aetiologies differ, but oxidative stress and chronic low-grade inflammation are recognised as common, convergent mechanisms of hepatocellular injury, fibrogenesis and progression. Current pharmacological management is largely supportive or specific to the cause, and relatively few agents are directed at the oxidative and inflammatory injury that the conditions share. This gap has sustained interest in hepatoprotective phytopharmaceuticals, among which silymarin occupies a uniquely well-studied position [2,3].

Silymarin is nevertheless a clear example of an active whose pharmacology has outrun its delivery. It is poorly soluble, it is extensively conjugated during first-pass metabolism, and its oral bioavailability is correspondingly low, which is why standardised products are given at a high dose two or three times a day for what is usually long-term therapy [2,4]. A

range of formulation strategies has been explored to overcome these limitations, including nanoemulsions, liposomes, polymeric nanoparticles and phospholipid complexes, and the last of these has the distinction of having reached the market as a silybin-phosphatidylcholine complex [2,5].

Phospholipid complexation, known as the phytosome approach when applied to plant actives, treats the absorption problem of a polar phenolic as an amphiphilicity mismatch and corrects it by forming a hydrogen-bonded complex with phosphatidylcholine [6,7]. A silymarin phytosome made with the solvent evaporation method has been reported to give a marked improvement in solubility, bioavailability and hepatoprotective efficacy over plain silymarin in rats [8]. What the literature offers much less of is systematic, risk-based optimisation of such a complex. Studies on silymarin phytosomes have tended to use empirical or simple factorial optimisation, whereas the Quality by Design approach defined by the ICH guidelines, with a Box-Behnken response-surface design, has been applied to phytosomes of other actives [9,10,11].

This short review sets out the scientific case for a silymarin-phospholipid phytosome developed and optimised within a Quality by Design framework. It begins with the hepatic pharmacology and biopharmaceutical limitations of silymarin, describes phytosome chemistry and preparation, and then considers the formulation variables, the precedent in the literature, the optimisation design, and the characterisation, dissolution, dosage-form and stability work needed to demonstrate improvement. A framework relating in vitro gain to in vivo exposure and hepatoprotection is proposed, and the remaining challenges are identified. The material is drawn from the primary and review literature on silymarin, on phospholipid complexes and on pharmaceutical Quality by Design, with foundational and regulatory references added to the core literature to provide context. Where a study is cited, only what its authors reported is attributed to it, and the review is a discussion of rationale and design that presents no new experimental data.

II. SILYMARIN: CHEMISTRY AND HEPATOPROTECTIVE PHARMACOLOGY

2.1 Source and composition

Silymarin is a standardised extract obtained from the fruits and seeds of Silybum marianum (milk thistle, family Asteraceae), and it is a complex mixture of flavonolignans together with minor flavonoid and polyphenolic components. The principal and most active constituent is silybin, also called silibinin, with a molecular formula of C25H22O10 and a molecular weight of about 482.44 g/mol, accompanied by isosilybin, silychristin and silydianin [3,12]. Silybin itself is not a single compound. It is an approximately equimolar mixture of two diastereomers, silybin A and silybin B, and the corresponding isosilybins also occur as a pair, a point that matters for analysis because a method that does not resolve the diastereomers reports them together [13].

In the solid state silymarin is a light yellow to beige powder of amorphous or crystalline habit. It is practically insoluble in water, with the aqueous solubility of its flavonolignan constituents reported in the range of roughly 50 to 430 micrograms per millilitre, sparingly soluble in most organic solvents, and soluble in dimethyl sulfoxide, methanol and polyethylene glycol 400 [4]. The phenolic hydroxyl groups of silybin are the structural feature that makes complexation with a phospholipid possible, and they are also the sites at which the molecule is conjugated in vivo, so that the same functional groups govern both the opportunity and the problem.

2.2 Mechanisms of hepatoprotection

The hepatoprotective activity of silymarin is attributed to several convergent mechanisms. It scavenges free radicals directly and chelates pro-oxidant metal ions, which limits lipid peroxidation of the hepatocyte membrane. It stabilises membrane structure and permeability and so restricts the entry of hepatotoxic agents into the cell. It activates the nuclear factor erythroid 2-related factor 2 pathway, raising the cellular content of glutathione, superoxide dismutase and catalase. It also inhibits nuclear factor kappa B-driven transcription of pro-inflammatory cytokines such as tumour necrosis factor alpha and interleukin 6, and it has been reported to stimulate ribosomal RNA

synthesis and so promote hepatocyte regeneration [2,14].

These actions have been examined in reviews of both the basic pharmacology and the clinical use of silymarin and silybin in liver disease, which describe a rational mechanistic basis and an encouraging but heterogeneous clinical evidence base, with trials differing in preparation, dose and patient population [15,16]. This heterogeneity is relevant to the present discussion because variable and low systemic exposure is one plausible contributor to inconsistent clinical results, and a formulation that raises and stabilises exposure is a legitimate and measurable objective whose success can be judged by pharmacokinetic and biochemical endpoints without first resolving every clinical question.

2.3 Marketed and investigational silymarin products

Silymarin products on the market and under investigation illustrate the range of strategies applied to the problem. Standardised extract capsules and tablets, including products of the Legalon type, are used as adjunct hepatoprotective therapy in chronic liver disease, and over-the-counter milk thistle preparations are used for general hepatic support under traditional use. Oily soft-gelatin capsule formulations of the standardised extract aim at improved dissolution, silymarin nanomicellar and nanoemulsion formulations are investigational, and the silybin-phosphatidylcholine complexes marketed internationally as Siliphos and Silipide are the established example of a complexation approach to enhanced bioavailability [2,5]. The safety record of silymarin is favourable, and it is generally well tolerated at the doses used in clinical practice, so that the case for a new formulation is one of exposure and dose economy and not of tolerability [3,15].

III. THE BIOPHARMACEUTICAL PROBLEM

3.1 Poor solubility and low permeation-limited absorption

Silymarin is classified as a Biopharmaceutics Classification System Class II compound, characterised by low aqueous solubility and comparatively higher permeability, and the extent to which it is absorbed from the gastrointestinal tract is therefore governed in large part by how much of the dose can be brought into solution at the absorptive surface [4,8]. Only a fraction of the administered dose, estimated at about 20 to 50 per cent, is absorbed. Because the dissolution of a crystalline or poorly wetted solid is slow compared with the transit time through the absorptive region of the intestine, increasing the apparent solubility or the rate of dissolution is a necessary, although not a sufficient, condition for improving exposure [4].

3.2 First-pass conjugation and elimination

The second limitation operates after absorption. Silybin undergoes extensive phase II metabolism, principally glucuronidation and sulfation, in the intestinal mucosa and in the liver, and the conjugates are eliminated rapidly in the bile and, to a lesser extent, the urine. The combination of limited absorption and extensive first-pass conjugation gives an absolute oral bioavailability of silybin that has been estimated at well below 1 per cent [4,8]. Reviews of formulation strategies for silymarin draw the same conclusion about the combined effect of poor solubility and extensive conjugation on systemic exposure [2,4].

The practical consequence is a high and frequent dosing requirement. Conventional standardised silymarin is given at about 140 mg, calculated as silybin, two to three times daily, to achieve a modest hepatoprotective exposure, and in a typically chronic and long-term therapy this burden is likely to reduce convenience and adherence. A formulation that increases the extent of absorption, and that does so reproducibly, could in principle allow a lower or less frequent dose, which is the benefit sought in the marketed phospholipid complexes [2,5].

3.3 Why a complexation strategy rather than particle engineering alone

A reduction of particle size or a change of the solid state can raise the dissolution rate of a poorly soluble flavonolignan, and several nanocarrier approaches have been explored for silymarin [2,4]. A phospholipid complex differs from these in that it changes the molecular environment of the drug itself. The complex is amphiphilic, it disperses in water to give a colloidal system whose properties differ from those of the drug alone, and it carries the drug in a lipid-compatible form that may interact more favourably with the lipid-rich intestinal membrane

[6,17]. Because the hydroxyl groups that make silybin vulnerable to conjugation are also those that interact with the phospholipid, the complexation chemistry is directly relevant to the pharmacokinetic problem, although whether and to what extent it alters first-pass conjugation is a question for the in vivo programme, not a conclusion to be assumed.

IV. PHYTOSOME (PHOSPHOLIPID COMPLEX) TECHNOLOGY

4.1 Chemistry of complex formation

The phytosome is a complex of a phytoconstituent with a phospholipid, most often phosphatidylcholine, in which the polar head group of the phospholipid interacts with the active through hydrogen bonding. A review of the supramolecular interactions between phospholipids and polyphenols described the mechanism in which the phenolic hydroxyl groups of the phytoconstituent interact with the phosphate and choline groups of phosphatidylcholines, while the two fatty acid chains of the lipid remain free, so that the complex acquires an amphiphilic character that the parent compound lacks [6]. Complexes are commonly formed at a molar ratio of 1:1 or 1:2 in an aprotic solvent under controlled temperature, and the stoichiometry and the solvent are among the variables that determine how completely the active is complexed [6,18].

Bhattacharya introduced the technology as a means of improving the bioavailability of botanicals and nutraceuticals, starting from the observation that many bioactive plant constituents are poorly absorbed because they are too polar to cross lipid membranes readily and often too large to be absorbed by simple diffusion, and presenting phospholipid complexation as a rational solution [7]. The approach was extended to a range of plant actives, and the phyto-phospholipid complexation technique was later reviewed from the standpoint of its effect on the pharmacokinetic profile of plant actives [17,19].

4.2 Comparison with liposomes and physical mixtures

A phytosome is frequently confused with a liposome, but the two differ in an essential way. In a liposome the active is physically entrapped in the aqueous core or in the bilayer of a pre-formed vesicle, and the loading depends on the properties of the vesicle and may be limited by leakage and fusion on storage. In a phytosome the active is chemically integrated with the phospholipid molecule through hydrogen bonding, so that the loading is defined by the stoichiometry of the complex and the physical stability of the product is not dependent on retaining a cargo within a vesicle [6,19]. A simple physical mixture of the drug and the phospholipid, prepared without solvent or heat treatment, is not a complex, and it is therefore the appropriate control with which to separate true complexation from simple co-formulation. These distinctions are summarised in Table 1 and illustrated in Figure 1.

Table 1. Comparison of plain silymarin, a physical mixture, a liposome and a phytosome complex [6,8,19]

Attribute	Plain silymarin	Physical mixture	Liposome	Phytosome complex
Bonding	None (free drug)	None (simple blend)	Physical entrapment in bilayer or core	Stoichiometric hydrogen-bonded complex
Aqueous solubility	Very poor (BCS Class II)	Marginally improved	Improved; vesicle-dependent	Markedly improved; amphiphilic complex
Lipid solubility	Poor	Poor	Moderate	Markedly improved
Physical stability	Not applicable	Poor; segregation on storage	Prone to leakage and fusion	Good; chemically integrated
Reported oral bioavailability gain	Baseline	Minimal	Variable; formulation-dependent	About 4- to 6-fold reported for silymarin

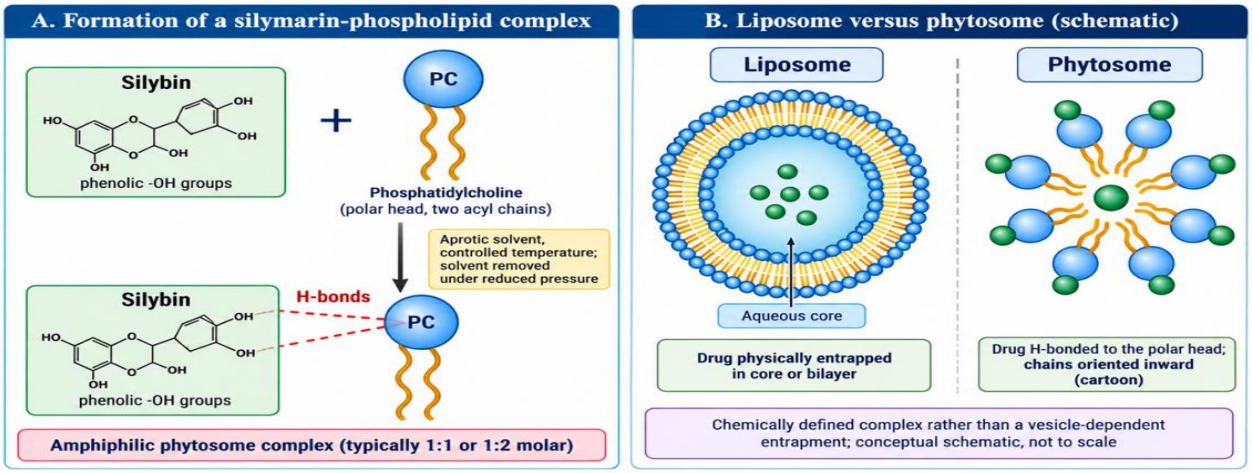


Figure 1. (A) Schematic formation of a silymarin-phospholipid complex: silybin and phosphatidylcholine (PC) react in an aprotic solvent, the phenolic hydroxyl groups of silybin hydrogen-bond with the PC head group, and the solvent is removed under reduced pressure. (B) Conceptual contrast between a liposome, in which the drug is entrapped, and a phytosome, in which it is bound to the polar head; both panels are schematic and not to scale [6,19].

4.3 Preparation methods

Phospholipid complexes are prepared by several methods, of which solvent evaporation is the most widely used. The drug and the phospholipid are dissolved together in an aprotic solvent such as dichloromethane, acetone or tetrahydrofuran, refluxed at a controlled temperature for a defined time to allow complexation, and the solvent is then removed under reduced pressure to leave a dry, waxy film of complex, which is dried under vacuum and stored in a desiccator. The influence of the ratio of phospholipid to drug and of the choice of solvent on complexation efficiency and on downstream delivery performance has been reviewed [18,20]. Alternatives include thin-film hydration, anti-solvent precipitation and spray drying, and the choice among them affects the particle characteristics, the residual solvent and the scalability of the product [17,20].

The precedent for silymarin illustrates two of these routes. Maryana and colleagues prepared silymarin phytosomes by the thin-layer hydration technique and identified a 2 per cent silymarin-phospholipid complex at a molar ratio of 1:5 as optimal, reporting vesicles of 133.5 nm with an entrapment efficiency of 97.2 per cent, with infrared spectroscopy and differential scanning calorimetry confirming interaction between silymarin and phospholipid [21]. Shriram and colleagues used solvent evaporation and optimised the formulation by a two-factor, three-level full factorial design, and their optimised phytosome of 218.4 nm showed an improvement in aqueous solubility of about 360 micrograms per millilitre [8]. The two routes differ in how the complex is generated and in the form in which it is presented, a difference that makes direct comparison of their numerical results inappropriate.

V. FORMULATION COMPONENTS AND CRITICAL PARAMETERS

5.1 Components

The phytosome and the solid dosage form made from it use a short list of components, summarised in Table 2 together with the role of each. The selection of the complexing phospholipid is the most consequential choice. Soya phosphatidylcholine of high purity, available as grades such as Phospholipon 90G and Lipoid S-100, is the usual choice, and its purity, its fatty acid composition and its phosphatidylcholine content influence the complex. The reaction solvent must dissolve both components, must not itself compete for hydrogen bonding in a way that prevents complexation, and must be removable to acceptable residual levels, and an aprotic solvent is therefore used. A non-solvent such as n-hexane may be used to precipitate and purify the complex from uncomplexed reactants [6,18].

The excipients of the solid dosage form are conventional, but they are not inert with respect to a lipid complex. The waxy, low-melting nature of a phospholipid complex can lead to poor flow and sticking, and the glidant and lubricant are included to address this. The proportion of a hydrophobic lubricant such as magnesium stearate should be kept low and its effect on dissolution checked, because a hydrophobic coating of the powder can slow wetting of a material whose purpose is to improve it [20].

5.2 Critical material and process attributes

Four variables are generally regarded as the main determinants of complex formation, and they are the ones proposed for the optimisation study. The molar ratio of phospholipid to drug controls whether the drug is fully complexed. At too low a ratio, uncomplexed drug remains, while at too high a ratio the product contains excess lipid, which dilutes the drug content and may alter the properties of the dispersion. The reports on silymarin illustrate how different the optima can be, with one thin-layer hydration study identifying a ratio of 1:5 as optimal and the general literature describing complexes at 1:1 or 1:2 [6,18,21].

Table 2. Components of the silymarin phytosome and its oral solid dosage form, with their functional roles [6,18,21]

Component	Category	Role in the formulation
Soya phosphatidylcholine (Phospholipon 90G, Lipoid S-100)	Complexing phospholipid	Forms a stoichiometric hydrogen-bonded complex with the phenolic hydroxyl groups of silymarin, conferring amphiphilic character
Dichloromethane, acetone, tetrahydrofuran, dioxane	Aprotic reaction solvent	Medium for complexation; removed by controlled evaporation after the reaction
n-Hexane, diethyl ether	Non-solvent	Precipitates and purifies the complex from residual uncomplexed reactants
Microcrystalline cellulose	Diluent	Bulking agent for the capsule fill
Colloidal silicon dioxide	Glidant	Improves flow of the complex during capsule filling
Magnesium stearate	Lubricant	Reduces friction during filling or compression
Hard gelatin or HPMC capsule shell	Dosage-form container	Encapsulates the optimised complex powder for oral use
Simulated gastric and intestinal fluids	Dissolution medium	Assesses solubility and dissolution behaviour of the complex

The solvent system affects solubility of both components and the strength of the interaction. The reaction temperature and the reaction time govern the kinetics of complexation, and each has a ceiling: excessive temperature risks oxidative degradation of both the phenolic drug and the unsaturated phospholipid, and the practical upper limit is set by the boiling point of the solvent under reflux. Stirring rate and the rate of solvent removal are further process variables that influence the physical form of the product, although they are usually controlled instead of being optimised [18,20]. These variables interact. A higher temperature may shorten the time required, and the optimum ratio may depend on the solvent, which is the reason why one-factor-at-a-time experimentation can miss the optimum and why a statistical design is preferable [22,23].

5.3 Drug loading and the physical state of the product

A phytosome is a defined complex and not a solution, so its drug loading is a consequence of the stoichiometry. A complex of silybin with phosphatidylcholine at a 1:1 molar ratio contains a much larger mass of lipid than of drug, because the molecular weight of phosphatidylcholine is of the same order as that of silybin or larger and the mass ratio therefore scales with the molar ratio, and the practical consequence is a low drug content per unit mass of complex. The dose per capsule is limited by the capsule volume, and a capsule of a size acceptable to patients may therefore deliver less silybin than the conventional dose unless the improved bioavailability allows the dose to be reduced. The drug content of the optimised complex, and the dose it would imply, should be calculated early in development and not treated as an afterthought [5,6].

The physical state of the product also matters. A dry complex film is waxy and may be sticky or hygroscopic, and the low melting range of phospholipids restricts the temperatures to which it can be exposed in drying and milling. The product may be prepared as a free-flowing powder by adsorption, sieving or lyophilisation, and the method chosen should be reported with the complex, because the same complex presented in different physical forms may behave differently in dissolution and in the capsule [17,20].

VI. PRECEDENT IN THE LITERATURE

The case for an optimised silymarin phytosome rests on the body of work summarised in Table 3. It includes direct precedent for silymarin phospholipid complexes in animals and humans, the review literature on phospholipid complexation, and the statistically designed optimisation of phytosomes of other actives. The summary reports, for each study, only the outcome stated by its authors.

Table 3. Representative precedent for silymarin phospholipid complexes, phytosome technology and design-based optimisation

Active / topic	Approach	Reported outcome	Ref.
Silymarin	Phytosome by solvent evaporation; two-factor full factorial design	218.4 nm; improved solubility; about 6-fold oral bioavailability and better hepatoprotection than plain silymarin in CCl4-treated rats	[8]
Silymarin	Phytosome by thin-layer hydration	2% complex at 1:5 molar ratio optimal; 133.5 nm; 97.2% entrapment; FTIR and DSC showed interaction	[21]
Silybin	Silybin-phospholipid complex	Preparation described; improved oral bioavailability of silybin in rats	[24]
Silybin	Silybin-phosphatidylcholine complex (IdB 1016)	Pharmacokinetics described in healthy human subjects	[25]
Milk thistle	Silybin-phosphatidylcholine phytosome (Siliphos)	Review of bioavailability and clinical efficacy	[5]
Naringenin	Phytosome optimised by Box-Behnken design	Ratio, temperature and time optimised by desirability; entrapment efficiency and yield as responses	[9]
Antidiabetic polyherbal	Phytosome optimised by Box-Behnken design	Phospholipid concentration, temperature and time varied; efficacy comparable to metformin	[10]
Phospholipid complexes	Reviews	Methods, characterisation and oral bioavailability enhancement consolidated	[17,18,20]

Three patterns emerge from Table 3. First, the evidence for a silymarin or silybin phospholipid complex is consistent across species and across laboratories, from rat studies of a silybin complex to human pharmacokinetic work on a marketed silybin-phosphatidylcholine complex and a phytosome of silymarin that improved bioavailability and hepatoprotection in the rat [8,24,25]. Second, the optimisation of silymarin phytosomes has used a two-factor full factorial design or a single-variable approach, which cannot estimate the interactions among ratio, temperature and time that a three-factor response-surface design can [8,21]. Third, Box-Behnken optimisation of phytosomes has been demonstrated for naringenin and for a polyherbal system, which supplies a methodological template but not the answer for silymarin [9,10].

These patterns define the gap precisely. A study that applied a risk-based, three-factor Box-Behnken design to silymarin, defined its design space and characterised the product against both plain silymarin and a physical mixture, and then tested it in vivo, would add what is missing. It would also be informative whichever way it turned out. If the optimised complex gave no greater exposure than the simpler published preparations, that would show that the earlier preparations had already approached the limit of what complexation can achieve for this molecule.

VII. QUALITY BY DESIGN AND BOX-BEHNKEN OPTIMISATION

7.1 The Quality by Design framework

Quality by Design is a systematic approach to development that starts from predefined objectives and emphasises product and process understanding and control, based on sound science and quality risk management [11,26]. The development begins with a Quality Target Product Profile, identifies the critical quality attributes that must be controlled to meet it, uses risk assessment to identify the material attributes and process parameters likely to affect those attributes, and then explores their relationships experimentally to define a design space within which quality is assured. A review of the approach in pharmaceutical development emphasised that it replaces trial-and-error development with an understanding of the relationships between variables and quality [23].

For the silymarin phytosome, the Quality Target Product Profile would include a high complexation efficiency, a particle size that favours dissolution, a narrow size distribution, adequate colloidal stability as indicated by zeta potential, and a markedly improved aqueous solubility relative to the plain drug. The critical quality attributes that follow are the complexation efficiency, the particle size, the polydispersity index, the zeta potential and the aqueous solubility of the complexed silymarin. A polydispersity index of about 0.3 or lower is generally regarded as acceptable for lipid-based carriers [27].

7.2 Risk assessment

An Ishikawa, or fishbone, diagram maps the possible material and process variables against each critical quality attribute. For the phytosome these include the phospholipid-to-drug molar ratio, the type of solvent, the reaction temperature, the reaction time and the stirring rate, together with raw-material variables such as the phospholipid grade and the quality of the silymarin. A risk-estimation matrix then ranks the variables by their anticipated effect and the uncertainty about it, and the highest-ranked variables are carried into the experimental design, as set out in the quality risk management guideline [23,26]. The ranking is a judgement based on prior knowledge, and it should be recorded together with the evidence on which it rests, so that the choice of three factors can be defended and revisited.

7.3 The Box-Behnken design

Box and Behnken introduced three-level designs for fitting second-order response surfaces, which allow main effects, two-factor interactions and quadratic effects to be estimated from a modest number of runs and without the extreme combinations of all factors that a full three-level factorial would require [28]. The design has been discussed as an alternative for optimisation in settings where the extreme corner combinations are impractical or undesirable [29]. For three factors the design has twelve runs at the mid-edges of the cube and replicated centre points, and with three centre points the design has fifteen runs, which provides an estimate of pure error.

The independent variables proposed are the phospholipid-to-drug molar ratio (X1), the reaction temperature (X2) and the reaction time (X3), at three levels each, as set out in Table 4. The synopsis on which this review is based does not fix the levels, which are to be set from the preliminary trials. The levels shown are illustrative working ranges, drawn from the ranges reported in the literature for the ratio and bounded for temperature by the solvent used, and they should not be regarded as results. The responses are the five critical quality attributes. Complexation efficiency and aqueous solubility are to be maximised, particle size and polydispersity index minimised, and zeta potential kept at a magnitude that indicates adequate colloidal stability [9,10,27].

Table 4. Proposed variables, illustrative levels and responses for the three-factor Box-Behnken design (15 runs, including three centre points); levels are to be fixed from preliminary trials [9,28]

Variable / response	Low (-1)	Medium (0)	High (+1)	Constraint
X1: phospholipid: drug molar ratio	1:1	1:2	1:3	Independent variable
X2: reaction temperature (°C)	40	50	60	Independent variable
X3: reaction time (h)	1	2	3	Independent variable
Y1: complexation efficiency (%)	-	-	-	Maximise
Y2: particle size (nm)	-	-	-	Minimise

Y3: polydispersity index	-	-	-	Minimise
Y4: zeta potential (mV)	-	-	-	Adequate magnitude
Y5: aqueous solubility (µg/mL)	-	-	-	Maximise

7.4 Analysis, design space and confirmation

The data from the runs are fitted to second-order polynomial models, and analysis of variance is used to establish the significance of each model and of its terms, with a lack-of-fit test against the pure error from the centre points and the coefficient of determination as a further check. Three-dimensional response-surface and contour plots show how each response changes with the variables and where interactions lie, and numerical optimisation by the desirability-function approach combines the responses into a single objective, as has been done for phytosomes of naringenin [9,22]. The predicted optimum is then prepared experimentally and its responses are compared with the predictions, which is the test of the validity of the design space [23].

One practical caution applies to Table 4. The choice of reaction solvent fixes the usable temperature range, since the temperature of a reflux cannot exceed the boiling point of the solvent, and a design with levels that are not attainable in the chosen solvent cannot be run. The solvent should therefore be fixed before the design is finalised, or the design run separately for each solvent considered, which is the reason that the solvent is treated as a screening variable ahead of the respons`W111111e-surface study [18].

VIII. CHARACTERISATION, DOSAGE FORM AND STABILITY

8.1 Preformulation and analytical method validation

Preformulation begins with the organoleptic and physicochemical evaluation of the silymarin, including colour, odour, crystal habit and melting point, and with equilibrium solubility by the shake-flask method in water, phosphate buffers of different pH, methanol, ethanol, dichloromethane and polyethylene glycol 400, which confirms the Class II solubility profile and guides the choice of complexation solvent and dissolution medium [4]. Drug-excipient compatibility is assessed by infrared spectroscopy of the drug, the phospholipid and their physical mixture, looking for the appearance, disappearance or shift of characteristic bands, in particular the phenolic O-H and the phosphate and carbonyl bands, and the findings are corroborated by differential scanning calorimetry [20,21].

A UV-spectrophotometric method for routine estimation, with a reverse-phase HPLC method for complexation efficiency, drug content and dissolution samples, should be developed and validated according to ICH Q2(R1), which defines the validation characteristics for different types of analytical procedure [30]. The characteristics to be examined are specificity, linearity and range, accuracy as percentage recovery, precision at the levels of repeatability and intermediate precision, the limits of detection and quantitation, and robustness. Because silymarin is a mixture of flavonolignans and silybin is a pair of diastereomers, the protocol should state which constituent or constituents the method quantifies, whether silybin A and B are resolved, and whether the result is expressed as silybin or as total silymarin, since this decides the meaning of every drug-content and dissolution figure that follows [13,30].

8.2 Physicochemical characterisation

Confirmation that a complex has formed, and not merely a mixture, rests on several complementary techniques. Infrared spectroscopy examines shifts in the phenolic O-H and phosphate bands. Differential scanning calorimetry looks for the disappearance or marked shift of the sharp melting endotherm of the drug in the complex compared with the physical mixture, which would indicate molecular-level interaction. Proton nuclear magnetic resonance spectroscopy probes the hydrogen-bonding interaction between the phenolic protons of silymarin and the phospholipid protons, and powder X-ray diffraction assesses the loss of crystallinity, since the absence or reduction of the characteristic drug peaks suggests amorphisation [17,20].

No single one of these is conclusive. A drug that is dispersed or dissolved in molten phospholipid can lose its melting endotherm without forming a hydrogen-bonded complex, and a loss of diffraction peaks shows amorphisation but not the interaction responsible for it, so that the strongest evidence comes from agreement among the techniques and from the comparison with the physical mixture [6,20]. Particle

size, polydispersity index and zeta potential of the dispersion in water are determined by dynamic and electrophoretic light scattering, and the morphology by scanning or transmission electron microscopy, and the size and polydispersity together influence the stability and the biological behaviour of lipidic carriers [27].

Complexation efficiency is the central quality attribute of the optimisation, and the way in which it is measured affects every result. A common approach is to separate uncomplexed drug from the complex by washing with a solvent in which the drug dissolves poorly and the complex is precipitated or retained, and to determine the drug in the complex and in the washings by the validated method, expressing efficiency as the proportion of the added drug that is recovered in the complex. The washing conditions should be fixed, because a drug that is only weakly bound may be removed by a vigorous wash and a drug that is simply adsorbed may be retained by a gentle one, and the method should be checked against the physical mixture, which should give a low value if the procedure discriminates complex from mixture [18,21].

8.3 Solubility and dissolution

The aqueous and n-octanol solubility of the complex are compared with those of plain silymarin and the physical mixture, which gives a measure of the improved amphiphilicity, and in vitro dissolution is studied in simulated gastric and intestinal fluids with a pharmacopoeial paddle apparatus, with samples analysed by the validated method. The release data are fitted to zero-order, first-order, Higuchi and Korsmeyer-Peppas models, a set of descriptive models whose use for comparing dissolution profiles has been reviewed [31,32,33]. The Higuchi model was derived for release from matrices and the Korsmeyer-Peppas exponent was developed for polymeric systems, so for a self-dispersing lipid complex the fitted parameters are descriptive tools for comparing profiles and not proof of a particular release mechanism [33].

A feature of lipid complexes makes the dissolution measurement itself delicate. A dispersion of a phospholipid complex may contain colloidal particles small enough to pass through a filter, so the separation of dissolved from particulate drug needs to be validated, otherwise the amount in solution may be overestimated. The sink conditions chosen should be stated, because a medium in which the plain drug is nearly insoluble will exaggerate the apparent advantage of any solubilising formulation compared with a medium of more physiological composition [2,4].

8.4 Solid dosage form

The optimised complex is blended with microcrystalline cellulose, colloidal silicon dioxide and magnesium stearate, and the powder blend is evaluated for bulk and tapped density, Carr's index, Hausner ratio and angle of repose before it is filled into hard gelatin capsules. The filled capsules are evaluated for weight variation, disintegration time and in vitro dissolution. This step is more than a convenience. A complex that shows excellent solubility as a laboratory powder has no clinical meaning unless it can be filled reproducibly and unless its dissolution survives the excipients, and the dissolution profile of the capsule, not that of the neat complex, is the one that corresponds to the dosage form to be tested in animals [18,20].

8.5 Stability

Short-term stability of the optimised complex and capsules is assessed under the ICH accelerated condition of 40 ± 2 °C and 75 ± 5% relative humidity and the intermediate or long-term condition of 25 ± 2 °C and 60 ± 5% relative humidity, with samples withdrawn at fixed intervals such as 0, 30 and 60 days and assessed for appearance, drug content, dissolution and integrity of the complex by infrared spectroscopy [34]. Phospholipids are susceptible to hydrolysis and oxidation, and a phenolic drug is susceptible to oxidation, so that the stability programme should include a measure of lipid degradation and drug content in addition to the physical attributes, and the packaging should protect against moisture and, where relevant, light [20,34]. The short observation period supports only a preliminary view of stability that long-term studies would have to confirm.

IX. A FRAMEWORK FOR RELATING FORMULATION IMPROVEMENT TO IN VIVO PERFORMANCE

Published phytosome studies usually report an improvement in solubility or dissolution in vitro and, separately, an improvement in exposure or efficacy in

vivo, and attribute the second to the first. A design that allows the attribution to be tested is of particular value, and the framework in Figure 2 treats the quality-by-design pathway as the route to an optimised complex, and then proposes that evidence of complex integrity, solubility and release from the physical and spectroscopic characterisation be combined into a single biopharmaceutical descriptor that is compared with the in vivo outcome across the formulations tested [8,17].

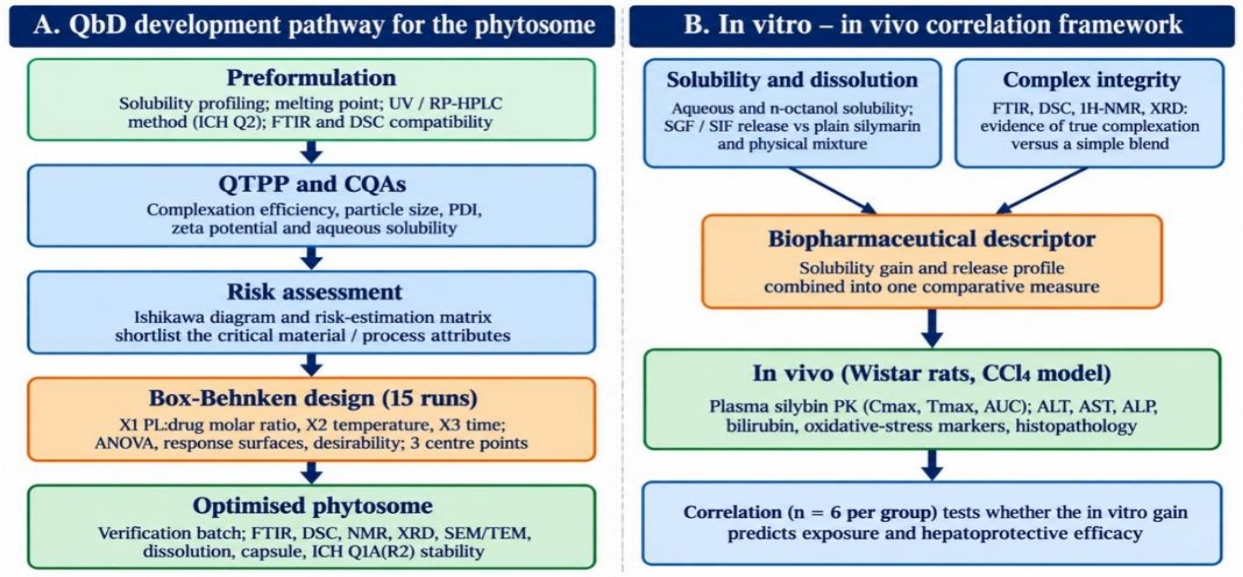


Figure 2. (A) Proposed Quality by Design pathway from preformulation, Quality Target Product Profile and risk assessment through Box-Behnken optimisation to the optimised phytosome. (B) Proposed framework correlating solubility, dissolution and complex integrity with in vivo pharmacokinetics and hepatoprotection in rats (n = 6 per group) [11,26].

The descriptor is a proposal of this review and is not an established measure. In its simplest form it ranks the plain drug, the physical mixture and the optimised complex, and any intermediate batches from the design, by solubility gain and by release at defined times, and examines whether the ranking corresponds to the ranking of plasma exposure and of the biochemical markers of protection. If it does, the relationship supports the mechanism and offers a screening tool, and if it does not, the result is equally informative, because it would show that the in vitro tests do not capture the limiting step in the animal, whether this is conjugation, biliary elimination or the behaviour of the complex in the gut [2,4].

9.1 The carbon tetrachloride model

The in vivo evaluation uses the carbon tetrachloride model of hepatotoxicity in rats, in which the toxin is bioactivated in the liver by cytochrome P450 enzymes, mainly CYP2E1, to the trichloromethyl radical, which initiates lipid peroxidation, membrane damage, steatosis and necrosis [35]. The mechanism is well matched to the antioxidant and membrane-stabilising actions attributed to silymarin, which is why the model has been widely used in this field and was used in the silymarin phytosome precedent [8,35]. A recent re-examination of the acute model identified an intraperitoneal dose of 2.0 mL/kg with evaluation 12 hours after exposure as an optimal protocol for the reliable and reproducible assessment of hepatoprotective compounds without inducing irreversible injury, and this informs the choice of protocol [36].

The model also has limits that should be stated. It is an acute chemical injury and it does not reproduce the chronic metabolic, viral or alcohol-related conditions for which silymarin is used clinically, and a protective effect in it supports the antioxidant and membrane-stabilising mechanisms but does not demonstrate efficacy in chronic human disease [15,35]. The results should be described accordingly, as evidence of hepatoprotection in a defined acute model.

9.2 Study design and endpoints

Healthy adult Wistar rats are used, following approval of the Institutional Animal Ethics Committee and in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals. The groups proposed are a normal control, a toxin control, a standard reference, plain silymarin, a physical mixture where feasible, and the optimised phytosome, at equal doses expressed as silymarin or silybin, with six animals per group. The endpoints are the serum markers of hepatic injury, namely alanine and aspartate aminotransferases, alkaline phosphatase and bilirubin, together with the tissue markers of oxidative stress and the histopathology of the liver, and the pharmacokinetic profile of silybin after oral dosing, with the peak concentration, the time to peak and the area under the concentration-time curve [8,36]. Results are expressed as mean and standard error of the mean and compared by one-way analysis of variance followed by Tukey's or Dunnett's post-hoc test, with a probability below 0.05 taken to indicate statistical significance. Two measurement issues deserve attention. Silybin is largely conjugated in plasma, so the protocol should state whether free silybin, total silybin after enzymatic hydrolysis of the conjugates, or both are measured, because the answer influences the interpretation of relative bioavailability. The pharmacokinetic and the efficacy arms should also be considered together, since a formulation can change the exposure to the conjugates without a proportionate change in the free compound that is generally regarded as the active species [4,8].

X. CHALLENGES AND FUTURE PERSPECTIVES

Several challenges can be identified in advance. The first is the heterogeneity of the starting material. Silymarin is a mixture of flavonolignans, the proportions vary between extracts, and the diastereomers of silybin may not interact with phospholipid identically, so that the phytosome made from one lot may not match that made from another unless the silymarin is standardised and the constituents are quantified [3,13]. The second is the residual solvent. Dichloromethane, tetrahydrofuran and dioxane are objectionable solvents on safety grounds, and an acceptable product requires either the use of a less hazardous solvent such as acetone or ethanol where complexation allows, or a validated demonstration that residual solvent is below the permitted limits [18,20].

The third is the question of what the complex does in the gut. A phospholipid complex disperses in the gastrointestinal fluids and may be affected by bile salts, by lipase and by dilution, and the improvement observed in simple media may not be reproduced under digestion. Biorelevant media and, where feasible, a lipolysis model would give a more realistic view than a simple buffer [17,19]. The fourth is the extent to which complexation alters the metabolic fate of silybin. The complex has been shown to improve exposure, but the contribution of improved absorption, altered distribution and reduced conjugation has not been separated, and a study that measures both free and conjugated silybin would help to resolve this [2,4]. The fifth is translation. Silymarin is used in chronic disease, whereas the model proposed is acute, the rat differs from the human in conjugation and biliary excretion, and a rat study should be seen as a proof of concept [15,35]. Looking forward, the approach is a platform and not a single product. A validated workflow that combines a risk-based design with a Box-Behnken response surface, a physical-mixture control and a correlation of in vitro and in vivo behaviour would be reusable for other flavonoids and polyphenols with similar limitations, and the conversion of the complex into a stable, well-flowing solid dosage form, the assessment of long-term stability and the extension to a chronic disease model are logical next steps [17,19].

XI. CONCLUSION

Silymarin has a well-documented hepatoprotective pharmacology and a biopharmaceutical profile that limits its oral delivery, namely poor aqueous solubility, extensive first-pass conjugation and a very low oral bioavailability, which together explain the high and frequent dose of conventional products. Phospholipid complexation addresses the underlying amphiphilicity mismatch, has been shown to improve the exposure and the hepatoprotective activity of silymarin and silybin in animal studies and to improve the pharmacokinetics of a silybin complex in humans, and has produced marketed products [5,8,24,25].

A study that develops a silymarin phytosome within a Quality by Design framework, using a risk assessment

and a three-factor Box-Behnken design with the phospholipid-to-drug ratio, temperature and time as variables and five quality attributes as responses, and that characterises and tests it against plain silymarin and a physical mixture in vitro and in a carbon tetrachloride model, is scientifically coherent and experimentally tractable [9,23,28]. The challenges that remain, including the heterogeneity of silymarin, residual solvent, behaviour under digestion and the translation from an acute rat model to chronic human disease, are identifiable and tractable, and addressing them would yield a rationally designed, quality-assured oral phytosome of a widely used hepatoprotective.

Conflict of interest. The authors declare that they have no conflict of interest.

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