DEVELOPMENT OF LIPOSOMAL DRUG FORMULATIONS: QUALITY ATTRIBUTES AND METHODS FOR QUALITY CONTROL

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The use of nanostructured components in drug manufacturing and, more specifically, targeted drug delivery has recently become a major pharmacy trend. Nanodrugs encompass a wide range of pharmaceutical agents containing dendrimers, nanocrystals, micelles, liposomes, and polymer nanoparticles. Liposomes are the most well-studied nanoparticles and effective drug carriers. However, the more complex their structure is, the more process controls are needed and the more quality attributes have to be monitored, including the chemical properties of the liposomal fraction such as the shape, size and charge of the nanoparticle, conjugation efficacy, and distribution of the active ingredient. We believe that quality control of key liposome characteristics can be carried out using dynamic and laser light scattering coupled with electrophoresis, differential scanning calorimetry, cryo-electron microscopy, nuclear magnetic resonance, laser diffraction analysis, and gel filtration chromatography.

Keywords: liposomes, nanodrugs, quality control, guidance documents

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РАЗРАБОТКА ЛИПОСОМАЛЬНЫХ ФОРМ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ: МЕТОДЫ ОЦЕНКИ И ПОКАЗАТЕЛИ КАЧЕСТВА

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Одним из трендов фармации на сегодняшний день является применение наноструктурных компонентов для производства лекарств, в частности для направленной доставки лекарственных средств в заданную область организма, органа или клетки. К нанопрепаратам авторы относят средства, содержащие дендримеры, нанокристаллы, мицеллы, липосомы, а также полимерные наночастицы. В настоящее время липосомы — одни из наиболее исследованных наночастиц, которые рассматривают как современные и эффективные средства доставки различных препаратов. Однако увеличение сложности структуры препарата неизбежно приводит к увеличению числа критических точек производства, а также к расширению списка показателей качества. Наряду с классическими показателями качества авторы считают необходимым оценивать также физико-химические свойства липосомной фракции: форму, размер и заряд частиц; эффективность конъюгации маркеров; равномерность распределения действующего вещества. Мы полагаем, что для контроля ключевых параметров липосом целесообразно использовать динамическое и лазерное светорассеяние в сочетании с электрофорезом, дифференциальную сканирующую калориметрию, криорасщепляющую электронную микроскопию, ядерный магнитный резонанс, лазерную дифракцию и гель-фильтрацию.

Ключевые слова: липосомы, нанопрепараты, контроль качества, нормативные документы

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Some of the major current challenges before the pharmaceutical industry are regulation of all pharmacokinetic parameters of a drug (absorption, distribution, clearance and biotransformation), ensuring its safety and selective action on target organs and other targets, minimization of undesirable reactions and side effects. Using nanostructured components in general and to deliver drugs to a given body part, organ or cell in particular is one of the trends that sees development today. Russian legislation does not describe the concepts of “nanopreparations” or “nanodrugs”; in reality, all drugs that are nanoparticles or contain them are considered to be such. This definition mainly applies to the drugs based on liposomes and micelles, where nanostructures enable transportation of the active pharmaceutical ingredient inside the body, prolong its absorption, increase stability, etc. Another case are drugs considered to be nanostructured due to the physicochemical characteristics of their active ingredients, an example of which are antianemic iron preparations that may contain iron (III) atoms stabilized by a carbohydrate complex, which defines their nanocolloidal structure. Currently, we are developing quality assessment and research guidelines applicable to drugs based on liposomes and micelles, as well as containing nanoparticles.

Features of the nanodrugs' compositions define the individual approaches to assessing their quality. For example, quality of liposomal preparations largely depends on their individual specific attributes (size of nanoparticles, surface morphology, surface charge), which can affect the following pharmacokinetic and pharmacodynamic properties *in vivo*:

• rate of release of the active ingredient from liposomes, a factor that has an effect on pharmacokinetics (PK) and pharmacodynamics (PD) and, consequently, drug's safety profile and efficacy;

• bioavailability of the active pharmaceutical ingredient in liposome, its biotransformation and clearance.

PK of the encapsulated active ingredient depends on that of the carrier, which is determined by the physicochemical properties of the nanoparticle material; interactions between the nanoparticle's components, active ingredient and biological environment (body) should also be taken into account.

We define nanodrugs as drugs that contain dendrimers, nanocrystals, micelles, liposomes and polymeric nanoparticles. Currently, liposome is one of the best-studied nanoparticles among those considered as effective carriers for various drugs. In the recent years, global pharmaceutical industry has developed and released over 20 liposomal drugs primarily used to treat cancer (Dauno Xome (Gilead, NeXstar), Doxil (Alza, Sequus), Couloux (Schering-Plow), Muocet (Elan, TLS)) and fungal infections (AmBisome, ABELSET (Gilead, NeXstar)) [1]. Specific capabilities related to transportation, translocation through histohematogenous barriers and cell membranes, as well as metabolic transformations, provide liposome-based drugs with unique properties that improve their PK.

This article summarizes and analyzes the data describing the use of various types of liposomes for drug delivery and defines the specifics of the liposome-based nanodrugs quality assessment.

Varieties of liposomes and their use by pharmaceutical industry

Liposomes are vesicles with a lipid bilayer built of amphiphilic molecules enclosing their contents. Recently, liposomes have evolved from a simple model that mimics cell membranes into an object of active research and practical application [2]. In the context of drug delivery, liposomes enable selective accumulation of the active ingredient in pathological lesions (tumors, inflamed tissues) due to their passive targeting ability. This ability is the results of the difference in distance between capillary cells in lesions/tumors and normal tissue: the former, which is 210 to 1000 nm, is significantly greater than the latter, which is approximately 40 nm. Thus, liposomes less than 200 nm in size cannot escape the bloodstream anywhere except the lesions (with the exception of the brain, where tumors typically have pores of 7–100 nm [3, 4]), and the active pharmaceutical ingredient, which can be toxic, is unlikely to contaminate anything but the target. For example, liposomal doxorubicin is 2–3 times less toxic than the solution of this drug [5].

Using target (endothelial) protein antibodies, which are specific to vessels of various organs, allows manifold improvement of precision of the nanoparticle-enabled delivery of active pharmaceutical ingredients and DNA [6–9].

To date, various researchers have described liposome-based preparations carrying a plethora of active ingredients, X-ray and scintigraphic tracers, toxins, peptides, proteins and nucleic acids. The overwhelming majority of studies in this field has to do with anticancer drugs (most often, anthracycline-based) [8]. There are five types of liposomes, different in composition and action *in vivo*, that the researchers preferred, namely: simple liposomes; sterically stabilized liposomes; directed liposomes (immunoliposomes); cationic liposomes; liposomes sensitive to physical and chemical stimuli, such as temperature, light, and changes in pH [2, 10] (Table 1).

When progress in biotechnology and genetic engineering allowed developing a new generation of drugs, such as recombinant proteins, peptides (biotechnological drugs), drugs based on nucleic acids (gene therapy drugs), liposomes acquired a special significance due to the susceptibility of these medicines to chemical and enzymatic hydrolysis [8, 39–41]. In gene therapy, liposome nanocontainers may carry a plasmid with a therapeutic gene sequence, antisense oligonucleotides or small interfering RNAs [42-44]. The volume of the liposomes allows them to contain genes of various sizes [45]. Vector molecules attached to the outer surface of the liposomes target delivery, a mechanism similar to that used for cytotoxic drugs and paramagnetic contrast agents.

When liposomes are used as DNA vaccines, they hold the antigen in their capsule and double as an immunomodulator [46, 47]. In one of the studies, S-antigen sequence of HBV (pRc / CMV HBS) enclosed in cationic liposomes was used as a DNA vaccine [47]. Balb/c mice received a vaccine of 10 μg of plasmid DNA (i.m., per mouse) twice on days 0 and 21. After administration of the native HBsAg, the levels of detectable cytokines in spleens of mice immunized with the liposome-based preparation were 4 times higher than those registered in intact mice and animals vaccinated with DNA, which suggests a possibility of using this liposomal construct as a Hepatitis B vaccine.

Both cationic or anionic liposomes and those with a neutral surface charge can be loaded with DNA. Neutral liposomes circulate in the bloodstream for a much longer period of time than the charged ones; moreover, their advantages are lesser toxicity and non-specific persorption in organs and tissues. However, it is much harder to load them with DNA. In case of passive loading, which is a plain emulsification of lipid components in the presence of DNA, only 10% of the total amount of DNA gets into the liposomes. There are special techniques that allow increasing the number to 40%, but, as a rule, they also increase the size of the liposomes [45]. Charged liposomes can be loaded with more DNA, which is their key advantage. However, cationic and anionic liposomes have higher levels toxicity and non-specific penetration into organs and tissues than neutral liposomes.

Specifics of the liposome-based drugs quality and production control

The main stages of production of liposomal drug formulations and the controlled parameters thereof are listed below [48].

1) Lipid film production and its dispersion/degradation. Controlled parameters: amount of residual organic solvents in the lipid film; active pharmaceutical ingredient integration rate and size of the liposomes after lipid film dispersion; stability; pH value.

2) Production of liposomes of the required size, separating the non-integrated active ingredient, sterilization by filtration. Controlled parameters: amount of the integrated active pharmaceutical ingredient; size of the liposomes; concentration of the lipid components; stability; pH value.

3) Lyophilization. Controlled parameters: residual moisture; stability and percentage of drug integration into the liposomes after lyophilisate rehydration.

The above-listed stages of the technological process allow a conclusion that the critical liposome-based drug quality checks imply determination of its crucial physicochemical properties; therefore, state registration applications for such formulations should provide the following information (Fig. 1).

Table 1. Use of different types of liposomes for drug delivery

Types of liposomes	Simple	Sterically stabilized	Immunoliposomes	Cationic (lipoplexes)	Thermosensitive and photosensitive
Composition specifics	Phospholipids (neutral and/or negatively charged) and/or cholesterol	Phospholipids + polyethylene glycol (PEG)	Modified PEG-vesicles conjugated with monoclonal antibodies or their fragments, peptides, growth factors, glycoproteins, etc.	Positively charged lipids	Phospholipids the phase transition temperature of which exceeds body temperature (thermosensitive). 1,2-Bis(4-(n-butyl)phenylazo-4'-phenylbutyroyl)phosphatidylcholine (Bis-Azo PC) in low concentrations is part of the vesicles of the photoisomerized lipid molecule. May be conjugated with PEG or antibodies (AB)
Route of administration	Oral, injection, inhalation, local, endovitreal	Injection, oral	Injection	Injection, intranasal	Injection
Half-life	Several minutes to 2-3 hours	6–8 hours to several days		Several minutes to 4-6 hours	Several days
Key accumulation sites	Liver, spleen, lungs		Determined by the attached ligands, liver, lungs	Liver, lungs	Tumor cells
Mode of action	Passive targeting	Passive targeting	Directed transport	Passive targeting	Directed transport
Examples of use	– part of the virus, antibacterial, parasitic infection vaccines [11]; – delivery of immunomodulators, cytotoxic and antimicrobial compounds to macrophages; – treatment of metastases after surgical removal of primary tumors [12, 13]; – delivery of drugs against intracellular pathogens [14], systemic fungal infection, HIV, mycobacterial infection [13]; – carrying radioisotopes and contrast agents for visualization purposes [12, 13]; – carrying antigens [12, 15]	– accumulation of drugs in solid tumors [16–18]; – treatment of small cell lung cancer and cutaneous melanoma [19], leukemia and lung carcinoma [20, 21]	– delivery of drug to the tumor [10, 22–26]; – treatment of chronic B-lymphocytic leukemia and acute T-cell leukemia [23], various lymphomas [27]; – treatment of breast, thyroid gland, ovarian cancer, that of uterus, lung, esophagus, stomach, colon and rectum, kidney [23, 26, 28]	– delivery of the genetic material to the liver, cell therapy of endothelial pulmonary tumors [2, 29, 30]; – antiangiogenic therapy; – treatment of tumors of neck and head, melanomas [30]	– delivery of drug to the tumor [2, 31]
Key advantages	Penetrate into the relatively inaccessible lesions (e.g., in the brain) due to their negative charge [32, 33]	Contain PEG, which prevents liposome opsonization, hinders their recognition by the reticuloendothelial system cells and increases the time of their persistence in the bloodstream [34, 35]	Antibodies allow modulating distribution of the liposomes in organs and tissues. Optimization of the drug's therapeutic properties. Correction of the effective dose	Penetrate into the tumor's vessels (as opposed to neutral or negatively charged liposomes) [36]	Offer greater selectivity of action compared to the free drug [31, 37, 38]

The behavior of the active pharmaceutical ingredient in a physiological environment is one of the main parameters influencing the liposome-based drug's PK and PD. Therefore, for the purposes listed below it is necessary to develop reliable, validated methods of assessment of the active ingredient release *in vitro*.

– Monitoring of imitation of the active ingredient release from liposomes in the body; a test for "leakage" *in vitro* in the relevant environment under various conditions (e.g., in a certain range of temperatures and pH) can be conducted given there are grounds for that.

– Monitoring of stability in storage to ensure consistency of lots;

– Investigation of stability and review of the production process in the intended conditions of use.

Table 2 provides an example of the certificate data (key parameters and quality indicators) describing liposomes [49, 50] used for delivery of the therapeutic genes' DNA.

We believe that, depending on the specific function of the liposomes (e.g., modification of the active ingredient's distribution by encapsulation in order to improve the safety profile), the following additional parameters should also be evaluated in the development of the drug:

– maintaining the integrity of the liposomal formulation in plasma;

– characteristics of the lipid bilayer phase transition process (transition temperature and enthalpy);

– determination of the surface charge of the liposomes;

– pH of the inner chamber of the liposomes filled by the pH gradient;

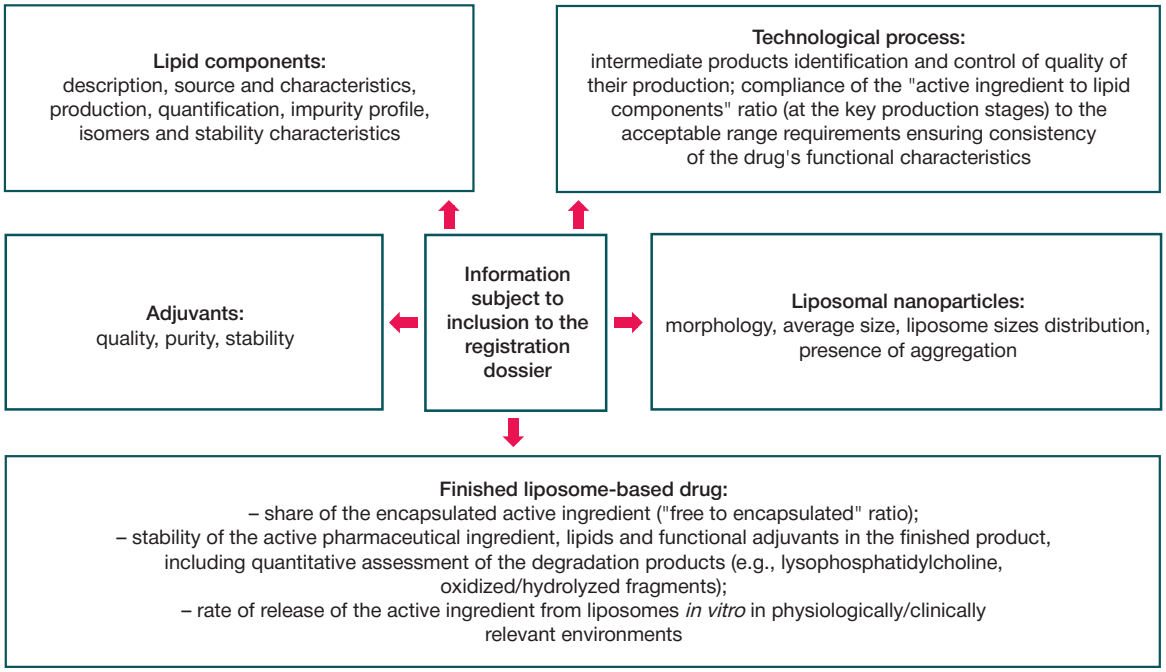

Fig. 1. Information about the quality characteristics of liposomal drug formulations

– if significant, determination of characteristics of the active pharmaceutical ingredient's physical state inside the liposome (e.g., formation of a precipitate for doxorubicin);

– distribution of the active ingredient (e.g., on the surface of liposomes, in the bilayer, internal environment, etc.);

– for conjugated (eg, pegylated) liposome-based preparations: the quality and purity of the pegylated starting material, molecular weight of the conjugated lipid and size distribution (dispersion), location of PEG on the surface, stability of the conjugate.

It is necessary to compile a list of tests each lot should routinely be subjected to. This list should be based on the parameters used to characterize the drug in accordance with the requirements described above.

Legal regulation of liposome-based drugs in the world

Table 3 provides the examples of requirements regulator bodies from various countries of the world impose on the production, quality control, preclinical and clinical studies of liposome-based forms of drugs.

CONCLUSIONS

Liposome-based drug delivery systems give a drug designer control over the active ingredient's absorption and release parameters. As a rule, liposome-based drugs are less toxic, pose a lower risk of adverse reactions and allow delivering

Table 2. Liposome-based drugs characteristics

Parameters		Analytical/instrumental methods
Physical characteristics		
1	Vesicle size and surface morphology	Electron microscopy
2	Distribution of the vesicles sizes (submicron and micron ranges)	Dynamic and laser light scattering, exclusion chromatography (gel filtration)
3	Surface charge	Dynamic light scattering
4	Surface pH	pH sensitive samples
5	Integrated DNA/free preparation percentage	Methanol-chloroform extraction and centrifugation in separation columns, ion exchange chromatography, spectrophotometry, radioactive labeling
Chemical characteristics		
1	Phospholipid concentration	Extraction and centrifugation in separation columns
2	Cholesterol concentration	Extraction and centrifugation in separation columns
3	Osmolality	Osmometry
Biological characteristics		
1	Sterility	Pharmacopoeial sterility test
2	Pyrogenicity	LAL test (Limulus amebocyte lysate test)
3	Toxicity	*In vitro* and *in vivo* monitoring, histology

Table 3. Regulatory documents containing requirements to liposomal drug

State	Document	Selected aspects
EU countries	Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product/21 February 2013 EMA/CHMP/806058/2009/Rev. 02, Committee for Human Medicinal Products (CHMP)	Quality control specifics: – composition and authenticity of the components (lipids, adjuvants); – active pharmaceutical ingredient to lipids ratio; – liposomes morphology, average size and size distribution, aggregation; – fraction of the encapsulated active ingredient (free/integrated amount); – stability of the active ingredient, lipids, adjuvants, critical decomposition products; – *in vitro* rate of release of the ingredient from liposomes in physiologically/clinically significant environments; – stability; – recovery; – maintaining integrity of the liposomal formulation in plasma
	Recommendations. Commission recommendation of 18 October 2011 on the definition of nanomaterial (Text with EEA relevance) (2011/696/EU)	Definition of nanomaterials
	Reflection paper on surface coatings: general issues for consideration regarding parenteral administration of coated nanomedicine products/22 May 2013, EMA/325027/2013, Committee for Medicinal Products for Human Use (CHMP)	Key critical quality indicators, as well as the requirements for clinical and preclinical studies, are included. Special attention is paid to the following aspects: – presence of a coating can affect the critical properties of the nanodrugs from the points of view of their safety and efficacy. The physico-chemical nature of the coating, uniformity of its surface coating and stability (both in terms of attachment and in terms of degradation) will determine the drug's PK and biodistribution; – in some cases, the coating material may cause new biological reactions that are not observed either for the coating material or for the active pharmaceutical ingredients separately
USA	Guidance for Industry. Liposome Drug Products Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation. — U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research, 2002	Brief description of the liposomes, critical stages of their production and quality control, recommendations for conducting research on PK and bioavailability of liposome-based drugs and labeling requirements. The guideline contains general principles and recommendations for registration of the drugs of this class.
	USP41-NF36 <1> Injections and implanted drug products (parenterals)-product quality tests	Contains definition of liposomes and liposome-based drugs and states that in the case of liposomes, quality control implies both general and special tests.
China	Pharmacopoeia of the Peoples Republic of China. Beijing: People's Medical Publishing Hous. 2010; (2). p. A244–245	Definitions of various nanoparticles, requirements, nanodrugs quality control criteria and methods are provided. The attributes that should be monitored in production and storage of the drugs (e.g., residual amounts of organic solvents, shape, particle size and distribution, encapsulation rate and amount of drugs in liposomes, liposome oxidation degree, etc.) are listed.

the active ingredient to the target part of the body. Innovative drugs containing liposomes conjugated with antibodies can be targeted with maximum effectiveness and release the active ingredient where needed. However, the more complex the drug's structure becomes, the more crucial stages its production acquires. Moreover, the list of parameters to control, those that determine the quality of the drug, grows. Evaluation of the liposomal fraction's physicochemical properties is added to the classic quality control methods: the shape, size, and charge of the particles are being assessed, as well as marker conjugation effectiveness and uniformity of distribution of the active ingredient. Key methods for estimating the liposome parameters make use of the optical effects: dynamic and laser light scattering, electron microscopy.

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