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CHAPTER 1

HEPATIC SENESCENCE AS AN IMMUNOMETABOLIC DISORDER: EMERGING ROLES OF cGAS-STING AND NLRP3

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1. Introduction

The liver is an immunologically active organ enriched in innate immune populations, including Kupffer cells, dendritic cells, natural killer cells, natural killer T cells, and infiltrating macrophages. These cells continuously survey pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), allowing the liver to respond rapidly to infection and tissue injury while maintaining metabolic and immune homeostasis. Aging, obesity, alcohol exposure, viral infection, metabolic dysfunction, and environmental stress can disturb this balance and promote persistent sterile inflammatory signaling (Li et al., 2024; Yan et al., 2026).

Cellular senescence is a stable cell-cycle arrest in which cells remain metabolically active but undergo profound transcriptional, metabolic, and secretory remodeling. It can be induced by telomere shortening, DNA damage, oxidative stress, mitochondrial dysfunction, oncogenic signaling, metabolic stress, and chronic inflammation. Senescent cells commonly develop a senescence-associated secretory phenotype (SASP), characterized by context-dependent secretion of cytokines, chemokines, growth factors, proteases, and other bioactive mediators. In the liver, persistent accumulation of senescent cells is increasingly implicated in aging and chronic liver diseases, although its consequences vary substantially among hepatic cell populations (Li et al., 2024; Wu et al., 2024; Xing & Zhu, 2025; Yan et al., 2026).

A key mechanistic feature of senescence is genomic instability accompanied by the appearance of nuclear and mitochondrial DNA fragments in the cytosol. Cytosolic DNA is sensed by cyclic GMP-AMP synthase (cGAS), which activates stimulator of interferon genes (STING) signaling and downstream inflammatory programs. cGAS-STING can reinforce senescence through TBK1-IRF3 and NF- κ B signaling and can interact with inflammasome pathways, particularly NLRP3. This chapter therefore examines hepatic senescence as an immunometabolic state centered on cGAS-STING/NLRP3 crosstalk, while emphasizing the cell-specific and potentially bidirectional consequences of senescence in chronic liver injury (Gong et al., 2024; Liu et al., 2024; Wang et al., 2024).

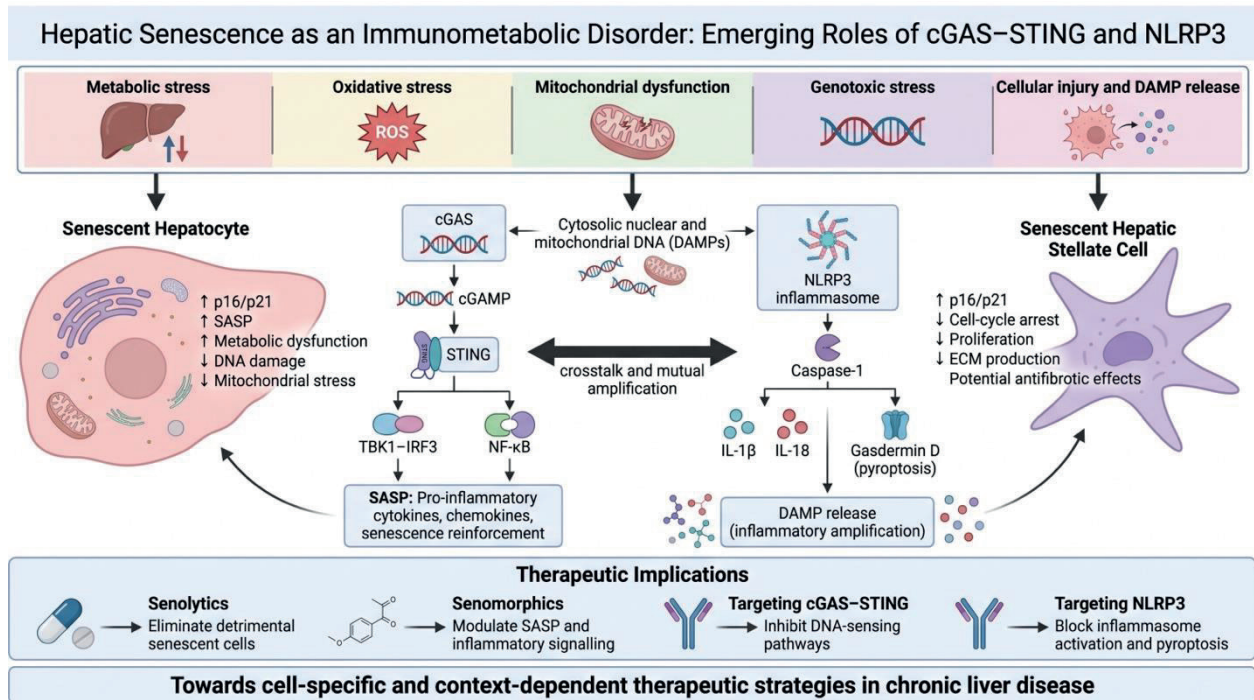


Figure 1. Proposed cGAS–STING/NLRP3 signaling network linking hepatic senescence, chronic inflammation, and cell-specific outcomes. Metabolic, oxidative, mitochondrial, and genotoxic stress, together with cellular injury, promote DNA damage, mitochondrial dysfunction, and the release of damage-associated molecular patterns (DAMPs). Cytosolic nuclear and mitochondrial DNA activate the cGAS–cGAMP–STING pathway, leading to TBK1–IRF3 and NF-κB signaling and reinforcement of the senescence-associated secretory phenotype (SASP). In parallel, mitochondrial dysfunction, reactive oxygen species (ROS), cytosolic DNA, and other stress-associated signals facilitate NLRP3 inflammasome activation, resulting in caspase-1 activation, IL-1β and IL-18 maturation, gasdermin D-mediated pyroptosis, and further DAMP release. Reciprocal crosstalk between cGAS–STING and NLRP3 may establish a feed-forward inflammatory circuit that sustains senescence-associated sterile inflammation. The biological consequences are cell- and context-dependent: persistent hepatocyte senescence may contribute to metabolic dysfunction and inflammatory signaling, whereas senescence of activated hepatic stellate cells may restrict proliferation and extracellular matrix (ECM) production, potentially exerting antifibrotic effects. Potential therapeutic approaches include senolytics, senomorphics, and selective modulation of cGAS–

STING or NLRP3 signalling, emphasising the need for cell-specific and context-dependent strategies in chronic liver disease.

Abbreviations: cGAS, cyclic GMP–AMP synthase; cGAMP, cyclic GMP–AMP; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; IRF3, interferon regulatory factor 3; NF- κ B, nuclear factor kappa B; NLRP3, NLR family pyrin domain containing 3; SASP, senescence-associated secretory phenotype; DAMPs, damage-associated molecular patterns; ROS, reactive oxygen species; ECM, extracellular matrix; GSDMD, gasdermin D.

2. Hepatic Senescence as an Immunometabolic State

Hepatic senescence arises at the intersection of cellular injury, metabolic dysfunction, and innate immune activation. Obesity, high-fat diet, alcohol misuse, oxidative stress, endoplasmic reticulum stress, impaired autophagy, mitochondrial dysfunction, and environmental toxicants can induce genomic instability and persistent damage responses. These stressors favor activation of canonical senescence programs, including p53/p21 and p16INK4a/RB, while simultaneously altering cellular metabolism and inflammatory signaling. Accordingly, hepatic senescence is better viewed as an immunometabolic phenotype than as an isolated proliferative defect (Xing & Zhu, 2025; Yan et al., 2026; Zhao et al., 2025).

Mitochondria are particularly important in this transition. Mitochondrial DNA (mtDNA) is highly vulnerable to oxidative injury, and mitochondrial dysfunction can promote mtDNA release into the cytosol. Nuclear DNA fragments can also accumulate following genomic damage. These nucleic acids function as DAMPs and connect cellular stress to cytosolic DNA sensing. In parallel, senescent hepatic cells acquire a SASP that may include IL-6, IL-1 α , IL-1 β , TNF- α , TGF- β , CCL2, CXCL chemokines, matrix metalloproteinases, and growth-regulatory factors. These mediators recruit and reprogram immune cells, thereby sustaining a chronic inflammatory microenvironment and linking metabolic dysfunction to innate immune activation (Li et al., 2025; Li et al., 2024; Liu et al., 2024).

Senescence is not biologically uniform across the liver. Hepatocytes, hepatic stellate cells (HSCs), Kupffer cells, liver sinusoidal endothelial cells, and cholangiocytes can all acquire senescent features. Persistent hepatocyte senescence is generally associated with impaired tissue function and progression of chronic liver disease. In contrast, senescence of activated HSCs may limit fibrosis by restricting proliferation and extracellular-matrix production. Senescent HSCs can display stable cell-cycle arrest, increased p16 and p21 expression, RB

activation, senescence-associated β -galactosidase activity, and a remodeled secretory phenotype. This cell-specific duality is essential when considering senescence-directed therapy: a pathway that is maladaptive in one hepatic cell population may be protective in another (Li et al., 2024; Wu et al., 2024).

3. cGAS–STING Signaling in the Senescent Liver

The cGAS-STING pathway is a major innate immune sensor of aberrantly localized DNA. During hepatic injury, oxidative stress, mitochondrial damage, endoplasmic reticulum stress, infection, and other forms of cellular damage can generate cytosolic nuclear DNA and mtDNA. Double-stranded DNA binding activates cGAS and promotes synthesis of cyclic GMP-AMP (cGAMP), which binds and activates STING. Activated STING recruits TANK-binding kinase 1 (TBK1), leading to phosphorylation of interferon regulatory factor 3 (IRF3), while also engaging NF- κ B-dependent inflammatory signaling. These transcriptional programs promote type I interferons and inflammatory mediators that can become incorporated into the SASP and reinforce a persistent sterile inflammatory state (Gong et al., 2024; Wang et al., 2024; Wang et al., 2025; Zhang et al., 2024).

cGAS-STING signaling is therefore positioned at the interface between DNA damage and cellular senescence. Genetic or pharmacological disruption of components of this pathway can attenuate DNA-damage-induced senescence in experimental settings, whereas restoration of downstream IRF3 signaling can re-establish senescence-associated responses. Importantly, IRF3 also intersects with the RB-E2F cell-cycle machinery. Activated IRF3 can stabilize hypophosphorylated RB and enhance RB-E2F interactions, thereby supporting durable cell-cycle arrest. This cGAS-STING-IRF3-RB connection provides a mechanistic route through which innate DNA sensing can directly reinforce the senescent phenotype (Wu et al., 2024; Zhang et al., 2024).

The consequences of cGAS-STING activation in the liver depend on context. Prolonged signalling can promote inflammatory cytokine production and contribute to chronic liver injury, including metabolic and alcohol-associated disease. Conversely, DNA-damage-induced cGAS-STING signalling in activated HSCs may promote a senescent state that restricts fibrogenic activity. Thus, the pathway cannot be classified simply as pathogenic or protective. Its net effect depends on the responding cell type, the duration and intensity of activation, and the stage of liver injury (Li et al., 2024; Wu et al., 2024; Xu et al., 2021).

4. NLRP3 Inflammasome and Its Crosstalk with cGAS–STING in Hepatic Senescence

The NLRP3 inflammasome is a key mediator of sterile inflammation and can be activated by many of the same stress signals that accompany hepatic senescence. Mitochondrial dysfunction, excessive reactive oxygen species, mtDNA release, lysosomal injury, ionic disturbances, and persistent inflammatory signalling all favour NLRP3 activation. Once assembled, the inflammasome activates caspase-1, which processes pro-IL-1 β , and pro-IL-18 into their mature forms and cleaves gasdermin D (GSDMD). GSDMD membrane pores enable inflammatory mediator release and can drive pyroptotic cell death (Chen et al., 2025; Tan et al., 2026).

cGAS-STING and NLRP3 should not be viewed as isolated pathways. cGAS-STING-dependent IRF3/NF- κ B signalling and SASP-associated inflammatory mediators can create a permissive environment for inflammasome activation, while mitochondrial injury releases mtDNA and ROS that can stimulate both systems. NLRP3-dependent caspase-1 activation and GSDMD-mediated membrane damage may, in turn, increase the availability of extracellular and cytosolic DAMPs. These DAMPs can further stimulate cGAS-STING signalling, creating a feed-forward circuit that links DNA sensing, inflammasome activation, inflammatory cell death, and persistent sterile inflammation (Liu et al., 2024; Xu et al., 2021).

Lysosomal stress offers another point of convergence. STING trafficking and stress-induced lysosomal dysfunction can compromise membrane integrity and ionic homeostasis, including potassium efflux, a recognised upstream signal for NLRP3 activation. Together with mitochondrial ROS and mtDNA release, these events can intensify inflammasome activity. The resulting inflammatory amplification is particularly relevant to chronic liver disease, in which repeated cellular injury can perpetuate cycles of DAMP release, innate immune activation, and tissue remodelling (Chen et al., 2025; Liu et al., 2024).

An integrated model can therefore be proposed: metabolic or genotoxic stress causes mitochondrial and nuclear DNA damage; cytosolic DNA activates the cGAS–cGAMP–STING axis; STING engages TBK1–IRF3 and NF- κ B; inflammatory mediators reinforce the SASP and facilitate NLRP3 activation; NLRP3 activates caspase-1, leading to IL-1 β and IL-18 maturation and GSDMD cleavage; and pyroptotic or sublethal membrane injury releases additional DAMPs that can re-engage cGAS–STING. This self-amplifying network provides a

mechanistic explanation for how transient stress sensing may evolve into persistent senescence-associated inflammation (Figure 2).

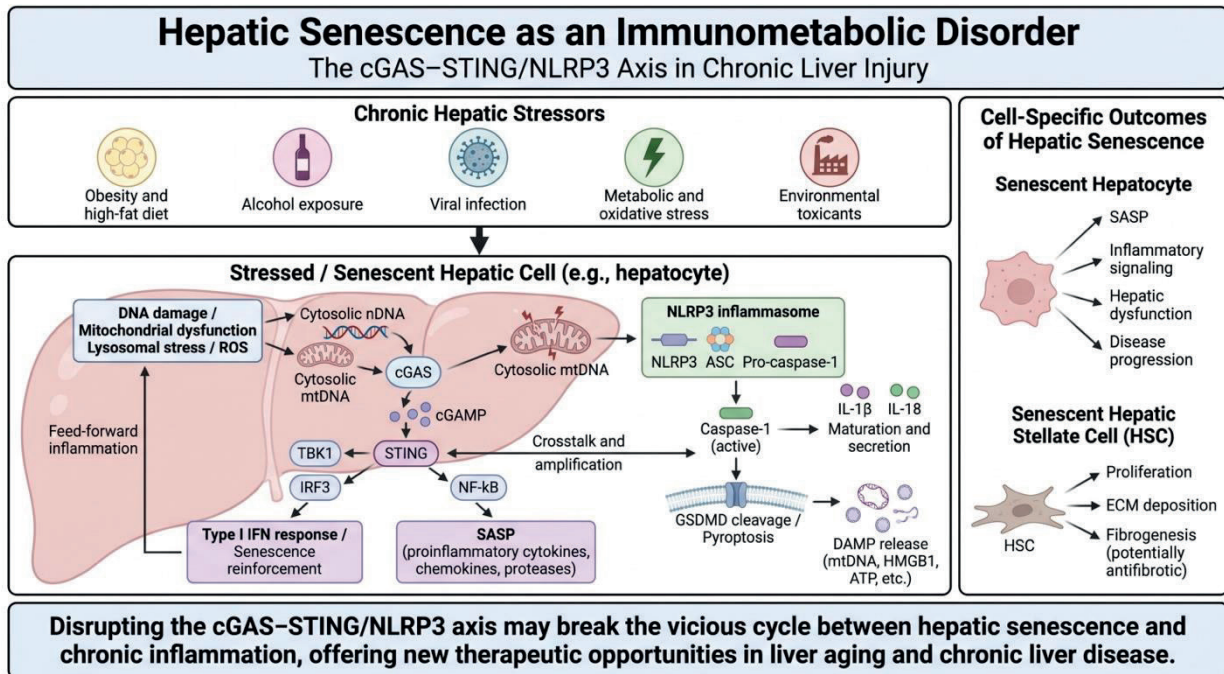


Figure 2. cGAS–STING/NLRP3 signaling axis in hepatic senescence. Hepatic stress promotes DNA damage and mitochondrial dysfunction, triggering cGAS–STING signaling, SASP formation, and NLRP3 inflammasome activation. Subsequent caspase-1 activation, IL-1β/IL-18 maturation, and GSDMD-mediated pyroptosis promote DAMP release and establish a feed-forward inflammatory loop.

5. Therapeutic Implications and Future Perspectives

The cGAS-STING/NLRP3 axis offers several potential intervention points, but its cell-specific biology argues against indiscriminate pathway suppression. In settings dominated by maladaptive hepatocyte senescence and chronic sterile inflammation, inhibition of cGAS-STING signalling could theoretically reduce DNA-sensing-driven inflammatory programs and SASP reinforcement. Likewise, modulation of NLRP3, caspase-1, IL-1 family signalling, or GSDMD-dependent inflammatory cell death may interrupt downstream amplification. However, cGAS-STING also contributes to host defence and can promote potentially antifibrotic senescence in activated HSCs; therefore, systemic and prolonged inhibition may

eliminate beneficial functions as well as pathogenic ones (Liu et al., 2024; Wang et al., 2024; Wu et al., 2024).

Senescence-directed strategies add another layer of complexity. Senolytics aim to selectively eliminate senescent cells, whereas senomorphic approaches seek to attenuate maladaptive SASP signaling without necessarily removing the cells. For liver disease, therapeutic success will likely require identification of the dominant senescent cell population and disease stage before intervention. A hepatocyte-focused strategy designed to reduce persistent inflammatory senescence may have different consequences from an HSC-directed intervention in which senescence can restrict fibrogenic activity. Future studies should therefore integrate cell-specific lineage tracing, spatial and single-cell profiling, and temporal assessment of cGAS-STING/NLRP3 activity (Gong et al., 2024; Hussin et al., 2026).

Several mechanistic questions remain unresolved. The relative contribution of nuclear DNA versus mtDNA to cGAS activation across liver diseases requires clarification, as does the extent to which NLRP3 activation is directly dependent on STING versus shared upstream stress signals. It will also be important to distinguish canonical inflammatory signaling from context-specific noncanonical effects and to determine whether pathway activity can serve as a biomarker of maladaptive hepatic senescence. These questions are central to translating the cGAS-STING/NLRP3 framework into precision therapies that suppress chronic inflammation while preserving immune surveillance, tissue repair, and antifibrotic responses. Table 1 summarises the principal molecular events linking hepatic senescence to cGAS–STING/NLRP3-dependent inflammatory amplification.

Table 1. Key Molecular Components of the cGAS–STING/NLRP3 Axis in Hepatic Senescence

Molecular component/process	Major upstream trigger	Role in hepatic senescence	Major downstream consequence
DNA damage and mitochondrial dysfunction	Aging, obesity, alcohol exposure, oxidative and metabolic stress	Promote accumulation/release of cytosolic nuclear DNA (nDNA) and mitochondrial DNA (mtDNA)	DAMP accumulation and activation of innate immune signaling
cGAS	Cytosolic nDNA and mtDNA	Functions as a cytosolic DNA sensor and catalyzes cGAMP production	Activation of STING
STING	cGAMP	Links aberrant cytosolic DNA sensing to inflammatory and senescence-associated signaling	Activation of TBK1–IRF3 and NF-κB pathways
TBK1–IRF3	STING activation	Reinforces senescence-associated signaling; IRF3 interacts with the RB–E2F cell-cycle machinery	Stable cell-cycle arrest and inflammatory transcriptional responses
NF-κB	STING-dependent signaling and cellular stress	Promotes inflammatory components of the SASP	Sustained sterile inflammatory signaling
SASP	Persistent senescence-associated signaling	Reshapes the hepatic inflammatory microenvironment through cytokines,	Immune-cell recruitment/reprogramming and amplification of inflammation

		chemokines and other mediators	
NLRP3 inflammasome	Mitochondrial dysfunction, ROS, mtDNA, lysosomal stress and persistent inflammatory signaling	Functions as an inflammatory effector of senescence-associated cellular stress	Caspase-1 activation
Caspase-1	NLRP3 inflammasome assembly	Processes pro-inflammatory cytokines and cleaves GSDMD	Maturation of IL-1 β /IL-18 and GSDMD activation
GSDMD-mediated pyroptosis	Caspase-1-dependent GSDMD cleavage	Promotes membrane pore formation and inflammatory cell death	Release of inflammatory mediators and DAMPs
DAMP release/feed-forward signaling	Pyroptotic or persistent cellular injury	Re-engages innate immune sensing and sustains the inflammatory microenvironment	Potential reactivation of cGAS–STING and perpetuation of sterile inflammation

6. Conclusion

Hepatic senescence is a complex, context-dependent immunometabolic state in which persistent cellular stress, metabolic dysfunction, mitochondrial damage, and genomic instability converge with innate immune signalling. Rather than functioning solely as an endpoint of irreversible cell-cycle arrest, senescence can actively reshape the hepatic microenvironment through the SASP and sustained inflammatory signalling. Within this framework, cGAS-STING provides a critical molecular link between cytosolic DNA accumulation and the establishment and maintenance of the senescent phenotype. The interaction between cGAS-STING and the NLRP3 inflammasome extends this response into a self-amplifying inflammatory network. Mitochondrial dysfunction, ROS generation, mtDNA release, lysosomal stress, and SASP-associated signalling can promote NLRP3 activation, leading to IL-1 β and IL-18 release and GSDMD-mediated pyroptosis. Subsequent DAMP

release may reinforce cGAS-STING signalling and sustain chronic sterile inflammation. However, the biological consequences remain strongly cell-specific: persistent hepatocyte senescence may promote tissue dysfunction, whereas senescence of activated HSCs may restrict extracellular matrix production and fibrosis. Collectively, these findings position the cGAS-STING/NLRP3 axis as a mechanistic interface linking DNA damage, mitochondrial stress, cellular senescence, and hepatic inflammation. Future work should define the temporal and cell-specific regulation of this axis rather than relying on global inhibition of senescence or innate immune signalling. Such precision will be essential for determining whether selective modulation can suppress maladaptive senescence-associated inflammation while preserving the potentially protective functions of cellular senescence.

Author Contributions

SK: Conceptualization, Literature Review, Supervision, Writing –Original Draft, Figure/Table Preparation, Review & Editing, Critical Revision, Final Approval. **HD:** Conceptualization, Writing –Original Draft, Final Approval.

Data Availability

No new data were generated or analysed for this review. This article is based exclusively on previously published literature.

Ethical Statement

This article is a literature-based review and does not involve any original studies with human participants or animals performed by the authors. Therefore, ethical approval and informed consent were not required.

Conflict of Interest: None declared.

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CHAPTER 2

LC-MS/MS ANALYSES OF FREE AND DOXORUBICIN LOADED- NANOPARTICLE: A REVIEW¹

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¹ Yapıcı, B. (2026). LC-MS/MS Analyses of Free and Doxorubicin Loaded-Nanoparticle. Istanbul Health and Technology University, Faculty of Pharmacy, Department of Analytical Chemistry, Graduation Project, Istanbul. Supervisor: Prof Dr Serap Sağlık Aslan

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1. INTRODUCTION

Cancer remains one of the primary health concerns worldwide, leading in morbidity and mortality rates. In chemotherapy, doxorubicin (DOX) serves as a first-line agent in the treatment of lymphomas, leukemias, Kaposi's sarcoma, and various solid tumors. Doxorubicin is an anthracycline derivative antibiotic isolated from the bacterium *Streptomyces peucetius*. DOX exerts its main mechanism of action by blocking DNA and RNA synthesis and inhibiting the topoisomerase II enzyme through intercalating between the DNA strands of tumor cells. However, despite this strong antitumor activity, the clinical use of free DOX has very serious limitations and disadvantages. Since free doxorubicin has a highly hydrophilic character, it is easily absorbed by healthy tissues without showing selectivity following intravenous administration. This situation leads to the rapid elimination of the drug from the body, a low rate of reaching the tumor tissue, and consequently, the administration of higher doses to the patient in order to achieve the targeted therapeutic effect (1). In recent years, focus has shifted toward smart drug targeting strategies and nanoparticle approaches to overcome toxicity problems and pharmacokinetic disadvantages. The encapsulation of doxorubicin into nanostructures such as liposomes, polymeric micelles, dendrimers, quantum dots, carbon nanotubes, silica-based or metallic nanoparticles is one of the most important milestones of modern oncology. Doxil is the first liposomal nanoparticle system approved by the FDA for Kaposi's sarcoma. These nanoparticle systems extend the half-life of the drug by protecting it from early degradation in systemic circulation, provide controlled release, and allow the drug to selectively accumulate in the tumor region via the Enhanced Permeability and Retention (EPR) effect developed due to the leaky vasculature in tumor tissues. Attachment of the drug to nanoparticles via covalent or non-covalent bonds directly affects the in vivo stability of the formulation; particularly, premature drug leakage observed in systems with weak bonds can lead to undesired toxicities in off-target healthy tissues. Therefore, it is crucial to clearly elucidate how the biodistribution and pharmacokinetic profiles of these developed next-generation smart drug delivery systems behave in living systems.

The main objective of this study is to analyze the accumulation in target tissues, plasma concentration, time-dependent concentration in the body, and toxic effects of free doxorubicin and its metabolites, as well as various nanoparticle-loaded doxorubicin systems, through compiled literature data; thereby providing a basis with the aid of LC-MS/MS analyses for the design of more targeted, next-generation nanoparticle systems that will exhibit maximum efficacy in the tumor region in the future (2).

2. GENERAL INFORMATION

2.1 DOXORUBICIN

Doxorubicin is a broad-spectrum cytotoxic chemotherapeutic drug belonging to the group of anthracycline antineoplastic antibiotics. Isolated from the actinomycete bacterium *Streptomyces peucetius* and subsequently obtained chemically through semi-synthetic methods, this compound has been used since the 1970s in the treatment of various cancer types ranging from hematologic malignancies to solid tumors. Its chemical structure consists of a four-ring anthracenone backbone combined with daunosamine, an amino sugar. This unique structure confers upon the molecule the ability to insert between DNA double strands, namely the property of intercalation. Doxorubicin also inhibits the topoisomerase II enzyme, preventing the unwinding of the DNA helix and ultimately arresting cell division. Owing to its efficacy and broad therapeutic spectrum, doxorubicin was approved by the FDA in 1974 for the treatment of various malignancies, including breast, lung, stomach, ovarian, thyroid, Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, sarcomas, and pediatric cancers (3).

It has become one of the main "first-line" cancer drugs, maintaining this feature today as well. However, like most other chemotherapy drugs, doxorubicin also has toxicities and side effects associated with its use. Therefore, while doxorubicin can be used clinically, significant limitations such as dose restrictions and the accumulation of dose-related toxicities have been influential in selecting doxorubicin as a drug to be administered via liposomal formulations. The liposomes used must be nanoscale and effective, a therapeutically sufficient amount of drug must be loaded into the tumors. The liposomal formulation must be designed to have a long plasma circulation time in order to optimize drug accumulation in tumor tissue, thereby enhancing targeted efficacy (4).

2.1.1. Chemical Structure and Physicochemical Properties

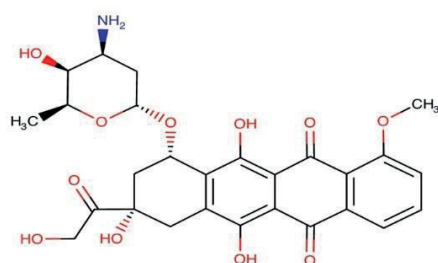


Figure 1 Doxorubicin and Its Amphiphilic Chemical Structure (5)

Doxorubicin is an amphiphilic molecule with the chemical formula $C_{27}H_{29}NO_{11}$ (Figure 1). The molecule features a planar core consisting of four aromatic rings. Doxorubicin is soluble in water owing to its hydroxyl and amino groups and is pH-sensitive due to its weakly basic character; this situation can affect its bioavailability. The planar and amphiphilic structure of the molecule enables its stable encapsulation and controlled release in lipid-based delivery systems, particularly in liposomes. Thereby, nano-liposomal formulations extend the circulation time of the drug in plasma, increasing its targeted accumulation in tumor tissue and reducing its systemic toxicity (6).

2.1.2. Mechanism of Action

Anthracyclines exert their effect by intercalating between the DNA double helix through the rings in their anthraquinone structure. During this interaction, rings B and C of the four-ring structure overlap with adjacent base pairs in DNA, while ring D extends toward the space between the DNA strands. The sugar moiety of the molecule (the daunosamine group) is directed toward the minor groove of DNA. Since this positioning shares the same region as the histones surrounding the DNA, it can cause spatial displacement with the histones. This intercalation not only stabilizes the Topoisomerase II–DNA complex, but also directly affects the structural integrity and function of DNA. Doxorubicin tightly binds to DNA, inhibiting both DNA synthesis and RNA synthesis. This situation prevents the accurate replication and transfer of genetic information, thereby slowing down or completely arresting cell division (7).

Oxidative Stress and Free Radical Formation

The quinone ring in the structure of doxorubicin is reduced to a semiquinone form by various oxidoreductase enzymes (such as cytochrome P450 reductase, xanthine oxidase, and mitochondrial NADH dehydrogenase). This semiquinone structure is unstable and, upon reoxidation, generates reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). As a result of the iron-mediated Haber–Weiss reaction, these compounds transform into highly harmful hydroxyl radicals ($\cdot OH$). These radicals cause lipid peroxidation in the cell membrane and DNA damage, resulting in severe cellular toxicity. Furthermore, doxorubicin can also enhance ROS production by disrupting iron metabolism; it accelerates iron release from ferritin, thereby aggravating oxidative stress (7).

Immune Modulation

In addition to directly destroying tumor cells, anthracyclines can also enhance the antitumor response by activating the immune system. During cell death, danger signals known as tumor antigens and damage-associated molecular patterns (DAMPs) are released into the tumor microenvironment. These molecules initiate an inflammatory response, enabling the

recruitment of immune cells to the site and making tumor cells more easily recognized by the immune system. This process is termed immunogenic cell death (ICD). Anthracyclines such as doxorubicin have been shown to trigger ICD and thereby induce a dendritic cell-mediated, tumor-specific CD8⁺ T cell response in murine colon cancer models. Furthermore, doxorubicin has been reported to selectively deplete myeloid-derived suppressor cells in the tumor microenvironment and reduce their immunosuppressive effect. This effect contributes to the enhancement of the immune response, particularly in breast cancer models (7).

2.1.3 Pharmacokinetic Properties

2.1.3.1. Pharmacokinetics of free doxorubicin

Doxorubicin is an anthracycline derivative that exhibits linear pharmacokinetic behavior when administered intravenously. It shows extensive distribution between plasma and tissues; the volume of distribution of the drug is above 500 L/m², and its plasma protein binding rate ranges between approximately 50% and 85%. Doxorubicin is mainly metabolized in the liver. While doxorubicinol (DOXol), a metabolite formed via aldo-keto reductases, retains its antitumor activity to a certain extent, reactions mediated by NADPH-dependent cytochrome P450 reductase lead to the generation of drug aglycones, and these derivatives play a key role in the development of cardiotoxicity. Clearance of the drug from the body is defined by a triphasic elimination curve. The half-life of the initial rapid distribution phase is approximately 5 minutes, that of the intermediate phase is about 2.6 hours, and that of the terminal elimination phase is around 48 hours. Fecal excretion of doxorubicin and its metabolites holds clinical importance; 10–20% of the total dose is eliminated via feces within the first 24 hours, and 40–50% after approximately 150 hours. The relationship between the pharmacokinetic properties of doxorubicin and hematologic toxicity has been demonstrated through various pharmacodynamic models. In studies utilizing linear or sigmoidal Emax models, a significant negative correlation was identified between the area under the curve (AUC) of the drug and the decrease in leukocyte count ($r \approx -0.69$) (8).

2.1.3.2. Pharmacokinetics of Pegylated Liposomal Doxorubicin (Doxil)

There are extremely large differences in plasma levels between free doxorubicin and pegylated liposomal doxorubicin. In the liposomal form, the AUC increases at least 60-fold, while a few hours after injection, plasma doxorubicin concentrations can reach hundreds of times higher than the levels observed in animals given the free drug. Overall, Doxil® administration leads to a significant increase in doxorubicin-equivalent AUC (9).

2.1.4. Pharmacological Toxicity and Safety Profile

There is evidence in the literature indicating that the presence of certain factors may increase the risk of developing DOX-induced cardiotoxicity. Gender, age, ethnicity, diabetes, dyslipidemia, obesity, hypertension, cardiovascular disease, and concomitant drug use are determinants in this toxicity profile. It has been demonstrated by various clinical studies that the cardiotoxic effect of doxorubicin, particularly the development of chronic heart failure, is closely related to the maximum plasma concentration ($C_{\max}$) of the drug, and that this risk can be significantly reduced by extending the infusion duration. For instance, a study conducted in pediatric patients showed that 24-hour (60 mg/m^2) and 72-hour (75 mg/m^2) infusions of doxorubicin provided a decrease in the rate of congestive heart failure compared to traditional bolus administration. While heart failure developed in only one of the 97 children receiving prolonged infusion, this rate reached approximately 13% in patients receiving bolus therapy. These findings support that long-term cardiotoxicity is associated more with $C_{\max}$ than with AUC. On the other hand, prolonging the infusion duration may lead to some new complications. Specifically, both the frequency and severity of mucositis and bone marrow suppression increase, and these side effects become the dose-limiting toxicities of the treatment. Some clinical data suggest that extended infusion duration increases the steady-state volume of distribution (V_{ss}) of the drug, potentially allowing greater drug penetration into tissues, particularly into tumor tissue, compared to short-term intravenous administrations. Furthermore, there are findings indicating that high $C_{\max}$ values of doxorubicin and its major metabolite, doxorubicinol, may be associated with longer remission periods in patients with acute non-lymphocytic leukemia (10). Trastuzumab and diclofenac sodium can be given as examples (11). Trastuzumab is an agent targeting human epidermal growth factor receptor 2. It is widely used in order to reduce tumor formation. During the drug's approval by FDA in 2001, a warning was issued against its concomitant use with anthracyclines, including DOX. The simultaneous administration of trastuzumab with anthracyclines was found to increase the risk of congestive heart failure approximately fourfold compared to the use of either drug alone (12). Digoxin is a cardiac glycoside used in the treatment of arrhythmia such as atrial flutter and symptoms of heart failure; co-administration of digoxin with DOX led to more pronounced myocardial damage in rabbits compared to the administration of either drug alone. In recent studies, metformin has been shown to attenuate the DOX-induced decrease in LVEF and cardiomyocyte damage. At the cellular level, metformin provides a protective effect during DOX treatment by increasing the levels of antioxidants such as glutathione and superoxide

dismutase (10). Another drug class used in diabetes treatment is sodium-glucose cotransporter-2 (SGLT2) inhibitors. These drugs demonstrate cardioprotective effects in patients not receiving chemotherapy. Specific SGLT2 inhibitors used in current animal models, namely empagliflozin and dapagliflozin, have shown the ability to reduce myocardial fibrosis and improve LVEF in the presence of DOX (13).

2.1.5 Doxorubicin's Metabolic Profile and Biotransformation Mechanisms

It is reported that approximately half of the doxorubicin administered to humans is eliminated from the body without undergoing metabolic transformation, whereas the remaining portion undergoes biotransformation through three primary metabolic pathways. The metabolism of anthracycline-class drugs involves processes such as hydroxylation reactions, the formation of semiquinone intermediates, or the generation of deoxyaglycone derivatives; the products resulting from these metabolic pathways can either enhance or diminish the anticancer efficacy of the drug (14).

A) Two-Electron Reduction of Anthracyclines (Hydroxylation): Anthracycline derivatives hydroxylated at the C13 position possess higher polarity and consequently better water solubility compared to the parent compound. However, despite these physicochemical properties, the elimination rates of these hydroxylated metabolites from certain tissues are known to be lower than that of the parent compound. They exhibit a more pronounced cardiotoxic effect, whereas their antineoplastic activity is reported to be weaker than that of the parent compound. Carbonyl Reductase 1 (CBR1) is a reductase that hydrolyzes doxorubicin in the liver, kidney, and gastrointestinal tract (GIT). Aldo-keto Reductases (AKR) reduce ketones and aldehydes to alcohols in an NADP-dependent manner. Heibein and colleagues demonstrated that exogenously administered doxorubicin predominantly localized in the nuclear region in breast tumor cells, whereas doxorubicinol mainly accumulated in the lysosomal compartment. In the same study, it was shown that the DNA-binding affinity of doxorubicinol was markedly lower compared to doxorubicin; additionally, β -cholanic acid, an AKR inhibitor, enabled the relocalization of doxorubicin to the nucleus in doxorubicin-resistant cells. This finding suggests that the inhibitor suppresses the hydroxylation of doxorubicin. In line with this interpretation, intracellular doxorubicin concentration was observed to increase in doxorubicin-resistant cells in the presence of β -cholanic acid (14).

B) One-Electron Reduction of Anthracyclines (Semiquinone Formation): Enzymes catalyzing the one-electron reduction of anthracyclines include NADPH-cytochrome P450 reductase, endothelial nitric oxide synthase, and NADH dehydrogenase (ubiquinone) Fe-S.

According to studies, there is strong evidence for DOX semiquinone formation in cardiac and tumor tissues, which is associated with increased cytotoxicity (14).

C) Reductive Deglycosylation of Anthracyclines (Deoxyaglycone Formation): The third and secondary pathway in anthracycline metabolism is the conversion of these compounds into 7-deoxyaglycone metabolites via a reduction-dependent deglycosylation process. The 7-deoxyaglycone derivatives of anthracyclines are structurally formed by the removal of the glycosidic sugar moiety. This requires a two-electron reduction reaction, which occurs exclusively under anaerobic conditions and in the presence of NADPH. In general, the formation of 7-deoxyaglycone metabolites is known to largely abolish the biological activity of the parent compound (14).

3. REVIEW OF ANALYTICAL METHODS

Jin et al. developed an HPLC-MS/MS system to quantify doxorubicin accumulation across various mouse tissues, including plasma, heart, spleen, and kidney. A C18 column operating at a flow rate of 0.2 mL/min under gradient elution conditions was utilized as the chromatographic column. The mobile phase was ammonium formate containing 0.1% formic acid and acetonitrile. Daunorubicin (DNR) was selected as the internal standard (IS). Doxorubicin and daunorubicin analytes were analyzed using a triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. Electrospray ionization (ESI) served as the ionization source. Additionally, the 4T1 mouse breast cancer cell line was used as the experimental model and cultured in RPMI-1640 medium supplemented with fetal bovine serum. The analytical method was validated according to ICH guidelines across various parameters, including specificity, recovery rate, matrix effect, and linearity. Evaluation of chromatographic conditions revealed that gradient elution yielded more symmetrical and intense peaks compared to isocratic elution. On the HPLC system, the retention times for doxorubicin and daunorubicin were observed at 6.95 and 7.83 minutes, respectively. To evaluate method selectivity, blank mouse plasma and kidney tissue matrices were analyzed. As shown in Figure 2, no interfering peaks originating from the biological matrices were observed at the retention times of DOX and the Internal Standard (7 and 8 minutes, respectively). Clear peak separation was achieved at the lower limit of quantification (LLOQ). Furthermore, upon intravenous administration to actual tissues, the analytes were clearly identified; thus, the peaks resulting from doxorubicin administration exhibited strong alignment between in vitro and ex vivo tissue settings. In this study conducted across six different mouse plasma samples, the

tissue matrix effect ranged from 12.2% to 15.5%, while the recovery rate fell between 8.3% and 12.5%. These values satisfied the acceptance criterion requiring the RSD of recovery and matrix effect for DOX and IS in plasma and tissue to be less than 15.5%. Moreover, the correlation coefficient was examined to assess linearity; the R^2 value was 0.9987 for plasma and remained above 0.99 for all other tissues, staying well within acceptable limits. Intra-day and inter-day precision evaluations demonstrated that deviation among values did not exceed 10.3%, remaining below the criteria threshold of $\pm 20\%$, which highlights the reliability of their findings. Regarding the MS system, ionization performed via ESI in positive ion mode yielded higher sensitivity compared to negative ion mode. The precursor and product ion mass spectra of the analyzed compounds were obtained as displayed in the plot. The mass transitions for DOX and the internal standard daunorubicin were observed as m/z 544.28 $\rightarrow$ 397.10 and m/z 528.35 $\rightarrow$ 321.08, respectively. This study was not limited strictly to plasma doxorubicin levels but also investigated its biodistribution across various organs; in vivo concentration levels ranked from highest to lowest as kidney, liver, spleen, lung, heart, and tumor cells, respectively. Through this developed method, comprehensive insights were gained regarding the plasma and tissue concentrations of doxorubicin as well as its pharmacokinetic behavior in living organisms (15).

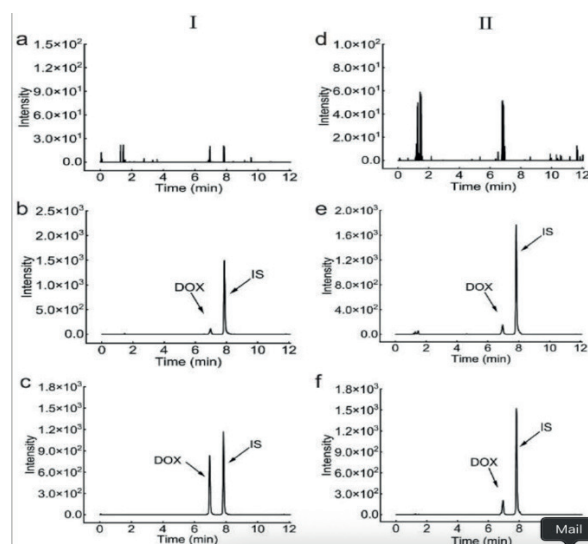


Figure 2 HPLC-MS/MS chromatograms of doxorubicin and IS; (1) Plasma (2) Kidney tissue (a) Blank plasma (b) Plasma spiked with DOX (LLOQ) and IS (100 ng/mL) (c) Mouse plasma 2 hours after IV injection of 5 mg/kg DOX (d) Blank kidney (e) Blank kidney spiked with DOX (LLOQ) and IS (100 ng/mL) (f) Mouse kidney 2 hours after intravenous injection of DOX (5 mg/kg) and IS (15).

In a study conducted by Choi et al., the simultaneous determination of doxorubicin and its various metabolites was achieved using an LC-MS/MS system. Using their developed method, they precisely analyzed the doxorubicin metabolites, namely doxorubicinol, doxorubicinone, doxorubicinolone, and 7-deoxydoxorubicinone. Doxorubicinol, the alcohol metabolite of doxorubicin, is the metabolite with the highest cardiotoxic potential. This method was developed to elucidate the pharmacokinetic behavior of doxorubicin and its metabolites following intravenous administration of doxorubicin at a dose of 1.3 mg/kg to female BALB/c mice. Furthermore, they successfully performed this analysis using as little as 10 μ L of mouse plasma. To prepare stock standard solutions, 1 mg of doxorubicin and each of its metabolites were dissolved in 1 mL of dimethyl sulfoxide; working standards were subsequently prepared by dilution with methanol. Daunorubicin, used as the internal standard (IS), was diluted with methanol to a concentration of 100 ng/mL. Using these stock standards, calibration standards spanning 8 different concentration levels were prepared in drug-free blank mouse plasma. Accordingly, the linear ranges were established as 0.5–200 ng/mL for doxorubicin, 0.1–200 ng/mL for doxorubicinol, and 0.01–50 ng/mL for doxorubicinone, doxorubicinolone, and 7-deoxydoxorubicinone metabolites. Quality control (QC) samples were also prepared to confirm method accuracy; these were set at 1.5 (low), 20 (medium), and 150 ng/mL (high) for doxorubicin; 0.3, 10, and 150 ng/mL for doxorubicinol; and 0.03, 1, and 37.5 ng/mL for the remaining three metabolites, respectively. For sample preparation, 10 μ L volumes were taken from blank plasma, calibration standards, and QC samples. A C18 column was utilized for the analysis. The mobile phase comprised ammonium formate containing formic acid (Mobile Phase A) and methanol containing formic acid (Mobile Phase B), operated under gradient elution at a flow rate of 0.2 mL/min. The chromatographic retention times for the five analytes were observed as 1.9 min for doxorubicinol, 2.0 min for doxorubicin, 2.2 min for daunorubicin (IS), 2.3 min for doxorubicinolone, 2.5 min for doxorubicinone, and 2.8 min for 7-deoxydoxorubicinone, the most nonpolar metabolite (Figure 3). Ionization of the analytes in the mass spectrometer was performed via electrospray ionization (ESI) in positive ion mode, with nitrogen gas utilized to fragment ions in the Q2 collision cell. Additionally, selected reaction monitoring (SRM) mode was employed for high-selectivity analysis of the analytes. Upon evaluating the validation parameters of this developed method, the linearity across all metabolites yielded a coefficient of determination of $r^2 \geq 0.9933$, demonstrating a linear relationship between concentrations and peak areas. Using chloroform-methanol extraction, the recovery rates in mouse plasma were calculated as 81.7–86.4% for doxorubicin, 84.1–87.9% for doxorubicinol, 77.0–90.4% for doxorubicinone, 81.6–94.7% for doxorubicinolone, 81.3–

87.7% for 7-deoxydoxorubicinone, and 108.8% for daunorubicin (IS). Furthermore, matrix effects for the analytes ranged between 94.8% and 119.7%, with the %RSD across six different samples remaining below 14.2%. This low matrix effect indicated that residual substances in the plasma did not hinder analyte ionization in the MS system. In intra-day and inter-day precision studies, when comparing the prepared standards with QC samples, the relative error (%RE) values ranged from -7.7% to +11.3%, staying within the acceptable limit of $\pm 15\%$. Through this study, the biodistribution and elimination profiles of doxorubicin administered at a dose of 1.3 mg/kg to mice were successfully evaluated with results aligning closely with the developed HPLC-MS/MS method (16).

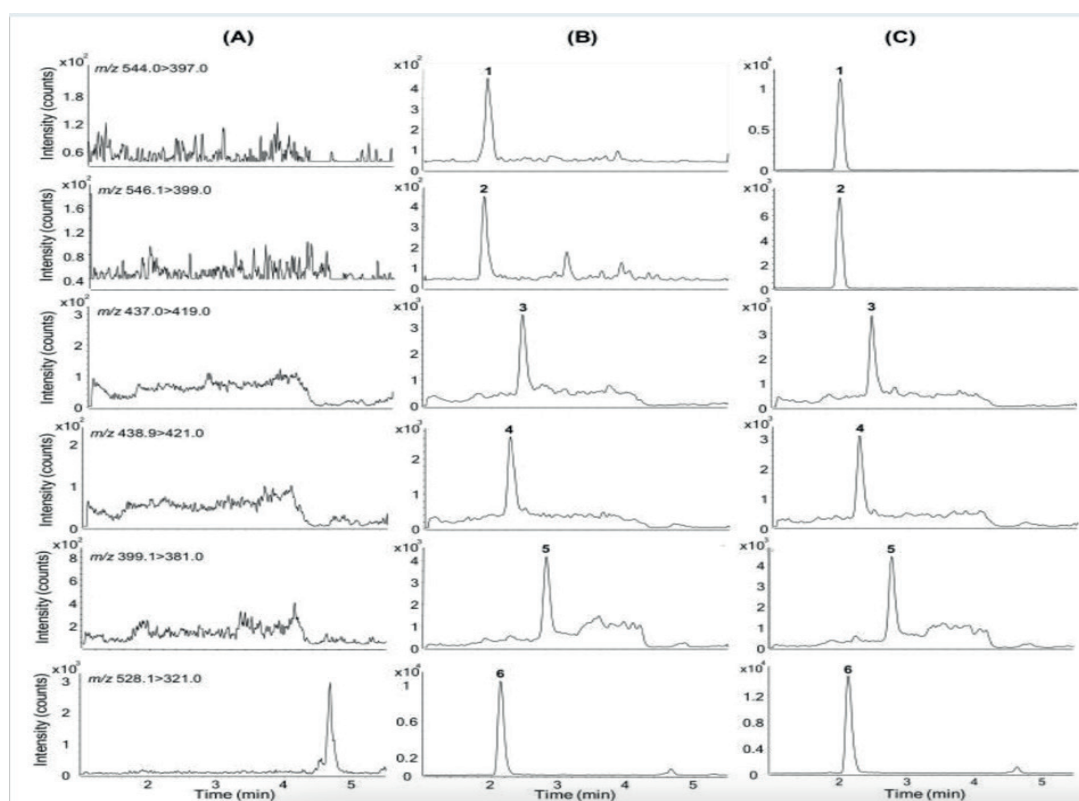


Figure 3 (A) Selected reaction monitoring (SRM) chromatogram of mouse plasma; (B) Doxorubicin (0.5 ng/mL), doxorubicinol (0.1 ng/mL), doxorubicinone (0.01 ng/mL), doxorubicinolone (0.01 ng/mL), 7-deoxydoxorubicinone (0.01 ng/mL), and daunorubicin (IS) (100 ng/mL) at LLOQ levels; (C) Mouse plasma 15 minutes after IV administration of 1.3 mg/kg doxorubicin to female BALB/c mice, showing in sequence: 1. Doxorubicin 2. Doxorubicinol 3. Doxorubicinone 4. Doxorubicinolone 5. 7-Deoxydoxorubicinone 6. Daunorubicin (16).

In a study conducted by Liu et al., an LC-MS/MS method was developed for the quantitative determination of pegylated liposomal doxorubicin and its metabolite, doxorubicinol, in rat plasma. In previous studies, limited research had been conducted on doxorubicinol, a metabolite responsible for the cardiotoxic effects of doxorubicin; thus, this

study addresses both the pharmacokinetic behavior of doxorubicinol and the extent to which liposomal encapsulation alters its pharmacokinetics. For standard preparation in the analysis, 0.8 mg/mL primary stock solutions of doxorubicin, doxorubicinol, and daunorubicin were prepared. Additionally, quality control (QC) samples were prepared at equivalent proportions to monitor the system and prevent errors during weighing and dilution procedures. Using these stock solutions, plasma calibration standards were prepared at nine different concentration levels, alongside quality control samples at five different levels (LLOQ, low, medium, high, and dilution QC). The concentration ranges of these plasma calibration samples were established as 20.0--8000 ng/mL for doxorubicin and 0.0500--20.0 ng/mL for doxorubicinol, with standards prepared at nine distinct concentration levels within these ranges to serve as references for comparing concentrations in animal samples. Subsequently, the daunorubicin internal standard was added to these prepared calibration standards, QC samples, and blank plasma. A silica-based pentafluorophenylpropyl (PFPP) bonded phase column was utilized for the analysis on the LC-MS/MS system. Distilled water containing formic acid was used as Mobile Phase A, and acetonitrile containing formic acid served as Mobile Phase B, with the flow rate set at 400 μ L/min. A triple quadrupole mass spectrometer was used as the mass analyzer, operating in multiple reaction monitoring (MRM) mode with electrospray ionization (ESI) in positive ion mode. The precursor-to-product ion transitions (m/z) were determined as 546 $\rightarrow$ 363 for doxorubicinol, 544 $\rightarrow$ 361 for doxorubicin, and 528 $\rightarrow$ 321 for daunorubicin (IS). Method validation tests were subsequently conducted, establishing the accuracy limits of the method over analyses performed across three distinct days. The intra-assay coefficient of variation (%CV) was determined as 14.7% for doxorubicinol and 3.2% for doxorubicin, successfully remaining below the 15% threshold. Because plasma composition (lipids, proteins, salts) varies among individual animals in bioanalytical studies, matrix effect was evaluated to determine how much the method was influenced by these variations; blank plasma was collected from six different rats and spiked with low concentrations of doxorubicin (60.0 ng/mL) and doxorubicinol (0.150 ng/mL). For doxorubicin, measured concentrations across all levels ranged from 57.4 to 65.2 ng/mL, with relative error (%RE) values ranging between -4.3% and +8.7%. For doxorubicinol, measured values were between 0.138 and 0.160 ng/mL, with %RE values ranging from -8.0% to +6.7%. The results obtained from these six distinct samples demonstrated minimal matrix effects, confirming the reliability of the method. Upon examining method linearity, the coefficients of determination (r^2) were greater than or equal to 0.995 for doxorubicin and 0.992 for doxorubicinol. Additionally, the stability

of the compounds at -70°C over 3 months was evaluated; both compounds were deemed stable, though the doxorubicinol concentration decreased to 85.5% within the 3-month period. This validated HPLC-MS/MS method was subsequently applied to pharmacokinetic studies to obtain time-dependent concentration profiles following the administration of pegylated liposomal doxorubicin to rats. The results revealed that the mean area under the curve (AUC) calculated for doxorubicinol in rat plasma accounted for only 0.011% of the value reached by doxorubicin. This ratio demonstrates that PEG encapsulation of doxorubicin substantially suppresses its conversion to the cardiotoxic metabolite doxorubicinol. Furthermore, the maximum plasma concentration of doxorubicinol remained exceptionally low throughout the analysis (C_{max} : 6.7 ng/mL), with an elimination half-life of $t_{1/2}$: 25.2 hours, confirming the success of the pegylated liposomal doxorubicin formulation (17).

In a study conducted by Zhang et al., an LC-MS/MS method was developed to precisely and selectively quantify both released free doxorubicin and total doxorubicin concentrations in plasma samples collected from Chinese breast cancer patients receiving liposomal doxorubicin infusion therapy. In the developed method, an Agilent Zorbax C18 column was utilized as the analytical column, with distilled water containing formic acid as Mobile Phase A and acetonitrile containing formic acid as Mobile Phase B. The flow rate was held constant at 0.60 mL/min. The mass spectrometry system comprised an electrospray ionization (ESI) source operating in positive ionization mode and a triple quadrupole mass analyzer. During the preparation of standard solutions, buspirone was used as an internal standard. Working standard solutions were prepared in the range of 2.00--5000 ng/mL for total doxorubicin and 2.00--2000 ng/mL for free doxorubicin. Subsequently, 50 μL of plasma from breast cancer patients was used to dilute the working standards, establishing linear calibration ranges of 0.2--500 ng/mL for total doxorubicin and 1.0--1000 ng/mL for free doxorubicin, with coefficients of determination (r^2) exceeding 0.995. According to the MS analysis, based on highest ion abundance in the Q1 compartment, the mass-to-charge ratios (m/z) for precursor ions were selected as 544.2 for doxorubicin and 386.3 for buspirone. For target product ion identification in the Q3 compartment, transitions of m/z 544.2 $\rightarrow$ 379.1 for doxorubicin and m/z 386.3 $\rightarrow$ 122.2 for buspirone were selected. Regarding method validation parameters, the intra-day and inter-day accuracy and precision performance of the method were evaluated at the LLOQ and various quality control (QC) concentration levels. For precision, the relative standard deviation (%RSD) values ranged from 1.20% to 8.40%, while for accuracy, all recovery data fell between 91.30% and 106.80%. These results demonstrated that the method

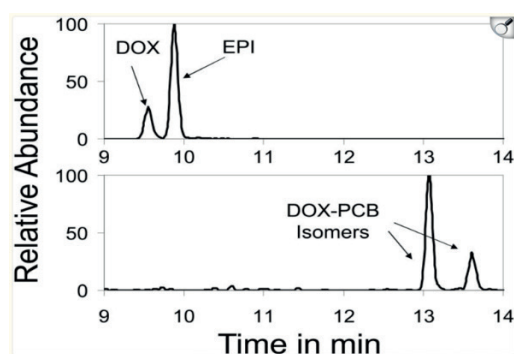
possesses high reliability and reproducibility. Furthermore, stability evaluations indicated that the analytes remained stable at room temperature (25°C) for 4 hours; across three consecutive freeze-thaw cycles between -80°C and 25°C, analyte loss was restricted to only 5%. This developed method served as a reference for calculating blood concentrations of liposomal doxorubicin in pharmacokinetic studies. Peak areas obtained from the analysis were converted into time-dependent net plasma concentration data (ng/mL) using validated calibration curves. By plotting these concentration values against time, geometric calculation of the pharmacokinetic profiles (area under the curve, AUC) was facilitated. In this study involving breast cancer patients, the total doxorubicin exposure in blood was determined as $AUC_{inf} = 5370 \pm 1002 \text{ h} \cdot \mu\text{g/mL}$, whereas free doxorubicin released from the liposome yielded $AUC_{inf} = 1175 \pm 458 \text{ h} \cdot \mu\text{g/mL}$. This demonstrates that total drug exposure in the body was approximately 5-fold higher compared to free drug. This difference indicates that doxorubicin is largely protected within the liposomal capsule in systemic circulation, thereby suppressing free drug levels and preventing uncontrolled distribution into healthy tissues (18).

In a study conducted by Desai et al., an LC-MS/MS method was developed for the identification of various metabolites of a peptide-doxorubicin conjugate used in prostate cancer treatment. Human, rat, and dog plasmas were utilized in this study. For the HPLC system, a C8 column and a flow rate of 0.2 mL/min were selected. Mobile Phase A comprised an ammonium acetate buffer, while Mobile Phase B consisted of an acetonitrile-water mixture. MS conditions were operated in both positive and negative electrospray ionization (ESI) modes to achieve superior structural characterization, with a triple quadrupole analyzer chosen as the mass analyzer. Multiple reaction monitoring (MRM) mode was selected for quantification, and neutral loss scanning was also performed to screen for unknown structures. For doxorubicinol, the precursor ion mass-to-charge ratio (m/z) was observed at 546.5, and following collision-induced dissociation in the collision cell (Q2), the characteristic product ion was found at m/z 398.7. This value represents the reduced aglycone core where the doxorubicin ring is reduced to a hydroxyl group. Furthermore, the fragment at m/z 130.1 corresponds to the daunosamine amino sugar moiety in the doxorubicin structure. The precursor ion m/z value for L-doxorubicinol was determined as 659.3, and the fragment at m/z 242.9 was identified as the leucine amino acid bound to the amino sugar structure. In this negative mode mass spectrum, the ion at m/z 351.2 corresponds to 7-deoxydoxorubicinol, formed via the loss of the amino sugar ring (daunosamine) from doxorubicin. Upon evaluating the method validation parameters, the chosen MRM transitions (546.2 $\rightarrow$ 399.3 and 659.2 $\rightarrow$ 243.3) completely

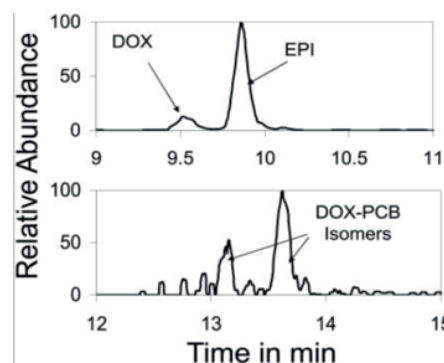
eliminated protein and lipid interferences originating from the plasma, thereby preventing matrix suppression and achieving high selectivity. Across consecutive intra-day and inter-day injections, the Relative Standard Deviation (%RSD) values calculated for both retention times and peak areas remained below 5.0%, falling well within acceptable limits. In conclusion, using this developed LC-MS/MS methodology with switching positive/negative ionization modes, non-standardized doxorubicin catabolites (doxorubicinol, leucine-doxorubicinol, and 7-deoxydoxorubicinol aglycone) which traditional fluorescence systems fail to resolve chromatographically and structurally were unambiguously identified in biological fluids for the first time through mass fragmentation (19).

In an LC-MS/MS method developed by Ibsen et al., exceptionally low limits of detection (LOD) were achieved in tissue, reaching 7.8 pg for doxorubicin and 0.36 ng for the doxorubicin prodrug. In this method, the DOX-PCB (photocleavable biotin-conjugated doxorubicin) prodrug was investigated. This prodrug exhibits 200-fold lower systemic toxicity compared to free DOX and is activated by light at a wavelength of 365 nm. Pure chemical standards and *in vivo* xenograft tumor tissue samples were utilized for this methodology. For standard solution preparation, DOX and epirubicin were diluted to 0.1 mg/mL with methanol, while DOX-PCB was diluted to 0.1 mg/mL with acetonitrile. For tumor tissue extraction, 30 mg of tumor tissue was extracted from mice bearing A549 human lung cancer xenografts. To release DOX intercalated between tissue DNA strands, AgNO₃ was added to the medium to denature the DNA. A C18 column was selected for the LC system, with an aqueous methanol solution containing formic acid used as Mobile Phase A and pure methanol containing formic acid used as Mobile Phase B, operated at a flow rate of 0.2 mL/min. A quadrupole ion trap analyzer was employed for the MS system, with ionization performed via electrospray ionization in positive ion mode, and selected reaction monitoring (SRM) mode was utilized for analysis. The chromatogram clearly demonstrates the resolution of the DOX and epirubicin compounds, with retention times observed at approximately 9.6 and 9.9 min, respectively. In the corresponding chromatogram, the retention times for the two isomers of the DOX-PCB prodrug appeared at 13.1 and 13.6 min. Due to its higher hydrophobicity, this prodrug exhibited longer retention on the column. This chromatogram confirmed that the developed extraction method effectively resolved components from the tissue matrix. Both DOX and EPI yielded a precursor ion at m/z 544. Following fragmentation in the collision cell, the most stable product ion fragment was determined at m/z 396.85. The precursor ion mass for DOX-PCB was found at m/z 1244.4; upon collision-induced dissociation, cleavage of the PEG moiety within the

prodrug yielded a product ion peak at m/z 848.28, which was selected as the target mass in SRM mode to evaluate the tissue behavior of the prodrug. Subsequently, the behavior of the DOX-PCB prodrug in real tumor tissues harvested from mice was analyzed using this method. Furthermore, with the newly developed salt precipitation method, DOX—which was intercalated within DNA helices and thus unextractable with acetonitrile/methanol—successfully generated analytical signals following DNA denaturation. Chromatographic results revealed a retention time of 9.5 min for free DOX, while non-photocleaved DOX-PCB isomers in the same sample were detected at 13.2–13.7 min. Upon evaluating the validation parameters, the coefficient of determination for linearity yielded an r^2 of 0.999. The limits of detection (LOD) were determined as 7.8 pg for DOX and 0.36 ng for the DOX-PCB prodrug. The lower limits of quantification (LOQ) were established as 13 pg and 0.6 ng for DOX and DOX-PCB, respectively. Extraction recoveries were approximately 80% for DOX and around 60% for DOX-PCB. In conclusion, the chromatograms of standard solutions and those obtained from *in vivo* analyses using this developed method demonstrated high consistency in retention times and peak profiles. This method serves as a valuable guide for evaluating the tissue biodistribution of smart drug delivery systems and prodrug designs in poorly vascularized tumor models (Figure 4a and Figure 4b) (20).



a



b

Figure 4a LC-MS/MS chromatograms of DOX and DOX-PCB (20).

Figure 4b LC-MS/MS chromatograms of tumor tissues obtained from mice injected with DOX-PCB (20).

In a study conducted by Simeka et al., the *in vivo* behavior of doxorubicin and hyaluronan (HA-C18:1) polymeric micelles carrying doxorubicin was simultaneously evaluated using an LC-MS/MS method. Regarding LC conditions, a C18 column was utilized for DOX and its metabolite DOXol, with pure acetonitrile containing formic acid prepared as

the mobile phase at a flow rate of 0.4 mL/min. For the hyaluronan fragments, a C4 column was chosen, and an aqueous acetonitrile solution containing formic acid was prepared as the mobile phase. A triple quadrupole mass analyzer equipped with an electrospray ionization (ESI) source was selected for the MS system. In positive ionization mode, DOX and DOXol were analyzed in MRM mode; the precursor ion for DOX was determined at m/z 544.0, and the resulting product ion fragment was found at m/z 397.0. Conversely, the HA-C18:1 polymer was analyzed in MRM mode using negative ionization, with the MRM ion transition channel establishing the precursor ion at m/z 642.3 and the product ion at m/z 175.0. Furthermore, endogenous hyaluronan naturally produced in the body was observed at a precursor ion value of m/z 378.1, allowing it to be clearly distinguished from the synthetic hyaluronan under analysis. For the *in vivo* study, a mouse model bearing a metastatic cell line was employed. When this developed method was applied to the mouse model, the chromatographic results demonstrated high consistency and were verified through method validation parameters. This approach enabled the simultaneous and quantitative tracking of both the polymeric matrix and the encapsulated drug. Owing to the method's exceptionally low lower limit of quantification (LLOQ) values 5 ng/mL for DOX and 0.10 μ g/mL for the HA-C18:1 polymer the presence of doxorubicin at measurable concentrations within the target tumor tissue was clearly detected even at 72 hours, when systemic concentrations had significantly declined. Although the diverging pharmacokinetic profiles of the drug and the hyaluronan matrix indicated that the polymeric micelles dissociated in the bloodstream during early stages, this method confirmed that encapsulating DOX within the micellar structure is an essential strategy for extending the drug's systemic circulation time and enhancing tumor targeting (21).

In a study conducted by Ma et al., an innovative LC-MS/MS method was developed and validated for the simultaneous determination of doxorubicin (DOX) and curcumin (Cur), a potent antioxidant, in rat plasma. For chromatographic separation, a C18 column and a gradient elution program were employed. A triple quadrupole analyzer was selected for the MS system. Ions were generated via electrospray ionization and monitored using multiple reaction monitoring (MRM) mode. The observed transitions were m/z 544.1 $\rightarrow$ 397 for DOX, m/z 369.3 $\rightarrow$ 177 for Cur, and m/z 494.1 $\rightarrow$ 368.9 for glibenclamide (IS). To evaluate method selectivity and matrix effects, the resulting chromatograms were evaluated. Comparing blank rat plasma with spiked calibration standards, no endogenous interferences from plasma components were observed at the retention times of DOX (3.63 min) and Cur (4.08 min). Most importantly, the chromatogram obtained from authentic rat plasma following combination drug

administration demonstrated complete parallelism and consistency with the peak profiles, sensitivity, and selectivity of the laboratory-prepared standard calibration samples. Even at high concentrations, the mass signals of the two active substances did not interfere with each other, thereby verifying the specificity of the method. During validation studies, the method exhibited linearity across broad working ranges: 2-8000 ng/mL ($r = 0.9942$) for DOX and 5--2000 ng/mL ($r = 0.9910$) for Cur. Lower limits of quantification (LLOQ), indicative of analytical sensitivity, were determined as 2 ng/mL for DOX and 5 ng/mL for Cur. Extraction recoveries from plasma ranged between 89.17% and 100.45%. In conclusion, this LC-MS/MS method provides a foundation for the development of safer and synergistic oncological therapies by enabling the evaluation of plasma and target tissue distribution, mutual effects on toxicity and absorption, and precise lower limits of quantification (LLOQ) for drug combinations such as curcumin and doxorubicin used in cancer treatment (22).

CONCLUSION

The literature data evaluated within the scope of this review that the application of LC-MS/MS methods is of critical importance for the analytical monitoring of doxorubicin (DOX) and its next-generation carrier systems. The analytical platform presented in this study paves the way for a faster and more accurate standardization of *in vivo* stability, release kinetics, and biodistribution processes of targeted next-generation nanocarrier systems via LC-MS/MS. Moreover, investigating simultaneous LC-MS/MS parameters for combination therapies designed to enhance treatment efficacy provides strong bioanalytical guidance for modern oncological strategies. The high sensitivity and selectivity offered by LC-MS/MS will advance patient-specific smart dosing approaches, thereby enhancing clinical success and enabling the bioanalytical detection and prevention of irreversible cardiac damage the major bottleneck of therapy at early stages. In conclusion, by leveraging the powerful analytical capabilities of LC-MS/MS, this review aims to contribute to the development of safer, targeted, and more effective cancer therapies by determining the distribution, toxicity profiles, and lower limits of quantification of novel drug delivery systems in plasma and target tissues.

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CHAPTER 3

CEFIDEROCOL: A NEW-GENERATION SIDEROPHORE CEPHALOSPORIN AGAINST CARBAPENEM-RESISTANT GRAM- NEGATIVE BACTERIA

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1. Introduction

The growing burden of antimicrobial resistance poses a substantial challenge to the effective management of infectious diseases worldwide. The World Health Organization (WHO) has identified carbapenem-resistant *Enterobacterales*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* as pathogens for which the development of novel antimicrobial agents is an urgent priority (Tacconelli et al., 2018). In addition, *Stenotrophomonas maltophilia* has been recognized as a difficult-to-treat opportunistic pathogen because of its intrinsic resistance to multiple antimicrobial agents (Looney et al., 2009).

In Gram-negative bacteria, carbapenem resistance may arise through several mechanisms, including carbapenemase production, loss or alteration of outer membrane porins, and overexpression of efflux pumps, which may occur either independently or in combination. In recent years, novel β -lactam/ β -lactamase inhibitor combinations, including ceftazidime-avibactam, meropenem-vaborbactam, imipenem-cilastatin-relebactam, and ceftolozane-tazobactam, have been introduced into clinical practice. However, their limited activity, particularly against strains producing metallo- β -lactamases such as New Delhi metallo- β -lactamase (NDM), Verona integron-encoded metallo- β -lactamase (VIM), and imipenemase (IMP), together with reports of emerging resistance during their clinical use, has further emphasized the need for alternative therapeutic options (Nordmann & Poirel, 2019; Watkins & Van Duin, 2019).

Cefiderocol is the first siderophore cephalosporin to be actively transported into bacterial cells by exploiting bacterial iron uptake systems. By chelating ferric iron through its catechol moiety, cefiderocol gains access to Gram-negative bacteria via their iron uptake pathways, an entry mechanism commonly described as the “Trojan horse” strategy (Page, 2013). In addition, its high stability against hydrolysis by a broad range of serine β -lactamases and metallo- β -lactamases has positioned cefiderocol as a promising antimicrobial agent for the treatment of severe infections caused by carbapenem-resistant Gram-negative pathogens (Sato & Yamawaki, 2019).

2. Discovery and Chemistry of Cefiderocol

The worldwide increase in carbapenem-resistant Gram-negative pathogens, together with the scarcity of effective therapeutic options, has driven the search for antimicrobial agents with alternative modes of action. Cefiderocol was developed by Shionogi & Co., Ltd. to address this therapeutic gap and became the first siderophore cephalosporin introduced into clinical use, employing bacterial iron acquisition pathways to facilitate its entry into the bacterial cell. The development of cefiderocol was aimed at achieving stability against clinically relevant β -lactamases, particularly metallo- β -lactamases (MBLs), while maintaining potent in vitro antibacterial activity supported by favorable pharmacokinetic/pharmacodynamic (PK/PD) properties (Ji, Juárez-Hernández, & Miller, 2012; Plaisance et al., 2024).

Cefiderocol produces its bactericidal effect through interaction with penicillin-binding proteins (PBPs), which disrupts peptidoglycan synthesis and ultimately compromises bacterial cell wall integrity. PBP3 is considered its primary target, and the high affinity of cefiderocol for this protein results in disruption of peptidoglycan synthesis, ultimately leading to bacterial cell lysis (Bonomo, 2019).

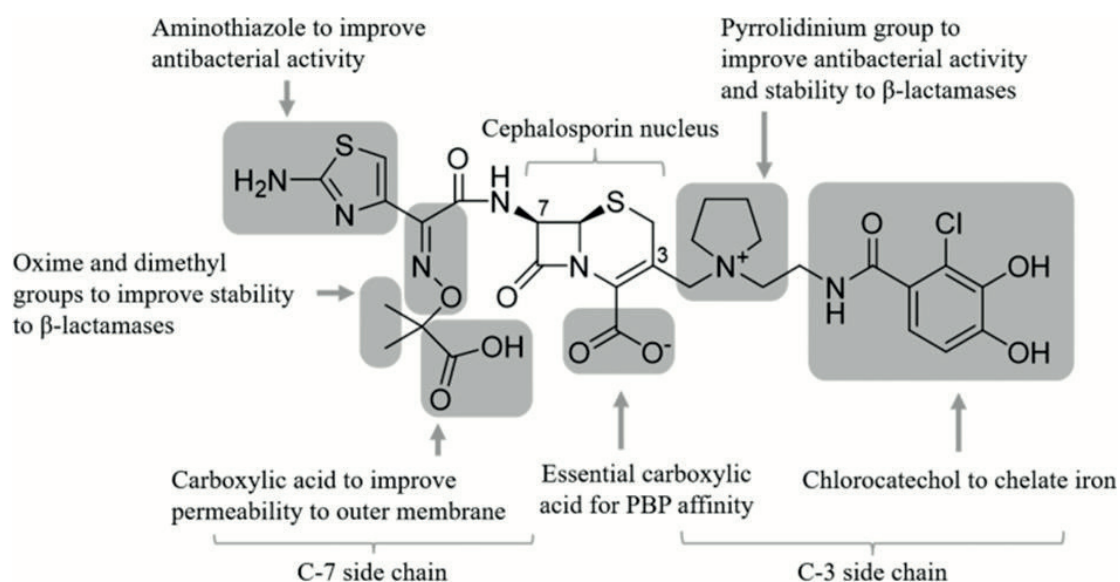


Figure 1. Structural components of cefiderocol associated with antibacterial activity, β -lactamase stability, and iron chelation. PBP, penicillin-binding protein.

In vitro studies have demonstrated that the *CirA* and *Fiu* receptors in *Escherichia coli* and the *PiuA* receptor in *Pseudomonas aeruginosa* play important roles in the cellular uptake of cefiderocol. Mutations affecting these transporter proteins have been associated with increased minimum inhibitory concentrations (MICs), suggesting that additional iron transport systems may also contribute to the uptake of cefiderocol into bacterial cells (Katsube et al., 2019).

The active transport mechanism of cefiderocol not only facilitates its efficient access to the periplasmic space but also substantially reduces the impact of resistance mechanisms commonly encountered in Gram-negative bacteria, such as porin loss and efflux pump overexpression. In *Klebsiella pneumoniae*, loss of the OmpK35 and OmpK36 porins has been shown to have only a limited effect on cefiderocol susceptibility, whereas the same alterations markedly reduce susceptibility to meropenem (Sato & Yamawaki, 2019). Similarly, in *P. aeruginosa*, loss of OprD or overexpression of the MexAB-OprM efflux pump has been reported to exert only a minimal effect on cefiderocol activity (McCreary et al., 2021).

3. Stability Against β -Lactamases

One of the major advantages of cefiderocol is its high stability against a broad range of β -lactamases. Cefiderocol has been shown to be poorly hydrolyzed by serine carbapenemases, including KPC- and OXA-type enzymes, as well as by metallo- β -lactamases such as NDM, VIM, and IMP. Moreover, hydrolysis by OXA-23 and OXA-48-like carbapenemases has been reported to be negligible (Kocer et al., 2023).

Cefiderocol also exhibits low affinity for AmpC β -lactamases and is less affected by the overexpression of these enzymes than many other cephalosporins. Furthermore, its weak induction of AmpC production may represent an additional advantage in terms of limiting the emergence of resistance. Thus, the activity of cefiderocol against carbapenem-resistant Gram-negative bacteria appears to be attributable not only to its active uptake through bacterial iron transport systems but also to its high stability against β -lactamase-mediated hydrolysis (Poirel et al., 2022).

4. In Vitro Susceptibility Testing

Unlike other β -lactam antibiotics, antimicrobial susceptibility testing of cefiderocol requires the use of iron-depleted cation-adjusted Mueller–Hinton broth (ID-CAMHB). The Clinical and Laboratory Standards Institute (CLSI) recommends the use of this medium to mimic the low free-iron conditions encountered in the host during infection, thereby allowing the siderophore-mediated active uptake of cefiderocol to be more accurately reproduced under laboratory conditions. Studies performed using ID-CAMHB have demonstrated highly reproducible minimum inhibitory concentration (MIC) results for cefiderocol, with a strong correlation between in vitro susceptibility and in vivo efficacy. According to current CLSI recommendations, *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 are recommended as quality-control strains (Huband et al., 2017; Wayne, 2008).

The antibacterial spectrum of cefiderocol predominantly encompasses aerobic Gram-negative organisms, including clinically important multidrug-resistant pathogens such as *Enterobacterales*, *P. aeruginosa*, and *A. baumannii*, whereas its activity against Gram-positive and anaerobic organisms remains limited. Potent in vitro activity has been demonstrated particularly against *Enterobacterales*, *P. aeruginosa*, *A. baumannii*, *Burkholderia* spp., and *Stenotrophomonas maltophilia*. Activity has also been observed against respiratory pathogens such as *Haemophilus influenzae* and *Moraxella catarrhalis*. In contrast, cefiderocol exhibits limited activity against Gram-positive bacteria and anaerobic microorganisms. The reduced activity observed against anaerobic bacteria has been suggested to be associated with their lower dependence on siderophore-mediated iron uptake systems (Ito et al., 2018).

Cefiderocol exhibits strong in vitro activity, with low MIC values, against Gram-negative bacteria producing extended-spectrum β -lactamases (ESBLs), AmpC β -lactamases, serine carbapenemases, and metallo- β -lactamases. Previous studies have reported cefiderocol MIC values of ≤ 2 $\mu\text{g/mL}$ for the majority of clinical isolates harboring various β -lactamases, including CTX-M, TEM, SHV, CMY, DHA, KPC, OXA-48, NDM, VIM, and IMP. The low MIC₉₀ values observed among IMP-6-producing *Enterobacterales* isolates further support the activity of cefiderocol against bacteria harboring different types of carbapenemases. Nevertheless, PBP3 alterations and certain mutations involving KPC enzymes have been reported to reduce susceptibility to cefiderocol, highlighting the importance of continued surveillance for the emergence of resistance (Ito et al., 2018; Kanazawa et al., 2017).

Clinical breakpoints for cefiderocol have been established by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). However, differences exist among the breakpoints proposed by various standard-setting organizations, partly because of differences in the interpretation of pharmacokinetic/pharmacodynamic (PK/PD) data and the available clinical evidence. In general, EUCAST has adopted lower susceptibility breakpoints than CLSI, while the breakpoints recommended by the U.S. Food and Drug Administration (FDA) and the United States Committee on Antimicrobial Susceptibility Testing (USCAST) may also differ according to the microorganism and type of infection (European Committee on Antimicrobial Susceptibility Testing, Cefiderocol Rationale Document, Version 1.1, 2022; U.S. Food and Drug Administration).

EUCAST has not established species-specific clinical breakpoints for *Acinetobacter* spp. and *Stenotrophomonas maltophilia* because of insufficient clinical efficacy data and instead provides guidance based on PK/PD considerations. In contrast, CLSI has published susceptibility breakpoints for these organisms and remains one of the standards widely used in clinical microbiology laboratories (European Committee on Antimicrobial Susceptibility Testing, Cefiderocol Rationale Document, Version 1.1, 2022).

Unlike the other standard-setting organizations, USCAST has established separate breakpoints for pneumonia and non-pneumonia infections. This approach is intended to account for differences in antimicrobial exposure according to the field of infection. As evidence from clinical studies and real-world data continues to accumulate, further refinement of cefiderocol breakpoints may be warranted, particularly for organisms for which clinical data remain limited (United States Committee on Antimicrobial Susceptibility Testing, Breakpoint Tables for Interpretation of MIC and Zone Diameter Results, Version 7.0, 2021).

Table 1. Clinical breakpoints for cefiderocol published by breakpoint committees and/or regulatory agencies in 2022.

Microorganisms and non-species related PK/PD breakpoints	EUCAST-EMA			USCAST		CLSI				FDA	
	MIC, mg/L	Inhibition zone diameter, mm ^a	MIC, mg/L	MIC, mg/L	Inhibition zone diameter, mm ^a	MIC, mg/L	Inhibition zone diameter, mm ^a	MIC, mg/L	Inhibition zone diameter, mm ^a	MIC, mg/L	Inhibition zone diameter, mm ^a
	≤S	>R	≥S	<R	ATU ^b	≤S	≥R	≤S	≥R	≤S	≥R
Enterobacterales	2	2	22	22	18-22	2 ^c (4) ^d	4 ^c (8) ^d	4	16	4	16
<i>P. aeruginosa</i>	2	2	22	22	14-22	2 ^c (4) ^d	4 ^c (8) ^d	4	16	1	4
<i>Acinetobacter</i> türleri	IE ^e	IE	-F	-F	-	IE	IE	4	16	1	4
<i>Stenotrophomonas maltophilia</i>	IE	IE	-H	-H	-	IE	IE	1 ⁱ	-	-	-
PK/PD	2	2	-	-	-	-	-	-	-	-	-

^aCefiderocol disk containing 30 µg; ^bATU, area of technical uncertainty; ^c interpretive criteria applicable to pneumonia; ^dinterpretive criteria applicable to infections other than pneumonia; ^eIE, insufficient evidence; ^fWith a 30-µg cefiderocol disk, inhibition zones ≥17 mm are associated with MICs below the PK/PD susceptibility threshold (S ≤2 mg/L); ^g Inhibition zones ≤14 mm should not be assigned an interpretive category because susceptible, intermediate, and resistant isolates may all yield values within this range. Therefore, cefiderocol results for isolates with zones ≤14 mm should only be reported after MIC determination; ^h With a 30-µg cefiderocol disk, inhibition zones ≥20 mm are associated with MICs below the PK/PD susceptibility threshold (S ≤2 mg/L); ⁱ Breakpoint determination is primarily supported by PK/PD parameters, with only limited clinical evidence available.

5. Mechanisms of Resistance

Among carbapenemase-producing organisms, the loss of OmpK35 and OmpK36 porins in *Klebsiella pneumoniae* and the increased expression of the MexAB-OprM efflux pump in *P. aeruginosa* have been shown to have no substantial effect on cefiderocol activity (Ito et al., 2018). In contrast, mutations that impair the function of genes encoding TonB-dependent receptors involved in iron transport, such as *piuA*, *pirA*, and *fiuA*, may contribute to the development of cefiderocol resistance by reducing its uptake into bacterial cells. In addition, the presence of PER- and NDM-type β -lactamases has been associated with elevated cefiderocol minimum inhibitory concentrations (MICs). These findings suggest that cefiderocol resistance may result from the combined effects of alterations in iron transport systems and β -lactamase production (Malik et al., 2020).

Alterations in AmpC β -lactamases may compromise the activity of ceftolozane-tazobactam and ceftazidime-avibactam against *P. aeruginosa*. These alterations may also reduce susceptibility to cefiderocol while increasing susceptibility to imipenem-relebactam (Simner et al., 2021). Furthermore, cross-resistance between ceftazidime-avibactam and cefiderocol has been reported in ceftazidime-avibactam-resistant KPC β -lactamase mutants (Hobson et al., 2021).

Resistance to cefiderocol is not restricted to alterations in bacterial iron transport systems. In addition to KPC carbapenemase variants, certain extended-spectrum β -lactamases (ESBLs), including PER, BEL, and SHV-12, as well as some metallo- β -lactamase (MBL) variants, including NDM and SPM, have been shown to increase cefiderocol MICs. This effect has been reported to be more pronounced in *P. aeruginosa* isolates than in *E. coli* and *K. pneumoniae* (Poirel et al., 2022).

Another mechanism contributing to the development of cefiderocol resistance involves structural alterations in AmpC β -lactamases. Deletions and point mutations within the R2 loop of the AmpC enzyme identified in *Enterobacter cloacae* have been shown to increase the MICs of both cefiderocol and ceftazidime-avibactam. These findings suggest that structural modifications of AmpC may reduce susceptibility to both antimicrobial agents (Kawai et al., 2020).

6. Conclusions

Cefiderocol is a novel parenteral siderophore cephalosporin that combines siderophore-mediated iron transport with the bactericidal activity of β -lactam antibiotics. Through its catechol side chain, which forms a complex with ferric iron, cefiderocol exploits the iron uptake systems of Gram-negative bacteria to facilitate active transport across the outer membrane. At the same time, it exhibits high stability against hydrolysis by a broad range of β -lactamases, including both serine β -lactamases and metallo- β -lactamases. These unique structural and functional characteristics enable cefiderocol to overcome several major β -lactam resistance mechanisms commonly encountered in Gram-negative bacteria, including carbapenemase production, porin loss, and increased efflux pump activity.

Evidence from in vitro and in vivo investigations supports the activity of cefiderocol against carbapenem-resistant Gram-negative organisms. Although resistance can emerge, current evidence does not suggest a greater tendency for resistance development compared with other β -lactam agents. Its clinical efficacy has been demonstrated in complicated urinary tract infections and other serious systemic infections. The broad spectrum of activity of cefiderocol, particularly against metallo- β -lactamase-producing *Enterobacterales*, multidrug-resistant or carbapenem-resistant *P. aeruginosa*, and *A. baumannii*, supports its role as an important therapeutic option for the management of severe Gram-negative infections for which effective treatment alternatives remain limited.

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CHAPTER 4

ENTERAL NUTRITION PRACTICES AND NURSING MANAGEMENT: CURRENT APPROACHES

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Nutrition and Enteral Nutrition

Nutrition refers to the adequate and balanced intake of organic and inorganic nutrients, such as proteins, carbohydrates, fats, vitamins, minerals, and water, to sustain vital functions. Because nutrient composition varies, it is not possible for a single food item to fully meet the requirements of other nutrients (Kıskacı et al., 2023). Adequate nutrient intake improves overall health by supporting physical and mental potential (Alija et al., 2024), ensuring the continuation of essential life-sustaining functions, and serving as an indicator of health in society (Demir Kıray, 2026; Sánchez-Sánchez et al., 2021). Nutritional issues, whether stemming from deficient or excessive intake, pose significant global health risks (Alija et al., 2024). Therefore, regular assessment of nutritional status and adjustments based on tolerance and requirements are vital for optimizing care (Smith et al., 2024). Systematic assessment of nutritional status is recommended starting from the initial hospital admission (Demir Kıray, 2026). The most common nutritional deficiency encountered in patients is protein-energy malnutrition. If left untreated, it can cause general weakness, increase the risk of complications, delay wound healing, and impair immune function. In adults, nutritional support is provided through enteral or parenteral routes. (Sarigül, 2026).

Enteral nutrition is the administration of nutrients via the gastrointestinal system (GIS) in patients whose GIS is functional but whose daily energy, protein, and macro- and micronutrient requirements cannot be adequately met orally (Compher et al., 2022; Kurt, 2026). It is reported that this approach helps maintain the natural functions of the GIT by physiologically supporting digestive and absorptive processes, preserving the integrity of the intestinal mucosa, reducing bacterial translocation, and supporting microbial diversity. Furthermore, enteral nutrition has been shown to provide significant benefits, including maintaining gut-associated immune responses, reducing systemic inflammation, regulating stress-induced hypermetabolic processes, and preserving organ function (Moghaddam et al., 2024; Kurt, 2026).

Enteral Nutrition Indications and Contraindications

According to the published Enteral Nutrition Guidelines, enteral nutrition is recommended for patients who were previously well-nourished but have received inadequate nutrition for the last 7 days, for those who have been inadequately nourished for longer than 7 days, or for those with malnutrition (Singer et al., 2023).

Various medical conditions and physiological states can lead to inadequate nutrition. Enteral nutrition is often preferred for patients who cannot receive adequate nutrition due to altered levels of consciousness, mental status changes, and related factors (Singer et al., 2023; Fugazza et al., 2022; Yılmaz, 2026).

For cachectic oncology patients with inadequate oral intake, enteral nutrition is recommended when oral intake is less than 60–75% of the planned nutrient intake for 7–14 days, or when adequate oral intake cannot be tolerated due to side effects such as nausea and vomiting (Bechtold et al., 2022; Yılmaz, 2026).

For some individuals who develop short bowel syndrome after surgery, enteral nutrition is recommended to support intestinal adaptation. This approach is an important therapeutic option that increases the absorptive capacity and functional efficiency of the remaining bowel tissue (Doley, 2022). However, severe intestinal bleeding, perforation, or obstruction; severe diarrhea or vomiting; and a high risk of aspiration are contraindications (Demir Kıray, 2026; Hu et al., 2025).

Routes of Enteral Nutrition

Enteral nutrition is delivered through naso(oro)gastric or nasointestinal feeding tubes, jejunostomy, or gastrostomy (Kaşıkçı & Akın, 2021). The most common methods for short-term enteral nutrition include nasogastric, nasoduodenal, nasojejunal, and orogastric tubes (Singer et al., 2023). These tubes are generally recommended for nutritional plans lasting less than 30 days, and NG tubes placed proximal to the gastric outlet are reported as the most frequently used method in clinical practice (Compher et al., 2022; Singer et al., 2023). Suitable alternatives, such as PEG or jejunostomy, are indicated for patients requiring long-term enteral nutrition (Kurt, 2026; Seyhan et al., 2024). Feeding tubes are placed through the abdominal wall into the stomach (gastrostomy) or jejunum (jejunostomy) using surgical or laparoscopic techniques. Placement of an enterostomy tube into the stomach via a percutaneous endoscopic approach is termed percutaneous endoscopic gastrostomy (PEG). Extending a PEG tube into the jejunum is termed PEG/J, whereas direct percutaneous endoscopic jejunostomy is termed PEJ (Kaşıkçı & Akın, 2021; Yüce, 2026).

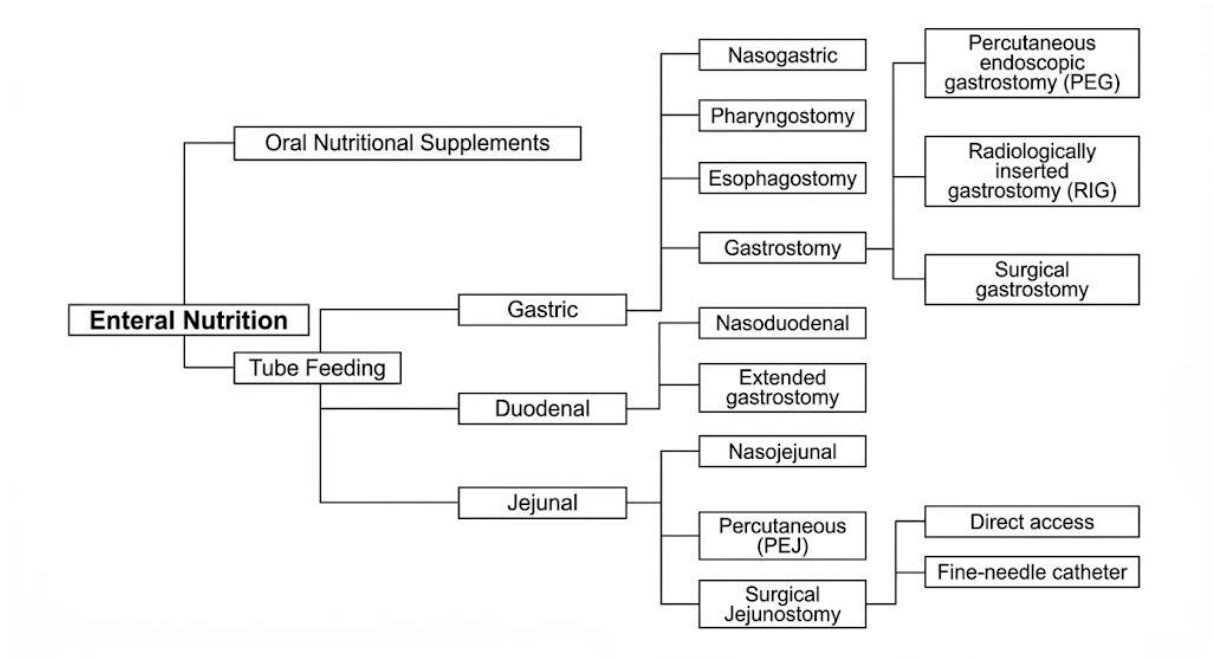


Figure 1. Routes of enteral nutrition (Ünlüsoy & Bahar, 2012).

Methods of Administering Enteral Nutrition

When selecting the enteral nutrition administration method, decisions are based on the patient's diagnosis, mode of nutrition, administration method, and tolerance status. Additionally, the availability of resources and the status of equipment play important roles. In the literature, nutrition administration methods are generally classified as continuous, bolus, and intermittent (Doley, 2022; Yilmaz, 2026).

Continuous Nutrition

In the continuous nutrition method, nutritional products are administered to the patient via an infusion pump over a specified period. Feeding solutions are generally administered at a low, continuous rate for 16–24 hours (Zhou & Zhou, 2026). To prevent intolerance, continuous nutrition is initiated at 20–50 mL/h and increased by 10–25 mL/h every 4–6 hours until the target rate is reached. Because it reduces high gastric residual volume (GRV) and the risk of reflux-associated aspiration while ensuring controlled delivery of nutrients to the intestines, this method is widely preferred for intubated, sedated, and critically ill intensive care patients who cannot tolerate intermittent or bolus feeding. On the other hand, it can be disadvantageous due to the limited hanging time of nutritional solutions in the feeding bag (up to 24 hours for closed systems and 8 hours for open systems) and the need for continuous monitoring (Doley, 2022).

Bolus Nutrition

The bolus infusion method is an enteral nutrition approach that involves administering nutritional products in high volumes and rapidly, at short intervals. In this method, nutrients are delivered to the patient, usually in 5–10-minute intervals, using a syringe or gravity (Abdelbaky et al., 2024). Bolus nutrition is widely used in conscious, mobile patients who do not wish to be tethered to a feeding pump. Because diarrhea and aspiration can occur with this route of administration, it is recommended for use in clinically stable patients fed via a gastric tube (Demir Kiray, 2026).

Intermittent Nutrition

It is stated that the intermittent nutrition method is based on dividing the total 24-hour nutritional intake into 3–6 equal meals (De Lazzaro et al., 2022). It is recommended that each meal be administered over 30–90 minutes, typically delivering 150–250 mL during this period. Nutrition should be administered in a controlled manner, preferably drop-wise or via a feeding pump whenever possible (Goksu et al., 2025; Kurt, 2026).

Enteral Nutrition Complications

It is reported that a significant portion of complications during enteral nutrition stems from largely preventable factors, such as incorrect administration techniques, inappropriate feeding rates, unsuitable solution characteristics, and inadequate clinical monitoring. Therefore, the most effective approach to reducing complications is to conduct the procedure in accordance with standardized protocols and to monitor the nutritional process regularly and carefully (Turan et al., 2024; Zare-Kaseb et al., 2025). The literature emphasizes that not only medical interventions but also a multidisciplinary approach, and particularly the quality of nursing care, are decisive elements in preventing complications (Batar et al., 2021). Complications related to enteral nutrition are noted to present across a wide spectrum, ranging from mild GI discomfort to life-threatening clinical pictures (Batar et al., 2021; Kurt, 2026). Thus, understanding the types of complications that may develop during enteral nutrition and their mechanisms of occurrence is of paramount importance for both identifying prevention strategies and intervening in a timely and appropriate manner when complications occur (Watkin et al., 2025).

The most common complications of enteral nutrition are classified into three categories (Ayık & Enç, 2019; Yıldız, 2025).

Gastrointestinal Complications: GIS complications, including nausea, vomiting, diarrhea, constipation, and abdominal distension, may develop (Demiray et al., 2020).

Mechanical Complications: Mechanical complications, including tube displacement, tube occlusion, aspiration pneumonia, and irritation, may occur (Demiray et al., 2020; Elhamdi et al., 2023; Yıldız, 2025).

Metabolic Complications: Metabolic complications, including hyperglycemia, fluid-electrolyte imbalances, and overhydration, may develop (Yıldız, 2025).

The most common complications in individuals undergoing enteral nutrition include diarrhea, constipation, nausea, vomiting, distension, pulmonary aspiration, feeding tube occlusion, and hyperglycemia (Elhamdi et al., 2023; Jordan & Moore, 2020; Yıldız, 2025). The occurrence of such complications and the interruption of enteral nutrition lead to inadequate fulfillment of patients' nutritional requirements, resulting in increased mortality and morbidity rates, elevated costs, and prolonged hospital stay (Demiray et al., 2020).

Nursing Care in Enteral Nutrition

Nurses play a critical role in planning, implementing, and continuing enteral nutrition (Wangari et al., 2024). Intensive care nurses, as effective members of the nutritional support team, play a significant role in determining energy and nutrient requirements, selecting the appropriate enteral access route, implementing nutritional protocols, and preventing complications (Zanten et al., 2019; Kurt, 2026). In this process, core nursing responsibilities include verifying tube placement, administering solutions at appropriate rates and volumes, monitoring GRV, reducing aspiration risk, and detecting GIS complications early (Jenkins et al., 2024).

Prior to initiating enteral nutrition, nurses are advised to plan the composition, administration rate, and total volume of the solution to be used, taking into account the patient's clinical condition, metabolic requirements, and GIS tolerance (McClave et al., 2016; Kurt, 2026). During the implementation stage, nurses are reported to assess whether the prepared solution is suitable for the patient's GI tolerance and to make their selection based on this evaluation (McClave et al., 2016). Furthermore, it is indicated that the tube must be flushed appropriately before and after feeding to prevent tube occlusion, with flushing requirements varying by administration method, whether continuous, intermittent, or bolus feeding (Watkin et al., 2025).

To reduce the risk of aspiration, elevating the head of the bed to 30–45° during feeding is recommended. During the monitoring phase of enteral nutrition, nurses regularly assess GRV measurements, abdominal distension, defecation frequency, and signs of GI tolerance. Based on these data, they may adjust the feeding regimen (Turan et al., 2024; Fırat Turan & Katar, 2023). In addition, it is emphasized that the skin around the tube should be assessed daily and monitored for the risk of infection, irritation, and pressure injury (Seyhan et al., 2024; Kurt, 2026).

Nurses are also responsible for providing education and counseling to patients and their families. Providing information on the purpose and administration of enteral nutrition, potential complications, and the home care process is indicated to help establish a post-discharge monitoring plan (Akçay et al., 2020). It is reported that nursing care does not consist solely of technical applications; non-pharmacological, integrative methods can also be used to enhance patient comfort and prevent complications. These methods are noted to include interventions that support GIS motility, such as abdominal massage, warm application, and appropriate positioning. In this regard, nurses are expected to deliver holistic care during enteral nutrition by employing both pharmacological and non-pharmacological approaches (Yang et al., 2025; Wangari et al., 2024).

Current Approaches in Enteral Nutrition

- In intensive care, early enteral nutrition (EN) should be initiated within 48 hours for patients who cannot be fed orally and are hemodynamically stable (Singer et al., 2023; Doğanay et al., 2023; Mutlu et al., 2026).
- Preferential early enteral nutrition over parenteral nutrition is classified as ESPEN Level of Evidence A, whereas initiating enteral nutrition without delay within the first 48 hours is classified as ESPEN Level of Evidence B (Singer et al., 2023; Doğanay et al., 2023).
- Enteral nutrition should be delayed in cases of uncontrolled shock, life-threatening hypoxemia, hypercapnia, or acidosis; active upper GI bleeding; overt intestinal ischemia; abdominal compartment syndrome; high-output intestinal fistulas; or when gastric residual volume exceeds 500 mL in 6 hours (ESPEN Level of Evidence B) (Mutlu et al., 2026).

- Enteral nutrition should be administered at low doses to patients undergoing therapeutic hypothermia, those with intra-abdominal hypertension without abdominal compartment syndrome, and those with acute liver failure (ESPEN Level of Evidence B) (Singer et al., 2023).
- Gastric access should be the standard approach to initiate enteral nutrition (ESPEN Level of Evidence- Good Practice Point). However, if the risk of aspiration is very high or gastric feeding intolerance (e.g., gastroparesis) cannot be overcome despite prokinetic agents, postpyloric (nasojejunal) feeding should be preferred (ESPEN Level of Evidence GPP and B) (Singer et al., 2023).
- Continuous infusion should be preferred over bolus feeding for critically ill intensive care patients (ESPEN Level of Evidence B). Continuous infusion enhances gastrointestinal tolerance, reduces the risk of diarrhea, and helps balance physiological metabolic responses (Singer et al., 2023; Doğanay et al., 2023).
- In the early phase, hypocaloric nutrition should be provided at a level not exceeding 70% of estimated or measured energy requirements (ESPEN Level of Evidence B) (Singer et al., 2023).
- Nutrition should be initiated at a low rate (10–20 mL/h) and increased gradually based on patient tolerance to reach the target dose within 3–5 days (Mutlu et al., 2026).
- To reduce the risk of aspiration, the head of the bed should be elevated by at least 30 degrees (Mutlu et al., 2026).
- Routine measurement of Gastric Residual Volume in every patient is not recommended. Evidence shows that this measurement does not reduce the risk of pneumonia; on the contrary, it delays the achievement of nutritional goals and causes unnecessary interruptions of enteral nutrition. In patients undergoing GRV monitoring, enteral nutrition can be safely continued when the volume is up to 500 mL (Singer et al., 2023; Mutlu et al., 2026).
- Before initiating nutrition, the correct placement of the feeding tube (nasogastric/nasoenteral) must be verified by chest radiograph (Mutlu et al., 2026).
- To prevent occlusion, the tube should be flushed with at least 30 mL of clean water every 4 hours during continuous feeding and before and after each feeding during intermittent feeding. If occlusion occurs, a guidewire must not be used; the tube should

be cleared with warm water delivered under syringe pressure (Singer et al., 2023; Doğanay et al., 2023; Mutlu et al., 2026).

Conclusion

Enteral nutrition plays a fundamental role in caring for patients at risk of malnutrition by maintaining GI integrity, supporting the immune response, and reducing complication rates. The safe execution of the procedure depends on determining the appropriate tube route and feeding method (continuous, bolus, or intermittent) suited to the patient's clinical picture and on managing the procedure in accordance with current clinical guidelines. Alongside standard practices such as positioning the head of the bed at 30–45° and routine tube care, innovative approaches such as avoiding routine GRV monitoring and initiating early feeding within the first 48 hours directly shape the quality of care. In this process, nurses who detect complications early, incorporate patient and family education into planning, and collaborate with the multidisciplinary team play a critical role in achieving nutritional goals, ensuring patient comfort, and reducing length of hospital stay and mortality.

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