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RESEARCH PAPER

Methods for the Recovery and Purification of Polyene Antifungals

David R. Worthen, Michael Jay, and Paul M. Bummer*

Division of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington

ABSTRACT

Despite the development of newer antifungal drugs, the polyene antifungals continue to be the most potent broad-spectrum fungicides available for clinical use. The incidence and severity of fungal infections are on the rise, underscoring the need for new and more effective antifungal drugs. Thus, the search for new polyene antifungals is ongoing. The limited solubility, polymorphic character, and inherent chemical instability of these compounds make their economical recovery and purification from mass culture challenging problems in biotechnology. This article provides a comprehensive review of the methods that have been developed for the recovery and purification of amphotericin B and nystatin, the two most important polyenes currently in clinical use.

KEY WORDS: *Amphotericin; Fermentation extraction; Nystatin; Polyene.*

INTRODUCTION

Invasive and disseminated fungal infections are serious clinical problems, difficult to diagnose, often lethal to patients, and increasing in occurrence (1,2). Despite the development of newer antifungal drugs, the polyene antifungals, including amphotericin B (AMB; Fig. 1) and nystatin A_1 (NYST; Fig. 1), continue to be the most potent broad-spectrum fungicides available for clinical use (3,4). AMB, produced by *Streptomyces nodosus* (5), is available in a deoxycholate complex for injection, as well as in lipid complex and lipid dispersion formulations that possess superior therapeutic and toxicity profiles (1,2,6). Isolated from *Streptomyces noursei*, NYST is employed topically for the treatment of a variety of fungal infections (4,7,8) and is also being developed for systemic use in several lipid-based formulations (9,10). The urgent need for new and improved antifungal agents is underscored by the considerable amount of research devoted to polyene antibiotics. A large number of novel polyenes

* Corresponding author. 514 College of Pharmacy, University of Kentucky, Rose Street, Lexington, KY 40536-0082. Fax: 606-257-7585; E-mail: pbumm01@pop.uky.edu

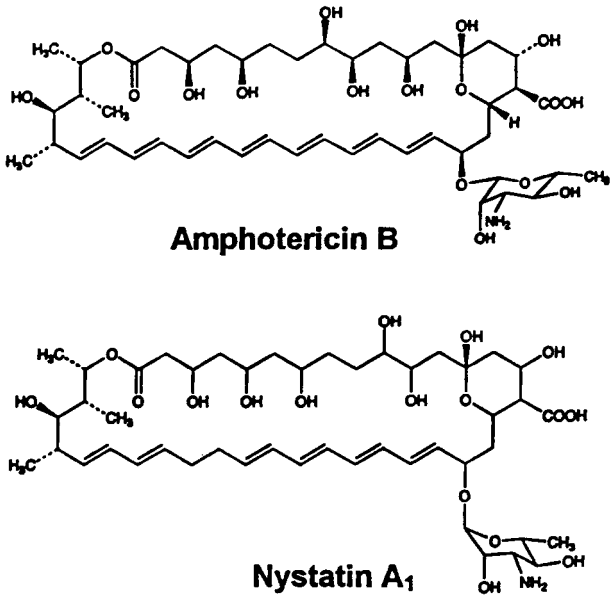

Figure 1.

and polyene formulations are currently being developed for use as antifungal drugs in humans and animals (11–18).

Produced as secondary metabolites by several species of fungi, the production and purification of polyene antifungals are confounded by their inherent chemical instability. The polyenes typically possess one or several potentially unstable structural functionalities, including hydrolyzable esters, acetals, and hemiacetals, as well as conjugated polyene systems vulnerable to oxidation (19). Thus, all of the polyene antifungals, to a certain extent, are subject to inactivation or frank degradation by conditions routinely encountered during their production in and recovery from mass culture. Most notable of these potentially degradative conditions include moisture, elevated temperature, atmospheric oxygen, polyvalent metals, and exposure to light (19,20).

In addition to their potential to degrade polyenes during recovery, current polyene production methods typically require the extensive use of organic solvents, complexing agents, acids, and salts (19,21). These represent both significant material expense and a potential environmental concern for the manufacturer. Further complicating polyene purification is the fact that virtually all crude polyene isolates from *Streptomyces* contain several distinct, although physicochemically similar, isoforms, only one of which may be desirable for clinical use. NYST, for example, consists of at least three distinct components, NYST A_1, A_2, and A_3 (19,22). Similarly, an initial *Streptomyces nodosus* extract typically contains a mixture of two major forms of amphotericin, the chemically unstable tetraene, amphotericin A, and the clinically useful heptaene, amphotericin B (AMB) (19,23). The economical recovery of such similar polyene isoforms from one another, as well as from related impurities devoid of biological activity, while minimizing loss or degradation of product, is an ongoing industrial challenge.

As a consequence of the continuing search for new polyenes, as well as the limitations of current polyene production methods, research continues to be directed toward the improvement of polyene production and purification techniques (24,25). Notably, these include methods for the recovery of polyenes from industrial effluent and formulation waste streams, as well as those for traditional fermentation recovery. A great deal of work has gone into developing and refining techniques for the recovery, separation, and purification of polyene antifungals. Nevertheless, each has several disadvantages that contribute significantly to the economic and environmental cost of producing polyenes.

This article provides a review of the processes and methods used to recover and purify the two most important polyene antifungals, AMB and NYST. A compilation of these methods will serve as a useful reference for those individuals and enterprises concerned with optimizing the production of polyenes already in clinical use, as well as of novel polyenes currently in development.

AMPHOTERICIN B

Standard Methods of Purification

Initially reported by Gold et al. (26), an intensely yellow material with powerful antifungal activity was isolated from *Streptomyces nodosus* by extracting sedimented culture solids with *n*-propanol, isopropanol, or butanol. Significant amounts of the new antibiotic could also be recovered from the fermentation broth. The recovery and purification of this material, found to be a mixture of two antifungal agents designated amphotericin A (AMA) and amphotericin B (AMB), was further refined by Vandeputte et al. (5).

These antibiotics, found to be much more soluble in hydroalcoholic mixtures of lower alcohols as opposed to either pure alcohols or water, were extracted from the filtered fungal cake using water-saturated butanol. Alternatively, the wet mycelial cake was extracted with methanol or propanol, and the antibiotic was precipitated on evaporation of the aqueous concentrate. Good recovery was also afforded by extracting the fermentation solids

with a water-miscible organic solvent, such as dimethylsulfoxide (DMSO) or *N*,*N*-dimethylformamide (DMF). Extraction was followed by precipitation with ethyl acetate or another less-polar solvent, and the precipitate was then washed with aqueous acetone, giving a crude product.

When employing these methods, the crude material recovered contained up to 70% mixed amphotericins. To separate AMA and AMB from this crude material, it was slurried in 2% w/v methanolic $CaCl_2$ for 2–3 h. The undissolved material consisted primarily of AMB, while an initial AMA product was recovered from the filtrate by precipitation with water. This precipitate, up to 95% AMA, was further purified by methanolic $CaCl_2$ solubilization, followed by fractional precipitation with water to remove the remaining AMB. The undissolved material from the original preparation consisted primarily of AMB and some biologically inactive impurities. After a 30-min treatment with acidified DMF, the filtered solution was diluted with methanol. By adding water while maintaining a constant pH 5, AMB, 80% pure and containing only 1%–2% AMA, was recovered.

This process of heating and extracting culture broth and sediment with lower alcohols, followed by alkaline metal or acid salt formation, then precipitation and crystallization, was the basis for the earliest commercially viable process for large-scale AMB production (27). Most early improvements to amphotericin production were directed at increasing the specific biological activity of the preparation while removing impurities and were achieved by manipulating the pH and solvent systems employed during the purification of the crude product. Precipitating crude AMB from a DMF solution at neutral pH (6.5–7.0), followed by an acetone-propanol wash, resulted in significant increases in purity with minimal loss of biological activity (28).

Maintaining neutral pH is not tantamount to efficient purification and recovery, however. Etingov et al. (29) described a process in which crude AMB could also be purified from aqueous DMF at acidic (2.0–3.6) pH, followed by a 50% (v/v) propanol wash to give the final product. By employing serial pH adjustments, Kul'bach et al. recovered 8 g of AMB with an activity of 800 μg/mg from 25 g of crude AMB with a specific biological activity of 500 μg/mg (30). Crude AMB was stirred in DMF at pH 7.2, then the pH was adjusted with HCl to 3.2 to precipitate impurities. The AMB precipitated from the filtrate on pH adjustment to 6.5 with NaOH. Washing for 2 h with propanol, followed by an acetone rinse, yielded purified AMB.

Michel et al. (31) devised a method for producing purified, crystalline AMB by adjusting both solvent and pH parameters while maintaining controlled temperature to maximize product stability. Crude AMB, suspended in a mixture of methanol, DMF, and citric acid at near-neutral pH and at 15°C–20°C, was agitated and filtered. After adding methylene chloride to the filtrate and adjusting the pH to 6 with triethanolamine (TEA), the slurry was gently heated to 45°C for 30 min to convert the AMB into its crystalline form. The product, isolated by filtration, was washed with cold aqueous methanol, then slurried and washed with acetone. Thus, pure, crystalline AMB could be recovered in high yield.

Certain contaminants, including biologically inactive heptaenes, pigments, metals, and fluorescent impurities, are undesirable by-products of AMB biosynthesis. These not only reduce the specific activity of AMB preparations, but also may promote product degradation. As summarized by Teresin (19), certain pigments and fluorescent impurities may promote oxidation and polymerization of the olefinic polyenes, rendering them inactive. The rate of autooxidation is significantly enhanced in the presence of heavy metals, particularly Cu^{++} and Fe^{+++}. Further, the presence of certain metabolic by-products, such as amines and organic acids, may promote polyene decomposition by catalyzing the hydrolytic cleavage of their lactone and acetal functionalities. In light of the deleterious effects of such impurities on product stability, Kul'bach et al. modified their method (reported above) by extending the pH range used in the AMB recovery process to 0.5–7.0, while employing successive precipitation and centrifugation steps (32). This method, similar to another improved method reported by Etingov et al. (33), afforded more efficient removal of inactive heptaenes and other impurities from the final product. The complete removal of fluorescent and other impurities from even semipurified AMB preparations has been achieved through the application of advanced, reverse-phase high-performance liquid chromatography (HPLC) techniques to AMB purification (34). Unfortunately, the cost of employing such a process on an industrial scale would likely be prohibitive.

The solubility of relatively hydrophobic compounds, such as AMB, can often be enhanced in a particular solvent system by forming a salt or complex. Further, depending on their hydration tendency, polarizability, and capacity to destructure water, many types of organic and inorganic electrolytes are routinely added to aqueous preparations to ''salt-in'' and increase the solubility of otherwise poorly soluble compounds (35,36). An electrolyte-induced increase in solubility has been useful for improving the production and purification of many com-

pounds as raw materials, as well as in their pharmaceutical formulation. When complexed with Ca^{++} ions or with oxalic or succinic acid, the solubility of AMB in water is greatly enhanced, while that of inactive impurities remains low. Thus, the complexation of crude AMB with one of these agents in methanol, followed by redissolution in water and filtration to remove particulate impurities, was found to be an efficient method of AMB purification by differential complex solubilization (37). Insoluble, Ca^{++}-complexed impurities could be removed by filtration and AMB recovered on concentration of the filtrate.

Solubility enhancement in organic solution has also been explored as a means of purifying AMB. AMB is fairly soluble in methanol or acetone previously saturated with either NaI or sodium thiocyanate (NaSCN), while undesirable contaminants tend to precipitate out of the same solutions (38). Michel and Fralick (38) took advantage of this observation by dissolving crude AMB in one of these salt-saturated solvents, then filtering the precipitated contaminants. The filtrate was combined with additional methanol, water, and DMF, then heated to promote crystallization. Pure crystalline AMB was thus collected.

A number of processes have been reported for producing purified crystalline AMB from fermentation extracts and solids (31,38). To simplify these elaborate recovery processes, which include costly filtration, solvent extraction, and recrystallization steps, Ko and Szarka (39) devised a process for forming AMB crystals directly in the fermentation medium. Here, lysis of *Streptomyces nodosus* in the fermentation broth was induced by heating the medium to temperatures ranging from 70°C to 130°C over various lengths of time, which ranged from 1 min to 10 h. The heating process induced the formation of AMB crystals directly in the fermentation medium. Alternatively, lysis of the organism was induced by the use of enzymes, such as lysozyme. In both cases, crystallization could also be induced by seeding the medium with AMB crystals (0.1 g/L medium). When utilizing centrifugation to recover the AMB crystals, yields as high as 95% were reported. A schematic diagram of the general steps involved in polyene recovery and purification from fermentation culture is shown in Fig. 2.

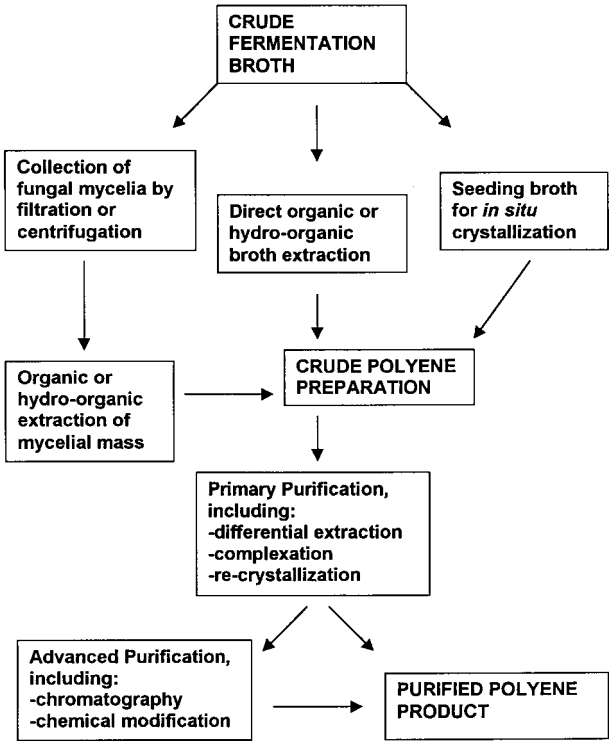

Figure 2. Schematic diagram of the processes involved in the recovery and purification of polyenes from fermentation culture.

NOVEL APPROACHES TO PURIFICATION

Several other methods have been developed for the removal of fermentation by-products and other contaminants, thereby purifying AMB. To remove commonly encountered bacteria from crude AMB, Tang (40) first collected the fermentation broth solids by filtration, then extracted the crude AMB with methanol. After adjusting the pH from 2.5 to 4, then filtering the resulting slurry, the methanolic solution of AMB was passed through a column containing Ambergard XE-352 ion-exchange resin, which effectively removed both gram-positive and gram-negative bacteria. The purified AMB was easily crystallized using standard techniques.

Liquid ion-exchange techniques have also been employed at the laboratory scale for recovering and purifying AMB from *S. nodosus* whole broth. AMB was produced directly from whole fermentation broth by extracting the basified (pH 10.5) broth with 1-butanol in the presence of the liquid ion-exchange reagent Aliquat 336. AMB precipitated from the organic phase on acidification, leaving AMA in solution. After washing the crude product, AMB more than 90% pure was produced (41). The authors noted that the AMB recovered was in a crystalline form different from that normally recovered using other techniques. However, this material was readily converted to that form commonly encountered by

recrystallization from appropriate solvents. These observations suggest that polymorphic changes, solvate formation, or both may have occurred. Polyene polymorphism has been well described in the literature (42). In light of the potential solubility, stability, and toxicity differences between polymorphs (42,43), the development of a purification process that reliably produces AMB of the same polymorphic form might be of particular interest in pharmaceutical manufacture.

As noted previously (25), chromatographic techniques, well established for the purification, validation, and assay of pharmaceuticals, have been extensively employed for the purification and recovery of the polyenes. Reversed-phase HPLC (RP-HPLC) has been employed for the recovery and assay of AMB from a number of preparations, including the crude fermentation product and various AMB formulations. Moreno and colleagues (44) employed a simple extraction process, followed by RP-HPLC, for the recovery, purification, and quantification of AMB from oil-water-lecithin AMB microemulsioins. A solid-phase chromatographic extraction method, designed for separating and recovering AMB and the lipids used in a liposomal AMB formulation, was reported by Stewart et al. (45). The crude AMB-lipid mixture left in the formulation waste stream was applied to a silica sorbent column, and the lipids were eluted with a basic $CHCl_3$-methanol mobile phase. Lipid-free AMB could then be eluted using slightly acidified methanol, affording AMB recoveries up to 95%. Although effective at recovering AMB from AMB-lipid mixtures, the method requires the extensive use of chlorinated solvent and expensive silica solid phase, limiting its potential for industrial scale-up.

While the purpose of this section is to review methods for recovering and purifying AMB proper for use in pharmaceutical manufacture, the results of a number of other studies may be useful to those involved in other aspects of AMB research. Ishii et al. (46) reported a process for purifying AMB derivatives, specifically, AMB methyl ester. AMB methyl ester, produced by esterification in methanol with diazomethane, was purified by dissolving it in acidic solution in the presence of urea. The AMB methyl ester dissolved, while insoluble impurities were removed by filtration or other appropriate means. The methyl ester was subsequently precipitated on adjustment of the pH to 12 or more. Presumably, the methyl ester could then be hydrolyzed back to AMB. While the removal of fermentation by-products has been the focus of most of the work reported here, advanced molecular biology and biochemical techniques have been explored to minimize the production of undesirable impurities during *S. nodosus* fermentation. Schaffner and Kientzler (47) devised a method for increasing the purity of AMB produced in culture by selectively inhibiting AMA biosynthesis with specific metabolic inhibitors.

NYSTATIN A_1

Standard Methods of Purification

The first report of a novel antifungal agent from a particular soil actinomycete, later designated *Streptomyces noursei*, came several years earlier than that for AMB, in 1950 (48). Hazen and Brown, who named this broad-spectrum antifungal fungicidin (NYST), extracted it in crude form from the surface growth of a liquid culture using lower alcohols. This extractive recovery procedure represents one general method most commonly employed for recovering NYST from culture. Typically, this involves the admixture of one or more water-miscible solvents into the entire fermentation medium (with or without pH adjustment), followed by removal of insoluble particulates by filtration, centrifugation, or another method. Separation of crude NYST is then afforded by precipitation, extract concentration, or some other means.

One representative process employing whole broth extraction is that developed by Renella (49). Here, whole *S. noursei* fermentation mash was extracted with *n*-butanol and centrifuged, and the solid crude NYST was filtered from the supernatant liquid. The solids were treated with either $CHCl_3$ or methyl isobutyl ketone to remove impurities. Depending on which solvent was used, NYST yield and specific antifungal activity were 71.5% and 3815 U/mg compared to 85.4% and 4020 U/mg for $CHCl_3$ and methyl isobutyl ketone, respectively.

Kubec et al. (50) employed a more elaborate whole broth extraction process for producing crystalline NYST. After diluting the medium with a 0.1% detergent solution, the dense bottom layer was spray-dried, and the remainder was extracted with 3% acetic acid in methanol. After adjusting the pH to 6.5 with oxalic acid and heating to 55°C, the methanol extract was added to an emulsion consisting of 55 parts water, 4.2 parts *n*-butanol, and 0.1 part sodium ethylenediaminetetraacetic acid (NaEDTA). The crystalline NYST that separated out was recovered by filtration, washed with methanol and acetone, and dried in vacuo at 50°C. The NYST product, recovered in 85% yield, had an activity of 4850 U/mg, and a low mineral content.

Since the majority of polyene antifungals produced by *Streptomyces* are localized in the fungal mycelia, the second general crude polyene production method, known as

mycelial extraction, affords efficient extraction while minimizing contamination by broth impurities. Lower alcohols were once again employed by Vandeputte and Nager (51) to produce crude NYST, this time from wet mycelia collected from the fermentation medium by filtration. The wet mycelia were extracted with methanol using a carboxylic acid and an organic amine, such as triethylamine, to maintain the pH at 6.0–6.5. The extract was filtered, the filtrate was concentrated to a 20^{th} of its original volume, and crude NYST was precipitated on dilution with an equal volume of 40% aqueous acetone and heating the mixture to 45°C. The crude material was washed repeatedly, first slurrying it in 40% aqueous acetone, then centrifuging the slurry to recover the solid product. When dried, the product had a potency of 4000 U/mg and minimal ash content. NYST yields from the methanolic mycelial extraction were even higher when freeze-dried mycelia were extracted with methanol in a blender (52). The process resulted in a fine, dry powder that did not require further grinding before formulation.

Methanolic solutions of divalent cations, such as $CaCl_2$, were as effective at solubilizing and extracting NYST as they were for AMB (53,54). The addition of water at controlled pH (about 6.0) induced precipitation and crystallization of crude NYST from such methanol-salt extracts. The purity of NYST extracted with methanolic $CaCl_2$ was enhanced by first prewashing the wet mycelia with acetone (55). After several acetone washings, the mycelia were extracted exhaustively with the methanol solution, the extracts were combined and diluted with water to precipitate impurities. After concentrating the filtrate by evaporation, then washing the product with water, 3430 g of crystalline NYST, with a specific activity of 4720 U/mg, were obtained from 8300 g of wet mycelia with an antifungal activity of 250 U/mg. Further improvements to this general methanolic extraction process, directed at stabilizing and preserving NYST during isolation, were developed by Culik et al. (56). The addition of 0.3% formaldehyde and Na_2SO_3 to the fermentation medium, followed by extraction with methanol and an anionic surfactant at pH 9.6 or with oxalic or citric acid, afforded NYST in 85% yield.

As a consequence of their water miscibility, good solvent characteristics, and capacity to be redistilled from and reused in the extraction process, alcohols are the solvents most commonly employed in industrial polyene extraction (19). Although they are relatively expensive and much more difficult to remove from the final product, other solvents, particularly DMF and DMSO, are even more efficient solvents for mycelial extraction of polyenes. Maidanov et al. (57) separated fungal mycelia from the fermentation medium, dried it at a modest temperature, then extracted the dry mycelium with pure DMSO or with 20%–35% aqueous solutions of it. DMSO appeared to be a particularly good solvent for NYST extraction. Indeed, NYST yields from DMSO extraction were as much as 40-fold higher than those obtained using other solvents.

Despite the careful, methodical refinement of these broth and mycelial NYST extraction protocols, present NYST recovery techniques still tend to produce relatively impure, low-potency products that require further purification (23). In light of the inherent chemical instability of NYST, the removal of impurities, including the removal of oxidation-promoting metals, is of particular concern (19). Lokshin and coworkers (58) discovered that the removal of metal ions from crude NYST with sodium hexametaphosphate in aqueous isopropanol significantly improved its stability in the presence of elevated humidity. A highly purified, crystalline NYST product was obtained when Mendelsohn (59) dissolved crude NYST in acetone saturated with NaI, NaSCN, or KSCN or in mixtures of these salts, useful for salting-in NYST into acetone solution. Pure NYST was subsequently precipitated by adding water, then recovered by filtration. In this manner, 20 g of crude NYST with a specific activity of 2202 U/mg were purified to give 10 g of NYST with an activity of 4113 U/mg.

Keseleski and Michel (60) carefully manipulated a number of solution parameters to purify crude NYST. After suspending the crude NYST in a solution of methanol, 2-methoxyethanol, and water at pH 3.5, the mixture was stirred until all of the NYST dissolved. Silica was added to flocculate impurities, the solution was filtered, and the filtrate was cooled to 10°C. After adjusting the pH to neutrality with TEA in cold water, the ionic strength was raised with NaCl, and the resulting suspension was heated to 45°C to induce crystallization. The crystal slurry was then chilled and held at 5°C for 2 h to complete crystallization. The product was collected by filtration, then washed with cold water and acetone. Purified NYST, 5640 U/mg in activity, was thus produced in 94% yield from starting material with an activity of 4960 U/mg.

The addition of chlorinated hydrocarbon solvents to alcoholic solutions of NYST, followed by precipitation with water, is another useful method for purifying crude NYST. A suspension of 15.4 g of crude NYST was slurried for 30 min in 300 ml of methanol and citric acid. Solid impurities were removed by filtration through diatomaceous earth, and a mixture of 75 ml methylene chloride, 12 ml TEA, and 588 ml cold water was added to

the filtrate. The seeded NYST crystals were collected by filtration to give 10.72 g (70%) purified NYST, 5360 U/mg in activity (61). Metzger also employed a mixture of methylene chloride and detergent to purify crude NYST further (68). In this case, 2 g crude NYST was suspended in 200 ml of sodium lauryl sulfate solution (aqueous) containing 8 ml methylene chloride. Then, 20 ml of 1 M *t*-octylamine HCl (aqueous) was added, producing a milky, amorphous NYST precipitate. Seed crystals were added to promote crystallization; the crystallized product was collected by filtration, then washed with methylene chloride to produce 1.56 g of purified NYST (86.6% yield).

Novel Approaches to Purifying Nystatin A_1

As with AMB, other, more complicated methods have been explored for purifying crude NYST, at least at the laboratory scale. Tang employed the same ion-exchange method previously described for AMB purification when analogously purifying crude NYST (40). To prepare a dry NYST precipitate for injection, Oita et al. (63) dissolved 1000 g NYST in a suspension of activated charcoal in 2500 ml DMF. The NYST was precipitated with diethyl ether, then filtered. The precipitate was then subjected to ion exchange with 400 g 2-ethylhexanoate sodium salt in 2500 ml methanol, followed by a second precipitation with 8000 ml diethyl ether. The purified NYST thus recovered was dissolved in water, then lyophilized in vials, ready for injection.

Other methods for purifying crude NYST, such as those employing preparative HPLC, have been extensively explored (25,64). Milhaud et al. (65) successfully separated and purified NYST and NYST complexes using semipreparative and microbore HPLC, methods also successfully applied to the recovery and purification of filipin, another polyene. Other chromatographic methods for purifying NYST have also been explored, including simulated moving bed chromatography, a countercurrent continuous separation process that usually requires less solvent than conventional chromatographic methods (66). Though capable of purifying NYST to a high degree, the expense and complexity of such costly chromatographic methods limit their usefulness at the industrial scale.

One promising method for recovering and purifying NYST from bulk aqueous dispersions was recently reported by Worthen et al. (67). These researchers employed foam fractionation, an adsorptive bubble separation technique (68,69), to recover NYST from a variety of solutions and suspensions. When employing foam fractionation, as much as 93% of the NYST in the bulk liquid could be recovered from dilute aqueous solutions. Moreover, this NYST was recovered in as little as 5% of the starting bulk liquid volume. Thus, the NYST concentration in the solutions recovered by foam fractionation was over 18-fold greater than that in the starting solution. Such high recovery and concentration of NYST by foam fractionation could be achieved at low temperature (10°C), neutral solution pH, and in an atmosphere of nitrogen gas, conditions ideal for maximizing NYST stability. The authors also demonstrated that the process could be scaled up for processing much larger volumes of NYST solutions and suspensions, suggesting a role for foam fractionation as a cost-effective alternative for the industrial production of polyene antifungals.

CONCLUSIONS

Polyene antifungals, including AMB and NYST, the mainstay of antifungal therapy, are becoming more and more important clinically. As the need for new antifungal drugs continues to expand (70,71), so does the search for novel polyene antifungals. The recovery and purification of these amphiphilic compounds, with their limited solubility, inherent chemical instability, heat lability, and multiple polymorphic forms (42,72), represent a great research and industrial challenge. Thus, careful review and consideration of established polyene production methods may be useful for limiting the cost and complexity of isolating and purifying novel polyenes, as well as for refining polyene production methods currently in use. Newer recovery and purification methods, such as foam fractionation, may prove to be cost-effective alternatives to current polyene production techniques. It is hoped that this review will be particularly useful to researchers and manufacturers engaged in the development and production of polyene antifungals.

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