acetonide-21-palmitate preparation as a selective drug delivery system for cutaneous administration may be optimal.

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Quantitation of Amphotericins by Reverse-Phase High-Performance Liquid Chromatography

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Abstract □ A reverse-phase high-performance liquid chromatographic (HPLC) method was developed for simultaneous potency determination of amphotericin A and amphotericin B in bulk amphotericin B preparations. This single, rapid, specific, and simple method can be used for qualitative and quantitative analyses and is proposed to replace a combination of cumbersome official tests presently in use. This new HPLC method employs an isocratic acidic phosphate buffer–methanol mixture in a reverse mode on an octadecylsilane-bonded silica column at ambient temperature, using UV detection at 313 nm. Results obtained by HPLC compare favorably with the official assay results. During these studies, an apparent heptaenic component, not previously described, was detected in commercial amphotericin B preparations at concentrations ranging from ~6 to 14%.

Keyphrases □ Quantitation—amphotericin B, amphotericin A, reverse-phase HPLC □ Amphotericin B—quantitation with amphotericin A, reverse-phase HPLC

Amphotericin B (1) (CAS registry 1397-89-3), produced by a strain of *Streptomycetes nodusus*, is an amphoteric macrocyclic polyene antibiotic that has become a valuable therapeutic agent in the treatment of fungal and monilial infections in humans (1). Polyene antibiotics are characterized chemically by strong absorption in the UV and visible regions of the spectrum, ascribed mainly to conjugated unsaturation with four (tetraenes) to seven (heptaenes) double bonds (1, 2). These substances are relatively unstable, and the heptaenes, in particular, are insoluble in most solvents.

Bulk amphotericin B is commercially available in two forms—one is purified for use in parenteral products (type I) and the other is a cruder grade for topical applications (type II). According to the official monograph (3), the potency of each form must be ≥750 μg/mg on the anhydrous basis. Both types may also contain amphotericin A, a cofermented tetraene very similar to nystatin; however, type I may contain ≤5% amphotericin A, whereas type II may contain ≤15% amphotericin A. Characteristics of amphotericin B are extensively discussed and reviewed with respect to physicochemical and analytical properties by Asher *et al.* (1) and Thomas (2).

Official requirements for the premarketing certification of amphotericin B (3) include a microbiological agar diffusion assay, a quantitative differential UV spectrophotometric procedure for the determination of the amphotericin A content, and a qualitative UV spectrophotometric identity test for amphotericin B. The microbiological assay measures the total activity of substances responding to a particular organism in comparison with an established standard of defined activity. Selectivity, precision, and reliability of this microbiological assay are inadequate, especially because of the deleterious effect of the high pH (4, 5) needed to solubilize the substance during this assay.

Reverse-phase high-performance liquid chromatography (HPLC) was demonstrated by Mechlinski and Schaffner (6) to be well suited to the qualitative analysis of such intractable

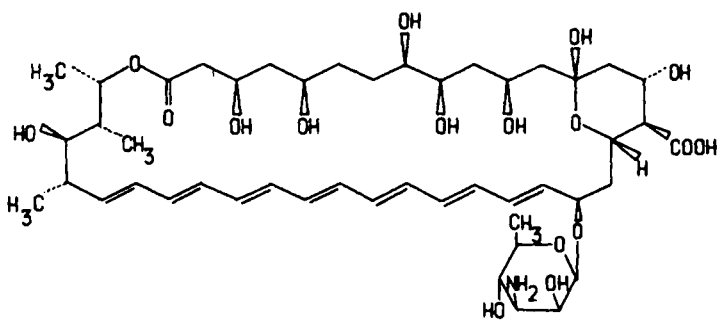

Table I—Comparison of the Code of Federal Regulation (CFR) and Liquid Chromatographic Methods for the Quantitation of Amphotericin B

Sample[a]	Potency, μg/mg[b] Mfg. Cert.	CFR Method	HPLC Method
Standard[c]	954	954	954
1[c]	952	924 ± 23 (4)	936 ± 33 (9)
2[d]	784	720 ± 63 (24)	784 ± 33 (9)
3[d]	807	755 ± 46 (6)	819 ± 42 (5)
4[d]	798	749 ± 53 (8)	754 ± 68 (15)
5[c]	939	840 ± 65 (7)	886 ± 37 (6)

[a] Batch numbers for samples 1–5 obtained from E. R. Squibb & Sons, Inc. are 58022-002, 58334-404, 58334-406, 53834-405, and 58366-003, respectively. [b] Mean ± *SD;* multiply by 0.1 to convert to percent potency. The number of analyses is given in parentheses. [c] Type I. Standard labeled at 954 μg of amphotericin B/mg. [d] Type II.

Table II—Comparison of the Code of Federal Regulations (CFR) and Liquid Chromatographic Methods for the Quantitation of Amphotericin A

Sample[a]	Amphotericin A Content, % Mfg. Cert.	CFR Method	HPLC Method
Standard[b]	0.24	0.24	0.24
1[b]	0.4	NT[c]	0.52
2[d]	0.27	0.32	0.26

[a] Batch numbers for samples 1–5 obtained from E. R. Squibb & Sons, Inc. are 58022-002, 58334-404, 58334-406, 53834-405, and 58366-003, respectively. [b] Type I. Standard amphotericin B containing 0.24% amphotericin A. [c] NT = Not tested. [d] Type II.

substances as the polyene antibiotics. Subsequently, Nillson-Ehle *et al.* (7) used this technique to quantitate amphotericin B in biological fluids. An HPLC system showing a partial separation of amphotericin A and amphotericin B was also described by Asher *et al.* (1). However, none of these methods provide the separation needed for a reliable assay of the components present in the bulk material. The method described and discussed here is proposed to replace the three methods (3) that are currently used for the certification of amphotericin B bulk preparations.

Instrument—A liquid chromatograph[1] equipped with a septumless valve injector[2] and a fixed-wavelength UV detector[3] with a 313-nm filter was used for all quantitative analysis. This was connected to a recording device[4] and an electronic integrator[5]. A special system[6] described by DiCesare *et al.* (8) was used for the qualitative analysis.

Column—A stainless steel column (4.6 × 150 mm) commercially packed with fully porous 7- to 8-μm silica particles to which a monomolecular layer of octadecylsilane was chemically bonded[7] was used in the quantitative phase of this study. The inlet of this analytical column was connected to a guard precolumn (4.6 × 100 mm) gravity-packed with an octadecylsilane-bonded silica of ~40-μm particle size[8]. The column system was equilibrated and conditioned with passage of ~10–15 void volumes of mobile phase, and tested for initial performance and suitability as described previously (7). A stainless steel column (4.6 × 100 mm) commercially packed with 3-μm octadecylsilane-bonded silica especially designed for high-speed analysis[9] was used in the qualitative study.

Chemicals and Reagents—Amphotericin B[10], labeled to contain 954 μg of amphotericin B/mg of powdered standard (containing 0.24% amphotericin A), was used as the external standard. Standards of amphotericin A[11] of unknown purity and nystatin[12] were also used. The dimethyl sulfoxide was spectro grade, phosphoric acid and sodium dihydrogen phosphate were reagent grade, and methanol was distilled-in-glass quality[13]. All the water used was deionized[14].

Stock Solutions—Approximately 6.3 g of sodium dihydrogen phosphate was dissolved in 950 mL of deionized water in a 1-L volumetric flask. The pH was adjusted to 2.6 with concentrated phosphoric acid, and the solution was diluted to volume with water.

Mobile Phase—A 300-mL aliquot of the phosphate solution was diluted to 1.00 L with methanol. This solution was mixed and deaerated by passing it through a 0.5-μm filter and ultrasonicating it for 2 min before use. As an added precaution, helium was allowed to sparge perceptibly through a 2-μm metal filter into this mobile phase during analysis to ensure maximum elimination of dissolved air. If necessary, the ratio of methanol to aqueous buffer can be adjusted to obtain adequate retention and peak separation.

Standard Solution and Sample—Approximately 25 mg of the amphotericin B standard was accurately weighed into a 25-mL volumetric flask, which was then filled to volume with dimethyl sulfoxide. The solution was mixed, shaken, and ultrasonicated until totally clear. The amphotericin B samples were prepared in a manner identical with that of the standard with ~25 mg of amphotericin B type I and ~30 mg of amphotericin B type II. All solutions of amphotericin B should be prepared fresh just before use.

Conditions for Quantitation—A constant operating temperature (ambient) was maintained, and the mobile phase flow rate of 2.0–2.5 mL/min was adjusted to give peaks of satisfactory configuration. The detector sensitivity was controlled to produce peak heights >50% full scale deflection with a chart speed of 2.5 mm/min. Chromatograms used for calculating performance parameters were obtained at a chart speed of at least 25 mm/min to allow better visual measurement of peak geometry for greater accuracy. The column was rinsed with methanol for 1 min and equilibrium was reestablished with the mobile phase before each injection.

Assay and Calculations—Carefully measured 15.0-μL aliquots of standard and sample solutions were injected sequentially into the chromatograph. Quantitation was achieved by comparing peak heights (and/or areas) of samples to that of the standard according to the following equations:

tericin B sample, C_A is the concentration of amphotericin A in the amphotericin B sample solution, P_B is the height (or area) of the amphotericin B peak in the amphotericin B sample, C_B is the concentration of amphotericin B in the amphotericin B sample solution, P_S is the height (or area) of the corresponding component peak in the amphotericin B standard reference material, and C_S is the concentration of the corresponding component in the solution of amphotericin B standard reference material. An APL time-sharing computer system[15] was used to plot all graphs and to perform all calculations and data reduction.

Amphotericin B is quite insoluble in most organic solvents; for this analysis it was dissolved in neat dimethyl sulfoxide. Initial attempts to dilute the dimethyl sulfoxide solutions with methanol frequently caused gelatinous precipitates, particularly with methanol–dimethyl sulfoxide ratios >9:1. One beneficial effect of neat dimethyl sulfoxide injections was slightly better peak resolution. On the other hand, the inactivating effect of dimethyl sulfoxide and residual sample (retained solutes) in the column diminished retention time with each subsequent injection. However, the original retention time was restored rapidly with a 1-min methanol rinse. One may alternate injections of samples and methanol rinses to maximize replication of retention; however, the actual frequency of column rinsings will depend on the total amount of solute injected. For samples analyzed within 1 d, peak heights for seven injections of standard interspersed with samples and rinses showed a coefficient of variation of 1.46%.

The stability of amphotericin B is greatest at a neutral pH where it exists as a zwitterion; however, better separation was achieved with ion suppression of the acid function. The use of acidic media in the HPLC procedure tends

[1] Model 6000A solvent delivery system; Waters Associates, Milford, Mass.
[2] Model U6K; Waters Associates.
[3] Model 440; Waters Associates.
[4]
[5]
[6] Model 3B solvent delivery system, Sigma 15 chromatography data station, model LC-85 spectrophotometric detector, and autocontrol; Perkin-Elmer Corp., Norwalk, Conn.
[7] Zorbax ODS; DuPont Instruments, Wilmington, Del.
[8] Bondapak (C_{18}/Corasil); Waters Associates.
[9] HS-3-C18; Perkin-Elmer Corp.
[10] USP reference standard.
[11] USP reference standard, lot no. HV-718.
[12] USP reference standard, lot F.
[13] Burdick & Jackson Laboratories, Inc., Muskegon, Mich.
[14] Milli-Q Reagent-Grade water system; Millipore Corp., Bedford, Mass.

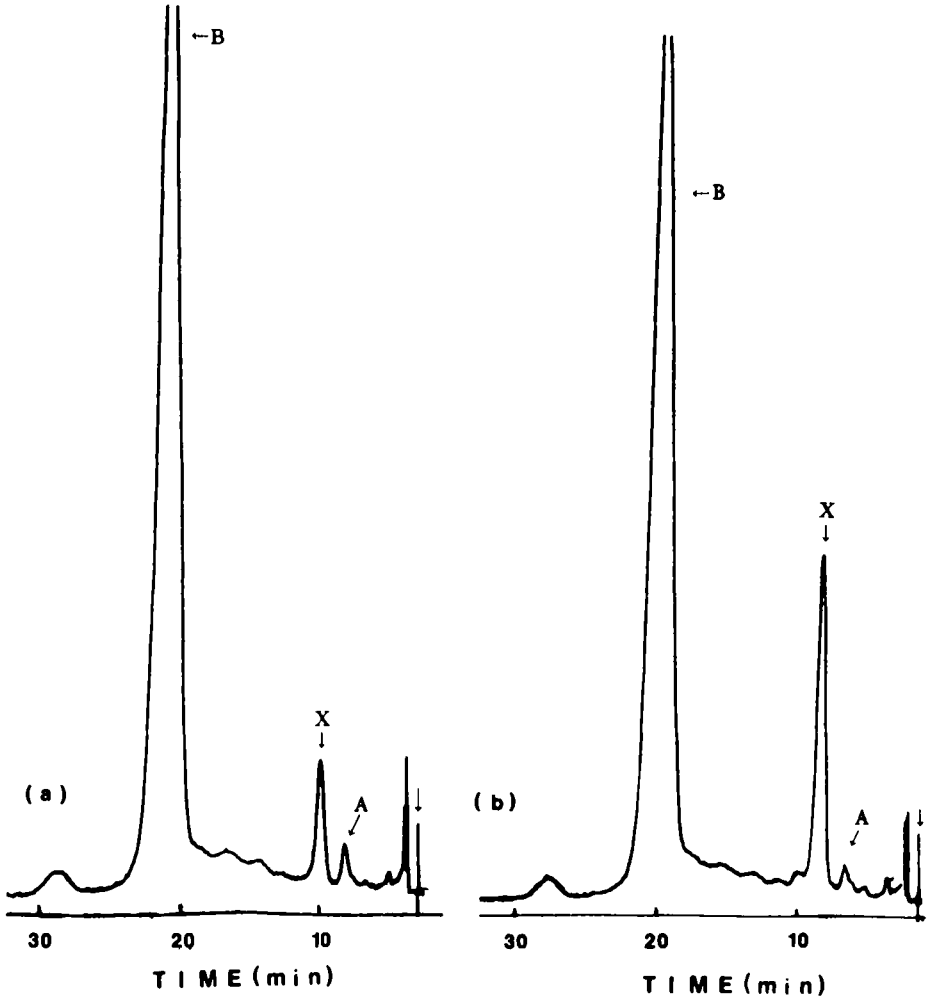

Figure 1—*Chromatograms of bulk amphotericin B from E. R. Squibb & Sons, Inc., type I (a) (batch #58022-002) and type II (b) (batch #58334-404). Key: (A) amphotericin A; (B) amphotericin B; and (X) amphotericin X.*

to approximate the method of isolation of the antibiotic from the broth in commercial production. The adverse effect of the acidity of the buffer is diminished by the high concentration of methanol in the mobile phase.

The capacity factor for amphotericin B is 25, but in spite of its magnitude, the column efficiency calculated as the reduced plate height is ~32. The separation factors for amphotericin A and amphotericin X (unidentified heptaene component) compared with amphotericin B are 4.3 and 2.7, respectively, with resolution factors of 4.4 and 3.4 between the same pairs of peaks. It is noted that although the column of 3-μm particles yielded substantially more plates, it provided about the same absolute efficiency as the column of larger particles (7–8 μm).

Qualitative Analysis—Samples of type I and type II amphotericin B yielded the chromatographic profiles shown in Fig. 1a and b, respectively, showing peaks attributed to amphotericin A and one labeled amphotericin X. These chromatograms are meant merely to provide a visual example of a separation previously unavailable from a qualitative standpoint and to indicate the relatively large quantitative difference between the two types (I and II) of commercial amphotericins. However, note that these chromatograms were not necessarily obtained the same day.

To the authors' knowledge, the presence of this newly discovered component in amphotericin B has never been described in the literature. Although the chromatograms of the type I and type II samples appear to be very similar from a qualitative standpoint, the type I sample is purer because it has a higher potency and contains less than half the amount of amphotericin X.

Amphotericin A and nystatin were believed for a long time to be identical. Consequently, because the spectral characteristics of both of these substances are so similar and because nystatin is more readily available than amphotericin A, the former is used as a standard to assay for the latter. Although these two substances had been separated under different conditions[16], they were not resolved by the method described here, demonstrating again their close similarity[17].

Chromatographic peaks were examined on-line by UV spectrophotometry (6) with the system[6] previously described. This system allows rapid elution (4 *versus* 30 min) with good resolution of peaks because of the short column packed with smaller particles (3 μm) and because of the accessory spectrophotometer especially designed with a 2.4-μL cell. This system also provides fast response time and a very low dead volume. Several peaks were subjected to UV scanning in an attempt to confirm or identify them. The resultant spectra indicated correctly that the peak attributed to amphotericin A is that of a tetraene; four other peaks including those labeled amphotericin B and amphotericin X were characterized as heptaene peaks on the basis of UV characteristics identical to those of known heptaenes.

Polyene antibiotics exhibit multiplet absorption peaks in the UV region; absorption maxima for the tetraene amphotericin A occur with decreasing intensity at 305, 318, 290, and 280 nm and at 406, 382, 363, and 356 nm for heptaenes (*e.g.*, amphotericin B) with this system[6]. The 313-nm filter of a photometer with a band spread of 10 ± 2 nm allows good sensitivity for minor tetraene peaks while simultaneously enabling adequate detection of heptaenes. Spectra of the amphotericins have also been published (1, 2).

Quantitative Analysis—Table I lists the calculated potency of several lots of commercial amphotericins; Table II gives the content of amphotericin A in the same samples.

A calibration curve for amphotericin B was obtained by plotting amounts ranging from ~5 to 26 μg of the injected drug against peak height and peak area. A rectilinear relationship was observed with corresponding correlation coefficients of 0.9999 and 0.9991 and corresponding relative percentage errors of 0.58 and 1.50. Optimum quantitation is obtained when injections of both standard and sample solutions are of approximately the same concentration and volume because errors due to nonlinearity of the detector[3] and the injector[2] are minimized (9). The addition of an internal standard to improve precision is advisable, if possible, but the complexity of the chromatogram precluded its use here.

Because the standard addition method did not prove satisfactory, the content of amphotericin A in commercial samples was calculated from peak heights by direct comparison to the content of amphotericin A in the amphotericin B standard. This standard had been established to contain 0.24% of amphotericin A by the official procedure, which is based on differential spectrophotometry of a standard solution of amphotericin B and a standard solution

of nystatin. The latter has been shown to possess spectral characteristics nearly identical to that of amphotericin A[16,17].

The paired t test was used to compare bioassay results from the manufacturer with those from the HPLC method. No significant statistical difference was found at the 95% confidence level. It would be difficult and impractical at this juncture to make further comparisons among sample results because of the poor precision of microbiological assays for antifungal substances.

It can, therefore, be concluded that the commercial bulk material is predominantly amphotericin B containing a limited amount of the A congener and several heptaenes. The potency of type I amphotericin B may measure >900 μg/mg with ~6% amphotericin X, whereas type II shows a potency of ~800 μg/mg with ~11-14% amphotericin X.

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In Vitro Stability of Sodium Nitroprusside Solutions for Intravenous Administration

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Abstract □ A sensitive high-performance liquid chromatographic assay for nitroprusside using an ion-exchange column and UV detection was developed to evaluate the stability of aqueous solutions of sodium nitroprusside in light-protected glass and plastic containers and during simulated infusions. The results showed that sodium nitroprusside is stable in 5% dextrose, normal saline, and lactated Ringer's solutions in light-protected glass or plastic containers. In addition, there was no decrease in the delivered potency of sodium nitroprusside solutions during simulated infusions lasting up to 24 h.

Keyphrases □ Sodium nitroprusside—*in vitro* stability, solutions, intravenous administration, HPLC □ Stability—*in vitro*, sodium nitroprusside solutions, intravenous administration, HPLC

Sodium nitroprusside [$Na_2Fe(CN)_5NO{\cdot}2H_2O$] is a valuable agent in the treatment of congestive heart failure (1), cardiogenic shock complicating acute myocardial infarction (2), and hypertensive crisis (3). This drug is also used for the production of intraoperative hypotension (4). However, there are several problems associated with the clinical use of sodium nitroprusside, including tolerance (5), tachyphylaxis (6), and the toxicity of its metabolites, cyanide and thiocyanate (7). Some of these problems might be alleviated by an understanding of the pharmacokinetics of nitroprusside in humans. To derive pharmacokinetic parameters from data collected during a constant infusion, the amount of sodium nitroprusside delivered must be known. The purpose of this study was to investigate the stability of sodium nitroprusside in various intravenous solutions and during simulated infusions.

EXPERIMENTAL

Reagents and Chemicals—Sodium nitroprusside[1] was used as received. Analytical-grade KH_2PO_4 and H_3PO_4 and deionized water were used to prepare the mobile phase. All glassware used for sampling or storage of stock solutions was silanized[2].

Instruments and Chromatographic Conditions—An anion-exchange column[3] and a 0.5 M KH_2PO_4 buffer (pH 3.0) with H_3PO_4, were used to achieve the chromatographic separation. The high-performance liquid chromatography (HPLC) was performed with a solvent pumping system[4], a variable-wavelength UV detector[5], and an injector equipped with a 50-μL sample loop[6]. The absorbance was measured at 230 nm with a 0.1 AUFS deflection and was recorded on a three-pen recorder[7]. The flow rate was 1.4 mL/min, and the mobile phase was filtered and deaerated prior to use.

Stability Studies—Sodium nitroprusside (50 mg) was dissolved in 5 mL of water and then added to 500 or 1000 mL of a test solution, resulting in a final concentration of 100 and 50 μg/mL, respectively. The stability of sodium nitroprusside was tested in 5% dextrose, normal saline (0.9% NaCl), and lactated Ringer's solutions[8] in both glass[8] and plastic[8] containers. After the addition of the sodium nitroprusside concentrate, the test solution was mixed and a sample was withdrawn for use in the construction of a standard curve. The bottle or plastic bag was wrapped in aluminum foil and left exposed to laboratory (fluorescent) light for 48 h. Sodium nitroprusside standards were protected from light and stored at 4°C. The stability of the standard solutions was verified by comparing their concentrations with freshly prepared 100-

[1] Roche Laboratories, Nutley, N.J.
[2] Prosil-28; PCR Research Chemicals, Gainesville, Fla.
[3] Partisil 10-SAX; Whatman Inc., Clifton, N.J.
[4] Constametric I; Laboratory Data Control, Riviera Beach, Fla.
[5] Spectromonitor III; Laboratory Data Control, Riviera Beach, Fla.
[6] Model 7120; Rheodyne Inc., Berkeley, Calif.
[7] Linear Instruments, Irvine, Calif.
[8] Abbott Laboratories, North Chicago, Ill.

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