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RESEARCH PAPER

Differences in the Method by Which Plasma Is Separated from Whole Blood Influences Amphotericin B Plasma Recovery and Distribution Following Amphotericin B Lipid Complex Incubation Within Whole Blood

Manisha Ramaswamy and Kishor M. Wasan*

Division of Pharmaceutics and Biopharmaceutics, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

ABSTRACT

A previous investigation suggested that the use of plasma as the biological fluid for measurement of amphotericin B (AmpB) concentrations greatly underestimates the concentrations of AmpB in the total blood circulation following amphotericin B lipid complex (ABLC) administration to humans. The purpose of this study was to determine if differences in the method used to obtain plasma from whole blood influences the percentage of AmpB recovered in plasma following ABLC incubation in whole blood. ABLC (5 μg AmpB/ml; peak blood concentration observed in rabbits following intravenous bolus of ABLC at a dose of 1 mg/kg) was incubated in whole blood for 5 min at 25°C. These conditions were used to mimic the sample retrieval conditions used when blood is obtained from animals and human patients. Following incubation, plasma was obtained from whole blood using five different methods: (A) Whole blood was centrifuged for 5 min at 23°C, and the plasma was separated; (B) whole blood was stored at 4°C

*Corresponding author. Fax: (604) 822-3035; E-mail: Kwasan@interchange.ubc.ca

for 18 h, and the plasma was separated by gravity; (C) whole blood was stored at 23°C for 18 h, and the plasma was separated by gravity; (D) whole blood was stored at 37°C for 18 h in a water bath, and the plasma was separated by gravity; and (E) whole blood was stored at 30°C for 18 h in a water bath, and the plasma was separated by gravity. All samples were protected from light throughout the duration of the experiment. AmpB concentration in each plasma sample was determined by high-performance liquid chromatography (HPLC) using an external calibration curve. The whole blood : plasma Amp B concentration ratio and the percentage of AmpB partitioned into plasma following incubation of ABLC in whole blood for each plasma separation procedure was as follows: (A) 6.5 : 1 blood : plasma AmpB concentration ratio, 15.4% ± 1.6% AmpB in plasma; (B) 2.98 : 1 blood : plasma AmpB concentration ratio, 33.6% ± 7.7% AmpB in plasma; (C) 1.5 : 1 blood : plasma AmpB concentration ratio, 67.6% ± 10.3% AmpB in plasma; (D) 1.5 : 1 blood : plasma concentration ratio, 68.1% ± 1.1% AmpB in plasma; and (E) 1.2 : 1 blood : plasma AmpB concentration ratio; 83.4% ± 5.5% AmpB in plasma. These findings suggest that when measurement of AmpB in plasma is required following ABLC administration, incubation of whole blood at 30°C for 18 h appears to be the most effective method.

Key Words: *Amphotericin B; ABLC; Lipoprotein distribution; Whole blood*

INTRODUCTION

Amphotericin B (AmpB) is a polyene macrolide antibiotic used for the treatment of systemic fungal infections commonly found in immunocompromised patients (i.e., those with acquired immunodeficiency syndrome [AIDS]), cancer patients, and diabetics (1–6). The conventional amphotericin B–deoxycholate micellar formulation, Fungizone (Bristol Myers Squibb, NJ) has been used for over 45 years, and despite its dose-dependent kidney toxicity, it remains the most widely used drug for the treatment of most systemic fungal infections (1,4,5). Incorporation of AmpB into liposomes and lipid-associated formulations (e.g., AmBisome, Abelcet, Amphocil) has been proved to reduce AmpB-induced kidney toxicity (6–11) and provide site-specific delivery of a high concentration of the drug.

Recently, our group and others (12) have suggested that determining the pharmacokinetics of AmpB after administration of one of these lipid formulations, Abelcet (commonly known as amphotericin B lipid complex [ABLC]), is influenced by the biological fluid that is used to measure the systemic exposure of the AmpB. Previous studies in vitro have shown that incubating ABLC into whole blood followed by centrifugation to separate the plasma results in the majority of AmpB in ABLC being recovered as a lipid/protein pellet formed at the bottom of the test tube on centrifugation (12). However, incubation of the conventional AmpB formulation (Fungizone) resulted in the complete recovery of the drug in the plasma fraction.

These observations suggest that the use of plasma as the biological fluid for the measurement of AmpB concentrations greatly underestimates the concentrations of AmpB in the total blood circulation following ABLC administration (12). Thus, the purpose of this study was to determine if differences in the procedure that is used to obtain plasma from whole blood influence the percentage of AmpB recovered in plasma following ABLC incubation in whole blood.

EXPERIMENTAL

Materials

Chemicals

Amphotericin B–deoxycholate (Fungizone) was purchased from Bristol–Myers Squibb Canada, Incorporated, and reconstituted with 10 ml of distilled water (5 mg/ml final concentration). ABLC (Abelcet; Liposome Company, NJ), which contains dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) was

purchased from the Vancouver General Hospital Department of Pharmacy (Vancouver, BC, Canada).

Rabbit Whole Blood and Plasma

Whole blood and plasma for this study were obtained from New Zealand white female rabbits (2.5 to 3.0 kg; Jeo-Bet Rabbits, Ltd., Aldon, BC, Canada). This was an "ideal animal model" because no kidney or liver function and hematological profile abnormalities were observed in age-matched New Zealand white rabbits, and 3-ml blood samples were obtained without significant changes in blood flow (13). Furthermore, the rabbit was the appropriate experimental animal to use in these studies because the behavior and structure of their systemic proteins and lipoproteins are similar to those of humans (14).

Plasma Lipoprotein Separation

The strategy for separating plasma into lipoprotein (high-density lipoproteins [HDLs], low-density lipoproteins [LDLs], triglyceride-rich lipoprotein [TRL], and lipoprotein deficient [LPDP]) fractions was by step-gradient ultracentrifugation (11). Rabbit plasma samples (3.0 ml) from the whole blood collection were placed into centrifuge tubes, and their solvent densities were adjusted to 1.25 g/ml by the addition of solid sodium bromide (0.34 g/ml of serum). Once the sodium bromide had dissolved into the serum, 2.8 ml of the highest density sodium bromide solution (density of 1.21 g/ml, which represents the HDL fraction) was layered on top of the serum solution. Then, 2.8 ml of the second sodium bromide solution (density of 1.063 g/ml, which represents the LDL fraction) was layered on top of the sample, followed by 2.8 ml of the third sodium bromide solution (density of 1.006 g/ml, which represents the TRL fraction and contains very low density lipoproteins and chylomicrons). On completion of layering with the sodium bromide density solutions, four distinct regions of progressively greater densities (from top to bottom of the tube) were observed.

All sodium bromide solutions were kept at 4°C prior to the layering of the density gradient. The centrifuge tubes were placed in an SW 41 Ti swinging bucket rotor (Beckman Canada) and centrifuged at 40,000 rpm (288,000 *g*; *k* factor = 128) at a temperature of 15°C for 18 h (L8-80 M, Beckman Canada). Following ultracentrifugation, each density layer was removed using a Pasteur pipette, and the volume of each lipoprotein fraction measured.

To ensure that the lipoprotein distribution of AmpB was a result of its association with each lipoprotein and not a result of the density of the formulation, the distribution of AmpB following ABLC reconstituted in normal saline within LPDP was determined. The majority of AmpB (> 90%) was found in the density range greater than 1.21 g/ml, suggesting that the AmpB distribution within the ultracentrifuge tubes following incubation in rabbit plasma is not a function of formulation density (11).

Characterization of Lipoproteins

Lipoprotein preparations were characterized with respect to lipid and protein composition. Cholesterol (esterified and unesterified), triglyceride, and protein were quantitated by established colorimetric and fluorometric techniques as previously described (6,9–11).

Measurement of Amphotericin B

Whole blood and plasma samples were obtained and processed for AmpB analysis as previously described (9–11). AmpB levels in whole blood, plasma, and lipoprotein fractions were determined by high-pressure liquid chromatography (HPLC) using an external calibration curve as previously described (9–11).

Experimental Design

ABLC (5 μg AmpB/ml; peak blood concentration observed in rabbits following intravenous bolus of ABLC at a dose of 1 mg/kg) (11) was incubated in whole blood for 5 min at 25°C. Following incubation, the plasma was obtained from whole blood using five different methods:

A. Whole blood was centrifuged for 5 min at 23°C, and the plasma was separated.
B. Whole blood was stored at 4°C for 18 h, and the plasma was separated by gravity.
C. Whole blood was stored at 23°C for 18 h, and the plasma was separated by gravity.
D. Whole blood was stored at 37°C for 18 h in a water bath, and the plasma was separated by gravity.

E. Whole blood was stored at 30°C for 18 h in a water bath, and the plasma was separated by gravity.

Total recovery and lipoprotein distribution of AmpB in each plasma sample were determined using HPLC as described above.

Statistical Analysis

Ratio of whole blood to plasma AmpB concentrations, percentage of AmpB partitioned into plasma, and the plasma lipoprotein distribution of AmpB following ABLC incubation were compared among the five different methods by analysis of variance (INSTAT; GraphPad). Critical differences were assessed by Tukey post hoc tests. A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($P < .05$). All data are expressed as mean plus or minus standard deviation (SD).

RESULTS AND DISCUSSION

Differences in the partitioning of AmpB into plasma following the incubation of ABLC within whole blood and separation of plasma using five different methods were observed (Table 1). The percentage of AmpB recovered in plasma was the greatest when the whole blood was stored at 30°C for 18 h with the plasma separated by gravity (Fig. 1). No significant differences in AmpB retention time and AmpB peak shape and no unknown peaks were observed in HPLC chromatographs obtained when using this method compared to the other methods tested (data not shown). These observations suggest that no apparent chemical breakdown of AmpB occurred when using these methods of plasma separation.

However, the other methods of plasma separation (methods A through D) resulted in poor recovery of AmpB from whole blood (Table 1 and Fig. 1). At a low temperature (i.e., 4°C) and with centrifugation, the lipid complex was found to be associated with the pellet formed at the bottom of the test tube. However, at higher temperatures (i.e., 30°C and 37°C) above the transition temperature (25°C) of the lipids (DMPC and DMPG) used in this formulation (8), the complex broke down, and more free AmpB was released into the plasma. It should be noted that the whole blood : plasma ratio presented in Table 1 represents the concentration of AmpB within whole blood prior to plasma separation and the concentration of AmpB recovered in the plasma following separation from whole blood.

Previous investigations by our laboratory have reported that AmpB predominantly associates with HDL and LPDP in human plasma when

Table 1

Ratio of Whole Blood to Plasma Amphotericin B (AmpB) Concentrations Obtained Following Incubation of Amphotericin B Lipid Complex (ABLC) in Whole Blood and Separation of Plasma by Different Methods

Method	Whole Blood : Plasma AmpB Concentration
A, centrifugation at 23°C	6.54 ± 0.69: 1
B, by gravity at 4°C	$2.98 \pm 0.07 : 1^a$
C, by gravity at 23°C	$1.50 \pm 0.23 : 1^a$
D, by gravity at 37°C	$1.47 \pm 0.02 : 1^a$
E, by gravity at 30°C	$1.20 \pm 0.08 : 1^a$

Data are expressed as mean ± standard deviation ($n = 3$).
[a] $P < .05$ vs. method A.

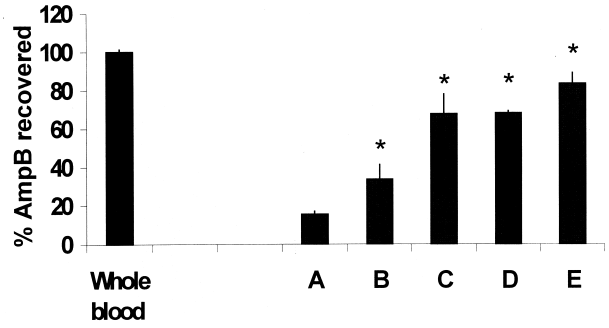

Figure 1. Percentage of amphotericin B (AmpB) partitioned into plasma fraction following incubation of amphotericin B lipid complex (ABLC) in whole blood and separation of plasma by five different methods: A, whole blood was centrifuged for 5 min at 23°C, and the plasma was separated; B, whole blood was stored at 4°C for 18 h, and the plasma was separated by gravity; C, whole blood was stored at 23°C for 18 h, and the plasma was separated by gravity; D, whole blood was stored at 37°C for 18 h in a water bath, and the plasma was separated by gravity; E, whole blood was stored at 30°C for 18 h in a water bath, and the plasma was separated by gravity. * $P < .05$ vs. method A; $n = 3$.

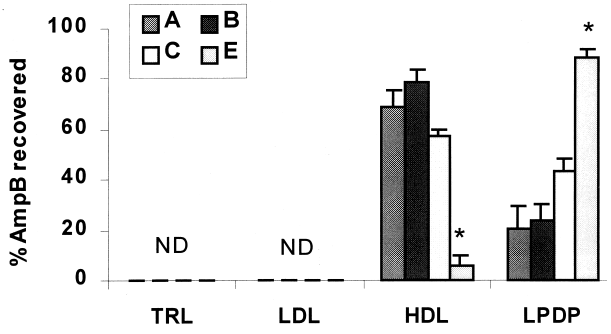

Figure 2. Amphotericin B (AmpB) distribution into plasma lipoprotein (high-density lipoproteins [HDL]; low-density lipoproteins [LDL]; triglyceride-rich lipoproteins [TRL], which includes very low-density lipoproteins and chylomicrons) and lipoprotein-deficient (LPDP) fractions following incubation of amphotericin B lipid complex (ABLC) in whole blood and separation of plasma by five different methods: A, whole blood was centrifuged for 5 min at 23°C, and the plasma was separated; B, whole blood was stored at 4°C for 18 h, and the plasma was separated by gravity; C, whole blood was stored at 23°C for 18 h, and the plasma was separated by gravity; D, whole blood was stored at 37°C for 18 h in a water bath, and the plasma was separated by gravity; E, whole blood was stored at 30°C for 18 h in a water bath, and the plasma was separated by gravity. * $P < .05$ vs. method A; $n = 3$. Note: Due to red blood cell lysis and plasma hemolysis, method D could not be completed. ND, below the detectable limit of the AmpB high-performance liquid chromatographic-assay used in this study.

incorporated into liposomes or as lipid complex (ABLC) containing DMPC and DMPG (6,11). The preferential association of the drug-lipid complex with HDL rather than LDL appears to be related to the composition of the lipid complex. The DMPG component of ABLC predominantly distributes into HDL because of its interaction with the apolipoproteins AI and AII of HDL (6). In this study, at low temperatures and with centrifugation, the drug was still in the form of a lipid complex, causing it to predominantly associate with HDL (Fig. 2). At a higher temperature (e.g., 30°C), since most of the drug was in the free form, it predominantly associated with LPDP. However, at 23°C, it was equally distributed between HDL and LPDP since only a part of the drug was released from the lipid complex into its free form (Fig. 2). Due to red blood cell lysis and subsequent plasma hemolysis, the plasma distribution of AmpB could not be determined when using method D.

Taken together, these finding suggest that different methods of plasma separation may influence the conclusions drawn about the plasma distribution of ABLC. Based on the observations from this study, it appears that incubation of whole blood at 30°C for 18 h is the most effective method for measurement of AmpB in plasma following the administration of ABLC.

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