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Pharmacokinetics and mechanisms of plasma removal of hemoglobin-based oxygen carriers

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Abstract

The circulatory persistence, distribution, and metabolism of hemoglobin-based oxygen carriers (HBOCs) is a major determinant of their safety and efficacy. In this communication, published data on the pharmacokinetics and routes of plasma elimination of HBOCs are summarized and evaluated. The circulating half-life of HBOCs is dose-dependent in both animals and humans. Half-life also increases with molecular weight in animals, at least up to the MDa range. The functional half-life of HBOCs is diminished by as much as 40% due to oxidation of the heme group relative to the overall rate of removal of hemoglobin (Hb) from plasma. Kidney excretion of HBOCs is greatly diminished compared to that of unmodified Hb, but the liver remains a primary site of catabolism. Both hepatocytes and Kupffer cells have been implicated in receptor-mediated HBOC uptake. Removal also occurs in the spleen and/or bone marrow and probably at dispersed sites in the endothelium as well. HBOCs extravasate into the lymph at a rate inversely proportional to their molecular weight and are taken up by monocyte/macrophage CD163 receptors, both as free Hb and in complexes with haptoglobin (Hp). The interactions with both Hp and the CD163 receptor are altered by Hb modification. However, monocyte/macrophage uptake may not be a quantitatively important route for the removal of clinically relevant doses of HBOCs. The relative contributions of different removal pathways have yet to be comprehensively determined, particularly in humans.

Keywords: crosslinked hemoglobins, dose effects, kidney excretion, liver uptake, metHb, plasma half-life, polymerized hemoglobins

Introduction

Hemoglobin-based oxygen carriers (HBOCs) are being developed to facilitate oxygen transport to ischemic or hypoxic tissues (Kim and Greenburg 2013). Potential indications include resuscitation from hemorrhagic shock, cardiac bypass pump priming, patient support when blood is unavailable, perfusion of infarcted tissues, maintenance of organ viability for transplant, and facilitation of radiation and chemotherapy (Winslow 1993). The active principles of HBOCs are mammalian origin or are recombinantly produced hemoglobins (Hbs), which have been modified to facilitate efficacy and minimize toxicity. The advantages of HBOCs include low risk of pathogen transmission, long-term storage stability, absence of need for immunologic matching to recipients, and enhanced ability to perfuse ischemic tissues (Mozzarelli et al. 2010). Although regulatory approval for human use has yet to be obtained due to safety concerns with the current generation of products, limited approval for veterinary indications has been granted and work continues on the development of improved formulations.

The distribution and metabolism of protein therapeutics strongly influences their efficacy and safety. As a consequence, the measurement of these parameters is both a regulatory requirement and a prudent means to understand whether a given formulation addresses the needs of specific clinical indications (Lin 2009, Shargel et al. 2012). For these reasons, the intravascular persistence of HBOCs and their routes of its elimination from circulation have been frequently studied. An overview of this topic is presented in the following text. Data on the pharmacokinetics of liposome-encapsulated Hb are not included, since a review of this subject was recently published (Taguchi et al. 2011).

Considerations and limitations

One convention explicitly used in this review is the characterization of intravascular persistence in terms of a "half-life", which is defined as that period of elapsed time after the completion of intravenous administration during which the HBOC concentration in plasma falls to one half that measured, or inferred, immediately after infusion. Although useful, this definition suffers from the fact that half-life is only rigorously defined mathematically for concentrations that decay in exponential fashion, that is, concentrations which decrease in a first-order manner. While published data for

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HBOC disappearance from plasma frequently appear exponential, this is not universally the case (DeVenuto 1983, Sehgal et al. 1984, Berbers et al. 1991, Baek et al. 2012).

A second limitation is that most published studies have not measured blood volume concomitant with HBOC concentration. This can be problematic because the doses of infused HBOC solutions are often a significant fraction of the total blood volume, and, in addition, tend to further expand this volume due to oncotic effects (Friedman and DeVenuto 1982, Bleeker et al. 1986, Bakker et al. 1986, Migita et al. 1997, Conover et al. 1997a,b, Fischer et al. 1999, Caron et al. 2001, Buehler et al. 2007). This is evident in human clinical studies in which total Hb concentrations were raised less after HBOC administration than after infusion of the same total amount of Hb as packed red blood cells (LaMuraglia et al. 2000). The reason is that packed red cells exhibit very little oncotic pressure, while that of HBOCs is often significant (Migita et al. 1997). An important consequence of this initial blood volume expansion is that blood volume will decrease as the HBOC disappears from circulation. Thus, the concentration of HBOC in blood will decrease more slowly than the total dose of HBOC, resulting in an overestimate of the circulating half-life if half-life is calculated only on the basis of changes in concentration. A dramatic example of this was reported by Migita and coworkers who demonstrated that an apparent half-life of greater than 200 h in rats for a polyethylene glycol (PEG)-modified Hb based on plasma Hb concentrations was in fact approximately three hours when data were corrected for blood volume changes (Migita et al. 1997). These and other scientists have also noted an apparent elevation in the circulating half-life of crosslinked and/or polymerized HBOCs due to blood volume changes (Bleeker et al. 1986, Bakker et al. 1986, Migita et al. 1997).

A third complication is that many HBOCs are infused in exchange transfusion protocols, while blood is simultaneously withdrawn. This results in some removal of HBOC during infusion, making the determination of administered dose more difficult. It may also obscure initial distribution or elimination processes. However, such protocols are necessitated by the large fluid volumes required for certain indications. In some of these experiments, net doses were calculated as the difference between the infused dose and the amount recovered in the withdrawn blood. Alternatively, the initial HBOC concentration in the blood measured or extrapolated immediately after infusion is taken as the starting point for analyses. Since approximately half of all HBOC studies reporting circulatory persistence have utilized exchange transfusion protocols, data from these studies are included despite the inherent difficulties in their interpretation.

A final point is the distinction between the circulatory persistence of functional HBOC versus that of total HBOC, since Hbs can only transport oxygen when the heme iron is in the reduced (+2) oxidation state. Generally speaking, HBOC heme irons undergo oxidation to the inactive (+3) methemoglobin (metHb) form during storage and after intravenous infusion (Kramlova et al. 1976, DeVenuto 1978, Sehgal et al. 1981, Buehler et al. 2007, Baek et al. 2012). Thus, the amount of functional HBOC is always less than the total plasma Hb content. The fraction of circulating reduced HBOC has not been consistently reported, but comparisons between functional and total HBOC content will be discussed on the basis of available data.

Plasma half-life—summary data and trends

Unmodified mammalian Hbs are rapidly excreted through the kidneys, resulting in short intravascular persistence and renal toxicity (Baker and Dodds 1925). This is primarily due to rapid dissociation of the 64 kDa Hb tetramer into two 32 kDa αβ dimers which readily permeate the glomerular membrane. Bunn, Esham, and Bull demonstrated that kidney excretion is substantially reduced by chemical crosslinking of the dimers to form stabilized $\alpha_2\beta_2$ tetramers (Bunn et al. 1969). As a result, a guiding principle in HBOC development has been to inhibit renal excretion by stabilization of the tetrameric conformation and/or incorporation of HBOCs into larger molecular complexes by polymerization, encapsulation, surface modification, or the utilization of naturally occurring high molecular weight heme proteins. In this review, intramolecularly stabilized Hb tetramers are denoted as crosslinked, while intermolecularly linked HBOCs are denoted as polymerized.

Building on the seminal work of Bunn and coworkers, a number of researchers have shown that crosslinking with a variety of reagents leads to increased intravascular persistence (Mok et al. 1975, Greenburg et al. 1977, Greenburg and Maffuid 1983, Bleeker et al. 1986, Snyder et al. 1987, Hess et al. 1989, Keipert et al. 1989a, Keipert et al. 1989b, Urbaitis et al. 1991, Dittmer et al. 1992, Bakker et al. 1993, Keipert et al. 1993, Keipert et al. 1994, Bush et al. 1994, Bucci et al. 1996, Migita et al. 1997, Ship et al. 2005). Polymerization further increases half-life (Tam et al. 1978, DeVenuto and Zegna 1983, Keipert and Chang 1983, Sehgal et al. 1984, Keipert and Chang 1987, Snyder et al. 1987, Berbers et al. 1991, Lenz et al. 1991, Bleeker et al. 1992, Hsia et al. 1993, Bakker et al. 1993, Anderson et al. 1993, Menu et al. 1994, Lee et al. 1995, Pearce et al. 2003, Wicks et al. 2003, Bonegio et al. 2006, Buehler et al. 2007, Buehler et al. 2010, Baek et al. 2012, Elmer et al. 2012). Most reported values have not been corrected for blood volume changes, but the increased half-life of crosslinked and/or polymerized HBOCs relative to unmodified Hb is not an artifact of such changes because these modifications do not increase the oncotic pressure at a given Hb concentration (DeVenuto 1983, Sehgal et al. 1984, Berbers et al. 1991, Vandegriff et al. 1997). On the other hand, PEG modification markedly increases the oncotic pressure of Hbs (Vandegriff et al. 1997). For this reason, preclinical data obtained with PEG-modified Hbs are considered separately (Conover et al. 1997a,b, Migita et al. 1997, Vandegriff et al. 1997, Conover et al. 1999, Vandegriff et al. 2003, Vandegriff et al. 2006). HBOC half-life has also been measured during human trials (Hughes et al. 1995, Swan et al. 1995, Hughes et al. 1996, Przybelski et al. 1996, Viele et al. 1997, Standl et al. 1998, Carmichael et al. 2000, O'Hara et al. 2001, Olofsson et al. 2006, Vandegriff et al. 2006). In considering both preclinical and clinical data, several trends are apparent:

Infusion of increasing HBOC doses results in an apparent prolongation of half-life (Bleeker et al. 1986, Keipert and

Chang 1987, Hess et al. 1989, Keipert et al. 1989a, Bleeker et al. 1992, Hughes et al. 1996, Przybelski et al. 1996, Viele et al. 1997, Carmichael et al. 2000, Pearce et al. 2003) in both animals and humans (Figures 1 and 2). The effect is modest with unmodified Hbs, but more pronounced with crosslinked and polymerized HBOCs (Bleeker et al. 1986, Bleeker et al. 1992, Keipert 1992). Bleeker and coworkers demonstrated that the apparent increase of half-life with increasing doses of unmodified Hb was greatly diminished, though not eliminated, when blood volume changes were considered (Bleeker et al. 1986). Unfortunately, blood volume change corrections have not been applied to dose-response series with crosslinked or polymerized HBOCs. The half-life of PEG-modified Hb in humans did not exhibit a dose dependence (Vandegriff et al. 2006, Olofsson et al. 2006), albeit from data collected over a restricted dosing range. The reason for this difference is unclear.

A second trend is that increasing the size of the HBOC complex is associated with an increase in half-life (Sehgal et al. 1984, Snyder et al. 1987, Berbers et al. 1991, Bleeker et al. 1992, Keipert et al. 1992, Bakker et al. 1993, Hsia et al. 1993, Wicks et al. 2003, Baek et al. 2012), an effect which does not depend on a particular modification chemistry (Figure 3). The increase in half-life seems to approach a maximum as the molecular weight approaches one MDa. With bovine polymerized HBOCs, a 31-h half-life was observed with a 1.3 MDa average molecular weight product, compared to 19 and 15 h for 0.75 and 5.5 MDa products, respectively (Baek et al. 2012). Sufficient comparative data have not been reported in humans to determine whether there is a dependency of circulatory persistence on the molecular weight of HBOCs similar to that observed in animal models, although one would be expected.

A third trend is the variation in circulatory persistence with species (Hess et al. 1989, Pearce et al. 2003). While the nature and relative paucity of comparable data do not permit a rigorous statistical analysis, two qualitative conclusions seem warranted. At similar doses with the same modified HBOC, the half-life in humans is approximately three-fold greater than that in rats and is comparable to that in intermediate sized mammals (Figure 4). These results suggest that reasonable expectations of human circulatory persistence may be predictable for new HBOCs on the basis of animal data.

Functional half-life

As noted in "Considerations and limitations", the concentration of functional HBOC is reduced by oxidation to the inactive metHb form during storage and circulation (Kramlova et al. 1976, DeVenuto 1978, Tam et al. 1978, Sehgal et al. 1981, Snyder et al. 1987, Bush et al. 1994, Lee et al. 1995, Caron et al. 2000, O'Hara et al. 2001, Sprung et al. 2002, Wicks et al. 2003, Olofsson et al. 2006, Vandegriff et al. 2006, Buehler et al. 2007, Buehler et al. 2010, Baek et al. 2012, Elmer et al. 2012). While the predominant oxidant during storage is oxygen, other oxidants are important intravenously, including hydrogen peroxide and nitric oxide (NO) (Brooks 1932, Buehler et al. 2007). Although NO is much less abundant than oxygen, the reaction with Hb is extremely rapid (Cassoly and Gibson 1975, Doyle and Hoekstra 1981). On the other hand, several reductants are also present in blood that can reduce metHb (Ames et al. 1981, McGown et al. 1990, Harrington et al. 2000). Ascorbate is known to be a potent metHb reductant in the presence of red cells which can recycle the oxidized dehydroascorbate to ascorbate (Frei et al. 1989, Mendiratta et al. 1998, Dorman et al. 2002, Dunne et al. 2006, Buehler et al. 2007). Oxidation/reduction rates of Hbs vary with species, the presence of organic phosphates, pH, oxygen partial pressure, degree of Hb dissociation, extent of polymerization, and location of the heme group on alpha or beta subunits (Brooks 1935, Mansouri and Winterhalter 1973, Harvey and Kaneko 1976, Tomoda et al. 1976, Kikugawa et al. 1981, Zhang et al. 1991, Alayash et al.

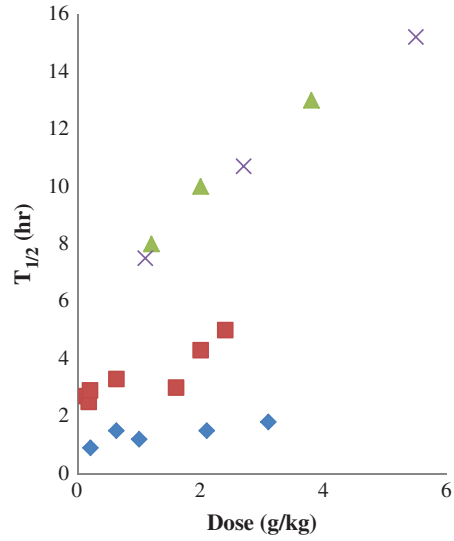

Figure 1. Circulating half-life of HBOCs in rats as a function of dose: unmodified Hb (♦), crosslinked tetrameric Hb (■), NFPLP-crosslinked, glutaraldehyde-polymerized Hb (▲), glutaraldehyde-polymerized bovine Hb (x). Data from (Bleeker et al. 1986, Snyder et al. 1987, Keipert et al. 1989b, Bleeker et al. 1992, Keipert et al. 1993, Keipert et al. 1994, Migita et al. 1997, Pearce et al. 2003).

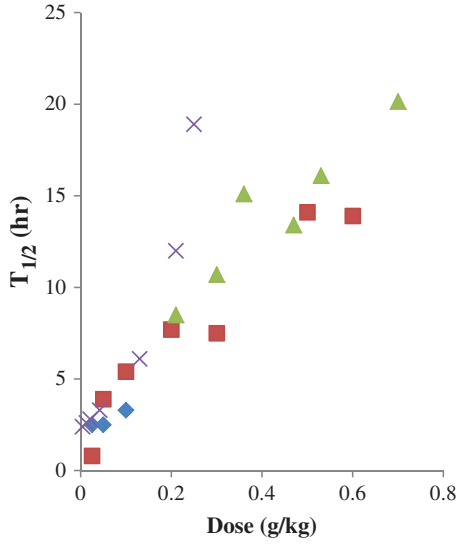

Figure 2. Circulating half-life of HBOCs in humans as a function of dose: crosslinked tetrameric human Hb (♦), raffinose-polymerized human Hb (■), glutaraldehyde-polymerized bovine Hb (▲), recombinant crosslinked tetrameric human Hb (x). Data from (Hughes et al. 1995, Hughes et al. 1996, Przybelski et al. 1996, Viele et al. 1997, Carmichael et al. 2000).

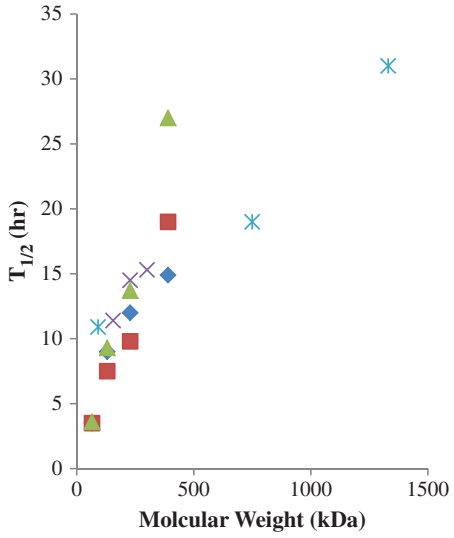

Figure 3. Circulating half-life of HBOCs in animals as a function of molecular weight: raffinose-polymerized human Hb—50% exchange transfusion in rats (♦), NFPLP-crosslinked and glutaraldehyde-polymerized human Hb—35% exchange transfusion in rats (■), NFPLP-crosslinked and glutaraldehyde-polymerized human Hb—50% exchange transfusion in rats (x), NFPLP-crosslinked and glutaraldehyde-polymerized human Hb—70% exchange transfusion in rats (▲), glutaraldehyde-polymerized bovine Hb—3 g/kg in guinea pigs (✳). Data from (Berbers et al. 1991, Bleeker et al. 1992, Hsia et al. 1993, Baek et al. 2012).

2001, Baek et al. 2012). The functional state of HBOCs after infusion is therefore a product of a myriad of variables, including metHb content upon infusion, intrinsic reactivity with oxidants and reductants, and relative concentrations of these reactants (Faivre-Fiorina et al. 1998). Due to this complexity, metHb formation rates after infusion cannot be predicted *a priori*. Not surprisingly, several researchers noted a poor correlation between oxidation rates *in vitro* and *in vivo* (Snyder et al. 1987, Vandegriff et al. 2006, Buehler et al. 2010).

A consistent finding from measurement of the *in vivo* oxidation of different HBOCs is that plasma metHb levels increase

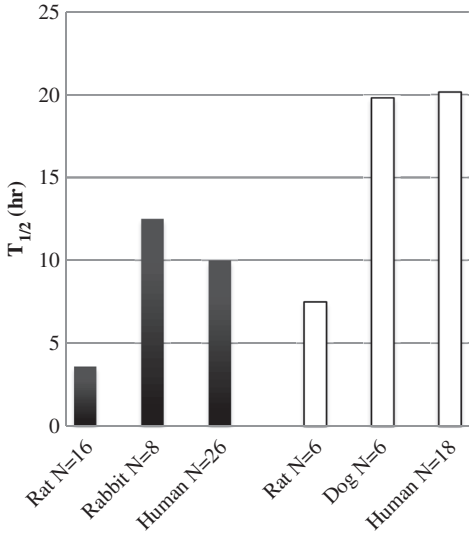

Figure 4. Comparison of intravascular half-life of two HBOCs in human and non-human species at 0.6–1.6 g/kg doses: crosslinked tetrameric Hb (■), glutaraldehyde-polymerized bovine Hb (□). Data from (Snyder et al. 1987, Hess et al. 1989, Hughes et al. 1995, Migita et al. 1997, O'Hara et al. 2001, Pearce et al. 2003).

immediately after infusion due to the oxidized Hb contained in the preparation. Subsequently, if the metHb content is initially low, the percentage of oxidized Hb usually increases as the total plasma Hb concentration decreases, resulting in 20–60% of the plasma HBOC being in the met form after several half-lives (Tam et al. 1978, Lee et al. 1995, Conover et al. 1997a, Buehler et al. 2007, Buehler et al. 2010). This reduces the effective functional half-life by as much as 40% (Table I), although this can be mitigated by the co-administration of reducing agents (Sehgal et al. 1981, Faivre-Fiorina et al. 1998). The rate of metHb formation is also dose-dependent. When den Boer and coworkers (den Boer et al. 1992) infused a polymerized Hb solution with 6% metHb into rats, plasma metHb content remained below 15% at exchange transfusion levels of 40% or 70% of blood volume, but after a 90% exchange metHb increased to 30%. The latter result probably reflects the removal of a substantial portion of the endogenous reduction capacity in blood during the high-volume exchange. Conversely, when fully oxidized Hb was infused, metHb levels decreased to 20% after 5% or 40% blood volume exchanges, but only 40% after a 70% exchange. A 40–80% reduction of high levels of oxidized HBOC was also observed by other authors in rats or guinea pigs, reinforcing the notion that mammalian blood exhibits substantial reducing potential (Snyder et al. 1987; den Boer et al. 1992, Faivre et al. 1994). It should be noted that while the percentage of plasma metHb usually increases with time after HBOC infusion, the absolute concentration may increase, decrease, or stay relatively constant until the inevitable ultimate decrease (Tam et al. 1978, Snyder et al. 1987, Bush et al. 1994, Lee et al. 1995, O'Hara et al. 2001, Sprung et al. 2002, Wicks et al. 2003, Dunne et al. 2006, Olofsson et al. 2006, Vandegriff et al. 2006, Buehler et al. 2007, Buehler et al. 2010, Baek et al. 2012, Elmer et al. 2012), depending on dose, recipient species, and HBOC type.

One question which arises is whether HBOC oxidation facilitates plasma removal. Snyder et al., reported that the half-life of completely oxidized diaspirin-crosslinked hemoglobin (DCLHb) was the same as that of reduced DCLHb, although these results are confounded by the fact that 73% of the oxidized Hb was reduced after five hours (Snyder et al. 1987). Vandegriff and coworkers argued on kinetic grounds that preferential clearance of the met form of PEG-conjugated Hb was unlikely because the overall rate of clearance of this HBOC from the circulation is significantly faster than the rate of metHb formation (Vandegriff et al. 2006). On the

Table I. Comparison of Total and Functional Half-Lives of HBOCs.[a]

HBOC Type	Test Species	$T_{1/2}$ Total HBOC (h)	$T_{1/2}$ Functional HBOC (h)
Human dextran-coupled	Dog	58	46
Bovine glutaraldehyde-polymerized	Guinea Pig	11.1	6.9
"	"	7.3	5.6
Bovine glutaraldehyde-polymerized	Rat	15.6	13.4
"	Guinea Pig	15.7	12.9
Bovine glutaraldehyde-polymerized	Sheep	53	30
Bovine PEG-derivatized	Rat	17.7	15.6

[a]Data from (Tam et al. 1978, Lee et al. 1995, Conover et al. 1997b, Buehler et al. 2007, Buehler et al. 2010).

other hand, Buehler et al. suggested that the difference in plasma persistence between bovine Hbs polymerized in the oxygenated versus the deoxygenated states may be due to the fact that the *in vivo* oxidation rates are different (Buehler et al. 2010); however, these two preparations also differed in a number of other physical and chemical properties. Overall, the extant literature is not definitive on this point, but suggestive that conversion of reduced Hb to the oxidized form is not a primary step in the Hb elimination process.

Overview of distribution and plasma elimination

Several laboratories have investigated the distribution and metabolism of HBOCs. General results will be summarized in this section, followed by detailed discussion of individual removal pathways in the next. One caution in interpreting these data is that the amount of HBOC present in an organ at any given time is a function of the difference between the rate of uptake and the rate of catabolism. Thus, if uptake rates are similar, but the catabolism rate is greater in one organ versus another, the amount of Hb removed by the latter may be similar even though the concentration of Hb at any particular time point will be lower. As a consequence, the relative amount of HBOC present may not quantitatively reflect the relative contributions of organs toward Hb removal.

Chromatographic analysis of HBOCs in plasma suggests that these proteins are not substantially degraded within the circulation (Bleeker et al. 1989, Hsia et al. 1993, Bush et al. 1994). Supporting this is the fact that the disappearance of radioactively labeled HBOCs from plasma corresponds closely to their rate of disappearance as determined by a spectrophotometric assay (Keipert et al. 1993, Keipert et al. 1994). In addition, when low doses of tritiated HBOC were recirculated through isolated, perfused rat liver, most of the radioactivity in the perfusate remained associated with intact Hb (Chow et al. 2008). On the other hand, low molecular weight metabolites rapidly appeared in the bile. Thus, the primary sites of HBOC catabolism are probably extravascular.

Three published studies investigated the plasma elimination and catabolism of human Hb intramolecularly crosslinked with 2-nor-2-formylpyridoxal 5'-phosphate (NFPLP) in rats (Bleeker et al. 1989, Keipert and Triner 1989, Keipert et al. 1989b). Using ^{99m}Tc labeling, Bleeker and coworkers found that the concentration of crosslinked HBOC in the kidney was greatly reduced relative to that seen with unmodified Hb two hours after high-dose exchange transfusion (Bleeker et al. 1989). Some HBOC accumulation was detected in the liver and spleen. However, scintigrams indicated that much of the protein was diffusely distributed throughout the body. One concern with these data is that technetium labeling demonstrably affected the kidney filtration of Hb, suggesting that the technique may influence the protein distribution.

Keipert and coworkers labeled this same HBOC with ^{3}H in a manner which would not be expected to alter chemical or physical properties (Keipert and Triner 1989, Keipert et al. 1989). Rats were monitored, and urine and feces collected, for up to nine days after the infusion of a relatively low (0.15 g/kg) dose. Organs were collected from exsanguinated and saline-flushed animals to quantify label accumulation. Nine hours after infusion, the highest concentration of label was detected in the kidneys, liver, and spleen, although the total amount of label in muscle was comparable to that in the liver and kidneys due to the large total muscle mass. Lesser amounts of label were detected in other organs and tissues. Label concentration rapidly decreased in the liver and kidney, suggesting that HBOC is actively catabolized in these organs. Radioactivity was found in urine several hours after infusion and throughout the monitoring period, albeit at a declining rate. A similar trend was seen in the feces. Ultimately, nearly 55% of the infused label was recovered in the urine and 28% in the feces. Ultrafiltration and chromatographic analyses demonstrated that radioactivity in the urine and feces was recovered predominantly in low molecular weight compounds. No intact HBOC could be detected in urine by spectrophotometric analysis.

Qualitatively similar results were obtained from studies of the distribution and elimination of DCLHb (Dittmer et al. 1992, Keipert et al. 1993, Bush et al. 1994, Keipert et al. 1994). After a 50% exchange transfusion into rats with ^{14}C-labeled protein, Keipert and coworkers found the highest radioactivity in the kidney, spleen, bone marrow, and liver, 24 h after infusion (Keipert et al. 1993, Keipert et al. 1994). However, the highest overall label recovery was in the muscle and skin. Brain radioactivity was particularly low. Tissue and organ concentrations peaked 5 to 24 h after infusion and declined thereafter. Radioactivity appeared in the urine within an hour of infusion, peaking on the second day of collection, and at detectable, but decreasing, levels thereafter. Approximately 59% of the administered radioactivity was recovered in the urine and 9% in feces. Electrophoretic and chromatographic analyses of urine demonstrated the presence of intact DCLHb in early collected samples, suggesting that some crosslinked Hb crosses the glomerular membrane. Subsequently, intact Hb was undetectable in the urine 12 h after infusion. Dittmer et al., reported a more diffuse distribution of radioactivity after the infusion of ^{51}Cr-labeled DCLHb into mice; however they also demonstrated some label elution from the protein (Dittmer et al. 1992), an artifact which was previously noted (Keene and Jandl 1965). After the infusion of a 2 g/kg dose of DCLHb into swine, Bush and coworkers found increased iron concentrations in the kidney and liver, consistent with Hb catabolism in these organs (Bush et al. 1994). Serum iron levels increased to a maximum eight hours after infusion and decreased to baseline by 96 h, never exceeding the total serum iron binding capacity.

Collectively, studies of crosslinked Hb tetramers suggest that this class of HBOCs distributes into numerous tissues and extravascular spaces after infusion. Small amounts may also be filtered by the kidney, though such filtration is markedly reduced from that of unmodified Hb. These HBOCs are catabolized by the liver and probably other organs of the reticuloendothelial system (See Mechanisms of plasma elimination).

Polymerization of Hbs to form higher molecular weight entities alters their distribution and elimination patterns, although interpretation of these data is confounded by the fact that these preparations contain components with a vari-

ety of molecular weights, including unmodified Hb (Lenz et al. 1991, Keipert et al. 1992, Anderson et al. 1993, Hsia et al. 1993, Baek et al. 2012). This difficulty was addressed by Hsia et al., who studied the distribution of human Hb polymers of different molecular weights synthesized by reaction with oxidized, ring-opened raffinose (Hsia et al. 1993). Differences were evaluated using both size-exclusion high-performance liquid chromatography and tritium-labeled polymer fractions separated and purified by preparative scale chromatography. After a 50% exchange transfusion into rats, kidney uptake decreased and liver uptake increased with increasing molecular weight, as measured ten hours after infusion. As expected, urinary excretion of the various polymerized Hb fractions was much lower than that of unmodified Hb. The similarity of plasma clearance rates for the different fractions determined by HPLC and labeled polymer clearance implies that polymers were chemically stable in plasma.

Anderson et al., radiolabeled unmodified human Hb with ^{3}H-formaldehyde and purified glutaraldehyde-polymerized human Hb with ^{14}C-formaldehyde (Anderson et al. 1993). The latter contained less than 2% of unmodified Hb, with more than 95% of the material exhibiting an apparent molecular weight in excess of 440,000 Da. These labeled fractions were then spiked into an unfractionated mixture of polymerized Hb which was infused into dogs at a dose of 0.8 g/kg. Once again, there was greater kidney accumulation of the unmodified Hb and greater liver uptake of the polymerized Hb. There was also greater extravasation of the former into other tissues, as evidenced by a higher specific radioactivity in the heart than would be expected on the basis of vascular volume. In a comparison of different molecular weight average glutaraldehyde-polymerized bovine Hb preparations in guinea pigs, Baek and coworkers noted an inverse dependence of iron deposition in the kidneys relative to molecular weight, implying a decreasing Hb metabolism in this organ (Baek et al. 2012). This was also corroborated by microscopic analysis for evidence of Hb deposition and heme oxygenase activity in the kidney, both of which are surrogate markers for the presence of intracellular Hb. Increases in iron content in the liver and spleen were noted for all three of the different molecular weight classes evaluated—91, 749, and 1330 kDa. The authors concluded that the 749 and 1330 kDa preparations were predominantly metabolized in the liver and spleen, while the 91 kDa Hb was also eliminated by the kidney. Lenz et al. found siderosis of Kupffer cells after the biopsy of chimpanzees partially exchange-transfused with a glutaraldehyde-polymerized human Hb, consistent with liver catabolism of this HBOC (Lenz et al. 1991). These authors observed renal excretion of approximately 7% of the administered dose of this preparation, which was expected due to the presence of 15% non-crosslinked Hb.

Mechanisms of plasma elimination

Haptoglobin and liver uptake of bound and free hemoglobin

Haptoglobin (Hp) binds unmodified Hb with near irreversible affinity, thereby incorporating it into high molecular weight complexes which prevent glomerular filtration (Laurell and Nyman 1957, Keene and Jandl 1965, Nagel and Gibson 1971). Infusion of preformed Hb–Hp complexes, or Hb at doses not exceeding the plasma Hp binding capacity, results in primary uptake by the liver (Murray et al. 1961, Keene and Jandl 1965, Bissell et al. 1972, Bunn 1972, Kino et al. 1980, Kino et al. 1987, Ship et al. 2005). At doses of Hb which exceed the limited plasma Hp binding capacity, the liver continues to be a significant organ for Hb removal, although substantial amounts are also excreted by the kidney (Keene and Jandl 1965, Goldfischer et al. 1970, Bissell et al. 1972, Bunn 1972, Greenburg 1983, Kino et al. 1987). Within the liver, the relative contribution of hepatocytes versus resident macrophages (Kupffer cells) to Hb removal has been debated. After infusion of 0.15 g/kg of Hb into rats, histologic staining revealed the presence of Hb only in Kupffer cells (Goldfischer 1970). However, after the infusion of higher doses, Hb was found primarily in endocytic vacuoles in hepatocytes (Goldfischer 1970). Likewise, after the infusion of ^{59}Fe-labeled Hb, 85–95% of the radioactivity associated with the liver was calculated to be in hepatocytes, regardless of association with Hp (Bissell et al. 1972). *In vivo* studies imply that Hb is taken up by hepatocytes in a receptor-mediated process (Kino et al. 1980, Kino et al. 1987), and that isolated hepatocytes can assimilate both Hp–Hb complexes and free Hb (Weinstein and Segal 1984, Zuwała-Jagiello and Osada 1998). Other studies have also shown that Hp is not required for rapid *in vivo* Hb clearance or hepatocyte uptake (Hershko et al. 1972, Weinstein and Segal 1984, Lim et al. 1998). On the other hand, an autoradiographic study of Hp–Hb uptake found that these complexes were primarily found in Kupffer cells (Wada et al. 1970), and Friedman and coworkers only detected Hb microscopically in Kupffer cells after the infusion of high doses of Hb (Friedman et al. 1978).

Many, but not all, HBOCs are also bound by Hp, although the strength of binding varies, depending on the particular modification chemistry utilized (Bunn 1967, Lockhart and Smith 1975, Benesch et al. 1976, Panter et al. 1994, Ship et al. 2005, Schaer et al. 2006, Buehler et al. 2008, Baek et al. 2012, Jia et al. 2013). These complexes are taken up by the liver, as are the much higher doses of free HBOC which are more representative of the anticipated clinical uses of these formulations (Bleeker et al. 1989, Keipert et al. 1989b, Smith et al. 1990, Dittmer et al. 1992, Hsia et al. 1993, Bush et al. 1994, Keipert et al. 1994, Ship et al. 2005, Baek et al. 2012). Only limited data are available concerning the specific liver cells involved with this uptake. After infusion of crosslinked Hb, light microscopy confirmed the presence of diaminobenzidene (DAB)-positive material within hepatocytes 15 min after exchange transfusion, presumably due to HBOC uptake (Bleeker et al. 1989). A small amount was detected in Kupffer cells. Smith and coworkers also detected Hb in both hepatocytes and Kupffer cells after the infusion of a different crosslinked Hb (Smith et al. 1990).

In comparing the clearance of unmodified and crosslinked HBOC in isolated, perfused rat liver with that observed *in vivo*, Chow and coworkers determined that liver uptake could account for 30–50% of the total whole body clearance of the latter, demonstrating both the importance of the liver in HBOC metabolism and the significance of other pathways

(Chow et al. 2008). Hp modestly facilitated the uptake of either Hb type, and the removal of the crosslinked Hb was slower than that of the unmodified counterpart. Breakdown of Hb into lower molecular weight metabolites excreted into bile began soon after liver uptake. Two caveats of this study are that it was performed with a low dose of Hb in mice. Similar comparisons at higher doses with other species would be of interest.

Kidney excretion

As summarized in the preceding rapid renal excretion of unmodified Hb is substantially mitigated by crosslinking and polymerization. Still, mild hemoglobinuria has been observed after the administration of modified HBOCs to both animals and humans (Keipert and Chang 1983, Bakker et al. 1986, Bleeker et al. 1986, Lenz et al. 1991, Keipert 1992, Keipert et al. 1992, Anderson et al. 1993, Keipert et al. 1993, Gilbert et al. 1994, Keipert et al. 1994, Lee et al. 1995, Conover et al. 1997, Schubert et al. 2002, Wicks et al. 2003, Bonegio et al. 2006, Olofsson et al. 2006, Baek et al. 2012). While this hemoglobinuria has been widely attributed to the presence of residual unmodified Hb, intact tetramers were detected in urine as well (Keipert et al. 1989b, Urbaitis et al. 1991, Keipert et al. 1992, Keipert et al. 1993, Keipert et al. 1994). This was somewhat surprising since it was historically believed that albumin, a protein only slightly larger than tetrameric Hb, undergoes little to no filtration by the kidney glomerulus (Pitts 1968, Guyton 1976). However, recent work has demonstrated that albumin indeed passes through the glomerular membrane, after which it is reabsorbed in the renal tubules where it may be degraded and/or transcytosed intact back into the circulation (Comper and Russo 2009, Menaka et al. 2009, Tojo and Kinugasa 2012, Tenten et al. 2013). Glomerular filtration is dependent on molecular size, charge, and shape, with reduced filtration favored by large size, negative charge, and globular conformation (Venkatachalam and Rennke 1978, Ghitescu et al. 1992, Haraldsson et al. 2008). Although albumin is a more elongated molecule than tetrameric Hb, it is much more negatively charged at physiologic pH and has a slightly greater molecular weight (Pitts 1968, Gros et al. 1978, Thomas et al. 1997, Tojo and Kinugasa 2012). In light of this, it is not surprising that some Hb tetramers are filtered. There is also evidence that, like unmodified Hb, some HBOCs passing through the glomerulus are reabsorbed by the kidney tubules, but the capacity of this system is limited (Lathem et al. 1960, Miller 1960, Ericsson 1965a,b, Friedman and DeVenuto 1982, Bleeker et al. 1989, Lenz et al. 1991, Urbaitis et al. 1991, Conover et al. 1997a,b, Gburek and Osada 2000, Gburek et al. 2002).

Extant data suggest that polymerization or conjugation of Hb into polymers of molecular weight greater than 100 kDa further reduces, and possibly, eliminates renal excretion, at least in animals with an intact glomerular filtration barrier (Berbers et al. 1991, Keipert 1992, Keipert et al. 1992, Hsia et al. 1993, Lee et al. 1995, Conover et al. 1997, Wicks et al. 2003, Bonegio et al. 2006, Baek et al. 2012). Thus, kidney excretion is considered to be a minor contributor to the removal of HBOCs from the intravascular circulation. On the other hand, studies performed with radioactively labeled HBOCs demonstrate substantial renal excretion of HBOC breakdown products, which probably derive from catabolism in various organs and tissues (Keipert et al. 1989b, Keipert et al. 1992, Anderson et al. 1993, Keipert et al. 1993, Keipert et al. 1994).

CD163 and monocyte/macrophage phagocytosis

It has been known for decades that macrophages pinocytose Hb (Ehrenreich and Cohn 1968), but it was only in 2001 that a macrophage protein, CD 163, was identified as the receptor which mediates the endocytosis of Hp–Hb complexes (Kristiansen et al. 2001). CD 163 is only expressed in the monocyte/macrophage cell lineage, including tissue-resident macrophages such as Kupffer cells (Graversen et al. 2002, Moestrup and Møller 2004, Polfliet et al. 2006). In addition to the high affinity binding of Hp–Hb complexes, CD163 was more recently shown to interact directly with Hb with lower affinity to effect receptor-mediated endocytosis in the absence of Hp (Schaer et al. 2006). CD163 interacts with modified HBOCs to varying degrees, depending on the nature of the chemical derivatization (Schaer et al. 2006, Buehler et al. 2008). In light of these results, it is tempting to infer a significant role for the CD163-mediated uptake of HBOCs into macrophages as a primary route for Hb clearance from the circulation (Schaer et al. 2006). This is consistent with the observation that Hb clearance was impaired in two patients with intravascular hemolysis concomitant with a treatment regimen for leukemia which reduced the number of CD163-expressing monocytes (Maniecki et al. 2008). However, Hb clearance was not markedly reduced in mice which were genetically depleted of CD163 compared to controls (Etzerodt et al. 2013). Comparison of these seemingly conflicting results is confounded by species differences and possible indirect effects of the aggressive therapy required in the treatment of the human subjects; however, the recent observation that Hb clearance was little affected in dogs in which the circulating pool of CD163-expressing macrophages was increased with glucocorticoid stimulation again suggests that this pathway may not be primary in the clearance of HBOCs (Boretti et al. 2014). A similar conclusion has been articulated by several of the authors involved with the characterization of the CD163–HBOC interaction (Schaer et al. 2007). A possibly related set of observations is that the clearance of Hb and the clearance of particulate challenges by the reticuloendothelial system do not interfere with one another, suggesting different clearance pathways (Keene and Jandl 1965, Greenburg 1983, Kim et al. 1993).

Extravasation and distributed endocytosis

Plasma components extravasate through the endothelium of blood vessels by mechanisms which are dependent on capillary structure, disease state, and blood and lymph flows (Takakura et al. 1998). Macromolecule extravasation is dependent on size, shape, charge, and hydrophilic/lipophilic balance (Gandhi and Bell 1992, Takakura et al. 1998, El-Sayed et al. 2001, Lin 2009). Although the mechanisms of extravasation have been debated for decades, caveolae-mediated transcytosis through the endothelial cell interior of capillaries is believed to be an important pathway for albumin, along

with passage through pericellular junctions between cells (Szabó and Magyar 1982, Malik et al. 1989, Schnitzer et al. 1994, Simionescu et al. 2002, Aird 2007, Lin 2009, Kumari et al. 2010). Measurements of radioiodinated albumin efflux indicate that 5–10% of plasma albumin extravasates per hour (Szabó and Magyar 1982, Fleck 1985). It is therefore not surprising that HBOCs also extravasate (Keipert et al. 1989a, Dittmer et al. 1992). Velky and coworkers noted that both unmodified and ATP-modified Hb leaked into the peritoneal cavity after intravenous infusion, with accumulation of the latter significantly inhibited by *m*-dansyl cadaverine, an endocytotic blocking agent (Velky et al. 1987). Bleeker et al. detected crosslinked Hb in thoracic duct lymph (Bleeker et al. 1989). Other researchers have detected crosslinked Hb in the hilar, lung, and soft tissue lymph (Conhaim et al. 1998, Matheson et al. 2000), and Burhop and Doyle reported that the appearance of genetically polymerized Hbs in rat thoracic lymph was inversely correlated with molecular weight (Burhop and Doyle 2002).

An interesting question is the fate of extravasated Hb. Albumin has a plasma half-life of several hours, but an overall circulating life span of approximately three weeks, implying that it constantly recirculates between the intravascular and extravascular space (Szabó and Magyar 1982, Fleck 1985). While the initial intravascular half-life of the comparably sized crosslinked Hb is similar to that of albumin, the overall circulatory persistence is much less, implying that Hb is subject to a catabolic process which does not operate on albumin. Similarly, IgG also exhibits an overall circulatory persistence which is much greater than like-sized HBOC polymers (170 kDa) (Morell et al. 1970, Peppard and Orlans 1980). On the other hand, other plasma proteins exhibit a more rapid catabolism which is qualitatively similar to that of HBOCs (Jarnum 1975, Bouma 1982). A large body of work has demonstrated that this difference is due to the fact that many plasma proteins are subject to catabolism in endothelial cells by endocytosis followed by lysosomal degradation, but that albumin and IgG are largely spared from this fate by a pH-specific binding to the FcRn receptor which redirects these proteins back into the circulation (Ghetie et al. 1996, Israel et al. 1996, Junghans and Anderson 1996, Chaudhury et al. 2003). It is likely that HBOCs are also subject to this catabolic pathway, and, while this has not been specifically evaluated, several lines of evidence are consistent with this hypothesis:

- HBOC clearance cannot be quantitatively explained by catabolism in the liver and monocytes/macrophages (Schaer et al. 2007, Chow et al. 2008, Etzerodt et al. 2013, Boretti et al. 2014).
- Kidney filtration of crosslinked and polymerized HBOCs is minimal (Berbers et al. 1991, Keipert 1992, Keipert et al. 1992, Hsia et al. 1993, Lee et al. 1995, Conover et al. 1997a, Wicks et al. 2003, Baek et al. 2012).
- Hb has been detected inside endothelial cells (Faivre-Fiorina et al. 1999).
- The inhibition of peritoneal accumulation of Hb by the endocytotic inhibitor *m*-dansyl cadaverine implies that Hb is subject to endocytosis (Velky et al. 1987).
- It seems highly unlikely that a rescue receptor for Hb would have evolved, since several pathways for Hb removal have been favored by evolution (Murray et al. 1961, Keene and Jandl 1965, Goldfischer et al. 1970, Bissell et al. 1972, Bunn 1972, Kino et al. 1980, Kino et al. 1987, Ship et al. 2005).
- The wide detection of the Hb label throughout the body in distribution studies is consistent with a dispersed catabolism component (Dittmer et al. 1992, Keipert et al. 1994, Bleeker et al. 1989).
- FcRn is detected in many tissues, but particularly in endothelial cells, indicating that these are important sites of plasma protein catabolism (Bouma 1982, Ghetie et al. 1996, Borvak et al. 1998).

One implication of this hypothesis is that HBOC circulatory half-life may be improved by association of the Hb with proteins or protein fragments that bind to the FcRn receptor. This strategy has been successfully employed to improve the circulatory persistence of therapeutic proteins by as much as 4-fold (Dall'Acqua et al. 2006, Andersen and Sandlie 2009, Andersen et al. 2011), and conjugation of Hb to albumin was reported to enhance intravascular half-life (Bonhard and Boysen 1982). However, the latter study employed nonspecific crosslinking and was not directed to exploitation of the FcRn receptor. Thus, it is unclear whether the observed increase was due to FcRn binding or a simple increase in molecular weight. Similar experiments, coupled with measurement of the interaction of the complex with the FcRn receptor, would be of interest, both to confirm the role of distributed endocytosis in HBOC catabolism and as a possible route for enhancing therapeutic efficacy.

Immune system-mediated HBOC removal

Generation of neutralizing antibodies to therapeutic proteins may accelerate their clearance (Porter 2001, Lin 2009). Human Hb A_1 is only weakly antigenic, requiring co-administration with adjuvant to solicit a significant response (Chernoff 1953, Heller et al. 1962, Rachmilewitz et al. 1963). It was found that crosslinked human Hb was not antigenic in rhesus monkeys or human patients after intravenous infusion (Estep et al. 1992, Patel et al. 1998). Hertzman and coworkers found that polymerization increased the antigenicity of heterologous Hbs (Hertzman et al. 1986), and a number of studies have further demonstrated that polymerized or conjugated heterologous Hbs stimulate antibody generation in several species, including humans (Cunnington et al. 1981, Marks et al. 1987, Bleeker et al. 1995, Hamilton et al. 2001, Hamilton and Kickler 2007). However, the toxicologic consequences of such antibody generation appear to be modest, and no assessment has been reported on the effects of such antibodies on pharmacokinetics. Thus, any effect of antibody generation on the circulating half-life of HBOCs is currently unknown.

Summary

The circulatory persistence of HBOCs ranges from less than one hour to several days, depending on dose, species, and

Figure 5. Identified and hypothetical routes of elimination of HBOC from plasma.

molecular weight. Half-lives of up to 20 h have been demonstrated in humans. Functional half-life is diminished relative to the overall circulatory persistence by as much as 40% due to oxidation of the HBOC heme. HBOCs are eliminated from the circulation and metabolized by several of the same pathways as unmodified Hb, but their relative importance varies depending on the physical and chemical characteristics of the given formulation (Figure 5). There is also direct and indirect evidence that one or more additional pathways may be important, especially endocytosis by the endothelium. A complete accounting of the quantitative contribution of each of the possible pathways to HBOC removal from the circulation would be of interest to rigorously define the metabolism of these compounds.

Declaration of interest

Timothy Estep is a consultant for Omniox, Inc., an early stage biopharmaceutical company developing new medicines for hypoxic diseases. No financial, editorial, or writing support was received from Omniox for the production of this manuscript.

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