Toxicological Quandary of the Use of Bis (2-diethylhexyl) phthalate (DEHP) as a Plasticizer for Blood Bags

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ABSTRACT

Plastic bags are very useful containers for the storage of blood and blood products since they are relatively transparent, hard to break, can be sealed aseptically with a radio-frequency current, and can be centrifuged for the isolation of blood components. In order to make the plastic more flexible, various agents are added, of which the most common is di- (2-ethylhexyl) phthalate (DEHP). This plasticizer has been found to leach from the plastic into the blood components during the storage period. Some animal studies have shown that this chemical can produce cancers and various tissue abnormalities. The human data from multi-transfused patients do not clearly indicate any specific damage; however, because of the animal studies, work has been carried out to find a non-leachable plasticizer. Several have been found: unfortunately, when survival studies are done, the red cell life span of the stored blood is decreased. Current work seems to indicate that DEHP has a membrane stabilizing function that prolongs the storage time of the red cell. Therefore, there currently is a trade off between plasticizer presence and red cell life span that must be considered when designing new blood storage bags.

Introduction

In the early days of blood transfusion, all transfusions were given directly from the donor to the recipient, so there was no need for a storage container for blood. However, following the discovery of anticoagulants, blood could be stored in a container until needed. The first containers used were glass bottles, Erlenmeyer flasks or Salvarsan flasks; however, during World War II, the evacuated glass bottle was established as the standard. The glass bottle had a number of limitations. It broke when dropped and often when it was being centrifuged. Because of this, it was relatively impossible to use for the isolation of blood components. The vacuum could be lost, thus making bleeding difficult. In order to draw blood, the donor's tubing had a needle on both ends, and the bottle top as well as the donor's arm had to be aseptically prepared before the needles were inserted. This doubled the...
possibility of contamination. Finally, glass bottles are heavy and a case full of blood is a significant burden to move.

In the late 1940s and early 1950s, Dr. Carl Walters and his associates\textsuperscript{44} began work on a bag made of plastic that would eliminate some of these problems. The final product was relatively resistant to breakage, drew blood using gravity instead of a vacuum, had an integral donor tubing, and was light weight. When empty, they were small and did not represent a major storage problem. The plastic used for the manufacture of these bags was a polymer of polyvinyl chloride (PVC) that contained about 45 percent by weight of di (2-ethylhexyl) phthalate (DEHP) as a plasticizer. This bag produced post transfusion red cell survival that was equivalent or superior to that found in glass bottles with the same anticoagulant and would preserve platelets which did not occur with the unmodified glass bottle. Finally, the bag could be centrifuged, and the various blood components could be extracted aseptically. For this reason, blood component therapy, as we know it today, was a result of this plastic bag.

Little thought was given to the plasticizer used. Most transfusionists did not even know the amount of this material that was in each bag nor did they consider it a problem. In 1967, however, Guess\textsuperscript{10} showed that some of the plasticizer could leach from PVC bags into the blood. In 1970, Jaeger and Rubin,\textsuperscript{14} while studying the isolated rat liver, found three different acidic ultraviolet-absorbing materials with chromatography that had leached from the plastic tubing into the perfusate medium. One of these materials was identified as glycolyl phthalate, and investigation showed that the plasticizer used in the plastic tubing was butyl glycolylbutyl phthalate. Subsequently, other tubing was tested that used DEHP for a plasticizer and it was found that it too would leach into the medium. When this tubing was used in the rat liver model, it was found that the material was cleared from the blood and absorbed by the rat liver. Salt solutions did not extract the DEHP but four percent bovine serum albumin removed about 40 percent as much DEHP as was removed by whole blood. Units of stored human blood were then studied, and a significant amount of DEHP was found in the plasma fractions, specifically those associated with the lipoprotein fraction. Finally, tissues from two patients who had received blood transfusions were analyzed and DEHP was recovered from many of the tissues. Jaeger and Rubin’s final discussion introduces the possibility that this material may be responsible for “subtle toxicity” in blood products.

This study was expanded in a subsequent article.\textsuperscript{15} Analyses were performed on whole human and dog blood stored in plastic bags with DEHP plasticizer. It was shown that the DEHP was extracted at a rate of 0.25 mg per 100 ml per day and was present primarily in whole blood. Washed red cells contained very little of the DEHP; however, the plasticizer was found in both the lipid-containing and the lipid-free portions of plasma. Seven of 12 lung-tissue specimens taken at autopsy from patients who had received transfusions contained detectable amounts of plasticizer; one patient, after open heart surgery, excreted a urinary metabolite of the plasticizer in greater amounts than he could have received from the blood transfused. The authors felt that the excess might have been supplied by the tubing of the cardiopulmonary apparatus. From these data, it was calculated that a whole-body exchange transfusion in a 70 kg man with 21-day-old blood would result in the intravenous administration of approximately 300 mg of DEHP, or a dose of four to five mg per kg of body weight. Marcel\textsuperscript{22} raised com-
parable concerns of overdosage with DEHP in hemophiliacs who receive large amounts of cryoprecipitate isolated in plastic bags.

A subsequent letter to the editor by Rubin33 stated that in an expanded study of patients who had or had not been transfused, over 50 percent of the non-transfused patients had qualitative amounts of DEHP similar to those of the patients who had been transfused. Technical problems did not allow him to make quantitative comparisons; however, it was felt that some of this contamination might be due to the environment containing significant amounts of the plasticizer that were subsequently absorbed by the patient. A report by DeHaan6 demonstrated that serum containing solutions perfused through PVC administration tubing were highly toxic to chick embryo heart cells isolated in tissue culture. Further work by Jaeger and Rubin16 showed that platelet concentrates contained a calculated concentration of approximately 19 mg of DEHP per 100 ml platelet concentrate. The platelets contained about 66 percent of this concentration, and the platelet-poor plasma contained the rest. These levels were much higher than would have been anticipated given the accepted diffusion rate of 0.25 mg per 100 ml per day for whole blood. Jaeger and Rubin mentioned that this level might be an artifact owing to the centrifugation process having concentrated the material. It was felt that if it were not, it would be possible to administer 57 to 144 mg of DEHP per 100 ml of DEHP in their supernatant at the end of five days storage at room temperature, while the platelet poor plasma contained 5.82 mg per 100 ml. Platelet concentrates stored at 22°C contained more DEHP than did those at 4°C. Contreras and coworkers felt that platelets were involved in some manner with the extraction process itself, but the process of extraction probably did not employ phagocytosis since the washed platelets themselves contained little DEHP. Furthermore, it was felt that the accumulation of amorphous debris which some authors had been invoking as a cause of pulmonary insufficiency was not primarily related to DEHP extraction.

Work presented by Carmen et al3 showed that the extracted amount of DEHP correlated fairly well with the amount of agitation applied to the platelet concentrates during room temperature storage. Unfortunately, this agitation is necessary to increase gas exchange across the plastic membrane and extend their storage life.

Miriopol and Stern23 studied the accumulation of DEHP in packed red cells and found that after three weeks of storage, there were about three times more whole blood stored in Citrate-Phosphate-Dextrose (CPD) anticoagulant increased by 0.268 mg per 100 ml per day. Plasma separated from ACD blood also had more DEHP in it than did that of CPD blood. Measurements on PVC containers with various anticoagulants in them, stored at room temperature for 218 to 427 days, showed no DEHP in the solution. Washing of a unit of blood removed about 98 percent of the DEHP. If the blood was frozen in either birefringent polylefin or polyvinyl chloride plastic containers at either −80°C or −150°C, the thawed red cells contained about the same amount of DEHP as had been present before freezing. Platelet concentrates were found to contain an average of 44.26 mg per 100 ml of DEHP in their supernatant at the end of five days storage at room temperature, while the platelet poor plasma contained 5.82 mg per 100 ml. Platelet concentrates stored at 22°C contained more DEHP than did those at 4°C. Contreras et al5 measured the residual levels of DEHP from a number of blood components. It was shown that whole blood preserved with Acid Citrate Dextrose (ACD) anticoagulant experienced an increase in the level of DEHP of about 0.36 mg per 100 ml per day while
plasticizer in whole blood. It was felt this was due to the smaller amount of plasma and also to restricted diffusion.

Studies reported by Gilbo and Coles\(^9\) showed that in plasma fractionation pools prepared from outdated blood, DEHP was present in the pools at contents ranging from 0.78 to 7.42 mg per 100 ml. High levels were found primarily in Cohn Fractions III and IV which contain most of the plasma lipoprotein. The therapeutic fractions (I, II, V, and SPPS [stable plasma protein solution]) contained only small amounts. For example, 100 ml of Fraction V (albumin) contained 0.23 mg of DEHP.

The detection of DEHP in plasma was complicated by the observations of Vessman and Rietz\(^{42}\) that a substantial amount of mono (ethylhexyl) phthalate (MEHP) could also be found in blood products. It was shown that from 4 to 56 \(\mu\)g per ml were found in ten bags of frozen plasma with ACD anticoagulant that had been stored for plasma fractionation. When eight of the samples were transferred to glass bottles, the MEHP content increased to between 14 and 44 \(\mu\)g per ml, while DEHP correspondingly decreased. Thus, there was active conversion of DEHP to MEHP during the storage period. The distribution of the MEHP in commercial fractions of plasma showed that it was less lipophilic than DEHP and, therefore, was present in larger quantities in the supernatant. Albumin was found to contain an average of 112 \(\mu\)g MEHP per g of albumin. Vessman and Rietz felt that DEHP, MEHP, and possibly phthalic acid levels should be totalled to determine the level of contamination.

This distribution was studied further by Albro and Corbett.\(^1\) One of their most useful discoveries was a method of introducing the DEHP into the plasma. Since DEHP is poorly soluble in most solutes, the majority of studies had been made by injecting plasma stored in plastic bags. Since the time and amount of leaching was variable, the DEHP levels used for toxicity studies were often not comparable. Reliable quantitation was thus difficult. These authors found that if the DEHP is added to Celite and this mixture was added to plasma that much more reproducible and rapidly established levels of DEHP that could be reproduced in the plasma. Using this model, it was determined that DEHP attached to lipoprotein in the order of LDL > VLDL > HDL > chylomicrons. Mono- (2-ethylhexyl) phthalate was in equilibrium between being freely soluble in solution and adsorbed to albumin. None was bound to lipoprotein.

Rock et al\(^{30}\) studied stored blood at periodic intervals and found that during the storage period both DEHP and MEHP increased, although the level of DEHP is always higher. The DEHP level increased greatly during the first 72 hours after which an approximate equilibrium was established. Mono (ethylhexyl) phthalate showed a progressive increase during the entire storage period. Although their early study showed an increase in the DEHP content of platelet concentrates compared to whole blood, when corrections were made for the different volumes, DEHP was found to be equally distributed in all blood fractions. Mono (ethylhexyl) phthalate was found to accumulate in whole blood stored in PVC bags; however, little MEHP is found in the bag itself. Further, when DEHP was added to blood stored in glass bottles, the DEHP decreased while the MEHP increased. Therefore, the authors concluded that there is some plasma component that metabolizes some DEHP into MEHP although it was not possible to determine what it was; however, it was not felt that platelets were responsible. Peck et al\(^{28}\) showed that the
DEHP→MEHP conversion can occur even when plasma samples are stored in glass while awaiting analysis. Heating the samples at 60°C for 25 to 30 minutes blocked this conversion. Therefore, it was felt that this conversion is caused by some type of enzyme. Carter et al. studied the in vitro hydrolysis of DEHP to MEHP and found an esterase (or esterases) present in rat liver, lung and kidney that were believed to be responsible for this reaction.

Toxic Effects of Plasticizers

A number of studies have been performed to show the toxic effects of the plasticizers in blood bags, but many of these are difficult to interpret and to determine if they are applicable. The basic problem encountered when studying DEHP administered intravenously is that it is poorly soluble and, thus, a standard concentration cannot be prepared. One must rather depend on the material leaching from the blood bag into the blood products. Thus, the levels achieved in the blood not only vary with the blood product used for testing, but also with the amount of storage time and the bag composition. The presence of multiple toxic metabolites further complicates the picture. To avoid this variable, many of the toxicity studies have been performed using the time honored method of feeding huge doses of the material to the experimental animals. This may or may not be directly correlatable with the intravenous injection of these materials, which is the method that humans will receive the material. Human studies are complicated by the finding that about 70 percent of all humans transfused die within six months of having received the blood or blood products. A certain mortality is to be expected since much blood is given to seriously ill patients, but the problem of following multi-transfused patients and determining their causes of death does complicate any chronic toxicity studies in patients.

Early toxicity studies were mostly concerned with the effects found in tissue culture or isolated organs. The works of Rubin and Jaeger previously cited, as well as that of DeHaan, showed that some of the material that would leach from polyvinyl chloride tubing and bags was toxic to cells grown in tissue culture or isolated organs.

Turner et al. countered other reports of the toxic effect of DEHP producing chromosomal abnormalities in tissue culture by stating that the major effect of DEHP on lymphocytes is an inhibition of mitosis and growth rather than genetic damage.

The effect on tissue culture cells is a highly sensitive method for measuring the toxicity, but it may not be toxic in some systems owing to its low solubility. Gesler has given an excellent review of the toxicity of phthalic acid plasticizers in animals. He stresses that acute intravenous toxicity of this group is hard to determine because of their limited solubility in water. Intraperitoneal and oral toxicities have been determined, but they are greatly variable, have a low order of toxicity, and may reflect contamination with free phthalic acid. In Gesler’s laboratory, the acute LD50 found in mice with intraperitoneal injections was >75 g per Kg. Only a few of the phthalate esters had been evaluated in long term toxicity studies at that time and Gesler felt that these studies needed to be done.

Very extensive reviews of the biological effects of the phthalates have been written by Thomas and Thomas and Autian. The Ph.D. dissertation of Jaeger is quoted as stating that an increase in microaggregates occurred...
owing to DEHP (cited in reference 8), but these data are not readily available. The subsequent paper by Rubin and Jaeger\textsuperscript{34} did mention the correlation between DEHP levels in platelet packs and the presence of microaggregates; however, these studies have been neither confirmed or expanded.

In a rather extensive review of the toxicity of DEHP, Wallin et al\textsuperscript{43} discuss many of the problems in the determination of the toxic effects of this chemical of which the first major problem is the limited solubility of the product in most common solutes. Because of this, most intravenous toxicity studies have been performed with the drug incompletely solubilized. Studies reported by this group showed that in rats, after the injection of a six percent solution of C-14 labelled DEHP in a fat emulsion, after 10 minutes there was a rather marked accumulation in the lungs after which it quickly migrated to the heart, liver, spleen, and the proximal small intestine. At 24 hours, there was a clearing of the drug from the lungs and liver, and the appearance of the label throughout the intestinal tract. Wallin and cohorts' interpretation is that the lipid suspension was first taken up by organs with a known reticuloendothelial function, then mobilized to the liver, and finally excreted in the bile either as intact DEHP or as one of its metabolites. Schulz and Rubin\textsuperscript{35} have shown that DEHP is excreted in the bile. With side chain C-14 labelled DEHP injected intravenously, they found that within 24 hours more than half of the DEHP was recovered in the urine, while the feces and gastrointestinal content accounted for more than 25 percent of the remainder. Counting techniques showed that about 81 percent of the injected dose was excreted within a 24 hr period. Significantly, only 1.7 percent of the administered dose remained in body fat at 24 hrs post injection. After oral administration of either ring or side chain C-14 labelled DEHP to rats, the majority was again found to be excreted either in the gastrointestinal content or in the urine. Comparable results were found in the dog; however, a larger amount remained in the carcass. Schultz and Rubin felt these data showed that there is significant absorption of intact DEHP from the gastrointestinal tract and that the reported low order of oral toxicity is a characteristic of the compound and not of its poor solubility.

Tavares and Vine\textsuperscript{38} isolated peritoneal leukocytes from rats and exposed them to dibutyl phthalate (DBP), MEHP, and DEHP. They found that these esters inhibited the formation of both cyclooxygenase and lipoxygenase arachidonate products by leukocytes. The authors felt that the phthalate esters have some structural resemblance to prostaglandins, and for this reason might inhibit arachidonate metabolism and alter cell functions such as platelet aggregation.

Lake et al\textsuperscript{20} showed that if rats were fed a daily oral dosage of 2000 mg per Kg of DEHP for 21 days the relative liver weights of the animals increased progressively. Alcohol dehydrogenase activity and microsomal protein and cytochrome P-450 content showed a marked initial increase followed by a decrease as the treatment progressed. In contrast, some of the other enzymes decreased throughout the exposure. Electron microscopic studies showed a progressive dilation of the smooth and rough endoplasmic reticulum, mitochondrial swelling and an increase in microbodies. There was no indication of storage of phthalate residues in the liver. Mono (ethylhexyl) phthalate produced comparable changes in similar dosages. The results indicate that the hydrolysis of DEHP to MEHP was the step that determined the hepatic changes produced by DEHP. Changes encountered in the ferret by Lake et al\textsuperscript{21} were compa-
rable. Kluwe et al\textsuperscript{19} showed that if rats or mice were fed diets containing 6000 mg per Kg DEHP, their survival rate was unaffected nor did it affect the amount of food eaten. Seminiferous tubular degeneration and hypertrophy of cells in the anterior pituitary were observed in male rats. The treatment also caused liver tumors in both sexes of mice and rats. About 50 percent of these tumors metastasized to the lungs. An interesting new aspect of toxicity has been introduced by Wierda et al\textsuperscript{45} who noted that infrequently some individuals (i.e., hemodialysis patients) experience unexplainable anaphylactoid reaction or eosinophilia after long term exposure to PVC leachates. These authors attempted to study whether or not extracts from PVC blood bags would result in sensitization or cytotoxicity or spleen cells as measured by growth of and uptake of tritium by spleen cell cultures. They found that the extractable products themselves might be somewhat cytotoxic, but no immunological response was shown.

Almost all of these articles showed that toxicity of DEHP is hard to determine because: (1) the drug is poorly soluble, (2) the effects, if any, are subtle and slow to take place, and (3) there is a considerable amount of DEHP contamination in the environment. For this reason, when Vox Sangunis devoted an International Forum to the toxicity of this material,\textsuperscript{13} the contributors, all of whom were engaged actively in blood banking, unan­imously felt that there was little danger to the patient from receiving blood or blood products stored in this material. Nowicki and Klos\textsuperscript{27} arrived at comparable conclusions after a review of the literature and some new experimental data; however, they felt that it would still be best to use other plasticizers if at all possible. Comparable conclusions were reached by Thomas et al\textsuperscript{39} who have published a very extensive and fairly current literature review of the biological effects of DEHP.

The Search for a New Plastic

With a disturbing number of studies showing that there might be some effect of DEHP on the patient, the bag manufacturers began to search for a better plastic that used no or a non-leachable plasticizer. The major studies involved non-PVC plastics, such as polyoylefin or a PVC plastic with a non or poorly leachable plasticizer. One such was the plasticizer triethylhexyl trimellitate (TEHTM or TOTM). This compound is composed of a phthalate ring triply substituted at the 1, 2, and 4 positions with ethylhexyl groups. Films made from either of these plastics showed little or no leaching of the plasticizer and very good flexibility. They further were found to allow good diffusion of gases from the blood component (especially platelets), – thus ensuring a more physiological pH. Soon, studies began to be reported showing that the storage time of platelets could be substantially increased at room temperature,\textsuperscript{29} while other studies showed that red cell antigens and plasma coagulation factors were well preserved in this plastic.\textsuperscript{36} The platelet storage times were eventually increased from two or three to seven days,\textsuperscript{11} although subsequent fear of bacterial growth finally caused the FDA to limit it to five.

The usual configuration for a multiple set of plastic bags is for the donor tubing to go directly into the primary bag. From there, a series of satellite bags is attached so that the entire assembly can be centrifuged and the desired component then expressed aseptically into the satellite bags. Manufacturing is simplified if all the bags are made out of the same material. Therefore, testing was carried out to determine whether or not red cells would survive well in this new plastic. This was especially important, since the new anticoagulants had increased the storage time of refrigerated whole blood and red cells from 21 days to 35 days, with 42 and 49 days
being considered. Stern and Carmen showed that there was increased hemolysis in stored blood with non-DEHP plasticizers and that this hemolysis could be decreased by the supplementary addition of some DEHP.

The in vivo human studies of Myhre et al showed that the survival of autologous red cells stored at 4°C in CLX plastic (a PVC plastic with TOTM added as the plasticizer) and using CPDA-1 as an anticoagulant was less than 75 percent at the end of 35 days. On the other hand, if the cells were stored for 21 days with CPD anticoagulant, the post transfusion survival was acceptable. The conclusion was that red cells should not be stored in TOTM plasticized films for longer than 21 days.

Horowitz et al recently studied the changes occurring in whole blood stored in containers fabricated from ethylene ethylacrylate (EEA) film and compared them with blood stored in PVC film. It was found that red cells stored in PVC film were osmotically more stable and lost one-third less hemoglobin than red cells stored in EEA. Incorporation of some DEHP into the EEA film significantly reduced erythrocyte osmotic fragility. This degree of stabilization correlated with the level of DEHP in the cell phase. Estep et al studied the cell morphology, plasma hemoglobin accumulation, microvesicle production, and electrolytes of erythrocytes stored at 4°C with and without DEHP. It was found that if sufficient emulsified DEHP was mixed with blood to give a final concentration of 300 μg per ml, the plasma hemoglobin accumulation was reduced by an average of 70 percent, the percentage of cells exhibiting normal morphology was enhanced by at least 20 fold, and the volume of microvesicles released from red blood cells was reduced by 50 percent after 35 days of storage when compared with corresponding samples stored without added phthalate. Estep and coworkers felt that the data are consistent with the hypothesis that DEHP inhibits the deterioration of the red blood cell membrane that results from the refrigerated storage of whole blood. Subsequently, Rock et al showed that about 25 percent of the DEHP binds immediately to sites in both the membrane and cytosol fractions of the red cells and does not change significantly over a seven day period. When red cell concentrates were stored with or without DEHP using either polyolefin bags or glass, there was a definite reduction in osmotic fragility when DEHP was absent.

Miyamoto and Sasakawa have been using a method of glow discharge treatment to decrease the leaching of the DEHP from the plastic. In a recent study that compared DEHP and TOTM plasticizers, it was found that DEHP in a granulocyte suspension caused a decrease in chemotaxis and bactericidal activity, but cell counts and phagocytosis were not affected. Glow treatment of the plastic causes the DEHP to remain fixed in the plastic.

Summary

Currently, the blood banking community is faced with a quandary. Some studies show that DEHP plasticizer is toxic to some tissue systems, and others show that ingestion of DEHP may cause tumors or testicular degeneration. Injection of the product is difficult because it is poorly soluble. Thus, the amounts of DEHP in blood products are nowhere near the amounts used in the usual toxicity study. Further, many patients with multiple transfusions who might receive large doses of DEHP do not survive. Therefore, it is difficult to quantitate the long term effects in human studies. However, with the concern generated by the animal studies, and the possibility of long term toxic effects occurring in surviving multi-transfused patients, it would appear prudent to find substitutes
for the plasticizer, if at all possible. However, when survival studies have been carried out on non-DEHP plastics, the red cell life span is decreased, and it appears that the DEHP has a stabilizing effect on the cell membrane. One is therefore caught in the quandary that if the plasticizer is eliminated, the stored life span of the red cell must be shortened. If this is shortened, the outdating will stabilize the membrane and at the same time not be toxic.

References


