의학박사 학위논문

Evaluation on erythrocyte preservation performance, plasticizer leaching, and clinical feasibility of DINCH as a substitute for phthalate plasticizer in blood bags

혈액백의 적혈구 보존 성능, 가소제 성분 유출, 및 프탈레이트 가소제 대체제로서 DINCH 의 임상적 타당성 평가에 대한 연구

2023 년 8 월

서울대학교 대학원

의학과 검사의학 전공

김 형 석

혈액백의 적혈구 보존 성능, 가소제 성분 유출, 및 프탈레이트 가소제 대체제로서 DINCH의 임상적 타당성 평가에 대한 연구

지도 교수 박 경 운

이 논문을 의학박사 학위논문으로 제출함
2023년 4월

서울대학교 대학원
의학과 검사의학 전공
김 형 석

김형석의 의학박사 학위논문을 인준함
2023년 7월

위 원 장 강 형 진 (인)

부위원장 박 경 운 (인)

위 원 전 윤 석 (인)

위 원 송 은 영 (인)

위 원 조 덕 (인)

Abstract

Evaluation on erythrocyte preservation performance, plasticizer leaching, and clinical feasibility of DINCH as a substitute for phthalate plasticizer in blood bags

Hyungsuk Kim

College of Medicine, Laboratory Medicine

The Graduate School

Seoul National University

Background: Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer commonly used in blood bags to convert polyvinyl chloride (PVC) into a flexible and elastic material. DEHP enhances red blood cell (RBC) stability during storage and prolongs its shelf-life by decreasing hemolysis. However, concerns about DEHP-related reproductive toxicity and potential endocrine dysfunction have been debated for many years. DEHP can leach out from the material to its content or surroundings because it is not

covalently bound to PVC. Because of this characteristic, humans and animals can be easily exposed to DEHP through various routes. Evidence of DEHP toxicity has been mostly found in animal models, and direct toxicity in humans is still unclear. However, patients undergoing certain clinical procedures, such as blood transfusion, can be at risk of high DEHP exposure. Several previous studies have investigated potential substitutes for DEHP in blood bags for RBC storage, but there is not enough data on the validation of non-DEHP systems in diverse settings. Di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) is an alternative plasticizer with a good toxicology profile, and DINCH-PVC has similar mechanical and physical properties to DEHP-PVC. This study aims to investigate the *in vitro* quality of RBCs stored in DINCH-plasticized blood bags with citrate phosphate dextrose adenine (CPDA-1) anticoagulant solution, develop an assay to measure DEHP and DINCH concentrations using ultra-high-performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS/MS), and analyze the plasticizer levels in blood bags.

Methods: Using a pool-and-split study design, we produced 20 matched homogenous quintets of RBC concentrates in two DINCH bags and three DEHP bags with CPDA-1 anticoagulant. RBC storage quality and plasticizer concentrations were assessed weekly for 35 days by performing a panel of *in vitro* tests. Samples for DEHP and DINCH measurement were prepared by liquid-liquid extraction, evaporation in N_2 for 30 min, and resuspension in 70% methanol with 0.1% formic acid. Samples were loaded into a UHPLC system equipped with the Synergi trap and

BEH columns, and multiple reaction monitoring transitions such as *m*/*z* 391 → 149 (DEHP) and *m*/*z* 425 → 281 (DINCH) were used for triple quadrupole MS/MS.

Results: On day 35, the median hemolysis levels in the DINCH bags (DINCH-GCMS, 0.297%; DINCH-TC, 0.342%) were marginally higher ($P < 0.05$) than the DEHP bags (DEHP-FK, 0.204%; DEHP-GCMS, 0.240%; DEHP-TC, 0.222%). However, all individual DINCH bags showed < 0.8% hemolysis. The DEHP-FK bag showed higher hemoglobin, hematocrit, and cell counts (RBC, white blood cell, platelet) than DINCH bags. RBCs in the DINCH bags showed increased mean corpuscular volume and decreased mean corpuscular hemoglobin concentration and eosin-5'-maleimide binding than the DEHP bags. Higher pO_2 and lower pCO_2 levels in the DINCH bags indicated better gas permeability than in DEHP bags. Other metabolic parameters were comparable in both bags. The within-run imprecision for DEHP and DINCH measurement were 11.11–14.01% and 2.08–6.15%, respectively. Calibration curves were linear over a range of 1–20 mg/L for DEHP ($R^2 = 0.9994$) and 0.0625–1.0 mg/L for DINCH ($R^2 = 0.9993$). The Synergi trap column was successful in separating the DEHP contamination in the mobile phase. The median plasticizer levels of the blood bags on day 35 increased to a range of 37.1–58.9 mg/L (DEHP) and 0.89–1.22 mg/L (DINCH).

Conclusions: The quality of RBCs stored for 35 days in DINCH-plasticized blood bags with CDPA-1 is comparable to those in DEHP bags. We successfully developed a UHPLC-MS/MS assay to measure DEHP and DINCH concentrations in blood

samples. DINCH leaches at a much slower rate into blood than DEHP during storage. Hence, DINCH is a promising alternative to DEHP in blood bags for RBC storage, even without the use of next-generation additive solutions to improve RBC preservation quality.

Keywords: DEHP, DINCH, Plasticizer, Phthalate, Blood component transfusion, Red blood cells

Student Number: 2010-30515

CONTENTS

ABSTRACT ····· i

CONTENTS ····· v

LIST OF TABLES ····· viii

LIST OF FIGURES ····· x

LIST OF ABBREVIATIONS ····· xii

PART I ····· 1

1. INTRODUCTION ····· 2

2. MATERIALS AND METHODS ····· 5

2.1 Donor Recruitment ····· 5

2.2 Blood Collection and Component Production ····· 8

2.3 Blood Storage, Sample Preparation, and *in vitro* Testing Schedule ····· 12

2.4 RBC Concentrate Quality Analysis ····· 15

2.4.1 Cell counts, RBC indices, and hemolysis ····· 15

2.4.2 RBC membrane integrity ····· 16

2.4.3 2,3-DPG measurement ····· 17

2.4.4 ATP measurement ····· 20

2.4.5 Other chemistry tests ····· 23

2.5 Statistical Analysis ····· 25

3. RESULTS ····· 26

3.1 Donor Recruitment and Study Progress ····· 26

3.2 Component Integrity ····· 28

3.3 RBC Concentrate Quality ····· 28

3.3.1 Hemolysis and plasma hemoglobin ····· 28

3.3.2 Cell counts and RBC indices ····· 33

3.3.3 RBC membrane integrity ····· 50

3.3.4 2,3-DPG and ATP ····· 53

3.3.5 pH, pCO_2, and pO_2 ····· 58

3.3.6 Glucose, lactate, K^+, and Na^+ ····· 65

4. DISCUSSION ····· 74

PART II ····· 84

1. INTRODUCTION ····· 85

2. MATERIALS AND METHODS ····· 87

2.1 Reagents ····· 87

2.2 Calibration Standard Preparation ····· 87

2.3 Sample Preparation ····· 89
2.4 UHPLC-MS/MS Analysis ····· 89
2.5 Evaluation of Analytical Performance ····· 92
2.6 Identification of Plasticizer Contamination ····· 92
2.7 Plasticizer Measurement in Donors and Blood Bags ····· 92
2.8 Statistical Analysis ····· 92

3. RESULTS ····· 94
3.1 UHPLC MRM Chromatogram ····· 94
3.2 Analytical Performance ····· 95
3.3 Identification of Plasticizer Contamination ····· 97
3.4 Plasticizer Measurement in Donors and Blood Bags ····· 99

4. DISCUSSION ····· 104

REFERENCES ····· 111

ACKNOWLEDGEMENTS ····· 119

CONFLICT OF INTEREST ····· 119

ABSTRACT IN KOREAN ····· 120

LIST OF TABLES

Table 1. Inclusion and exclusion criteria of blood donation participants. ····· 7

Table 2. *In vitro* testing plan schedule throughout the study course. ····· 14

Table 3. Information on reagent contents for 2,3-DPG measurement. ····· 19

Table 4. Information on reagent contents for ATP measurement. ····· 22

Table 5. Plasma dilution rates for the measurement of analytes using Vitros 5600. ·· 24

Table 6. Characteristics of the donors. ····· 27

Table 7. Hemolysis (%) results of RBC concentrates. ····· 31

Table 8. Plasma hemoglobin (mg/dL) results of RBC concentrates. ····· 32

Table 9. Hemoglobin (g/dL) results of RBC concentrates. ····· 42

Table 10. Hematocrit (%) results of RBC concentrates. ····· 43

Table 11. RBC count ($\times 10^6/\mu L$) results of RBC concentrates. ····· 44

Table 12. WBC count ($\times 10^3/\mu L$) results of RBC concentrates. ····· 45

Table 13. Platelet count ($\times 10^3/\mu L$) results of RBC concentrates. ····· 46

Table 14. MCV (fL) results of RBC concentrates. ····· 47

Table 15. MCH (pg) results of RBC concentrates. ····· 48

Table 16. MCHC (g/dL) results of RBC concentrates. ····· 49

Table 17. EMA binding test (%) results of RBC concentrates. ····· 52

Table 18. 2,3-DPG (μmol/g Hb) results of RBC concentrates. ····· 56

Table 19. ATP (μmol/g Hb) results of RBC concentrates. ····· 57

Table 20. pH results of RBC concentrates. ····· 62

Table 21. pCO_2 (mmHg) results of RBC concentrates. ····· 63

Table 22. pO_2 (mmHg) results of RBC concentrates. ····· 64

Table 23. Glucose (mg/dL) results of RBC concentrates. ····· 70

Table 24. Lactate (mmol/L) results of RBC concentrates. ····· 71

Table 25. K+ (mmol/L) results of RBC concentrates. ····· 72

Table 26. Na+ (mmol/L) results of RBC concentrates. ····· 73

Table 27. Chemical structure and molecular weight of plastic materials. ····· 88

Table 28. UHPLC-MS/MS settings and MRM transition information. ····· 91

Table 29. Within-run precision for DEHP and DINCH measurement. ····· 95

Table 30. DEHP concentration (mg/L) results of RBC concentrates. ····· 102

Table 31. DINCH concentration (mg/L) results of RBC concentrates. ····· 103

LIST OF FIGURES

Figure 1. Advertisement poster to recruit blood donors. ····· 6

Figure 2. Study design. ····· 11

Figure 3. Hemolysis of RBC concentrate units. ····· 29

Figure 4. Median values of plasma hemoglobin levels. ····· 30

Figure 5. Median values of hemoglobin levels. ····· 34

Figure 6. Median values of hematocrit levels. ····· 35

Figure 7. Median values of RBC count. ····· 36

Figure 8. Median values of WBC count. ····· 37

Figure 9. Median values of platelet count. ····· 38

Figure 10. Median values of MCV. ····· 39

Figure 11. Median values of MCH. ····· 40

Figure 12. Median values of MCHC. ····· 41

Figure 13. Median values of EMA binding test. ····· 51

Figure 14. Median values of 2,3-DPG. ····· 54

Figure 15. Median values of ATP. ····· 55

Figure 16. Median values of pH. ····· 59

Figure 17. Median values of pCO_2. ····· 60

Figure 18. Median values of pO_2. ····· 61

Figure 19. Median values of glucose. ····· 66

Figure 20. Median values of lactate. ····· 67

Figure 21. Median values of K^+. ····· 68

Figure 22. Median values of Na^+. ····· 69

Figure 23. Gradient information of UHPLC. ····· 90

Figure 24. Representative chromatogram for plasticizer measurement. ····· 94

Figure 25. Linearity for plasticizer measurement. ····· 96

Figure 26. Comparison between blank and 50 mg/L DEHP chromatograms. ·· 98

Figure 27. Median values of DEHP. ····· 100

Figure 28. Median values of DINCH. ····· 101

LIST OF ABBREVIATIONS

2,3-DPG	2,3-diphosphoglycerate
5OH-MEHP	Mono(2-ethyl-5-hydroxyhexyl) phthalate
5oxo-MEHP	Mono(2-ethyl-5-oxohexyl) phthalate
ABGA	Arterial blood gas analysis
ATP	Adenosine triphosphate
BTHC	Butyryl trihexyl citrate
CBC	Complete blood count
CLP	Classification, Labelling and Packaging
CMR	Carcinogenic, mutagenic or toxic for reproduction
CPDA-1	Citrate phosphate dextrose adenine
CV	Coefficient of variation
DEHP	Di(2-ethylhexyl) phthalate
DEHP-d_4	Di(2-ethylhexyl) phthalate-3,4,5,6-d_4
DEHT	Di(2-ethylhexyl) terephthalate
DINCH	Di(isononyl) cyclohexane-1,2-dicarboxylate
EMA	Eosin-5'-maleimide
FK	Fresenius Kabi
GCMS	Green Cross Medical Science
HPLC	High-performance liquid chromatography
HS	Hereditary spherocytosis
IARC	International Agency for Research on Cancer

LLOQ	Lower limit of quantification
LOD	Limit of detection
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MEHP	Mono(2-ethylhexyl) phthalate
MFI	Mean fluorescence intensity
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
PBS	Phosphate-buffered saline
PRP	Platelet-rich plasma
PVC	Polyvinyl chloride
RBC	Red blood cell
TC	Taechang
TOTM	Trioctyl trimellitate
UHPLC	Ultra-high-performance liquid chromatography
US FDA	United States Food and Drug Administration
WBC	White blood cell

Part I

In Vitro Evaluation of DINCH-Plasticized Blood Bags for Red Blood Cell Storage with CPDA-1 Anticoagulant

1. INTRODUCTION

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used to convert polyvinyl chloride (PVC) into a flexible and elastic material. DEHP has long been used to make various items commonly used in our everyday lives, such as plastic toys, cosmetic containers, housewares, and product packaging materials [1]. It is also used to make medical products, including intravenous bags, tubing, catheters, disposable gloves, and especially blood bags. The revolutionary introduction of DEHP for making blood bags in the 1950s significantly improved blood transfusion safety by preventing the breakage of blood bags and facilitating sterile manufacture and separation of blood components, leading to the complete replacement of conventional glass bottles [2]. DEHP is also unique in that it enhances red blood cell (RBC) stability during storage and prolongs its shelf-life by increasing the *in vivo* recovery and decreasing hemolysis [3]. It is unclear how DEHP reduces RBC deterioration, but it is believed that DEHP gets directly incorporated into the RBC membrane and stabilizes it [4, 5].

However, concerns about DEHP-related reproductive toxicity and potential endocrine dysfunction have been debated for many years [6, 7]. While DEHP-related reproductive toxicity has been identified in animal models, its safety in humans remains unknown [7, 8]. Following a 2002 safety assessment of DEHP for medical devices, the United States Food and Drug Administration (US FDA) recommended that non-DEHP alternatives be considered for certain procedures (e.g., exchange transfusion, hemodialysis, total parenteral nutrition, and extracorporeal membrane oxygenation) in high-risk patient groups including male neonates, pregnant women

carrying male fetuses, and peripubertal males [9, 10]. However, they also emphasized that not performing a necessary procedure because of the DEHP-associated risks may pose a greater danger to these patients. Some countries are legally restricting the use of DEHP for human products. France has banned DEHP tubings in hospitals for neonates, children, and maternity wards [9]. Europe will cease the exemption for using DEHP in blood bags and prohibit its use in all medical devices by May 2025 [11, 12]. South Korea has also banned its use in intravenous sets and restricts the amount of DEHP in blood bags to 150 ppm [13]. Because DEHP leaches from the bag to its contents [8], patients receiving blood transfusions are at risk of being exposed to DEHP. Many blood bags for platelet and plasma storage have successfully shifted from using DEHP to other plasticizers such as trioctyl trimellitate (TOTM) or butyryl trihexyl citrate (BTHC) [2]. However, replacing DEHP in RBC storage bags has been difficult because of its aforementioned beneficial and protective effects on RBC stability.

Transitioning to non-DEHP blood collection and storage systems without deterioration in blood component quality is an important goal for blood suppliers. Several previous studies have investigated potential substitutes for DEHP in RBC storage bags [9, 14-19]. Plasticizers such as di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH), BTHC, and di(2-ethylhexyl) terephthalate (DEHT) have been evaluated under different conditions including different preparation methods, product volumes, storage, and additive solutions, with varying results. Unfortunately, blood product manufacturing processes can differ greatly among countries, and there is not enough data in the literature on the validation of non-DEHP systems in diverse

settings.

This study aimed to investigate the *in vitro* quality of RBCs stored in DINCH-plasticized blood bags for up to 35 days in citrate phosphate dextrose adenine (CPDA-1) anticoagulant solution. Using a pool-and-split study design, we manufactured and compared adult-sized RBC concentrates in two DINCH bags and three DEHP bags.

2. MATERIALS AND METHODS

This study was ethically approved by the Institutional Review Board at Seoul National University Bundang Hospital (B-1705-395-309, B-1707-406-301) and performed in accordance with the Declaration of Helsinki.

2.1 Donor Recruitment

We recruited blood donors through poster advertisement at Seoul National University Bundang Hospital (Figure 1). Donor inclusion and exclusion criteria were determined based on the legal regulations for blood donation with partial revision to the content (Table 1).

Each donor was assessed through a health survey, blood donation history interview, and laboratory tests on their first visit. ABO/Rh blood typing and unexpected antibody detection tests were performed using an automated immunohematology analyzer (Qwalys 3, Diagast, Loos, France). Hemoglobin and hematocrit were assessed using an automated hematology analyzer (XN-9000, Sysmex, Kobe, Japan). All study participants provided written informed consent to participate in the study. After reviewing the information from the donors' first visit, we individually notified each donor whether it was appropriate for them to participate in the study.

To organize twenty ABO-matched groups of four donors, eighty eligible donors were required. We expected that the scheduling process of blood collection from four ABO-matched donors on the same day would not be easy. Hence, we aimed to recruit 120 participants to secure a sufficient number of available donors.

Figure 1. Advertisement poster to recruit blood donors for study participation.

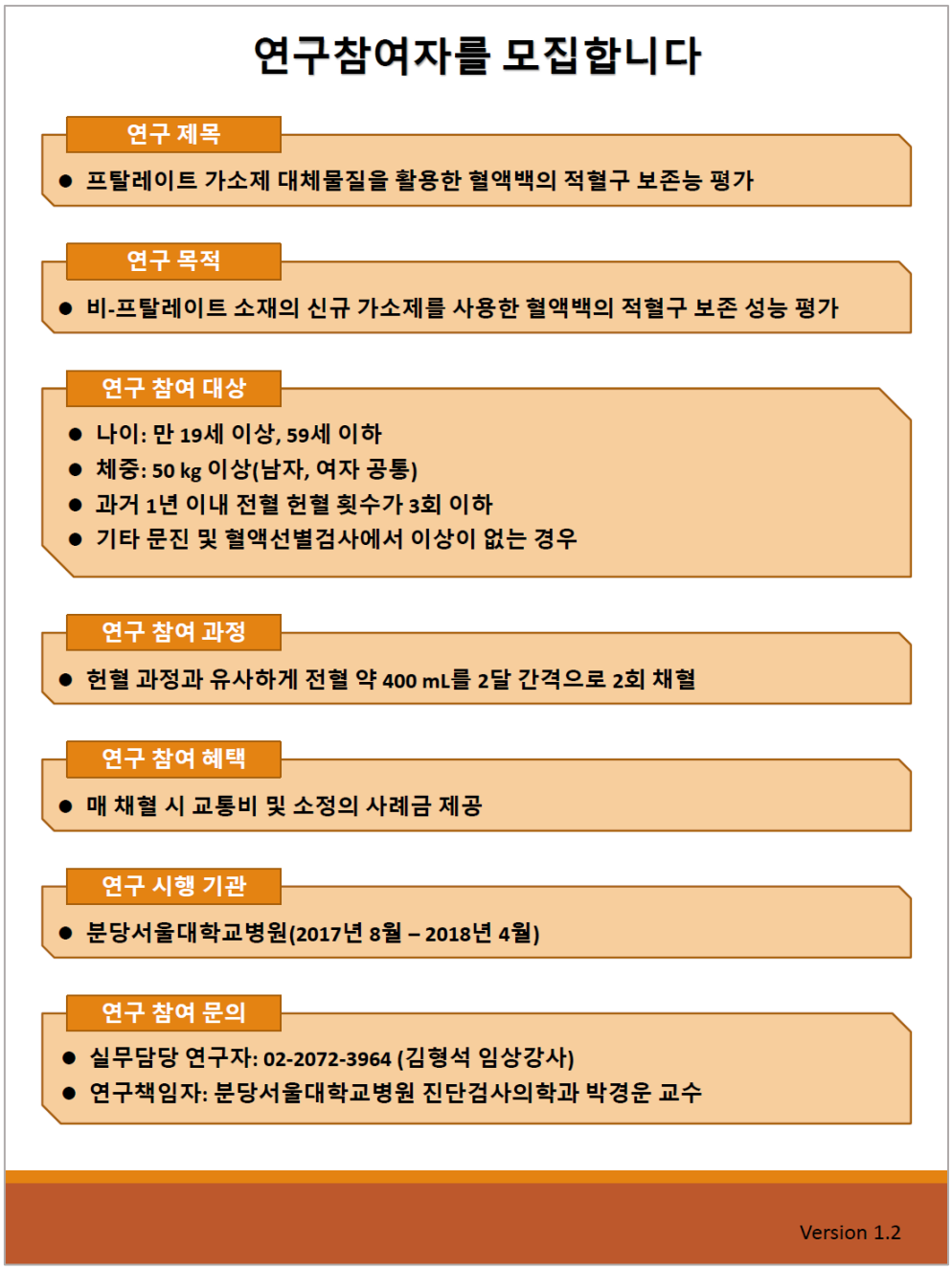


Table 1. Inclusion and exclusion criteria of blood donation participants.

Category	Item
Inclusion criteria	Weight ≥ 50 kg
	Body temperature ≤ 37.5°C
	Systolic blood pressure ≥ 90 mmHg and ≤ 180 mmHg
	Diastolic blood pressure < 100/min
	Age ≥ 19 and ≤ 59
	Hemoglobin ≥ 12.5 g/dL
	Hematocrit ≥ 38%
	Blood donation ≤ 3 times during the previous year
	Estimated blood volume ≥ 3.4 L (by Nadler's equation)
	Negative for unexpected antibody detection test
Exclusion criteria	Not meeting the above items
	Other medical issues not appropriate for blood donation

2.2 Blood Collection and Component Production

A pool-and-split study design was implemented to compare DINCH and DEHP blood bags (Figure 2). We arranged for a group of four ABO-matched donors to make a visit on the morning of each blood collection day. Approximately 407 mL ± 10% of whole blood was collected into non-DEHP prototype DINCH-plasticized blood bags (Green Cross Medical Science [GCMS], Yongin-si, South Korea) containing 57 mL of CPDA-1 anticoagulant from each donor. At the beginning of blood collection, the first 8 mL of blood was additionally collected into the diversion pouch to reduce the risk of bacterial contamination in the primary bag. The diversion pouch was promptly sealed and disconnected. The blood sample in the diversion pouch was then poured into a 10 mL glass test tube which was later used for testing baseline DEHP and DINCH concentrations in the donors.

Subsequently, the four units of whole blood collected from the group of ABO-matched donors were pooled in a 2 L non-DEHP, DINCH-plasticized pooling bag (GCMS) to reduce donor-dependent variability of blood components. Before transferring the pooled blood from the pooling bag to individual triple bags, preexisting CPDA-1 in the primary bag of the triple bag was poured over into one of the empty secondary satellite bags, and that secondary satellite bag was sealed and disconnected to minimize the addition of excessive anticoagulant. Then, from the 2 L pooling bag, 365 mL of anticoagulated whole blood was transferred to five different conventional top and top blood bags: two non-DEHP prototype DINCH-plasticized triple blood bags manufactured by GCMS and Taechang (TC) Industry (Gongju-si, South Korea) and three DEHP-plasticized triple blood bags

commercially available in South Korea which were manufactured by GCMS, TC, and Fresenius Kabi (FK) (Bad Homburg, Germany). The pooling bag was gently shaken continuously during this process to prevent RBC sedimentation and maintain homogeneity. DINCH-GCMS bags initially received 373 mL of whole blood, but we promptly removed 8 mL using a 10 mL glass syringe connected to an 18 G needle and transferred the blood to a 10 mL glass test tube which was later used for testing DEHP and DINCH concentrations on the pooled blood. After distributing the pooled blood into five different types of triple blood bags, the remaining blood sample in the pooling bag was transferred to a 15 mL conical tube and was used for *in vitro* testing on the pooled blood.

We produced RBC concentrates from each triple blood bag using the platelet-rich plasma (PRP) method without leukoreduction. The triple blood bags were centrifuged at 2000 g for 4 minutes using a high-capacity centrifuge (Model 8730, Kubota, Osaka, Japan) and then positioned on a manual plasma extractor. Supernatant PRP was extracted into the satellite bag until the target weight (DEHP-FK, 165 g; DEHP-GCMS, 163 g; DEHP-TC, 160 g; DINCH-GCMS, 163 g; DINCH-TC, 160 g) was reached to achieve optimum hematocrit and volume of the RBC concentrates in the primary bag. The predesignated target weight of the satellite bag for each triple blood bag was calculated after measuring the blank weight of the satellite bags. After PRP extraction, the satellite bag was sealed, disconnected, and discarded.

Homogenous quintets of matched RBCs were produced within 4 hours of blood collection. Aseptic techniques were used for all procedures, and a sterile connection

device (TSCD II, Terumo Corporation, Tokyo, Japan) was used to make connections between blood bags. Twenty-one ABO-matched quintets were prepared for this study. Among them, the first one was used for a pilot study to establish a standard operating procedure for the handling of research materials to maintain consistency throughout the study and minimize unnecessary trial and error. The other twenty quintets were used for blood bag evaluation.

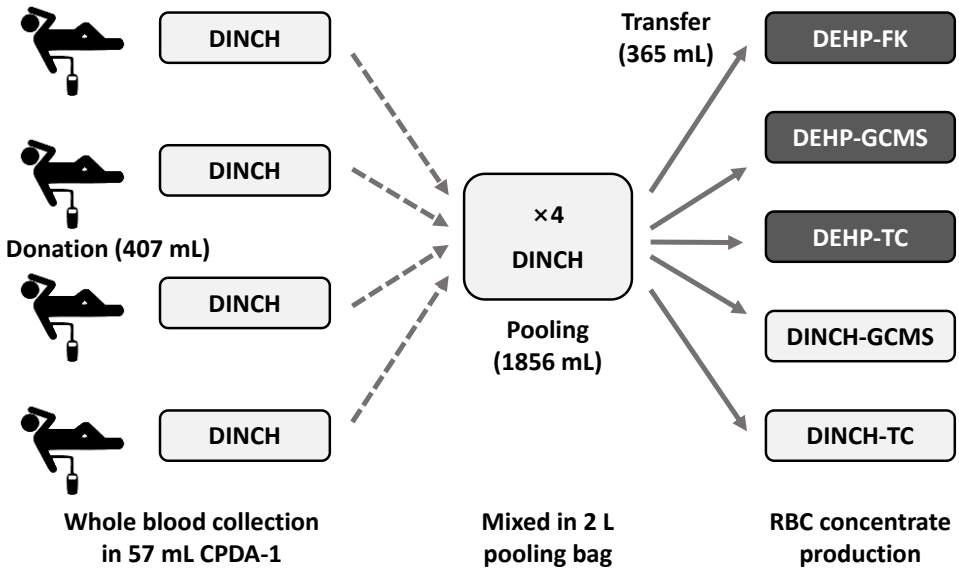


Figure 2. Study design. A pool-and-split study was used to evaluate DINCH-plasticized blood bags for red blood cell storage with CDPA-1 anticoagulant. Whole blood collected from four ABO-matched donors was pooled and equally distributed into five blood bags to produce RBC concentrates. Twenty matched quintets of RBC concentrates were compared during 35 days of storage.

2.3 Blood Storage, Sample Preparation, and *in vitro* Testing Schedule

RBC concentrates were stored for 35 days in a standard blood bank refrigerator at 1° – 6 °C. At the end of 35 days of storage, each unit was tested for sterility using an automated microbial detection system (BacT/ALERT 3D, bioMérieux, Marcy l'Étoile, France) with both aerobe and anaerobe culture bottles.

All units were sampled on days 1, 7, 14, 21, 28, and 35 of storage to perform a panel of *in vitro* tests to analyze RBC concentrate quality. For DEHP-FK bags, blood samples were drawn through sampling site couplers (4C2405, Fenwal Inc., Lake Zurich, IL, USA). DEHP-GCMS, DEHP-TC, DINCH-GSMC, and DINCH-TC bags were manufactured with a sampling site attached to the blood bag during the production stage. All RBC concentrate units were manually mixed gently and thoroughly for 30 seconds before each sampling. After carefully cleaning the sampling site with an alcohol swab, 8 mL of blood was drawn using a 10 mL glass syringe connected to an 18 G needle and transferred to a 10 mL glass test tube which was used for testing DEHP and DINCH concentrations. An additional 16 mL of blood was drawn using a 25 mL plastic syringe connected to an 18 G needle. From that sample, 1 mL was promptly transferred to an arterial blood gas analysis (ABGA) testing syringe, and the syringe was sealed. The remaining 15 mL of blood was transferred to a 15 mL conical tube and processed by the following procedure to be used for a panel of *in vitro* testing. The remnant blood from the pooling bag after RBC concentrate production that was transferred to a 15 mL conical tube was also processed in the same manner.

From the 15 mL of blood, 1.0 mL was transferred to a 1.5 mL microcentrifuge tube and used for a complete blood count (CBC) and eosin-5'-maleimide (EMA) binding test. For adenosine triphosphate (ATP) measurement, 1.5 mL was transferred to a 2 mL microcentrifuge tube. For 2,3-diphosphoglycerate (2,3-DPG) measurement, 3 mL was transferred to two 2 mL microcentrifuge tubes. The samples for 2,3-DPG measurement were stored at -70°C in a freezer until analysis. The remaining blood was centrifuged at 1690 g (3000 rpm) for 10 minutes, and the supernatant plasma was used for testing plasma hemoglobin, glucose, lactate, K^+, and Na^+. The *in vitro* testing plan schedule throughout the study is summarized in Table 2.

Table 2. *In vitro* testing plan schedule throughout the study course.

Test item	Donor screening	Blood donation		RBC storage					
		Donor	Pooled blood	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35
ABO/Rh	●								
Unexpected antibody detection	●								
Sterility testing									●
CBC	●		●	●	●	●	●	●	●
Plasma hemoglobin			●	●	●	●	●	●	●
EMA binding			●	●	●	●	●	●	●
2,3-DPG			●	●	●	●	●	●	●
ATP			●	●	●	●	●	●	●
pH			●	●	●	●	●	●	●
pCO_2			●	●	●	●	●	●	●
pO_2			●	●	●	●	●	●	●
Glucose			●	●	●	●	●	●	●
Lactate			●	●	●	●	●	●	●
K^+			●	●	●	●	●	●	●
Na^+			●	●	●	●	●	●	●
DEHP		●	●	●	●	●	●	●	●
DINCH		●	●	●	●	●	●	●	●

2.4 RBC Concentrate Quality Analysis

2.4.1 Cell counts, RBC indices, and hemolysis

CBC parameters, including total hemoglobin, hematocrit, RBC count, white blood cell (WBC) count, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were assessed using an automated hematology analyzer (XN-9000).

Supernatant plasma Hb was measured using the Fairbanks method [20]. In a 4.5 mL disposable cuvette, 0.2 mL plasma was mixed into 2.0 mL of 0.001% Na_2CO_3 solution. Absorbance values were measured at 415 nm, 450 nm, and 700 nm using a spectrophotometer (DU 730, Beckman Coulter, Brea, CA, USA). Blank absorbance was set by using 2.0 mL of 0.001% Na_2CO_3 solution without adding the plasma sample. Plasma hemoglobin was calculated according to the following formula:

$$\text{Plasma hemoglobin (mg/dL)} = 155.0\ A_{415} - 130.0\ A_{450} - 124.0\ A_{700}$$

By using the above measurements, hemolysis was calculated according to the following formula [21]:

$$\text{Hemolysis (\%)} = (100 - \text{Hematocrit}) \times \frac{\text{Plasma hemoglobin (g/dL)}}{\text{Total hemoglobin (g/dL)}}$$

2.4.2 RBC membrane integrity

The EMA binding test was performed to assess RBC membrane stability. From the 1.5 mL microcentrifuge tube, 10 μL of blood was taken and mixed into 2 mL phosphate-buffered saline (PBS) in a 5 mL test tube. After vortexing gently, the tubes were centrifuged at 620 g (1800 rpm) for 3 minutes, and the supernatant was discarded. Next, the RBC pellet at the tube bottom was gently tapped, and the RBCs were washed again with 2 mL PBS in the same manner. After washing twice, 5 μL of the RBCs were incubated with 25 μL of EMA (Sigma-Aldrich, St. Louis, MO, USA) in a new 5 mL tube for one hour at room temperature in the dark. During mid-incubation, the tubes were gently tapped at 30 minutes. After staining with EMA, the RBCs were washed twice with PBS, and 2 mL PBS was added and vortexed gently. The final suspension was analyzed on a flow cytometer (FACSCanto II, BD, Franklin Lakes, NJ, USA). For each sample, 50,000 events were acquired, and RBCs were gated by forward and side scatter parameters. The ratio (%) of the mean fluorescence intensity (MFI) of the test sample to the mean MFI of a panel of six normal controls was calculated to determine EMA binding.

$$\text{EMA binding (\%)} = 100 \times \frac{\text{FITC} - \text{A MFI (sample)}}{\text{mean FITC} - \text{A MFI (control)}}$$

2.4.3 2,3-DPG measurement

2,3-DPG was measured using a commercialized kit (Cat. No. 10148334001, Roche, Basel, Switzerland). Detailed information on the reagent contents is summarized in Table 3. Before analysis, the frozen tubes were allowed to stand at 20°–25°C for at least 30 minutes to melt. From the 2 mL microcentrifuge tube, 1 mL of blood was taken and mixed into 5 mL of 0.6 M perchloric acid in a 10 mL test tube. The tubes were centrifuged at 3000 g, 4°C for 10 minutes. After centrifugation, 4 mL of the supernatant was transferred to a new 10 mL tube. The supernatant was neutralized by adding 0.5 mL of 2.5 M potassium carbonate and kept at 4°C for 1 hour. The tubes were then centrifuged at 3000 g, 4°C for 5 minutes. From the supernatant, 0.1 mL of the sample was taken and added into a 4.5 mL cuvette containing a mixture of 2 mL of triethanolamine buffer, 0.05 mL of solution 2, and 0.05 mL of solution 3. For the blank sample, 0.1 mL of distilled water was added instead of the supernatant. After mixing and allowing to stand at 20°–25°C for 5 minutes, absorbance values (A_1) were measured at 340 nm using a spectrophotometer (DU 730). Then, 0.02 mL of solution 4 and solution 5 were added to the cuvettes. After mixing and allowing to stand at 20°–25°C for 25 minutes, absorbance values (A_2) were measured at 340 nm. The concentration of 2,3-DPG was calculated according to the following formula:

$$\Delta A = (A_1 - A_2)_{\text{sample}} - (A_1 - A_2)_{\text{blank}}$$

$$c = \frac{V \times MW \times F}{\varepsilon \times d \times v \times 1000 \times 2} \times \Delta A \ [\text{g/L blood}]$$

V = assay volume [mL] = 2.24 mL

v = sample volume [mL] = 0.1 mL

MW = molecular weight of 2,3-DPG = 266.037 g/mol

d = light path [cm] = 1 cm

ε = absorption coefficient of NADH at 340 nm = 6.3 [L × $mmol^{-1}$ × cm^{-1}]

F = dilution factor for blood = 6.582

The calculation of 2,3-DPG concentration in blood can be summarized as 11.70 × ΔA (mmol/L) or 3.112 × ΔA (g/L). For final data analysis, 2,3-DPG concentrations were normalized by hemoglobin values according to the following formula:

$$2{,}3-\mathrm{DPG}\ \ (\mu\mathrm{mol/g\ Hb})\ =\ \frac{100\times 2{,}3-\mathrm{DPG}\ (\mathrm{mmol/L})}{\mathrm{Hemoglobin}\ (\mathrm{g/dL})}$$

2,3-DPG was measured only until day 21, as very low levels were expected on days 28 and 35 [14, 15].

Table 3. Information on reagent contents for 2,3-DPG measurement.

Solution	Content	Preparation	Final concentration
1	• 48 mM triethanolamine buffer (pH 7.6) • 5.2 mM EDTA • 5.3 mM $MgCl_2$	Ready to use (70 mL)	
2	• 2 bottles • 24 mg ATP • 8.2 mg NADH	Dissolve in 1 mL DW	• 40 mM ATP • 9.6 mM NADH
3	• Lyophilizate • 25 U PGM • 1600 U PGK • 25 U GAP-DH • 870 U TIM • 230 U GDH	Dissolve in 1.75 mL triethanolamine buffer (solution 1)	• 14×10^3 U/l PGM • 94×10^4 U/l PGK • 14×10^3 U/l GAP-DH • 50×10^4 U/l TIM • 13×10^4 U/l GDH
4	• Lyophilizate • 620 U PGM	Dissolve in 0.7 mL triethanolamine buffer (solution 1)	• 88×10^4 U/l PGM
5	• 16.5 mg G2P	Dissolve in 0.7 mL DW	• 48 mM G2P

EDTA, ethylene-diamine-tetraacetic acid; NADH, nicotinamide adenine dinucleotide hydrogen; DW, distilled water; PGM, phosphoglycerate mutase; PGK, phosphoglycerate kinase; GAP-DH, glyceraldehyde-3-phosphate dehydrogenase; TIM, triosephosphate isomerase; GDH, glycerol-3-phosphate dehydrogenase; G2P, glycolate-2-phosphate.

2.4.4 ATP measurement

ATP was measured using a commercialized kit (ATP Hexokinase FS kit, DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Detailed information on the reagent contents is summarized in Table 4. From the 2 mL microcentrifuge tube, 1 mL of blood was taken and mixed into 1 mL of 12% (w/v) trichloroacetic acid in a new 2 mL microcentrifuge tube. After vortexing gently, the tubes were kept at 4°C for 5 minutes and centrifuged at 3000 g, 4°C for 5 minutes. After centrifugation, 250 μL of the supernatant was transferred to a new 1.5 mL microcentrifuge tube. The tubes were then loaded on an automated immunoassay analyzer (ARCHITECT i2000SR Plus, Abbott Laboratories, Chicago, IL, USA) to proceed with the remaining assay procedure. The samples were mixed with 2.4 mL of solution 1. For the blank sample, 250 μL of distilled water was used instead of the supernatant. After mixing and allowing to stand at 20°–25°C for 5 minutes, absorbance values (A_1) were measured at 340 nm. Then, 600 μL of solution 2 was added. After mixing and allowing to stand at 20°–25°C for 15 minutes, absorbance values (A_2) were measured at 340 nm. The concentration of ATP was calculated according to the following formula:

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

$$c = \frac{V \times f \times 100}{\varepsilon \times v \times d} \times \Delta A \; [\mu\text{mol/dL}]$$

V = assay volume [µL] = 3250 µL

f = dilution factor of sample preparation = 2.0

d = light path [cm] = 1 cm

v = sample volume [µL] = 250 µL

ε = coefficient of NADH at 340 nm = 6.3 [L × $mmol^{-1}$ × cm^{-1}]

The calculation of ATP concentration in blood can be summarized as 412.70 × ΔA (µmol/dL). For final data analysis, ATP concentrations were normalized by hemoglobin values according to the following formula:

$$\text{ATP (µmol/g Hb)} = \frac{\text{ATP (µmol/dL)}}{\text{Hemoglobin (g/dL)}}$$

Table 4. Information on reagent contents for ATP measurement.

Solution	Content	Preparation
1	• pH 7.8 • 0.1 mol/L TRIS-buffer • 4 mmol/L $Mg2^{+}$ • 20 mmol/L glucose • 2.1 mmol/L NAD	Ready to use
2	• pH 7.0 • 4 mmol/L Mg^{2+} • ≥ 7.5 kU/L hexokinase • ≥ 7.5 kU/L G6P-DH	Ready to use

NAD, nicotinamide adenine dinucleotide; G6P-DH, Glucose-6-phosphate-dehydrogenase

2.4.5 Other chemistry tests

The Cobas b 221 (Roche) blood gas analyzer was used to measure pH, pCO_2, and pO_2. pH was equilibrated to standard values at 37°C.

Glucose, lactate, K^+, and Na^+ were measured using an automated chemistry analyzer (Vitros 5600, Ortho Clinical Diagnostics, Raritan, NJ, USA). These analytes can reach certain ranges that are outside of normal physiological reference values throughout the storage period. Due to limitations in the analytical measurement range of the instrument, plasma samples were diluted with distilled water for the measurement of lactate and K^+ from day 7 and afterward. Specific dilution rates are summarized in Table 5.

Table 5. Plasma dilution rates for the measurement of analytes using Vitros 5600.

	Glucose	Lactate	K^+	Na^+
Day 0	None	None	None	None
Day 1	None	None	None	None
Day 7	None	1/4	1/4	None
Day 14	None	1/4	1/4	None
Day 21	None	1/4	1/4	None
Day 28	None	1/4	1/6	None
Day 35	None	1/4	1/6	None

2.5 Statistical Analysis

Statistical analysis was conducted with receiving consultation from the Medical Research Collaborating Center at Seoul National University Hospital Biomedical Research Institute. Each DINCH bag (DINCH-GCMS, DINCH-TC) was compared with each of the three DEHP bags (DEHP-FK, DEHP-GCMS, DEHP-TC) and also with the other DINCH bag. Non-parametrical matched analysis using the Wilcoxon signed-rank test was performed using IBM SPSS Statistics 25 (Armonk, NY, USA). A P value of < 0.05 was considered significant.

3. RESULTS

3.1 Donor Recruitment and Study Progress

We received 176 inquiries about study participation from November 2017 to February 2018. A total of 108 (61.4%) people were assessed as suitable for study participation, 36 (20.5%) were ineligible, and 32 (18.2%) did not visit for evaluation. Detailed information on the donor characteristics is summarized in Table 6.

Among the 108 donors who qualified for participation, 56 (51.9%) donors were able to participate in blood donation at least once (20 [18.5%] donors participated twice, and 4 [3.7%] donors participated thrice during the study period), but 52 (48.1%) donors did not participate because of difficulties in making schedule arrangements. Blood collection was conducted in 21 groups (blood group: A = 7, B = 6, O = 6, AB = 2) of ABO-matched donors consisting of four participants.

Blood collection, component production, sampling, and *in vitro* testing were conducted from May 2018 to November 2019. Testing for 2,3-DPG from frozen samples was continued until October 2020 due to delays in reagent shipping.

Table 6. Characteristics of the donors that were qualified for study participation.

	Total (n = 108)	Male (n = 59)	Female (n = 49)
Participation in blood donation[1]	56 (51.9%)	34 (57.6%)	22 (44.9%)
Age (y)	35 (19–57)	31 (19–56)	39 (19–57)
Height (cm)	168.4 (150.3–188.5)	174.7 (164.0–188.5)	160.9 (150.3–175.4)
Weight (kg)	70.3 (50.8–107.0)	75.7 (54.1–107.0)	63.8 (50.8–85.0)
Estimated blood Volume (mL)	4449 (3400–6506)	5000 (4315–6506)	3785 (3400–4427)
Hemoglobin (g/dL)	14.6 (12.6–17.9)	15.6 (13.9–17.9)	13.5 (12.6–15.7)
Hematocrit (%)	43.9 (38.1–52.8)	46.3 (41.7–52.8)	40.9 (38.1–47.7)
ABO blood group[1]			
A	33 (30.6%)	16 (27.1%)	17 (34.7%)
B	31 (28.7%)	17 (28.8%)	14 (28.6%)
O	32 (29.6%)	20 (33.9%)	12 (24.5%)
AB	12 (11.1%)	6 (10.2%)	6 (12.2%)

Data is presented as mean (range) if not otherwise specified.

[1]Data is shown as n (%).

3.2 Component Integrity

Tube seals and sampling site coupler connections for all RBC concentrate units showed no leakage and functioned adequately throughout the storage period. There were no visual or physical differences between the DEHP and DINCH blood bags. All units tested negative for aerobic and anaerobic bacterial culture at the end of storage.

3.3 RBC Concentrate Quality

3.3.1 Hemolysis and plasma hemoglobin

Hemolysis increased in all bags throughout the storage period (Figure 3A). On day 35, the hemolysis rates in all bags of each study arm were below the regulatory limit of 0.8% (Figure 3B). Both of the DINCH bags showed higher hemolysis than the DEHP-GCMS and DEHP-TC bags starting as early as day 7 and the DEHP-FK bag starting from day 14 (Table 7). This difference was maintained throughout the storage period. Although the median hemolysis in the DINCH-TC bag on day 35 was higher than in the DINCH-GCMS bag, the difference was not statistically significant.

Plasma hemoglobin generally showed similar results with hemolysis. Plasma hemoglobin increased in all bags throughout the storage period (Figure 4). DINCH-TC showed higher plasma hemoglobin than the DEHP bags from day 7, and DINCH-GCMS bags also showed higher plasma hemoglobin than the DEHP bags from day 14 (Table 8).

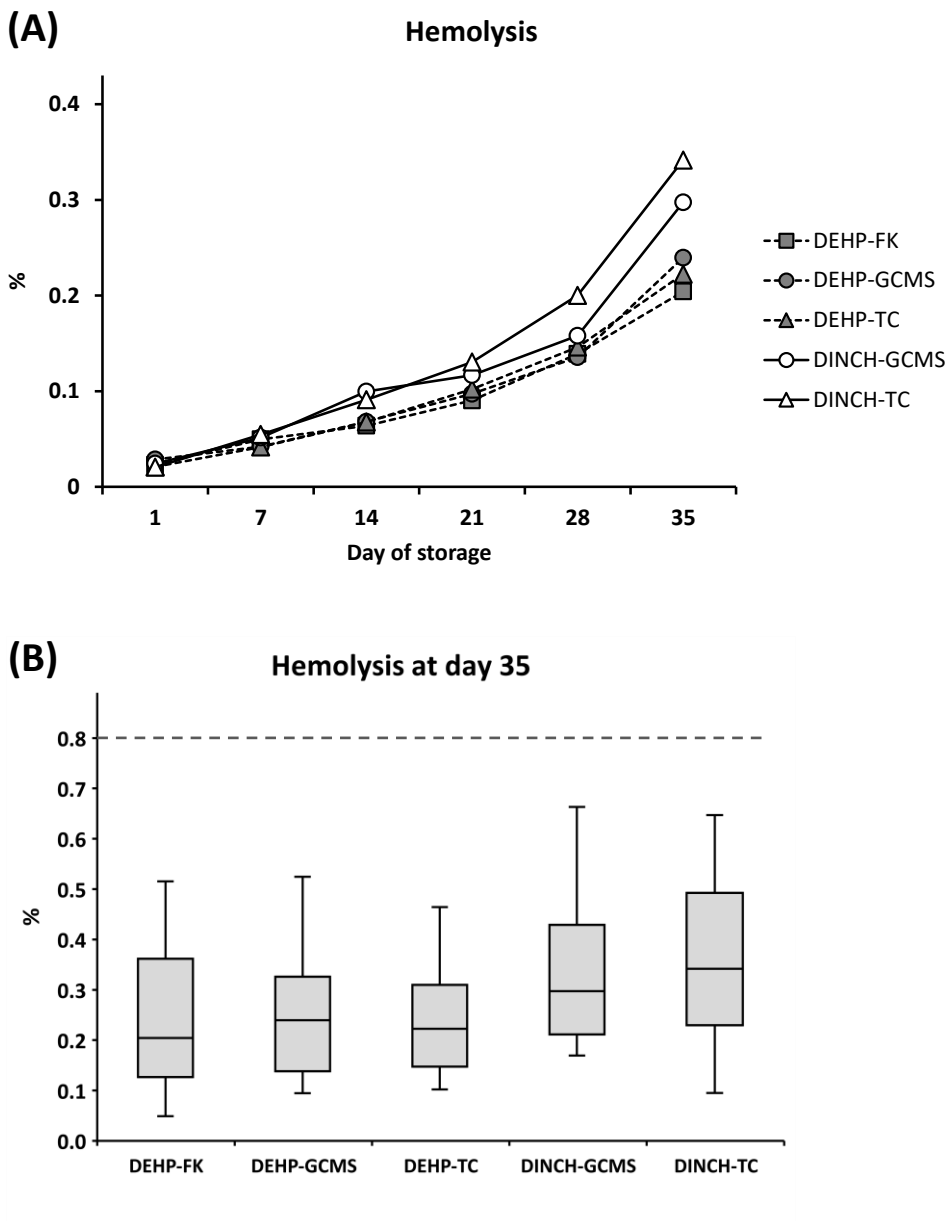


Figure 3. Hemolysis of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20). (A) The DINCH bags showed increased median values of hemolysis compared with DEHP bags. (B) Box and whisker plot shows a < 0.8% hemolysis rate in all the bags in each study arm on day 35.

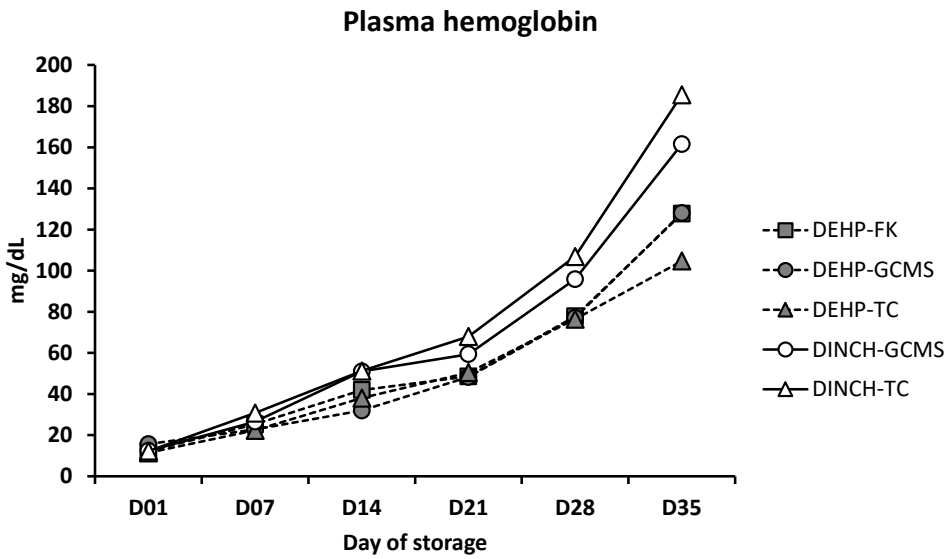


Figure 4. Median values of plasma hemoglobin levels of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20). The DINCH bags showed increased levels of plasma hemoglobin compared with DEHP bags.

Table 7. Hemolysis (%) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	0.018 (0.011–0.030)					
Day 1		0.023 (0.020–0.057)	0.029 (0.016–0.070)	0.021 (0.015–0.050)	0.024 (0.016–0.035)	0.021 (0.017–0.040)
Day 7		0.050 (0.032–0.060)	0.042 (0.035–0.059)	0.042 (0.034–0.051)	0.051 (0.039–0.074)[2,3]	0.055 (0.045–0.070)[2,3]
Day 14		0.064 (0.047–0.104)	0.068 (0.047–0.095)	0.068 (0.049–0.095)	0.100 (0.071–0.131)[1,2,3]	0.091 (0.079–0.145)[1,2,3]
Day 21		0.090 (0.064–0.159)	0.097 (0.071–0.130)	0.102 (0.063–0.139)	0.117 (0.103–0.210)[1,2,3,4]	0.130 (0.109–0.266)[1,2,3,5]
Day 28		0.139 (0.086–0.189)	0.136 (0.101–0.213)	0.146 (0.099–0.203)	0.158 (0.146–0.293)[1,2,3]	0.200 (0.156–0.316)[1,2,3]
Day 35		0.204 (0.127–0.362)	0.240 (0.138–0.326)	0.222 (0.147–0.310)	0.297 (0.212–0.429)[1,2,3]	0.342 (0.230–0.493)[1,2,3]

Data is presented as median (Q_1–Q_3).

[1]$P < 0.05$ compared to storage in DEHP-FK.

[2]$P < 0.05$ compared to storage in DEHP-GCMS.

[3]$P < 0.05$ compared to storage in DEHP-TC.

[4]$P < 0.05$ compared to storage in DINCH-TC.

[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 8. Plasma hemoglobin (mg/dL) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	3.81 (2.22–6.00)					
Day 1		12.27 (10.09–25.16)	15.66 (6.99–58.15)	11.43 (7.29–22.76)	12.38 (6.97–31.11)	12.26 (8.06–31.54)
Day 7		25.27 (17.69–38.87)	22.75 (17.94–34.66)	22.32 (18.45–32.16)	26.56 (20.38–49.67)	30.74 (22.45–39.04)[2,3]
Day 14		41.95 (26.56–60.86)	31.94 (26.36–46.47)	38.03 (26.11–52.55)	50.99 (37.16–74.38)[1,2,3]	51.20 (40.13–79.03)[1,2,3]
Day 21		48.55 (39.88–96.60)	48.28 (36.12–64.83)	50.46 (35.58–88.58)	59.33 (50.99–97.79)[2,3,4]	67.96 (59.61–137.69)[1,2,3,5]
Day 28		77.80 (52.53–101.06)	77.11 (56.29–102.36)	76.42 (50.72–104.52)	95.86 (76.31–164.89)[1,2,3]	106.89 (81.06–152.59)[1,2,3]
Day 35		127.70 (73.76–190.34)	128.03 (79.57–181.42)	104.71 (87.21–198.00)	161.56 (114.85–250.61)[1,2,3]	185.53 (129.03–282.02)[1,2,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

3.3.2 Cell counts and RBC indices

Hemoglobin maintained a steady level throughout the study period in all of the study arms (Figure 5). DEHP-FK bags showed higher hemoglobin levels than both DINCH bags from day 14 onward (Table 9). Hematocrit gradually increased throughout the study period in all of the study arms (Figure 6). DEHP-FK bags showed higher hematocrit levels than both DINCH bags from day 14 onward (Table 10).

Similar to hemoglobin, the RBC count maintained a steady level throughout the study period in all of the study arms (Figure 7). In addition, DEHP-FK bags also showed higher RBC counts than the DINCH bags from day 14 onward (Table 11). The WBC and platelet counts decreased during the storage period in all of the study arms (Figures 8 and 9). DEHP-FK bags showed higher WBC counts than both DINCH bags from day 21 onward (Table 12). For platelets, DINCH bags showed lower platelet counts than DEHP bags throughout the storage period (Table 13).

The MCV increased during the storage period in all of the study arms, indicating RBC swelling (Figure 10). The MCV for both the DINCH bags was higher than the DEHP-FK bag starting from day 7 and DEHP-GCMS and DEHP-TC bags starting from day 14 (Table 14). The MCH maintained a steady level throughout the study period in all of the study arms (Figure 11), and there was not much difference between DINCH and DEHP bags (Table 15). The MCHC decreased during the storage period in all of the study arms (Figure 12), showing an inverse relationship with MCV. DINCH bags showed lower MCHC than DEHP bags (Table 16).

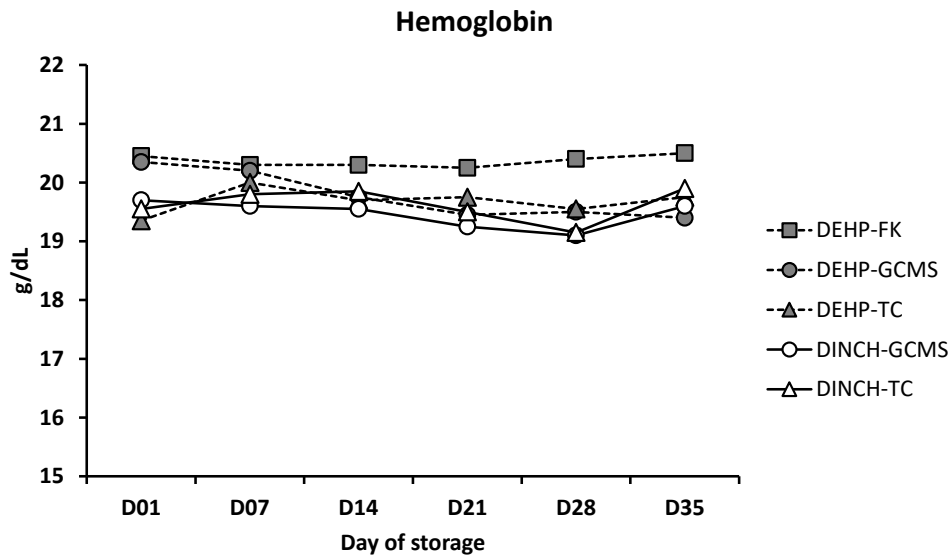


Figure 5. Median values of hemoglobin levels of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

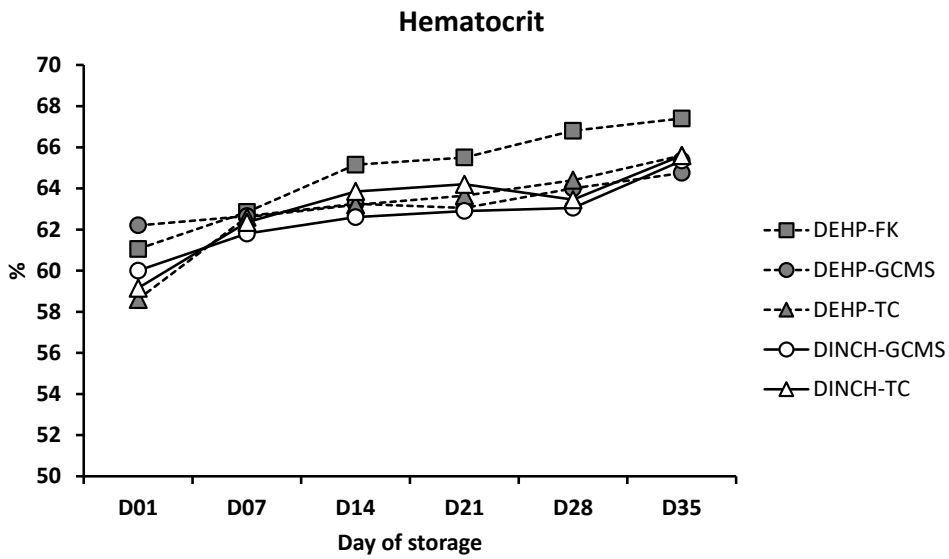


Figure 6. Median values of hematocrit levels of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

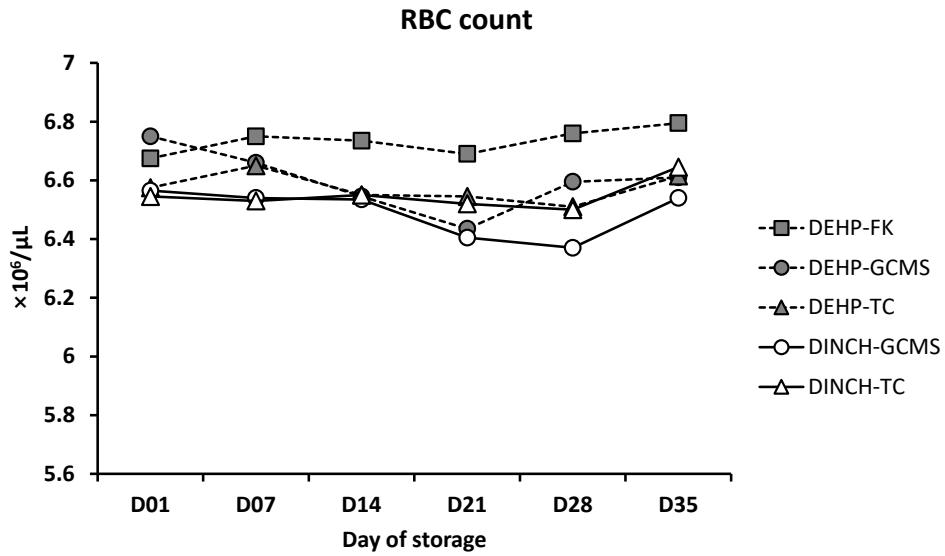


Figure 7. Median values of RBC count of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

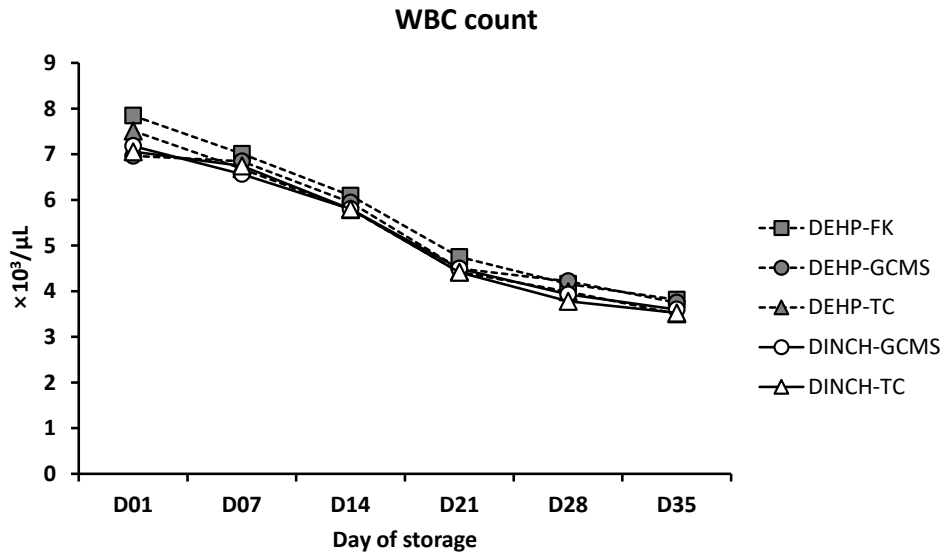


Figure 8. Median values of WBC count of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

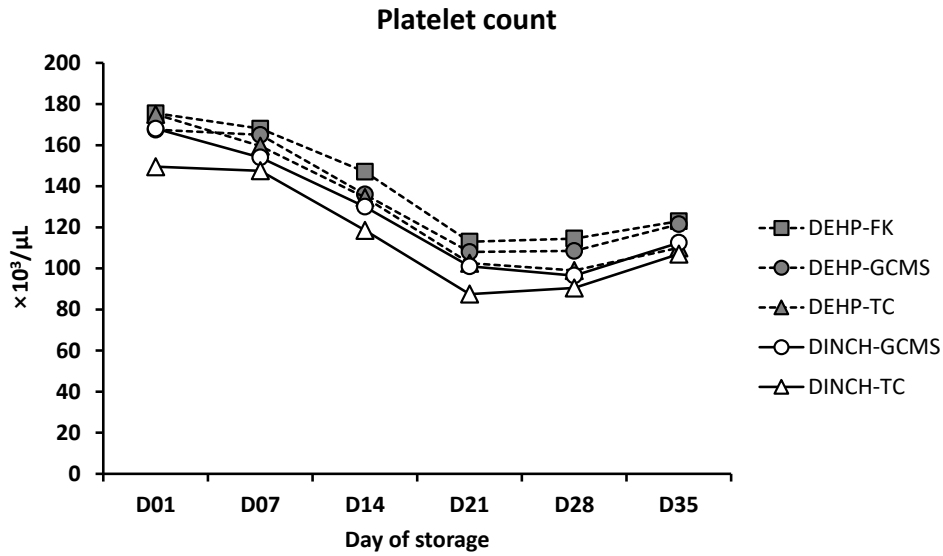


Figure 9. Median values of platelet count of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

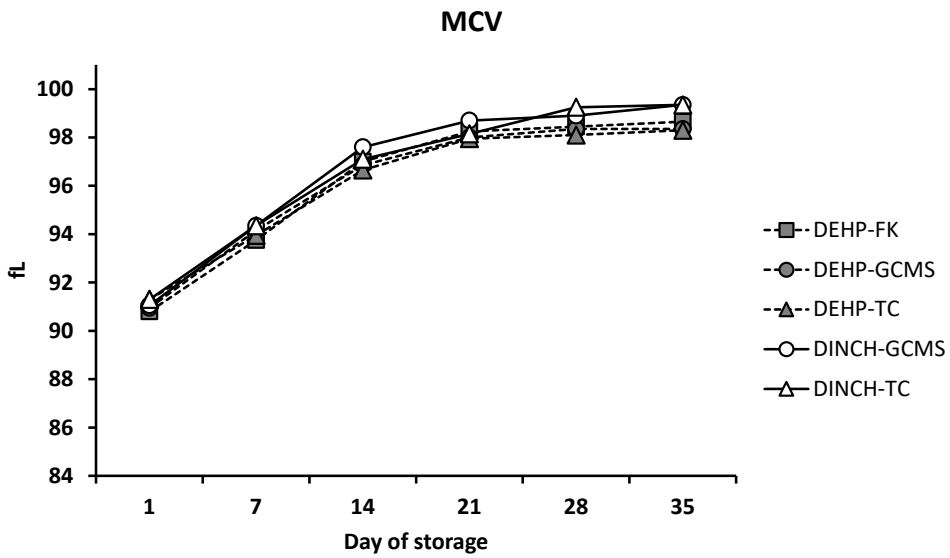


Figure 10. Median values of MCV of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

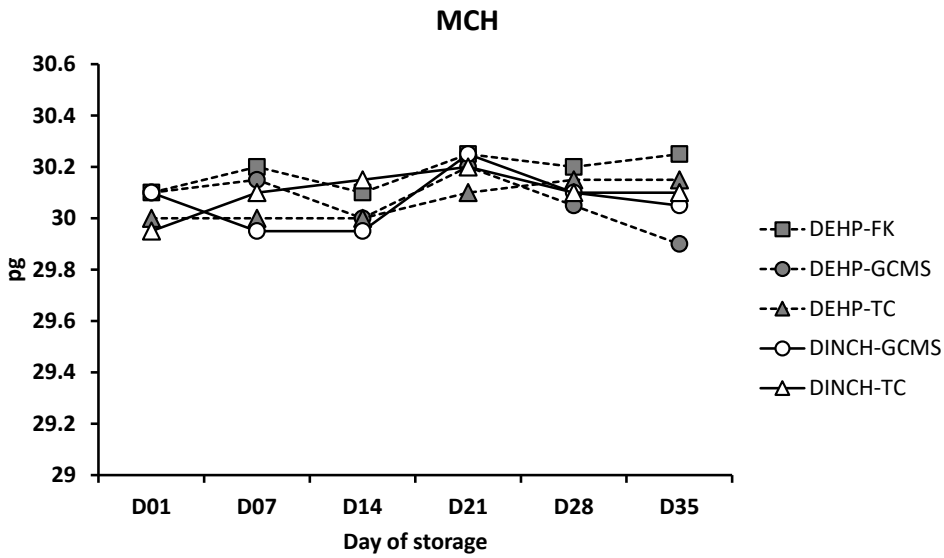


Figure 11. Median values of MCH of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

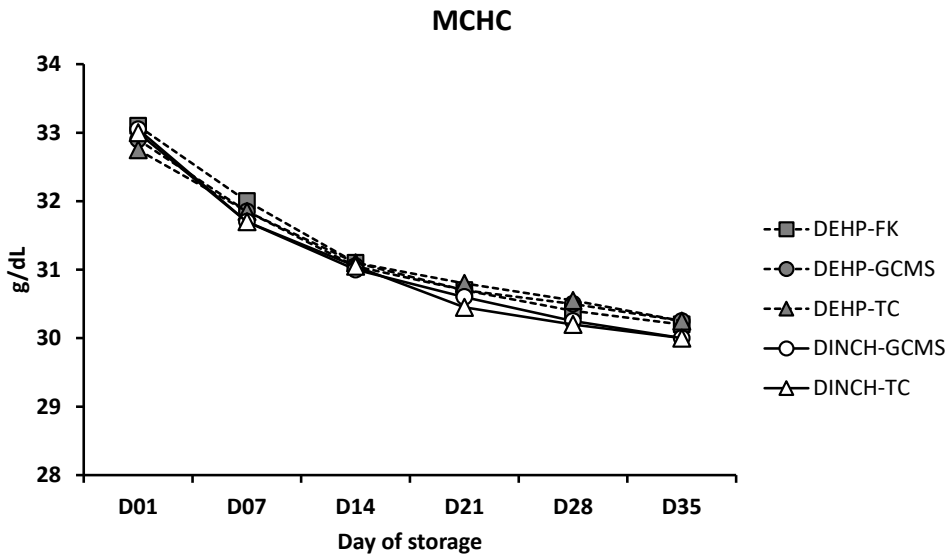


Figure 12. Median values of MCHC of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

Table 9. Hemoglobin (g/dL) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	12.4 (12.1–13.1)					
Day 1		20.5 (19.0–21.0)	20.4 (19.3–21.1)	19.4 (18.7–20.5)	19.7 (18.7–20.6)[2]	19.6 (18.9–20.7)
Day 7		20.3 (18.8–20.8)	20.2 (18.2–21.0)	20.0 (19.0–20.9)	19.6 (18.3–21.1)	19.8 (18.6–21.0)
Day 14		20.3 (19.3–21.0)	19.8 (18.1–20.4)	19.7 (18.7–20.7)	19.6 (18.1–20.5)[1]	19.9 (17.6–20.7)[1]
Day 21		20.3 (19.3–20.8)	19.5 (18.5–20.1)	19.8 (18.8–20.2)	19.3 (18.2–20.4)[1,3]	19.5 (18.5–20.5)[1]
Day 28		20.4 (19.3–20.9)	19.5 (18.8–20.3)	19.6 (18.8–20.4)	19.1 (18.3–20.5)[1,3]	19.2 (18.3–20.5)[1,3]
Day 35		20.5 (19.5–21.1)	19.4 (18.8–20.5)	19.8 (18.9–20.9)	19.6 (18.5–20.7)[1]	19.9 (18.5–20.8)[1]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 10. Hematocrit (%) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	36.8 (36.0–38.6)					
Day 1		61.1 (57.1–63.3)	62.2 (59.2–63.7)	58.6 (57.0–63.1)	60.0 (57.4–62.3)	59.2 (57.7–63.6)
Day 7		62.9 (60.0–65.4)	62.7 (57.4–66.6)	62.6 (58.9–64.5)	61.8 (57.4–66.1)	62.4 (59.1–65.4)
Day 14		65.2 (63.4–67.8)	63.3 (58.3–66.2)	63.2 (60.3–67.1)	62.6 (58.4–66.2)[1]	63.9 (57.0–66.6)[1]
Day 21		65.5 (64.1–67.6)	63.1 (61.1–65.4)	63.7 (62.2–65.2)	62.9 (59.7–65.5)[1]	64.2 (61.4–67.2)[1]
Day 28		66.8 (64.1–68.4)	64.0 (62.4–67.0)	64.4 (61.8–66.4)	63.1 (60.8–67.5)[1]	63.5 (61.1–67.1)[1]
Day 35		67.4 (65.2–68.9)	64.8 (62.7–67.0)	65.6 (63.0–66.9)	65.4 (62.0–67.7)[1]	65.6 (62.8–68.2)[1]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 11. RBC count ($\times 10^6/\mu L$) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	4.13 (4.02–4.28)					
Day 1		6.68 (6.32–7.02)	6.75 (6.45–6.99)	6.58 (6.14–6.87)	6.57 (6.22–6.88)	6.55 (6.24–6.97)
Day 7		6.75 (6.41–7.02)	6.66 (6.23–7.01)	6.65 (6.37–6.94)	6.54 (6.16–7.09)	6.53 (6.14–6.92)
Day 14		6.74 (6.45–7.00)	6.55 (5.94–6.86)	6.55 (6.20–6.91)	6.54 (6.06–6.84)[1]	6.55 (5.75–6.88)[1]
Day 21		6.69 (6.41–6.84)	6.44 (6.27–6.73)	6.55 (6.25–6.77)	6.41 (6.04–6.70)[1,3]	6.52 (6.13–6.74)[1]
Day 28		6.76 (6.48–7.02)	6.60 (6.19–6.81)	6.51 (6.20–6.77)	6.37 (6.15–6.79)[1]	6.50 (6.10–6.75)[1]
Day 35		6.80 (6.49–7.00)	6.61 (6.24–6.77)	6.62 (6.46–6.83)	6.54 (6.19–6.83)[1,3]	6.65 (6.33–6.81)[1]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 12. WBC count ($\times 10^3/\mu L$) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	4.88 (4.46–5.03)					
Day 1		7.85 (7.00–8.39)	6.97 (6.62–7.77)	7.51 (7.16–7.68)	7.18 (6.21–7.54)[1,3]	7.06 (6.46–7.79)[1]
Day 7		7.01 (6.29–7.70)	6.85 (6.23–7.47)	6.69 (6.10–7.21)	6.57 (6.06–7.15)	6.75 (6.26–7.35)
Day 14		6.10 (5.27–6.59)	5.95 (5.11–6.27)	5.79 (5.09–6.03)	5.80 (4.71–6.04)[1]	5.80 (4.96–6.24)
Day 21		4.75 (4.19–5.45)	4.50 (2.05–5.07)	4.43 (3.87–5.02)	4.50 (2.07–4.89)[1]	4.42 (3.95–5.08)[1]
Day 28		4.16 (3.89–4.71)	4.22 (3.75–4.54)	3.99 (3.68–4.36)	3.93 (3.59–4.32)[1,2,4]	3.78 (3.42–3.98)[1,2,3,5]
Day 35		3.82 (3.55–4.30)	3.75 (3.29–4.12)	3.51 (3.29–3.80)	3.60 (3.24–4.09)[1]	3.53 (3.17–3.73)[1,2]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 13. Platelet count ($\times 10^3/\mu L$) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	171 (162–185)					
Day 1		176 (158–209)	168 (141–197)	175 (151–198)	168 (148–180)[1]	150 (128–184)[1,3]
Day 7		168 (152–206)	165 (152–185)	160 (146–179)	154 (138–191)[1,4]	148 (128–183)[1,2,5]
Day 14		147 (118–175)	136 (109–163)	135 (112–160)	130 (99–167)[1,4]	119 (88–161)[1,2,3,5]
Day 21		113 (89–149)	108 (90–138)	103 (82–132)	101 (78–131)[1,2,4]	88 (72–123)[1,2,3,5]
Day 28		115 (93–137)	109 (93–130)	99 (83–109)	97 (81–118)[1,2,4]	91 (74–115)[1,2,3,5]
Day 35		123 (111–149)	122 (114–142)	110 (98–132)	113 (94–130)[1,2]	107 (92–131)[1,2]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 14. MCV (fL) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	89.8 (88.3–92.4)					
Day 1		90.8 (88.9–92.8)	91.0 (89.4–93.0)	91.3 (89.4–93.0)	91.1 (89.4–92.8)	91.3 (89.4–93.2)[1]
Day 7		93.8 (92.5–95.3)	94.2 (92.6–95.7)	94.0 (92.5–95.6)	94.4 (92.5–95.7)[1]	94.4 (92.6–95.9)[1]
Day 14		97.0 (95.1–98.0)	96.9 (95.1–97.9)	96.7 (95.1–97.9)	97.6 (95.1–98.3)[1,2,3]	97.1 (95.4–98.2)[1,2,3]
Day 21		98.3 (96.6–99.6)	98.0 (96.5–99.7)	98.0 (96.3–99.4)	98.7 (96.7–100.2)[1,2,3]	98.2 (97.0–100.2)[1,2,3]
Day 28		98.5 (97.4–100.7)	98.4 (97.3–100.3)	98.1 (97.2–100.1)	98.9 (97.9–101.0)[1,2,3]	99.3 (97.7–101.0)[1,2,3]
Day 35		98.7 (98.1–101.0)	98.4 (97.9–100.9)	98.3 (97.7–100.5)	99.4 (98.7–101.4)[1,2,3]	99.4 (98.7–101.5)[1,2,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 15. MCH (pg) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	30.3 (29.7–30.6)					
Day 1		30.1 (29.5–30.4)	30.1 (29.7–30.4)	30.0 (29.4–30.4)	30.1 (29.5–30.7)[3]	30.0 (29.7–30.3)
Day 7		30.2 (29.5–30.5)	30.2 (29.6–30.3)	30.0 (29.5–30.5)	30.0 (29.5–30.4)	30.1 (29.4–30.5)
Day 14		30.1 (29.7–30.5)	30.0 (29.6–30.3)	30.0 (29.6–30.4)	30.0 (29.6–30.5)	30.2 (29.7–30.6)[2,3]
Day 21		30.3 (29.8–30.5)	30.2 (29.6–30.6)	30.1 (29.7–30.5)	30.3 (29.6–30.5)	30.2 (29.5–30.5)
Day 28		30.2 (29.5–30.6)	30.1 (29.5–30.6)	30.2 (29.4–30.7)	30.1 (29.7–30.4)	30.1 (29.5–30.4)[3]
Day 35		30.3 (29.7–30.5)	29.9 (29.7–30.4)	30.2 (29.4–30.4)	30.1 (29.6–30.5)	30.1 (29.5–30.8)

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 16. MCHC (g/dL) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	33.4 (32.9–33.9)					
Day 1		33.1 (32.7–33.3)	32.9 (32.6–33.3)	32.8 (32.4–33.1)	33.1 (32.8–33.4)[3]	33.0 (32.3–33.2)
Day 7		32.0 (31.7–32.2)	31.9 (31.5–32.1)	31.9 (31.5–32.1)	31.7 (31.4–31.9)[1]	31.7 (31.5–32.3)
Day 14		31.1 (30.8–31.4)	31.1 (30.6–31.3)	31.1 (30.8–31.3)	31.0 (30.7–31.2)[4]	31.1 (30.9–31.5)[5]
Day 21		30.7 (30.4–31.0)	30.7 (30.2–31.0)	30.8 (30.5–31.1)	30.6 (30.3–30.8)	30.5 (30.2–30.8)[1,2,3]
Day 28		30.4 (30.1–30.8)	30.5 (30.2–30.7)	30.6 (30.3–30.8)	30.3 (30.0–30.6)[1,2,3]	30.2 (29.9–30.5)[1,2,3]
Day 35		30.2 (29.9–30.6)	30.3 (29.9–30.5)	30.3 (30.1–30.7)	30.0 (29.8–30.3)[1,3]	30.0 (29.7–30.4)[1,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

3.3.3 RBC membrane integrity

The EMA binding test results gradually decreased over the storage period in all of the study arms (Figure 13). The DINCH bags generally showed lower levels of EMA binding than the DEHP bags, and on days 28 and 35, the DINCH-TC bag showed lower EMA binding than all the other bags (Table 17).

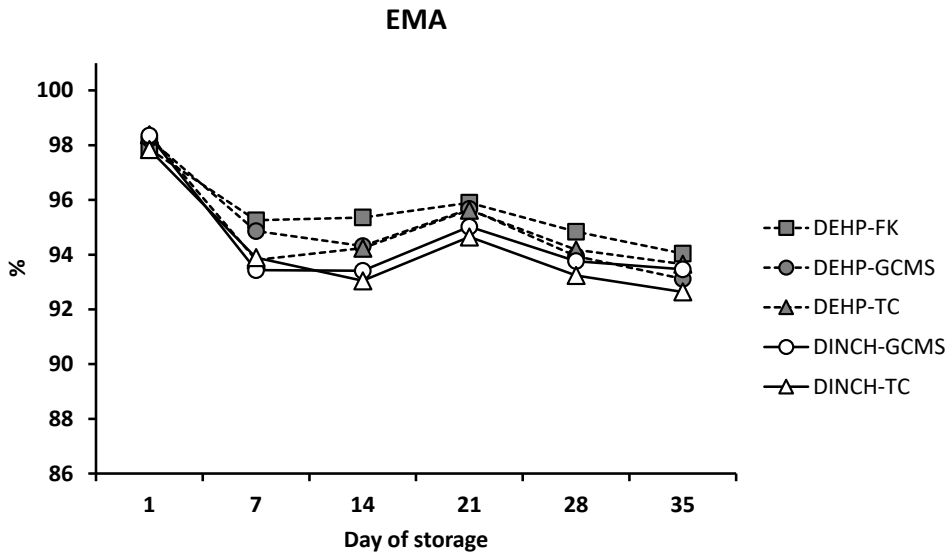


Figure 13. Median values of EMA binding test of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

Table 17. EMA binding test (%) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	95.5 (92.9–97.6)					
Day 1		97.9 (95.9–100.4)	98.2 (95.9–99.8)	98.4 (97.0–102.4)	98.4 (97.2–100.0)	97.8 (96.0–100.8)[3]
Day 7		95.3 (92.9–96.8)	94.9 (93.1–97.2)	93.8 (92.0–96.7)	93.4 (92.5–96.7)[1]	93.9 (92.1–97.2)[1,2]
Day 14		95.4 (93.9–98.4)	94.3 (92.9–97.0)	94.2 (91.9–97.1)	93.4 (92.1–96.8)[1,2]	93.0 (91.4–96.6)[1,2,3]
Day 21		95.9 (90.9–97.0)	95.7 (89.4–96.8)	95.6 (92.0–96.9)	95.0 (91.9–96.2)[4]	94.7 (91.1–96.1)[1,2,5]
Day 28		94.8 (90.1–97.1)	93.9 (92.2–97.1)	94.2 (91.6–96.7)	93.8 (91.1–96.0)[1,2,4]	93.2 (91.3–96.2)[1,2,3,5]
Day 35		94.0 (91.7–97.3)	93.1 (91.0–98.1)	93.7 (90.4–96.6)	93.5 (91.1–97.3)[2,4]	92.6 (89.9–95.6)[1,2,3,5]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

3.3.4 2,3-DPG and ATP

2,3-DPG levels decreased during the storage period, and the results were generally comparable between the study arms (Figure 14). The DEHP-FK bag showed a higher level of 2,3-DPG than the DINCH-GSMC bag on days 1 and 7 and the DINCH-TC bag on days 7 and 21, but the differences were small (Table 18). On day 21, 2,3-DPG was nearly depleted in all the bags. After a slight increase on day 7, ATP levels slowly decreased throughout the remaining storage period (Figure 15). Again, the results were generally comparable between the study arms. The DEHP-FK bag showed a slightly lower level of ATP than the DINCH bags on days 7, 21, 28, and 35, but the differences were very small (Table 19).

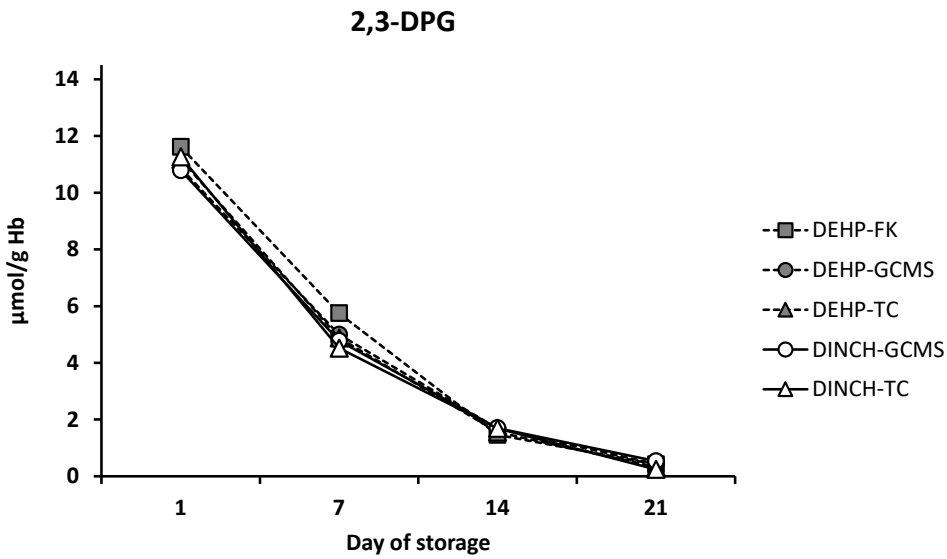


Figure 14. Median values of 2,3-DPG of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20). 2,3-DPG was not measured on days 28 and 35.

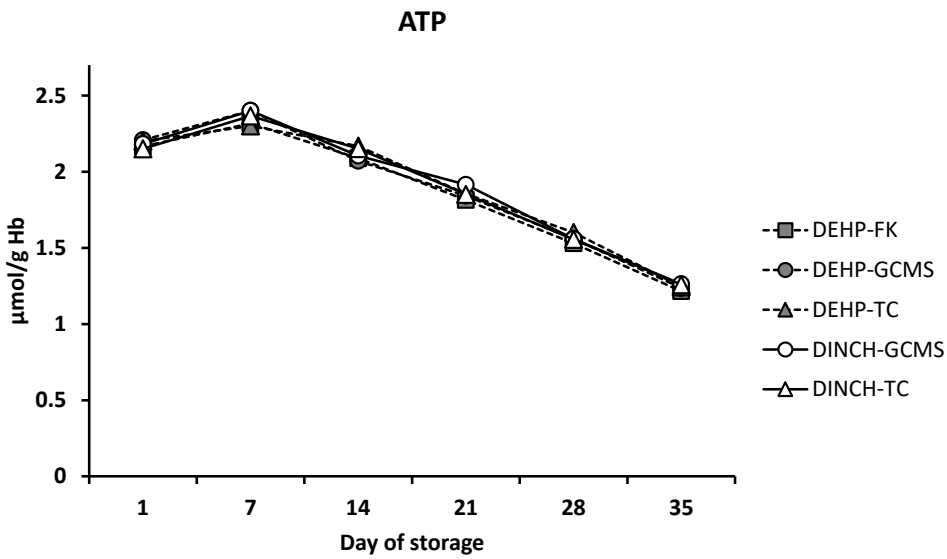


Figure 15. Median values of ATP of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

Table 18. 2,3-DPG (μmol/g Hb) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	11.1 (10.6–11.7)					
Day 1		11.6 (10.2–13.8)	10.9 (9.6–12.7)	11.2 (9.4–12.4)	10.8 (9.7–12.2)[1]	11.3 (9.3–12.2)
Day 7		5.7 (3.6–7.0)	5.0 (2.5–7.2)	4.9 (3.1–5.6)	4.8 (2.9–6.7)[1]	4.5 (3.2–6.5)[1]
Day 14		1.5 (0.7–3.0)	1.7 (0.5–2.6)	1.6 (0.1–2.3)	1.7 (0.5–2.5)	1.7 (0.6–2.1)
Day 21		0.4 (0.0–0.9)	0.4 (0.1–1.1)	0.3 (0.1–0.8)	0.5 (0.1–1.1)	0.2 (0.0–0.9)[1]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 19. ATP (μmol/g Hb) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	2.38 (2.29–2.57)					
Day 1		2.17 (2.03–2.36)	2.21 (2.07–2.33)	2.20 (2.13–2.32)	2.18 (2.05–2.36)	2.15 (2.11–2.34)
Day 7		2.32 (2.20–2.42)	2.40 (2.24–2.54)	2.30 (2.19–2.47)	2.40 (2.25–2.51)[1,3]	2.37 (2.25–2.45)[1]
Day 14		2.09 (1.97–2.22)	2.07 (1.97–2.30)	2.17 (1.91–2.32)	2.11 (1.95–2.28)	2.15 (2.07–2.25)
Day 21		1.81 (1.74–1.92)	1.84 (1.76–1.98)	1.85 (1.75–2.00)	1.92 (1.74–2.01)[1]	1.85 (1.73–2.01)
Day 28		1.53 (1.45–1.61)	1.56 (1.45–1.64)	1.60 (1.53–1.65)	1.56 (1.51–1.65)[1]	1.56 (1.52–1.63)
Day 35		1.22 (1.10–1.28)	1.24 (1.16–1.34)	1.25 (1.17–1.42)	1.26 (1.15–1.38)[1]	1.26 (1.18–1.39)[1]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

3.3.5 pH, pCO_2, and pO_2

pH decreased in all bags throughout the storage period, and the results were generally comparable between the study arms (Figure 16). The DINCH bags showed statistically significant differences with other bags at several points during the storage period, but the differences in median values were very trivial (Table 20).

The DINCH bags had better gas permeability than DEHP bags. While all bags showed an increase in pCO_2 until day 14, followed by a decrease, the decrease was more significant in the DINCH bags than the DEHP bags (Figure 17). Both DINCH bags showed lower pCO_2 levels than all three DEHP bags from day 14 onward (Table 21).

Similarly, all bags showed an increase in pO_2 throughout the storage period, and the increase was more significant in the DINCH bags than the DEHP bags (Figure 18**)**. Both DINCH bags showed higher pO_2 levels than all three DEHP bags from day 14 onward (Table 22). The DINCH-GCMS bag showed lower pO_2 levels than the DINCH-TC bag from day 1 to day 28, but there was no difference on day 35 (Table 22).

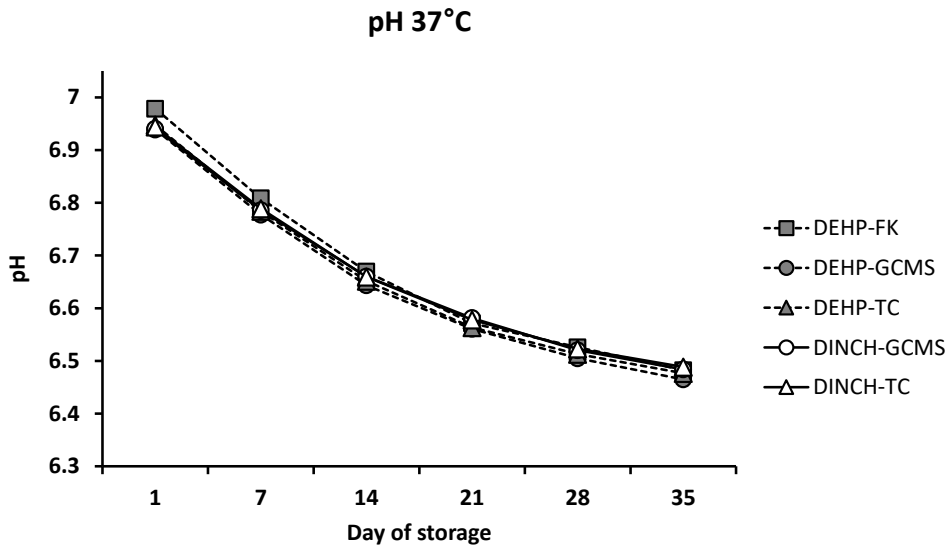


Figure 16. Median values of pH of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

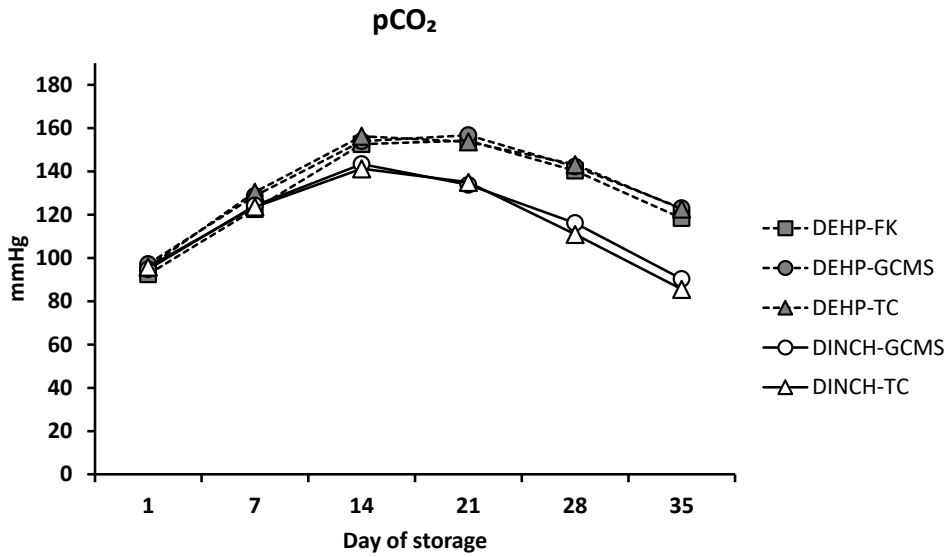


Figure 17. Median values of pCO_2 of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

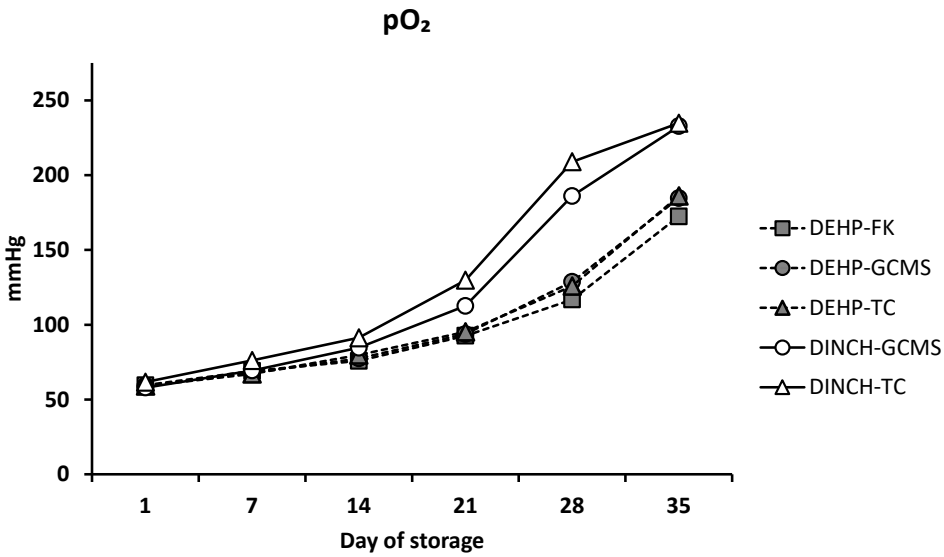


Figure 18. Median values of pO_2 of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

Table 20. pH results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	7.03 (7.02–7.07)					
Day 1		6.98 (6.96–6.98)	6.94 (6.93–6.96)	6.95 (6.93–6.96)	6.94 (6.93–6.96)[1]	6.94 (6.93–6.96)[1]
Day 7		6.81 (6.78–6.85)	6.78 (6.76–6.82)	6.78 (6.76–6.83)	6.79 (6.77–6.80)[1]	6.79 (6.76–6.82)[1]
Day 14		6.67 (6.65–6.69)	6.64 (6.62–6.67)	6.65 (6.62–6.67)	6.66 (6.63–6.68)[1,2,3]	6.66 (6.64–6.69)[1,2,3]
Day 21		6.57 (6.55–6.59)	6.56 (6.54–6.58)	6.56 (6.54–6.58)	6.58 (6.56–6.60)[2,3]	6.58 (6.56–6.60)[2,3]
Day 28		6.53 (6.49–6.54)	6.51 (6.49–6.52)	6.51 (6.48–6.54)	6.52 (6.49–6.54)[2,4]	6.52 (6.50–6.55)[1,2,3,5]
Day 35		6.48 (6.45–6.50)	6.47 (6.43–6.48)	6.48 (6.44–6.49)	6.48 (6.45–6.50)	6.49 (6.46–6.50)[1,2,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 21. pCO_2 (mmHg) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	76.2 (67.9–81.2)					
Day 1		92.5 (86.0–93.9)	97.3 (91.0–101.1)	95.8 (89.3–99.4)	94.8 (88.3–98.3)[1,2]	95.8 (90.6–99.3)[1,2]
Day 7		122.6 (116.2–129.6)	128.7 (123.6–136.9)	130.5 (122.7–134.6)	124.3 (120.0–128.4)[2,3]	123.6 (117.9–129.3)[2,3]
Day 14		152.6 (142.6–157.8)	154.0 (148.2–158.8)	156.3 (146.9–161.1)	143.4 (136.5–147.8)[1,2,3]	141.2 (136.0–147.9)[1,2,3]
Day 21		154.2 (148.0–163.4)	156.8 (145.4–163.6)	153.7 (145.0–163.2)	133.8 (128.0–141.6)[1,2,3]	135.0 (125.0–142.7)[1,2,3]
Day 28		140.3 (131.5–148.5)	142.2 (130.7–148.6)	143.2 (130.9–148.6)	116.2 (103.6–121.6)[1,2,3]	110.9 (103.9–123.1)[1,2,3]
Day 35		118.4 (102.7–126.4)	123.0 (106.3–126.0)	122.6 (107.4–127.8)	90.3 (75.8–98.5)[1,2,3]	85.5 (77.2–99.9)[1,2,3]

Data is presented as median (Q_1–Q_3).

[1] $P < 0.05$ compared to storage in DEHP-FK.

[2] $P < 0.05$ compared to storage in DEHP-GCMS.

[3] $P < 0.05$ compared to storage in DEHP-TC.

[4] $P < 0.05$ compared to storage in DINCH-TC.

[5] $P < 0.05$ compared to storage in DINCH-GCMS.

Table 22. pO_2 (mmHg) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	62.1 (56.7–85.5)					
Day 1		59.6 (52.1–62.9)	59.2 (52.5–64.5)	59.1 (52.5–64.9)	58.0 (50.9–65.4)[3,4]	61.6 (53.9–69.8)[2,3,5]
Day 7		69.1 (60.6–77.5)	67.8 (56.9–78.5)	67.1 (60.0–78.2)	69.4 (61.3–78.2)[2,4]	76.1 (65.6–87.3)[1,2,3,5]
Day 14		75.9 (64.3–89.0)	77.4 (59.4–88.4)	79.8 (62.9–89.2)	84.6 (71.4–93.8)[1,2,3,4]	91.3 (75.0–109.1)[1,2,3,5]
Day 21		92.6 (78.9–116.7)	93.8 (75.4–113.1)	95.2 (78.5–109.7)	112.6 (90.3–140.8)[1,2,3,4]	129.8 (97.6–165.7)[1,2,3,5]
Day 28		116.8 (100.5–181.6)	128.7 (92.9–163.9)	125.8 (107.3–178.0)	186.1 (142.5–225.1)[1,2,3,4]	209.0 (166.5–227.6)[1,2,3,5]
Day 35		172.5 (145.4–221.7)	184.7 (119.2–210.2)	186.2 (160.8–224.4)	232.8 (220.7–255.9)[1,2,3]	234.8 (226.9–245.1)[1,2,3]

Data is presented as median (Q_1–Q_3).

[1]$P < 0.05$ compared to storage in DEHP-FK.

[2]$P < 0.05$ compared to storage in DEHP-GCMS.

[3]$P < 0.05$ compared to storage in DEHP-TC.

[4]$P < 0.05$ compared to storage in DINCH-TC.

[5]$P < 0.05$ compared to storage in DINCH-GCMS.

3.3.6 Glucose, lactate, K^+, and Na^+

The glucose level decreased in all bags throughout the storage period (Figure 19). The DEHP-FK bag showed lower glucose levels than the other four bags. However, a difference of approximately 40 mg/dL of glucose was maintained between the DEHP-FK bag and the other bags throughout the storage period (Table 23), and the glucose consumption rate was comparable between the different study arms.

The lactate level increased in all bags throughout the storage period (Figure 20), showing an inverse relationship with glucose. Although the difference was minimal, the DEHP-FK bag showed higher lactate levels than the DINCH bags throughout the storage period (Table 24). The DINCH bags showed statistically significant differences with DEHP-GCMS and DEHP-TC bags at several points during the storage period. However, the differences in the median values were very small, and there was no consistent trend between them. The lactate production rate was comparable between the different study arms.

The K^+ level increased, and the Na^+ level decreased in all bags throughout the storage period (Figures 21 and 22), showing an inverse relationship similar to that of glucose and lactate. From day 7 onwards, the DEHP-FK bag showed increased levels of K^+ (Table 25) and decreased levels of Na^+ (Table 26) compared with the two DINCH bags. However, the differences in the median values were very small. The K^+ and Na^+ levels were generally comparable between the DINCH bags and the DEHP-GCMS and DEHP-TC bags.

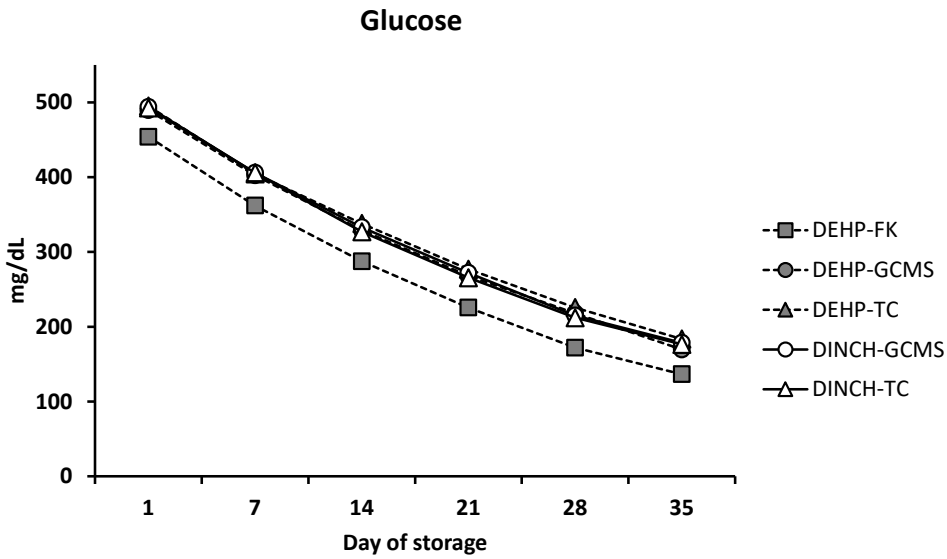


Figure 19. Median values of glucose of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

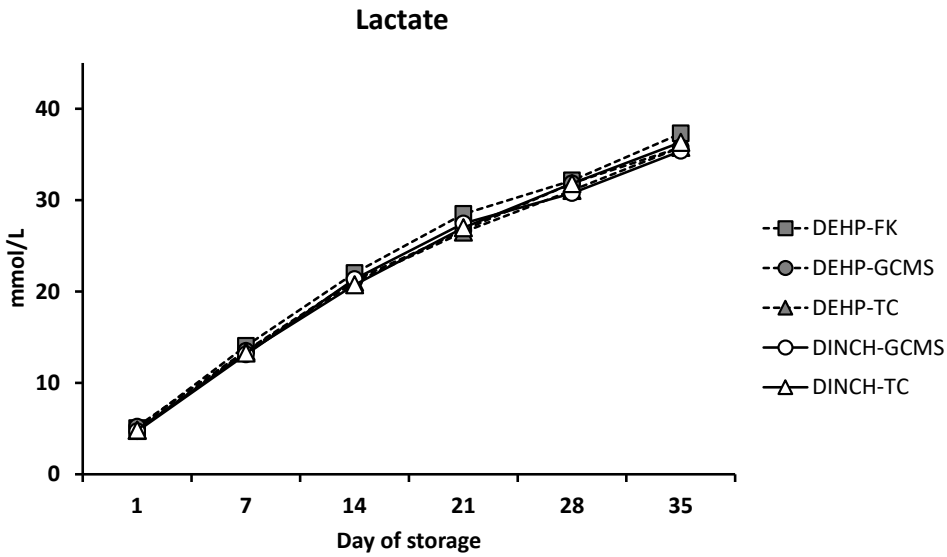


Figure 20. Median values of lactate of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

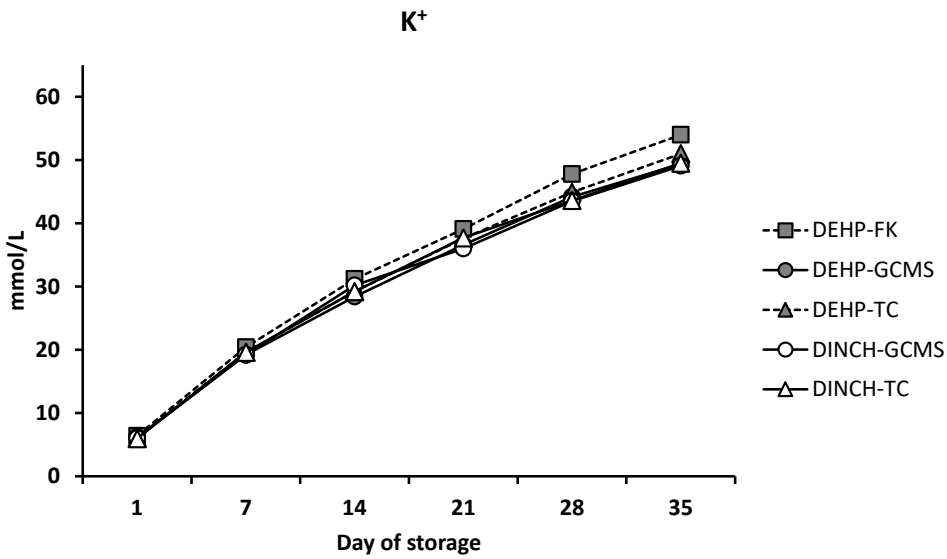


Figure 21. Median values of K^+ of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

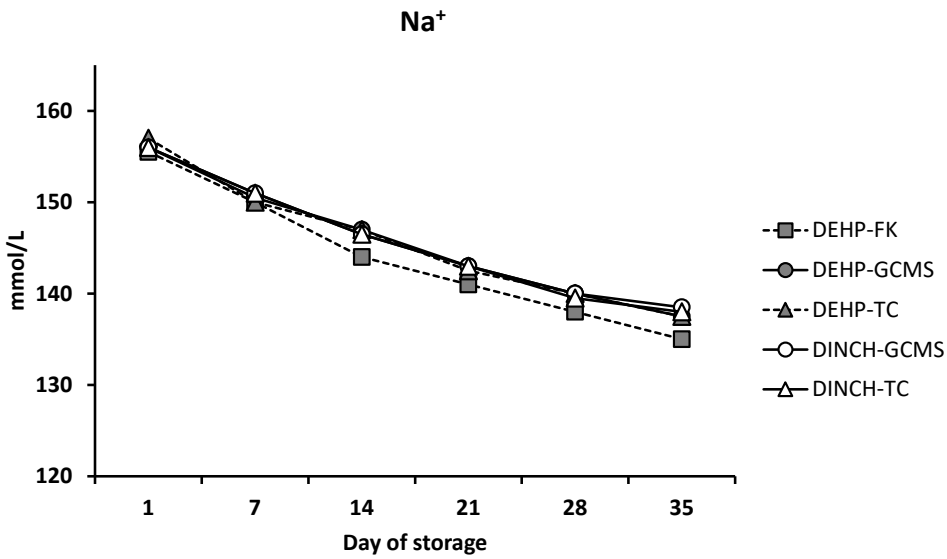


Figure 22. Median values of Na^+ of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20).

Table 23. Glucose (mg/dL) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	480 (454–499)					
Day 1		454 (445–473)	490 (468–504)1	495 (484–508)	494 (483–513)[1,2]	493 (483–503)[1]
Day 7		362 (357–377)	403 (385–410)1	405 (395–419)	406 (396–431)[1,2]	406 (390–432)[1]
Day 14		288 (280–303)	329 (315–342)	338 (315–350)	333 (310–362)[1]	327 (321–353)[1]
Day 21		226 (209–246)	268 (248–285)	277 (253–290)	272 (244–299)[1]	266 (255–289)[1]
Day 28		172 (156–192)	218 (196–238)	226 (205–247)	215 (195–252)[1]	212 (192–237)[1,3]
Day 35		137 (111–159)	170 (151–200)	184 (159–210)	179 (148–214)[1]	177 (151–197)[1,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 24. Lactate (mmol/L) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	2.10 (1.70–2.40)					
Day 1		5.05 (4.88–6.25)	5.21 (4.52–6.37)	4.81 (4.45–5.77)	4.70 (4.34–6.22)[1,2]	4.85 (4.38–5.75)[1,2]
Day 7		14.0 (12.2–15.1)	13.5 (11.3–14.6)	13.4 (11.2–14.3)	13.1 (11.6–14.5)[1]	13.3 (11.7–14.4)[1]
Day 14		22.0 (19.9–24.6)	21.2 (18.5–23.0)	21.0 (18.1–23.1)	21.4 (18.5–24.0)[1,3]	20.7 (19.0–24.0)[1]
Day 21		28.5 (26.3–30.8)	26.7 (23.7–28.3)	26.5 (23.6–29.1)	27.4 (24.6–29.2)[1,2,3]	27.0 (24.2–29.1)[1,3]
Day 28		32.1 (31.0–35.8)	31.8 (29.1–34.3)	31.1 (28.6–33.5)	30.8 (29.0–34.9)[1,3]	31.8 (29.5–35.0)[1,3]
Day 35		37.3 (34.6–40.0)	35.8 (32.8–38.0)	35.8 (32.9–37.9)	35.4 (33.3–38.8)[1,3]	36.3 (34.4–39.1)[2,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 25. K^+ (mmol/L) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	3.0 (3.0–3.1)					
Day 1		6.4 (5.7–7.2)	6.2 (5.6–7.5)	6.1 (5.5–6.9)	6.1 (5.3–8.9)	6.0 (5.5–8.2)
Day 7		20.4 (17.7–22.8)	19.2 (16.5–21.3)	19.6 (17.2–21.6)	19.2 (16.4–21.4)[1]	19.6 (16.5–21.2)[1]
Day 14		31.2 (27.8–35.9)	28.4 (25.7–33.1)	29.2 (25.8–33.7)	30.2 (24.9–33.0)[1]	29.2 (26.0–33.5)[1]
Day 21		39.1 (36.0–42.9)	36.6 (34.2–40.7)	37.6 (33.6–42.2)	36.0 (33.0–41.2)[1,3]	37.7 (33.4–40.8)[1]
Day 28		47.8 (41.7–50.3)	44.2 (41.6–47.3)	44.9 (40.5–48.5)	43.5 (40.2–47.9)[1]	43.6 (38.6–47.7)[1]
Day 35		54.0 (48.8–56.3)	49.4 (48.0–53.0)	51.0 (47.4–52.8)	49.1 (44.7–52.7)[1,3]	50.0 (46.4–52.6)[1]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 26. Na^+ (mmol/L) results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0	157 (156–158)					
Day 1		156 (153–157)	156 (154–158)	157 (155–158)	156 (154–158)	156 (153–158)
Day 7		150 (146–152)	151 (149–153)	150 (149–153)	151 (149–154)[1]	151 (149–153)[1]
Day 14		144 (143–148)	147 (146–150)	147 (145–151)	147 (145–151)[1]	147 (145–150)[1]
Day 21		141 (139–144)	143 (142–146)	143 (141–145)	143 (143–147)[1,3]	143 (141–146)[1]
Day 28		138 (136–141)	140 (137–143)	140 (137–143)	140 (139–144)[1,3]	140 (138–143)[1]
Day 35		135 (133–140)	138 (136–141)	138 (135–141)	139 (137–142)[1,2,3]	138 (136–142)[1,3]

Data is presented as median (Q_1–Q_3).
[1]$P < 0.05$ compared to storage in DEHP-FK.
[2]$P < 0.05$ compared to storage in DEHP-GCMS.
[3]$P < 0.05$ compared to storage in DEHP-TC.
[4]$P < 0.05$ compared to storage in DINCH-TC.
[5]$P < 0.05$ compared to storage in DINCH-GCMS.

4. DISCUSSION

This study evaluated DINCH as an alternative plasticizer to replace DEHP in blood bags for RBC storage with CDPA-1 anticoagulant. We used a pool-and-split study design to obtain homogenous matched quintets of adult-sized RBC concentrates for comparison. Some previous studies have used pediatric-sized units [15, 16], which may have different volume-to-area properties of the bags and concentrations of plasticizer leaching into the blood compared with adult-sized bags.

The RBC concentrate units in this study were not leukoreduced and were stored using CDPA-1 as the anticoagulant with no other additive solutions. Previous studies on DEHP alternatives were mainly based on leukoreduced components [9, 14-18]. Without a direct comparison, it is unclear if leukoreduction may affect RBC concentrate properties during storage in DINCH bags. However, prestorage leukoreduction has been reported to have various beneficial effects on RBC storage quality, including hemolysis [22, 23]. Therefore, it is possible that prestorage leukoreduction may have the potential to improve RBC storage quality in DINCH bags compared to the results in this study. Utilizing additive solutions is another option that can be used to improve RBC storage quality. In addition to the widely used SAGM, new additive solutions such as PAG3M, PAGGSM, PAGGGM, E-Sol 5, AS-1, AS-3, AS-5, and AS-7 have been developed for better preservation of RBC quality and to prolong the storage period [9, 17, 18, 24]. However, universal leukoreduction of blood components and routine use of additive solutions are not available in every jurisdiction or country. In South Korea, leukoreduction for RBCs

and platelets is allowed only for high-risk patients, and SAGM is only used when producing prestorage leukoreduced RBCs. Since most previous studies have been conducted with the use of leukoreduction and additive solutions, our study's results will be of value to areas where blood product manufacturing is based on conventional settings with limited use of these options.

The main issue when searching for a viable surrogate to replace DEHP in RBC storage bags is concerned with DEHP's protective effects on RBC stability. Alternative plasticizers should not only have a non-toxic biosafety profile, but also demonstrate sufficient ability to preserve RBCs. Hemolysis of RBCs during storage has profound clinical significance regarding the safety of transfused patients. Free hemoglobin released from RBCs dissociates into dimers which are then bound to haptoglobin and removed by the reticuloendothelial system [25]. Once the haptoglobin binding capacity has been exceeded, consumption of nitric oxide by excess plasma hemoglobin may lead to disruption in the regulation of smooth muscle tone resulting in dystonia [26]. This deterioration in smooth muscle function can give rise to various clinical problems such as pulmonary and systemic hypertension, erectile dysfunction, dysphagia, abdominal pain, and clot formation [26]. In addition, intravascular cell-free hemoglobin can facilitate oxidation reactions that induce lipid peroxidation, cellular and renal injury, and amplification of innate immune responses in sepsis [27].

Although the specific amount of intravascular cell-free hemoglobin that may induce clinical problems is uncertain, regulatory agencies have stated requirements regarding hemolysis in RBC concentrate units. The US FDA requires less than 1%

of hemolysis at the end of RBC storage [28], while the Council of Europe guideline has a more stringent requirement of 0.8% [29]. The degree of hemolysis in RBC concentrates is assessed by measuring the ratio (%) of cell-free hemoglobin to the total hemoglobin corrected by the hematocrit value [21, 25]. In this study, we found that the median hemolysis rates were higher in the DINCH bags than in the DEHP bags. Moreover, the difference between them gradually increased throughout the storage period. However, the hemolysis rates for all individual DINCH bags were well within the acceptable regulatory limits, demonstrating their potential as a feasible alternative to DEHP bags. Other previous studies on DINCH bags have similarly shown comparable or slightly increased levels of hemolysis compared with DEHP bags in various experimental settings [15-18].

Two interesting hemolysis-related factors assessed in these studies on DINCH bags were (a) mixing the contents of the RBC concentrate units during storage and (b) the additive solution used for RBC preservation. A study on DINCH bags with leukoreduced RBCs in Optisol (AS-5) reported better RBC characteristics, including hemolysis, when mixed weekly compared with being statically stored for 42 days [18]. The RBC hemolysis in DINCH bags mixed weekly was comparable to that seen in statically stored DEHP bags. In contrast, DINCH bags that were statically stored showed increased levels of hemolysis compared with statically stored DEHP bags. In this study, the DINCH and DEHP bags were mixed weekly before sampling, and the former showed slightly higher hemolysis than the latter, which was statistically significant. The additive solution used could also remarkably affect hemolysis in the DINCH bags. A study on DINCH bags with leukoreduced RBC concentrates and

four different additive solutions (SAGM, PAGGSM, PAGGGM, AS-3) reported increased hemolysis in RBCs stored with SAGM compared to the conventional DEHP/SAGM combination [17]. However, the hemolysis with PAGGSM, PAGGGM, and AS-3 was comparable to that with DEHP/SAGM [17]. Another study comparing DEHP/SAGM, BTHC/SAMG, BTHC/PAGGSM, and DINCH/PAGGSM systems reported that although storage of RBCs in BTHC/SAGM for 42 days without mixing showed significantly increased hemolysis than DEHP/SAGM, BTHC/PAGGSM showed no difference with DEHP/SAGM, and DINCH/PAGGSM was slightly inferior to BTHC/PAGGSM and DEHP/SAGM [19]. These findings suggest that although DINCH may be inherently inferior to DEHP in terms of preserving RBC stability, periodic mixing of RBC units during storage and/or using appropriate additive solutions may help compensate for this disadvantage.

The CBC result assessed by automated hematology analyzers gives comprehensive information on blood samples. While most of the parameters are actually measured values, some are derived from calculations using other results. In the case of the XN-9000, the MCV, MCH, and MCHC are parameters derived from calculation. While the hemoglobin and RBC count maintained a steady level throughout storage, the WBC and platelet counts gradually decreased. This deterioration is presumed to be the result of the shorter lifespan of WBCs and platelets compared to RBCs. Although the hematocrit gradually increased during storage in all study arms, one interesting finding was that the DEHP-FK bag showed generally higher hematocrit levels than other bags. Some instruments report

hematocrit derived from calculation using other parameters. The XN-9000 directly measures hematocrit using the 'cumulative pulse height method' by analyzing the cumulative volume of individual RBCs relative to the total volume of whole blood. In addition to hematocrit, hemoglobin and RBC, WBC, and platelet counts were also increased in the DEHP-FK bag compared to other bags. Although the reason for this finding is unclear, differences in the plastic compound or anticoagulant contents among manufacturers might be things to be considered.

In addition to the findings on hemolysis, DINCH bags also showed differences in MCV and MCHC in this study. Increased MCV indicating RBC swelling is commonly observed during RBC storage [30]. Based on the type of plasticizer and additive solution used, varying levels of increase in MCV have been reported in RBC bags [9, 14-19]. In this study, RBCs stored in DINCH bags with CPDA-1 showed a slight increase in MCV compared with DEHP bags. Two previous studies comparing RBCs stored with SAGM in DEHP, DINCH, and BTHC bags reported a more significant increase in MCV in the BTHC bags than in the other two bags [15, 16]. Another study reported a higher increase in MCV following RBC storage in DINCH bags with SAGM compared with PAGGSM, PAGGGM, AS-3, and the conventional DEHP/SAGM combination [17]. Because of the increased MCV, MCHC was decreased in DINCH bags compared to DEHP bags in this study. However, the MCH, indicating the average amount of hemoglobin in each RBC, maintained a steady level throughout storage, and there was no difference between DINCH and DEHP bags.

The EMA binding test is used to diagnose hereditary spherocytosis (HS). HS is characterized by a deficiency in RBC membrane proteins such as band 3, protein 4.2,

ankyrin, and α and β spectrin leading to detachment of the lipid bilayer from the cytoskeleton [31]. EMA binds to the ε-NH_2 group of lysine in band 3, and sulfhydryl groups in CD47 and the Rh-associated glycoprotein [32]. The intensity of fluorescence signal derived from EMA bound to RBCs measured by flow cytometry is an indicator of the RBC membrane integrity. EMA binding is reported as either the absolute MFI value or the ratio (%) of sample MFI to normal controls. Based on an EMA binding reference range of 86.9-118.7%, one study reported the estimated cutoff value for HS to be < 86.9% [33]. In this study, EMA binding decreased in all study arms throughout the storage period, but the values mainly remained above 90%. The DINCH bags showed a small yet significant decrease in EMA binding compared to the DEHP bags on day 35. Another parameter used to measure the loss of RBC membrane integrity is the RBC microvesicle (microparticle). Previous studies comparing DINCH or DEHT bags to DEHP bags have reported an increase in microvesicle count throughout storage, consistent with an increase in hemolysis [14-16]. The EMA binding and microvesicle counts are based on opposing effects on the RBC membrane. This relationship is well demonstrated in Figures 3 and 13**,** which show an inverse relationship between hemolysis and EMA binding. Compared to the DEHP bags, the DINCH bags had increased hemolysis and decreased EMA binding, while the DINCH-TC and DEHP-FK bags generally showed the corresponding maximum or minimum median values.

In this study, the DINCH and DEHP bags showed no remarkable differences in the levels of 2,3-DPG, ATP, and pH. These findings are consistent with a previous study comparing RBCs with SAGM in BTHC, DINCH, and DEHP bags [15]. 2,3-

DPG and pH affect the binding of oxygen to hemoglobin. 2,3-DPG increase and pH decrease move the oxyhemoglobin dissociation curve to the right, which leads to a decrease in the oxygen affinity to hemoglobin [34]. If the body tissue requires more oxygen, RBCs produce more 2,3-DPG to dissociate oxygen from hemoglobin to the tissue. 2,3-DPG deficiency in stored RBCs leads to a decreased capacity of the RBCs to release and supply oxygen to the tissues. In this study, 2,3-DPG rapidly dropped during RBC storage, and the measurements were nearly at undetectable levels on day 21. ATP is important in maintaining RBC viability. RBCs rely on ATP synthesized from glycolysis and lactic acid fermentation as the energy source instead of the oxygen they carry because they do not have mitochondria [35]. Reduced ATP in RBCs also results in the loss of membrane lipids, increased cellular rigidity, and the shape change from discs to spheres [36]. The gradual drop of ATP in this study resembles that of EMA binding, which is another parameter representing membrane integrity.

Significant differences were seen in the blood gas partial pressure between DINCH and DEHP bags. The DINCH bags had higher pO_2 and lower pCO_2 levels than the DEHP bags. Previous studies comparing the DINCH, BTHC, and DEHT bags with DEHP bags have reported similar results [14, 15]. Consistent with the previous studies, we found no correlation between the pH values and blood gas partial pressures, suggesting that the changes in pO_2 and pCO_2 are based on the gas permeability properties of the bags rather than the RBCs' metabolic activities. It is unclear whether this increased gas permeability of DINCH-PVC compared to DEHP-PVC has any clinical impact on RBC transfusion for patients. Although pO_2

is known to increase during RBC storage due to changes in oxygen affinity caused by the decline in 2,3-DPG, the reason why DINCH-PVC presented such high values of pO_2 needs further research to be explained. DEHP-PVC has been reported to have low permeability to O_2 and CO_2, limiting its capability for platelet storage [37], and the replacement of DEHP has been shown to have little to no effect on the quality of platelets [14, 17]. Therefore, DEHP is no longer widely used as a plasticizer for platelet storage containers, and nowadays PVC containers plasticized with BTHC or TOTM are preferred by having higher gas permeability, which allows for longer storage of PLTs compared to DEHP-PVC [2].

While the DEHP-FK bag showed decreased glucose and slightly increased lactate levels, the other four had comparable levels of these metabolites. The noticeable gap in the glucose level observed as early as day 1 in the DEHP-FK bag might be due to differences in the composition of CPDA-1 from different manufacturers. Although we removed excess CPDA-1 from the primary bag of the triple bags before distributing the whole blood from the pooling bag during the production process, some remnant CPDA-1 may have affected the results. However, as shown in Figures 19 and 20, the five study arms had comparable rates of glucose consumption and lactate production. The relationship between hemolysis and K^+ in the blood bags is another interesting issue. Hemolysis in blood samples is known to increase K^+ and decrease Na^+ concentrations in plasma [38, 39]. In this study, although hemolysis was the lowest in the DEHP-FK bag, it had the highest K^+ and the lowest Na^+ levels. This lack of correlation between hemolysis and K^+ levels has also been observed in previous studies on DINCH and DEHT bags [9, 14, 15, 17, 18]. The reason for this

unexpected yet repetitive finding is unclear, and further research is required to better understand this phenomenon.

This study has some limitations. First, we did not validate the effect of additive solutions. We could not utilize various types of additive solutions because of the limited access in our jurisdiction. As observed in previous studies, the choice of proper additive solutions has varying beneficial effects during RBC storage. Second, the magnitude of the differences in many of the test results, even when statistically significant, was not considerably large. It is unclear whether they are clinically relevant. Third, the gradual reduction in RBC concentrate unit volume is another factor to consider. Because we took considerable amounts of samples from the units for testing every week, the volume of blood where the plasticizer is being accumulated decreased throughout the storage period. A simple comparison of days 1 and 35 might have produced more straightforward results. Fourth, although the cross-contamination of DINCH into the DEHP study arms was very low (data shown in part II of this study), their impact on the results of this study cannot be totally ruled out. Finally, other differences, such as collection volume, time delay between collection and processing, leukoreduction, irradiation, centrifugal force, centrifugation time, and production method (buffy coat vs. PRP), can all contribute to variability in results.

In conclusion, this study demonstrates that RBCs stored for 35 days in DINCH-plasticized blood bags with CDPA-1 are of comparable quality to those stored in DEHP bags. While RBCs in DINCH bags showed marginally higher hemolysis than those in DEHP bags, hemolysis was below the current regulatory limit in all the bags.

Our findings indicate that DINCH is a promising alternative to DEHP in blood bags for RBC storage, even without the use of next-generation additive solutions. More accumulative information on the validation of alternative plasticizers, in combination with various factors including additive solutions, component production methods, storage conditions, irradiation, and washing, is needed to better understand this issue and derive a suitable solution.

Part II

Analysis of DEHP and DINCH Concentrations in Blood Donors and Red Blood Cell Concentrates

1. INTRODUCTION

A plasticizer is a chemical substance that is added to a material, such as PVC, to increase its flexibility, durability, and workability [40]. It works by reducing the stiffness and brittleness of the material, making it easier to process and shape, and it can be added to as high as 40% of the total weight formulation [41]. Plasticizers have been commonly used in the production of a wide range of products, including children's toys, household items, medical devices, clothing, food packaging materials, construction materials, and cosmetics [42]. They are also used as solvents in the manufacture of paints, glue, and insect repellent [40]. However, plasticizers can leach out from the material to its content or surroundings because they are not covalently bound to the products [8, 43]. Hence, plasticizers accumulate in our environment and ecosystem, making them a ubiquitous material detected in aquatic systems, drinking water, air dust, soils, and sewage treatment plants at considerable concentrations [40].

Because of this characteristic, humans and animals can be easily exposed to plasticizers through various routes, such as inhalation, ingestion, and skin contact. For this reason, there has been growing concern over the safety of certain plasticizers, particularly phthalates, which have been linked to health issues. The biological harmfulness of DEHP, the most widely used phthalate, has been mainly observed in reproductive and developmental toxicity through studies on rats, mice, hamsters, and guinea pigs [7, 8, 44]. Numerous studies have attempted to determine the direct human toxicity of DEHP. These studies have investigated various fields such as

testosterone production, breast tumors, hypospadias, cryptorchidism, decreased anogenital distance, infant and adolescent growth, endometriosis, neurological behavior, obesity, insulin resistance, and type 2 diabetes [45]. However, the conclusions regarding DEHP's human toxicity have not been clear or consistent. Based on these data, DEHP is classified as a carcinogenic, mutagenic or toxic for reproduction (CMR) substance of category 1B (scientific evidence based on animals) by the CLP (Classification, Labelling and Packaging) regulation in Europe [45]. The International Agency for Research on Cancer (IARC) has also acknowledged sufficient evidence for the carcinogenicity of DEHP in experimental animals and has classified DEHP as group 2B (possibly carcinogenic to humans) [46].

One of the factors that make it difficult to interpret the results of DEHP-related studies is that, as previously mentioned, DEHP is used not only in medical devices but also in various everyday items and is commonly present in our environment, such as air, dust, water, soil, and food. However, there is a lack of data on the concentration of DEHP and its alternatives in humans and medical devices. Analyzing plasticizer levels is a difficult task, and these tests are not routinely performed in clinical laboratories. To achieve a better understanding of this issue, it is important to develop reliable methods to measure these materials and generate data that can support clinical studies being conducted in this field of medical research [41].

This study aimed to develop an assay to measure DEHP and DINCH using ultra-high-performance liquid chromatography (UHPLC) and tandem mass spectrometry (MS/MS) and analyzed the plasticizer levels in donors and blood bags.

2. MATERIALS AND METHODS

This study was ethically approved by the Institutional Review Board at Seoul National University Bundang Hospital (B-1705-395-309, B-1707-406-301) and performed in accordance with the Declaration of Helsinki.

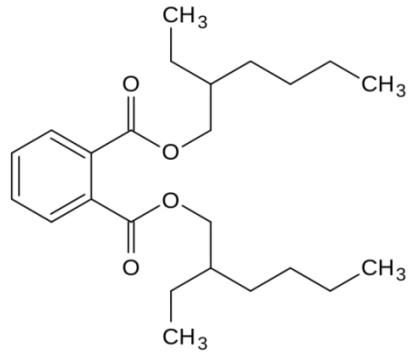
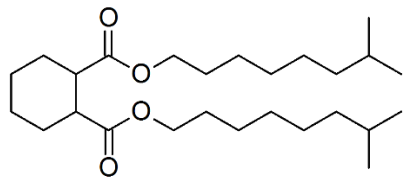
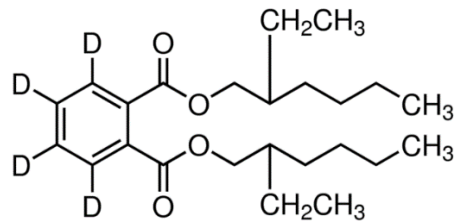
2.1 Reagents

DEHP, di(2-ethylhexyl) phthalate-3,4,5,6-d_4 (DEHP-d_4), and formic acid were purchased from Sigma-Aldrich. DINCH was obtained from BOC Sciences (Shirley, NY, USA). High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile, n-hexane, and distilled water (J.T. Baker, Phillipsburg, NJ, USA) were used in the experiment. The chemical structure and molecular weight of DEHP, DINCH, and DEHP-d_4 are shown in Table 27.

2.2 Calibration Standard Preparation

To prepare the stock solutions of DEHP and DINCH, their compounds were dissolved in methanol. Afterward, a series of five working solutions was prepared by diluting the necessary volume of stock solutions with water/methanol (30:70, v/v), resulting in a concentration range of 1–20 mg/L for DEHP and 0.0625–1 mg/L for DINCH. The solutions were stored at -70°C until use for constructing calibration curves.

Table 27. Chemical structure and molecular weight of plastic materials.

Compound	Structure	Molecular weight
DEHP		390.56
DINCH		424.666
DEHP-d_4		394.58

2.3 Sample Preparation

To avoid unnecessary contact with plastic materials that may contain DEHP as possible, we used only glass syringes, test tubes, and volumetric pipettes during the sample preparation process. The 10 mL glass tubes containing 8 mL of blood sample were centrifuged at 1690 g (3000 rpm) for 7 minutes. From the supernatant, 2 mL of plasma was transferred to another 10 mL glass tube using a glass syringe. We prepared a 100 mg/L DEHP-d_4 working solution as the internal standard and added 50 μL of the solution to each tube containing the calibrators and samples. Then, 5 mL of n-hexane was added for extraction using a glass volumetric pipette, and the tubes were vortexed. The tubes were centrifuged at 1690 g (3000 rpm) for another 7 minutes. After centrifugation, 4 mL of the supernatant was transferred to a new glass tube, and the solvent was evaporated in N_2 gas for 30 minutes. The dried tubes were stored at -70°C in a freezer. Before analysis, the tubes were allowed to stand at 20–25°C for 30 minutes, and the sample was reconstituted in 300 μL of 70% methanol with 0.1% formic acid. The samples were loaded into a UHPLC system in glass vials, and 5 μL of the samples were injected.

2.4 UHPLC-MS/MS Analysis

We used the LC-30A Nexera (Kyoto, Japan) UHPLC system equipped with the Synergi trap column (50.0 mm × 2 mm, 4 μm; Phenomenex, Torrance, CA, USA) and BEH column (50.0 mm × 2.1 mm, 1.7 μm; Waters, Watford, UK). The mobile phases were 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in methanol (solvent B). The total run time was 12 minutes, and the gradient

information applied to the column is summarized in Figure 23.

We used the AB Sciex API 6500 (AB Sciex LLC, Framingham, MA, USA) triple quadrupole tandem mass spectrometer for quantitation. The UHPLC-MS/MS settings and multiple reaction monitoring (MRM) transition information is summarized in Table 28.

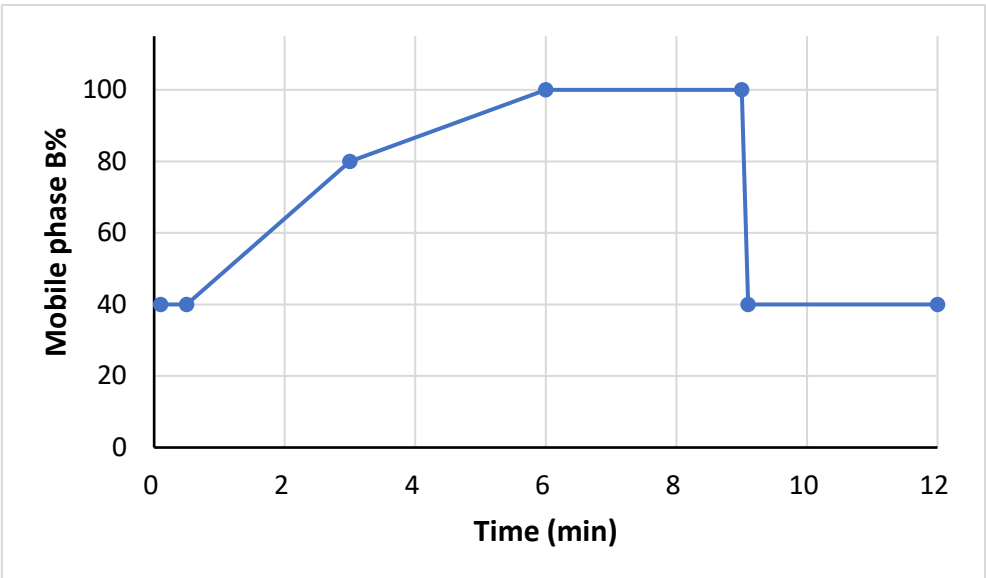


Figure 23. Gradient information of UHPLC for DEHP and DINCH measurement.

Table 28. UHPLC-MS/MS settings and MRM transition information.

Compound	DP (V)	CE (V)	CXP (V)	Q1 (m/z)	Q3 (m/z)	Function
DEHP	80	30	10	391	149	Quantifier
	80	17	10	391	279	Qualifier
	80	28	10	391	167	Qualifier
DINCH	236	13	26	425	281	Quantifier
	236	23	22	425	155	Qualifier
	236	17	8	425	127	Qualifier
D4-DEHP	66	23	10	395	153	IS

Abbreviations: DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; IS, internal standard.

2.5 Evaluation of Analytical Performance

The within-run precision for DEHP and DINCH measurements was evaluated by performing five replicated analyses on low, medium, and high concentration controls. The criteria for passing were set as a coefficient of variation (CV) within ±20% for each concentration. Linearity was assessed using blank plasma and calibration standard solutions.

2.6 Identification of Plasticizer Contamination

To identify plasticizer contamination caused by the mobile phase, chromatograms derived from blank and a 50 mg/L DEHP solution were compared.

2.7 Plasticizer Measurement in Donors and Blood Bags

Blood samples obtained from part I of this thesis were used for this study. We analyzed the donors' baseline DEHP and DINCH concentrations from the blood sample taken from the diversion pouch. Plasticizer concentrations of the pooled blood during component production were analyzed using the excess 8 mL of blood taken from the DINCH-GCMS bag before centrifugation and PRP extraction. Plasticizer concentrations were also measured throughout the storage period of RBC concentrate units every week.

2.8 Statistical Analysis

Statistical analysis was conducted with receiving consultation from the Medical

Research Collaborating Center at Seoul National University Hospital Biomedical Research Institute. Excel (Microsoft, Redmond, WA, USA) was used to evaluate the within-run precision and linearity. For the measurement of plasticizers in blood bags, each DINCH bag (DINCH-GCMS, DINCH-TC) was compared with each of the three DEHP bags (DEHP-FK, DEHP-GCMS, DEHP-TC) and also with the other DINCH bag. Non-parametrical matched analysis using the Wilcoxon signed-rank test was performed using IBM SPSS Statistics 25 (Armonk, NY, USA). A *P* value of < 0.05 was considered significant.

3. RESULTS

3.1 UHPLC MRM Chromatogram

DEHP, DINCH, and the internal standard were all clearly separated and identified by the UHPLC-MS/MS system without noticeable ion suppression. The retention time was approximately 6.2 min for DEHP and 6.9 min for DINCH (Figure 24).

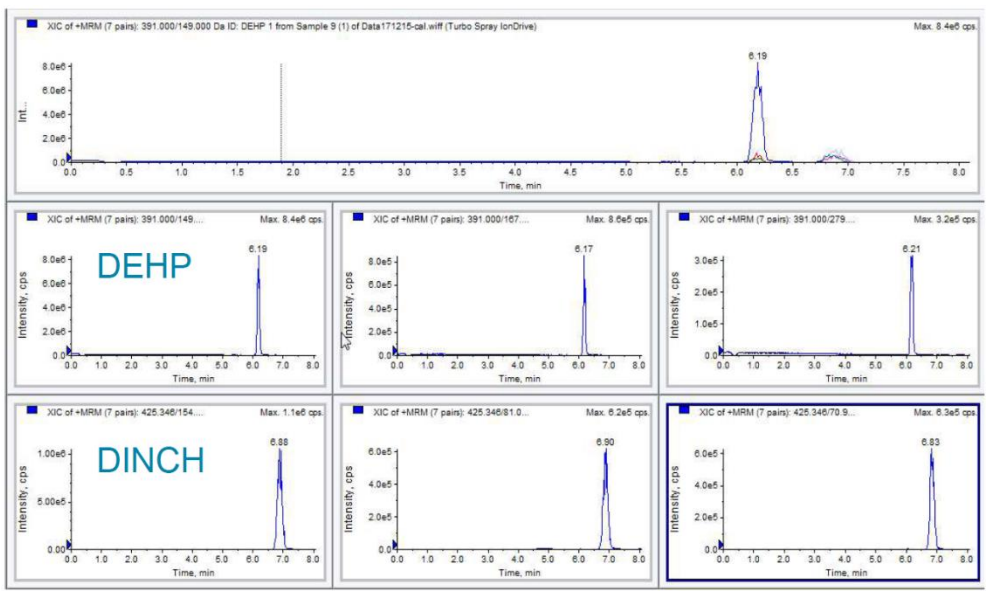


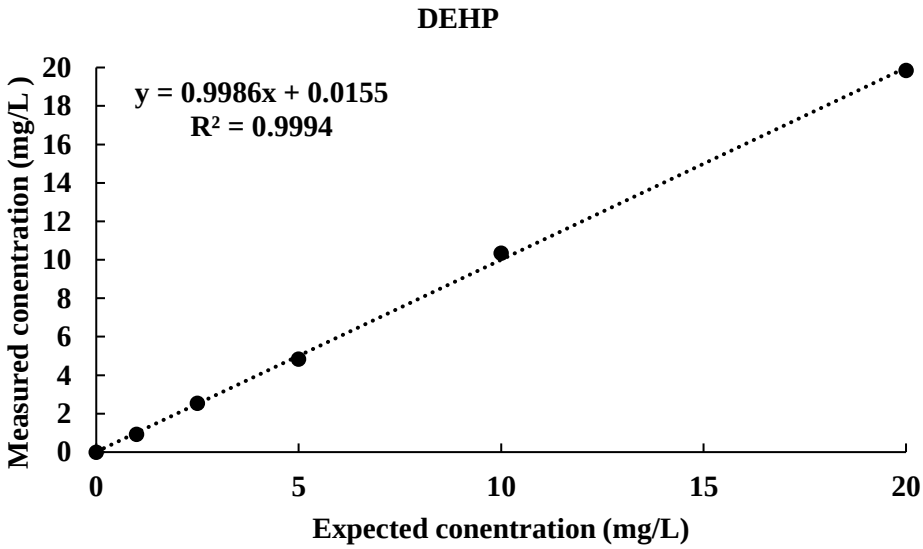
Figure 24. Representative chromatogram for the measurement of DEHP and DINCH

3.2 Analytical Performance

The within-run precision evaluated using three concentration levels for DEHP and DINCH were 11.11–14.01% and 2.08–6.15%, respectively (Table 29). The CV for all concentration levels for both plasticizers were within the acceptable limit of ±20%. Calibration curves were linear over a range of 1–20 mg/L for DEHP (R^2 = 0.9994) and 0.0625–1 mg/L for DINCH (R^2 = 0.9993) (Figure 25).

Table 29. Results of within-run precision for the measurement of DEHP and DINCH

	Within-run CV (%) (mean concentration, mg/L)		
	Low level	Medium level	High level
DEHP	11.11 (6.6)	13.98 (330.9)	14.01 (529.9)
DINCH	6.15 (0.9)	2.08 (42.5)	2.70 (76.9)



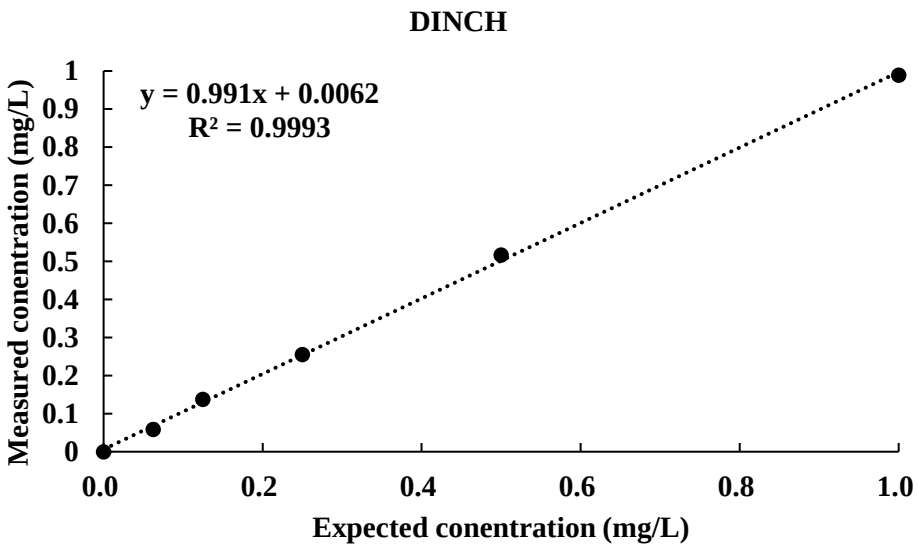
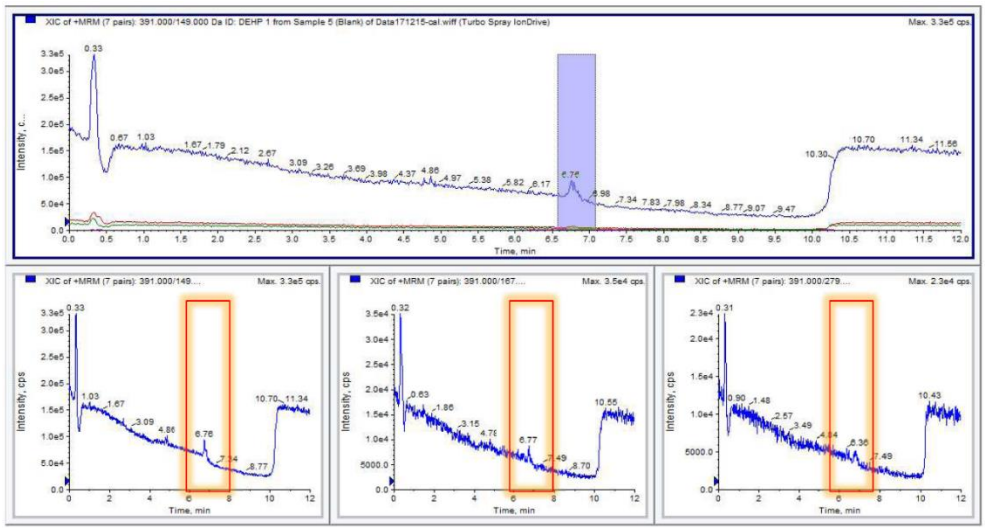


Figure 25. Linearity for DEHP and DINCH measurement.

3.3 Identification of Plasticizer Contamination

By comparing the chromatograms between blank and 50 mg/L DEHP solutions, a weak signal was identified at a retention time of approximately 6.8 min that was assumed to be originated from the mobile phase (Figure 26). This peak was observed in both chromatograms and could be differentiated from the main peak observed only in the 50 mg/L DEHP solution at a retention time of 6.2 min.

A) Blank (mobile phase)



B) 50 mg/L DEHP

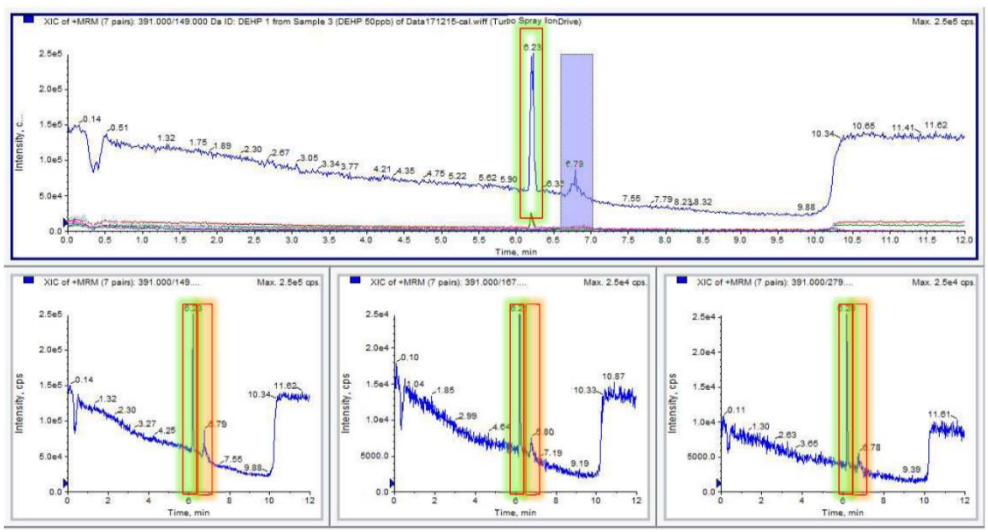


Figure 26. Comparison between blank and 50 mg/L DEHP solution chromatograms.

3.4 Plasticizer Measurement in Donors and Blood Bags

Plasticizer concentrations were measured in a subset of the donors and RBC concentrate unit quintets. The median (Q_1–Q_3) levels of DEHP (mg/L) and DINCH (mg/L) in the donors' blood samples (n = 40) were 0.57 (0.28–0.89) and 0.00 (0.00–0.00), respectively. The median (Q_1–Q_3) levels of DEHP (mg/L) and DINCH (mg/L) in the pooled blood (n = 10) during component production were 0.30 (0.24–1.50) and 0.01 (0.00–0.02), respectively. The DEHP levels gradually increased throughout the storage period in the DEHP bags, while remaining nearly undetectable in the DINCH bags (Figure 27). The DEHP bags showed significantly higher DEHP levels than the DINCH bags from day 1 onward (Table 30). Conversely, the DINCH levels gradually increased throughout the storage period in the DINCH bags, while remaining nearly undetectable in the DEHP bags (Figure 28). The DINCH bags showed significantly higher DINCH levels than the DEHP bags from day 1 onward (Table 31). Compared to DINCH, DEHP displayed considerably higher levels of plasticizer leaching into blood bags.

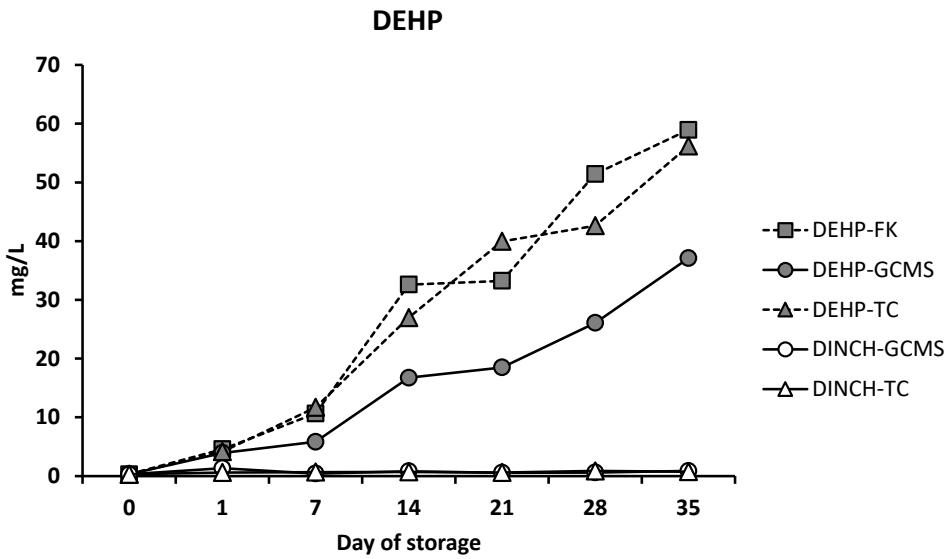


Figure 27. Median values of DEHP in pooled blood (day 0) and RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days.

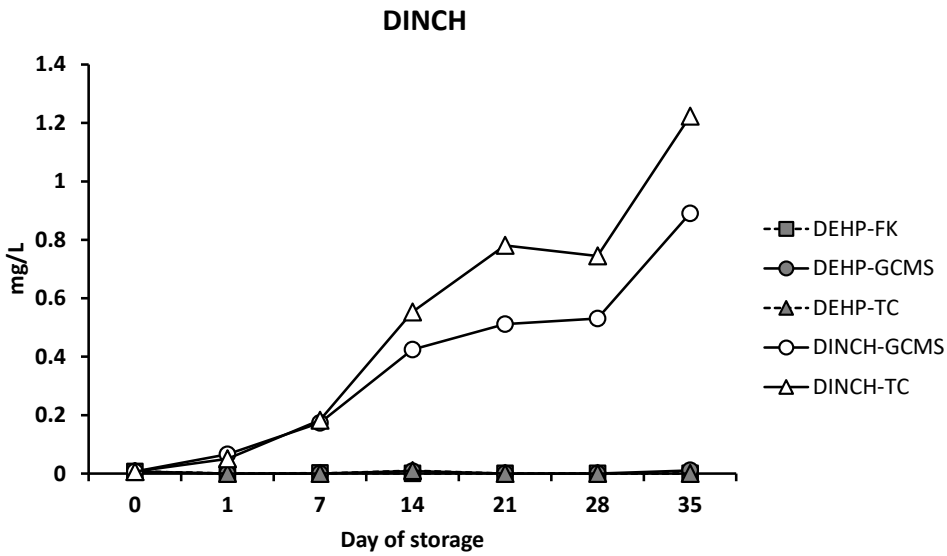


Figure 28. Median values of DINCH in pooled blood (day 0) and RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days.

Table 30. DEHP concentration (mg/L) results for matched quintets of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0 (n = 10)	0.3 (0.2–1.5)					
Day 1 (n = 11)		4.6 (1.9–6.4)	3.9 (2.0–5.2)	4.1 (3.1–6.4)	1.3 (0.2–2.3)[1,2,3]	0.6 (0.2–1.4)[1,2,3]
Day 7 (n = 11)		10.7 (5.1–20.2)	5.8 (4.0–13.1)	11.7 (1.9–15.8)	0.4 (0.3–1.5)[1,2,3]	0.7 (0.3–1.3)[1,2,3]
Day 14 (n = 12)		32.6 (16.4–34.5)	16.7 (12.0–24.9)	27.0 (21.7–42.0)	0.8 (0.3–1.3)[1,2,3]	0.7 (0.5–1.9)[1,2,3]
Day 21 (n = 13)		33.2 (17.3–50.5)	18.5 (12.5–37.1)	40.0 (23.9–52.9)	0.6 (0.2–1.6)[1,2,3]	0.6 (0.3–1.5)[1,2,3]
Day 28 (n = 13)		51.4 (23.5–81.1)	26.1 (18.1–35.7)	42.6 (25.1–61.2)	0.6 (0.3–1.5)[1,2,3]	0.9 (0.3–2.1)[1,2,3]
Day 35 (n = 12)		58.9 (37.3–64.0)	37.1 (29.1–58.3)	56.2 (44.6–70.1)	0.9 (0.5–1.3)[1,2,3]	0.7 (0.5–1.2)[1,2,3]

Data is presented as median (Q_1–Q_3).

[1]$P < 0.05$ compared to storage in DEHP-FK.

[2]$P < 0.05$ compared to storage in DEHP-GCMS.

[3]$P < 0.05$ compared to storage in DEHP-TC.

[4]$P < 0.05$ compared to storage in DINCH-TC.

[5]$P < 0.05$ compared to storage in DINCH-GCMS.

Table 31. DINCH concentration (mg/L) results for matched quintets of RBC concentrates stored in DEHP and DINCH bags with CPDA-1.

	Pooled blood	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 0 (n = 10)	0.01 (0.00–0.02)					
Day 1 (n = 11)		0.00 (0.00–0.03)	0.00 (0.00–0.03)	0.00 (0.00–0.01)	0.07 (0.02–0.14)[1,2,3]	0.05 (0.01–0.21)[1,2,3]
Day 7 (n = 11)		0.00 (0.00–0.01)	0.00 (0.00–0.01)	0.00 (0.00–0.01)	0.17 (0.05–0.29)[1,2,3]	0.18 (0.12–0.38)[1,2,3]
Day 14 (n = 12)		0.00 (0.00–0.05)	0.01 (0.00–0.06)	0.01 (0.00–0.05)	0.42 (0.29–0.96)[1,2,3,4]	0.55 (0.30–1.37)[1,2,3,5]
Day 21 (n = 13)		0.00 (0.00–0.05)	0.00 (0.00–0.08)	0.00 (0.00–0.06)	0.51 (0.22–0.89)[1,2,3]	0.78 (0.27–1.15)[1,2,3]
Day 28 (n = 13)		0.00 (0.00–0.06)	0.00 (0.00–0.04)	0.00 (0.00–0.06)	0.53 (0.31–1.13)[1,2,3,4]	0.74 (0.32–1.42)[1,2,3,5]
Day 35 (n = 12)		0.00 (0.00–0.04)	0.01 (0.00–0.26)	0.00 (0.00–0.13)	0.89 (0.68–1.65)[1,2,3]	1.22 (0.56–1.58)[1,2,3]

Data is presented as median (Q_1–Q_3).

[1]$P < 0.05$ compared to storage in DEHP-FK.

[2]$P < 0.05$ compared to storage in DEHP-GCMS.

[3]$P < 0.05$ compared to storage in DEHP-TC.

[4]$P < 0.05$ compared to storage in DINCH-TC.

[5]$P < 0.05$ compared to storage in DINCH-GCMS.

4. DISCUSSION

Evidence of DEHP toxicity has been mostly found in animal models, and direct toxicity in humans is still unclear. DEHP has proved to be carcinogenic due to its induction of liver tumors in rats and mice, but the mechanism is considered to be specific for those animals and not relevant for humans [7]. Male neonates and male preterm neonates of several animals (rats, mice, hamsters, ferrets, and marmosets) showed age-dependent testicular effects and were susceptible to testicular dysgenesis syndrome (hypospadias, cryptorchism and decreased anogenital distance) if exposed to high levels of DEHP, particularly during gestation [7]. DEHP's acute toxicity studies indicate low toxicity, with LD50 (lethal dose for 50% of tested animals) values of >25 g/kg for oral administration in rats and mice, and about 250 mg/kg for intravenous administration in rats [7]. DEHP is converted into several metabolites, including its hydrolytic cleavage to mono(2-ethylhexyl) phthalate (MEHP) mainly in the liver [7, 47]. DEHP and its metabolites are well excreted through urine, and there is no evidence of their accumulation in the human body. However, DEHP toxicity affects the liver, kidneys, and testes in laboratory animals, with similar exposure levels observed in neonates undergoing intensive clinical procedures including blood transfusion [7]. In a safety assessment of DEHP released from PVC medical devices, the US FDA concluded that patients under the following indications may be at risk of exceeding the tolerable limit of DEHP: adults and infants undergoing extracorporeal membrane oxygenation, infants undergoing exchange transfusions, all patients receiving enteral nutrition, infants receiving total parenteral

nutrition, infants receiving medical therapy in neonatal intensive care units where exposures to DEHP may come from multiple sources simultaneously, adults undergoing cardiopulmonary bypass, and nursing infants of mothers on hemodialysis [10].

DINCH, along with BTHC and DEHT, is being investigated as a potential non-toxic substitute for DEHP in blood bags used for RBC storage [9, 14-19]. Although data from human studies are limited, DINCH has shown low or no relationship to toxicity, genotoxicity, carcinogenicity, or toxicity to reproduction in animal models [41, 48-50]. DINCH has been approved by the European Union for food packaging and has been widely used in toys and childcare products [50, 51]. Several companies have introduced commercially available DINCH-PVC blood bags for RBC storage [2, 19, 50]. DINCH-PVC showed similar viscosity and mechanical properties to DEHP-PVC and high resistance to degradation to steam sterilization, allowing the potential to be a promising alternative to DEHP for medical device industrialization [52, 53].

Gas and liquid chromatography separation combined with mass spectrometry detection are one of the most major methods proposed for measuring DEHP and its alternative plasticizers [9, 17, 19, 41, 47, 54]. DEHP and DINCH have similar molecular weights and some structural resemblance but differ in that DEHP has a benzene ring while DINCH has a cyclohexane ring. DINCH is produced by hydrogenating the benzene ring present in phthalates, and its aliphatic cyclohexane ring gives a chair structure, compared to the flat structure of the planar aromatic benzene ring in DEHP [17, 18, 52]. This chemical difference enables the separation

of their chromatogram peaks, leading to different retention times of 6.2 min for DEHP and 6.9 min for DINCH in our assay using UHPLC-MS/MS. Although the within-run CVs for measuring both plasticizers were below 20%, the precision for measuring DINCH was better than DEHP, showing CVs below 10% at all three concentrations. Our assay showed excellent linearity for measuring both DEHP and DINCH.

While baseline DEHP levels were detected in the donors' blood samples at low concentrations, indicating its ubiquitous presence as an environmental contaminant, DINCH was not detected. DINCH concentrations also remained at nearly undetectable levels in the DEHP bags throughout the storage period. The levels of DINCH in the DINCH bags on day 35 in this study were lower, but within a comparable range to the results observed in previous studies which reported a mean level of 4.5–7.5 mg/L on day 42 of RBC storage [17, 19]. The DEHP concentrations in the DINCH bags remained consistent with the donors' baseline levels throughout the storage period. The median DEHP levels of the three different DEHP bags on day 35 in this study increased to a range of 37.1–58.9 mg/L, which is comparable to the results observed in previous studies [9, 17, 19, 47]. DEHP levels measured in RBC concentrates on day 20 ranged upto 36.5 mg/L [47], and the mean levels of DEHP measured on day 42 were reported to be 27.6–41.6 mg/L [9, 17, 19]. DEHP-FK and DEHP-TC bags showed higher levels of DEHP leaching than the DEHP-GCMS bag. Variations in DEHP and DINCH levels among studies can be attributed to differences in analytical methods and chemical composition of the blood bags.

Since DEHP is widely used in many plastic items and can easily leach from the

product, there is a potential risk of DEHP contamination during the assay process utilizing UHPLC-MS/MS. Although we put effort into avoiding unnecessary contact with plastic materials that may contain DEHP as possible by utilizing glass syringes, test tubes, and volumetric pipettes during the sample preparation process, it was technically and financially difficult to replace the various tubing in the UHPLC-MS/MS system to make a DEHP-free condition. To help this problem, we used the Synergi trap column to delay the flow between the mobile phase mixer and the autosampler. This measure was taken to minimize the potential contamination of the sample with DEHP leached from the tubing, which could be present in the mobile phase. We were able to identify a relatively small peak at a retention time of approximately 6.8 min in chromatograms from both blank and 50 mg/L DEHP solutions which is presumed to represent DEHP contamination in the mobile phase. The Synergi trap column was successful in separating the DEHP contamination and preventing an over-estimation of DEHP levels in blood samples. A liquid chromatography-MS based assay utilizing a column-switching technique was also shown to be capable of preventing this problem [47].

When the primary objective is to assess DEHP exposure in human samples, this problem can also be partially addressed by measuring the levels of DEHP metabolites. MEHP can be measured in various biological samples, such as blood, urine, and breast milk, and is used as a biomarker of exposure to DEHP. Along with the hydrolytic metabolite MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) and mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP) are oxidative metabolites commonly found in human samples after exposure to DEHP. The levels

of 5OH-MEHP and 5oxo-MEHP are higher in urine compared to serum, and their urine levels are 10-fold higher than that of MEHP [55]. Serum enzymes cannot generate 5OH-MEHP and 5oxo-MEHP through hydrolysis of DEHP introduced during blood collection and storage. However, because DEHP and MEHP have low water solubility compared to 5OH-MEHP and 5oxo-MEHP, they are the primary residual compounds in blood products [56]. MEHP can be detected in *in vitro* samples from RBC storage bags as a degradation product of DEHP [9, 47].

As DEHP can easily migrate from the tubing and bag into the blood, we used DEHP-free prototype collection systems and pooling bags. These bags were made using DINCH as the plasticizer to eliminate DEHP contamination during blood collection and production of RBC concentrates. Since DEHP is well known for its protective effect on RBC stability, we believed that using collection systems and pooling bags made with DINCH to prevent DEHP cross-contamination would be more important than allowing RBCs to be exposed to DEHP, as noted as a limitation in some previous studies [9, 15, 16, 18]. Nonetheless, there is a possibility of DINCH cross-contamination into the DEHP study arms. However, because RBC concentrates were produced within four hours of blood collection and DINCH leaches at a much slower rate into the blood than DEHP [17], we assumed there would be a very low level of DINCH contamination in the DEHP study arms. This was confirmed by the DINCH measurements in the donors, pooling bag, and RBC concentrate units. DINCH was not detected in most of the donor samples and remained at a nearly undetectable level throughout the storage period in DEHP bags. Therefore, the levels of DINCH would have a negligible impact on the hemolysis

results of the DEHP bags.

Our study has some limitations. First, the linearity needs to be evaluated in a wider range of concentration. During the initial pilot studies, we encountered some challenges in optimizing the assay's experimental settings. We later noticed that the DEHP concentrations detected during the later periods of storage exceeded the range of linearity that we had evaluated. Although we considered diluting the samples before the assay, we refrained from doing so due to concerns about potential mobile phase contamination affecting the results when applying the dilution factor. Second, we were not able to evaluate the limit of detection (LOD) and lower limit of quantification (LLOQ) of the assay. LOD is the lowest concentration of an analyte in a sample that can be consistently detected with a certain stated probability and is usually set as the minimum concentration with a signal-to-noise ratio (S/N ratio) of 10 or higher. LLOQ is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy of less than 20%. Because the main focus of DEHP and DINCH measurement in blood bags for this study was to identify their trend in increase during the storage period, the process of thoroughly evaluating LOD and LLOQ was not a top priority among the research activities. However, proper assessment of LOD and LLOQ is recommended to ensure that the results are valid and reliable when measuring substances at very low concentrations. Further efforts to improve and validate the performance of this assay is needed in future research.

In conclusion, we successfully developed a UHPLC-MS/MS assay to measure DEHP and DINCH concentrations in blood samples and analyzed their levels in

donors and RBC concentrate units during storage for 35 days. Although DINCH was not detected, a small amount of DEHP was found in the blood of the donors due to its ubiquitous nature. Throughout the storage period, the levels of DEHP and DINCH only increased in the blood bags in which they were used to produce. Compared to DEHP, DINCH presented a much slower rate of leaching into blood during storage.

REFERENCES

1. Almizraq RJ and Acker JP. Closing in on DEHP-free red blood cell concentrate containers. Transfusion 2018;58:1089-92.
2. Prowse CV, de Korte D, Hess JR, van der Meer PF, Biomedical Excellence for Safer Transfusion C. Commercially available blood storage containers. Vox Sang 2014;106:1-13.
3. AuBuchon JP, Estep TN, Davey RJ. The effect of the plasticizer di-2-ethylhexyl phthalate on the survival of stored RBCs. Blood 1988;71:448-52.
4. Horowitz B, Stryker MH, Waldman AA, Woods KR, Gass JD, Drago J. Stabilization of red blood cells by the plasticizer, diethylhexylphthalate. Vox Sang 1985;48:150-5.
5. Estep TN, Pedersen RA, Miller TJ, Stupar KR. Characterization of erythrocyte quality during the refrigerated storage of whole blood containing di-(2-ethylhexyl) phthalate. Blood 1984;64:1270-6.
6. Shaz BH, Grima K, Hillyer CD. 2-(Diethylhexyl)phthalate in blood bags: is this a public health issue? Transfusion 2011;51:2510-7.
7. Sampson J and de Korte D. DEHP-plasticised PVC: relevance to blood services. Transfus Med 2011;21:73-83.
8. van der Meer PF, Reesink HW, Panzer S, Wong J, Ismay S, Keller A, et al. Should DEHP be eliminated in blood bags? Vox Sang 2014;106:176-95.
9. Graminske S, Puca K, Schmidt A, Brooks S, Boerner A, Heldke S, et al. In vitro evaluation of di(2-ethylhexyl)terephthalate-plasticized polyvinyl chloride blood

bags for red blood cell storage in AS-1 and PAGGSM additive solutions. Transfusion 2018;58:1100-7.

10. Center for Devices and Radiological Health. Safety Assessment of Di(2-ethylhexyl)phthalate (DEHP) Released from PVC Medical Devices. US Food and Drug Administration 2002.
11. Razatos A, Acker JP, de Korte D, Begue S, Noorman F, Doyle B, et al. Survey of blood centre readiness regarding removal of DEHP from blood bag sets: The BEST Collaborative Study. Vox Sang 2022.
12. Council of the European Union. Annex to the Commission Regulation (EU) amending Annex XIV to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Available from: https://data.consilium.europa.eu/doc/document/ST-6893-2021-ADD-1/en/pdf
13. National Institute of Food and Drug Safety Evaluation. Study on the aggregated risk assessment of phthalates to human health. Korean Ministry of Food and Drug Safety. 2019.
14. Larsson L, Sandgren P, Ohlsson S, Derving J, Friis-Christensen T, Daggert F, et al. Non-phthalate plasticizer DEHT preserves adequate blood component quality during storage in PVC blood bags. Vox Sang 2021;116:60-70.
15. Serrano K, Levin E, Chen D, Hansen A, Turner TR, Kurach J, et al. An investigation of red blood cell concentrate quality during storage in paediatric-sized polyvinylchloride bags plasticized with alternatives to di-2-ethylhexyl phthalate (DEHP). Vox Sang 2016;110:227-35.

16. Bicalho B, Serrano K, Dos Santos Pereira A, Devine DV, Acker JP. Blood Bag Plasticizers Influence Red Blood Cell Vesiculation Rate without Altering the Lipid Composition of the Vesicles. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie 2016;43:19-26.
17. Lagerberg JW, Gouwerok E, Vlaar R, Go M, de Korte D. In vitro evaluation of the quality of blood products collected and stored in systems completely free of di(2-ethylhexyl)phthalate-plasticized materials. Transfusion 2015;55:522-31.
18. Dumont LJ, Baker S, Dumont DF, Herschel L, Waters S, Calcagni K, et al. Exploratory in vitro study of red blood cell storage containers formulated with an alternative plasticizer. Transfusion 2012;52:1439-45.
19. Vermeulen C, den Besten G, van den Bos AG, Go M, Gouwerok E, Vlaar R, et al. Clinical and in vitro evaluation of red blood cells collected and stored in a non-DEHP plasticized bag system. Vox Sang 2022;117:1163-70.
20. Fairbanks VF, Ziesmer SC, O'Brien PC. Methods for measuring plasma hemoglobin in micromolar concentration compared. Clin Chem 1992;38:132-40.
21. Han V, Serrano K, Devine DV. A comparative study of common techniques used to measure haemolysis in stored red cell concentrates. Vox Sang 2010;98:116-23.
22. Antonelou MH, Tzounakas VL, Velentzas AD, Stamoulis KE, Kriebardis AG, Papassideri IS. Effects of pre-storage leukoreduction on stored red blood cells signaling: a time-course evaluation from shape to proteome. J Proteomics

2012;76 Spec No.:220-38.

23. Pertinhez TA, Casali E, Baroni F, Berni P, Baricchi R, Spisni A. A Comparative Study of the Effect of Leukoreduction and Pre-storage Leukodepletion on Red Blood Cells during Storage. Front Mol Biosci 2016;3:13.
24. Lagerberg JW, Korsten H, Van Der Meer PF, De Korte D. Prevention of red cell storage lesion: a comparison of five different additive solutions. Blood Transfus 2017;15:456-62.
25. Sowemimo-Coker SO. Red blood cell hemolysis during processing. Transfusion medicine reviews 2002;16:46-60.
26. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA 2005;293:1653-62.
27. Gladwin MT, Kanias T, Kim-Shapiro DB. Hemolysis and cell-free hemoglobin drive an intrinsic mechanism for human disease. J Clin Invest 2012;122:1205-8.
28. Dumont LJ and AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. Transfusion 2008;48:1053-60.
29. European Directorate for the Quality of Medicines & HealthCare. Guide to the preparation, use and quality assurance of blood components. 20th ed. Council of Europe. 2020.
30. Acker JP, Hansen AL, Kurach JD, Turner TR, Croteau I, Jenkins C. A quality monitoring program for red blood cell components: in vitro quality indicators

before and after implementation of semiautomated processing. Transfusion 2014;54:2534-43.

31. Bolton-Maggs PH, Langer JC, Iolascon A, Tittensor P, King MJ, General Haematology Task Force of the British Committee for Standards in H. Guidelines for the diagnosis and management of hereditary spherocytosis--2011 update. Br J Haematol 2012;156:37-49.
32. King MJ, Smythe JS, Mushens R. Eosin-5-maleimide binding to band 3 and Rh-related proteins forms the basis of a screening test for hereditary spherocytosis. Br J Haematol 2004;124:106-13.
33. Park SH, Park CJ, Lee BR, Cho YU, Jang S, Kim N, et al. Comparison study of the eosin-5'-maleimide binding test, flow cytometric osmotic fragility test, and cryohemolysis test in the diagnosis of hereditary spherocytosis. Am J Clin Pathol 2014;142:474-84.
34. MacDonald R. Red cell 2,3-diphosphoglycerate and oxygen affinity. Anaesthesia 1977;32:544-53.
35. Tilton WM, Seaman C, Carriero D, Piomelli S. Regulation of glycolysis in the erythrocyte: role of the lactate/pyruvate and NAD/NADH ratios. J Lab Clin Med 1991;118:146-52.
36. Karger R, Lukow C, Kretschmer V. Deformability of Red Blood Cells and Correlation with ATP Content during Storage as Leukocyte-Depleted Whole Blood. Transfus Med Hemother 2012;39:277-82.
37. Rock G, Sherring VA, Tittley P. Five-day storage of platelet concentrates. Transfusion 1984;24:147-52.

38. Delgado JA, Morell-Garcia D, Bauca JM. Hemolysis Interference Studies: The Particular Case of Sodium Ion. EJIFCC 2019;30:25-34.
39. Lippi G, Salvagno GL, Montagnana M, Brocco G, Guidi GC. Influence of hemolysis on routine clinical chemistry testing. Clin Chem Lab Med 2006;44:311-6.
40. Bereketoglu C and Pradhan A. Plasticizers: negative impacts on the thyroid hormone system. Environ Sci Pollut Res Int 2022;29:38912-27.
41. Bernard L, Decaudin B, Lecoeur M, Richard D, Bourdeaux D, Cueff R, et al. Analytical methods for the determination of DEHP plasticizer alternatives present in medical devices: a review. Talanta 2014;129:39-54.
42. Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, et al. NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-n-octyl phthalate. Reprod Toxicol 2002;16:721-34.
43. Bauer MJ and Herrmann R. Estimation of the environmental contamination by phthalic acid esters leaching from household wastes. Sci Total Environ 1997;208:49-57.
44. Scientific Committee on Emerging and Newly Identified Health Risks. Opinion on The Safety of Medical Devices Containin g DEHP plasticized PVC or Other Plasticizers on Neonates and Other Groups Possibly at Risk. 2015. (http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_047.pdf) (accessed Apr 7, 2023).
45. European Union, Regulation (EC) No. 1272/2008 of the European Parliament

and of the Council on Classification, Labelling and Packaging of Substances and Mixtures, Amending and Repealing Directives 67/548/EEC and 1999/45/EC, and Amending Regulation (EC) No. 1907/2006, 2008.

46. IARC Monographs on the Identification of Carcinogenic Hazards to Humans (https://monographs.iarc.who.int/list-of-classifications) (accessed Apr 7, 2023).
47. Inoue K, Kawaguchi M, Yamanaka R, Higuchi T, Ito R, Saito K, et al. Evaluation and analysis of exposure levels of di(2-ethylhexyl) phthalate from blood bags. Clin Chim Acta 2005;358:159-66.
48. Wadey BL. An innovative plasticizer for sensitive applications. Journal of Vinyl and Additive Technology 2003;9:172-6.
49. Koch HM, Schutze A, Palmke C, Angerer J, Bruning T. Metabolism of the plasticizer and phthalate substitute diisononyl-cyclohexane-1,2-dicarboxylate (DINCH((R))) in humans after single oral doses. Arch Toxicol 2013;87:799-806.
50. Klei TRL, Begue S, Lotens A, Sigurjonsson OE, Wiltshire MD, George C, et al. Recommendations for in vitro evaluation of blood components collected, prepared and stored in non-DEHP medical devices. Vox Sang 2023;118:165-77.
51. Biedermann-Brem S, Biedermann M, Pfenninger S, Bauer M, Altkofer W, Rieger K, et al. Plasticizers in PVC Toys and Childcare Products: What Succeeds the Phthalates? Market Survey 2007. Chromatographia 2008;68:227-34.
52. Crespo JE, Balart R, Sanchez L, López J. Substitution of di(2-ethylhexyl) phthalate by di(isononyl) cyclohexane-1,2-dicarboxylate as a plasticizer for industrial vinyl plastisol formulations. Journal of Applied Polymer Science

2007;104:1215-20.

53. Burgos N and Jiménez A. Degradation of poly(vinyl chloride) plasticized with non-phthalate plasticizers under sterilization conditions. Polymer Degradation and Stability 2009;94:1473-8.

54. D'Alessandro A, Nemkov T, Hansen KC. Rapid detection of DEHP in packed red blood cells stored under European and US standard conditions. Blood Transfus 2016;14:140-4.

55. Kato K, Silva MJ, Reidy JA, Hurtz D, 3rd, Malek NA, Needham LL, et al. Mono(2-ethyl-5-hydroxyhexyl) phthalate and mono-(2-ethyl-5-oxohexyl) phthalate as biomarkers for human exposure assessment to di-(2-ethylhexyl) phthalate. Environ Health Perspect 2004;112:327-30.

56. Koch HM, Bolt HM, Angerer J. Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP. Arch Toxicol 2004;78:123-30.

ACKNOWLEDGEMENTS

This study was supported by Green Cross Medical Science (Yongin-si, Gyeonggi-do, South Korea) and Taechang Industry (Gongju-si, Chungcheongnam-do, South Korea).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

국문 초록

배경: Di(2-ethylhexyl) phthalate (DEHP)는 polyvinyl chloride (PVC)를 유연하고 신축성이 있는 물질로 변환하기 위해 혈액백의 제조 시 흔하게 사용되는 가소제이다. DEHP는 적혈구의 안정성 향상에 기여하고 용혈률을 감소시켜 보존 기간을 늘려주는 성질을 가지고 있다. 그러나 생식기계 독성 및 내분비계 교란과 관련된 DEHP의 위험성에 대해 많은 우려가 수년간 제기되어 왔다. DEHP는 PVC에 공유결합되어 있지 않기 때문에 PVC 제품의 내용물이나 그 주변 환경으로 용출되어 나올 수 있다. 이러한 특성으로 인해 사람과 동물은 다양한 경로로 DEHP에 노출될 수 있다. DEHP 독성에 대한 증거는 대부분 동물 모델에서 발견되었으며, 인간에서의 직접적인 독성은 아직 명확하게 밝혀지지 않았다. 그러나 혈액제제 수혈과 같은 특정 치료를 받는 환자들은 고농도의 DEHP에 노출될 우려가 있다. 적혈구 보존을 위한 혈액백에서 DEHP 대체재에 대해 몇몇 연구가 시행된 바 있으나, 다양한 혈액제제 생산 조건에서의 DEHP 대체재에 대해 충분한 연구 결과가 축적되어 있지 않다. Di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH)는 우수한 독성학적 성질을 지닌 대체 가소제로, DINCH-PVC는 DEHP-PVC와 유사한 기계적 및 물리학적 특성을 가지고 있다. 본 연구에서는, citrate phosphate dextrose adenine (CPDA-1) 항응고제 하에서 DEHP의 대체제로 DINCH를 사용한 혈액백의 적혈

구 보존 성능을 평가하고, DEHP와 DINCH 농도를 측정할 수 있는 고성능액체크로마토그래피-탠덤질량분석기 검사법을 개발하여 혈액백에서의 가소제 농도를 측정하는 것을 목표로 하였다.

방법: 'Pool-and-split' 방법의 연구 설계를 이용하여, CPDA-1 항응고제와 혼합된 균질한 성상의 혈액을 2개의 DINCH 혈액백과 3개의 DEHP 혈액백에 나누어 농축적혈구를 생산할 수 있었다. 이렇게 짝지어진 5개의 농축적혈구를 총 20그룹 생산하였으며, 35일동안 보관하면서 매주 *in vitro* 실험실 검사를 시행하여 적혈구 보존 품질과 가소제 농도를 평가하였다. DEHP와 DINCH 측정용 검체는 액체-액체 추출 후 N_2 내에서 30분간 건조시키고, 70% 메탄올과 0.1% 포름산으로 재부유시켰다. 이후 Synergi trap 및 BEH column이 장착된 고성능액체크로마토그래피 기기에 검체를 주입하고, m/z 391 → 149 (DEHP) 및 m/z 425 → 281 (DINCH)와 같은 다중반응모니터링 조건 하에서 삼중 사중극자 질량분석기로 검사하였다.

결과: 35일째 DINCH 혈액백에서의 용혈률 중앙값(DINCH-GCMS, 0.297%; DINCH-TC, 0.342%)은 DEHP 혈액백(DEHP-FK, 0.204%; DEHP-GCMS, 0.240%; DEHP-TC, 0.222%)보다 증가되어 있었으나($P < 0.05$), 모든 개별 혈액제제에서의 용혈률은 0.8% 미만이었다. 혈색소, 적혈구용적률, 세포 수(적혈구, 백혈구, 혈소판)는 DINCH 혈액백에 비해 DEHP-FK 혈액백에서 증가되어 있었다. DINCH 혈액백에 보관된 적혈구는 DEHP 혈액백에 비

해 평균적혈구용적은 증가되어 있었으며, 평균적혈구혈색소농도와 eosin-5'-maleimide 결합성은 감소되어 있었다. DINCH 혈액백은 DEHP 혈액백보다 산소분압이 높고 이산화탄소분압이 낮아 우수한 기체투과도를 보여주었다. 적혈구대사와 관련된 다른 수치는 두 종류의 혈액백 사이에 비슷한 성질을 보여주었다. 검사차례내 정밀도는 DEHP와 DINCH 측정에 대해 각각 11.11–14.01% 및 2.08–6.15%로 확인되었다. DEHP와 DINCH 측정 시 각각 1–20 mg/L 및 0.0625–1.0 mg/L 범위에서 유의한 직선성을 보여주었다(DEHP, $R^2 = 0.9994$; DINCH, $R^2 = 0.9993$). Synergi trap column은 이동상에서의 DEHP 오염을 성공적으로 분리해낼 수 있었다. 농축적혈구 보관 35일째 DEHP와 DINCH 혈액백에서의 가소제 농도 중앙값은 각각 37.1–58.9 mg/L 및 0.89–1.22 mg/L 범위로 증가하였다.

결론: CPDA-1 항응고제 하에서 35일동안 보관된 DINCH 혈액백에서의 적혈구 품질은 DEHP 혈액백과 비교하여 뒤쳐지지 않는 수준을 보여주었다. 고성능액체크로마토그래피-탠덤질량분석기를 이용한 DEHP 및 DINCH 측정 검사법을 성공적으로 개발할 수 있었으며, 농축적혈구 보관 중 DEHP보다 DINCH가 느린 속도로 혈액 내로 유출됨을 확인할 수 있었다. DINCH는 혈액백 제조 시 차세대 적혈구 보존제를 사용하지 않는 조건 하에서도 DEHP를 대체할 수 있는 유망한 가소제라 평가할 수 있다.

주요어 : **DEHP, DINCH, 가소제, 프탈레이트, 혈액제제수혈, 적혈구**

학 번 : **2010-30515**