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Genapol X-080を含む溶血性試験用中程度陽性対照材料の開発及び性能評価

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Development and performance evaluation of a moderate positive reference material containing Genapol X-080 for hemolysis testing

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In the previously study, we developed plasticized polyvinyl chloride (PVC) pellets named "Y-2" or "Y-4" that show weak or strong hemolytic activity depending on the content of Genapol X-080, which is used in biological safety evaluations of medical devices. However, the hemolytic activity of Y-2 was weak and inferior in reproducibility tests. This study deals with the development and performance evaluation of a moderate positive reference material (Y-3) for hemolysis testing. Y-3 was prepared as PVC pellets consisting of 33.4% di(2-ethylhexyl)-phthalate (DEHP), 4.86% epoxidized soybean oil (ESBO), 3.04×10^{-2}% calcium and zinc stearates, and 0.91% Genapol X-080 as the hemolytic substance. Y-3 showed a hemolysis ratio around 40–60% with human and rabbit blood samples in American Society for Testing and Materials (ASTM) direct contact assays under the test sample/extraction vehicle ratio specified by ISO 10993-12 (0.2 g/mL). Y-3 also exhibited hemolytic activity in extract-based assays, but the hemolysis ratio varied greatly depending on the test conditions; this likely occurred because the solubility of Genapol X-080 decreases markedly at temperatures higher than the cloud point (74–76℃). As a result of optimizing the test sample/extraction vehicle ratio for each extraction condition used in the extract-based assays, a hemolysis ratio around 20–80% was yielded with human and rabbit blood when Y-3 extracts with phosphate-buffered saline were prepared under the following conditions: 0.08 g/mL at 37 or 50℃ for 72 h, 0.24 g/mL at 70℃ for 24 h, and 0.8 g/mL at 121℃ for 1 h. Y-3 may be useful as a positive reference material for hemolysis testing and may show advantages over PVC sheets spiked with 0.61% (Y-2: weak positive reference material) or 5.8% (Y-4: strong positive reference material) of Genapol X-080 that induce weak or complete hemolysis, respectively, as previously reported. Hatano Research Institute is planning to start distributing Y-3 as a first positive reference material for hemolysis test on a worldwide scale beginning in the spring of 2019.

Keyword: hemolysis test, positive reference material, biological safety evaluation, guidance

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1. Introduction

Biological safety evaluations of raw materials or medical devices must be conducted by using risk analysis techniques specified in ISO 14971[1], as described in detail previously[2]. The intended use or purpose and the safety properties of a medical device must be clarified, and additionally, known or foreseeable hazards must be identified and the risk of each hazard must be anticipated. Items to be evaluated for the biological safety of each medical device are selected according to the requirements specified in ISO 10993-1[3,4]. As a general rule, hemocompatibility testing is required for evaluations of the safety of a medical device or its material that comes into contact with blood[5–8]. There are both mechanical and biochemical factors that can affect hemolysis. For devices that have little mechanical impact on blood cells, established static or semi-static *in vitro* testing can be used. This type of testing may be also useful for devices with mechanical impacts on blood to evaluate the impacts of the materials on hemolysis. Biological testing methods have to be validated to confirm that test results meet the requirements for evaluating the safety of items in clinical use[3,4]. Negative and positive reference materials or positive control substances are widely used in biological safety evaluation tests such as cytotoxicity, sensitization, genotoxicity, and implantation tests to detect toxicity in test samples and to certify the validity of the test system.

Hemolysis is a phenomenon involving the rupturing of erythrocytes and the release of their cytoplasm into the surrounding fluid as a result of cell membrane damage originating from physical, chemical, or biological factors. However, no reference materials have been used for hemolysis testing. For a positive reference material to evaluate hemolysis based on chemical factors, we developed plasticized polyvinyl chloride (PVC) pellets named "Y-2" or "Y-4" that show weak or strong hemolytic activity depending on the content of Genapol X-080, which is non-ionic detergent selected as a candidate of hemolytic substance in survey of 23 kinds of chemical compounds[2]. Y-4, which contains large amounts of Genapol X-080, induced complete hemolysis regardless of the test conditions. However, the hemolytic activity of Y-2 was weak and inferior in reproducibility tests, and the hemolysis ratio sometimes was found to be less than 5%, which is the positive threshold, depending on the test conditions. These results revealed that the Genapol X-080 content must be optimized for preparing a truly useful positive reference material for hemolysis testing. Further, previous hemolysis testing was performed using rabbit blood.

In the present study, we improved the chemical composition of PVC pellets spiked with Genapol X-080 to prepare a moderate positive reference material (Y-3) for hemolysis testing, and the performance of Y-3 as a reference material was estimated by ASTM direct contact and/or extract-based assays with human and rabbit blood. In addition, the sample/extraction vehicle ratio specified by ISO 10993-12 was optimized for the extract-based assays.

2. Materials and methods

2.1 Materials, chemicals, and utensils

Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} were purchased from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan). Potassium cyanide, potassium ferricyanide, and potassium dihydrogen phosphate were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Triton X-100 and human hemoglobin were purchased from Nakarai Tesque, Inc. (Kyoto, Japan), and Sigma-Aldrich Co. (Tokyo, Japan), respectively. All utensils made of glass, metal, or Teflon® used to prepare the samples for hemolysis testing were heated at 250℃ for more than 16 h prior to use.

Cyanmethemoglobin reagent was prepared by dissolving 0.05 g of potassium cyanide, 0.2 g of potassium ferricyanide, and 0.14 g of potassium dihydrogen phosphate in 1,000 mL of the water for used injections, and then, 0.5 mL of Triton X-100 was added. The stock solution of the hemoglobin standard was prepared by dissolving 20 mg of human hemoglobin in 20 mL of cyanmethemoglobin reagent. After 30 min, the absorbance (Abs.) at a wavelength of 540 nm was measured, and the hemoglobin concentration was standardized according to the following formula[9].

Hemoglobin concentration (mg/mL) =
Abs. of stock solution × 0.1481 × dilution ratio

2.2 Animal and human blood

For the preliminary simple hemolysis tests using Y-3 sheets, the rabbit defibrinated blood (1 mL), which

was purchased from Kohjin Bio Co., Ltd. (Saitama, Japan), was placed into a screw-capped test tube and centrifuged at 700 × g for 5 min at 4℃. The supernatant was removed, and then, PBS (10 mL) was added to the sedimented red blood cells (RBCs) and the material was gently mixed. After centrifugation again at 700 × g for 5 min at 4℃, the supernatant was removed, and this step was repeated 2 times to wash the RBCs. The RBC sediment was finally suspended in PBS of equal volume to the rabbit defibrinated blood sampled and stored at 4℃ until use.

For hemolysis testing as described by the American Society of Testing and Materials (ASTM)[6], fresh-drawn rabbit blood was anticoagulated by adding sodium citrate at a final concentration of 0.38% and stored at 4℃ until use. Japanese White rabbits were used in this study, and the drawn blood was directly used for hemolysis testing without washing the blood cells. The procedure was performed in accordance with the ethical guidelines on animal experiments of the Hatano Research Institute, Food and Drug Safety Center (approval numbers 1150220A and 1150221A).

Human whole blood (9 mL) donated by a laboratory volunteer in Hatano Research Institute was treated in the same manner as the rabbit blood for the official methods provided by ASTM. Experiments using human blood were approved by the Ethics Committees of Hatano Research Institute, Food and Drug Safety Center (approval number H2014-04A), and the procedure was performed in accordance with the ethical standards of the Committees on Human Experimentation of Hatano Research Institute. Written informed consent was obtained from enrolled subjects.

2.3 Preparation of Y-3 sheets and pellets

The PVC powder (100 g) was added gradually to a mixture of di(2-ethylhexyl)-phthalate (DEHP) (55 g), ESBO (8 g), calcium and zinc stearates (0.05 g each), and Genapol X-080 (1.5 g; final concentration = 0.91%) while stirring with a spatula. The mixed powder was gently heated from room temperature to 100℃ in an oven and then stirred well. The powder was stirred a second time after heating at 100℃ for 5 min to completely plasticize the PVC. The plasticized powder was heat-pressed at 180℃ to prepare the PVC sheets (thickness = 0.4 mm). Each sheet was cut into small pieces (1 cm × 3 cm).

For preparing Y-3 pellets, a total of 25 kg of the mixture with the same composition was gently compounded at 140℃ with a kneading machine, and the pellets were prepared by extrusion molding as columnar or cube shapes having a height of 2–5 mm. Specific gravity and hardness, which were measured according to Japanese standards JIS K 7112 and JIS K 6253-3, were 1.22 ± 0.03 and 74.0 ± 3.0, respectively. The tensile strength, elongation percentage, and 100% modulus measured by tensile testing according to JIS K 6723 were >19.0 MPa, >360%, and 8.2 ± 2.0 MPa, respectively.

2.4 Hemolysis testing

These tests were performed by using the following two types of methodologies: a simple method[2] used to preliminarily evaluate the hemolytic activity of Y-3, and official methods provided by ASTM[6].

Simple hemolysis test was performed according to the method previously reported[2]. Briefly, Y-3 sheets were cut into small pieces (1 cm × 3 cm) and these pieces were placed into a screw-capped glass bottle. In simple hemolysis testing (n = 1), 1 mL of PBS and 20 μL of washed and suspended RBCs prepared according to the procedure described above were added to the bottle containing the test pieces for the direct contact hemolysis testing. In the extraction method, 1 mL of PBS was added to the bottle and test solutions were prepared under the following four conditions for extraction: 121℃ for 1 h, 70℃ for 24 h, 50℃ for 72 h, and 37℃ for 72 h. Subsequently, the washed RBC suspension (10 μL) was added to 0.5 mL of each test solution placed into another glass tube. After incubation at 37℃ for 1, 2, and 4 h, the hemolytic ratio of each sample was measured according to the same method described below.

According to a property of a medical device, official hemolysis tests provided by ASTM[6]: direct contact and/or extract-based assays, should be conducted to determine a hemolytic property of the device. Both tests were performed for Y-3 pellets. Calculated amounts of Y-3 pellets were placed into a screw-capped glass bottle. For direct contact hemolysis testing, PBS (7 mL) and 1 mL of rabbit or human blood anti-coagulated with sodium citrate were used to adjust the hemoglobin concentration to 10.0 ±1.0 mg/mL, and these solutions were added to the bottle

containing sample pieces so that the sample/extraction vehicle ratio was 0.2 g/mL as specified by ISO 10993-12[2]. For the extract method, PBS was added to the bottle so that the sample/extraction vehicle ratio was in the range of 0.02 to 1.0 g/mL. Test solutions were prepared under the following four conditions for wd 37℃ for 72 h. Citrated rabbit or human blood (1 mL) was added to 7 mL of the test solution placed into another glass bottle. Each sample was incubated at 37℃ for 3 h under static conditions followed by centrifugation at 750 × g for 15 min. The supernatant (1 mL) was mixed with cyanmethemoglobin reagent (1 mL), and the absorbance was measured at 540 nm after incubation at room temperature for 15 min. The hemoglobin concentration in each sample was calculated according to a standard curve.

PBS and distilled water were used as a negative and positive control, respectively, and the hemolysis ratio was calculated in accordance with the methods provided in the ASTM guideline. These tests were repeated in triplicate.

The hemolytic ratio was calculated in accordance with the following formula.

Blank corrected % hemolysis = (AS − AB) / (AT − AB) × 100

where

AS: Absorbance of the sample

AB: Absorbance of the blank (mean)

AT: Absorbance of the total blood hemoglobin concentration (mean)

3. Results

3.1 Hemolytic behavior of Y-3 sheets

To evaluate the validity of the Genapol X-080 content, Y-3 sheets were preliminarily prepared and the hemolytic activity was measured with simple hemolysis testing. As shown in Fig 1, the hemolysis ratio of Y-3 increased in proportion to the increase in the incubation time in the direct contact assays, and the ratios at 1, 2, and 4 h were 21.1, 69.7, and 93.7%, respectively. In the extract-based assays, Y-3 extracts prepared at 121℃ for 1 h and 70℃ for 24 h showed no hemolytic activity, and the extracts prepared at 37℃ for 72 h and 50℃ for 72 h exhibited complete hemolysis, regardless of the incubation time (Fig 1).

3.2 Hemolytic behavior of Y-3 pellets

Hemolytic activity of Y-3 pellets was estimated with direct contact assays by using human and rabbit blood with a sample/extraction vehicle ratio of 0.2 g/mL. As shown in Table 1 and Fig 2, the hemolysis ratio of Y-3

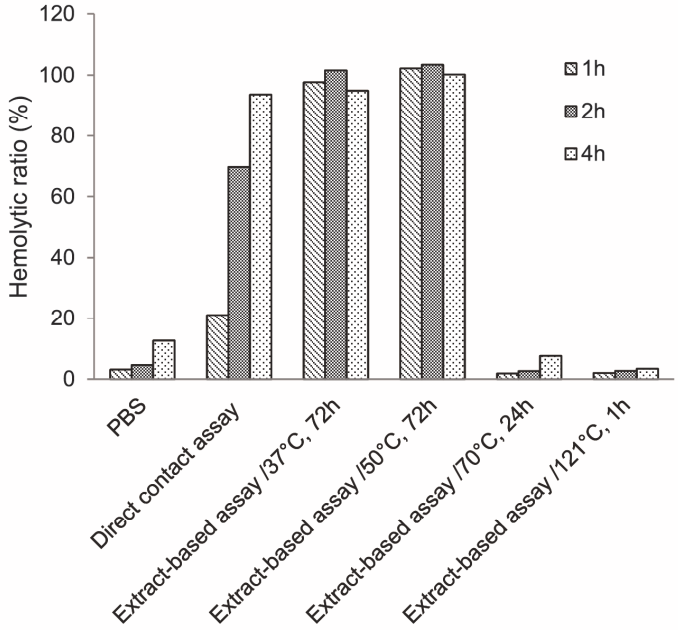

Fig 1. Hemolytic behavior of Y-3 sheets spiked with Genapol X-080

Hemolytic activity was estimated by direct contact and extract-based (37℃ for 72 h, 50℃ for 72 h, 70℃ for 24 h, and 121℃ for 1 h) assays using the simple method. PBS alone was used as the negative control.

was 42.9 ± 2.1% for human blood and 56.1 ± 9.3% for rabbit blood.

Hemolytic activity of Y-3 pellets was estimated with extract-based assays by using test samples prepared by combinations of various sample/extraction vehicle ratios and four extraction conditions. As shown in Table 1 and Fig 3a–d, the hemolysis ratio increased in proportion to the increase in the sample/extraction vehicle ratio, regardless of the extraction conditions. In the case of the extraction at 37℃ or 50℃ for 72 h, Y-3 showed no hemolytic activity toward human blood when the test sample was prepared with a sample/extraction vehicle ratio of 0.02 g/mL. Weak and moderate hemolytic activity amounting to 7.0 ±1.6% and 20.0 ± 3.3% was detected with the sample/extraction vehicle ratios of 0.06 and 0.08 g/mL, respectively. Strong or complete hemolysis was observed with a sample/extraction vehicle ratio of more than 0.1 g/mL. In the case of the extraction at 70℃ for 24 h or 121℃ for 1 h, moderate hemolysis amounting to 34.7 ± 3.6% and 86.1 ± 8.1% was detected with the sample/extraction vehicle ratios of 0.24 and 0.8 g/mL, respectively.

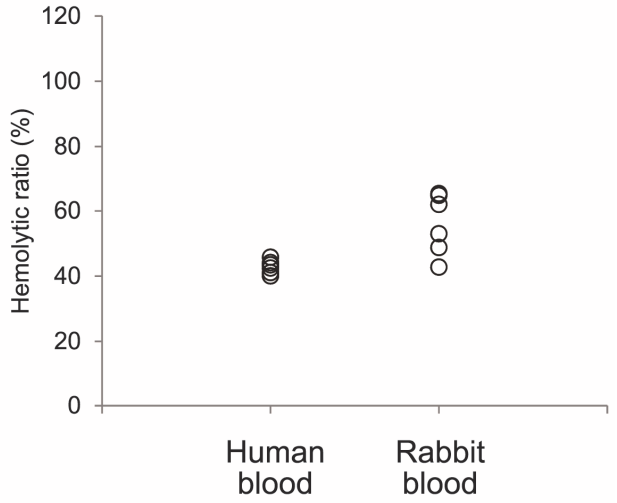

Fig 2. Hemolytic behavior of Y-3 pellets determined by ASTM direct contact assays using human and rabbit blood

Hemolytic activity was estimated with a sample/extraction vehicle ratio of 0.2 g/mL.

As shown in Table 1 and Fig 4a–d, the appropriate sample/extraction vehicle ratio to yield moderate hemolysis with rabbit blood was 0.08 g/mL at 37℃ or 50℃ for 72 h, 0.24 at 70℃ for 24 h, and 0.8 g/mL at 121℃ for 1 h.

4. Discussion

In this investigation, we attempted to develop a positive reference material exhibiting appropriate hemolytic activity for use in the hemolysis testing

Table 1. Summary for hemolytic behavior of Y-3 pellet

Sample/extraction vehicle ratio (g/mL)	Hemolytic ratio (%)									
	Extract-based assay								Direct contact assay	
	Human				Rabbit					
	Extraction condition				Extraction condition					
	37℃ 72 h	50℃ 72 h	70℃ 24 h	121℃ 1 h	37℃ 72 h	50℃ 72 h	70℃ 24 h	121℃ 1 h	Human	Rabbit
0.02	0.13 ± 0.1[1)]	0.37 ± 0.3								
0.06	7.03 ± 1.6	7.07 ± 2.4								
0.08	51.7 ± 40	20.0 ± 3.3			69.6 ± 26	41.7 ± 6.6				
0.10	91.7 ± 7.4	86.6 ± 7.9								
0.15	100 ± 0.8	95.9 ± 9.5								
0.20	97.4 ± 0.6	98.1 ± 0.9	4.17 ± 2.6	0.00 ± 0.0	100 ± 0.8	99.9 ± 1.3	4.60 ± 2.3	0.00 ± 0.0	42.9 ± 2.1	56.1 ± 9.3
0.24			34.7 ± 3.6				71.2 ± 3.1			
0.28			69.4 ± 8.7				85.6 ± 2.7			
0.30			95.8 ± 2.3							
0.40			98.7 ± 2.5							
0.50			101 ± 1.2							
0.75			99.8 ± 0.9	20.0 ± 2.8						
0.80				86.1 ± 8.1				78.6 ± 18		
1.00				87.2 ± 2.6						

[1)] Data are reported as mean ± SD

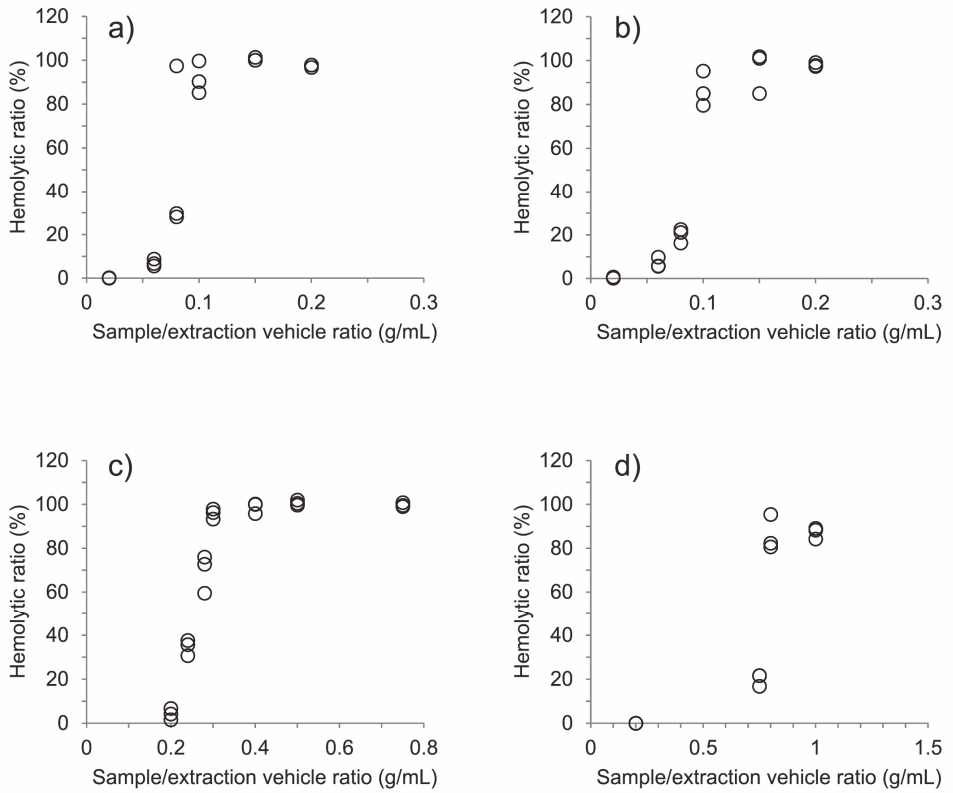

Fig 3 . Hemolytic activity of Y-3 pellets determined by ASTM extract-based assays using human blood

Hemolytic activity was estimated by using various sample/extraction vehicle ratios under 4 and the following conditions for extraction: a) 37℃ for 72 h, b) 50℃ for 72 h, c) 70℃ for 24 h, and d) 121℃ for 1 h.

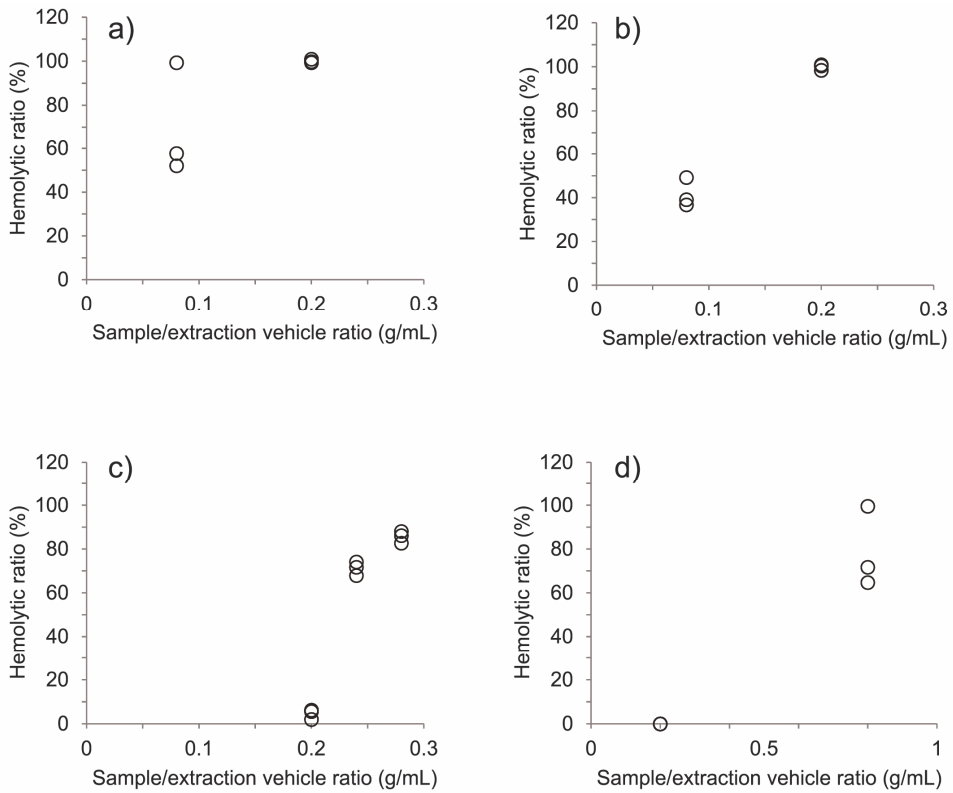

Fig 4 . Hemolytic activity of Y-3 pellets determined by ASTM extract-based assays using rabbit blood

Hemolytic activity was estimated by using various sample/extraction vehicle ratios under 4 and the following conditions for extraction: a) 37℃ for 72 h, b) 50℃ for 72 h, c) 70℃ for 24 h, and d) 121℃ for 1 h.

that is performed during safety evaluations of the hemocompatibility of medical devices.

In the preliminary simple hemolysis tests, Y-3 sheets exhibited ideal hemolytic activity as a moderate positive reference material in the direct contact assays, and this activity was found to be different from that of the previously developed Y-2 and Y-4 products, which are weak and strong positive reference materials, respectively[2]. The optimized property was also confirmed in Y-3 pellets made for industrial use. The hemolysis performance shown by the positive reference material was greatly improved by slightly increasing the Genapol X-080 content from 0.61% (Y-2) to 0.91% (Y-3). Genapol X-080 is a polyethyleneglycol (PEG) monoalkyl ether that is used as a general-purpose non-ionic detergent. Genapol X-080 induces hemolysis of RBCs by destroying the cell membrane through the detergent effect at concentrations higher than the critical micelle concentration (CMC) that is approximately 25 μg/mL. The effect changes dramatically with the CMC as the boundary. In the previous direct contact method tests, the amount of Genapol X-080 eluted into the test solution of suspended RBCs and Y-2 sheets increased over time and reached 25.6 μg/mL after a 4 h incubation period[2]. This concentration was almost equal to the CMC, but the hemolytic activity was weak because the detergent ability was weakened by the presence of blood proteins. The Genapol X-080 concentration eluted into the test solution with Y-3 was not measured in this study, but it can be easily speculated that Y-3 exhibited ideal performance behavior because the detergent effect was appropriately enhanced by improving the Genapol X-080 content. Thus, it was revealed that an appropriate concentration for Genapol X-080 spiking to PVC for preparing a moderate positive reference material for hemolysis testing might be 0.91%.

However, Y-3 sheets exhibited different hemolytic activities depending on the extraction conditions used for the extract-based assays with the simple method in which moderate hemolysis was not observed regardless of any of the conditions used for extraction. These differences may have originated from the physicochemical properties of Genapol X-080; notably, the solubility of the compound decreases markedly at temperatures higher than the cloud point (74–76℃)[2]. Therefore, we attempted to optimize the sample/vehicle ratio of Y-3 pellets and extraction solvent (PBS) for ASTM extract-based assays. According to the results, appropriate hemolytic activity was observed after adjusting the ratio for each extraction condition, and the optimized ratios to yield moderate activity were 0.08 g/mL for extractions at 37 or 50℃ for 72 h, 0.24 g/mL for extractions at 70℃ for 24 h, and 0.8 g/mL for extractions at 121℃ for 1 h.

Y-2 and Y-3 was provided to ISO/TC 194/WG 9 as one of several positive reference materials for international Round Robin (RR) testing to verify the performance of hemolysis testing and harmonize the methodology. In the RR tests, ASTM direct contact and extract-based assays, National Institute of Health Sciences (NIH) direct contact assays, and extract-based assays for hemolysis testing used by the Japanese Ministry of Health, Labor, and Welfare (MHLW) were selected as the official methods. The moderate hemolytic activity of Y-3 was verified in each method (data not shown). However, the MHLW method that uses defibrinated blood could not appropriately detect the activity of latex globes that served as a strong positive reference material for use in the RR tests, and these were different results from those derived with the ASTM and NIH methods using citrated blood; hence, the official Japanese method will be changed from the original procedure to the ASTM method. This was the reason why the ASTM method was used to optimize the sample/vehicle ratio in this study. The ratio for the NIH method was not confirmed in this study, but the optimized ratio for the ASTM method also may be applicable to the NIH method, as far as the results of the RR testing are concerned. The optimized sample/vehicle ratio to yield an appropriate hemolysis ratio in ASTM extract-based assays may be also informative for investigators working in medical device manufacturing facilities and pre-clinical contract research organizations with the goal of ensuring the safety and effectiveness of testing of medical devices and associated materials.

Our data clearly indicate that Y-3 may be useful as a moderate positive reference material for certifying the validity of hemolysis testing. The Hatano Research Institute, Food and Drug Safety Center (Kanagawa, Japan) will soon start to distribute Y-3 pellets that were manufactured by the Showa Kasei Kogyo Co., Ltd. (Tokyo, Japan), on a worldwide scale. The

sample/vehicle ratio to prepare the test solution for ASTM extract-based assays also will be provided to the users of Y-3.

5. Acknowledgments

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