Research

Standard Operating Procedure for One - Spin Individualized Therapeutic Plasma Based on a Mathematical Model and Test Spin

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Abstract:
Standard operating procedure (SOP) for one-spin centrifugation of blood to prepare plasma is presented. The aim of the procedure is to prepare plasma with highest content of platelets and extracellular vesicles (EVs) from an individual blood sample. As sedimenting erythrocytes push plasma that carries platelets and EVs in the opposite direction, the optimal time is defined at the point when the lower bound of the ascending plasma meets the upper bound of the sedimenting erythrocytes. This process is described by a mathematical model which has one adjustable parameter. The parameter is estimated by a test spin at low centripetal acceleration of the centrifuge rotor and short centrifugation time. By using the mathematical model and the estimated parameter, the optimal time of centrifugation at chosen centripetal acceleration of the centrifuge rotor can be set. In the presented contribution we describe the protocole and state the elements needed for safe preparation of optimized and individualized plasma intended for regeneration.

Keywords: Therapeutic plasma, Platelet rich plasma, Platelet and extracellular vesicles rich plasma; Regeneration; Extracellular vesicles; Extracellular particles; Exosomes; Sedimentation of erythrocytes

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1. Definitions

EVs: extracellular vesicles
PVRP: plasma rich with platelets and extracellular vesicles
SOP: standard operating procedure
QMS: Quality Management System

2. Background

Blood plasma that is rich with platelets and extracellular vesicles (PVRP) is widely used in clinical practice in various fields of medicine for regeneration and healing (Troha et al., 2023). The gold standard method for preparation of PVRP is sedimentation of erythrocytes in blood samples taken into tubes with anticoagulant, due to a force upon the particles (e.g. graviation or centrifugal force; **Figure 1**). Sedimentation of erythrocytes in blood is subjected to direct interactions between blood constituents and causes an increase of the plasma platelet concentration above its baseline level in whole blood (Božič et al., 2022). The preparation is made from patient's own blood following a procedure that is straightforward and easy to perform and can be administred directly to the damaged area. With autologous application there is low risk of complications such as infections or immune rejection (De Pascale et al., 2015). Initially, healing capacity of plasma preparations was attributed to platelets; it was found that activated platelets release different growth and inflammation factors to the extracellular millieu, which have been regarded as vectors of the healing process (Etulain et al., 2018). Later on, other features were considered, i.e. platelet membrane receptors, their effects on the immunomodulatory actions of the innate and adaptive immune system, effects of leukocytes and of mesenchymal stem cells (reviewed in Everts al., 2020), indicating complex interactions

between cells in the healing process. Recent developments in the field of biology and medicine have outlined nano-sized (ranging from 20 nm-1000 nm) membrane-enclosed cellular fragments (extracellular vesicles – EVs) which are abundant in preparations from processed plasma (Šuštar et al., 2011a,b; Božič et al., 2020) as important mediators of intercellular communication

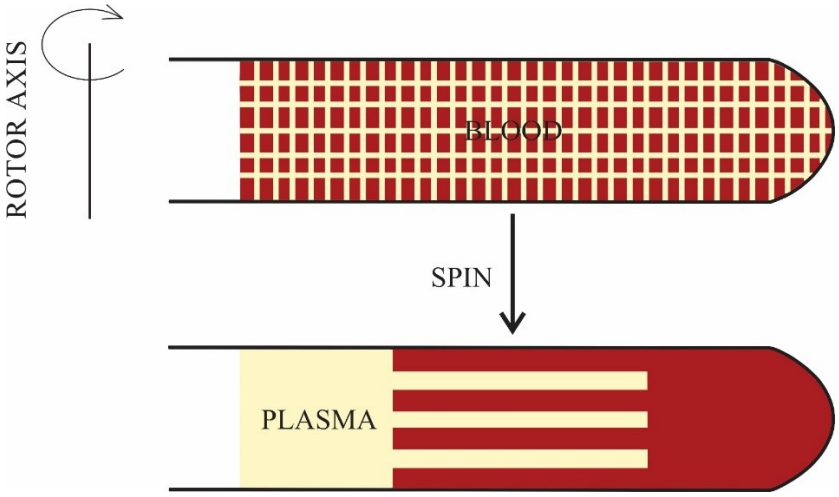

Figure 1. Preparation of PVRP by one-spin protocol. Blood with anticoagulant is centrifuged (Spin) to separate erythrocytes (pellet) from plasma (supernatant). The yellowish area denotes plasma and the red area denotes packed erythrocytes. Stripes indicate formation of channels during sedimentation of erythrocytes.

Plasma preparation was named »platelet- rich plasma« due to elevated platelet concentration. However, more recent results indicate that also EVs are abundant in plasma (Božič et al., 2022) so here we refer to the preparation as "platelet and EV – rich plasma" (PVRP). Both platelets and EVs seem to be important bioactive substances in plasma and the presented SOP is based on the desire to obtain plasma with maximal possible number density of platelets, EVs and concentration of growth factors. For the definition of the SOP, we chose the model identified in the Quality Management System (QMS) for EV studies developed within the H2020 Fet-Open project VES4US (Liguori and Kisslinger, 2021).

3. Purpose, Scope and Applicability

Despite pervasive use and extensive clinical and basic science study, many important questions regarding plasma preparation remain unanswered. Therapy with plasma - derived preparations appeared ineffective in about 20 to 40 percent of cases, depending on the injury and lack of standardization in preparation and dosage renders results difficult to interpret (Middleton et al., 2012). The presence and the role of EVs in PVRP are largely unknown. As the effects of the processing on the samples are multiple and complex, better understanding of the mechanisms taking place during plasma processing is urgently needed to define the key parameters in preparation protocols and thereby develop a better controlled and more effective therapeutic preparation.

The purpose of the SOP is to focus on the parameters of the sedimentation (the force that pulls the particles, the time of acting of the force, and the physical properties of the sample).

The scope of the SOP is to define the plasma preparation with highest yield of platelets, EVs and growth factors, starting from the point where the sample is transported to the laboratory in the vacutubes to the point when plasma leaves the centrifuge still staying in the vacutubes.

The applicability of the SOP is mainly targeted to plasma preparations for regeneration of tympanoplasty postoperative wounds, however, they can be used in many fields of medicine, for regeneration of different tissues, such as skin, fibrotic and neural tissues. It is being used in otorhinolaryngology (Steiner et al., 2022), where plasma preparations are especially effective in otology and skull-base surgery. Based on research conducted to date, it was shown that PVRP improves and accelerates healing of damaged tissues (Vozel et al., 2021a, Vozel et al., 2021b) and reduces likelihood of postoperative inflammation (Bielecki

et al., 2007). The therapeutic challenge considered is inflammation of the middle ear due to low regenerative capacity of the tissue. Surgical treatment of the inflammation of the middle ear aims at removing the destructive inflammatory process (ie ablative treatment) and restoring the physiological mechanism of sound wave transmission to the inner ear (ie reconstructive treatment - tympanoplasty). The success rate of tympanoplasty reported in the literature ranges between 75% and 98% (Bayram et al., 2020). Thus, post-operative healing presents a problem that has not yet been satisfactorily solved.

4. Health and safety warning

Working with blood and preparations from blood presents in general a risk for infection of the staff or anyone that comes in contact with the material. The hazards and safety measures regarding working with blood and its products are thoroughly described in Guidance on Working Safely with Human Blood and Plasma https://www.sgul.ac.uk/about/our-professional-services/safety-health-and-environment/documents/guidance-on-working-safely-with-human-blood-or-plasma.pdf. These risks and the relative preventive and corrective measures should be carefully acknowledged and taken into account by the staff. Vacutubes are used, so blood stays within the tubes during the processing considered in this SOP. This diminishes the risk of infection, however, the probability of mistakes or spills must be considered. Gloves, caps, clothes and footware protection should be used to protect the staff as well as the sample from infection. Dispose of used material should be in accordance with applicable laws and good research and laboratory practices.

Besides material, the hazards may derive from using the centrifuge. The hazards and safety measures regarding the use of the centrifuge are thoroughly described in Centrifuge Safety https://ehs.stanford.edu/reference/centrifuge-safety. They include mechanical failure of the centrifuge and dispersion of aerosols in case of spillout. **Mechanical failure** can cause ejection of pieces of metal, or the production of sparks and heat derived from friction. In preparation of PVRP, centrifugal pull is moderate (up to cca 1500 g, where g is the Earth gravity constant) and the rotor should be loaded in a balanced way to minimize vibrations causing sparks and local hot spots. **Dispersion of aerosol** from blood into the centrifuge chamber or into the room can occur in case of damaged or poorly closed tubes or if centrifuge is opened before they have settled.

5. Cautions

Dispose of used material and equipment should be in accordance with applicable laws and good research and laboratory practices. The centrifuge should be fixed to the working surface, regularly maintained and lubricated. For the correct use of the centrifuge, the rotor should be loaded in a balanced way, with centrifuge tubes adequately balanced, and centrifuge speed should be increased gradually. Possibility of aeration of the room should be provided. Chamber and rotor should be cleaned and desinfected before and after use. An electronic or paper register for user reservations should be provided. Written operating procedures of the centrifuge should be at hand available for the users.

6. Personnel Qualifications / Responsibilities

Blood sampling should be performed by a trained nurse. Staff who uses SOP should acknowledge the hazards connected to blood and its products and the hazards connected to operation of the centrifuge. The staff should undergo training on how to handle blood samples, use the centrifuge and apply SOP.

7. Materials, Equipment and Supplies

Blood samples taken into evacuated closed tubes with anticoagulant derive from hospital facilities. They should be gently (avoiding shaking or cooling) transported to the laboratory in a room temperature - saving container and processed immediately. The samples should be used as soon as possible.

Devices: Centrifuge Centric 400R with swinging rotor RS4/100 (Domel, Železniki, Slovenia).

Other equipment: A ruler to measure the dimensions of the centrifuge rotor and the sample tube and of the length of the plasma column after the Test spin.

8. Computer Hardware and Software

Use of the mathematical model (as described below) is key in SOP. Hardware: Office PC; software: Microsoft Excel; data saving and sharing: Cloud and/or Drive documents.

9. Mathematical Model of Plasma Formation

Mathematical model of the first generation was introduced in Božič et al. (2022) and of the second generation considering the effect of hematocrit on the sedimentation in Berry and Kralj-Iglič (2023). In this SOP we follow the general course of the previous models. For clarity we present again the basic equations (1) – (13) from (Berry and Kralj-Iglič, 2023) describing movement of erythrocytes. The model applies above the region of the forming sediment where the motion of erythtrocytes is not hindered by direct interactions between each other and with the tube walls. Upon centrifugal acceleration, erythrocytes move from a chosen slice (denoted by index *i* in **Figure 2**) into the one which is closer to the bottom of the tube (denoted by index *i*+1 in **Figure 2**). At the same time the slice receives the erythrocytes from the slice above – the one closer to the rotor axis (*i* -1, **Figure 2**).

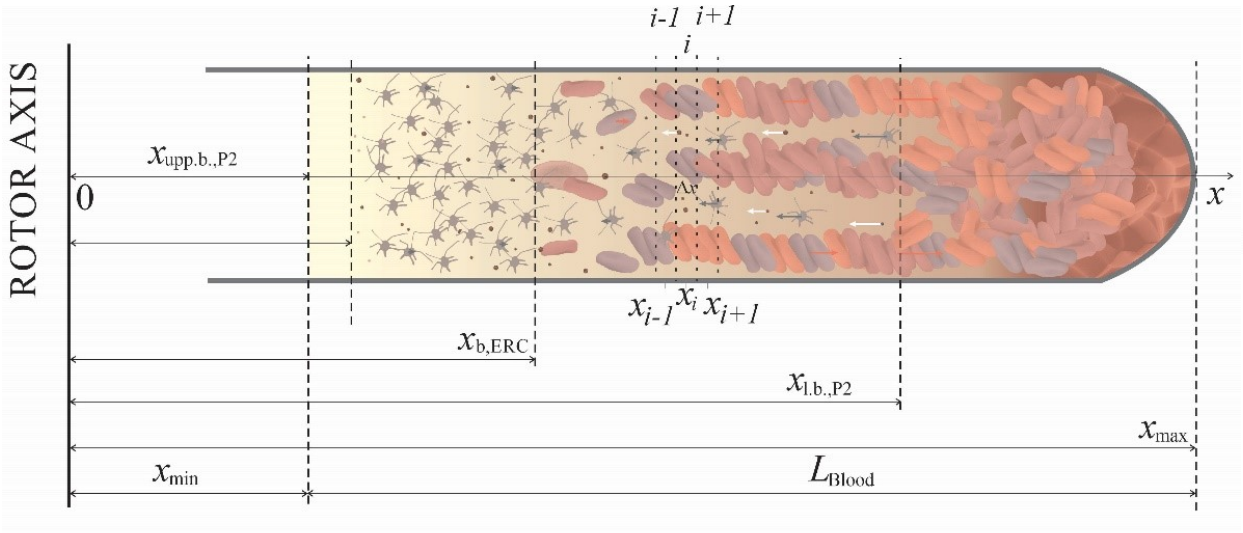

Figure 2. Modeling of the movement of blood cells during centrifugation. The sample is imagined as divided in thin slices with uniform properties.

The change of the number of erythrocytes in the i-th slice *N*i with time *t* is

$$dN_i/dt = c_{i-1}\, S\, u_{i-1}(t) - c_i\, S\, u_i(t)\,, \qquad (1)$$

where c_i is the number density (concentration) of particles, S is the cross section of the tube and u_i is the velocity of the particles in the i-th slice at the time t.

Following Božič et al. (2022) and Berry and Kralj-Iglič et al. (2023) it is assumed that the concentration and the velocity of erythrocytes change only slightly from one slice to another, so that the expansion can be used to approximate the changes,

$$c_i - c_{i-1} = dc_i/dx\, \Delta x \quad , \qquad (2)$$

$$u_i - u_{i-1} = du_i/dx\, \Delta x \quad . \qquad (3)$$

Neglecting the terms quadratic in Δx and performing the limit $\Delta x \rightarrow dx$ yields after rearrangement of Eqs. (1) – (3) a differential equation

$$dc(x,t)/dt = -\,(dc(x,t)/dx\, u(x,t) + du(x,t)/dx\, c(x,t)) \quad . \qquad (4)$$

It is assumed that the velocity of the platelets is proportional to the centripetal acceleration which is expressed by a multiplicity of the Earth gravity constant X

$$u(x) = \omega X x \quad . \tag{5}$$

where ω is a constant characteristic for the sample. It follows from Eqs. (4) and (5) that

$$dc/dt = - \omega X d(cx)/dx \quad . \tag{6}$$

By using dimensionless quantities: γ - concentration of erythrocytes divided by its initial value c_0,

$$\gamma(x,t) = c(x,t)/c_0 \quad , \tag{7}$$

ξ - distance divided by the maximal length from rotor axis to the bottom of the tube, x_{max}

$$\xi = x/x_{max} \quad , \tag{8}$$

and relative sedimentation time τ - time multiplied by ωX

$$\tau = \omega X t \quad , \tag{9}$$

the Eq. (6) is given in a dimensionless form

$$d\gamma_{p.}(\xi,\tau)/d\tau = - d(\gamma(\xi,\tau)\xi)/d\xi \quad . \tag{10}$$

An ansatz solution is

$$\gamma_{.}(\xi,\tau_p) = C \exp(-\tau) + D \exp(-\tau/2)\ \xi^{-1/2} \quad , \tag{11}$$

where C and D are constants. We chose D = 0 (Berry and Kralj-Iglič, 2023). The initial condition

$$\gamma_p(0) = 1 \tag{12}$$

implies that C = 1 and the relative number density of erythrocytes is therefore

$$\gamma(\xi.\tau) = \exp(-\tau) \quad . \tag{13}$$

The erythrocytes that were at the top of the sample are forming a boundary which is moving towards the bottom with the velocity $dx_{b,ERC}/dt$. According to Eq.(5)

$$dx_{b,ERC}/dt = u(x) = \omega X x \quad . \tag{14}$$

Using dimensionless expressions (8) and (9), Eq. (14) transforms into

$$d\xi_{b.ERC}/d\tau = \xi_{b.ERC} \tag{15}$$

with the solution

$$\xi_{b.ERC} = \xi_{min} \exp(\tau) \quad . \tag{16}$$

Here $\xi_{min} = x_{min}/x_{max}$ is the dimensionless distance of the surface level of the sample from the rotor axis.
The measurable quantity is the length of the plasma column formed after a certain sedimentation time $L_{plasma} = x_{max}\ l_{plasma}$, where

$$l_{plasma} = \xi_{b.ERC} - \xi_{min} \quad . \tag{17}$$

Using Eqs. (16) and (17) yields the dependence of the dimensionless length of the plasma column on dimensionless time

$$l_{plasma} = \xi_{min}\,(\exp(\tau) - 1) \quad . \tag{18}$$

Inserting the measurable quantities by using Eqs.(8) and (9) yields the dependence of the length of the plasma on time

$$L_{plasma} = x_{min}\,(\exp(\omega X t) - 1) \quad . \tag{19}$$

Measurement of L_{plasma}, x_{min} and t following a Test spin is used to determine the unknown parameter ω which is characteristic for the sample. By some rearrangement of Eq. (19) we get

$$\omega = \ln(1 + L_{plasma}/x_{min})/X\,t \tag{20}$$

where we insert the measured values $L_{plasma} = L_{plasma,TEST}$, X_{TEST} and $t = t_{TEST}$.
Within the model, it is assumed that the settling erythrocytes push plasma that carries platelets and EVs in the opposite direction (Božič et al., 2022). The optimal centrifuge setting is defined by the condition that the lower bound of the plasma $\xi_{l.b.\,PLASMA}$ given by

$$\xi_{l.b.\,PLASMA} = \xi_{max}\exp(-\tau) \tag{21}$$

meets the upper bound of the erythrocytes $\xi_{b.ERC}$ (Eq.(16)),

$$\xi_{max}\exp(-\tau) = \xi_{min}\exp(\tau) \quad . \tag{22}$$

After rearrangement of Eq.(22) we obtain

$$\tau_{OPT} = \ln(x_{max}/x_{min})^{1/2} \tag{23}$$

which yields with insertion of the measurable quantities (Eqs.(8) and (9)) and the parameter ω (Eq. (20)) the expression

$$X_{OPT}\,t_{OPT} = X_{TEST}\,t_{TEST}\ln(x_{max}/x_{min})^{1/2}/\ln(1 + L_{plasma,TEST}/x_{min}) \quad . \tag{24}$$

10. Step by Step Procedure

10.1. *Measurement of x_{max} and x_{min}*

Assuming horisontal position during the spin, the distance from the rotor axis to the bottom of the tube x_{max} and the distance from the rotor axis to the level of the blood x_{min} (**Figure 2**) are measured by the ruler.

10.2. Test Spin and Measurement of L_{TEST}

The purpose of this step is to estimate the physical properties of the blood comprised in the parameter ω in Eq. (5). One tube containing blood is placed into the centrifuge rotor and centrifuged mildly (e.g. at lowest possible centripetal acceleration for 5 minutes). In this time a small amount of plasma is expected to form (cca 1 cm). The length of the test plasma column

$$L_{plasma,TEST} = x_{b,ERC,TEST} - x_{min} \tag{25}$$

is measured by the ruler (**Figure 3**).

10.3. Determination of Centrifuge Setting

The product $X_{OPT}\,t_{OPT}$ is calculated by using Eq.(24),

$$X_{OPT}\,t_{OPT} = X_{TEST}\,t_{TEST}\ln(x_{max}/x_{min})^{1/2}/\ln(1 + L_{plasma,TEST}/x_{min}) \quad . \tag{26}$$

The expression should be inserted in a conventent software, e.g. Microsoft Excel, to minimize possibility of mistakes and minimize the time blood is waiting. In the model, the centripetal acceleration of the centrifuge rotor and the time of centrifugation stay linked which means that it is arbitrary to chose one of the two parameters and adjust the other accordingly. It should however be taken into consideration that the centrifuge must accelerate to reach the required centripetal acceleration and decelerate to stop and that the centripetal acceleration affects the angle of the tubes with respect to the horizontal if the swinging rotor is used. These issues have not yet been elaborated by experiments.

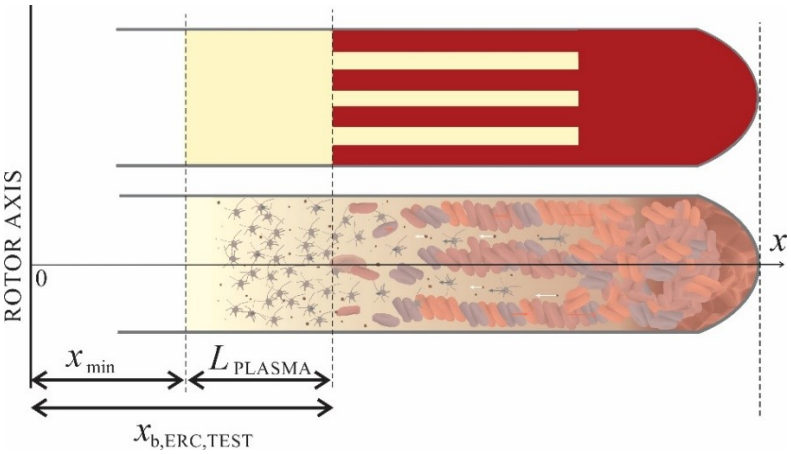

Figure 3. Measurement of the distance between the rotor axis and the bottom of the tube in the horizontal position of the tube with respect to the rotor axis x_{max}, the distance from the rotor axis to the level of the blood x_{min} and the length of the plasma column $L_{plasma,TEST} = x_{b,ERC,TEST} - x_{min}$ (Eq. (25)). The swinging buckett should be placed in the horizontal position to measure x_{min} and x_{max}.

10.4. Plasma preparation

Time and centripetal acceleration of the centrifuge are set by Eq. (24) and the blood in the tubes is centrifuged. If there are multiple tubes of blood acquired from the patient, the setting should be done as given below. The test tube should be centrifuged together with other tubes and blood in the test tube should not be re-suspended. This will somewhat deplete the plasma of the test tube with platelets. If the test tube is the only tube acquired then the time of the test spin t_{TEST} should be subtracted from t_{OPT}.

$t = t_{OPT}$ (for multiple tubes) or $t = t_{OPT} - t_{TEST}$ (for a single tube) (27)

10.5. Data acquisition

We report on processing of 6 samples from 2 donors according to the above SOP (**Table 1**).

Table 1. Comparison between optimal length of the plasma column L_{opt} predicted by the mathematical model and length of the plasma column measured in the experiment L_{exp}.

Donor	X	x_{min} (mm)	x_{max} (mm)	$\omega \times 10^{-5}$ (min^{-1})	L_{test} (mm)	t_{opt} (min)	L_{opt} (mm)	L_{exp} (mm)
1	300	95	142	4.08	6	17	21	15
1	300	96.5	142	4.35	6.5	15	20	14
1	300	100	145	4.82	7.5	13	20	13
2	300	95	142	9.77	15	7	21	15
2	300	96.5	142	9.03	14	7	20	18
2	300	102	145	8.57	14	7	20	15

Also shown are centrifuge centripetal acceleration setting (multiplicity of g) X, distance from the centrifuge rotor axis and the level of the blood sample x_{min} , distance from the centrifuge rotor axis and the bottom of the tube x_{max}, adjustable model parameter ω, length of the plasma column after the test spin L_{test} and optimal time of the spin estimated by the mathematical model t_{opt}. From Berry and Kralj-Iglič (2023).

Blood was donated by two of the authors (a female aged 64 years and a male aged 22 years, with no record of disease). Collection was established in the morning after fasting for a minimum of 12 h overnight. A G21 needle (Microlance, Becton Dickinson, Franklin Lakes. NJ, USA) and 2.7 mL evacuated tubes with trisodium citrate (BD Vacutainers, 367714A, Becton Dickinson, Franklin Lakes, NJ, USA) were used. Blood was processed fresh within 1 hour of sampling. While waiting to be centrifuged, the samples were gently mixed on a carousel at room temperature. The test spin was performed at 300 g, 5 minutes. Blood was centrifuged in the tubes in which it was sampled at 18 °C and 300g in the Centric 400R centrifuge (Domel, Železniki, Slovenia) with rotor RS4/100. The results are shown in Table 1. It can be seen that the model somewhat underestimated the length of the optimal plasma column. In donor 1 the estimated proportion of the column length was 69% while in donor 2 it was 79%. In both cases, X_{OPT} t_{OPT} was not overestimated which is positive. Namely too long or too strong sedimentation depletes plasma of platelets which is not desired. On the other hand, eventhough the volume of plasma does not reach the optimal, the plasma remains rich in platelets and EVs.

10.6. Troubleshooting

- In the test spin, the plasma does not form. The centripetal acceleration and/or the time of centrifugation should be increased and Test spin performed again.
- In the test spin, all erythrocytes reach the level of the hematocrit. If there are multiple tubes available, the Test spin should be performed with a new tube and chosing shorter time of centrifugation. If only one tube is available, plasma should be used as formed in the Test spin. Do not resuspend the sample and spin again.

11. Data and records management

All the experimental details are recorded within the lab journal carefully. All raw as well as treated data is stored in electronic form with physical backup for a minimum of 10 years after data generation.

12. Waste management

There is no waste in this SOP as regards the samples. Protective gloves, caps, clothes and footware shields used by the staff should be disposed in biological sanitary wastes, according with applicable laws and good research laboratory practices. Damaged samples should be disposed in yellow plastic containers for hazardous materials.

13. Related protocols or SOPS

There are many reported protocols for preparation of PVRP (reviewed for example by Dhurat and Sukesh, (2014), Chahla et al., (2017), Miranda et al., (2019)). However, the distinction of our protocole is in the modeling of an individual sample.

14. Quality control and quality assurance section

14.1 Instrument calibration

Not applicable.

14.2 Critical processes parameters and checkpoints

The test time and centripetal acceleration of the centrifuge are critical parameters. In particular, their product should not be too high. As the physical properties of the sample are not known at the time of the decision, especially in patients with higher erythrocyte sedimentation rate, setting the Xt_{OPT} too high may deplete plasma of platelets already at the test spin and no additional spin can be performed.

The temperature during the processing is also important. As the processing times are rather short, the rotor should be thermally pre-equilibrated to the desired temperature.

Quality control of plasma preparations should be continuously performed by collecting data on the volume and content of plasma in correlation with the measurement of hematocrit (proportion of the volume of the erythrocytes in blood) and blood cells count available for the individual patients.

Conclusions

Mathematical models, guidelines and SOPs are extremely useful tools for optimization and standardization of the procedures, enhancing reproducibility of the results, minimizing risks and failures, and therefore increasing experimental efficacy and efficiency (Bongiovanni et al., 2015; Digilio et al., 2016; Mancinelli et al., 2019; 2021, Mascia et al., 2021; Holmann et al., 2022). Guidelines and SOPs should be periodically monitored and eventually revised, to be constantly updated and ameliorated, according to scientific progress and research requirements (Digilio et al., 2016). The single tools are even more efficent if they are inserted in the context of a total QMS, as long as it is customised for research purposes and needs without curtailing the freedom of researchers (Liguori and Kisslinger 2020; 2022).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, blood was donated voluntarily by the authors of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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