ISSN 0030-400X, Optics and Spectroscopy, 2016, Vol. 120, No. 1, pp. 58–64. © Pleiades Publishing, Ltd., 2016.
Original Russian Text © V.A. Doubrovski, M.F. Medvedeva, S.O. Torbin, 2016, published in Optika i Spektroskopiya, 2016, Vol. 120, No. 1, pp. 68–75.

OPTICS AND SPECTROSCOPY IN BIOPHYSICS AND MEDICINE

An Acousto-Optical Method for Registration of Erythrocytes' Agglutination Reaction—Sera Color Influence on the Resolving Power

V. A. Doubrovski, M. F. Medvedeva, and S. O. Torbin

Razumovsky Saratov State Medical University, Saratov, 410012 Russia

e-mail: doubrovski43@yandex.ru

Received February 20, 2015

Abstract—The absorption spectra of agglutinating sera were used to determine blood groups. It was shown experimentally that the sera color significantly affects the resolving power of the acousto-optical method of blood typing. In order to increase the resolving power of the method and produce an invariance of the method for sera color, we suggested introducing a probing light beam individually for different sera. The proposed technique not only improves the resolving power of the method, but also reduces the risk of false interpretation of the experimental results and, hence, error in determining the blood group of the sample. The latter is especially important for the typing of blood samples with weak agglutination of erythrocytes. This study can be used in the development of an instrument for instrumental human blood group typing based on the acousto-optical method.

DOI: 10.1134/S0030400X16010070

INTRODUCTION

The automation of human blood typing in the AB0 system is an important task due to the prevalence of this type of laboratory test. There are many theoretical and experimental studies aimed at the improvement of existing and the development of new principles of designing such devices, for example [1–9]. In clinical practice, human blood typing is done using standard hemagglutinating sera of all blood groups ($0_{\alpha\beta}$(I), A_{β}(II), B_{α}(III), and if necessary AB_0(IV)) or using monoclonal antibodies. The studied erythrocytes are mixed with a standard serum (standard monoclonal antibodies) and reactions where the agglutination of erythrocytes occurred are detected. One of the main problems for us to solve is increasing resolving power of the developed method for the determination of blood types. The resolving power is the ratio of the measured signal P_+, corresponding to a positive agglutination test (erythrocyte agglutinates are formed); to the level of P_- signal for negative agglutination (formation of agglutinates is absent). Obviously, the increase of the resolving power $R = P_+/P_-$ increases the reliability of the determination of blood group.

In order to improve the resolving power of optical methods of registration of agglutination of erythrocytes [10], it has been suggested to use the effect of a standing ultrasonic wave on the mixture of "blood–serum–saline." Upon irradiation of this solution by ultrasound, a standing ultrasonic wave is formed in the cuvette, leading to a grouping of erythrocytes and their immune complexes in the area of nodes. As a result, the blood cell suspension disintegrates with a spatial period equal to the half of the ultrasonic wavelength ($\lambda/2$). The approximation of erythrocytes in the nodal areas increases the probability of their interaction, and hence agglutination occurs for a positive reaction. This increases both the reaction rate of agglutination and the sizes of immune erythrocyte complexes and, as a result, the rate of sedimentation (precipitation) of agglutinates upon stopping the ultrasonic generator (the end of the effect of levitation of erythrocytes and agglutinates). The optically investigated medium becomes more transparent.

With negative agglutination, erythrocytes are also grouped into nodal areas, forming "nonspecific" aggregates that are scattered into separate erythrocytes when the ultrasound is turned off. Naturally, the sedimentation rate of "free" erythrocytes is much lower than the sedimentation rate of agglutinates and the medium remained cloudy for a long time. The difference in the value of the optical transmission coefficient of the studied samples for the positive and negative reactions respectively carry the information whether agglutination occurred or not, and that is used for the determination of the blood group of the sample. This method is called the "acousto-optical method" of the registration of erythrocytes' agglutination and, hence, determination of blood group. It is based on the combined action of ultrasound on

a blood–serum reaction mixture with the optical sounding of the mixture. The detailed mechanism of the interaction of blood–serum mixture with ultrasound is described in [11, 12]. Registration of ultrasonically enlarged agglutinates by flow cytometry was performed in [13, 14], and turbidimetric registration using the using sedimentation phenomena was done in [15–19].

It is important to note that the haemagglutinating sera of the AB0 system produced by blood centers have different special colors. This technology is used in order to avoid mistakes using various sera during blood typing, and hence, in the determination of the blood group. Naturally, a different color of sera introduces an error in the photometric registration of sedimentation of erythrocytes and/or their agglutinates. This error is different for different sera, being most strongly manifested for registration of the positive agglutination reaction. It is obvious that resolving power R should depend on the type of the blood sample, the immunological matching of blood–serum pairs, agglutinating activity of erythrocytes etc., but it should not depend on the nature of serum color. The objective of this study is investigation of the effect of color of agglutinating sera on the resolving power of the acousto-optical method and, therefore, the accuracy of determining the group of a blood sample.

MATERIALS AND METHODS

The objects of the study were donor blood of $0_{\alpha\beta}$(I), A_{β}(II), and B_{α}(III) groups according to the AB0 system and agglutinated serum of all four blood groups, as well as their combination. Samples of donor blood were centrifuged (3000 rev/min, 5 min), and then the packed erythrocytes were used to determine the blood group of the sample. Donor erythrocytes preliminarily diluted with saline were mixed with $0_{\alpha\beta}$(I), A_{β}(II), B_{α}(III), and AB_0(IV) sera. The complex of preliminary experiments [18] allowed determining the optimal conditions for observation of agglutination of erythrocytes by the acousto-optical method. Thus, the optimal ratio of standard serum/investigated erythrocytes is 17 : 1, providing that packed erythrocytes were diluted with saline in a ratio of 1 : 78. In this case, the volume fraction of packed erythrocytes in the solution is 1.05% and the volume fraction of the standard serum is 17.8%. A negative agglutination reaction was observed for blood group $0_{\alpha\beta}$(I) in combination with all types of sera. Positive agglutination reaction was observed for blood groups A_{β}(II) and B_{α}(III) in combination with the sera of groups $0_{\alpha\beta}$(I), A_{β}(II), and B_{α}(III).

Registration of the sedimentation of erythrocytes and their agglutinates was performed using an optical setup (Fig. 1). The emission spectrum of the LED LXHL-G1S with $\lambda = 540 \pm 20$ nm corresponded to the absorption spectrum of hemoglobin in the green part of the spectrum. A rectangular cuvette with a volume of 2800 μL and 5-mm gap was used for the determination of the human blood group by turbidimetric method. The light beam passed through the tested suspension to a Logitech Quick Cam polychrome web-camera, used as the detector. The camera was connecting to the computer. All settings of the web-cameras were fixed and did not change during the experiment while photographing the bio objects.

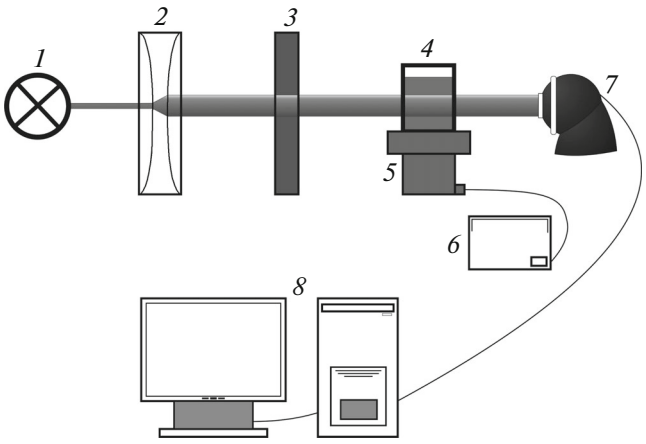

Fig. 1. The scheme of the device for recording agglutination of erythrocytes: (*1*) LED, (*2*) condenser, (*3*) neutral color filter, (*4*) cuvette, (*5*) ultrasonic vibrator, (*6*) ultrasonic generator, (*7*) digital camera, and (*8*) PC.

Immediately after the preparation, each sample was exposed to ultrasonic standing waves. The cuvette with the test mixture was placed on a piezoelectric transducer, and an ultrasonic standing wave was focused in the vertical direction. Preliminary experiments [18] allowed selecting the optimal duration of sonication on the standard serum–investigated erythrocytes–saline mixture equal to 60 s. The investigated suspension was photographed by a web camera 90 s after the ultrasound was then switched off (the incubation time of the sample). At this time, the sedimentation of erythrocytes and/or agglutinates occurred. Later, the supernatant was taken for spectral analysis.

During the computer processing of photo images of sedimentation of red erythrocytes and/or their agglutinates, decomposition of images into RGB components was done, and only the G component was subjected to statistical analysis. In the image, the square area was chosen for averaging of the brightness of pixels. The resulting average brightness values were recalculated into the luminous power of the light impinging on the camera. We used an experimentally obtained calibration curve: the curve of the correspondence of luminous power P to average brightness of the pixels B to the average camera aperture CCD (questions regarding calibration of digital microscopy devices were considered, for example, in study [19]). The value of resolving power R was calculated by the fol-

lowing method. For negative agglutination reactions, the average value of luminous power P_{av} was calculated based on the results of numerous measurements, while for all subsequent measurements value of resolving power R was calculated as $R = P/P_{av}$.

The registration of the absorption spectra of samples was carried out using a USB 4000-VIS-IR spectrometer (Ocean Optics, United States) using rectangular cuvette with a 1-mm gap. Absorption spectra of standard sera were obtained for sera from all four blood groups diluted with saline, the volume fraction of the serum being 17.8%. During the registration of the spectrum of the reference signal (suspension of erythrocytes of blood group $0_{\alpha\beta}$(I) in saline), the volume fraction of packed erythrocytes was 1.05%, which corresponded to the optimal value of the dilution of the suspensions identified by the preliminary experiments.

RESULTS

The absorption spectra of standard hemagglutinating sera are shown in Fig. 2. It can be seen that the optical density of sera of blood groups B_α(III) and AB_0(IV) has a strong dependence on the wavelength of the optical radiation of the visible range, and, in the sera of both spectra, there are absorption bands at wavelengths corresponding to absorption of hemoglobin. Obviously, in this spectral region, the effect of sera coloring is especially high. At the same time, for sera of blood groups $0_{\alpha\beta}$(I) and A_β(II), the dependence of the optical density and the corresponding absorption bands are practically absent.

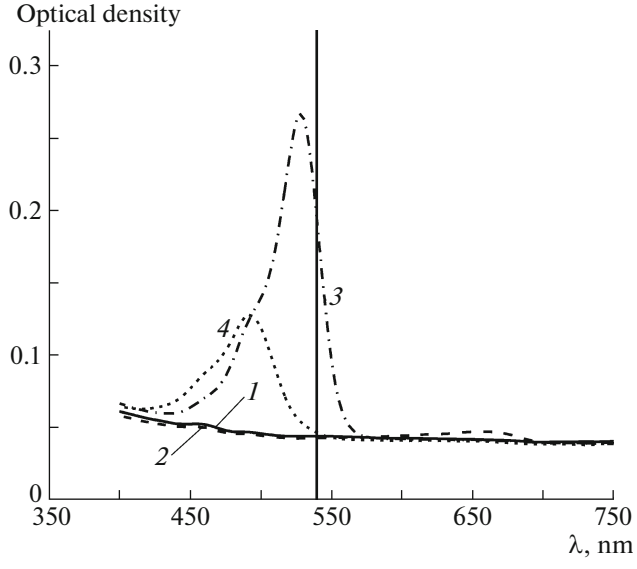

Fig. 2. The absorption spectra of standard hemagglutinating sera. (*1, 2, 3, 4*) Spectra of $0_{\alpha\beta}$(I), A_β(II), B_α(III), and AB_0(IV) sera, respectively. The cuvette gap size is 1 mm. The vertical line at $\lambda = 540$ nm indicates the position of the center line of the LED spectrum.

The absorption spectra of investigated suspensions for positive and negative agglutination of erythrocytes are shown in Figs. 3a and 3b, respectively. The high optical density of suspensions of erythrocytes in the green region of the spectrum (Fig. 3b, the reference graph, curve *5*) corresponds to the maximum of the absorption spectrum of hemoglobin. It should be noted that the center line of the spectrum of the probing light beam substantially coincides with one of the hemoglobin light absorption maxima in the green region. It is easy to see that, for the positive agglutination reaction, the analogous similar absorbance maxima of erythrocytes (hemoglobin) is less expressed due to the sedimentation of erythrocytes and their agglutinates (e.g., curve *1* in Fig. 3a). At the same time, it is obvious that the color of sera significantly affects the results of spectral measurements, especially at the wavelength of $\lambda = 540$ nm, with the strongest effect being caused by the color of the B_α(III) serum (curves *3* in Figs. 3a and 3b).

The results of measurements of transmittance factor T on the wavelength of $\lambda = 540$ nm for the positive and negative agglutination of erythrocytes with different sera are shown in Table 1 based on the spectra (Fig. 3). Table 1 also contains analogous results for the respective solutions of corresponding sera in the saline (optical density D was converted into transmittance factor T).

From the table, the ensuing follow.

1. The transmission factor for B_α(III) serum is almost 1.5 times lower than for the rest of the sera with approximately equal T values (column 1).

2. Transmission factors T for all sera, except B_α(III), are approximately equal both for the negative reaction (column 2) and for the positive agglutination reaction (column 3).

3. For the positive agglutination reaction, transmittances factors T for $0_{\alpha\beta}$(I) and A_β(II) sera (column 3) are close to the corresponding values of the same parameters for sera solutions (column 1). This indicates that, due to the effect of sedimentation of erythrocytes and their agglutinates enhanced by ultrasound, the number of cells and their associates is so small that they do not significantly contribute to absorption and scattering of light as compared with the colored sera.

Comparison of T for the B_α(III) serum solution and the suspension for a negative agglutination reactions with this serum (columns 1 and 2, respectively) shows that the sedimentation of erythrocytes and their nonspecific aggregates leads to transmittance factor T being higher (column 2) than the serum of the same group, but without cells (column 1). This can be interpreted as follows: as a result of the sedimentation of blood cells, part of the dye sediments with cells at the bottom of the

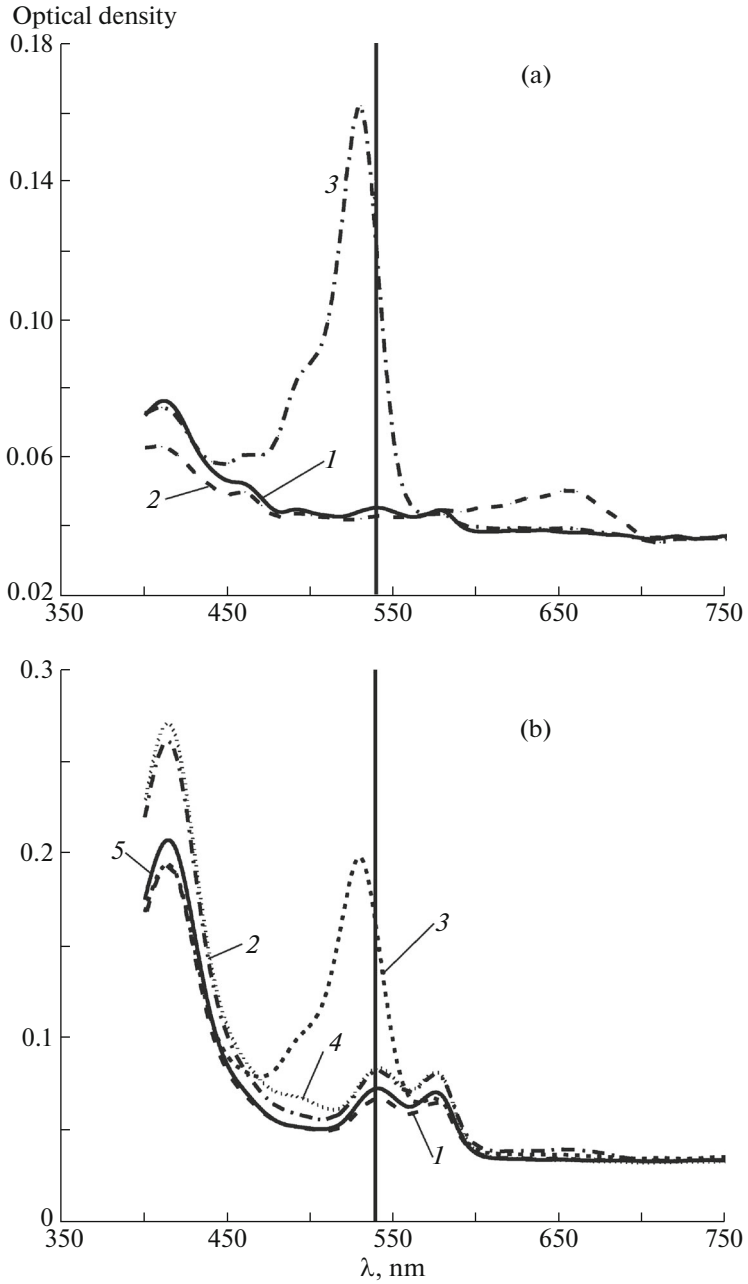

Fig. 3. The absorption spectra of the studied suspensions for (a) positive agglutination reactions (erythrocytes AB(IV)), (b) negative agglutination reactions (erythrocytes 0(I)) with corresponding hemagglutinating sera: (*1, 2, 3, 4*) react with $0_{\alpha\beta}$(I), A_{β}(II), B_{α}(III), and AB_0(IV) sera, respectively; (*5*) a spectrum of the reference signal (erythrocytes in saline). The cuvette gap size is 1 mm. The vertical line at $\lambda = 540$ nm indicates the position of the center line of the LED spectrum.

cuvette. The same effect would be observed for a positive agglutination reaction (column 3).

The latter conclusion is quite interesting, as in earlier studies, for example [10, 15–18], the clearing of the "blood–serum–saline" suspension both at the positive agglutination reaction and to a lesser extent at the negative was explained by only sedimentation of erythrocyte and agglutinates, but not by the change in the optical properties of the dye during the sedimentation of sedimentation of cells and their associates. This is due to the fact that the authors of those studies did not consider the color of the sera.

Naturally, the different effects of the colors of different types of sera on the optical density of suspensions of erythrocytes and their agglutinates affect the brightness of the images for both positive and negative agglutination reactions. This, in turn, changes the ratio between the resolving power values of acousto-optical method for blood typing of different sera and,

Table 1. The values of transmittance factor T (in %) at wavelength $\lambda = 540$ nm for positive and negative agglutination reactions of erythrocytes with different sera

Serum type	Serum solution	Negative agglutination reaction	Positive agglutination reaction
$0_{\alpha\beta}$(I)	90.2	85.2	90.0
A_β(II)	90.4	82.0	90.5
B_α(III)	64.0	69.3	76.6
AB_0(IV)	89.8	81.7	–

as a result, affects the accuracy of the determination of the blood group of the sample.

These considerations are illustrated by Fig. 4. Figure 4 shows the following.

1. The brightness of images for the positive reaction of erythrocyte with A_β(II) serum (Fig. 4b) is disproportionately higher than the brightness of the same image for negative agglutination reaction (Fig. 4a). This means that resolving power R of the method to the interaction of the studied blood with A_β(II) is high.

2. The ratio of brightness of images on Figs. 4c and 4a is significantly lower than in the previous case. This is due to significant absorption of light in the region of $\lambda = 540$ nm by serum due to its color. Naturally, it reduces resolving power R for the analyzed blood sample–B_α(III) serum reaction.

The decrease of resolving power R for the acousto-optical method of blood typing by color of sera, specificity of spectrum of dyes may lead to incorrect interpretations of experimental results, and even to an error in determination of the blood groups. In order to avoid the dependence of R from the color of sera we suggested to pass the probing light beam through a neutral color filter, and for each type of serum matched the filter with a certain transmittance factor should be selected. This will create the conditions under which the light input impinging the webcam would be the same for all four used sera. An image similar to the image in Fig. 4c (B_α(III) serum), but with the adjustment of the probing light beam is shown in Fig. 4d: the neutral density filter in the optical system is used for all other sera with transmittance factor $T_1 = 32\%$ (Fig. 1) was replaced by a neutral filter with an increased transmittance factor $T_2 = 65\%$. Thus, the value of the power of detected signal for B_α(III) serum was much closer to the corresponding values for $0_{\alpha\beta}$(I) and A_β(II) sera. It is clear that such an adjustment of the probing light beam significantly improves resolving power for the registration of agglutination of the studied red blood cells–B_α(III) serum. Most importantly, the photodetector part of the device (Fig. 1) is able to detect the presence or absence of erythrocytes agglutination independently from the specifics of colored sera.

The efficiency of the correction of probing light beam can be illustrated by the following experimental data: the resolving power of the method for the blood sample with A_β(II) serum A is $R(A_\beta(II)) = 242$, and under the same conditions with B_α(III) serum without correction of the impinging light R_0 (B_α(III)) = 106. At the same time, the correction of the probing light beam for the B_α(III) serum increases resolving power up to $R(B_\alpha(III)) = 215$. Not only the magnitude of resolving power R, but the proximity of its values for the various sera is very important. Correction of the probing light beam is particularly important for the weak agglutination of erythrocytes. In this case, the optical response of a positive reaction is not significantly different from the response of the negative reaction that could have a significant impact on the interpretation of the results and even lead to the registration of false-negative result and, consequently, to the incorrect determination of blood groups. For example, for AB(IV) erythrocytes with a weak agglutination ability with A_β(II) and B_α(III) sera, the following val-

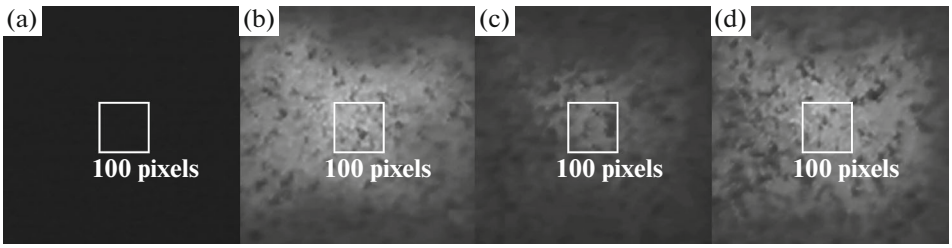

Fig. 4. Photos of the light-striking of the cuvette in a green RGB channel with the test solution: (a) negative reaction; (b), (c), (d) positive agglutination reactions; (b) reaction of erythrocytes B(III) with serum A_β(II); (c) and (d) the reaction of erythrocytes A(II) with serum B_α(III). Photo (c) was done without correction of the probing light beam by neutral color filters; (d) the correction of the light beam was implemented. Open squares with a size of 100 × 100 pixels indicate statistical processing of images.

Table 2. Variants of the blood–agglutinating serum interaction and the possibility of errors in blood typing by the acousto-optical method

Type of erythrocytes / Type of serum	0(I)	A(II)	B(III)	AB(IV)
$0_{\alpha\beta}$(I)	1	2	3	4
	–	+	+	+
A_{β}(II)	5	6	7	8
	–	–	+	+
B_{α}(III)	9	10	11	12
	–	+/–	–	+/–
AB0(IV)	13	14	15	16
	–	–	–	–

ues of the resolving power were obtained: $R(A_{\beta}(II)) = 31$ and $R_0(B_{\alpha}(III)) = 21$ without correction for the impinging light for B_{α}(III) serum; i.e., $R(A_{\beta}(II)) > R_0(B_{\alpha}(III))$. However, by using the correction $R(B_{\alpha}(III)) = 41$, then $R(A_{\beta}(II)) < R(B_{\alpha}(III))$.

Possible errors in determining blood groups by the acousto-optic method are demonstrated in Table 2. Here, "minus" shows a blood–serum combination in which agglutination is fundamentally negative, with the plus corresponding to a positive reaction. However, due to the color and, consequently, considerable light absorption by B_{α}(III) serum, the resolving power of R can be so low that the positive reaction (table, cells 10 and 12) can be interpreted as negative. This situation occurs for a weak agglutination of erythrocytes when the resolving power of R is below its threshold value $R < R_{thr}$; therefore, cells 10 and 12 contain the symbol +/–. If, for example, cell 12 corresponds to –, there is no difference between cells 3, 7 and 4, 8. Then, the B(III) blood group may be designated as AB(IV).

DISCUSSION

Due to the high specificity of the blood samples of various donors or recipients, the resolving power of the developed method for determining blood group may vary widely, even if the samples correspond to the same group. The specificity is determined by many factors, such as agglutination of erythrocytes, concentration of erythrocytes in the sample, concentration of hemoglobin, the viscosity of the analyzed blood, titer of hemagglutinating sera and others. That is why, in practice developers of devices for determining blood groups, in addition to the desire to maximize resolving power R, as the result of technical and biomedical tests establish certain thresholds $R_{thr}(max)$ and $R_{thr}(min)$. If, for the tested blood sample, the measured R value exceeds a predetermined threshold value $R_{thr}(max)$, than the agglutination occurred and it is positive, and if $R < R_{thr}(min)$, than the reaction is negative. Then, the blood group is determined by similar experiments with the given blood sample, but with different sera. If the measured R value in the experiment with at least one of the four sera is in the "uncertainty" area $R_{thr}(min) \leqslant R \leqslant R_{thr}(max)$, then it is impossible to draw a conclusion about the agglutination reaction. Then the blood group is not determined, and the device, and it is extremely important has the right to answer "the blood group is not defined." Such cases require an additional special test often performed manually, for example, using the crossover method. Naturally, the increase in the resolving power of the device for blood typing increases detuning of the measurement of R within the selected range $R_{thr}(min) - R_{thr}(max)$ and therefore, not only increases the reliability of determining blood groups, but also reduces the number of blood samples belonging to the unidentified group. In this context, the proposed adjustment of the probing light beam individually for each sera type not only improves the resolving power of the acousto-optical method of blood typing, but also create conditions for determining blood groups, regardless of sera color. In turn, this increases both the accuracy of the results of medical tests and the number of samples with the determined blood type.

CONCLUSIONS

It has been shown that the proposed adjustment of the probing light beam using filters individually selected for different types of sera significantly increases the resolving power of the acousto-optical method of blood typing, makes it more stable, and does not depend on the color of the sera. In turn, this reduces the possibility of incorrect interpretation of the results of medical tests and, consequently, the errors in determining the blood group. This suggestion is especially important when typing blood samples with a weak agglutination of erythrocytes. This study can be used in the development of a device for instrumental determination of human blood groups.

REFERENCES

1. G. N. Vyas et al., US Patent No. 5776711 (1998).
2. P. Sturgeon, Immunohematology **17**, 100 (2001).
3. T. R. Kline, M. K. Runyon, M. Pothiawala, and R. F. Ismagilov, Anal. Chem. **80**, 6190 (2008).
4. I. Muranyi et al., US Patent No. 4533638 (1985).
5. R. A. Steven, PhD Thesis (North Carolina State Univ., Raleigh, North Carolina, USA, 2005).
6. J. B. Lambert, PhD Thesis (North Carolina State Univ., Raleigh, North Carolina, USA, 2006).
7. P. Moncharmont, A. Plantier, et al., Immunohematol. **19** (2), 54 (2003).
8. D. Goldfinger et al., US Patent No. 4650662 (1987).
9. C. F. Battrell et al., US Patent No. 20100112723 (2010).

10. A. N. Alipov, V. Z. Vaninskii, L. B. Denisov, S. I. Donskov, V. A. Doubrovski, E. N. Zav'yalov, and N. N. Knyaz'kov, USSR Inventor's Certificate No. 1683760, Byull. Izobret., No. 38 (1991).
11. V. A. Doubrovski and K. N. Dvoretski, Ultrasound Med. Biol. **26**, 655 (2000).
12. V. A. Doubrovski, K. N. Dvoretskii, and A. E. Balaev, Acoust. Phys. **50**, 146 (2004).
13. Yu. A. Ganilova, V. A. Doubrovski, and I. V. Zabenkov, Proc. SPIE **7999**, 799903 (2011).
14. V. A. Doubrovski, Yu. A. Ganilova, and I. V. Zabenkov, Opt. Spectrosc. **115**, 212 (2013).
15. V. A. Doubrovski and A. A. Dolmashkin, Opt. Spectrosc. **109**, 263 (2010).
16. V. A. Doubrovski and A. A. Dolmashkin, Med. Tekh., No. 2, 24 (2012).
17. A. A. Dolmashkin, V. A. Doubrovski, and I. V. Zabenkov, Quantum Electron. **42**, 409 (2012).
18. V. A. Doubrovski and M. F. Medvedeva, in *Proceedings of the Saratov Fall Meeting SFM-13 on Problems of Optical Physics* (Novyi Veter, Saratov, 2013), p. 34.
19. Yu. A. Ganilova, A. A. Dolmashkin, V. A. Doubrovski, I. Yu. Yanina, and V. V. Tuchin, Opt. Spectrosc. **115**, 212 (2013).

Translated by V. Mittova