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Serum alkaline phosphatase assay with paired emitter detector diode

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ABSTRACT

A simple multicommutated flow system based on optoelectronic detector, three valves and peristaltic pump only has been developed for photometric determination of alkaline phosphatase activity in human serum. A miniaturized, compact flow-through detector dedicated to selective photometric detection of product formed in the course of the enzyme assay has been constructed using two paired light emitting diodes. The proposed analytical procedure based on kinetic methodology of enzyme activity detection and stopped-flow methodology of two-point measurements eliminates interferences caused by intense color of real samples and impurities present in commercial reagents. After optimization the system allows reproducible, mechanized analysis of human serum in relatively short time (8–9 samples per hour). Volume of serum required for single determination is 0.05 mL only. The system validated with real clinical samples is useful for determination of enzyme activity in human serum at physiological and pathological levels.

1. Introduction

Although optical detection dominates in analytical chemistry, for many practical applications highly advanced and expensive spectrophotometers are not necessary. For many dedicated analytical uses simple optical devices based on economic semiconductor components are sufficient. Light emitting diodes (LEDs), the cheapest optoelectronic components, providing low-cost, high-intensity and quite monochromatic light sources with low-voltage power requirements. Various compositions of the semiconductor gate of diode lead to diversity of LEDs which can cover a broad spectral range from UV to NIR. Because of that, LEDs can be really useful in reducing the complexity of photometric devices by eliminating the need of wavelength selection, and offering simple measurement systems for determination of specific analytes that absorb at the wavelength emitted by selected LED. The utility of LEDs for optical methods of analysis is well established and widely reported in the analytical literature [1]. Several applications of LEDs as light sources in flow analysis have been reviewed recently [2].

The electroluminescence process by which LED operates as a light source is reversible. Illuminated LED produces a small current proportional to the light. It means, that the internal photoelectric effect (opposite to electroluminescence phenomenon) allows the use of LED as light detector when it is applied in the reversed mode. LEDs will respond to fluctuations in light intensity of frequencies to more than several hundred kHz. This makes them potentially useful as fast and reversible light detectors. Concluding, two LEDs can form a paired-emitter-detector-diode (PEDD) – a complete optoelectronic device for absorbance measurements. Unfortunately, the current generated by illuminated LED is very low and rather difficult to precise measure without special instrumentation. To bypass this problem, an electromotive force generated by illuminated LED detector can be applied as an analytical signal of PEDD. In this approach, when PEDD is coupled with pH-meter [3] or voltmeter [4] the analytical signal (given in millivolts) is a linear function of absorbance. This linearity is easily explained by the compilation of Shockley equation for diodes and Lambert–Beer law for photometry [3]. For practical uses, instead of relatively expensive pH-meter, a low-budget voltmeter can be applied [4]. In such case, due to low resistance of voltmeter, a partial discharging of LED detector is observed. Therefore, after the instrument replacement, the linearity in the wide range of concentration is lost but in the narrow range the sensitivity significantly increases. It is worth to notice, that this range of maximal sensitivity can be adjusted by selection of current supplying PEDD [4].

PEDDs operating according to the reported methodology of analytical signal transduction have just found first analytical applications as extremely economic photometers for some cuvette enzyme assays [5], as hemoglobinmeters [6] as well as dedicated flow-through cells (additionally integrated with respective semiconductor light emitters and detectors) [3,4]. Recently, a flow-through sensor for redox species obtained by integration of sensing film with PEDD has been reported [7]. This sensor has been applied for glucose enzyme-based bioPEDD development [8]. In this

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communication, the PEDD-based flow system for enzyme activity assays developed for the needs of clinical diagnostics is demonstrated.

The phosphoesterase activity of serum is predominantly defined by the presence of alkaline phosphatase (ALP, EC 3.1.3.1) which belongs to the group of indicating enzymes mostly assayed in clinical practice [9]. A rise in ALP activity occurs with all forms of cholestasis, particularly with obstructive jaundice. Moreover, serum ALP significantly elevates in diseases of the skeletal system, such as Paget's disease, osteomalacia, fractures and rickets. Elevated ALP level in serum is evident indication for sarcoma and malignant tumors. Summarizing, determinations of ALP serum activity is particularly important for oncologic diagnostics. Clinically recommended spectrophotometric methods for ALP determination use *p*-nitrophenylphosphate (NPP) as a chromogenic substrate [9–12]. Nitrophenol (NP), the product of enzymatically catalyzed hydrolysis of NPP, has a deep yellow color (λ_{max} = 400 nm). Recently, a dedicated PEDD for NP detection has been developed [13]. As shown previously, this detector is useful for some bioanalytical uses, particularly for detection of ALP activity under flow conditions. The main goal of the present work is the development of compact and economic flow system based on the PEDD detector dedicated for fast and reproducible ALP assays in real clinical samples having complex matrix like human serum.

2. Experimental

The PEDD detector for *p*-nitrophenol (NP) was constructed using UV-LED and blue LED, as the light emitter and detector, respectively. Both LEDs (5 mm diameter, transparent lens, flat front; 140° view angle) were obtained from Optosupply (Hong Kong). Fabrication and analytical performance of PEDD detector used in this work was reported in detail elsewhere [13]. Electromotive force generated by PEDD, treated as an analytical signal, was measured and recorded with UNI-T multimeter (model UT70B, China) operating as voltmeter connected with data storage PC via RS232 interface.

A peristaltic pump (model Minipuls 3) was from Gilson (France). Three-way solenoid valves (product no.100T3MP12-62-5) were purchased from Bio-Chem Fluidics (Boonton, USA). These devices were PC-controlled by the KSP Measuring System (Poland). Flow manifold was arranged with PTFE Microbore tubings (ID 0.8 mm) from Cole-Palmer (USA).

Serum standards with physiological, and pathological ALP activity were obtained from Cormay (Poland). These control sera received in the lyophilized form were reconstituted with water 2 h before use and stored at room temperature no longer than 12 h. *p*-Nitrophenyl phosphate (NPP) was obtained from Sigma–Aldrich (USA). Other reagents of analytical grade were obtained from POCh (Poland). Doubly distilled water was used throughout.

Real human serum samples with physiological and pathological ALP levels were obtained from Central Military Hospital in Warsaw. For the reference spectrophotometric measurements as a color-forming ALP substrate NPP was applied. This method is officially recommended by International Federation of Clinical Chemistry and American Association for Clinical Chemistry [10,11]. The reference determinations of ALP activities in human serum samples were performed using clinical analyzer Cobas Integra 800 system (Roche Diagnostics, USA).

3. Results and discussion

3.1. The principle of assay mechanization

There are two important points in case of serum analysis using the photometric ALP assay with NPP substrate. First of all both, sample (serum) and the product of indicating reaction (NP), are yellow. Moreover, in many cases the colorless substrate (NPP) contains some NP impurities and additionally in alkaline solutions spontaneous non-enzymatic hydrolysis of NPP to color NP takes place. It means, that in case of serum analysis the measured absorbance will contain nonspecific component. Secondly, at pH of 9 ALP activity is maximal and the effects from other phosphatases present in serum are negligible [14]. Therefore, especially in case of serum analysis, the working buffer should have a large buffer capacity at this pH. However, for the detection the alkalization of sample is recommended because only alkaline form of the enzyme reaction product strongly absorbs the light (NP is a common pH indicator exhibiting pK_a near pH of 7). Of course, this alkalization interrupts enzymatic hydrolysis due to ALP inhibition. The flow system reported in this paper complies these circumstances.

The manifold and the program executing developed analytical procedure are shown in Fig. 1A and B, respectively. The sample is aspired through the valve V_1 playing the role of sample injector. After directing V_1 for water the sample segment is mixed with substrate in working buffer (0.1 M diethylamine, pH of 9.0) stream by the valve V_2 switched with 5 Hz frequency. Directly in the front of PEDD inlet the reaction stream is mixed with 0.25 M NaOH solution by the valve V_3 switched with 5 Hz frequency. The peristaltic pump (PP) is stopped when the alkalized sample stream arrives to detector. In consequence, the valve V_3 in NC position breaks the segment of serum mixed with NPP into two stopped fragments: the strongly alkalized segment located inside the PEDD detector and the still-reacting segment in the reaction coil (RC) located between V_2 and V_3 valves. After certain time (t), the reacting segment is moved from the reaction coil (RC) to PEDD through the valve V_3 where is mixed with NaOH solution and the pump (PP) is turn off again. In the meanwhile the valves V_1 and V_2 are directed for the washing step of the flow system with water, when PP will be turn on again. After the washing step, the measurement cycle (programmed according to the scheme shown in Fig. 1B) can be repeated for next sample of serum.

The corresponding idealized readout expected for the single determination cycle performed according to the above-mentioned program (Fig. 1B) is shown in Fig. 1C. After washing step, when the signal for water is recorded (step 1), the first part of sample (with NPP and just after alkalization) is delivered to PEDD detector (step 2). Then, the stream is stopped for certain reaction time (t). During this time (step 3) background signal is measured by PEDD, whereas the enzymatic reaction continuously takes place in the reaction coil (RC). After this time, the reacting segment is moved from the reaction coil (RC) through the valve V_3 (where is alkalized) to the PEDD detector (step 4) and again is stopped for the signal reading (5). Finally, the peristaltic pump is turn on and the system is washed with water (step 6). As can be seen from Fig. 1C, in the course of single measurement cycle three distinguish stationary PEDD-signals are recorded: for water (1), for sample segment after negligibly short time of reaction (3) and again for sample segment (5) but after defined reaction time (t).

In the reported approach, the difference between voltage generated by PEDD during steps 3 and 5 is treated as an analytical signal for sample (see Fig. 1C). This way, so-called two-point methodology, common for kinetic measurements, is adapted in the developed analytical system. Such approach is expected to be specially profitable in case of real sample analysis as the method for elimination of photometric interferences. In the course of preliminary PEDD investigations [13] some interferences from NPP were observed, although the UV-LED used for construction of the PEDD emits almost monochromatic light at λ_{max} = 405 nm, fully compatible with absorbance spectrum of NP but not NPP. It was found, that these interferences were caused by NP impurities almost always present in the reagent. In case of serum analysis, similar, but

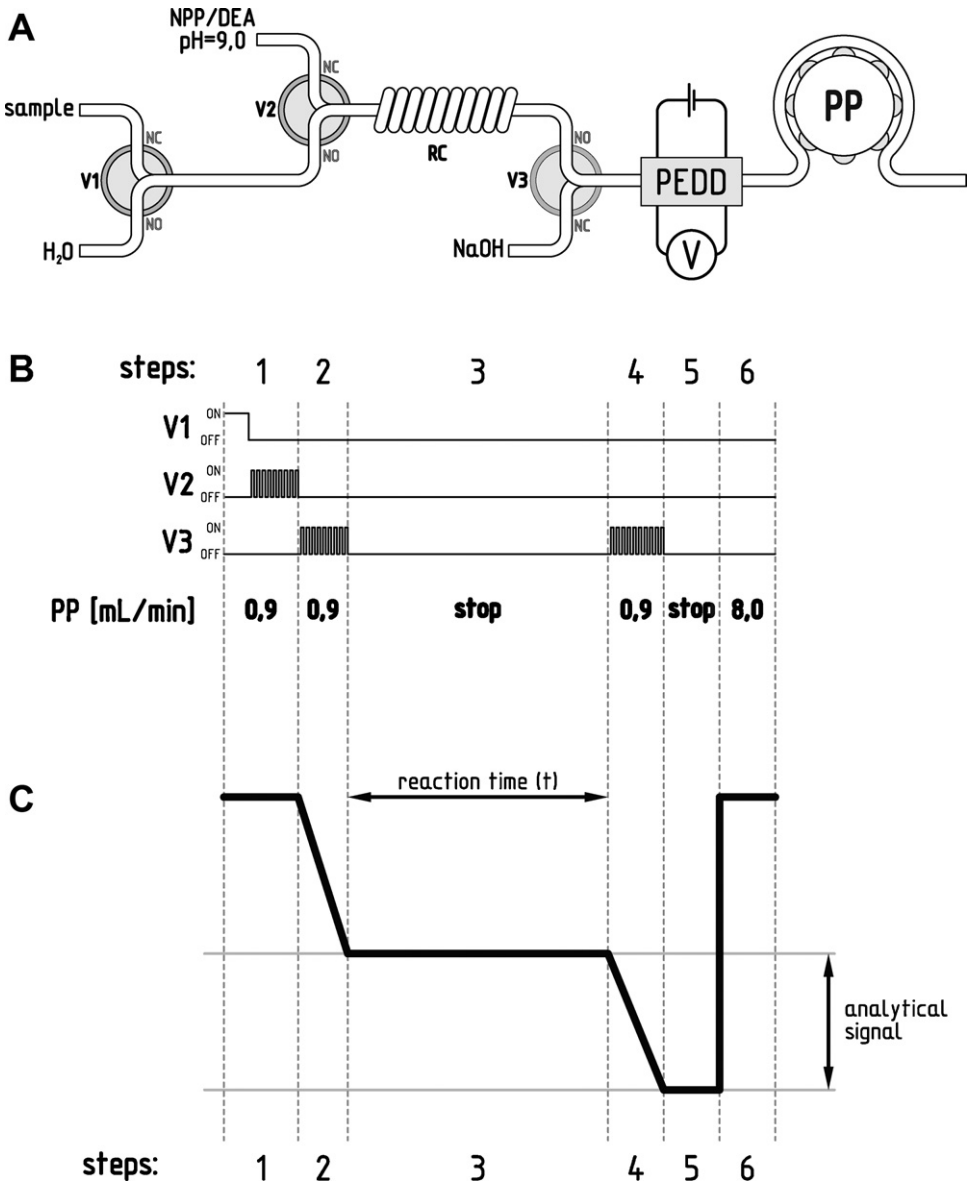

Fig. 1. Flow system set-up (A), program controlling the operation of system for single determination cycle (B) and corresponding idealized readout (C). Detailed description is given in the text.

significantly stronger effects are expected because protein matrix strongly absorbs the light in the UV–vis region of spectrum. As can be seen from Fig. 1C, the signal measured during third step is a "blank" measurement of absorbance for sample matrix and substrate impurities. The signal measured during fifth step of procedure results from total absorbance of sample matrix and the product formed in the course of reaction in the reaction coil. The stationary readouts for water (step 1) can be served as "blank signal of photometer" useful for periodic checking of PEDD operation stability.

3.2. Optimization of flow system with serum standards

The primary analytical characteristics of the developed flow system and program shown in Fig. 1 were performed using control sera five-fold diluted with water. These were two, physiological and pathological serum standards exhibiting low (L) and high (H) enzyme activities, respectively. Using these serum standards two effects were investigated. As reported previously [4], it is possible to fit the range of maximal sensitivity of PEDD detector to the required range of determination by the appropriate adjustment of power supplying this device. Thus, the effect of current driving the PEDD on its sensitivity was investigated. In the course of these experiments the analytical signal was defined as mentioned above (voltage difference between 3 and 5 steps – as indicated in Fig. 1C) and reaction time (t) was fixed at 3 min. As shown in Fig. 2A, the optimal current offering maximal sensitivity for ALP detection in both control serum was found to be 4.5 mA. This current was fixed for further measurements. Afterwards, the effect of reaction time (t) on sensitivity of detection was investigated. As expected, sensitivity is proportional to this time (Fig. 2B). It means, that there is no optimal time of the assay, so its selection is only the result of arbitrary compromise between the required sensitivity and time for single determination cycle. For further experiments the reaction time 210 s has been chosen.

Fig. 3A presents the readout recorded in the course of analysis performed for both control sera under optimized conditions. Each assay was three times repeated. The readout is consistent with

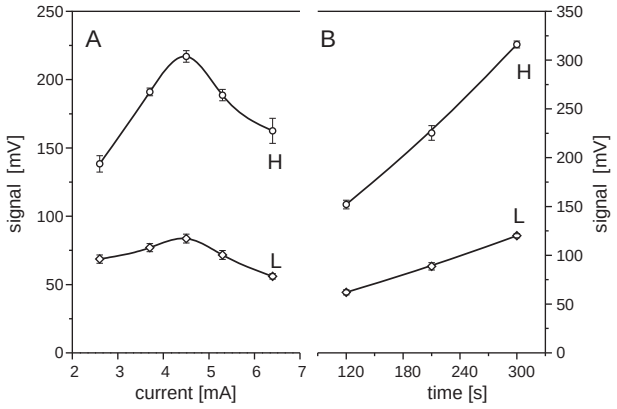

Fig. 2. Performance of the flow system: optimization of current driving PEDD detector (A), and the effect of enzyme reaction time (B). The measurements performed using serum standards with physiological (L) and pathological (H) levels of ALP.

this one expected as a result of program operation (Fig. 1C). The recorded signals are well reproducible and differences in signals for pathological and physiological serum standards are significant. The long-time operation stability test of the developed system and analytical procedure for simultaneous detection of both enzyme activities was performed using two control sera (L and H) and one additional intermediate (I) standard prepared by their mixing in 1:1 ratio. The detection cycles (Fig. 1B) were five times repeated for each standard (L, I and H). The results of the test are shown in Fig. 3B. The obtained results evidenced that the developed flow and detection system is robust and reproducible. Additionally, it can be concluded that in the course of almost 6-h test serum samples as well as reagent solutions did not change significantly. It is worth to notice that, the whole test was controlled by the program and

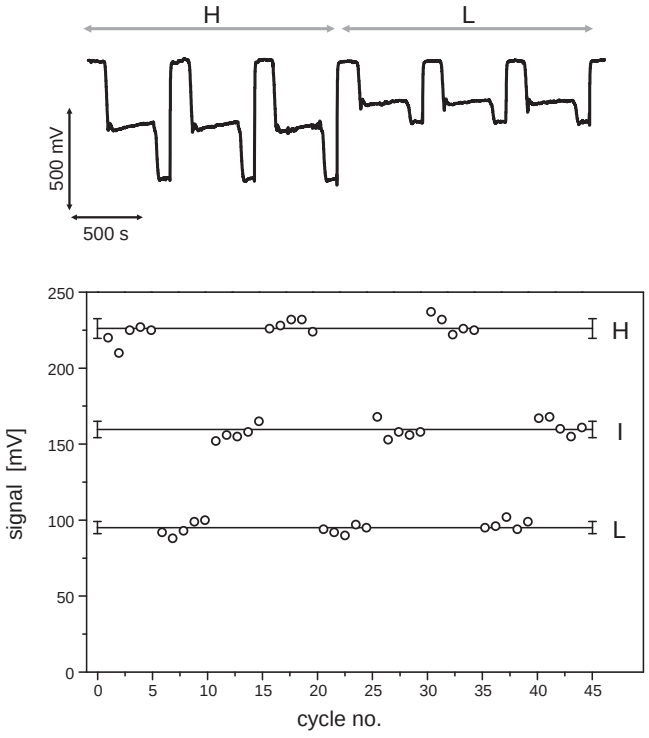

Fig. 3. Real readout for six measurement cycles (A) and the results of long-time reproducibility test (B). The measurements performed with serum standards having physiological (L) and pathological (H) levels of ALP and their 1:1 mixture (intermediate, I).

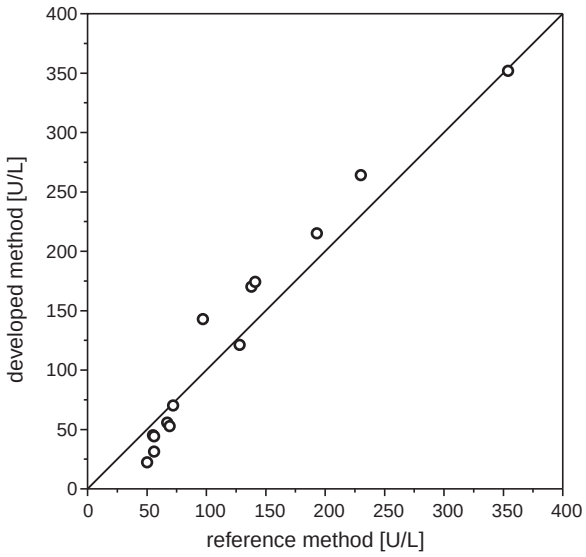

Fig. 4. Correlation of the results of human serum analysis using the developed bioanalytical system and reference method.

performed without any operator intervention because for samples changing additional valve (not shown in Fig. 1) was applied.

3.3. Human serum analysis

The results of above-reported experiments obtained with control sera indicate that the developed flow system can be useful for real clinical analysis. For single determination 0.05 mL of sample is required. The single determination cycle takes 7 min, thus flow-throughput of the system is 8–9 determinations per hour approximately. The final goal of this study was the analysis of real human serum. The system was validated using samples analyzed in clinical laboratory settings using recommended method and analyzer. The results of the validation are shown in Fig. 4. The correlation between results of human serum analysis obtained using the developed and routine instrumentations are satisfactory. The regression coefficient for ALP determination is 0.98 ($n = 14$). Student's t-test was used for comparing the means of reference and calculated values of the ALP activities. The calculated t value is 0.113 and does not exceed the tabulated value which is 2.056 (for 26 degrees of freedom and 95% probability for a correct statement). It means no statistically significant differences between results obtained by presented method and the reference method. It can concluded, that the developed bioanalytical system allows fast and accurate human serum analysis.

The demonstrated flow system after negligible modification (changing of carrier buffer) has been also tested as a tool for acid phosphatase (ACP) assays in human serum (results not shown). Serum ACP activities are approximately in 1–2 orders lower than ALP. Unfortunately, due to lower sensitivity (at the same assay time as for ALP activity determination) the precision of these assays was significantly worse: the regression coefficient for 14 samples of human serum was 0.72 only. However, the calculated value of t (0.012) from Student test does not exceed the tabulated value (2.056). It suggests that the difference between obtained and reference results is not significant. In fact, despite of the sensitivity decrease the system still allows evident differentiation of physiological and pathological levels of ACP. Probably, for more accurate measurements a significant increase of reaction time lowering of throughput of flow system is required. The PEDD-based flow systems for ACP assays need further investigations.

4. Conclusions

Multicommutated flow systems are intensively explored in modern analytics [2]. In this paper the bioanalytical application important for biomedical needs has been presented. The simple MCFA system demonstrated in this communication operates according to two-point methodology of kinetic measurements commonly applied in clinical diagnostics.

One of the current trends in analytical chemistry is the development of "multicommutation in spectrometry" [2], where the special attention is paid for the use of LEDs as dedicated, low-cost, robust and miniature light sources. This communication suggests that the next step in the evolution of this concept can be application of complete integrated photometric detectors made of paired LEDs.

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