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Porous membrane strip microsampling: a dried biofluid collection format and application for quantitative enzyme immunoassay

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Applicability of a new and simple membrane-strip microsampling format for the analysis of human blood serum in a strip-dried form for the presence of a range of model low and high molecular weight analytes by ELISA was demonstrated. Biofluid was applied onto a narrow strip of porous membrane, dried and analysed using a square piece of membrane (0.5 × 0.5 cm). An antigen distribution along a sampling strip and storage stability in strip-dried human serum samples were investigated in a quantitative assay. It was demonstrated that thyroxine, progesterone, thyroglobulin, prostate specific antigen and antibodies (IgG) against *H. pylori* were stable on the strip-dried sample for at least a week at ambient temperature and 37 °C. For all antigens, the concordance of the data obtained for liquid and strip-dried serum samples was observed (Bland–Altman data analysis). The proposed new strip microsampling format provides volumetric sample collection and can find application in quantitative immunoassays, for instance, in ELISA.

Introduction

Over the past few years, there has been an increasing interest in the use of dried spot technology for quantitative analyses of biofluid samples.[1–4] Conventional Dried Blood Spot (DBS) technology is based on dropwise application of blood onto a special sampling card made of cellulose material. The spots are then dried and a disc with a fixed quantity of dried blood sample is sub-punched and analysed for the presence of target haptens, proteins, and DNA/RNA.[1–4] DBS samples can be prepared under field conditions in remote areas, or by patients themselves, and then transported to an analytical laboratory by post due to the high storage and shipment stability of dried samples. All these advantages made DBS technology a promising alternative to conventional venous or capillary blood collection/sample pre-treatment techniques. DBS technology is also considered as a microsampling alternative to traditional blood, plasma and serum sampling (only 5–6 drops per card and 50 μL blood per drop compared to few millilitres of venous blood).[5] However, there are still some drawbacks. For instance, the hematocrit effect suppresses wide application of DBS for routine quantitative analysis.[2,6] Hematocrit is the volume percentage of red blood cells in whole blood and it has an influence on spot size, analyte recovery and blood-to-plasma ratio.[6] To provide an accurate quantitative assay, the biofluid volume taken for the analysis (the sub-punched membrane disc of fixed diameter with dried sample) should be constant and independent of the applied volume of the initial native sample. However, when applied onto a sampling card, a blood sample of the same volume with a low hematocrit displays larger spots than a sample with a higher haematocrit level. Thus, the sub-punch (fixed diameter) taken from those spots will have different blood volumes and therefore different analyte quantity recovered under analysis. This results in an assay bias. Moreover, the non-homogeneity of analyte across the spot on a cellulose card, chromatographic effect can be observed for any biofluid due to nature of cellulose fibers, which also absorb sample/target analyte inside the fibers.[6] All this leads to inaccuracies in quantitative assays.

In recent years, some approaches to overcome the hematocrit effect were described such as pre-cut or perforated discs for sample application,[2,6,7] new shapes for the application membrane,[8] new approaches for volumetric microsampling[9] and new sampling materials.[10] The volumetric sampling when a precise biofluid volume is absorbed by solid support (membrane, substrate) is one of the new approaches to obtain dried samples for quantitative bioanalysis. Volumetric microsampling combines the advantages of conventional DBS sampling while overcoming the area bias and homogeneity issues of when a sub-punch of the dried spot is taken for analysis. Denniff *et al.* described a novel approach called volumetric absorptive microsampling.[9] A porous polymeric tip was

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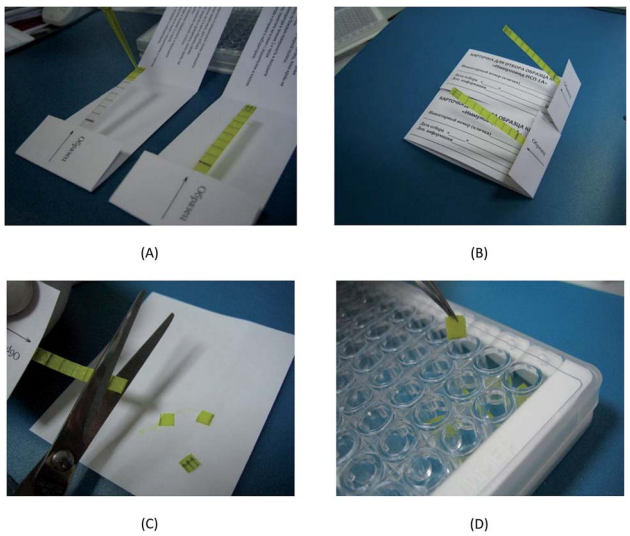

Fig. 1 New microsampling format: general scheme of work. (A) Application of a sample/standard solution onto a narrow strip of membrane material; (B) drying of sample/standard solution at room temperature; (C) cutting of square pieces of a strip with dried sample/standard solution; and (D) strip-dried samples/standard solutions are put into an ELISA microtiter plate for further analysis (one square piece of a strip per microtiter plate well).

used to absorb a fixed volume of blood with low variability across a wide hematocrit range. The volume of the sample was controlled by the properties and amount of polymeric material. It was demonstrated that the proposed sampler collects an accurate volume of blood for quantitative analysis. Dried spots technology was applied not only for blood, but also for other biofluids such as saliva, urine,[1–4] serum and plasma.[11] Serum and plasma were used as an alternative for whole blood to avoid the hematocrit problem. Recently, a new commercial DBS card manufactured from non-cellulose material was introduced.[12] It reportedly provides a homogeneous blood spot that is practically independent of the hematocrit level.[13]

This study describes a new and simple approach to volumetric collection and quantitative analysis of a dried sample. The approach involves application of liquid sample onto the end of a membrane strip of non-cellulose membrane material. In our previous studies, the successful application of this new format of sample pre-treatment for volumetric collection and preparation of the strip-dried form of samples, such as cows' whole milk for progesterone analysis[14] and whole blood for the determination of bovine leukemia provirus DNA,[15] was demonstrated. The purpose of the present study was to show the applicability of the microsampling format for the quantitative immunoanalysis (ELISA) of a few model low and high molecular weight antigens in strip-dried human blood sera.

Experimental

The following commercial ELISA kits were used for human sera analyses: "ELISA-progesterone", "ELISA-total thyroxine", "ELISA-thyroglobulin", "ELISA-prostate specific antigen" (Immunotek, Moscow, Russia) and "GAP *H. pylori* – IgG ELISA kit" (Biomerica, Irvine, USA). Standard solutions and serum samples were analysed in duplicates. Strip-dried human blood serum samples were prepared using a "DBS strip sampling card" (Immunoved, Moscow, Russia). Sample application strips were produced from glass fiber hydrophilic porous membrane (MAPDS-0300, Arista Biologicals, USA). Human blood serum samples were provided by Immunotek (Moscow, Russia). All experiments were performed in compliance with the relevant laws and institutional guidelines. Serum samples were stored at −20 °C.

Serum samples were analysed in native (liquid) and in strip-dried forms. The strip-dried form of a sample was prepared as follows: an aliquot of serum (100 μL) was applied in whole onto the end of a narrow strip (as part of the sampling card) placed horizontally and then dried for 2 hours at room temperature (Fig. 1). Strip-dried samples were stored in a sealed zip-lock plastic pack in the presence of a desiccant. Strip-dried standard solutions for progesterone, thyroxine, thyroglobulin and prostate

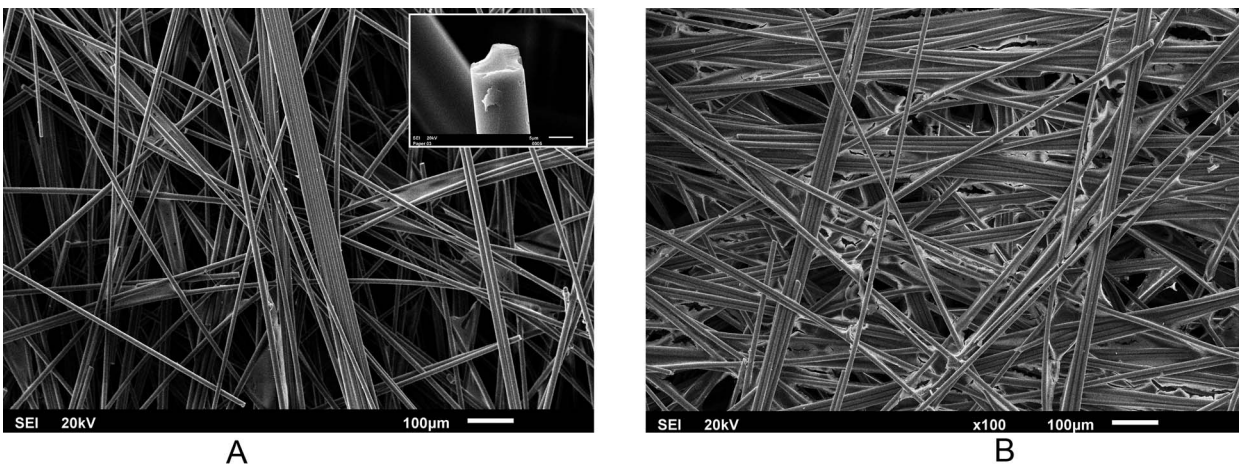

Fig. 2 Images of the fiberglass membrane: with no sample applied (A) and with dried serum sample (B). Images were obtained on scanning electron microscope JSM 6610LV (Jeol Ltd, Japan).

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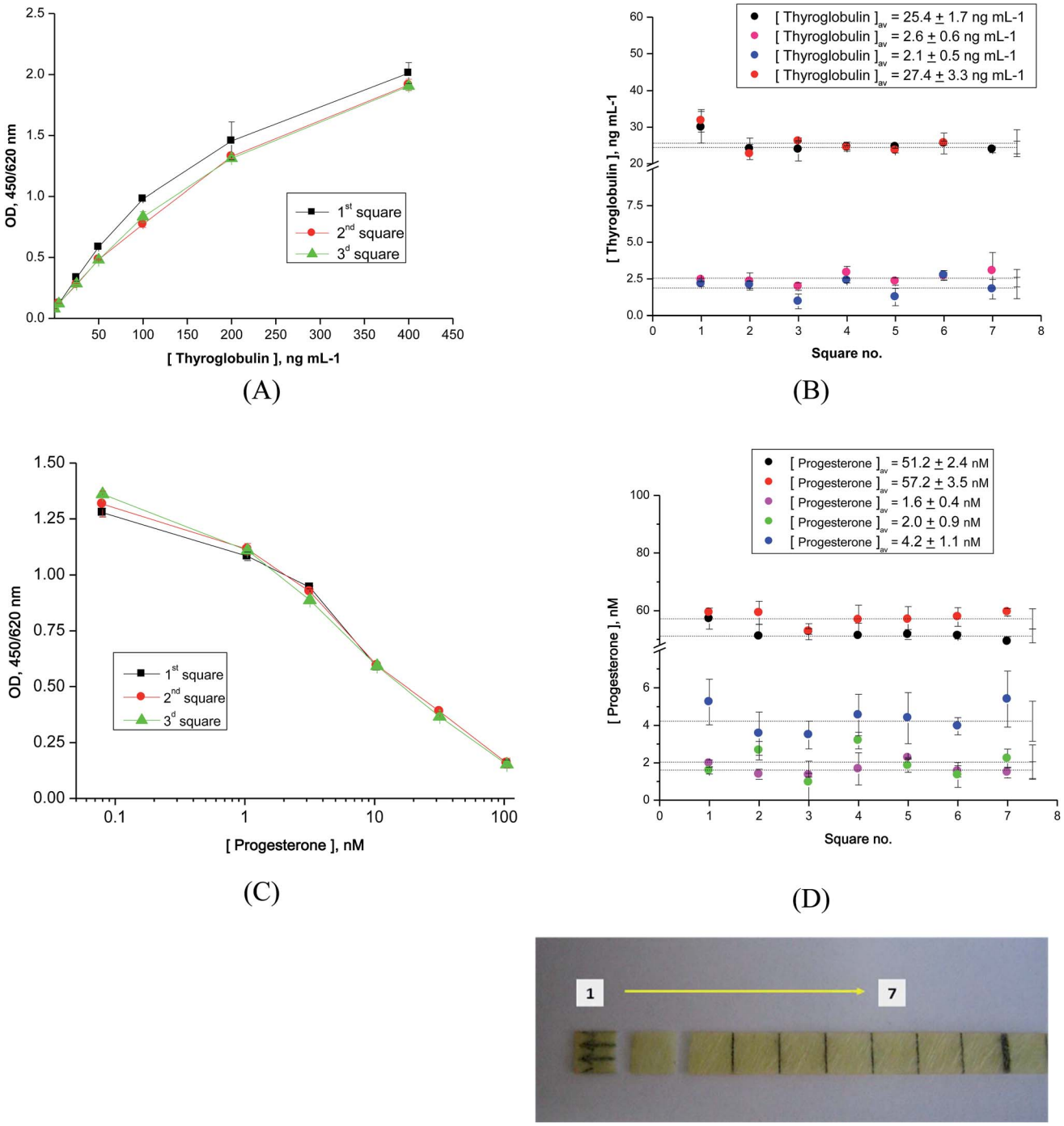

Fig. 3 ELISA calibration curves and antigen concentration detected in sequential pieces of a sampling strip (from no. 1 to no. 7) with strip-dried serum samples (thyroglobulin, A and B, and progesterone, C and D, correspondingly). For the generation of calibration curves (A and C) the following square pieces of a sampling strip with strip-dried standard solutions were used: ■ – square piece no. 1, ● – square piece no. 2, ▲ – square piece no. 3. (B) and (D): *x* axis – no. of a square piece of sampling strip with strip-dried serum sample analysed in ELISA, *y* axis – concentration of a target antigen detected in a square piece. An antigen concentration (mean ± SD) detected for square piece no. 2 to square piece no. 7 is presented as a line (B and D) (*i.e.* mean of measurements of pieces no. 2–7).

specific antigen ELISA were prepared analogously. For the analysis, a part of the membrane strip with dried standard/sample (0.5 × 0.5 cm) was cut-off with scissors along a marked line. ELISA for strip-dried samples was performed in accordance with the instructions from each kit with slight modifications. For progesterone, thyroxine, thyroglobulin and prostate specific antigen analysis, a part of the membrane (0.5 × 0.5 cm) with the strip-dried standard/sample was placed into a microtiter plate well followed by 20 μL of distilled water. Then, the kit procedure was followed. For analysis of antibodies (IgG) against *H. pylori*,

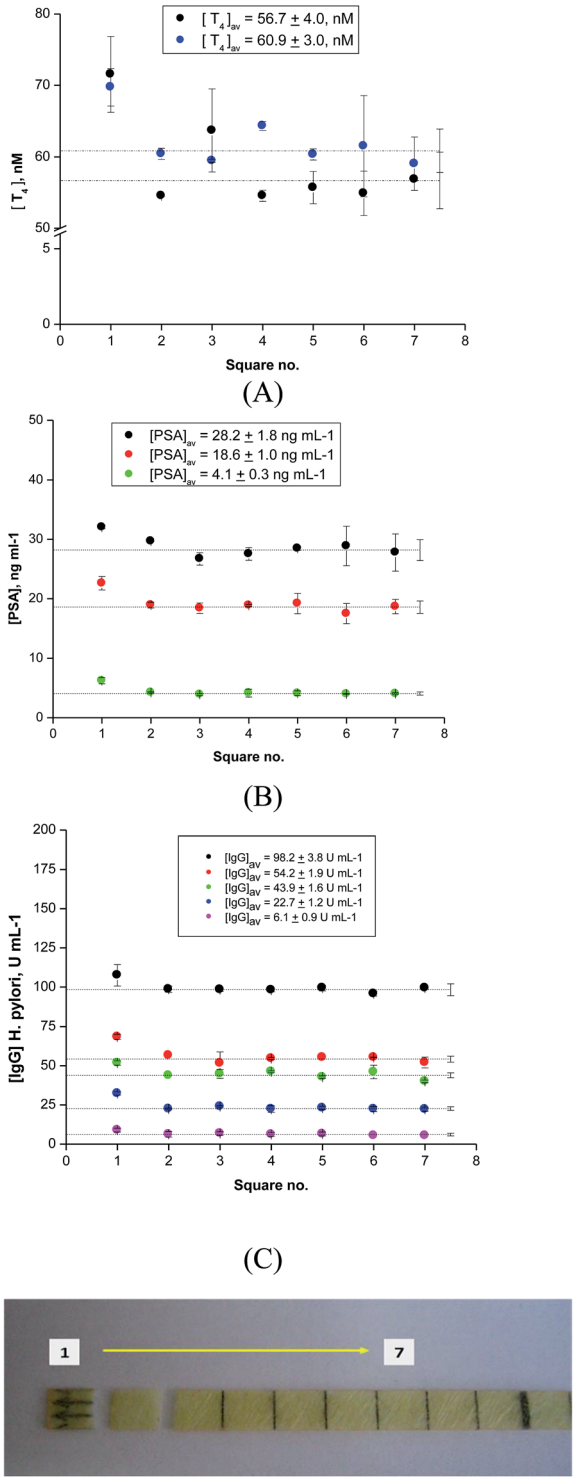

Fig. 4 Concentration of thyroxine (A), prostate specific antigen (B) and antibodies (IgG) against *H. pylori* (C) detected by ELISA in sequential pieces of a sampling strip (from no. 1 to no. 7) with strip-dried serum samples, *x* axis – no. of square piece of a sampling strip analysed in ELISA, *y* axis – concentration of a target antigen detected in a square piece. An antigen concentration (mean ± SD) detected for square piece no. 2 to square piece no. 7 is presented as a line (*i.e.* mean of measurements of pieces no. 2–7). T4 – thyroxine; PSA – prostate specific antigen.

serum samples were applied onto a strip and dried as described above. Dried sample (0.5 × 0.5 cm part of a strip) was placed into 1 ml dilution buffer and stirred for 15 min. 100 μL of the resulting solution was mixed with dilution buffer (200 μL) and analysed according to the kit instructions against the calibration curve obtained from the supplied liquid standards.

Optical density was measured on microplate reader Anthos 2010 (Labtec, Wals, Austria).

Results and discussion

New format of sample pre-treatment was applied for a range of model low and high molecular weight antigens such as progesterone, thyroxine, thyroglobulin, prostate specific antigen and antibodies against *H. pylori* utilizing commercially available ELISA kits and human serum samples in strip-dried form. Low molecular weight antigens were analysed by competitive ELISA; high molecular weight antigens were analysed by sandwich ELISA. Using of DBS technology for the preparation and analysis of target antigens in dried blood spots by immunoassay for the purposes of medical and veterinary diagnostics was described earlier.[16–21] DBS cards of conventional format are manufactured with cellulose fibres and samples are applied dropwise onto marked round parts of the card. After drying, the paper discs with applied sample are punched. A new format for sample pre-treatment proposed in the present study includes application of a liquid sample onto the end of a marked narrow strip of porous glass fiber membrane material (Fig. 1).[22] A sample is applied in whole, starting from the beginning (the first marked square), which flew up the strip. Alternatively, application of a sample onto a strip can be carried out by dipping the end of a strip into biofluid. Then, the applied liquid migrates along the strip under capillary forces and saturates the curtain length of the strip according to absorptive properties of the membrane material. The pore size is relatively big (around 50–100 μm) and biofluid moves between the glass fibers and does not penetrate inside them because of the solid structure (Fig. 2). Therefore, after drying, a biofluid residue is evenly distributed in the pores of the carrier. Both characteristics provide no chromatographic effect under fluid distribution along a strip. Thus, the proposed microsampling approach includes the combination of a new membrane support format in the form of a narrow strip and a new membrane material (fiber glass) is used to collect biological fluid samples. This combination provides volumetric sampling of a biofluid when equal amounts of a biological liquid (*i.e.* target analyte) are impregnated per single square unit of a membrane support. Each glass fiber strip used in the present study had a curtain length and black marks precisely put onto each strip at the 0.5 cm interval. For the analysis, a square piece of the prepared strip was cut-off. The membrane material used to fabricate a strip (glass fiber) soaks about 60 ± 4 mg of liquid (distilled water) per square centimetre. Therefore, one square piece of membrane (0.5 × 0.5 cm) contains enough quantity of strip-dried sample to perform conventional quantitative ELISA. Glass fibers have an advantage compared to cellulose (cotton) fibers because they do not absorb liquid inside the fibers (Fig. 2).

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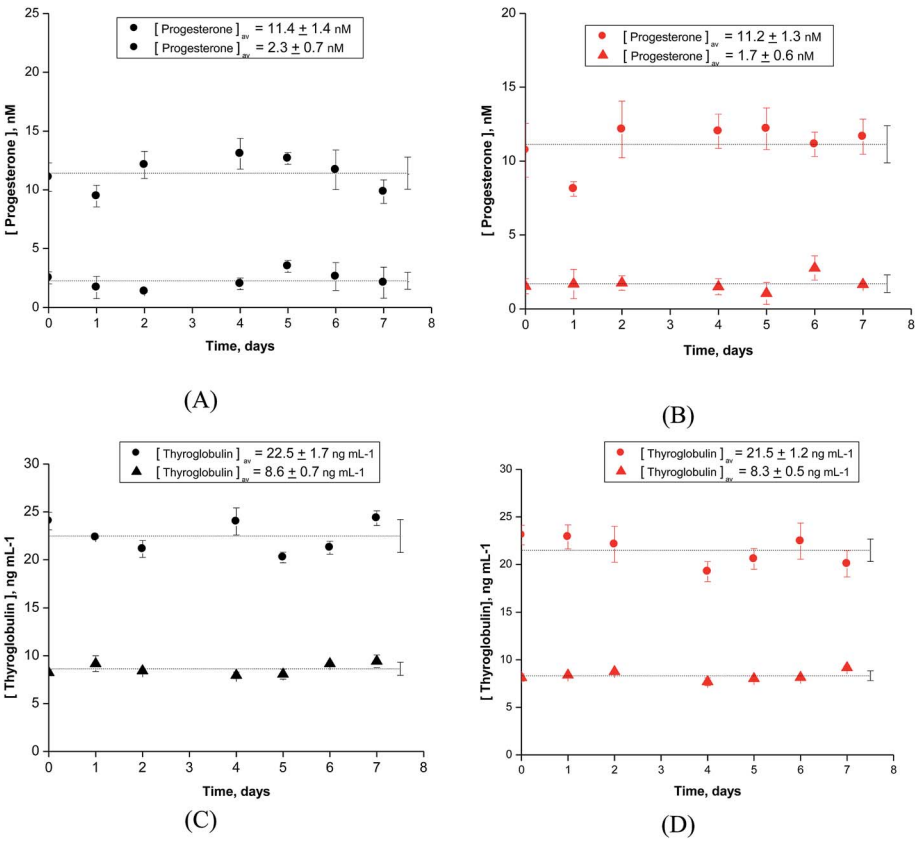

Fig. 5 Stability of progesterone and thyroglobulin in strip-dried human blood serum samples stored at room temperature (A and C) and 37 °C (B and D). *x* axis – days of sample storage, *y* axis – concentration of a target antigen detected by ELISA.

Therefore, a membrane can easily desorb the dried material under its solubilisation before/during sample analysis.[23] Glass fiber membranes are widely used as a conjugate pad in lateral flow immunoassay. The most important task of a conjugate pad is uniform transfer of specific reagents into the following analytical membrane. Transfer is provided from such glass fibre membrane properties as low non-specific binding, consistent flow characteristics and consistent bed volume.[23] These characteristics make the membrane suitable for the purposes of biofluid sampling in dried form followed by sample solubilisation and quantitative analysis. Non-cellulose (glass fiber) membrane, as part of a DBS conventional card format, was acclaimed to be less influenced by the hematocrit effect than cellulose membrane cards.[12,13] However, it was also reported that the glass fiber material used for the production of such cards was fragile towards manual punching and which made it less suitable for reproducible punching.[2] The proposed new format strip is easy to work with. After sample application and its drying, the strip becomes rigid and any square piece of it can be separated by scissors with little effort.

Investigation of an antigen distribution along a strip containing dried sample

For good assay reproducibility and accuracy, any part of a narrow strip used in the proposed microsampling format should contain equal amounts of the analysed material and therefore the target antigen. To investigate this issue, a few serum samples were applied onto a membrane strip and dried as described above. An aliquot of 100 μL was enough to saturate 7–8 marked pieces (squares) of the sampling strip (Fig. 1). Then, square pieces of a strip containing dried sample were cut-off sequentially (from square no. 1 to no. 7) starting from the point where a sample was applied and analysed by ELISA. Calibration curves for progesterone and thyroglobulin using square no. 1, no. 2 and no. 3 of strip-dried standard solutions were obtained (Fig. 3A and C). Calibration curves and results of thyroglobulin analysis of serum samples in different pieces of a strip are presented in Fig. 3A and 2B. It could be observed that for strip-dried standard solutions (Fig. 3A) and serum samples (Fig. 3B), a higher concentration of thyroglobulin was observed in the first square piece of a strip. Analogously, the strip-dried progesterone standard solutions and serum samples were analysed (Fig. 3C and D). In this case, the edge effect was not distinct. Similar edge effects were observed for other substances (Fig. 4). On average, the recovered antigen concentration on the first piece of a strip (square no. 1) was about 10% higher than that on other pieces of a strip. Probably, the effect observed relies upon the redistribution of liquid on a membrane strip while drying. As the narrow strip starts drying from its edges, redistribution of the sample results in a higher antigen concentration on the end of the strip. Therefore, for accurate

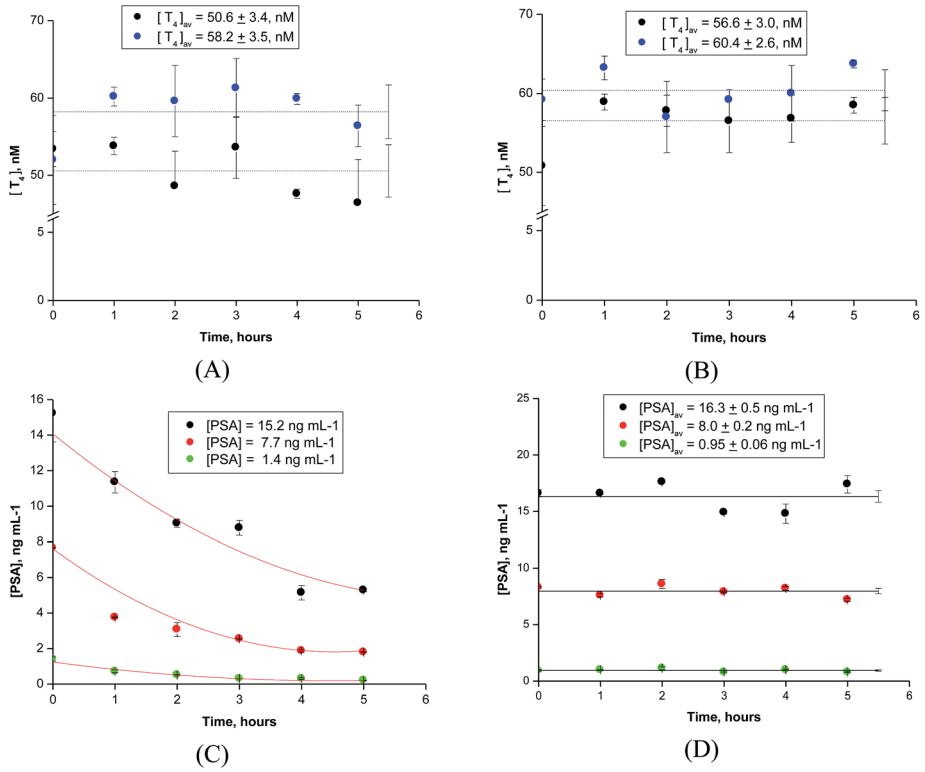

Fig. 6 Stability of thyroxine and prostate specific antigen in native (liquid) (A and C) and strip-dried human serum samples (B and D) stored at 60 °C. *x* axis – days of sample storage, *y* axis – concentration of a target antigen detected by ELISA. T4 – thyroxine; PSA – prostate specific antigen.

ELISA results, any square piece of the membrane with strip-dried standard solution/sample can be used for further analysis except for square no. 1, as shown Fig. 1, which can be easily eliminated before analysis. Moreover, a single strip that is completely wetted with biofluid can provide up to ten square pieces (0.5 × 0.5 cm) containing equal amounts of analyte. Volumetric sampling is an approach wherein a sample is volumetrically applied onto a carrier followed by whole sample analysis. In the proposed sampling format, volumetric sampling is provided by the properties of the porous membrane material used in the study (glass fiber). The membrane soaks a certain amount of liquid material per square unit. Moreover, solid glass fibers do not soak up reagents such as cellulose fiber membranes, which decrease non-specific analyte adsorption on the membrane and its penetration inside membrane fibers. Thus, this microsampling approach based on a narrow porous non-cellulose membrane strip provides volumetric sampling of a biofluid (blood serum). It was shown that fixed biofluid volume per one piece of membrane (in the form of a 0.5 × 0.5 cm square) is collected. That leads to accurate immunoassay results with low square-to-square variability of the determined antigen concentration even with manual cut-off (not more than 7.3% for square no. 2 and further), as can be observed from Fig. 3 and 4.

Investigation of antigen storage stability in strip-dried human serum samples

For transportation of biological fluids in the form of dried spots applied onto a membrane material, including postal delivery without particular conditions, for instance, cold chain, storage

Table 1 Bland–Altman method parameters for the results of ELISA analysis of native and strip-dried blood serum samples[a]

Antigen	Method	Number of samples	Bias	Upper agreement limit (+1.96 SD)	Lower agreement limit (−1.96 SD)
Progesterone	Competitive ELISA	34	0.65 nM	10.32 nM	−9.02 nM
Thyroxine	"—"	26	2.49 nM	30.61 nM	−25.63 nM
Thyroglobulin	One step sandwich ELISA	35	0.33 ng ml^{-1}	3.17 ng ml^{-1}	−2.50 ng ml^{-1}
Prostate specific antigen	"—"	24	0.53 ng ml^{-1}	1.61 ng ml^{-1}	−0.55 ng ml^{-1}
Antibodies (IgG) against *H. pylori*	Two step sandwich ELISA	38	−7.11 U ml^{-1}	7.83 U ml^{-1}	−22.05 U ml^{-1}

[a] SD – standard deviation. Bias is mean of the differences between the results of liquid and strip-dried samples. Upper and lower agreement levels calculated as bias ±1.96 SD. In Fig. 7, bias for thyroxine (A) and prostate specific antigen (B) is represented as a blue line. The upper and lower agreement limits are denoted by red lines.

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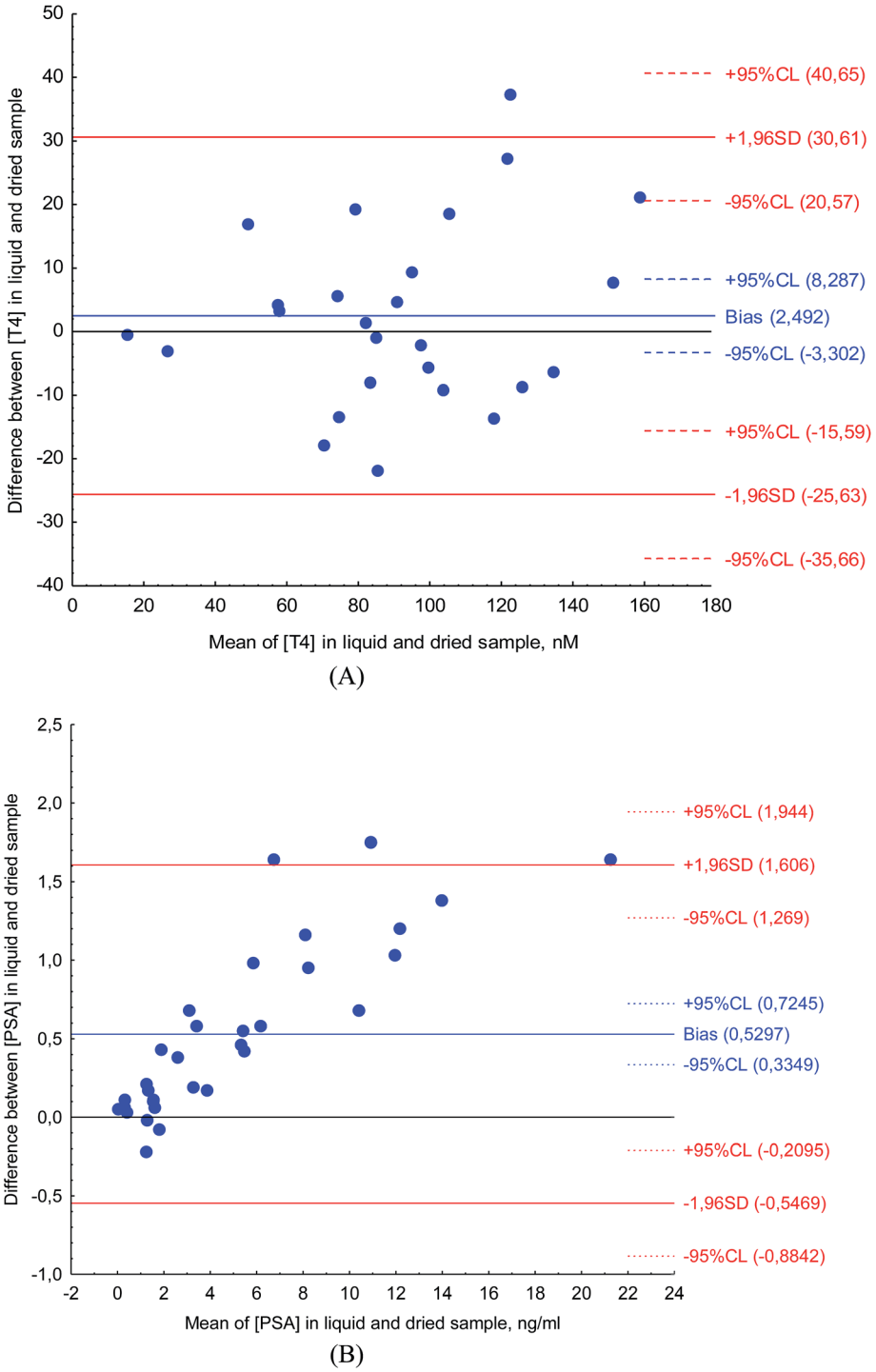

Fig. 7 Bland–Altman plot of ELISA results for liquid and strip-dried samples for thyroxine (A) and prostate specific antigen (B). Horizontal lines are drawn at the mean difference (bias) of antigen concentration in liquid and strip-dried samples and at the limits of agreement, which are defined as bias ±1.96 SD. SD – standard deviation, T4 – thyroxine; PSA – prostate specific antigen.

stability of the target antigen should be confirmed. On the next stage, the storage stability of one low (progesterone) and one high (thyroglobulin) molecular weight antigen in strip-dried human serum samples within 7 days (analogous transportation time to laboratory) was investigated (Fig. 5). The stability was investigated at ambient (20–25 °C) and elevated temperature (37 °C) taking into account the fact that shipment can be performed under any temperature conditions. During 7 days of storage at room temperature and 37 °C, the recovered antigen concentration (progesterone and thyroglobulin) did not change significantly (Fig. 5). The same pattern of 7 days storage stability was observed for other antigens such as thyroxine, prostate specific antigen and antibodies against *H. pylori* (data not shown).

As an example, the stability of low molecular weight thyroxine and high molecular weight prostate specific antigen in liquid and strip-dried samples were investigated in a model experiment with sample incubation at 60 °C (Fig. 6). It could be observed that low molecular antigen concentration in liquid and strip-dried serum samples was constant within 5 hours at 60 °C (Fig. 6A and B). While the concentration of prostate specific antigen (high molecular weight antigen of protein nature) in native (liquid) serum was reduced about two times in 5 hours at 60 °C, its concentration was stable in strip-dried samples (Fig. 6C and D). The results obtained demonstrated better storage stability of strip-dried samples over liquid samples.

Overall, the storage stability of the variety of antigens in strip-dried blood serum samples has demonstrated that the new sampling format completely meets all mentioned advantages of dried spots technology and therefore samples can be transported to the laboratory without cold chain support.

Concordance of ELISA results for native (liquid) and strip-dried samples of human blood sera

As a final step, the Bland–Altman method was used to assess the level of agreement between ELISA results for native/liquid (conventional format) and strip-dried blood sera samples prepared with using the proposed narrow strip microsampling format. A range of agreement was defined as bias ±1.96 SD. About 30 samples per each antigen (test system) were analysed (Table 1). In Fig. 7, the Bland–Altman plots for thyroxine and prostate specific antigen are presented. Bias of difference of ELISA results for antibodies against *H. pylori* was observed due to higher differences occurred for samples with IgG concentration of 30 U ml^{-1} and higher. The matter needs further investigation on a larger selection of samples with a particular check for optimal solubilisation conditions. For all other antigens, concordance data was obtained for liquid and strip-dried serum samples (Bland–Altman data analysis) with a bias value close to zero. The concordance data obtained has confirmed the applicability of this new format for the preparation of biological fluids in a strip-dried form for the quantitative enzyme immunoassay of low and high molecular weight antigens in human blood sera.

The new microsampling format, utilizing strip-dried samples, has the following advantages: the applied sample distributes along a strip evenly, except for the first square piece and it should be discarded. After drying, a square piece of membrane (0.5 × 0.5 cm) with the strip-dried sample can be easily cut-off by scissors along marked lines and analysed by ELISA. One strip with applied sample provides volumetric sampling because it gives up to ten pieces of carrier with equal amounts of dried sample for analysis. Moreover, while collecting non-coloured biofluids (for instance, urine, synovial fluid, cerebrospinal fluid) there is no need to saturate a carrier with visualizing agents as soon as the migrated biosample saturates the defined amount of marked pieces (squares) of the strip, and this could be easily controlled by the eye or by the volume of applied biofluid.

Conclusions

Due to homogeneity issues associated with analyte distribution across the spot and the hematocrit bias effect, dried spots technology needs to adopt new sampling formats and new portable devices for personal use, which makes the procedure more convenient for sample application and quantitative analysis. We believe that the microsampling procedure described here, utilizing strip-dried samples, meets the mentioned targets, provides a new volumetric microsampling technique, has an easy operational procedure that anyone can work with and can find potential application for quantitative analysis, for instance ELISA, in different areas such as biochemical, clinical or veterinary analysis. The proposed sample application microsampling strip is capable of collecting an accurate volume of biofluid (serum in the studied case) per square unit and any piece of a strip can be easily separated by scissors for further analysis. Moreover, a narrow strip can be directly applied into biofluid to absorb the sample so that the use of micropipettes could be omitted. Further assessment of the applicability of a new narrow strip microsampling format towards whole blood sample analysis should be performed and the hematocrit issue should be addressed in future study. Furthermore, the influence of plasma components, such as albumin and lipids on the distribution and recovery of analytes, should also be investigated.

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