Development of a one step strip test for the detection of sulfadimidine residues†

The Analyst

Ron Verheijen,* Piet Stouten, Geert Cazemier and Willem Haasnoot

State Institute for Quality Control of Agricultural Products (RIKILT-DLO), P.O. Box 230, 6700 AE Wageningen, The Netherlands

Received 22nd June 1998, Accepted 27th August 1998

The one step strip test described is a competitive immunoassay in which the detector reagent consists of colloidal gold particles coated with affinity purified polyclonal anti-sulfadimidine (SDD) antibodies. The capture reagent in the assay is an SDD–ovalbumin conjugate which is immobilised on the lateral flow membrane of the test device. In the test procedure, 150 μl (four drops) of a liquid sample (buffer, urine or milk) are brought into the sample well of the test device and allowed to migrate over the membrane. The more analyte present in the sample, the more effectively it will compete with the SDD immobilised on the membrane for binding to the limited amount of antibodies of the detector reagent. A sufficient amount of SDD in the sample will therefore prevent the binding of the detector reagent to the SDD immobilised on the membrane. Therefore, a positive sample will not show a test line in the read-out zone. With spiked buffer or calf urine this was obtained at a level of >10 ng ml^{-1} of SDD and with spiked (diluted) fresh cow milk at a level >20 ng ml^{-1} of SDD. At these levels, the test is applicable only as a qualitative assay. The presence or absence of a test line indicates lower or higher levels of SDD, respectively. The major advantages of the one step strip test are that results can be obtained within 10 min and that all reagents are included in the test device.

Introduction

In 1980, Leuvering *et al.*[1] reported the use of colloidal particles, not necessarily gold particles, as labels for immunoassays and proposed the term sol particle immunoassay (SPIA). Macromolecules such as proteins (*e.g.*, immunoglobulins) can be adsorbed on the dyed colloidal particles[2] [*e.g.*, gold (red), carbon (black), latex[3] (several colours)]. Such immunoglobulin loaded particles can be applied to make antigens directly visible. One step strip test devices, using antigen immobilised on a membrane and antibody–gold conjugates as mobile phase, are commercially available for an increasing number of antigens (high and low molecular mass). The first major target analyte for this test format was human chorionic gonadotropin (HCG) for the detection of pregnancy.

A great variety of test strip assays are now available,[2,3] *e.g.*, for the detection of hormones (pregnancy, fertility, ovulation, menopause, sexual disorder, thyroid functions), tumour markers (prostate, colorectal, *etc*.), viruses (HIV, hepatitis B and C), bacteria (*Streptococcus* A and B, *Chlamydia trachomatis*, *Treponema pallidum*, *Helicobacter pylori*, *etc*.), allergy (IgE) and cardiac monitoring (troponin T[4]). All these analytes are measured on the basis of their presence or absence. For such assays, a binding agent specific for the analyte (usually, but not necessarily, an antibody) is immobilised on the membrane. The detector reagent, typically an antibody coupled to latex or colloidal metal, is deposited (but remains unbound) in the conjugate pad. When liquid sample (urine, serum, plasma, whole blood, milk, *etc*.) is added to the conjugate pad, the detector reagent is solubilised and begins to move with the sample flow front up the membrane strip. The analyte present in the sample is bound by the antibody coupled to the detector reagent. As the sample passes over the zone to which the capture reagent has been immobilised, the analyte–detector reagent complex is trapped. Here, colour development is proportional to the amount of analyte present in the sample, which is only applicable for analytes with more than one epitope (high molecular mass analytes).

There are also commercially available assays for low molecular mass analytes, *e.g.*, for drugs of abuse, for steroid-based ovulation prediction and for progesterone in milk,[5] which are based on competitive immunoassay protocols. In this type of assay, the detector reagent can be the analyte bound to a protein or the antibody bound to latex or a colloidal metal. As the analyte containing sample and the detector reagent pass over the zone to which the capture reagent (antibody or analyte–protein conjugate) has been immobilised, some of the analyte and some of the detector reagent are bound and trapped. The more analyte present in the sample, the more effectively it will be able to compete with the immobilised analyte on the membrane for binding to the limited amount of antibodies of the detector reagent. Hence an increase in the amount of analyte in such competitive immunoassays will result in a decrease in signal in the read-out zone.

So far, these tests have been mainly intended for human diagnostics where, especially for the low molecular mass analytes, relatively high concentrations of analytes are measured. In food diagnostics, however, lower detection levels are needed. For the detection of veterinary drug residues, for example, detection limits at ppb level (nanograms of analyte per gram of sample) are necessary. In cooperation with a diagnostic company [Princeton BioMeditech (PBM), Princeton, NJ, USA], an SPIA based one step strip test for SDD, using polyclonal anti-SDD, was developed.[6] By adjusting the amount of essential reagents in the strip test device, two types of competitive tests (low- and high-level tests) were prepared. In the low-level test, almost complete disappearance of the signal in the read-out zone was obtained at a level of >100 ng ml^{-1} and in the high-level test at a level of >1000 ng ml^{-1}. The low- and high-level tests were intended for the monitoring of plasma samples and for urine samples, respectively. Owing to the higher levels of SDD and the presence of cross-reacting metabolites in plasma

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

and especially in urine compared with tissue, levels of 200 ng ml^{-1} in plasma and 1000 ng ml^{-1} in urine would compare with about 20–40 ng g^{-1} of the parent compound in tissue.[7] These action levels included safety margins for the variation of SDD concentrations and were recommended for the control on a maximum residue level (MRL) of 100 ng g^{-1}.

In this study, with SDD as the target analyte, all ingredients of the test device were reinvestigated and/or selected with the aim of acquiring expertise about all aspects of the test principle to be used and to develop as sensitive a test as possible. With a higher sensitivity, such tests could also be suitable for analytes with lower MRLs.

Experimental

If not stated otherwise, procedures were performed at room temperature.

Chemicals and materials

Sheep serum was obtained from the DLO Institute for Animal Science and Health (ID-DLO, Lelystad, The Netherlands). Bovine γ-globulins (BGG), bovine serum albumin (BSA), 1-ethyl-3-[3-(dimethylaminopropyl]carbodiimide hydrochloride (EDC), sodium azide, ovalbumin grade V, sulfadimidine (SDD), tetrachloroauric(III) acid trihydrate ($HAuCl_4 \cdot 3H_2O$), Igepal CA-630 (octylphenoxypolyethoxyethanol) and purified rabbit anti-sheep IgG (whole molecule) (2.5 mg ml^{-1}) were obtained from Sigma (St. Louis, MO, USA). Acetic anhydride, D-(+)-glucose monohydrate, glutaraldehyde (25% m/m solution in water), glycerol (extra pure), potassium carbonate (extra pure), lactose monohydrate, sodium acetate, sodium carbonate, sodium dihydrogenphosphate monohydrate, sodium ethylenediaminetetraacetate, sodium hydrogencarbonate, sodium hydroxide, trisodium citrate dihydrate and sucrose were supplied by Merck (Darmstadt, Germany). The bicinchoninic acid (BCA) protein assay and PharmaLink immobilization kit were obtained from Pierce (Rockford, IL, USA) and sodium dodecyl sulfate and Visking dialysis tubing (44110, 20/32, diameter 16 mm) from Serva (Heidelberg, Germany). Both a surfactant starter kit (manufactured by Pragmatics) and diagnostic materials kit were obtained from BioDot (Irvine, CA, USA). AE 100 (12 μm) nitrocellulose membrane strips (300 × 25 mm) on a Mylar backing were supplied by Schleicher and Schuell (Dassel, Germany), 5 mm Acrodisc filter by Gelman Sciences (Ann Arbor, MI, USA) and Centriprep-30 concentrators by Amicon (Beverly, MA, USA). Varian (Harbor City, CA, USA) supplied a Bond Elut reservoir (8 ml). Centriprep concentrators (Type 30) were supplied by Amicon.

Equipment

The BioDot system consisted of two BioJets Quanti3000 attached to a BioDot XYZ-3000 dispensing platform. An AZCON Sur-Size automatic guillotine cutter (Model SS-4) was supplied by AZCON (Elmwood Park, NJ, USA). Sealing equipment (Magneta, Model 421) was purchased from Kenosha (Amstelveen, The Netherlands).

Antibodies

Preparation of the immunogen, by coupling of succinylated SDD to BSA, and the raising of polyclonal antibodies in a rabbit against the SDD-BSA obtained conjugate, have been described previously.[7] Specific anti-SDD immunoglobulins were isolated from the obtained complete rabbit serum (pool 464B) by affinity chromatography with immobilised SDD.

Preparation of an affinity column with immobilised SDD

SDD was coupled to PharmaLink Gel, obtained from a PharmaLink immobilization kit, as follows. A 2 ml volume of gel slurry of 50% m/v DADPA (diaminodipropylamine)-activated 6% cross-linked agarose in 0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 4.7) (PharmaLink coupling buffer) was poured into a Varian column. After draining the storage solution, the gel was washed with 20 ml of demineralized water (water) and transferred into a PharmaLink reaction bottle. After addition of 2 ml of 0.2 M sodium acetate and 2 ml of acetic anhydride, the mixture was gently swirled for 30 min at 4 °C on a rock and roll shaking platform. Subsequently, another 1 ml of acetic anhydride was added, followed by another 30 min of incubation. After transferring the gel slurry into the column, the reaction mixture was drained completely and washed successively with 10 ml of water, 10 ml of 0.1 M sodium hydroxide and approximately 25 ml of water to pH 7. Coupling was performed in a PharmaLink reaction bottle by suspending the gel in 2 ml of 0.1 M MES (pH 4.7) (PharmaLink coupling buffer) and adding 20 mg of SDD in 500 μl of methanol and 60 mg of EDC. The reaction mixture was incubated for 24 h while being swirled on a rock and roll shaking platform. As the pH of the reaction mixture increased slightly during the first 8 h of the incubation, 1 M hydrochloric acid was added at regular intervals to maintain a pH of approximately 5. After the incubation, the gel slurry was once again transferred into the column. The reaction mixture was drained completely, then the gel was washed successively with 20 ml of water, 20 ml of 0.1 M sodium acetate, 20 ml of 0.5 M sodium hydrogencarbonate, 20 ml of water, 6 ml of 0.1 M acetic acid and 10 ml of phosphate buffered saline (PBS) (pH 7.4), containing 0.02% m/v sodium azide.

Isolation of rabbit anti-SDD specific antibodies

The SDD affinity column was washed with 20 ml of PBS to remove the sodium azide. For absorption, 2 ml of 464B serum were diluted with 3 ml of PBS and poured on to the column. The diluted serum was allowed to drain completely, then it was poured once again on to the column. Washing was performed with 10 ml of PBS, followed by desorption with 15 ml of 0.1 M acetic acid. During desorption, the pH of the eluate was kept between 5 and 7 by the addition of small amounts of 1 M sodium hydroxide. The entire eluate of approximately 15 ml was adjusted to pH 7.5 with 1 M sodium hydroxide solution and concentrated to about 2 ml in a Centriprep-30 concentrator according the manufacturer's operating instructions. Centrifugation was performed in a Roty III centrifuge with a swinging-bucket rotor. The purified and concentrated anti-SDD antibody preparation was stored in 1 ml aliquots at −20 °C. The protein concentration of the antibody preparation obtained was determined using the BCA protein assay. Regeneration of the column was performed directly after elution by washing with 10 ml of PBS until the pH of the eluate was neutral.

Isolation of total IgG from sheep serum

After mixing 10 ml of raw sheep serum with 20 ml of PBS, 30 ml of saturated ammonium sulfate solution (80 g in 100 ml of water) were slowly added with constant stirring. After standing for 30 min, immunoglobulins were collected by centrifugation for 10 min at 11 000 *g* in a Sigma-320K centrifuge with a swinging-bucket rotor. After dissolving the pellet in as small a volume of PBS as possible (a few millitres), the solution was dialysed for 48 h at 4 °C against PBS. The protein content was determined using the BCA protein assay. The IgG obtained was stored in small aliquots at -20 °C until used.

Protein quantification

The concentration of proteins in solution was determined by the BCA protein assay (Pierce) in microtitre plates using either ovalbumin or bovine γ-globulins (BGG) as standard proteins.

Preparation of colloidal gold particles (G40)

Colloidal gold with an average particle diameter of 40 nm (G40) was prepared by controlled reduction of gold chloride with sodium citrate using the procedure described by Frens.[8] The solutions of the reagents were prepared in water that was purified using a Milli-Q 185 Plus water purification system (Millipore, Bedford, MA, USA). The water obtained in this way had a resistivity of > 18.2 MΩ cm. The procedure for preparing a G40 suspension was as follows: 100 ml of 0.01% m/v tetrachloroauric(III) acid trihydrate were heated to boiling under reflux conditions, then 1 ml of 1% m/v trisodium citrate dihydrate solution was added under constant stirring. After about 25 s the slightly yellow solution turned faintly blue (nucleation). After approximately 70 s the blue colour then suddenly changed to brilliant red, indicating the formation of monodispersed spherical particles. The solution was allowed to boil for another 5 min to complete the reduction of the gold chloride. The absorbance, measured at 540 nm (A_{540}) of such G40 suspensions was about 0.9. The size of gold particles obtained in one such procedure was checked using transmission electron microscopy (IPO-DLO). The particle diameter was found to vary between 38 and 43 nm. When supplemented with 0.05% m/v sodium azide, the G40 suspensions obtained could be stored at 4 °C for several months.

Preparation of antibody-coated colloidal gold (detector and control reagent)

The pH of 50 ml of G40 sol with an A_{540} of approximately 0.9 was adjusted to 8.5 with 0.2 M potassium carbonate solution. Coating of the G40 particles was performed by incubating 7.5 μg of antibody per millilitre G40 sol for 45 min while being gently swirled on a rock and roll shaking platform. The antibodies used were either affinity purified rabbit anti-SDD antibodies obtained from serum 464B (test detector reagent) or rabbit anti-sheep IgG (control detector reagent). For both types of antibody, the minimum protecting amount (MPA), *i.e.*, the minimum amount of protein needed to protect the sol from salt-induced precipitation, was found to be approximately 5 μg per millilitre of G40 sol as determined by the procedure of Horisberger and Rosset.[9] After coating, the sol was further stabilised by adding 5 ml of 1% m/v bovine milk casein mixture (Na, K and Ca salts, obtained from the Department of Quality Control, RIKILT-DLO, Wageningen, The Netherlands) that had been adjusted to pH 8.5 with 0.2 M potassium carbonate. After incubation for 60 min on a rock and roll shaking platform, 27.5 ml of the detector or control reagent were centrifuged in a 50 ml Greiner tube over a discontinuous glycerol gradient consisting of 5 ml of 80% v/v glycerol in water (pH 8.5) and 7.5 ml of 50% v/v glycerol in water (pH 8.5). Centrifugation was performed for 45 min at 2000 *g* in a Roty III centrifuge with a swinging-bucket rotor. Under these conditions, the detector or control reagent was found entirely in the 50% v/v glycerol layer. The particles were harvested by piercing the centrifuge tube with a syringe needle and removing the dark red layer sideways out of the tube. The 4–5 ml of detector reagent obtained were then diluted to 15 ml with water (pH 8.5), transferred into a Centriprep-30 concentrator and centrifuged in a Roty III centrifuge for 15 min at 1500 *g*. This was repeated four times, then in an additional run the reagent was further concentrated to 2 ml. After addition of 400 μl of 6% m/v bovine milk casein (pH 8.5) in water (1% m/v end concentration) and 12 μl of 10% m/v sodium azide (0.05% m/v end concentration), both the detector and control reagents were stored at 4 °C until used.

The following composition of the reagent mixture was applied on the conjugate pads (Whatman Glass Fiber Paper with F 075-17 binder, 6 × 4 mm): 30 μl of anti-SDD antibody coated G40 sol (test detector reagent), 50 μl of rabbit anti-sheep coated G40 sol (control detector reagent), 20 μl of 12% m/v sucrose in water, 4 μl of 1% v/v surfactant S24 in water and 16 μl of water. Each conjugate pad was loaded with 12 μl of the detector reagent mixture and dried for 2 h at 40 °C. Thereafter, the pads were immediately assembled into the test devices.

Preparation of capture reagents

Test capture reagent. The SDD–ovalbumin conjugate that was used as the test capture reagent was prepared as follows: 10 mg of SDD were dissolved in 1 ml of methanol and added to 99 ml of PBS (solution I). Solution II was prepared by dissolving 300 mg of ovalbumin in 30 ml of PBS, after which the solution was filtered over a 5 μm Acrodisc filter (Gelman Sciences, Model 4489). The reaction mixture consisted of 30 ml of solution I, 30 ml of solution II and 30 ml of PBS to which 900 μl of 25% m/m glutaraldehyde solution were added slowly with constant stirring. Approximately 10 min after addition of the glutaraldehyde, the clear solution took on a yellowish appearance. The mixture was stirred for 4–5 h and exhaustively dialysed against PBS for 4–5 days.

The dialysate was then concentrated to 50 mg ml^{-1} protein using a Centriprep-30 concentrator and stored in 1 ml aliquots at −20 °C.

Control capture reagent. Ammonium precipitated total sheep immunoglobulin fraction was used as the control capture reagent.

Immobilization of capture reagents

Both the test and control capture reagent were dispensed on the nitrocellulose membrane strips (300 × 25 mm) at a concentration of 0.5 μg cm^{-1} protein using a BioJet Quanti3000, attached to a BioDot XYZ-3000 dispensing platform. After drying the membrane strips for 2 h at 40 °C, blocking was performed by incubating the strips for 30 min in 0.9 g l^{-1} sodium dihydrogenphosphate monohydrate, 2% m/v non-fat milk powder and 0.02% m/v SDS. After blocking, the membrane strips were washed three times for 5 min with 0.9 g l^{-1} sodium dihydrogenphosphate monohydrate containing 0.01% (v/v) surfactant (S24). After drying for 2 h at 40 °C, the membrane was divided into 6 mm strips using the cutter. The strips were stored under dry conditions (desiccator) at room temperature until used.

Test production

As shown in Fig. 1, the test strip consists of a backing plate on which the nitrocellulose membrane, detector conjugate pad, sample pad and absorbent pad are pasted. In all assays Gelman Type 133 cellulose was used for both the absorbent and sample pads. The test strip as shown in Fig. 1 was mounted in a plastic housing provided with a sample well and read-out zone and sealed in a pouch in the presence of a drying agent.

Test procedure

The principle of the assay is depicted in Fig. 2. Four drops (about 150 μl) of the liquid sample were pipetted into the sample well and allowed to migrate upwards. After 10 min, the test result was judged. When SDD was present in the sample, it

competed with the immobilised SDD on the capture line for the limited amount of antibodies of the detector reagent. When a sufficient amount of SDD was present, it therefore prevented the binding of detector reagent on the membrane. Hence a positive sample gave no visible test line in the sample read-out zone. When the test is performed properly, the control test line is always visible.

Results and discussion

A polyclonal rabbit antiserum raised against an SDD–BSA conjugate was used as the antibody source. This serum (pool 464B) was previously successfully applied for the development of a sensitive enzyme linked immunosorbent assay (ELISA) for SDD and its major metabolites.[7] In general, a polyclonal rabbit serum contains about 60 mg ml^{-1} of protein, of which approximately 10 mg is IgG and about 1 mg of this IgG is specific towards the hapten. The specific anti-SDD IgGs were isolated by affinity chromatography using a column with immobilised SDD as described in the Experimental section. In this way, two batches of affinity purified antibodies were prepared. For each batch the procedure was performed five times (10 ml of raw serum per batch). The amount of protein in these batches, as determined by the BCA protein assay, was found to be 0.76 mg ml^{-1} in an end volume of 8 ml (= 6.1 mg of specific IgG) and 0.5 mg ml^{-1} in an end volume of 14 ml (= 7.0 mg of IgG), respectively.

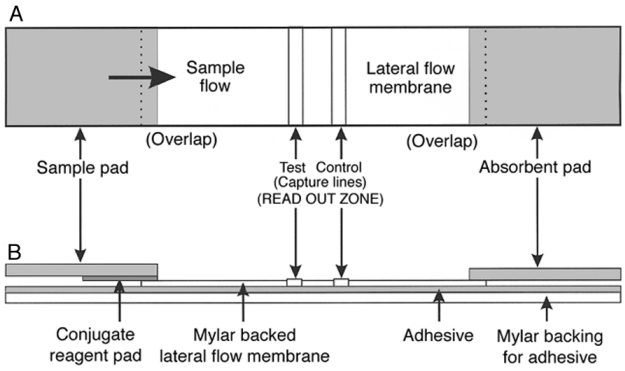

Fig. 1 Schematic diagram of a test strip showing its several components. A, Top view; B, cross-section. A complete one step strip test device consists of a test strip as shown here, packed in a plastic housing.

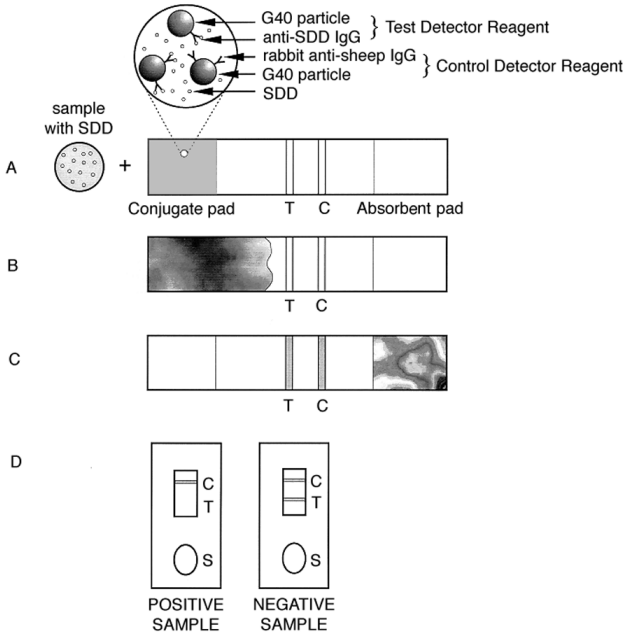

Fig. 2 Principle of a one step strip test for the detection of low molecular mass analytes. A, Sample is brought on to the sample pad (not shown) and SDD present in the sample is bound by the test detector reagent in the conjugate pad. This pad contains two distinct detector reagents: gold particles coated with specific anti-SDD antibodies (test detector reagent) and gold particles coated with rabbit anti-sheep IgG (control detector reagent). B, Control detector reagent, SDD-bound test detector reagent, free SDD or free test detector reagent migrates up the strip with the sample. C, As the sample passes over the capture zones, test detector reagent (anti-SDD IgG) that is free of analyte binds to the test capture reagent (SDD–ovalbumin conjugate) at test line T, whereas the control detector reagent (rabbit anti-sheep IgG) binds to the control capture reagent (sheep IgG) at control line C. D, Reading the results. Colour develops at the capture line T if the sample contains no SDD (negative sample), whereas no colour is seen if the sample contains SDD (positive sample). Colour develops at the negative control line (C) if the test has been used properly.

To prepare the detector reagent, 7.5 μg of the affinity purified antibodies were used per millilitre of G40 sol. After coating and stabilization with a casein mixture, the protein sol preparation obtained was centrifuged over a discontinuous glycerol gradient in a 50 ml Greiner tube. The conditions of centrifugation were chosen such that the coated G40 particles appeared entirely in the 50% v/v glycerol layer. In this way, the particles were separated from large and/or aggregated particles that were centrifuged through the 80% v/v glycerol layer on to the bottom of the tube and also from loosely or non-bound proteins (free antibody and casein proteins) that remained in the aqueous upper layer of the gradient. The particles in the 50% v/v glycerol layer were recovered by piercing the centrifuge tube with a syringe needle and removing the dark red layer sideways out of the tube. In this way, 4–5 ml of concentrated G40 sol in 50% v/v glycerol were obtained from each 25 ml of the original sol. For loading on the conjugate pad, the detector reagent had to be washed and further concentrated. This was achieved by four successive washes of the G40 sol with water (pH 8.5) to a 15 ml end volume, each wash followed by a concentration step in a Centriprep-30 concentrator. Removal in this way of (most of) the glycerol from the G40 sol appeared to be essential as, at too high a concentration, the glycerol caused problems in drying the conjugate pads and in running the test, *i.e.*, slow migration of the sol over the membrane and insufficient binding of the test detector reagent at the capture line in the case of a negative sample. Finally, in an additional run the G40 sol was concentrated to a volume of 2 ml. After addition of a bovine milk casein mixture, the sol was stored at 4 °C until further use. By this procedure, the initial sol was concentrated 10-fold. A comparable procedure was applied to produce the control sol. Both G40 sols were mixed in the appropriate ratio, followed by addition of sucrose and surfactant (see Experimental). Sucrose as additive was tested at concentrations of 0.5, 1.0, 2, 3 and 4% m/v respectively. The best results were obtained with 2% m/v sucrose: concentrations lower than 2% m/v resulted in poor solubilization of the detector reagent from the conjugate pad and consequently in less intensively stained capture lines, and at concentrations higher than 2% m/v the viscosity of the detector reagent apparently became too high, resulting in an irregular and slow moving flow front and capture lines that were not stained uniformly. The use of glucose or lactose as additives at concentrations ranging from 0.5 to 5.0% m/v resulted in poor solubilization of the detector reagent from the conjugate pad and consequently in weakly stained capture lines.

The influence of the surfactant (S24) concentration in the detector reagent mixture was also tested. It was found that solubilization of the detector reagent from the conjugate pad was optimum at S24 concentrations between 0.02 and 0.05% v/v.

Pads made from different materials (6 × 4 mm) were loaded with 12 μl of the detector reagent mixture and dried for 3–4 h at 40 °C. The following materials, present in the BioDot diagnostic materials kit, were tested for use as conjugate pads: rayon, non-woven 5S (Schleicher and Schuell), Cellulostic

Paper 593 (Schleicher and Schuell), Glass Fiber Matrix T5 NM-10 (Millipore), Glass Fiber Paper with F 075-17 binder (Whatman) and Glass Fiber 8980 (Gelman). Considering the ease with which the conjugate pad was wetted, the capacity of the pad to absorb fluid, the distribution of the detector reagent on the pad and the amount of detector reagent that remained on the pad after running the assay, the Whatman Glass Fiber Paper with F 075-17 binder was chosen as the most appropriate pad for further use.

After immobilization of the capture reagents on the nitrocellulose membrane, the reactive groups on the membrane had to be blocked. The effect of several types of blocking buffers on the lateral flow rate of the nitrocellulose strip and on the intensity of the capture line was determined. The best results were obtained with either Blocker BLOTTO in PBS (Pierce, 37526) or with 2% m/v non-fat milk powder supplemented with 0.02% m/v sodium dodecyl sulfate (SDS). In the present study, milk powder was chosen as the blocking agent, mainly because of its low cost. As an excess of blocking agent may dramatically interfere with the membranes capillary flow properties, unadsorbed blocking agent had to be removed by washing the membrane in a weak buffer solution. To promote uniform rewetting of the blocked membrane, the presence of a low concentration of surfactant is essential. Washing of the blocked membrane was performed by swirling the membrane three times for 5 min on a rock and roll platform in a solution of 0.9 g l^{-1} sodium dihydrogenphosphate monohydrate (pH 7.5), supplemented with different surfactants. The following surfactants (all at a concentration of 0.025% m/v) were tested: SDS, Standapol ES-1 (S7), Triton X-100 (S14), Triton X-305 (S15), Tween 20 (S19) and Surfactant 10G (S24). The S-numbers refer to the surfactants present in the BioDot surfactant starter kit. After washing, the excess of buffer was removed from the membranes with a piece of filter-paper. After drying for 2 h at 40 °C, each membrane (25 × 300 mm) was divided into strips (25 × 6 mm). When monitoring the appearance of the test capture line on the strips, the highest staining intensity and best sharpness of the line were obtained with washing buffer containing S24. In an additional experiment, washing buffer with different concentrations of S24 (0.01 , 0.025 and 0.05% v/v) was tested and no significant difference was found. Therefore, the lowest concentration tested, *i.e.*, 0.01% v/v, was applied in the washing buffer in all further experiments.

All test ingredients (nitrocellulose, gold conjugate pad and sample and solvent absorbent pads) were pasted on to the backing plate and mounted in the plastic housing. This ready to use strip test device was sealed in a pouch in the presence of a bag of drying agent.

The lower detection limit (LDL) is defined here as the amount of SDD in the sample solution that just causes total invisibility of the capture line. The LDL of the test was first determined using different amounts of SDD in 200 μl of PBS. Although the presence of 2.5 ng of SDD per millilitre of PBS already gave a considerable decrease in the assay signal, the LDL was estimated as 10 ng of SDD per millilitre of PBS (10 ppb).

Further, five calf urine samples (pH ≈ 7) and ten bovine urine samples (pH 7.8–8.4) were tested. Also with urine the LDL of the assay was found to be 10 ppb.

When running the assay with either PBS or urine samples, test results were visible in approximately 5 min.

When undiluted milk samples were tested, it took a long time for a 200 μl sample to be completely absorbed by the sample pad. Consequently, the assay running time was extended to 20–25 min. A sample volume of 100 μl, on the other hand, appeared to be insufficient for running the assay, as concluded from the incomplete solubilization of the detector reagent from the conjugate pad and the abrogated lateral flow on the membrane during the assay performance. The most appropriate sample volume for milk samples in these assays was found to be 150 μl. The assay could then be performed in approximately 10 min. Only when using diluted milk samples (*e.g.*, 1:1 in PBS), could the assay running time be brought back to 5–10 min.

For spiked milk samples, the LDL of the assay appeared to be approximately 20 ng of SDD per millilitre of milk. These observations, however, only apply to fresh milk. When milk samples were used that had been cooled, frozen or kept at room temperature for a long time (8 h or longer), the sample pads became silted up with milk-fat, resulting in a considerable redardation to even complete abrogation of the lateral flow. In an attempt to make old milk samples suitable again for use on a strip test, such samples were submitted to several treatments. In doing so, some improvement concerning the assay time was observed when the samples were first heated to 40 °C for about 10 min and then cooled to 20 °C. However, the use of such heated samples also resulted in unacceptably long assay times of 30 min or more. In another attempt to make old milk samples more suitable for being used in the strip test, several surfactants (SDS, S5, S22, S24 and Igepal CA-630) were tested at concentrations of 0.05 and 0.1% m/v or v/v. With none of these surfactants could the assay running time be improved. Dilution of the samples with PBS (1:4 v/v and higher) appeared to be the only method by which the assay time could be shortened to acceptable values (5–10 min).

The results of this study demonstrate that the one step strip test works for a veterinary drug at the low ppb level in buffer, urine and diluted milk. However, when using a polyclonal antiserum, the amount of tests to be produced is limited (approximately 2500–3000 tests per millilitre of raw serum). In the future, the work will be continued using monoclonal antibodies and combinations of assays in panel tests will be studied.

Acknowledgement

The authors thank the Department of Electron Microscopy of IPO-DLO (Wageningen, The Netherlands) for performing the transmission electron microscopy.

References

1 J. H. W. Leuvering, P. J. H. M. Thal, M. Van der Waart and A. H. W. M. Schuurs, *J. Immunoassay*, 1980, **1** (1), 77.
2 *Syllabus of a Two-day Seminar on Solid Phase Membrane-Based Immunoassays, Paris, September 25–26th, 1997*, Millipore Corporation, Bedford, MA, 1997.
3 *Syllabus of The Latex Course, London, October 1–3rd, 1997*, organised by Bangs Laboratories, Inc., Fishers, IN, USA, 1997.
4 M. Mullerbardorff, H. Freitag, T. Scheffold, A. Remppis, W. Kubler and H. A. Katus, *Circulation*, 1995, **92** (10), 2869.
5 M. P. A. Laitinen and M. Vuento, *Acta Chem. Scand.*, 1996, **50**, 141.
6 W. Haasnoot, K.-A. Kim, G. Cazemier, W. Kang and J. Kang, in *Proceedings of the EuroResidueIII Conference, Veldhoven, May 6–8, 1996*, ed. N. Haagsma and A. Ruiter, University of Utrecht, Faculty of Veterinary Medicine, Utrecht, 1996, p. 461.
7 W. Haasnoot, G. O. Korsrud, G. Cazemier, F. Maneval, H. J. Keukens and J. F. M. Nouws, *Food Addit. Contam.*, 1996, **13** (7), 811.
8 G. Frens, *Nature (London) Phys. Sci.*, 1973, **241**, 20.
9 M. Horisberger and J. Rosset, *J. Histochem. Cytochem.*, 1977, **25**, 295.

Paper 8/04695F