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An in-house ELISA method for measuring surfactant protein A

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Abstrcat

An in-house enzyme-linked immunoabsorbant assay (ELISA) for SP-A was successfully developed using in-house polyclonal anti SP-A and a commercial polyclonal anti-rabbit immunoglobulin horse-radish peroxidase conjugate system. The standard curve, generated by using 50 ng of SP-A to coat the plate and 1:500 dilution of polyclonal anti SP-A as a primary antibody, was linear for concentrations of SP-A ranging from 4 μg/l to 4000 μg/l and reproducible. Results of recovery study of SP-A from a known sample of tracheal aspirate ranged from 94% - 114%. Intra- and inter-assay coefficients of variations were 2.7% and 5.6% respectively for a known sample of tracheal aspirate. Interference study showed that tracheal aspirate did not interfere with the assay. The assay developed was intended to be used for SP-A measurement in tracheal aspirates obtained from neonates with and without respiratory distress syndrome.

Key words: SP-A, immunoassay.

INTRODUCTION

Pulmonary surfactant stablizes the alveoli by lowering the surface tension on expiration and preventing alveolar collapse. Deficiency of pulmonary surfactant in the airways of infants is associated with the development of respiratory distress syndrome (RDS).[1] Surfactant is a lipoprotein complex consisting of phospholipid (about 80%) and protein (about 10%) components.[2] The optimal activity of lung surfactant depends on the presence of specific proteins.[3] SP-A remains the major surfactant-associated protein found in human lungs.[4,5] Measurement of human SP-A levels is important in the investigation and diagnosis of SP-A deficiency. The aim of the present study was to develop an in-house enzyme-linked immunoassay for quantification of SP-A.

METHODS AND MATERIALS

Polyclonal anti-SP-A

Polyclonal anti SP-A was raised in two 3-4 month old rabbits by the modified methods of King *et al.*[6], using purified human SP-A obtained from amniotic fluid.[7] 1 mg of purified SP-A was added to 1 ml of Freund's complete adjuvant and injected intramuscularly into the rabbits. The first and second booster were given intramuscularly two and three weeks later using 0.2 mg SP-A without adjuvant. The third booster were given intravenously two weeks after the second booster and blood was collected one week after the third booster. Serum was obtained from blood by centrifuging at 1500 *g* for 15 minutes.

Commercial antibody

Polyclonal anti-rabbit immunoglobulin horse-radish peroxidase conjugate was purchased from Dako, Denmark. The commercial antibody is stable for more than one year if kept at 4°C at all times as recommended by the manufacturers.

Buffers

Chemicals used were purchased from Sigma Co., USA and consisted of:

- ***Phosphate buffered saline (PBS, pH 7.4):*** NaCl 120 mmol/l, KC1 2.7 mmol/l, phosphate buffer 10 mmol/l.
- ***Assay buffer:*** PBS containing 0.5% (w/v) bovine serum albumin, stable up to 1 month at 4°C.
- ***Coating buffer (pH 9.6):*** PBS, pH 7.4.
- ***Wash buffer:*** PBS containing 0.05% Tween 20. Stable up to 1 month at 4°C.
- ***Blocking buffer:*** PBS containing 3% (w/v) BSA. Prepared when required.
- ***Citrate-phosphate buffer (pH 5.0):*** Na_2HPO_4 7.1 g, citric acid 5.25 g. Stable up to 2 months at 4°C.
- ***Substrate buffer:*** Citrate-phosphate buffer 25

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ml, *o*-phenylene diamine dihydrochloride (OPD) 10 mg, H_2O_2 (30%, v/v) 10μl. Prepare within 30 minutes of use.
- Stopping solution: 1.25 M sulphuric acid.

ELISA procedure

The 96-well flat-bottomed microtitre plate was Nunc Immunoplate Maxisorp F96, purchased from Nunc, Roskilde, Denmark. Samples were assayed in triplicate and all incubations were done at room temperature. The principle of assay used was the antibody capture assay.[8] The assay procedure is shown in Fig. 1. Wells were first coated with a fixed concentration of SP-A and incubated for 2 hr. This was followed by 2 cycles of washing with wash buffer, thoroughly dried and the remaining binding sites were blocked with 200 p1 blocking buffer overnight. Blocking buffer was discarded and after 2 cycles of washing, the plate was tap-dried onto absorbant paper. 50 μl of standard SP-A solution/samples and 50 μl of serum anti SP-A (1:500) were added to each well. The plate was firmly covered with parafilm, and incubated for another 2 hr. Following 3 cycles of washing and thorough drying, 100 p1 of anti-rabbit immunoglobulin horse-radish peroxidase (1:1000) was added into each well. The plate was incubated for 2 hr. After another 3 cycles of washing and drying, 100 μl of freshly prepared substrate buffer was added to each well. The plate was incubated for 30 minutes in the dark. 50 μl of 1.25 M sulphuric acid was added immediately to terminate the chromogenic reaction. Absorbance was read at 492 nm with ELISA Reader EAR 400 AT (SLT Labinstrument, Austria).

Pipet 50 ng of standard SP-A into each well
Incubate for 2 hr
↓
Wash 2X with wash buffer
Pipet 200 μl 3% BSA/PBS into each well
Incubate overnight
↓
Discard BSA, tap plate dry
Pipet 50 μl standard SP-A/sample + 50 μl serum anti SP-A
Cover plate, incubate for 2 hr
↓
Wash 3X, pipet 100 p1 anti-rabbit HRP conjugate into each well
Incubate for 2 hr
↓
Wash 3X, pipet 100 μl substrate OPD buffer into each well
Incubate for 25 minutes
↓
Pipet 50 μl 1.25 M sulphuric acid into each well. Measure absorbance at 492 nm

FIG. 1: Flow-chart for SP-A ELISA.

Assay *optimisation*

A set of standard solutions of SP-A were used to coat the plate and react with a fixed amount of primary serum anti SP-A. The results were plotted (Fig. 2) and a suitable concentration that yielded a strong signal was chosen to coat the plate. Next, using this amount of SP-A concentration for coating, different dilutions of polyclonal rabbit anti SP-A were titrated. The curve was plotted (Fig. 3) and dilutions that were within the linear portion of the curve were considered suitable to be chosen.

Standard curve construction

The standard curve was generated using 50 ng of SP-A to coat the plate and 1:500 dilution of polyclonal rabbit anti SP-A as a primary antibody (Fig. 4).

Assay validation

(a) Recovery: A known tracheal aspirate sample was assayed after addition of standard SP-A at concentrations of 10, 100, 500, 2000, 3000 and 4000 μg/l. Recovery was obtained by the following formula:

$$\text{Recovery} = \frac{\text{expected concentration of SP-A}}{\text{measured concentration of SP-A}} \times 100\%$$

(b) Intra- and inter-assay precision: Triplicate study was performed for all samples and standards to reduce the imprecision of the method as we had found that single sample intra-assay precision was more than 10% and this was not acceptable.

Intra- and inter-assay precision study was performed by using a tracheal aspirate sample. For the intra-assay precision the same sample was assayed 10x in triplicate on the same day. For the inter-assay precision the sample was assayed in triplicate for the following five days. %CV was determined by using the following formula:

$$\%CV = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

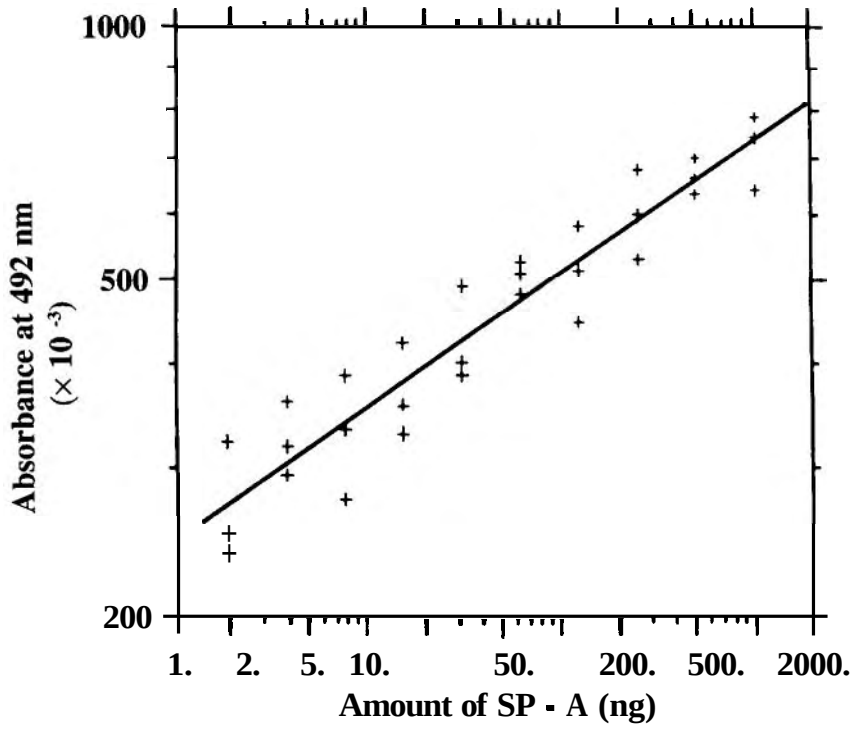

FIG. 2: Graph showing absorbance at 492 nm at different amounts of standard SP-A. SP-A chosen for coating was 50ng.

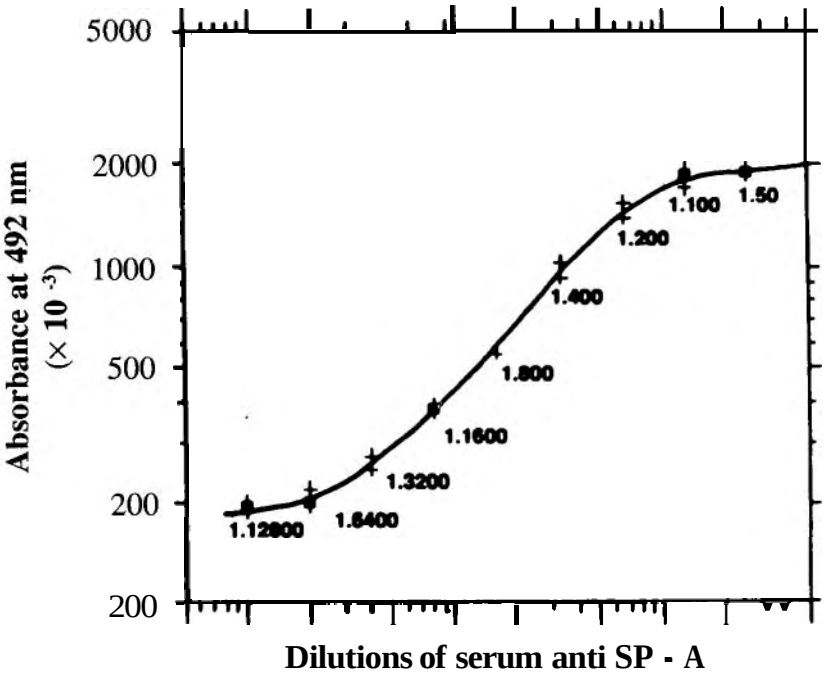

FIG. 3: Graph showing absorbance at 492 nm at different dilutions of serum anti SP-A. Suitable dilutions for the assay 1:250 to 1:800.

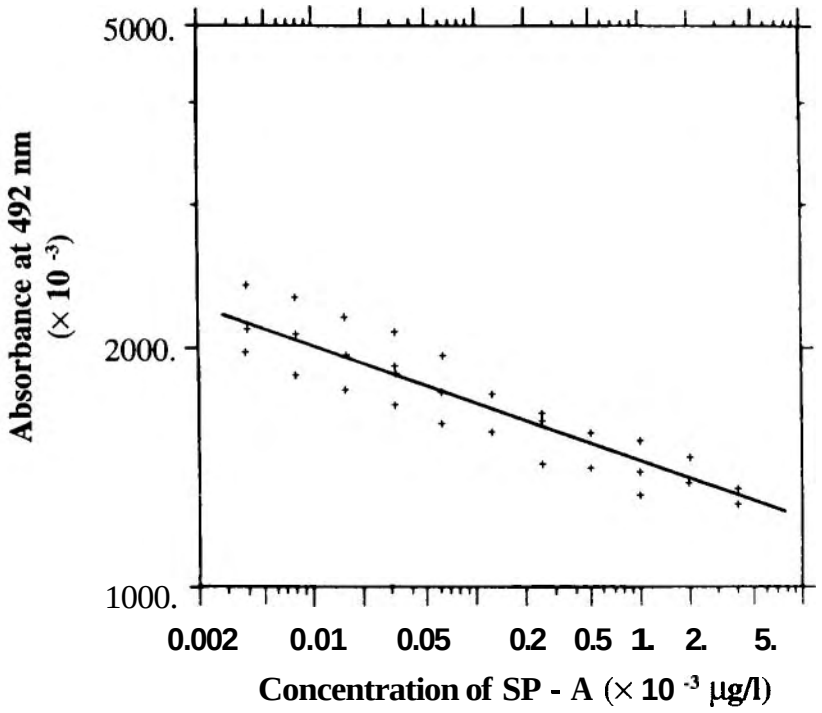

FIG. 4: Standard curve of SP-A at dilution of serum anti SP-A 1:500.

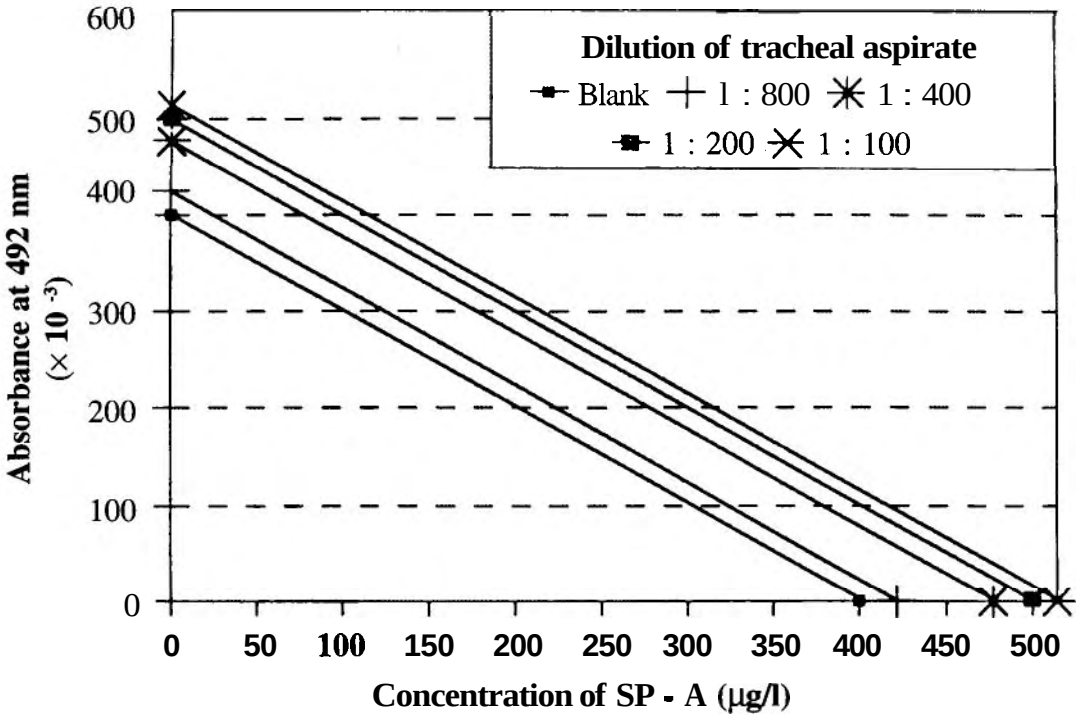

FIG. 5: Simple regression analysis on absorbance at 492 nm versus dilution of tracheal aspirate and blank.

TABLE 1: Recovery studies for the assay from a known sample of tracheal aspirate

Initial SP-A (μg/l)	Added SP-A (μg/l)	Expected (μg/l)	Measured (μg/l)	Recoveries (%)
58	10	68	64	94
58	100	158	180	114
58	500	558	589	105
58	2000	2058	2160	105
58	3000	3058	3081	101
58	4000	4058	4298	106

(c) Interference: Interference study was carried out by mixing SP-A standards with tracheal aspirate fluids at dilution of 1:100, 1:200, 1:400 and 1:800. The curves obtained were compared to the standard curve generated by using SP-A standard in assay buffer (blank) (Fig. 5).

If the tracheal aspirate contain subtances which interfered with the assay, the dilution curves would not be linear or parallel to the standard curve.

Statistics

RIA-CALC program (Wallac Oy, USA) was used to generate the standard curve and measure the samples later. Standard curve was generated by using log-log linear regresion and results of sample were obtained by calculating the mean concentration of triplicate based on the standard curve generated.

Unpaired t test was performed by using Statgraphic program (STSC,Inc, USA) for comparison between both mean concentration of SP-A in the tracheal aspirate sample used for intra- and inter-assay precision study and simple linear regression analysis was performed for the interference study of tracheal aspirate on SP-A standard.

RESULTS

We have chosen the coating SP-A at a concentration of 50 ng (Fig. 2) and the primary polyclonal rabbit anti SP-A at a dilution of 1:500 (Fig. 3) to construct the standard curve. Repeated assays showed that the standard curve was reproducible with a linear range of 4 μg/l to 4000 μg/l (Fig. 4). Progressive dilutions of a known tracheal aspirate when added to SP-A standard showed a linear standard curve parallel to that of the blank. This indicates that tracheal aspirates do not interfere with the SP-A assay.

TABLE 2: Intra- and inter-assay precision studies for the assay

Precision for a known Tracheal Aspirate	
Intra-assay precision	
Mean concentration of tracheal aspirate	%CV
'146.8 μg/l (n=10)	2.7
Inter-assay precision	
Mean concentration of tracheal aspirate	%CV
'134.2 μg/l (n=5)	5.6

* statistically significant at $p < 0.05$

Otherwise, the linear standard curve would not be linear or parallel.

Results of recovery and intra- and inter-batch precision study are shown in Table 1 and Table 2 respectively. Recoveries between 94% - 114% were obtained with addition of SP-A standards of concentration ranging from 10 μg/l to 4000 μg/l to a known concentration of tracheal aspirate. The intra-assay coefficient of variation (CV) for a mean concentration of tracheal aspirate at 146.8 μg/l was 2.7% (n=17) whilst the inter-assay CV for a mean concentration of tracheal aspirate at 134.2 μg/l was 5.6% (n=5) respectively. The mean concentrations from intra- and inter-assay precision studies showed a statistically significant difference ($p<0.05$).

DISCUSSION

Compared to the method performed by Wali et *al*,[9] the principle used in this paper was antibody capture assay. The microtiter plates were first saturated with SP-A. The SP-A added (in standard or samples) will compete with SP-A coated in the plate for anti SP-A. Higher

absorbance would be obtained if there was less SP-A in the solution and vice versa.

The %CV for intra- and inter-assay precision were 2.7% and 5.6% respectively. These results were comparable to the SP-A assay developed by Wali *et al*[9] in which the %CV obtained were less than 6%. The difference between the intra-assay CV and inter-assay CV is statistically significant. This may reflect the inherent variability of the inter-assay. The difference is small and would not have practical significance. This variability may be further reduced by a double sandwich ELISA. A suitable concentration of monoclonal guinea pig anti SP-A could be used for coating the plate. After washing SP-A could be added to the plate. This could be followed by an incubation step with a suitable dilution of rabbit anti SP-A after washing. After another cycle of washing procedure the plate could be incubated with a suitable dilution of goat anti-rabbit horse-radish peroxidase conjugate and the absorbance detected by an indicator. The colour change is proportional to the amount of SP-A in the second step. We intend to explore. this alternative approach for comparison with our current method and hope the new assay method could obviate the need to sample in triplicate.

Tracheal aspirate was chosen for assay validation as this is the body fluid targeted for measurement of SP-A. We intend to use this in-house assay to measure SP-A in tracheal aspirates of neonates with and without respiratory distress syndrome.

ACKNOWLEDGEMENT

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