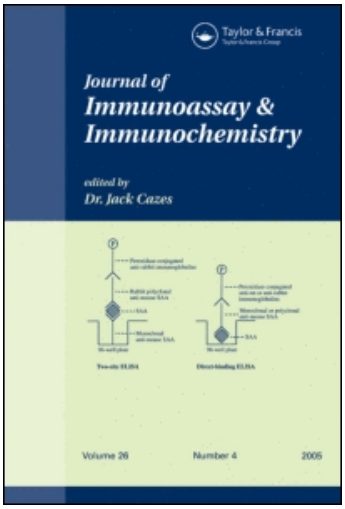

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IMMUNOCHEMICAL CONTROL OF THE SPECIES ORIGIN OF INTESTINAL MUCOSA USED FOR HEPARIN PURIFICATION

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IMMUNOCHEMICAL CONTROL OF THE SPECIES ORIGIN OF INTESTINAL MUCOSA USED FOR HEPARIN PURIFICATION

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ABSTRACT

Species specific antisera against bovine, ovine, and porcine serum albumin were produced in order to control the absence of bovine, ovine, or caprine tissues in the porcine intestinal mucosa used for heparin production. Two immunoassays were developed. An enzyme linked immunosorbent assay (ELISA) was very sensitive down to 1 ng/mL bovine albumin or 10 ppm bovine intestinal mucosa in porcine intestinal mucosa. For routine control, a more convenient single radial immunodiffusion assay (SRID) was found suitable to detect 2 μg/mL albumin or 3 p 1000 bovine, ovine, or caprine intestinal mucosa in porcine intestinal mucosa. Conditions of extraction of albumin from intestinal mucosa were optimized and a CV % of 4.1 was obtained for its quantitation.

Due to higher albumin concentrations, detection of bovine hashed gut and lung was more sensitive (1.5and 0.9 p 1000, respectively). Using antisera raised against porcine albumin the SRID can be applied to certify the porcine origin of the intestinal mucosa used for heparin purification and to control its adequate conservation before analysis.

INTRODUCTION

Heparin is a highly sulfated polysaccharide used clinically as an anticoagulant drug. It has usually been extracted from gut or lung obtained from pigs, cattle, and, to a lesser extent, sheep. Although bovine lung heparin displays a similar pharmacological profile to porcine mucosa heparin, it differs by a less heterogeneous structure (lower content in N-acetyl-glucosamine), a higher sulfation degree, and a higher molecular weight. By contrast, bovine mucosa heparin has long been considered structurally very close to porcine mucosa heparin.(1)

The outbreak of bovine spongiform encephalopathy in the United Kingdom in 1986, and its extension to other countries have led the regulatory authorities to restrict, as a precaution, the production of heparin for human use to porcine mucosa only. Thus, it became necessary to develop analytical methods able to control the animal origin of the heparin.

Differences in number and distribution of acetyl and sulfate groups in porcine and ruminant heparins have allowed their distinction by ^{13}C NMR(2-4) or by HPLC analysis of heparinase digests.(1,4,5,6) Intra-species variations in the heparin chemical structure and slight structural modifications due to the manufacturing processes(6) impact the sensitivity of these techniques for the analysis of mixtures of porcine and ruminant heparins.

As of today, the possibility of a direct control of the origin of the mucosa used for heparin purification has not been available. Immunochemical techniques, which utilize the exquisite specificity and sensitivity of the antigen-antibody reaction, have been successfully used over the last decades for the species characterisation of crude products such as meat or milk.(7,8) We describe here the development of an enzyme-linked immunosorbent assay (ELISA) and a simple and robust single radial immunodiffusion test (SRID) —both directed against ruminant albumins— which can be used to control the absence of ruminant tissues and to certify the porcine origin of the mucosa used for heparin production.

EXPERIMENTAL

Materials

Agar Noble (Difco) was supplied by Serlabo (Bonneuil sur Marne, France). Freund's complete and incomplete adjuvants were purchased from Miles (Puteaux, France), bovine albumin and fish gelatine from Sigma Aldrich Chimie (St-Quentin Fallavier, France). Mayer's buffer

(5 mM barbital-HCl, NaCl 0.15M, $CaCl_2$ 0.5 mM, pH 7.2) was supplied as Complement Fixation Test diluent tablets by Unipath (Dardilly, France).

Protein Purification

Immunoglobulins G_1 (IgG_1) and secretory immunoglobulins A (IgAs) were purified, respectively, from bovine colostrum and tears by a combination of gel permeation chromatography on Sephadex G200 (Pharmacia Biotech, Orsay, France) and ion exchange chromatography on DEAE Cellulose (Serva, Heidelberg, Germany) as previously described.(9)

Ovine serum albumin (OSA) and porcine serum albumin (PSA) were purified from serum by gel permeation chromatography on a 950 x 50 mm column of Sephadex G100 (Pharmacia Biotech) equilibrated in 0.02M Tris-HCl pH 7.0 and eluted at a flow rate of 50 mL/h. The albumin rich fractions were loaded onto a Mono-Q anion-exchange column (HR 10/10, Pharmacia-Biotech) equilibrated in the same buffer. Elution was performed at 1 mL/min over a 0-0.5 M NaCl gradient in 0.02 M Tris HCl buffer, pH 7.0, using a HPLC equipment (Gradient Former GF 425, Pump 420, Detector 430; Kontron Instrument, Montigny les Bretonneux, France).

Bovine serum albumin (BSA) was purchased from Sigma Aldrich and repurified by chromatography on Sephadex G-100 as described for ovine and porcine albumins. Purity of the purified proteins was checked by PAGE analysis.

Caprine serum albumin (CSA) was purchased from Sigma Aldrich and used without further purification.

Antisera

Different antisera were raised against the purified IgG_1, IgAs, BSA, OSA, and PSA. Rabbits or sheep were immunized at monthly intervals by multiple intradermal injections of antigen-adjuvant mixture prepared by emulsifying saline containing 1 mg purified protein per mL and complete (first injection) or incomplete (booster injections) Freund's adjuvant (1:1, by vol).(10) Animals were bled 7 to 9 days after each booster injection and the sera were analyzed for antibody activity and specificity by double immunodiffusion and immunoelectrophoresis.(11)

Cross-reacting antibodies between ruminants and porcine albumin were absorbed using porcine serum beads obtained by glutaraldehyde polymerization.(12)

The immunoglobulin G (IgG) fraction of the BSA-raised antiserum was obtained by anion-exchange chromatography on Q-Sepharose Fast-

Flow (Pharmacia Biotech) using a linear gradient of NaCl (0-0.5 M) in 0.02 M Tris-HCl buffer, pH 8.5. The purified IgG were conjugated with horseradish peroxidase using the $NaIO_4$ method of Tijssen & Kurstack.(13) The conjugates were further purified by size-exclusion chromatography on a Zorbax GF250-XL column (Dupont de Nemours, Wilmington, DE 19898 USA). Elution was performed at 5 mL/min using the HPLC equipment with the Gradient Former GF 425. Conjugated IgG were pooled, 5-fold concentrated by ultrafiltration, diluted with an equal volume of glycerol, and stored at -20°C.

Quantitative Precipitin Analysis

Antisera (0.1 mL, diluted 1/2 in Mayer's buffer) and albumins (0.1 mL, serial dilutions in Mayer's buffer) were mixed in flat bottom microwell plates and allowed to precipitate at room temperature for 30 min. Optical density (O.D.) was measured at 340 nm using a microplate reader (IEMS, Labsystem, Helsinki, Finland). Cross-reactivity was calculated as follows.

$$\% \text{ Cross reaction} = \frac{\text{Max. O.D. with heterologous albumin}}{\text{Max. O.D. with homolog albumin}} \times 100$$

Ruminants and Porcine Tissues

Bovine, ovine, caprine, and porcine lungs and small intestines were obtained from local slaughterhouses. Small intestine was emptied of its faecal content. Then, it was either cut open and the internal mucous membrane (intestinal mucosa) was scraped off, or it was cut into small pieces and chopped (3x5 sec) in a food processor to obtain hashed gut. Lung was processed as described for the hashed gut. Sodium metabisulfite was added as a preservative at a final concentration of 2 % (w/w).

Extraction of the Soluble Proteins from Tissues

5 g Hashed gut, intestinal mucosa, or hashed lung were mixed with 10 mL ethanol/water 45% (v/v) using a Polytron PT 3100 (Bioblock Scientific; Illkirch, France) fitted with a 12 mm diameter energetic axe for 7 s at 12000 rpm. The suspension was centrifugated at 11,000 g for 15 min. The aqueous phase was adequately diluted in Mayer's buffer containing 1% normal rabbit serum and 1 g/L sodium azide as a preservative for BSA, OSA,

CSA, and PSA quantitation by SRID. Samples were undiluted for bovine material detection in raw porcine material.

Sandwich ELISA

Polystyrene flat-bottom microwell plates (Maxisorp, Nunc, Kamstrup, Denmark) were coated overnight at 4°C with 100 μL of the capture antibody (IgG fraction of the rabbit antiserum to BSA) in a 0.05 M borate buffer, pH 9.2. The optimum coating concentration was determined by titration. All subsequent steps were performed at room temperature. Plates were washed 4 times with 0.01M phosphate buffer with NaCl 0.15M, pH 7.2 (PBS), containing 1 mL/LTween 20 (PBSTw). When used, blocking was performed with 200 μL of 10% (v/v) normal rabbit serum or 10 g/L fish gelatine in PBS. After four washes with PBSTw, 100 μL of albumin solutions or adequately diluted aqueous extracts of intestinal mucosa were added to the coated wells.

After mixing, the plates were incubated for 1 h and washed four times with PBSTw. After addition of 100 μL of adequately diluted peroxidase-labelled IgG to the wells, the plates were incubated for 1 h in the dark. After washing four times with PBSTw, 100 μL of 0.4 mg/mL *o*-phenylenediamine in 0.1 M phosphate-citrate buffer, pH 5.0, were added, running the colour reaction for 30 min before stopping it with 2.5 M H_2SO_4 (50μL/well).

Absorbance was measured at 492 nm using the microplate reader. A 10 mL/L solution of normal rabbit serum or 1 g/L fish gelatine with normal porcine serum (1/300, v/v) in PBSTw were used as diluent for the solutions. All analyses were made in duplicate.

SRID

Concentrations of IgG_1, IgAs, and albumins were determined by SRID assay(14) using 1.85 mm-thick agar plates containing 12 g/L agar Noble in Mayer's buffer, 1 g/L sodium azide, and suitable quantities of each specific antiserum. Circular wells (1.5 mm diameter) were punched out in the gel and filled with 3 μL portions of adequately diluted samples or 3 μL of known concentrations of purified proteins as standards. Plates were incubated in a moist box at 37 °C for 16-20 h. Diffusion was stopped by immersion of the plate in 2% (v/v) acetic acid for 1 min and the plates were rinsed with distilled water. The diameters of the ring-shaped precipitates were measured using dark-field oblique illumination and a magnifying video camera system.(15) Standard curves were constructed by plotting the

diameter of the precipitating ring *versus* the square root of the protein concentration. With a diffusion time of 16-20 h, a linear regression was always obtained. Samples and controls were analysed in duplicate.

For routine testing on industrial sites, the same technique was used with minor modifications: the agar (5.4 mL) containing suitable concentrations of the antisera was poured into Petri dishes (5 cm diameter). Circular wells of 3.5 mm diameter were punched out and filled with 15 μL samples solutions, or standards. The end of the procedure remained unchanged for PSA quantitation. For ruminant albumin quantitation in porcine material, the tissue extract was analysed undiluted. After immersion in acetic acid, the dishes were rinsed in 0.15 M NaCl for 5 to 10 min. Pictures of the dishes were taken as a record of each control, using a video printer (Sony UP 1200EPM, Manganelly, Clermont-Ferrand, France) coupled to a magnifying video camera (Sony XC003P).

RESULTS

Choice of the Target Protein

Suitable concentrations of the different antisera, raised against bovine IgG_1, IgAs, and albumin, were first determined to optimize the SRID quantitation of these proteins. We obtained 5, 20, and 2 μg/mL as detection limits for, respectively, IgG_1, IgAs, and albumin. The quantitation of these proteins in a preliminary extract of bovine intestinal mucosa gave, respectively, mean concentrations of 0.7, 1.0, and 2.0 mg/g. The differences in concentrations combined with the detection limits allowed us to conclude that quantitation of albumin was 7- and 20-fold more sensitive than the quantitation of IgG_1 and IgAs, respectively, for the detection of bovine intestinal mucosa.

The concentration of albumin was higher in bovine hashed gut extracts (3.9 ± 0.6 mg/g, $n = 6$) and in bovine lung extracts (6.7 ± 1.3 mg/g, $n = 6$) than in bovine intestinal mucosa (1.9 ± 0.7 mg/g $n = 15$) as a consequence of the higher blood content in these tissues.

Antisera

The different antisera raised against BSA and OSA were tested in double diffusion test (Figure 1) and in quantitative immunoprecipitation (Figure 2) as regards their cross-reactivity towards heterologous albumins. A higher cross-reactivity was observed with the anti-BSA towards OSA

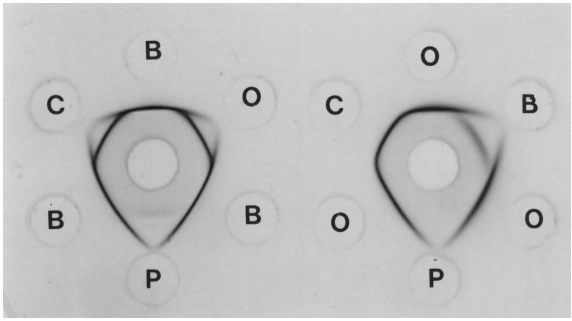

Figure 1. Analysis of the specificity of the rabbit antisera to BSA and OSA by agar gel double immunodiffusion. (a) Central hole: rabbit anti-BSA. (b) Central hole: rabbit anti-OSA. Peripheral holes B: BSA; O: OSA; C : CSA; P: PSA.

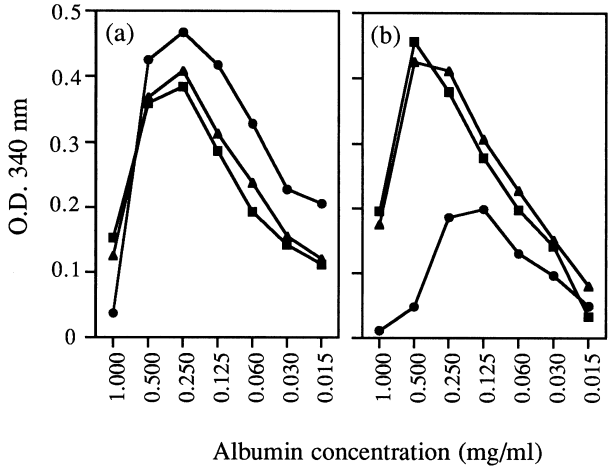

Figure 2. Quantitative precipitin curves. (a) and (b) denote the curves obtained with anti-BSA and OSA, respectively. Symbols indicate the optical density obtained with BSA (circles), OSA (squares), and CSA (triangles). Experiments were performed in duplicate.

(82.%) and CSA (85%)(Figures 1a and 2a) than with the anti-OSA towards BSA (43%)(Figures 1b and 2b). Moreover, the anti-BSA exhibited a very low cross-reactivity towards PSA which was not observed with the anti-OSA. This cross-reaction was removed by immunoabsortion of the antisera

with insolubilized PSA (not shown). Antiserum against OSA reacted with the same intensity towards OSA and CSA (96% cross-reactivity (Fig. 1 and 2).

Sample Extraction

During preliminary trials for bovine albumin quantitation in porcine raw material with the SRID test, some non-diluted extracts of porcine intestinal mucosa gave slight non-specific precipitates in agar which could mask specific precipitates of small diameter. Consequently, different extraction solutions were tested for their ability to reduce the interactions between the extracts and agar or agarose, either by a better solubilisation of the extract or by a partial elimination of proteins or lipoproteins.

The results reported in Table 1 indicate that the non-specific precipitation in agar or agarose, devoid of antiserum, could be totally eliminated

Table 1. Effect of the Diluent Used for the Extraction of Albumin from Intestinal Mucosa on the Apparition of Non-Specific Precipitates in Agar or Agarose Gels, Quoted from - (Negative) to + + + (Strong)

Diluent	Agar	Agarose
0.1M Tris-HCl pH 8.5	+ +	+ + +
0.1M Glycine-NaOH pH 9.5	+ +	+ + +
0.01 M PBS pH 7.4 + Tween 20 0.1%	+	+ +
0.01 M PBS pH 7.4 + Tween 20 1%	+ + + +	+
0.01 M PBS pH 7.4 + Triton X100 0.1%	+	-
0.01 M PBS pH 7.4 + Triton X100 1%	+	-
0.15 M NaCl	±	-
0.3M NaCl	±	±
0.5M NaCl	±	±
1M NaCl	±	-
Ammonium Sulfate 75% saturation	-	-
Ammonium Sulfate 90% saturation	-	-
Ethanol 30%	-	+
Ethanol 45%	-	-
Ethanol 60%	-	-
0.1M CaCl2	-	±
1M CaCl2	±	-
0.1M Tris-HCl pH 8.6 +0.1M Dithiotreitol	+	-
Water then 3 vol Chloroform	-	+
Water then 3 vol Frigen	+ + +	+ + + +
Control (Water)	+ +	+ + +

Table 2. Effect of the Diluent Used for the Extraction of Albumin from Intestinal Mucosa on the Apparition of Specific and Non-Specific Precipitates in Agar or Agarose Gels Containing a Rabbit Anti-BSA Antiserum. Specific Precipitates: A (Absent), B (Blurred), N (Normal). Non-Specific Precipitates: from - (Negative) to + + + (Strong)

	Agar		Agarose	
Diluent	Specific	Non-Specific	Specific	Non-Specific
0.01 M PBS pH 7.4 + Triton X100 1%	B	+ + +	B	+
0.15 M NaCl	N	+ +	N	+
1M NaCl	N	+ + +	N	±
Ammonium Sulfate 75% Saturation	A	+ + +	A	+ +
Ammonium Sulfate 90% Saturation	A	+ +	A	+
Ethanol 30%	N	-	N	-
Ethanol 45%	N	-	N	-
Ethanol 60%	A	-	A	-
0.1M CaCl2	N	+ +	N	±
1M CaCl2	N	+	N	+
Water then 3 vol Chloroform	N	+	N	-
Control (Water)	N	+ +	N	+

with ammonium sulfate at 75 or 90% saturation and ethanol at 45 or 60%. However, when tested with agarose or agar containing a rabbit antiserum against bovine albumin, ammonium sulfate at 75 or 90% saturation and ethanol at 60% totally inhibited the specific antigen-antibody precipitation (Table 2). Optimal results were obtained with ethanol at 30 and 45%, however, when using short diffusion times in SRID (1 or 4 h) 45% ethanol remained the best diluent for the sample extraction (Table 3).

The mechanical extraction of bovine and porcine albumin from intestinal mucosa was tested using 2 g sample and 4 mL ethanol 45% (v/v) in water. Reproducible and similar results were obtained using Polytron or Ultraturrax: 2.49 ± 0.03 mg/g and 2.5 ± 0.03 mg/g, respectively, for BSA (n =), 2.26 ± 0.06 and 2.16 ± 0.04, respectively, for PSA (n = 10).

Sandwich ELISA

Optimal concentrations of coating and conjugated antibodies were determined by checkerboard titration (illustrated in Figure 3).

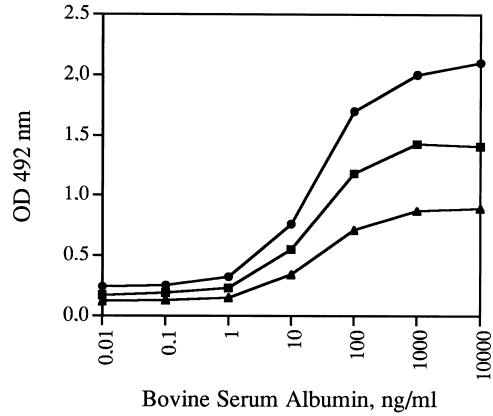

Figure 3. Quantification of BSA by sandwich ELISA. Polyclonal rabbit IgG (1 μg/mL) were used as capture antibody and polyclonal rabbit peroxydase labelled IgG diluted 1:250 (circle), 1:500 (square), and 1:1000 (triangle) as tracer antibody. Results are means of duplicate analyses.

Table 3. Effect of the Diluent Used for the Extraction of Albumin from Intestinal Mucosa on the Apparition of Non-Specific Precipitates in Agar or Agarose After 1 or 4 h Diffusion of a Porcine Mucosa Extract

	Agar		Agarose	
Diluent	1 h	4 h	1 h	4 h
0.15 M NaCl	+ + +	+ + + +	+ +	+ + +
Ethanol 30%	+ + +	+ + +	-	-
Ethanol 45%	+	±	-	-
1M CaCl2	+	+ +	-	-
Water, then 3 vol Chloroform	+ + +	+ + + +	+ +	+
Control (Water)	+ + +	+ + + +	+ +	+ + +

Optimal sensitivity was obtained using the capture IgG at 1 μg/mL and the labelled IgG at a 1:250 dilution. Blocking the non-specific binding sites of the plates with normal rabbit serum or fish gelatine had no effect on the background level. Consequently, the blocking step was omitted in the optimized assay.

Under the optimal conditions, the detection limit of the assay, estimated from the interpolated value at three SD above zero dose, was $\sim$ 10 ppm of bovine mucosa in porcine mucosa (Figure 4).

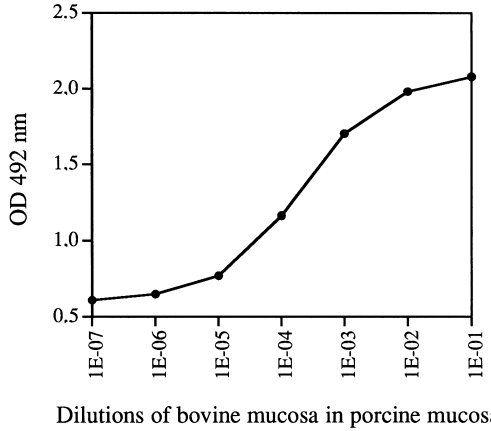

Figure 4. Reference curve for quantification of bovine mucosa in porcine mucosa by sandwich ELISA (same system as in Fig. 3). Results are means of duplicate analyses.

Single Radial Immunodiffusion

The effect of diffusion time on the precipitate diameters is shown in Figure 5a. Diffusion of the SRID plates can be stopped after 3-4 h diffusion for a quick control. At this time, the small precipitates which correspond to low albumin concentrations have reached their final diameter and can be detected. However, the large precipitates of the highest albumin concentrations have not reached their maximal diameters and the resulting standard curve is only fitted by a 3rd degree polynomial equation. After 16 h diffusion, a linear relationship between the square of the precipitate diameter and albumin concentrations was obtained in the range 2-40 μg/mL.

When the SRID is used for trace ruminants albumin quantitation, care must be taken to rinse the plates or dishes with NaCl 0.15 M instead of the usual distilled water, in order to avoid non-specific precipitates that could occur with non-diluted extracts. PSA quantitation on porcine samples is performed after dilution of the sample extracts; in such cases, distilled water is recommended for the rinsing step, since more contrasted precipitates are obtained.

Linear regressions with $r \geq 0.999$ were usually obtained for BSA concentrations ranging from 4 to 40 μg/mL BSA. In the range 2-40 μg/mL, r values were only ≥ 0.998 since the diameter of the 2 μg/mL concentration is slightly overestimated after 16 h diffusion (Figure 5b).

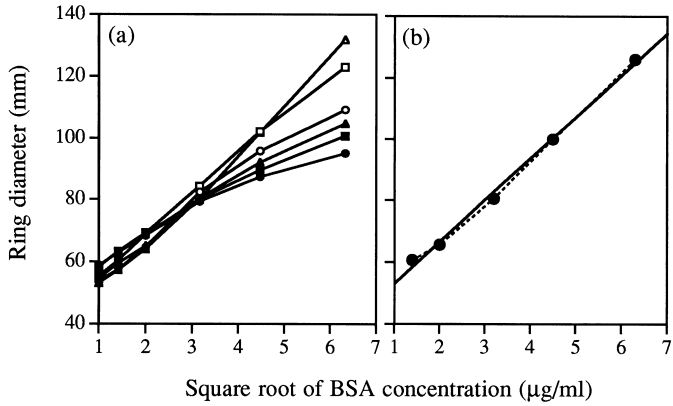

Figure 5. Quantification of BSA by single radial immunodiffusion. (a) Effect of diffusion time: 2 h (closed circles), 3 h (closed squares), 4h (closed triangles), 5h (opencircles), 8 (open squares), and 16 h (open triangles). (b) Reference curve for BSA quantification in porcine mucosa. Results are means of duplicate analyses.

The detection limit obtained was around 3 p 1000 bovine intestinal mucosa in porcine intestinal mucosa. The limit of detection obtained with ovine and caprine mucosa was of the same order of magnitude, since their albumin concentration was comparable to that of the bovine mucosa (2.3 ± 0.4 and 2.0 ± 0.6 mg/g, respectively, $n = 5$). For bovine hashed gut and lung the detection limits are, respectively, 1.5 and 0.9 p 1000, since albumin concentrations are higher in these tissues.

Repeatability of the BSA quantitation has been evaluated on extracts of porcine intestinal mucosa containing different proportions of bovine intestinal mucosa extract. Mean CV% was 4.11, with values ranging from 1.57 to 5.5 % (Table 4).

Results obtained for the analysis of extracts obtained from experimental contaminations of porcine intestinal mucosa by increased quantities of bovine intestinal mucosa are presented in Figure 6 and Table 5. The mean recovery was 99.5% and the correlation between the percentage of added bovine mucosa and bovine mucosa found by SRID analysis was 0.9996.

Because rabbit antisera raised against BSA recognize BSA better than OSA or CSA, overestimated results were obtained when OSA or CSA were quantified by SRID with agar containing only the antiserum raised against BSA. Consequently, suitable quantities of the anti-OSA were added to the anti-BSA in order to obtain precipitates with similar diameter values for the same concentration of BSA, OSA, or CSA, as illustrated in Figure 7.

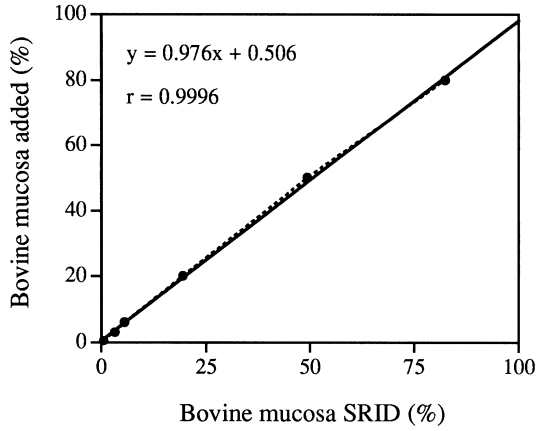

Figure 6. Correlation between the percentage of bovine mucosa added to porcine mucosa and the percentage of bovine mucosa recovered by SRID analysis.

Table 4. Repeatability of SRID Quantification of the BSA Content of Mixtures of Bovine and Porcine Mucosa Analyzed Diluted 1:1 or 1:20

% Bovine Mucosa	Dilution	CV%
80.0	1/20	2.57
50.0	1/20	5.31
20.0	1/20	4.69
6.0	1/1	1.57
3.0	1/1	5.05
0.5	1/1	5.51

Table 5. Recovery of the BSA Content of Mixtures of Bovine and Porcine Mucosa. Results Are Means of Duplicate Analysis

% Bovine Mucosa (Experimental)	% Bovine Mucosa (SRID)	% Recovery
80	82.4	103.0
50	49.3	98.6
20	19.4	97.0
6	5.5	91.7
3	3.2	106.7
0.5	0.5	100.0

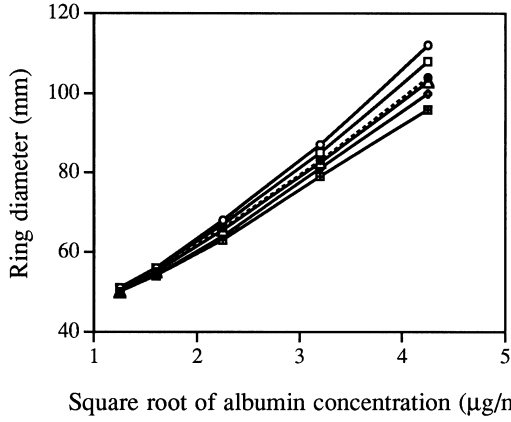

Figure 7. Quantification of BSA and OSA by SRID using different quantities of rabbit anti-OSA added to the rabbit anti-BSA. Closed circle with broken line: reference curve for BSA using the rabbit anti-BSA. Open symbols: curves obtained for OSA using the rabbit antisera against BSA and increasing quantities of rabbit anti-OSA (circle: 0 µL; square: 2 µL; triangle: 4 µL; lozenge: 6 µL; quadrated square: 8 µL). Results are means of duplicate analyses.

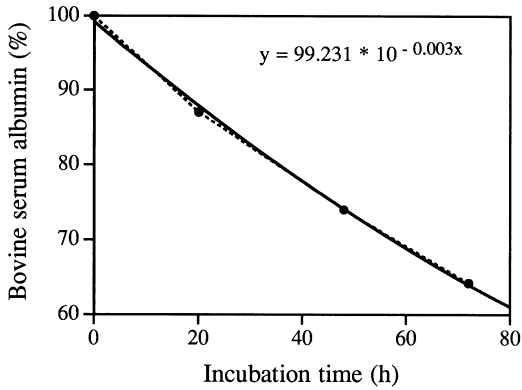

Figure 8. Effect of the incubation time at 37°C on the BSA content of a mixture of 20% bovine mucosa in porcine mucosa. The BSA content is expressed as % of the initial concentration. Results are means of duplicate analyses.

Reference albumin solutions, prepared as indicated in the Experimental section, are highly stable at 4°C since no modification of their immunological reactivity was observed throughout 60 days of storage. Moreover, no significant loss of BSA immunoreactivity was observed for

bovine mucosa stored 36 or 72 h at -20°C or at 4°C. When bovine mucosa was added to porcine mucosa (20%, w/w) and stored at 20°C, BSA immunoreactivity was not modified after 36 h and decreased by 9% after 72 h. However, storage at 37°C of the same mixture induced an exponential decrease of BSA immunoreactivity and only 64.3 % of its reactivity remained after 72 h incubation (Figure 8).

Albumin concentration in extracts of porcine intestinal mucosa was quantified using rabbit antisera against PSA. Mean concentration was 2.3 ± 0.36 mg/g of tissue ($n = 10$).

DISCUSSION

Albumin and IgG are the major proteins of mammals serum. By diffusion into the extravascular spaces, they are also present in numerous tissues, but at lower concentrations. In mammalian secretions, IgG are frequently substituted by the secretory immunoglobulin A (IgAs) which is specifically synthesized in the mucosas(16,17) and is particularly resistant to the proteolytic enzymes present in mucosas, such as the intestinal mucosa. However, in the ruminant species, the IgG_1 isotype plays a role similar to that of IgAs in secretions.(18) Thus, our preliminary trials were done using rabbit antisera prepared against purified bovine IgG_1, IgAs, and albumin. The results obtained indicated that the concentrations of IgG_1 and IgAs in the bovine intestinal mucosa were lower than that of albumin and, due to their higher molecular weight, their detection limits in SRID were higher. As a consequence, albumin quantitation was, respectively, 7- and 20-fold more sensitive than IgG_1 and IgAs quantitation. Moreover, ruminant IgG_1 and IgAs are not commercially available and must be purified from particular secretions such as colostrum and tears.(9) In contrast, ruminant albumins are commercially available at a low price, and the pure protein can be used as standard.

Antisera raised in rabbits against ruminant albumins display generally strong cross-reactivity between the ruminant species, but react slightly with porcine albumin.(19) Antisera specific for only one of the ruminant species can be obtained by inter-ruminant immunisation, i.e., bovine protein injected to goat or sheep, or ovine protein injected to goat.(20) For the control of the intestinal mucosa used in heparin production, we wished to develop a test which is able to detect, simultaneously, the bovine, ovine, and caprine species in the same assay and with the same sensivity. Consequently, we have first prepared a rabbit antiserum against BSA. This antiserum cross-reacted partially with OSA and CSA. Then, we have prepared a

rabbit antiserum against OSA which recognized OSA and CSA with the same sensivity, but was less reactive for BSA.

These results are in accordance with those obtained by Kamiyama(19) on the immunological cross-reactions and species-specificities of BSA, OSA, and CSA. Using single quantitative precipitation, SRID, rocket immunoelectrophoresis, and immunoadsorbent chromatography, this author reported 90% cross-reaction between OSA and CSA and 40-75% cross-reaction between BSA and OSA or CSA. However, in our results, the cross-reactivity of the rabbit antisera against BSA was greater for OSA than the cross-reactivity of the anti-OSA for BSA.

Using the obtained antisera, we have first developed a sensitive sandwich ELISA which can detect 10 ppm bovine mucosa in porcine mucosa. At this level, care must be taken to exclude any contamination by traces of BSA. For example, we have observed erroneous results occurring from traces of BSA adsorbed on magnetic stir bars. Moreover, ELISA techniques require high technicity and are very sensitive to matrix effects. In order to avoid false positive results, we have preferred the guarantee afforded by the direct visual examination of the SRID plates, which enable a non-ambiguous differentiation of a specific precipitate from a potentially non-specific one.

As opposed to ELISA, SRID is a very simple technique which can be used by non-experienced people without any special equipment. A few microliters of the sample to be tested and of reference samples or standards are put into the wells of an agar plate containing specific antibodies. Diffusion can be stopped after 3-4 h incubation for a quick response or after 16-24 h if a linear relationship between the precipitate area and the antigen concentration is needed. The ring diameters can be measured using a magnifying lens or on the monitor of a video camera. In its application to the control of intestinal mucosa batches, pictures can be obtained from the video camera signal and stored as raw data. For routine testing of large numbers of samples (more than 50-100 samples a day), a semi-automated version of the SRID(15) can be used. The obtained CV around 4% is consistent with CVs % previously obtained for the quantitation of other proteins by SRID(13,21,22) and is adequate for the purpose of albumin detection.

Using suitable proportions of antisera against BSA and OSA, we have developed SRID plates which give the same diameter for similar concentrations of BSA, OSA, and CSA. Lastly, the use of a sheep antiserum raised against PSA in a second plate allowed the quantitation of the PSA content of the sample. This quantitation is necessary to certify the porcine origin of the intestinal mucosa and to validate the adequate conservation and extraction of the intestinal mucosa prior to the SRID analysis.

Many other immunochemical techniques are available to detect albumin in intestinal mucosa using anti-albumin antisera. We have not evaluated techniques that use antigen-antibody reactions on nitrocellulose strips (e.g., dot immunoassay or immunochromatography) since they are not quantitative. Particle agglutination with antigen or antibody immobilized onto the surface of the particle can provide semi-quantitative results in a few minutes. Preliminary trials with latex particles coated with anti-BSA antibodies gave promising results (detection limit 0.5-1% bovine mucosa in porcine mucosa), but non-specific auto-agglutination has been observed with some mucosa samples (results not shown).

In conclusion, the proposed SRID method is suitable to detect ruminant albumins coming from different tissues (intestine or lung) in porcine intestinal mucosa, and can be used to certify the porcine origin of the intestinal mucosa used for heparin production. This technique, which is now commercially available as a kit, enables heparin manufacturers to routinely control their crude material and to obtain a better traceability of their heparin production as required by Health agencies.

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