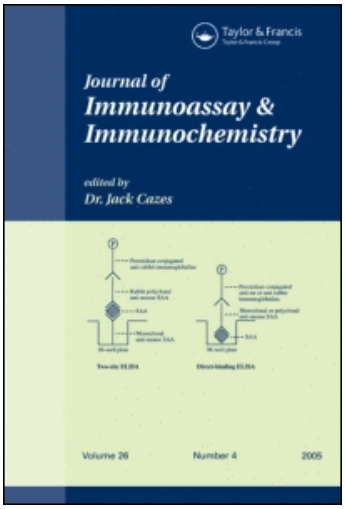

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 30 November 2001

To cite this Article Levieux, Annie , Rivera, Vincent and Levieux, Didier(2001) 'A SENSITIVE ELISA FOR THE DETECTION OF BOVINE CRUDE HEPARIN IN PORCINE HEPARIN', Journal of Immunoassay and Immunochemistry, 22: 4, 323 — 336
To link to this Article: DOI: 10.1081/IAS-100107398
URL: http://dx.doi.org/10.1081/IAS-100107398

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 22(4), 323–336 (2001)

A SENSITIVE ELISA FOR THE DETECTION OF BOVINE CRUDE HEPARIN IN PORCINE HEPARIN

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ABSTRACT

Heparin is an effective anticoagulant drug which has been purified for decades from bovine or porcine tissues. However, with the emergence of BSE, heparin purification is today restricted to porcine intestinal mucosa. To control the origin of crude heparins, polyclonal antibodies were raised against bovine contaminants. These antibodies were used to develop one sandwich and two competitive indirect ELISAs. Optimal results were obtained with competitive indirect ELISA, using bovine crude heparin for the coating and anti-bovine crude heparin as a detector antibody. The detection limit of the assay was 1 ppm of bovine crude heparin in a porcine heparin. Taking into account the variability of the background obtained for 10 crude and 9 pure porcine heparins from known origin, the detection level was 5 ppm. Ovine and caprine crude heparins cross-reacted slightly (6–17 ppm).

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INTRODUCTION

Heparin is a sulfated glycosaminoglycan consisting of repeating units of disaccharides containing hexuronic acids (D-glucuronic or L-iduronic acid) and D-glucosamine residues in alternate sequences. The aminosugar can either be N-sulfated or N-acetylated and, in addition, both the hexuronic acic and glucosamine moieties carry O-sulfate groups at varying positions.(1)

Heparin is a potent anticoagulant molecule, endowed with well-established antithrombotic activity. It enhances the inactivation of several serine proteases of the coagulation cascade, via specific binding to antithrombin III. As a result of its various biological properties, such as anti-inflamatory,(2) anti-lipemic,(3) and anti-angiogenic activities,(4) there is an increasing need for heparin in medicine.

Commercial heparin has been essentially extracted from gut or lung tissues obtained from pigs, cattle, and, to a lesser extent, from sheep. However, the outbreak of bovine spongiform encephalopathy (BSE) in 1986 prompted regulatory authorities to restrict the sourcing of heparin for human therapy to porcine intestinal mucosa. Accordingly, in order to control the application of this restriction, it became necessary to develop analytical methods able to identify the species origin of heparin and to exclude the presence of any ruminant tissues in the relevant industrial raw materials.

The simplest way to secure the whole heparin-supply chain would be to implement an analytical control on each industrial batch of the end-product, i.e., on pure heparins. Theoretically, the control of purified heparins can be implemented through physicochemical methods such as ^{13}C NMR(5–7) or HPLC analysis of heparinase digests.(8–10) Unfortunately, these methods are not fully satisfactory, due to two different drawbacks: a lack of sensitivity and, also, the impossibility to differentiate ovine and porcine pure heparins. Consequently, analysts were led to adopt an alternative and indirect approach which consisted in the development of analytical methods applied to the starting material (intestinal mucosa) and to the crude or semi-purified heparins.

To certify the porcine origin of the mucosa collected by heparin manufacturers, two immunochemical methods are now available: a very sensitive ELISA and, for routine control in plants, a more convenient single radial immunodiffusion assay (SRID) suitable to detect 3 p 1000 bovine, ovine, or caprine intestinal mucosa in porcine intestinal mucosa.(11)

For crude or semi-purified heparins, an immunochemical approach has been recently developed to characterize species-specific impurities which remain in these products.(12) Up to 13 antigenic components were

revealed in the eluted material (EM) issued from the chromatographic step of a bovine intestinal heparin preparation. In the final crude heparin, three of these contaminants were recovered. The major contaminant, called Ag1, was identified as strictly bovine-specific and was detected in a pulmonary EM as well as in intestinal crude heparins, whatever the process used for their purification.

In this report, we describe the development of sensitive ELISA methods using rabbit antisera raised against either EM or against the end-processed bovine crude heparin. A detection limit of 1–5 ppm of bovine crude heparin in porcine heparin was obtained using a competitive indirect technique.

EXPERIMENTAL

Chemicals

Freund's complete and incomplete adjuvants, bovine serum albumin (BSA), human serum albumin (HSA), porcine heparin-agarose, and heparin-BSA were purchased from Sigma Chemical Company (St Louis, MO, USA). Crude and pure porcine mucosal heparins were supplied by Valori-5 (Ploërmel, France). Peroxidase-labelled goat anti-rabbit immunoglobulin was from Nordic Immunology (Tebu, France). Amdex goat anti-rabbit IgG conjugate was supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). Glutaraldehyde was from Serva (Heidelberg, Germany).

Crude Heparin Preparation

Bovine, ovine and caprine crude heparins were purified from hashed gut according to the procedure of Griffin et al.(13) Briefly, the tissues were homogeneized and proteolyzed at 55–65°C with a proteolytic enzyme from *Bacillus subtilis* until total liquefaction. After filtration, they were made 1 M NaCl and incubated with a macroporous strong anion-exchange resin. The resin was washed with 1 M NaCl and the adsorbed material was eluted with 3 M NaCl. Heparin was then selectively precipitated from the eluted materials (EM) with 50% methanol and vacuum dried (process 1).

For antisera production, portions of the eluted materials were ten-fold concentrated by ultrafiltration and then extensively dialysed against 0.1 M Tris-HCl buffer, pH 7.5.

Bovine crude heparin was also obtained by proteolysis, heat coagulation at 90°C, and cetylpyridinium chloride complexation of heparin, as described by Scott(14) (process 2).

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Polyclonal Antibodies

Antisera were produced against concentrated EM or 50 mg/mL bovine crude heparin in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Rabbits (four for each antigen) were immunized at monthly intervals by multiple intradermal injections of antigen-adjuvant mixture prepared by emulsifying 1 mL antigen sample with 1 mL complete (first injection) or incomplete (booster injections) Freund's adjuvant.(15) Animals were bled seven to nine days after each booster injection and the sera were analyzed for antibody activity and specificity by double immunodiffusion, immunoelectrophoresis, and line immunoelectrophoresis as previously described.(12)

Antibodies reacting with BSA and heparin in the anti-bovine crude heparin were absorbed by BSA beads obtained by glutaraldehyde polymerization(16) and by affinity chromatography on a porcine heparin-agarose column (3 mL settled bed for 8.5 mL antiserum).

Purification and Peroxidase Labeling of Rabbit Serum Immunoglobulins

The immunoglobulin G (IgG) fraction of rabbit antisera 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.(17) The conjugates were further purified by size-exclusion chromatography on a Zorbax GF250-XL column (Dupont de Nemours, Wilmington, USA). Elution was performed at 5 mL/min using a HPLC equipment (Gradient Former GF 425, Pump 420, Detector 430; Kontron Instrument, France). The conjugated IgG were pooled, 6.6-fold concentrated by ultrafiltration, diluted with an equal volume of glycerol, and stored at $-20^{\circ}C$.

Sandwich ELISA

Polystyrene flat-bottomed microwell plates (Maxisorp, Nunc, Denmark) were coated overnight at $4^{\circ}C$ with 100 µL of the capture antibody (IgG fraction of the rabbit antisera against EM) in 0.01 M phosphate buffer with NaCl 0.15 M, pH 7.2 (PBS). The optimum coating concentration was determined by titration. All subsequent steps were performed at room temperature. Plates were washed five times with PBS containing 0.1% (v/v) Tween 20 (PBSTw). Blocking was performed with 200 µL of 3% (v/v)

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HSA. After five washes with PBSTw, 100 μL of serial dilutions of EM or crude heparins were added to the coated wells. After mixing, the plates were incubated for 1 h and washed five times with PBSTw. After addition of 100 μL of the adequately diluted peroxidase-labelled IgG to the wells, the plates were incubated for 1 h in the dark. After five washes 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 (50 μL/well). Absorbance was measured at 492 nm using a microplate reader (IEMS, Labsystem, Finland). PBSTw with 0.5% (v/v) HSA (PBSTw-HSA) was used as diluent for the solutions. All analyses were made in duplicate. The detection limit was calculated as the mean blank signal + 3 SD.

Competitive Indirect ELISA

The microtiter plates were coated overnight at 4°C or 1 h at room temperature with 100 μL of EM or bovine crude heparin in PBS. The optimum coating concentrations were determined by titration. All subsequent steps were performed at room temperature. Plates were washed five times with PBSTw. A 1 h blocking step was performed with 200 μL of 1% (v/v) HSA in PBS and the plates were washed five times with PBSTw. While coating and blocking, one volume of the adequately diluted antiserum (anti-EM or anti-bovine heparin) was incubated in test tubes with one volume of serially diluted solutions of the inhibitors (EM and heparin solutions). After 90 min incubation, 100 μL of the antigen–antibody mixture were added to the wells for a 1 h incubation. After five washes with PBSTw, 100 μL of adequately diluted peroxidase labelled goat anti-rabbit antibody were added to the wells and the plates were incubated for 1 h in the dark. The final steps of enzyme revelation and blocking with H_2SO_4 were conducted as described above. PBSTw-HSA containing or not porcine heparin at 50 mg/mL was used as diluent for the solutions. All analyses were made in duplicate and the detection limit was calculated as the mean blank signal − 3 SD.

Indirect ELISA

The technique was performed, as described above, for the competitive indirect ELISA, but omitting the 90 min inhibition step. Heparin-BSA at 0.1 μg/mL in PBS was used as the coating antigen. The antisera were tested

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from 10^{-3} to 10^{-6} before and after absorption. All subsequent steps were unchanged.

RESULTS

Sandwich ELISA with Anti-EM Antiserum

The IgG fraction of the rabbit antisera against EM was purified by anion-exchange chromatography and conjugated with horseradish peroxidase. A sandwich ELISA was developed using non-labelled IgG as a capture antibody and labelled IgG as a detector antibody. Optimal concentration of coating and conjugated antibodies were determined by checkerboard titration. High sensitivity was obtained using the capture antibody at 8 μg/mL and the labelled antibody at a 1 : 600 final dilution (Figure 1). A positive reaction was still obtained for a 1 : 200,000 dilution of concentrated EM (Figure 1a). However, porcine EM was also positive at a 1 : 100 dilution.

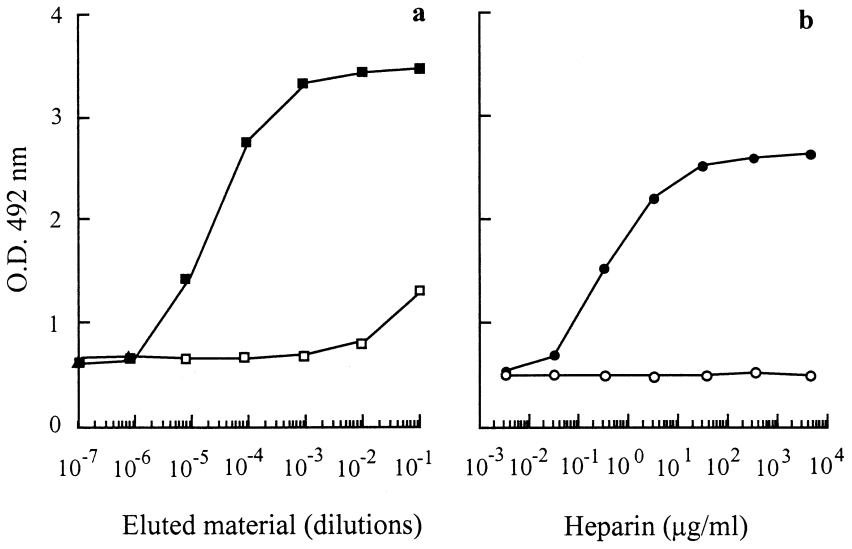

Figure 1. Quantitation, by sandwich ELISA, of bovine specific antigens in eluted materials (EM) from batch chromatography of bovine proteolyzed hashed gut (a) or in bovine crude heparin (b). IgG from rabbit antiserum against EM were used as the capture antibody. The same IgG, after peroxidase labelling, were used as the tracer antibody. Closed symbols: bovine samples. Open symbols: porcine samples. Squares: eluted materials. Circles: crude heparin. Results are means of duplicates.

Using crude heparins, the detection limit was 30 ng/mL for bovine heparin and no reaction was observed for porcine heparin tested at a concentration of 5 mg/mL (Figure 1b).

Competitive Indirect ELISA with Anti-EM Antiserum

The competitive indirect ELISA was first tested using microtiter plates coated with bovine EM. Optimal conditions were obtained with bovine EM diluted 1 : 1000 in PBS and the rabbit anti-EM antiserum diluted 1 : 1000. However, the bovine crude heparin did not fully inhibit the reactivity of the anti-EM antisera as obtained for the concentrated EM (Figure 2). Such a result could be partially expected since 13 antigenic contaminants had been observed in concentrated EM instead of three in bovine crude heparin.(12) Consequently, microtiter plates were then coated with bovine crude heparin instead of EM. The optimal coating concentration was 20 μg/mL bovine

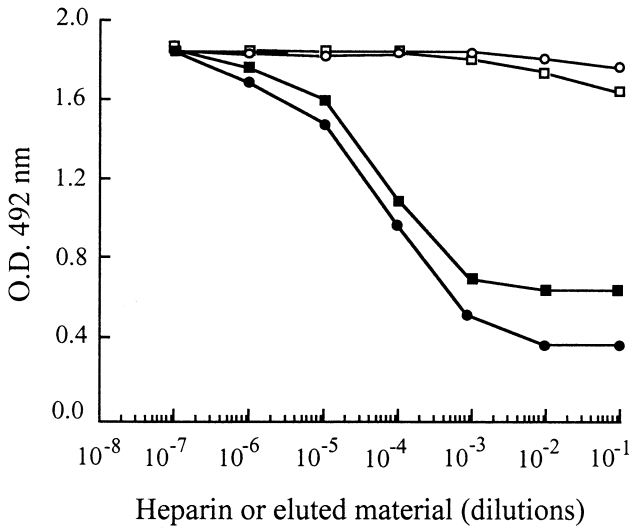

Figure 2. Quantitation, by competitive indirect ELISA, of bovine specific antigens in eluted materials (EM) from batch chromatography of proteolyzed hashed gut (circles) or in 10 mg/mL crude heparin (squares). Microtiter plates were coated with bovine EM. Rabbit IgG against EM were used as the primary antibody and peroxidase labelled goat anti-rabbit IgG as the tracer antibody. Inhibition of the primary antibody was performed with bovine (closed symbols) or porcine (open symbols) samples. Results are means of duplicates.

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crude heparin in PBS buffer. The optimal dilution of the anti-EM antiserum was 1 : 7500 and the inhibition time was fixed at 90 min since no significative improvement was observed between 90 and 145 min. Substituting the peroxidase conjugated goat anti-IgG by the Amdex conjugate increased markedly the sensitivity of the test (Figure 3a). Unexpectedly, higher optical density was observed when increasing the concentration of the porcine crude heparin used in the diluent (Figure 3b). In the optimal conditions, the detection limit (mean background −3 SD) was 150 ng/mL of bovine heparin, that is 3 ppm of bovine crude heparin in a porcine heparin analyzed at 50 mg/mL.

Competitive Indirect ELISA with Anti-Bovine Crude Heparin Antiserum

The anti-bovine crude heparin antiserum was essentially directed against the bovine Ag1. However, antibodies against BSA and heparin

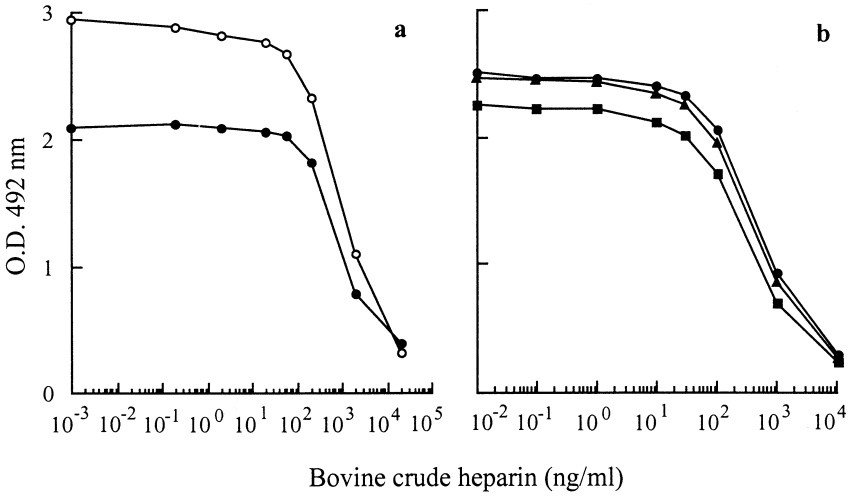

Figure 3. Quantitation, by competitive indirect ELISA, of bovine specific antigens in bovine crude heparin. Microtiter plates were coated with bovine crude heparin. (a) rabbit IgG against EM were used as the primary antibody and standard peroxidase labelled goat anti-rabbit IgG (closed circles) or Amdex multiperoxidase labelled goat anti-rabbit IgG (open circles) as the tracer antibody. (b) bovine crude heparin was diluted in increasing concentrations of porcine crude heparin: 20 (squares), 40 (triangles) and 60 (circles) mg/mL . Results are means of duplicates.

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were detected. In order to use a well defined antigen–antibody system, the antiserum was successively absorbed with polymerized BSA and porcine heparin bound to agarose. The efficiency of the absorption was controlled by indirect ELISA using a heparin-BSA coated plate. As shown in Figure 4, the absorbed antiserum did not react at a 1 : 1000 dilution, while the unabsorbed antiserum still reacted at a 1 : 100,000 dilution.

Microtiter plates were coated with bovine crude heparin at an optimal concentration of 20 μg/mL. The coating was equally efficient by incubation overnight at 4°C or 1 h at room temperature. The optimal concentration of the absorbed antiserum was found around 1 : 70,000 when using the Amdex conjugate diluted 1 : 1000 (Figure 5a). The detection limit was established at 1 ppm (50 ng/mL) of bovine crude heparin in porcine heparin (50 mg/mL). The intra-assay RSD was found to be 12% for a concentration of 2 μg/mL (40 ppm) of bovine crude heparin and 16% for the detection limit (1 ppm). However, when testing different batches of crude ($n = 10$) or pure ($n = 9$) porcine heparins, slight variations of the background level were observed and the detection limit was established at 5 ppm.

Serial dilutions of bovine crude heparin in PBSTw-HSA buffer containing 50 mg/mL porcine crude heparin were stored at 4°C for 14 days. As shown in Figure 5b, the same immunochemical reactivity was observed for freshly made or two weeks stored standards.

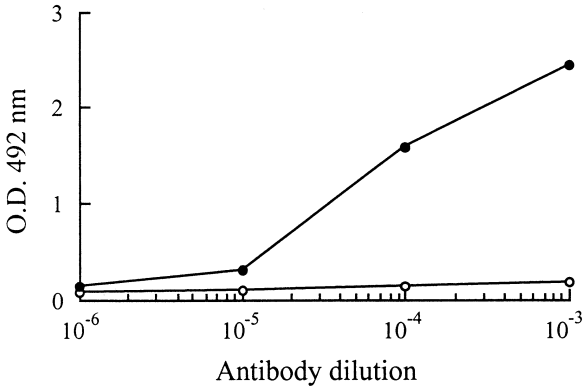

Figure 4. Quantitation, by indirect ELISA, of the antibody titer against heparin-BSA of a rabbit antiserum against bovine crude heparin before (closed circles) and after (open circles) immunoabsorption with polymerized BSA and heparin-agarose. Microtiter plates were coated with heparin conjugated to BSA and a peroxidase labelled goat anti-rabbit IgG was used as the tracer antibody. Results are means of duplicates.

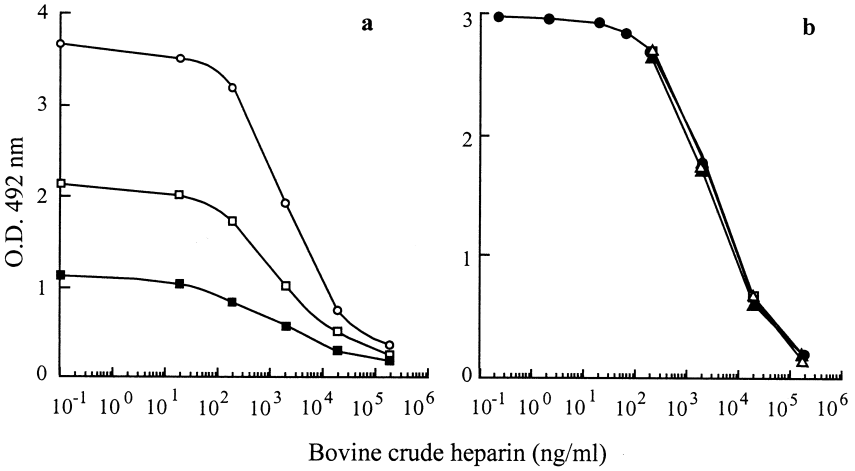

Figure 5. Quantitation, by competitive indirect ELISA, of bovine specific antigens in bovine crude heparin. Microtiter plates coated with bovine crude heparin. Rabbit anti-bovine crude heparin was used as the primary antibody and Amdex multi-peroxidase labelled goat anti-rabbit IgG as the tracer antibody. (a) optimisation of the Amdex conjugate dilution: 1 : 500 (open circles), 1 : 1000 (open squares), 1 : 2000 (closed squares). (b) effect of storage of bovine crude heparin standards for 7 (open triangles) or 14 (closed triangles) days at 4°C. Control: closed circles. Results are means of duplicates.

The cross-reactivities of experimental ovine ($n = 2$) and caprine ($n = 1$) crude heparins obtained by process 1 were 6–10 and 17 ppm, respectively.

Finally, a batch of bovine crude heparin produced by enzymatic proteolysis, heat coagulation at 90°C, and cetylpyridinium chloride complexation of heparin (process 2) was analyzed by the optimized competitive indirect ELISA. The immunochemical reactivity of this heparin was 22.1% of the reactivity of bovine crude heparin obtained by process 1.

DISCUSSION

Characterization of the animal origin of crude heparins is a major goal for manufacturers. Physicochemical methods such as ^{13}C NMR or HPLC analysis have been applied to heparinase digests in order to study the differences between porcine and ruminant heparins at the molecular level.(5–10) Intra-species variations in the heparin chemical structure and

slight structural modifications due to the manufacturing processes impact on the sensitivity of these techniques for the analysis of mixtures of porcine and ruminant heparins. Accordingly, an immunochemical approach has been developed to characterize species-specific contaminants in bovine crude heparin.(12) Up to 13 bovine-specific components have been detected in EM and three in the final crude heparin. The major one, called Ag1, is not gut specific since it has been also recovered in high concentration in lung, liver, spleen, and kidney.(12) The immunochemical characterization of rabbit antisera produced against EM or bovine heparin has been fully described and a single radial immunodiffusion test able to detect 6 p 1000 bovine crude heparin in porcine heparin at 50 mg/mL has been proposed.(12) However, the development of more sensitive techniques was required to better detect the presence of bovine tissues in porcine heparin.

ELISA techniques are roughly one thousand-fold more sensitive than precipitation techniques. Among them, heterogeneous enzyme immunoassays are considered more sensitive than homogeneous enzyme immunoassays, and the sensitivity of competitive assay is theorically lower than two site assays.(18) Consequently, a sandwich ELISA was first developed using the peroxidase labelled IgG fraction, purified from a rabbit anti-EM antiserum. The sensivity of the test (10 ppm) was satisfactory. However, a high background that could not be reduced by the blocking step was observed.

Competitive indirect ELISAs were then tested using EM or bovine crude heparin for the coating and anti-EM or anti-bovine crude heparin as detector antibodies. Substituting the standard peroxidase-labelled goat anti-rabbit IgG by Amdex conjugate resulted in approximately a ten-fold increase in sensitivity. In Amdex conjugate, the antibodies are conjugated to multiple horseradish peroxidase molecules linked to a dextran backbone. An amplified signal is thus obtained when revealing the peroxidase activity of Amdex conjugate bound to the primary antibody.

In the optimal conditions, a detection limit of 1–5 ppm was obtained using a coating with bovine crude heparin and either the anti-EM or anti-bovine crude heparin antiserum. This last combination was selected, since the antigen–antibody system was better defined: the anti-bovine-crude heparin contained a high titer of antibodies essentially directed against Ag1(12) and the low level of antibodies reacting against BSA and heparin were absorbed by immunoaffinity.

The competitive indirect ELISA presents some advantages over the sandwich type, since high titer goat anti-rabbit conjugates, such as the very sensitive Amdex conjugate, are commercially available and no purification and labelling of the specific antibody are required. Thus, laboratories not experienced in antibody purification and labelling techniques can more easily use the technique when the primary antibody is available.

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The optimized test was used to control a bovine crude heparin extracted according to the second type of the processes generally used.(14) The immunochemical reactivity of this crude heparin was only 22.1% of the reactivity obtained with the bovine crude heparin extracted by process 1. This result underlines a limit to the present approach: the detection level is directly dependent on the contaminant concentration of the crude heparin, which may vary with the extraction process. Thus, ELISA results obtained for unknown crude heparins cannot be related to accurate proportions of bovine heparin. However, even traces of bovine antigens, detected in a porcine heparin, are indicative of a contamination by bovine tissues and the heparin must be discarded. In this context, more important than the assay accuracy is the absolute specificity of the technique. No false positive reactivities were observed when 23 batches of porcine crude heparin of known origin were tested.

Unexpectedly, very slight reactivity was observed for ovine and caprine crude heparins. These species are phylogenetically close to the bovine and it is generallee difficult to obtain species-specific antisera without using interspecies immunisations(19,20) or monoclonal antibody production.(21) Thus, Ag1 must be considered as a strictly bovine specific antigen, which is an additional guaranty for the specificity of the developed ELISA. However, since all ruminant heparins are banned from heparin production, ELISAs for ovine and caprine crude heparins have been recently developed in our laboratory and their validation is being completed.

ACKNOWLEDGMENTS

The authors wish to thank Aventis Pharma for partial financial support and, particularly, J. Poirier for his advice and encouragements, L. Siret for careful revision of the manuscript, and T. Le Baron (Valori 5) for providing some of the heparins used in this work.

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Received February 26, 2001 Manuscript 3033
Accepted April 15, 2001

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