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Immunochemical control of the species origin of porcine crude heparin and detection of ovine and caprine materials

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Abstract

As a consequence of the outbreak of bovine spongiform encephalopathy (BSE), ruminants materials have been generally banned from the production of heparin. Immunochemical methods have been recently developed for the control of the raw materials used by manufacturers of materials such as porcine mucosa and for the detection of bovine crude heparins. To certify the porcine origin of crude porcine heparins and to exclude ovine or caprine materials, new ELISAs were developed. Rabbit antisera were produced against species-specific antigenic contaminants present in crude heparins or in eluted materials (EM) from the chromatographic step of the purification process. When analysed by line immunoelectrophoresis, these antisera revealed five to eleven antigenic contaminants in the EMs, the major one being the most anodic and predominant antigen in crude heparins. Using the best antisera, competitive indirect ELISAs were optimised. They allowed the detection of porcine, ovine and caprine crude heparins down to a dilution of 0.6 to 1.5 parts per 1000, with CVs ranging from 3 to 12%. These ELISAs complete the set of immunological techniques which can be routinely used by heparin manufacturers to secure their supply chain.

Keywords: Heparin; Bovine Spongiform Encephalopathy, BSE; Ovine antigen; Caprine antigen; Porcine antigen; Immunochemical detection; ELISA

1. Introduction

Heparin is a highly sulphated polysaccharide used for over half a century as an anticoagulant and antithrombotic drug. Approximately 500 million heparin doses are used world-wide each year for this purpose and the large number of favourable biologic activities unrelated to anticoagulation have led to a constantly growing interest for new therapeutic applications [1,2].

Heparin is synthesised in connective tissue mast cells as a proteoglycan and its commercial form has been purified from intestinal mucosa or lung of pig and cattle for decades. However, the outbreak of BSE in the UK in 1986 and its extension to other countries have led the regulatory authorities to ban ruminant heparins for human therapy. Accordingly, it became necessary to develop analytical methods intended to confirm the species origin of heparin.

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To certify the porcine origin of the mucosa collected by heparin manufacturers, immunochemical methods have been recently reported [3]. For routine control in plants, a particularly convenient single radial immunodiffusion assay has been found suitable to characterise the porcine origin of the mucosa with a detection limit of 3 parts per 1000 bovine, ovine or caprine intestinal mucosa in porcine intestinal mucosa.

However, manufacturers often have relied on external suppliers of crude heparin for their production. The control of the species origin of these heparins has been studied by ^{13}C and ^{1}H NMR or HPLC analysis of heparinase digests [4–9]. These techniques are currently not sensitive enough, notably as a consequence of intra-species variations in heparin structure and of desulphation during the storage of the mucosa and the purification process [7,9]. Moreover, none of these techniques can differentiate ovine heparin from porcine heparin.

Since the species origin of crude heparins cannot be certified with confidence by analysis of the polysaccharide chain structure, it has been recently proposed to characterise species-specific antigenic contaminants in raw materials using immunochemical methods. Using rabbit polyclonal antisera raised against bovine crude heparin a major contaminant (Ag1) has been found in bovine intestinal heparins and bovine pulmonary EM. The antisera produced against this antigen have been used to develop a sensitive ELISA which allows the detection of 5 ppm bovine crude heparin in porcine heparin [10].

Following the same immunological approach, this work reports the development of ELISAs able to ascertain the porcine origin of crude heparins and to detect ovine and caprine crude heparins which are also banned from heparin production.

2. Materials and methods

2.1. Chemicals

Freund's complete and incomplete adjuvants, human serum albumin (HSA) and porcine heparin–agarose were purchased from Sigma Chemical Company (St Louis, MO, USA). Crude and pure porcine mucosal heparins obtained by process 1 [11] and process 2 [12] were supplied by Valori-5 (Ploërmel, France). Amdex multiperoxidase labelled goat anti-rabbit IgG was supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). The Amdex conjugate was diluted 1:2 (v/v) with glycerol and stored at -20 °C.

2.2. Crude heparins preparation

Ovine and caprine crude heparins were purified from hashed gut according to process 1 [11]. Briefly, the tissues were homogeneised and proteolysed 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.

For antisera production, EM were 10-fold concentrated by ultrafiltration and then extensively dialysed against Tris–HCl buffer (pH 7.5, 0.1 M).

2.3. Polyclonal antibodies

Antisera were produced against concentrated EM or 50 mg/ml crude heparins in sodium phosphate buffer (pH 7.2, 0.01 M) containing 0.15 M NaCl (PBS). Rabbits (four for each antigen) were immunised 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 [13]. Animals were bled 7–9 days after each booster injection and the sera were analysed for antibody activity and specificity by double immunodiffusion and line immunoelectrophoresis.

Antibodies against heparin were removed by affinity chromatography on heparin–agarose (3 ml settled bed for 9 ml antiserum 10^{-2} diluted in PBS).

2.4. Immunoprecipitation techniques

Agar gel double immunodiffusion was performed using 1.8 mm thick agar gel containing 1.2% agar Noble in veronal buffer (pH 7.3, 0.05 M).

Line immunoelectrophoresis was carried out according to Kroll [14] using 1.2% agarose. Antigens and/or antisera to be compared were moulded into adjoining gel sections. Electrophoresis was run at 2.5 V/cm for 180 min.

After immunodiffusion or immunoelectrophoresis the gels were washed in 0.15 M NaCl containing 0.1% sodium azide for 24 h at 4 °C under continuous stirring to remove non-precipitated proteins. They were then slipped on a GelBond® film (Pharmacia Biotech), dried and stained in a 0.12% Coomassie Brilliant Blue R250 water/ethanol/acetic acid solution (65:30:5, v/v/v).

2.5. Competitive indirect ELISA

Microtitre plates were coated overnight at 4 °C or 1 h at room temperature with 100 μl of EM or crude heparins in PBS. The optimum coating concentration was determined by checkerboard titration. All subsequent steps were performed at room temperature. Plates were washed five times with PBS containing 0.1% Tween 20 (PBSTw). A 1 h blocking step was performed with 200 μl of 1% HSA (v/v) 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-crude heparins) was incubated in test tubes with one volume of serially diluted solutions of crude heparins solutions (20 mg/ml starting concentration). 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. After five washes with PBSTw, 100 μl of 0.4 mg/ml *o*-phenylenediamine in phosphate–citrate buffer (pH 5.0, 0.1 M) were added, running the colour reaction for 30 min before stopping it with 50 μl per well H_2SO_4 (2.5 M). Absorbance was measured at 492 nm using a microplate reader (IEMS, Labsystem, Finland). PBSTw containing 0.1% HSA (v/v) and crude or pure porcine heparin at 20 mg/ml was used as diluent for the solutions. All analyses were performed in duplicate and the detection limit was calculated as the mean blank signal minus 3 S.D.

3. Results

3.1. Immunochemical characterisation of rabbit antisera

The rabbit antisera raised against porcine EM detected one major antigen and one to two minor

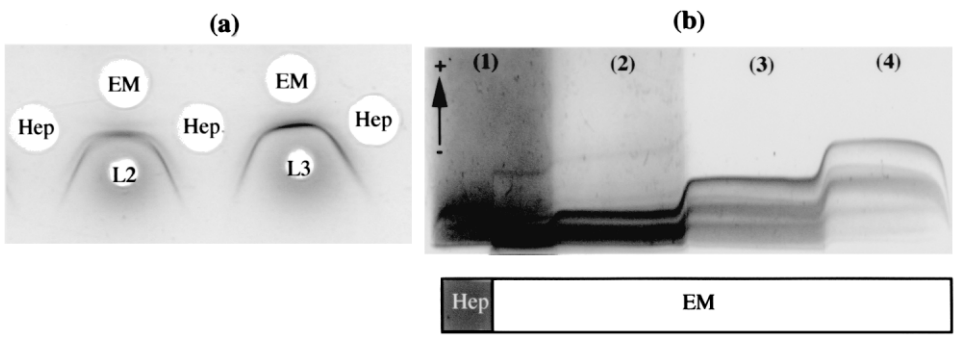

Fig. 1. Immunochemical characterisation of the rabbits anti-porcine EM by agar gel double immunodiffusion (a) and line immunoelectrophoresis (b). EM, eluted materials from the chromatographic step of the purification process of heparins. Hep, porcine crude heparin. L2 and L3, rabbit anti-porcine EM no. 2 and no. 3. In the line-immunoelectrophoresis (b), the rabbit anti porcine EM was used at 320 (1), 160 (2), 80 (3) and 40 (4) μl/2.5 ml of agar.

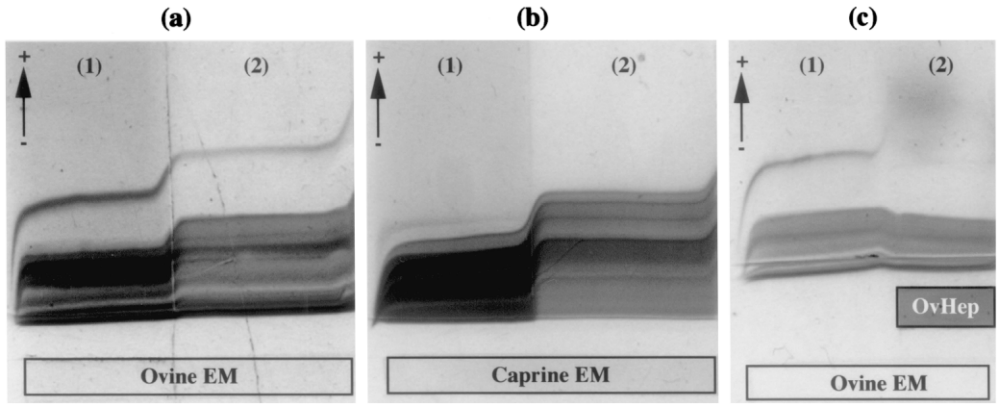

Fig. 2. Immunochemical characterisation of the rabbits anti-ovine and caprine EM by line immunoelectrophoresis. (a) rabbit anti-ovine EM at 320 (1) and 160 (2) μl/2.5 ml agar. (b) rabbit anti-caprine EM at 320 (1) and 160 (2) μl/2.5 ml agar. (c) rabbit anti-ovine-EM at 160 μl/2.5 ml. In (2), a sample of ovine crude heparin (OvHep) was inserted in the free agar gel zone between the antigen and antibody containing gels.

antigens against porcine EM or crude heparin when tested by double immunodiffusion (Fig. 1a). No precipitin line was detected against bovine, ovine and caprine EM or heparins (not shown).

Using the more resolving line immunoelectrophoresis, up to five precipitin lines were observed against porcine EM, the major one being the most anodic and the only one observed in porcine crude heparin (Fig. 1b). The rabbit antisera raised against ovine EM revealed up to eight precipitin lines when tested against ovine EM in line immunoelectrophoresis (Fig. 2a). Similarly, up to 11 precipitin lines were observed when the rabbit anti-caprine EM were tested against caprine EM in line immunoelectrophoresis (Fig. 2b). In the ovine system, when a sample of ovine crude heparin was inserted between the antigen and antibody zones, the most anodic precipitin line revealed with EM disappeared as a consequence of a large excess of antigen while the other precipitin lines were not modified (Fig. 2c). Thus, the most anodic antigen can be considered as the major antigen of ovine crude heparin. In the caprine system, all the precipitin lines were more anodic after insertion of caprine crude heparin (not shown). This indicates that the relative proportion of the contaminants detected has not been significantly modified by the methanolic precipitation of EM.

The rabbit antisera raised against ovine and caprine crude heparins generally exhibited higher antibody titres. Consequently these antisera were preferred for the development of the ELISA tests and they were used to study the cross reactivity between ovine and caprine crude heparins by double immunodiffusion. As shown in Fig. 3, strong precipitin lines were observed in both homologous systems. The precipitin line observed in the caprine system was only slightly deviated by the anti-ovine antisera, indicating a very faint cross reaction of the anti-ovine antiserum with caprine crude heparin. When tested against ovine crude heparin, the anti-caprine antiserum revealed a slight precipitate with complete antigenic iden-

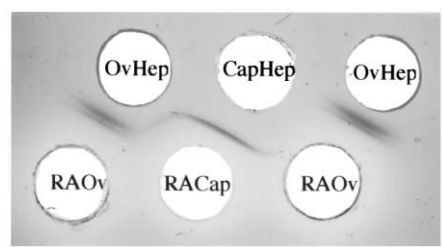

Fig. 3. Immunochemical characterisation of the rabbits anti-ovine and caprine crude heparins by agar gel double immunodiffusion. RAOv, rabbit anti-ovine crude heparin; RACap, rabbit anti-caprine crude heparin; OvHep, ovine crude heparin; CapHep, caprine crude heparin.

Table 1
Characteristics of the ELISA assays used to certify the porcine origin of heparins and to detect ovine and caprine materials

Heparin	Detection limit (parts per 1000)	Coefficient of variation	
		Concentration tested (parts per 1000)	CV%
Porcine	1.5	10	5.8
		100	2.9
Ovine	1.3	1.5	12
		15	8
Caprine	0.6	1	8.0
		10	8.5

tity with the caprine antigen but whose fusion with the ovine antigens was not clearly established.

3.2. Competitive indirect ELISA for porcine antigens

Optimal concentrations of the porcine crude heparin used for the coating, of the primary rabbit antibody and of the anti-rabbit conjugate were defined by checkerboard titration. Microtitre plates were coated with porcine crude heparin at 200 μg/ml in PBS buffer, the primary antibody and the Amdex conjugate were used diluted 1:2000 and 1:800, respectively. In these conditions the detection limit was 1.5 part per 1000 porcine crude heparin in porcine pure heparin (Table 1) and the cross-reactivities of the bovine, ovine and caprine crude heparins were all ≤ 1.5 parts per 1000 (Fig. 4). The intra-assays CVs obtained for 10 determinations are given in Table 1.

3.3. Competitive indirect ELISA for ovine antigens

Optimisation of this ELISA was particularly studied since the sensitive detection of ovine material in porcine heparins is of the upmost importance. Coating conditions were defined using different concentrations of ovine crude heparin in three different buffers: borate buffer (pH 9.4, 0.05 M), PBS (pH 7.2, 0.01 M) and acetate buffer (pH 5.0, 0.2 M) (Fig. 5a). Optimal results were obtained by coating at 200 μg/ml in PBS. The coating was equally efficient by incubation overnight at 4 °C or 1 h at room temperature. The inhibition step was studied using different concentrations of antiserum and conjugate, as exemplified in Fig. 5b. Optimal results were obtained with the rabbit antiserum against ovine crude heparin diluted 1:30 000 and the Amdex goat anti-rabbit peroxidase labelled conjugate diluted 1:800.

When testing dilutions of ovine crude heparins in mixtures of porcine crude and pure heparins we observed an inhibitory effect related to the proportion of porcine crude heparin (Fig. 6a). The

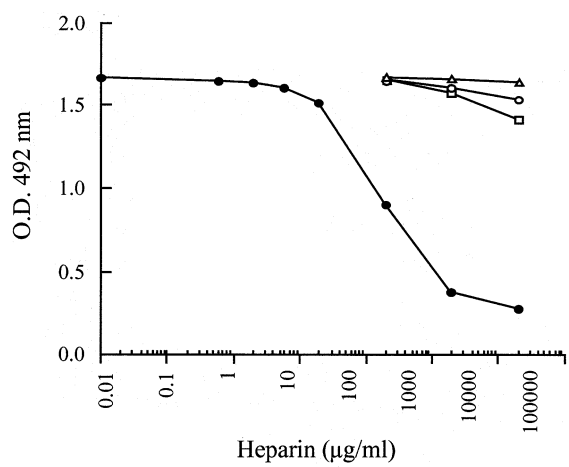

Fig. 4. Quantitation by competitive indirect ELISA of porcine specific antigens in crude heparins. Microtitre plates were coated with porcine crude heparin. Rabbit anti-porcine EM was used as primary antibody and Amdex multiperoxidase labelled goat anti-rabbit IgG as tracer antibody. Competitive antigens were crude heparins from pig (closed circles), cow (open circles), sheep (open triangles) and goat (open squares). Results are means of duplicates.

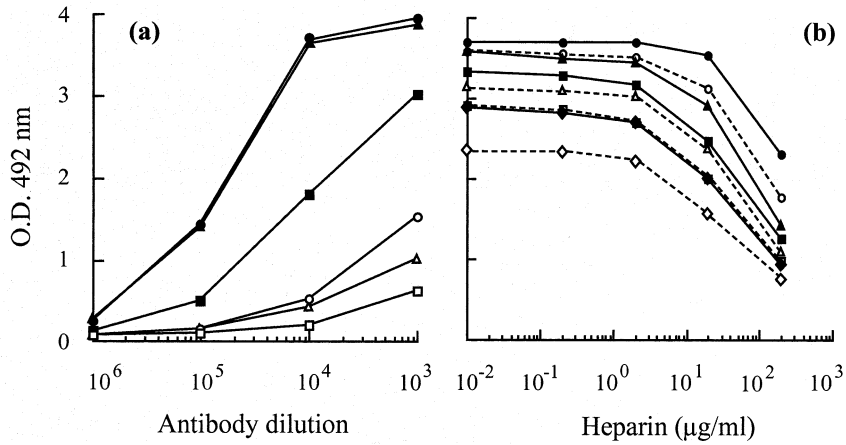

Fig. 5. Quantitation by competitive indirect ELISA of ovine specific antigens in crude heparins: optimisation of the test. Microtitre plates were coated with ovine crude heparin. Rabbit anti-ovine crude heparin was used as primary antibody and Amdex multiperoxidase labelled goat anti-rabbit IgG as tracer antibody. (a) Effect of the coating buffer of ovine crude heparin, PBS (circles), borate (triangles) and acetate (squares). Two concentrations of ovine crude heparin are presented, 200 (closed symbols) and 2 (open symbols) μg/ml. (b) Effect of the primary antibody dilution: 1:10 000 (circles), 1:20 000 (triangles), 1:30 000 (squares) and 1:45 000 (diamond). Effect of the Amdex conjugate dilution, 1:500 (closed symbols) and 1:1000 (open symbols). Results are means of duplicates.

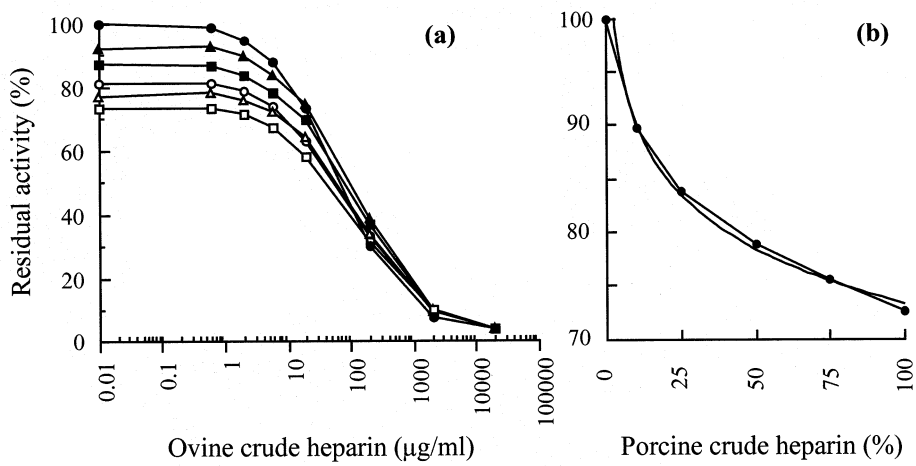

Fig. 6. Quantitation by competitive indirect ELISA of ovine specific antigens in crude heparins, inhibitory effect of porcine crude heparins. Microtitre plates were coated with ovine crude heparin. Rabbit anti-ovine crude heparin was used as primary antibody and Amdex multiperoxidase labelled goat anti-rabbit IgG as tracer antibody. The competitive ovine crude heparin was diluted in porcine heparin. (a) Effect of the percentage of porcine crude heparin added to the porcine pure heparin diluent. Closed symbols, 0 (circles); 10 (triangles); and 25 (squares)%. Open symbols, 50 (circles); 75 (triangles); and 100 (squares)%. Results are means of duplicates. (b) Curve fitting of the relation between the porcine crude heparin concentration (X axis) and the residual activity (Y axis): $Y = -16.9\log(x) + 107$.

inhibitory effect was fitted by the equation: $Y = -16.9\log(X) + 107$ (Fig. 6b) with Y as the percentage of residual activity and X the percentage of porcine crude heparin. Absorption of the rabbit antiserum with insolubilised porcine heparin did not result in a diminution of this inhibitory

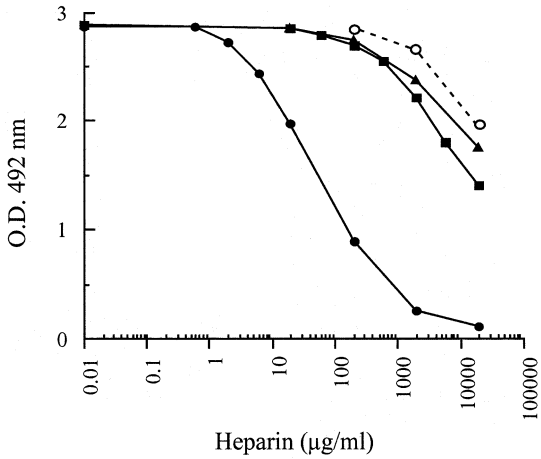

Fig. 7. Quantitation by competitive indirect ELISA of ovine specific antigens in crude heparins, cross-reactivity of porcine and ruminants heparins. Microtitre plates were coated with ovine crude heparin. Rabbit anti-ovine crude heparin was used as primary antibody and Amdex multiperoxidase labelled goat anti-rabbit IgG as tracer antibody. Competitive antigens were crude heparins from sheep (closed circles), goat (closed triangles), cow (closed squares) and pig (open circles). Results are means of duplicates.

effect. Consequently, the detection limit established at 0.2 part per 1000 ovine crude heparin in porcine pure heparins ($n = 13$) was increased to 1.3 part per 1000 ovine crude heparin in porcine crude heparins (Table 1). This last value was obtained by testing 13 and 11 porcine crude heparins obtained by process 1 and 2, respectively.

The intra-assays CVs obtained for 10 determinations are given in Table 1.

The cross-reactivities of bovine, caprine and porcine crude heparins were found at 2.8, 1.4 and 1.0 parts per 1000, respectively (Fig. 7).

3.4. Competitive indirect ELISA for caprine antigens

The development of this ELISA was conducted essentially as described for the ovine ELISA. Optimal results were obtained with caprine crude heparin coated at 200 μg/ml in PBS, the rabbit anti-caprine crude heparin diluted 1:30 000 and the Amdex conjugate diluted 1:800. A typical calibration curve three fold replicated is presented in Fig. 8a.

The detection limit was determined at 0.3 part per 1000 caprine crude heparin in porcine pure heparins ($n = 11$). However, a very slight inhibitory effect was observed with porcine crude

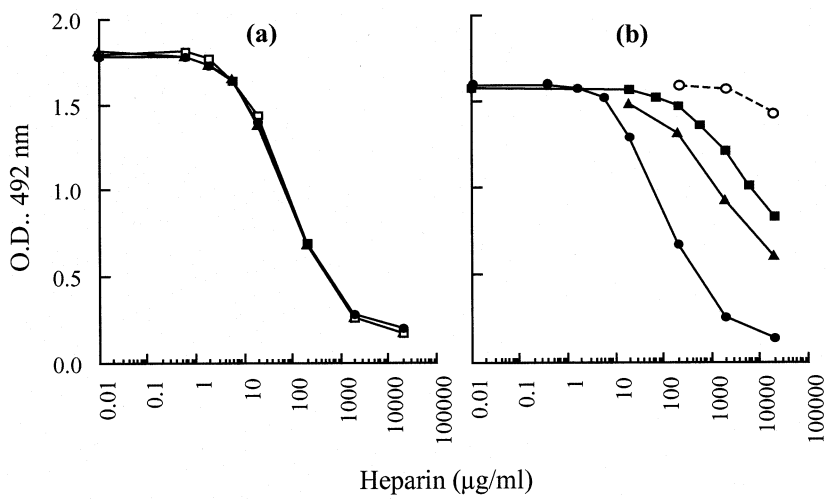

Fig. 8. Quantitation by competitive indirect ELISA of caprine specific antigens in crude heparins. Microtitre plates were coated with caprine crude heparin. Rabbit anti-caprine crude heparin was used as primary antibody and Amdex multiperoxidase labelled goat anti-rabbit IgG as tracer antibody. (a) Superposition of the results obtained for a triplicated standard curve. (b) Cross-reactivity studies: the competitive antigens were crude heparins from goat (closed circles), sheep (closed triangles), cow (closed squares), and pig (open circles). Results are means of duplicates.

heparins and the detection limit was finally established at 0.6 part per 1000 caprine crude heparin in porcine crude heparin at 20 mg/ml (Table 1).

The intra-assays CVs obtained for 10 determinations are given in Table 1.

The cross-reactivities of bovine, ovine, and porcine crude heparins were found at 4, 12 and 0.5 part per 1000, respectively (Fig. 8b).

4. Discussion

Immunochemical methods have been first developed to control the species origin of the gut mucosa used in plants for heparin purification [3]. More recently, an ELISA has also been proposed for the detection of bovine materials in crude heparins obtained from external suppliers [10]. In this case, the technique was based upon the production of rabbit antisera directed against the antigenic contaminants of bovine crude heparin. In the present work, a similar approach was developed for the detection of ovine and caprine materials since these species are also banned from heparin production. The ovine detection was of particular interest since this species is sensitive to BSE contamination and exhibits, upon infection, clinical symptoms similar to the 'scrapie' disease one.

Rabbit antisera were produced against the crude heparins as well as against the concentrated EMs since this intermediate product was expected to contain contaminants in higher concentrations than in the corresponding final crude heparin. The antibody responses were firstly analysed using immunoprecipitation techniques such as double immunodiffusion and the resolutive line immunoelectrophoresis. As previously observed for bovine species, up to 11 contaminants were revealed in EMs using the highly resolutive line immunoelectrophoresis, the major one being the most anodic and the predominant contaminant of crude heparins. The observation of a pattern roughly similar between the caprine EM and crude heparin indicated a lack of effect for the methanolic selective precipitation step and outlined the interest of such a technique for monitoring the process as well as for an intermediate quality control.

Double immunodiffusion was particularly useful for the cross-reactivities studies of the antisera directed to crude heparin. Surprisingly, the ovine and caprine antigens did not cross-reacted intensively as usually observed for different proteins of these species [3,15,16]. However, such a phenomenon has been previously observed with the major antigen of the bovine crude heparin (Ag1) which was not found in ovine or caprine materials.

Since the absence of clear precipitin lines in agar gel diffusion techniques is not a sufficient proof of the lack of cross-contaminating antibodies, the antisera specificity was also evaluated in the indirect competitive ELISA. As expected, cross-reactivities were very low and never exceeded 2 parts per 1000 between the porcine antisera and the ruminants crude heparins analysed at 20 mg/ml or between the caprine or ovine antisera and the porcine crude heparin.

The detection limits obtained for the ovine and caprine ELISAs allow an efficient control of porcine crude heparins in plants. The CVs% were also satisfactory. However, it must be kept in mind that the efficiency of this control depends on the contaminant concentration in the samples. Consequently, the crude character of the porcine heparin to be controlled for the absence of ruminant materials must be firstly assessed by the porcine ELISA.

Added to the SRID test and to the bovine ELISA already reported [3,10], the three new ELISAs here proposed constitute a complete set of simple and sensitive techniques which can be routinely used by heparin manufacturers to secure their supply chain.

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