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Title of the Project	:	ior-CEA-1: Labelling, Quality Control and Clinical Evaluation
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2.3.1. Introduction

Within the Co-ordinated Programme on Labelling, Quality Control and Evaluation of Monoclonal Antibodies, the IAEA has made a great effort to expand efficient labelling methods, mainly those with radioisotopes which have been used for radioimmunoscintigraphy. In this sense, more recently $^{99}Tc^{m}$ has been mostly employed in the majority of the investigations [1–4] due to its ideal physical characteristics. Efficient labelling of monoclonal antibodies depends on a number of factors including the method and way of the label incorporation into the protein. During the last years several direct labelling approaches have been developed [2, 4, 5], which led to attain simple and inexpensive methods for medical practice, as well as safe and stable techniques which bring accurate and good quality images.

Accordingly, this paper describes the results obtained during last five years which come from the comparison among different labelling systems, passing through the quality control to test the labelled monoclonal stability and the protein bioreactivity, to continue in the clinical evaluation of ior-CEA-1, as well as the evaluation of other antibodies. Upto now we have evaluated more than 70 patients with the anti-CEA monoclonal antibody (ior-CEA-1), examined in different clinical assays such as:pilot [6], phase I-II [7] and extensive phase III-IV trials, whose results are encouraging. It confirms that the employed labelling approach was safe and adequate.

2.3.2. Materials and methods

2.3.2.1. Monoclonal antibody (MAb)

This is a murine IgG-1 antibody secreted by hybridoma clone K3/15 obtained from the cell fusion between P/X63-Ag 8-653 myeloma cells and spleen cells derived from Balb/c mice immunized with purified cells from the liver metastasis of a colonic adenocarcinoma. This IgG is highly specific against protein epitope on cell bound CEA and it is included in the gold 1 group according to Hedin's classification [8].

2.3.2.2. Labelling

MAb labelling was tested by different approaches using 2-mercaptoethanol (2-ME), $SnCl_2.2H_2O$, ascorbic acid (AA), dithionite (DT) as reductants.

The 2-ME approach was described elsewhere [3,4]. Briefly, ior-CEA-1 was reduced with a molar ratio of 2000:1 of 2-ME:Antibody and allowed to incubate at room temperature for 30 min. At the end of the incubation, the reduced IgG was purified by gel filtration on Sephadex G-50 (Pharmacia) using PBS solution as mobile phase. Aliquots of 0.5–1 mg of reduced antibody were used for labelling. After reconstitution of the AMERSHAM MDP kit with 5 mL of 0.9% saline, 50 μL of this solution was added to reduced monoclonal antibody and it was labelled with the corresponding activity (74 MBq or 1850 MBq) and allowed to react for 15 min.

For the evaluation of the other reducing agents two different systems were employed, on one hand Sn(II)/Ascorbate were used as reducing agents, while Ascorbate/DT were assessed on the other. For the analysis of these systems, two experimental designs were carried out (type 33 and 3 respectively).

In the first system the influence of Sn(II), AA as well as the tartrate on labelling yield was studied. The Sn(II) ions amounts were varied ranging from 5 to 20 μg of $SnCl_2.2H_2O$, according to the molar ratio in relation to MoAb of 3:1, 6:1 and 13:1. This interval was chosen since at lower stannous concentration $^{99}Tc^mO_4^-$ was present in the reaction mixture, while at higher concentrations an appreciable amount of $^{99}Tc^m$-colloid could be observed. The ascorbate varied ranging from 0 to 500 μg for a molar ratio of 0–3500 in relation to MoAb and tartrate ranging from 50 to 200 ug. These three components were mixed according to corresponding amounts to conform a final volume of 70 μL of reducing solution. This solution was added to 250 μL of antibody solution. The mixture was incubated at room temperature for 30 min. and then the reduced antibody was immediately labelled with 74 MBq of $^{99}Tc^m$ and allowed to incubate for 15 min.

The third labelling approach is based on Thakur's method [5], where the ascorbate is added ranging in a molar ratio of 3500–35 000:1 in relation to antibody, and incubated for 1 h at room temperature. Then $^{99}Tc^m$-pertechnetate was reduced over 5 min with a freshly dithionite solution at pH11 and added to the antibody solution previously treated with AA to conform a DT final concentration ranging from 0.4 to 4 mg/mL in a final volume of 182 μL. The pH was varied ranging from 4.5 to 6.9 and the mixture was incubated for 45 min at room temperature.

2.3.2.3. Quality control

Quality control was measured by means of a combination of paper and instant thin layer chromatography (ITLC) as well as FPLC or HPLC. Whatman paper no. 1 (10 mm × 60 mm strips) was used for paper chromatography using methylethylketone as mobile phase. ITLC was carried out in 10 mm × 75 mm strips (German Inc., USA) in 0.9% saline as solvent (protein bound R_f = 0.0, MDP and $^{99}Tc^mO_4^-$ R_f = 0.9 and 1.0 respectively). FPLC (Pharmacia system) was performed in a Superose 6 HR 10/30 column and 0.2 M phosphate buffer in 0.15 M NaCl, pH7.4 as eluent, a flow rate of 0.5 mL/ min in a Mono S HR 10/10 cation exchange column with a stepwise elution from 0.05 M acetate pH5.0 (solution A) to 0.5 M sodium + 0.5 M Tris-hydrochloride pH8.0 (solution B). The salt concentration increase (solution B) was of 0.2 fold in every step and the flow rate was 180 mL/h. HPLC was carried out in a Beckman system using a Dupont GF-250 gel filtration column using 254 nm UV and radioactivity flow detectors and 0.1 M phosphate pH7.0 buffer as mobile phase.

2.3.2.4. Transchelation challenge test

The samples were submitted to two different challenge media, serum and cysteine solution, in order to test the "in vitro stability". **Serum**: 10 μg of the labelled antibody is added to 1 mL of fresh human serum. After a mild shaking, the sample was analysed by paper and ITLC chromatography, as well as electrophoresis if considered necessary. **Cysteine**:Two cysteine solutions were added to a solution of the radiolabelled antibody, such that the final molar ratio were 0.5:1 and 500:1 in relation to MoAb. The protein concentration was 350 μg/mL. After incubations at room temperature and 37°C for over 4 hrs the solutions were analysed by a paper chromatographic system [9], whose mobile phase was PBS, pH7.2.

2.3.2.5. Immunoreactivity studies

Micro ELISA system

The immunoreactivity of reduced and labelled ior-CEA-1 was determined in a competitive binding assay against the native antibody by a micro ELISA system, described previously [10]. Polystyrene plates (high binding, COSTAR), were coated with ior-CEA-1 diluted in coating buffer

(10 μg/mL) with 50 μL per well. The plate was incubated for 18 h at 37°C and then washed with washing buffer (phosphate buffer saline (PBS) with 0.05% of Tween 20) three times with 200 μL of buffer per well. The modified MoAb (reduced and labelled) and control antibodies were serially diluted in dilution buffer (sheep serum at 5% washing buffer) to obtain concentrations ranging from 10–0.325 μg/mL. Two hundred microliter of CEA (2 μg/mL) and modified or control antibody were incubated in Eppendorf vials to a final volume at 200 μL and vortexed for 10 sec. Fifty microlitres were added to wells coated with ior-CEA-1 as described above. The plate was incubated at 37°C for 1 h and washed in the same way as before. A sheep conjugate polyclonal anti-CEA antibody was added to each well (50 μL/well), according to the glutaraldehyde method [11]. After incubation at 37°C for 1 h the plate was washed and a solution of p-nitrophenyl phosphate in diethanolamine buffer 1 M pH9.8 (1 mg/mL) was added to each well (50 μL/well). The color was developed in 30 min at room temperature and the reaction was stopped with NaOH 3 M (50 μL/well). The absorbance values were measured in a ELISA plate reader (Organon, Teknica) at 405 nm.

Immunohistochemistry

A biotin-streptavidin peroxidase complex system (Amersham) was used for detection of the tissue bound ior-CEA-1. Briefly, deparaffined and rehydrated sections were treated with 3% H_2O_2 (aqueous or methanol solution) for 30 min. to block endogenous peroxidase activity, rinsed in buffer (PBS), incubated with 0.02 mg/mL of ior-CEA-1 MAb, followed by biotin-conjugated sheep antimouse immunoglobulin (diluted 1:100) and finally with the biotin-streptavidin-peroxidase complex (diluted 1:500).

2.3.2.6. Animal biodistribution study

Balb/c female mice weighing 18–25 gm were used in the experiment. About 100 μL of labelled MAb, corresponding to ~3.7 MBq, were injected intraperitoneally. Four h later, the animals were sacrificed. Radioactivity in each organ was counted in a gamma counter and recorded as percentage of dose/g tissue.

2.3.2.7. Clinical studies

Up to now over 70 patients have been studied with an age range of 44–73 years. All patients had documented malignancies of prior resection of colorectal carcinoma and in whom there is a high clinical suspicion of recurrence. The patients signed informed consent and were studied with the approval of the National Co-ordination Centre of Clinical Trials.

Each patient received 1 mg of ior-CEA-1 labelled with 2.25 GBq of $^{99}Tc^{m}$. The preparation was intravenously administered over a period of 2–3 min. One pre-administration and several post-administration blood samples were obtained along with a complete urine collection throughout the period of study (24 h). Quantitation of radioactivity of liver, spleen and kidneys was determined through regions of interest at 10 min, 4 h and 24 h post-administration by imaging on a planer camera equipped with a medium energy collimator and the attenuation correction according to [12, 13].

2.3.3. Results

We have explored different alternative variants for the labelling of ior-CEA-1 in order to attain our own experience in the reducing mechanism and the role that each agent plays. The labelling efficiency by the 2-ME reduction approach was measured by a combination of different chromatographic methods, such as paper chromatography, ITLC and HPLC. Figure 1 shows the HPLC results, where yields over 98% were obtained.

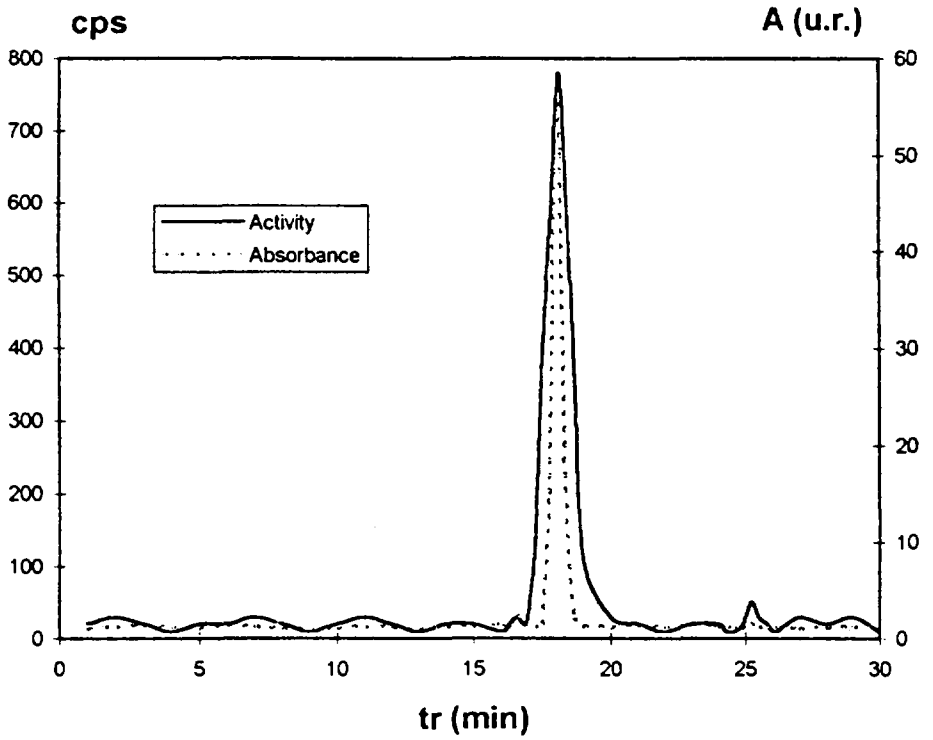

FIG. 1. HPLC Analysis of $^{99}Tc^{m}$-ior-CEA-1 antibody.

Figure 2a shows the influence of stannous ions, AA and tartrate in the Sn(II)/Ascorbate system It can be observed that the effect of Sn(II) is not significant. However, as may be seen in Fig. 2a ascorbate influences positively upto a point, and is thereafter minimal. Tartrate was used in the system as an appropriate complexing agent for preserving $^{99}Tc^{m}$ in the required redox state [16]. Although the influence is almost minimal, we observed a certain negative trend in the labelling yield while increasing tartrate concentration. Quality control showed that free and colloidal technetium were at a relatively low level. Nevertheless, although part of the radioactivity was associated with the peaks of identical retention time and area of the protein, FPLC results (Fig. 2b) show a profile which do not correspond to that expected.

The experimental design for the AA/DT system demonstrated that ascorbate as well as pH played no significant role in antibody labelling. The results for the AA/DT system are given in Fig. 3a, where all labelling values for low concentrations of DT were under 25%. The HPLC elution profile shows results similar to those obtained by Sn(II)/AA reduction.

Labelling stability was tested in vitro in the presence and absence of serum, determining the percentage of radioactivity associated with the protein as a function of time. Table I shows the results of challenging with serum after labelling with the three different approaches. After incubating the samples in serum for 24 h at 37°C, they were analysed by electrophoresis suggesting that although the majority of the radioactivity was associated with the protein, certain label dissociation can be observed, presumably TcO_4^-, especially, in the Sn(II)/AA and AA/DT samples. The ior-CEA-1 labelled via 2-ME was submitted to cysteine challenge (Fig. 4). This labelling technique attained the best stability result in serum and its possible in vivo application.

The designed micro ELISA system allows the assessment of biological activity of modified monoclonal antibody against control. By means of an inverse competitive assay using a fixed amount of CEA (1 μg/mL) and serial dilution curves of MAb, the antigen will react with the coated antibody, so the color is developed when the modified antibody is not able to bind with the CEA. Fig. 5 shows the binding curve trends of the native, reduced, labelled ior-CEA-1 and a non-specific MoAb. By means of a linear regression line, the slope of each one was determined, which by the ratio from control and modified antibody binding curves allows to determine the immunoreactivity index, defined as a ratio between the slopes from modified antibody and control binding curves. The results are shown in Table II.

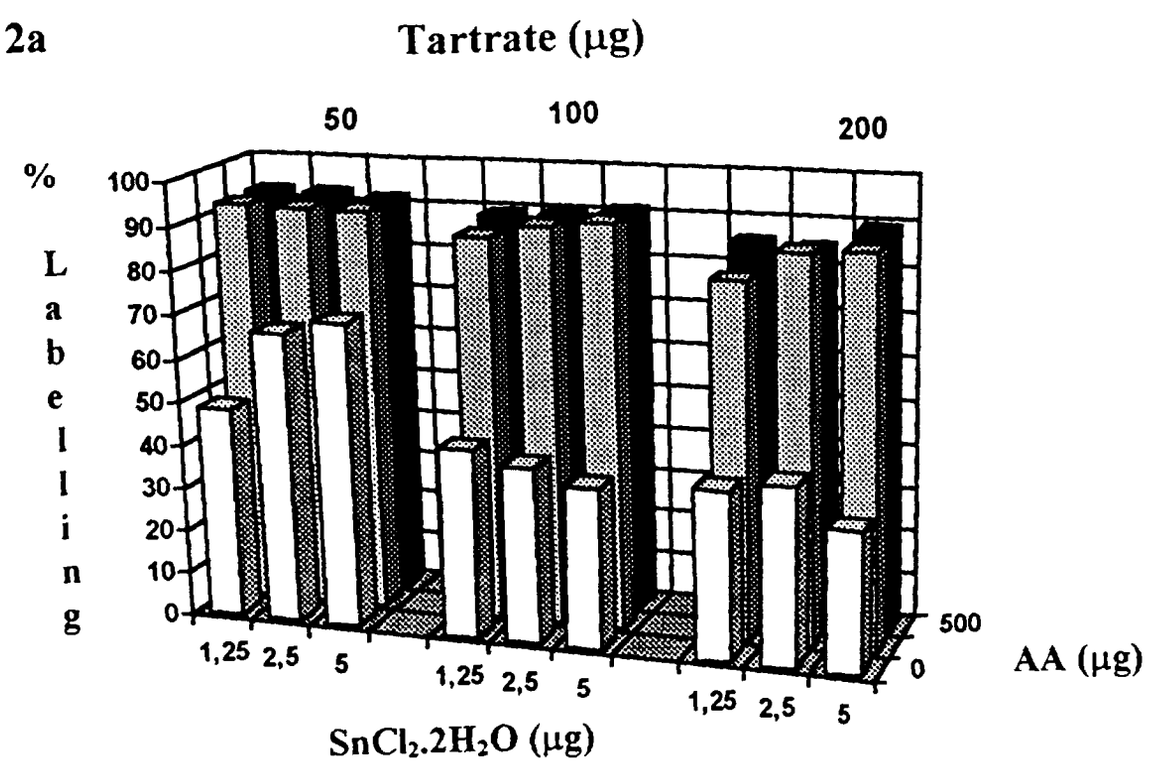

FIG. 2a. Influence of amount of reagents on $^{99}Tc^{m}$ labelling of antibody in Sn(II)/Ascorbate system.

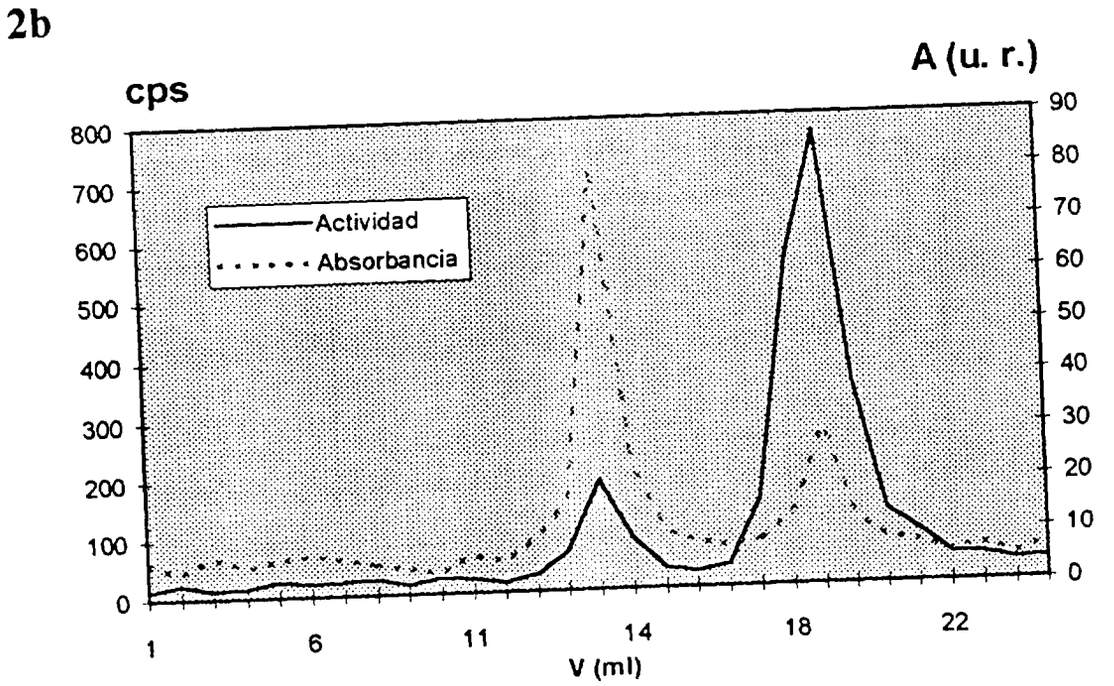

FIG. 2b. HPLC Analysis of $^{99}Tc^{m}$ labelled antibody.

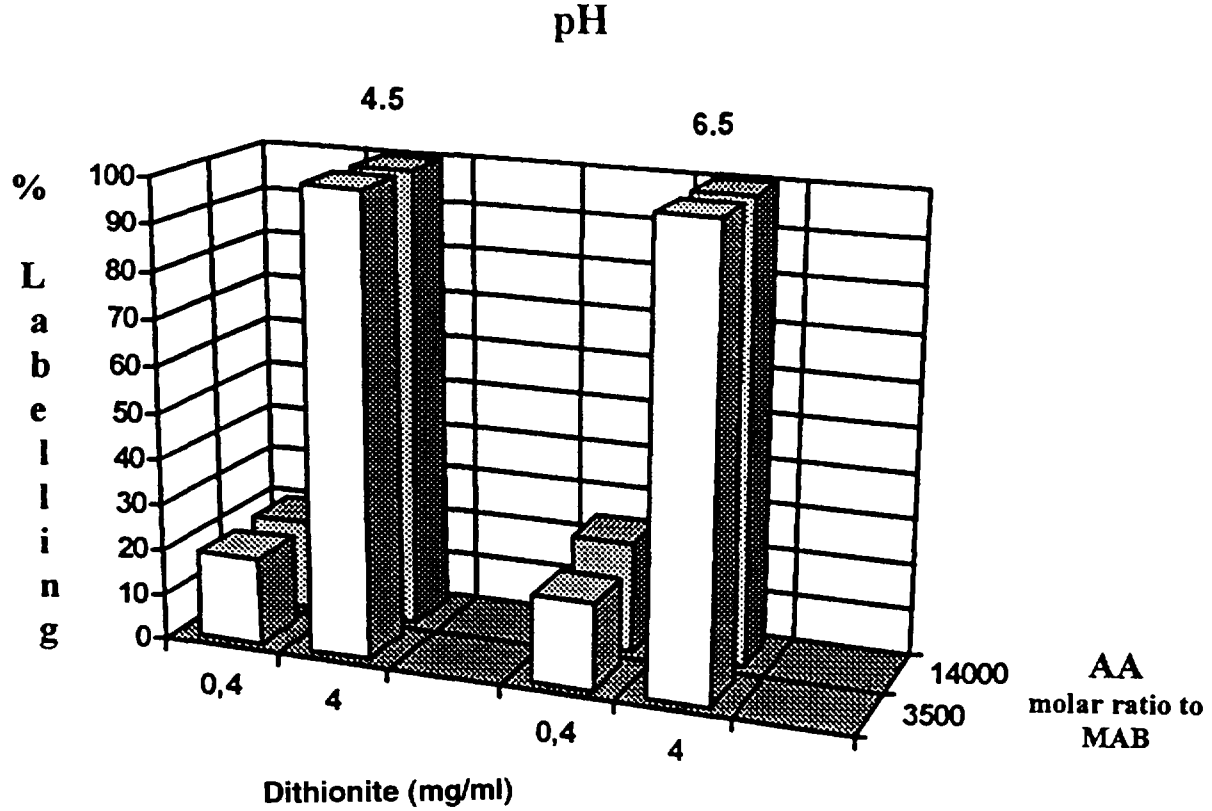

FIG. 3. Effect of amount of reagents on $^{99}Tc^{m}$ labelling of antibody in AA/DT system.

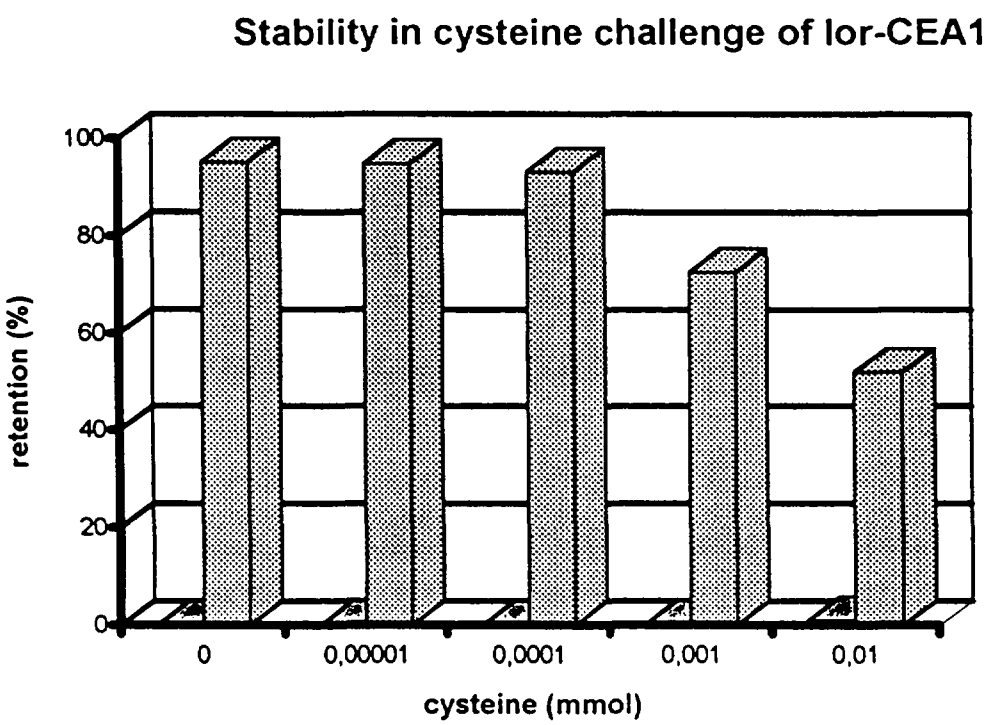

FIG. 4. Cysteine challenge test of $^{99}Tc^{m}$-ior-CEA-1 labelled using 2-ME method.

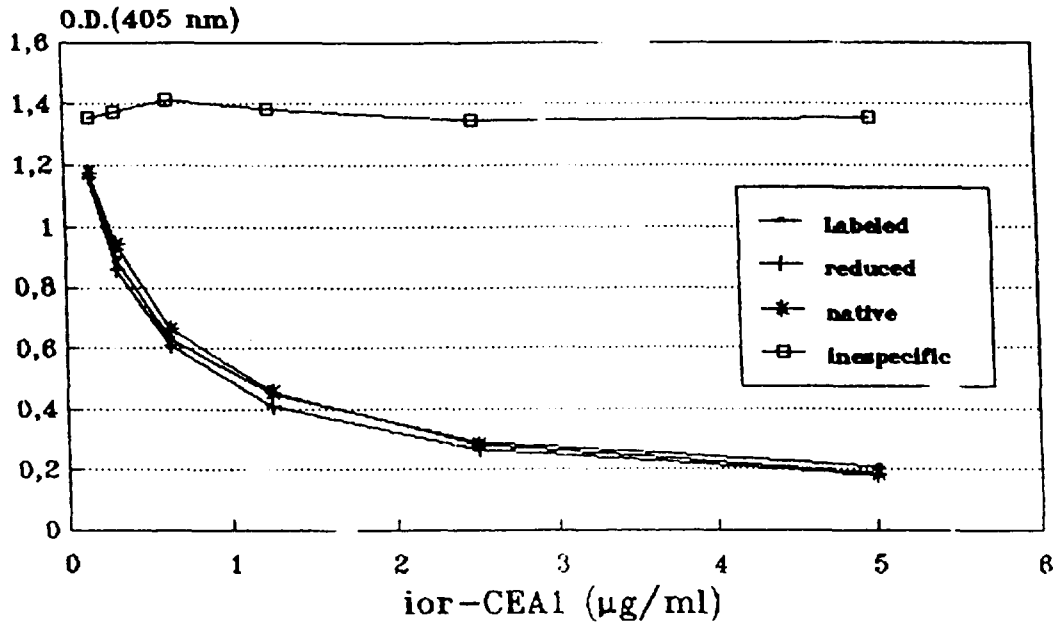

FIG. 5. Assessment of immunoreactivity of native and modified ior-CEA-1.

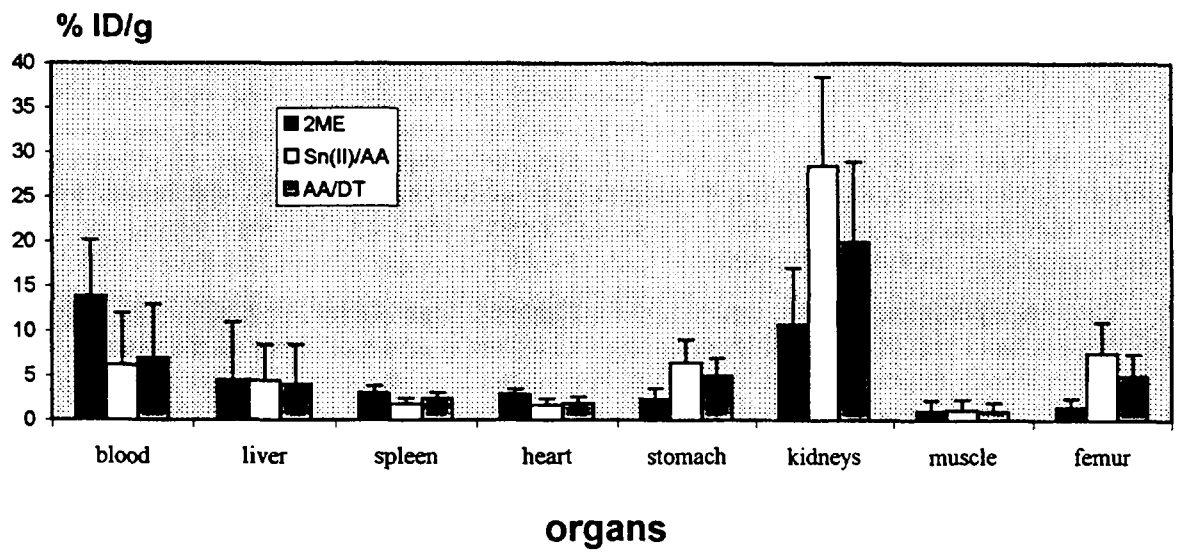

FIG. 6. Biodistribution of $^{99}Tc^{m}$-ior-CEA-1 prepared by different methods.

TABLE I. STABILITY IN SERUM OF ior-CEA-1 LABELLED BY DIFFERENT METHODS, EXPRESSED IN LABELLING PERCENTAGE

Method	15 min	1 h	2 h	4 h	11 h	24 h
2-ME	98.1	96.3	97.1	95.7	93.7	92.8
Sn(II)/AA	94.0	95.0	93.2	4.7	91.6	84.0
AA/DT	96.2	95.0	93.0	94.2	91.2	82.7

TABLE II. IMMUNOREACTIVITY INDEX OBTAINED FROM BINDING CURVES BY COMPETITIVE ASSAY

MAb	Intercept	Slope	R	Immunoreactivity Index
non-specific	0.32	0.004	-0.449	0.012
labelled	-0.015	-0.354	-0.924	0.985
reduced	-0.12	-0.239	-0.919	0.914
native	-0.08	-0.359	-0.936	1.00

As CEA antigen was not available in sufficient amounts, immunohistochemical studies were carried out in order to compare the immunoreactivity affectation by the labelling process. In case of Schwarz's method it can be observed that all conjunctive tissue is clear and only the tumour areas remained hot, so labelled ior-CEA-1 retained the same recognition capacity as that of native MoAb. This suggested that the immunoreactivity of the MoAb was preserved during the labelling procedure. However, although immunohistochemical studies indicated that $^{99}Tc^{m}$-ior-CEA-1 labelled by means of the Sn(II)/AA method maintains the same recognition pattern as the native MAb, a relatively high background was observed. This indicates an increase of non-specific binding sites.

The results obtained in the animal biodistribution by the Schwarz labelling approach, suggested that there is no significant accumulation in non-critical organs, the excreting organs behaved as normal (Fig. 6). However, since in vitro studies by Sn(II)/AA and AA/DT labelling suggested some structural changes in the protein, the biodistribution study could play a role in evaluating the catabolism at the sites of antibody localization [17] or an in vivo instability. The results reflect a relatively high accumulation in stomach, femur and kidneys (Fig. 6). The high activity in kidneys suggests some lower molecular structure.

The immunogammagraphic results were correlated with those of CAT, US, cytology, endoscopy, surgery and clinic, in order to obtain the sensitivity and specificity of the method, which are 86.2% and 69% respectively. Each injection was tolerated without any secondary effect. No acute toxicity was observed, whereby the preparation was assimilated without significant change. Figures 7 and 8 show some picture of patients with recurrence and metastasis of the colorectal lesion. Radioactivity levels in liver, kidneys and spleen for $^{99}Tc^{m}$ at 24 h post administration are shown in Fig. 9 for some representative patients, which were corrected for radioactivity decay values for spleen and kidneys showed no significant differences in all patients, though were lower than those of liver (Fig. 9). Cumulative urinary excretion of radioactivity was $10 \pm 3\%$ ID ($n = 7$) at 24 h (Fig. 10), while the activity level in both kidneys was $3.5 \pm 0.8\%$ ID ($n = 8$).

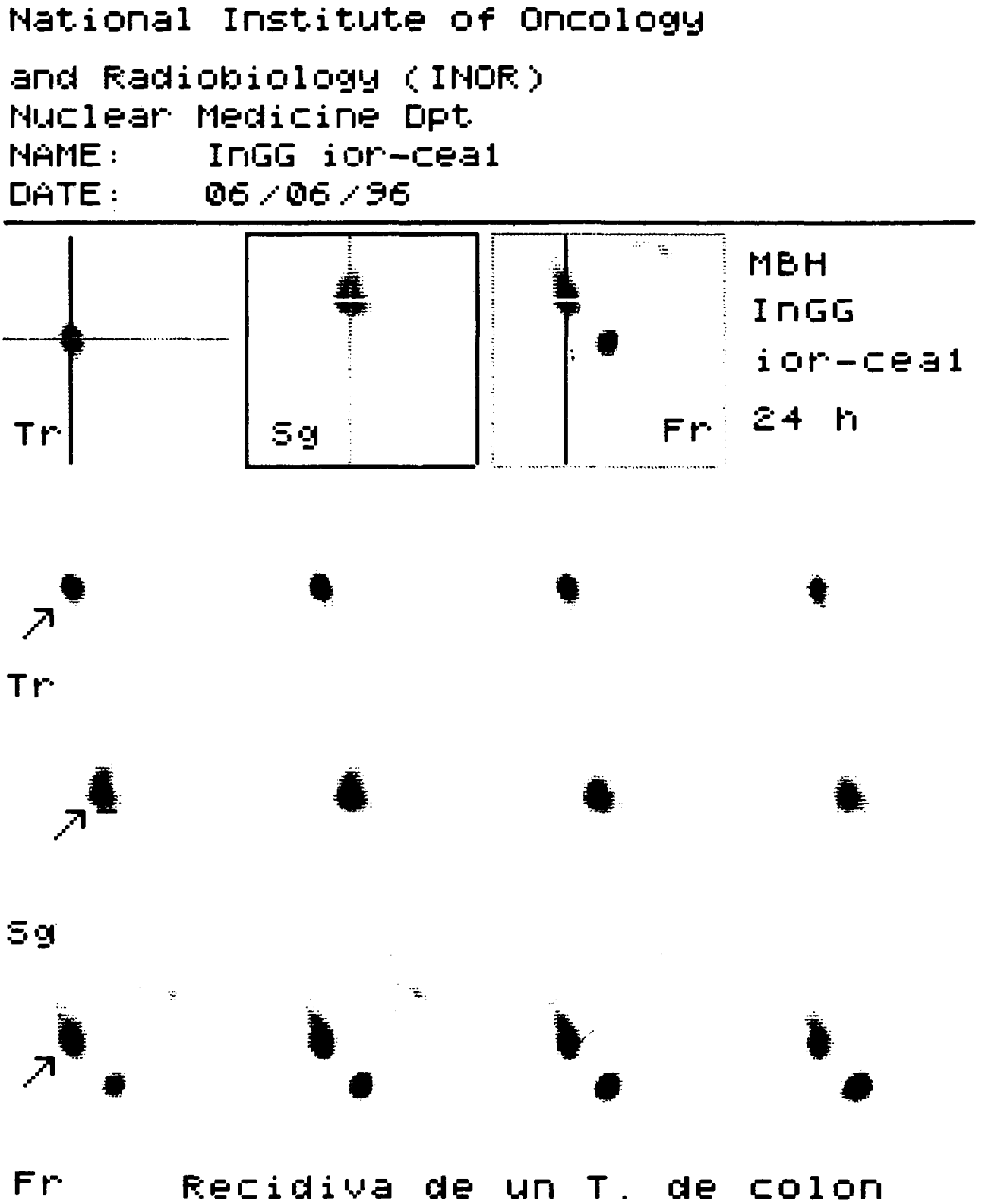

FIG. 7. Spot anterior image of lower abdomen obtained at 24 h postadministration of ior-CEA-1 labelled by 2-ME approach.

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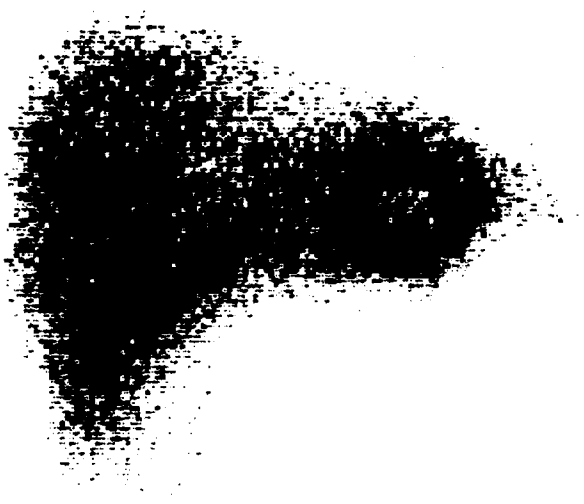

Liver metastases ("ring-type")

FIG. 8. Liver metastasis image (ring-type) obtained 22 h postadministration of ior-CEA-1 labelled by 2-ME approach.

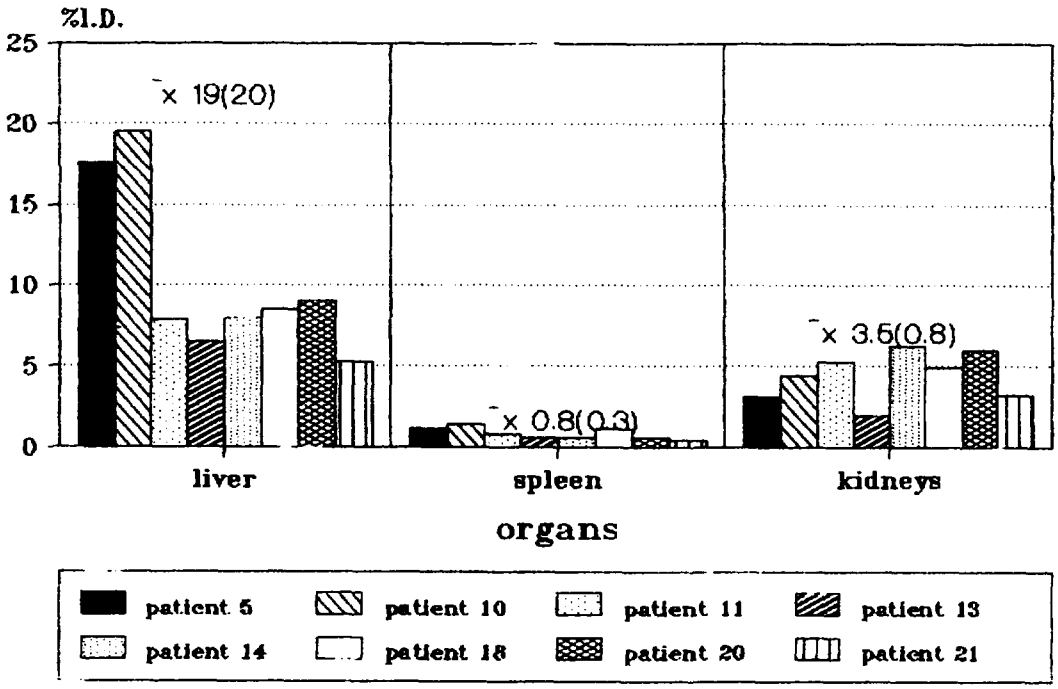

FIG. 9. Radioactivity levels in patents 24 h after administrations of $^{99}Tc^{m}$-ior-CEA-1.

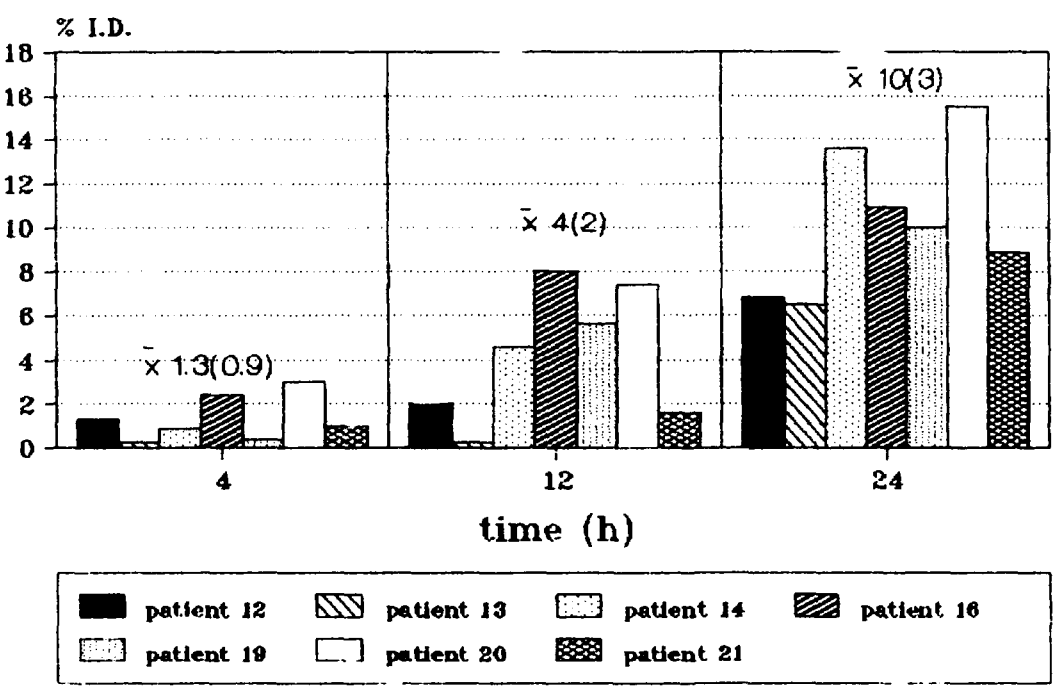

FIG. 10. Cumulative urinary excretion of radioactivity 24 h after administration of $^{99}Tc^{m}$-ior-CEA-1.

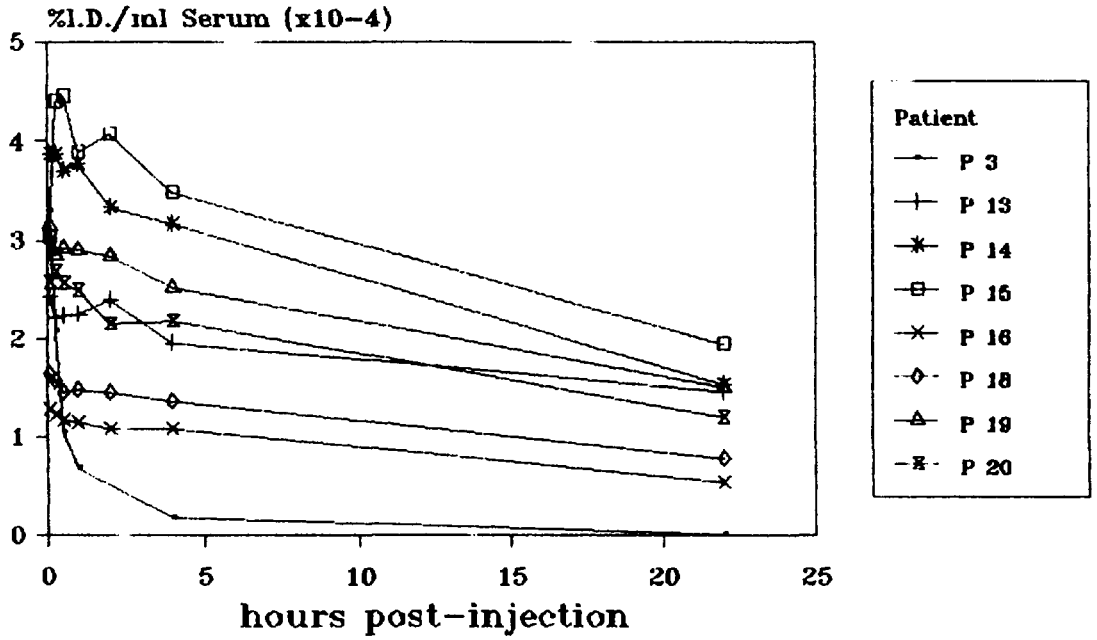

FIG. 11. Blood clearance of $^{99}Tc^{m}$-ior-CEA-1 in patients.

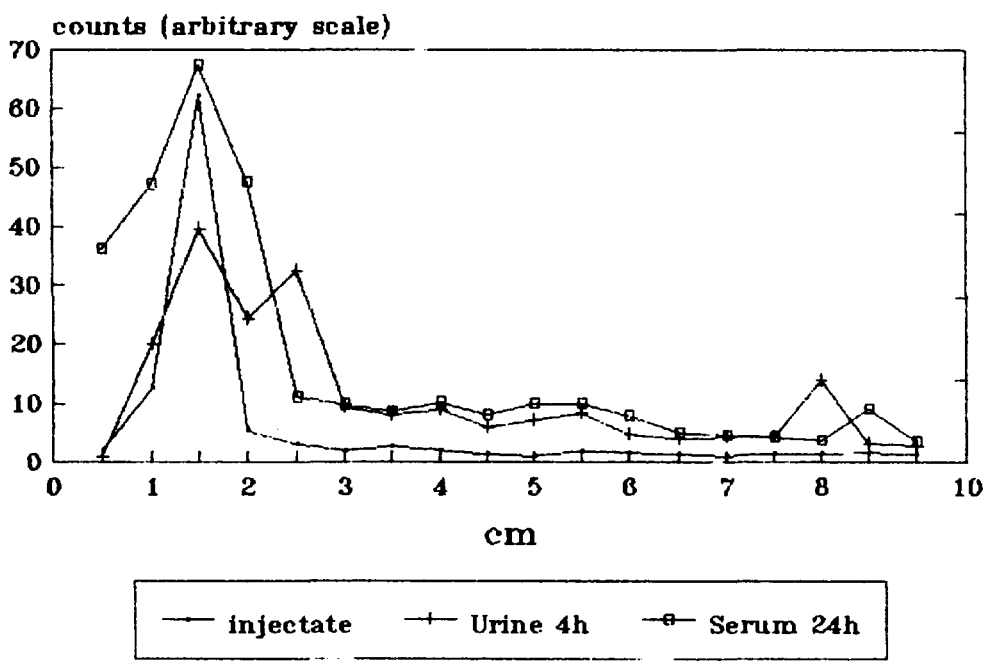

FIG. 12. SDS-PAGE analysis of serum and urine samples of patients.

TABLE III. PHARMACOKINETICS OF $^{99}Tc^{m}$-ior-CEA-1

Dose	1 mg, (1875 MBq $^{99}Tc^{m}$)
Cl (% ID mL/h)	0.0093(0.004)
V_d	3.5(1.8)
$T_{1/2a}$(h)	1(0.6)
$T_{1/2b}$(h)	22(4)

Blood clearance of the injected ior-CEA-1 is shown in Table III. The results were calculated by means of the FARMAC program. Clearance of radioactivity from blood was biexponential in most cases. A rapid decrease is observed in the percentage of serum radioactivity (Fig. 11), followed by a second, much slower decline. In some patient curves, a slight early increase can be observed, so that the initial values were sometimes re-established. Thus, the clearance was determined mainly by second decline.

Radiochromatograms obtained by analysis on FPLC and electrophoresis of serum samples, show a complex radiochromatogram with multiple peaks, which correspond with some peaks of the UV detection. Nevertheless the majority of radioactivity remained associated to the labelled antibody, as a proof of label stability. Significant additional peaks not present in the native antibodies analysis shows the presence of high molecular weight species which in some cases increase slightly with time (Fig. 12). Likewise several peaks were observed in urine sample radiochromatograms being its radiochromatographic profile much more complex. Part of $^{99}Tc^{m}$ activity ran near to the front, suggesting the formation of low molecular weight species, presumably cysteine.

2.3.4. Discussion

In this investigation three direct labelling methods, which are simpler and more easy to perform for the daily practice were evaluated. From our results, obtained by the different quality controls, may be observed that the 2-ME approach gave the best results to be considered for the clinical application. The Sn(II)/AA system as well as AA/DT showed some change in the protein during the labelling process.

With regard to the role played by each agent in the reduction process, no evidence was found to suggest that ascorbate was capable of reducing the antibody. Additional studies of this investigation employing the Schwarz procedure, but using ascorbate as reducing agent for 1 h attained a labelling efficiency of 3.9% only for a 3500:1 ascorbate to antibody molar ratio and 8.3% for 35000:1.

On the other hand, ior-CEA-1 was treated with dithionite but not ascorbate, gave labelling efficiencies over those obtained with ascorbate alone (72%). If these results are compared with the AA influence in the Sn(II)/AA system, it could be concluded, that although the ascorbate presence plays a insignificant role in the reductant process, emphasizes the reducing action of the other partner agent.

Another important aspect of the $^{99}Tc^{m}$-labelling methods is the protein stability. No noticeable changes were obtained for the labelled ior-CEA-1 by the 2-ME approach in the serum challenge (Table I). However, cysteine challenge experiments indicated that up to the molar ratio of 50 (cysteine to MAb) the cysteine influences significantly. Likewise, taking into account the immunohistochemical studies, the results reflected an increase of the non-specific binding sites by the Sn(II)/AA reducing system, while labelled ior-CEA-1 by the 2-ME approach retained the same recognition pattern as that of native MAb, suggesting that the effects on monoclonal biological activity due to reduction and labelling process was insignificant, coinciding so with that obtained with the micro ELISA assay.

We selected the 2-ME reducing method as an adequate labelling technique to be applied in human trials, since it accomplished with the required parameters to obtain a competent radiopharmaceutical, giving an injectable preparation of high radiochemical purity and unimpaired immunoreactivity.

The immunoscintigraphy has been able to detect not only the clinically suspected recurrent and metastatic lesions, but also unsuspected lesions, like an asymptomatic patient with negative laboratory tests, as well as the US and NMR. The immunoscintigraphy with ior-CEA-1 indicated a recurrent lesion and liver metastasis.

Pharmacokinetic behavior of ior-CEA-1 was similar to that observed with other monoclonal antibodies [19, 20]. The majority of the patient's data were fitted to a two compartmental model. The biodistribution results revealed that the largest accumulation was in liver (19 ± 2.05, $n=15$) of injected dose at 24 h post injection. However, excluding the patients who had liver metastases and high CEA level, due to correlation between high liver uptake and immune complex formation [21], the value for the liver uptake was 9 ± 4 % ID ($n=10$). At 24 h post administration activity in liver, spleen, kidneys and tumour accounts for about 26 % of the injected dose, remaining about 50% in circulating serum. Taking into account that the cumulative urinary excretion accounts about 10%, the remaining is distributed in the rest of the body, under the assumption that the major route of excretion is urine; faeces excretion was not measured (approximately 14% ID).

2.3.5. Conclusions

The FPLC chromatographic analysis of serum samples at different times did not show significant presence of $^{99}Tc^{m}$-pertechnetate neither in vitro nor in vivo assays. The $^{99}Tc^{m}O_4^-$ levels remained under 9% at 24 h, which were confirmed by the SDS-PAGE analysis (Fig. 12). Similar results were obtained previously [19, 22]. From the figure it can be observed that most of the label remained associated to the protein, at least within 24 h. This, together with the antibody capacity to detect the tumour [6–8], gives a measure of the ior-CEA-1 sensitivity to recognize the lesion, as well as that the selected labelling method accomplished the proposed objectives.

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