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Distribution study of radioactivity in rats after oral administration of the lipido/sterolic extract of *Serenoa repens* (Permixon®) supplemented with [1-^{14}C]-lauric acid, [1-^{14}C]-oleic acid or [4-^{14}C]-β-sitosterol

G. CHEVALIER[1], P. BENARD[1], H. COUSSE[2] and T. BENGONE[1]

[1]*Ecole Nationale Vétérinaire, Département des Sciences Biologiques et Fonctionnelles, Laboratoire de Radioéléments et d'Etudes Métaboliques, Toulouse, France*
[2]*Pierre Fabre Médicament, Direction Générale, Castres, France*

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SUMMARY

The study carried out on rats given orally the *n*-hexane lipido/sterolic extract of *Serenoa repens* (LSESR), supplemented with [^{14}C]-labelled oleic or lauric acids or β-sitosterol, demonstrated that radioactivity uptake in prostatic tissues shows the highest level in the case of administration of LSESR supplemented with [^{14}C]-labelled oleic acid. This was clearly demonstrated on a rat with an induced fibro-muscular hyperplasia of the prostate and by quantitative measurements of radioactivity. Ratios of radioactivity in tissues compared to plasma show an uptake of radioactivity greater in prostate as compared to other genital organs, i.e. the seminal vesicles or to other organs such as liver.

INTRODUCTION

The saw palmetto *Serenoa repens* is a scrub dwarf palm very common in the open areas through much of the South-Eastern part of the US (South Carolina, Florida and Alabama) (1,2).

The drupe-like fruits, green becoming yellowish and blue-black when ripe, are eaten by wild animals and were highly appreciated by the natives as a food, for giving an 'increase of fat, flesh and strength'. The anti-irritating properties of the fruits of *S. repens* were confirmed in the mid-XIXth century; the extracts demonstrated activity on mucosae and appeared to be efficient against catarrhal affections and chronic bronchitis.

The present major medicinal use of *S. repens* started in 1894 with a description of the beneficial and

Please send reprints request to : Pr. Patrick Benard, Ecole Nationale Vétérinaire, Département des Sciences Biologiques et Fonctionnelles, Laboratoire de Radioéléments et d'Etudes Métaboliques, 23 chemin des Capelles, F-31076-Toulouse-Cedex, France.

Footnote : Permixon® is marketed by Pierre Fabre Médicament (Castres, France) whose other trademarks include Capistan, Libeprosta and Sereprostat.

more specific action of the extracts on prostatic dysfunctions (3). These efficacious properties are described in several American books of materia medica (4–7). Since then, *S. repens* has entered different national pharmacopeia (8,9).

Now, the saw palmetto extracts have regained an important place in urological therapy thanks to their lenitive action on the functional symptoms of benign prostatic hyperplasia, associated with an absence of side effects. Numerous clinical confirmations were made in countries such as France (10–13), Germany (14,15), Italy (16–19) and Spain (20). The beneficial effects of the extracts (21) may be related not only to their anti-inflammatory effects (22,23), but also to their interference with hormonal metabolism, either at the level of androgen (24) and prolactin (25) receptors, or on the biosynthesis of 5α-dihydrotestosterone reductase (26).

The drupes of *S. repens* have been extracted with various solvents, such as ethanol, acetone, hypercritical carbon dioxide or hexane. The hexane extract of the entire drupe (pulp and seed), the basis of the French pharmaceutic speciality Permixon® appears as a complex mixture of essentially free fatty acids and their esters, small quantities of phytosterols (β-sitosterol, campesterol, stigmasterol), cycloartenol, aliphatic alcohols (C_{26}, C_{28}, C_{30}) and several polyprenic compounds. The chief more potent fatty acids, determined on 11 batches from two autumnal crops, are oleic (36 ± 3%), lauric (27.5 ± 3%) and myristic (12 ± 1%) acids (27).

In order to understand the mechanism of the pharmacological effects of the hexane extract of *S. repens*, the tissue distribution of its constituents must first be systematically investigated. In the experiments reported here, rats were administered orally the lipido/sterolic extract of *S. repens* (LSESR), to which was added [^{14}C]-labelled oleic, or lauric acid, or β-sitosterol.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]-Lauric acid, [1-^{14}C]-oleic acid and β-[4-^{14}C]-sitosterol were purchased through Amersham International. [1-^{14}C]-Lauric acid was available as a *n*-hexane solution and sealed under nitrogen in a borosilicate vial. Its specific activity was 2.18 GBq/mmol (59.5 mCi/mmol). The radiochemical purity was checked using radio-thin layer chromatography on silica gel plates (Merck F256) in ether:hexane:formic acid (25:75:2). It was found to be higher than 98.5%.

[1-^{14}C]-Oleic acid was available as a dry deaerated toluene solution sealed under nitrogen in a borosilicate vial. Its specific activity was 2.04 GBq/mmol (55 mCi/mmol). The radiochemical purity was checked using radio-thin layer chromatography on silica gel plates (Merck F256) in ether:hexane:formic acid (25:75:2). It was found to be higher than 98%.

[4-^{14}C]-β-Sitosterol was available as a solution in toluene:ethanol (9:1). Its specific activity was 2.07 GBq/mmol (56 mCi/mmol). The radiochemical purity was checked using radio-thin layer chromatography on silica gel plates (Merck F256) in cyclohexane:chloroform (50:50). It was found to be higher than 97%.

The n-hexane lipido/sterolic extract of *S. repens* (LSESR) was supplied by Pierre Fabre Médicament, Castres, France (batch no. 646).

Preparations

Each vial containing 9.25 MBq (250 μCi) of either [1-^{14}C]-lauric acid, [1-^{14}C]-oleic acid or [4-^{14}C]-β-sitosterol was evaporated under a gentle nitrogen flow. The vial was then filled with 2.5 ml of LSESR, so that the reactive volume was 100 μCi/ml. The vial was stirred for 30 min at room temperature in the darkness. Then, an aliquot was weighed in a vial which was filled with 10 ml of Instagel® (Packard, France). Radioactivity counting was carried out 5 times in a liquid scintillation counter (Packard 2200 CA) for 1 min.

Animals

The study was performed on male Long Evans rats, weighing 150 g upon arrival in the animal unit. They were purchased through Elevages Janvier (Le Genest-Saint-Isle, France). They were maintained in the animal unit for 15 days before being included in the protocol. They were housed, one rat per cage, under a 12 h light-12 h dark lighting regime. They received a controlled diet for rats (Vilemoisson-sur-Orge, France) and water *ad libitum*.

The rats were randomized into 3 groups:

Group A

18 animals were included in the protocol of the distribution study carried out by whole-body autoradiography. They were divided into 3 groups of 6 animals.

Group B

14 animals were included in the protocol of the quantitative study. They were divided into 2 groups of 7 animals.

Group C

Two animals were considered.

One was prepared according to the method described for inducing a fibromuscular proliferation in the lateral prostate (28). Under surgical conditions, tubing implants filled with either estradiol-17β or dihydrotestosterone were implanted subcutaneously in the dorsal area between the scapula. Castration was performed via the scrotal route and the epididymal fat pad was removed with the testes.

The other rat served as a control: it was castrated but did not receive any hormonal implantation.

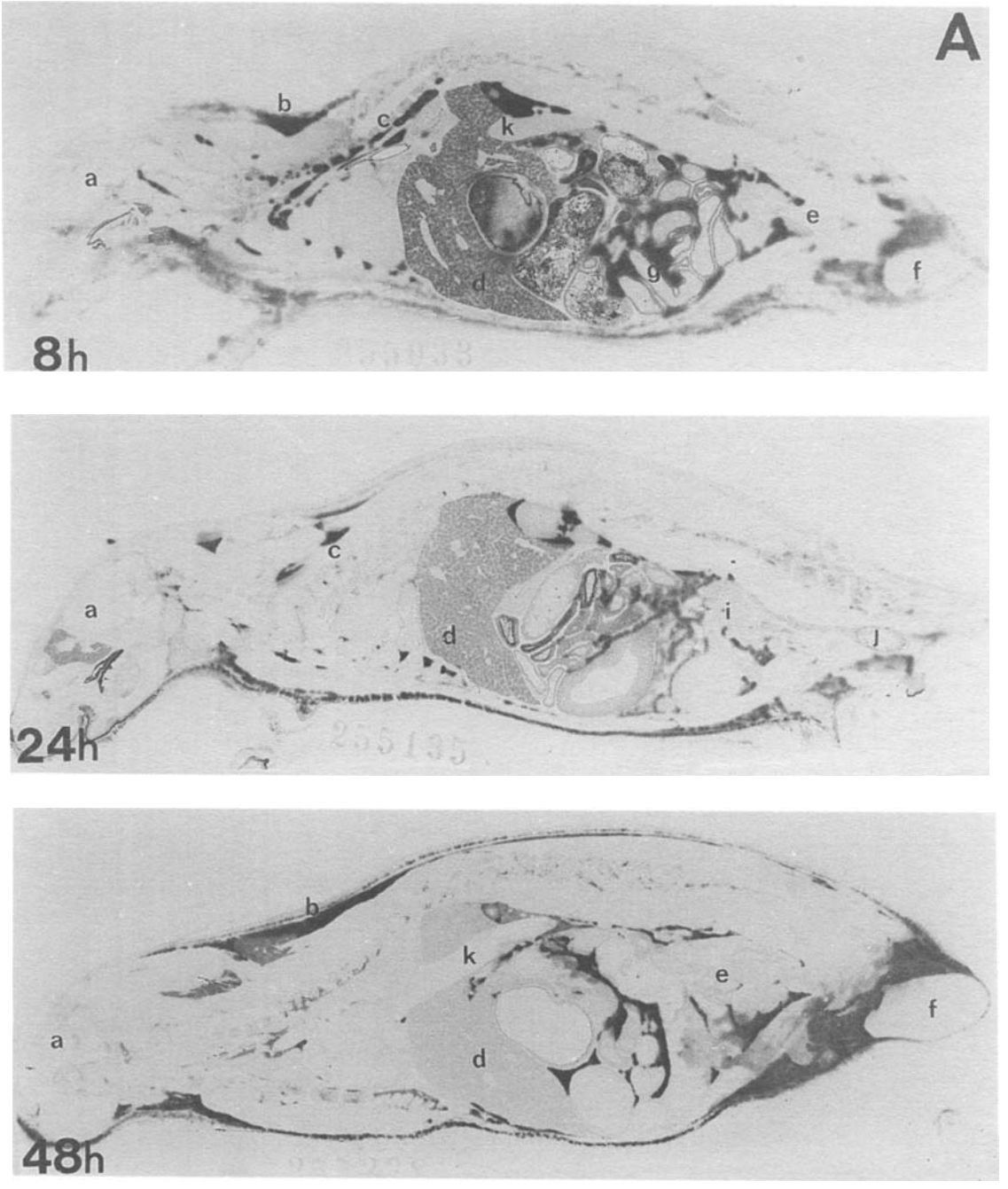

Fig. 1A : Whole body autoradiograms of rats sacrificed 8, 24 and 48 h after an oral administration with the LSESR supplemented with lauric acid (A). Radioactivity appears in black. Key: a, brain; b, brown fat; c, thoracic duct; d, liver; e, prostate; f, testes; g, fat; i, urinary bladder; j, rectum; k, blood.

Experimental

The day before administration, food was withdrawn at 5 pm. The rats were dosed orally the next day between 8 and 9 am. All the animals were given 10 mg of LSESR by stomach tubage, to which was added either [1-^{14}C]-lauric acid, [1-^{14}C]-oleic acid or [4-^{14}C]-β-sitosterol. The activity by weight was 3.7 kBq/kg (100 μCi/kg).

In the first phase, animals of group A were randomized into 3 groups. Animals of the first group received LSESR supplemented with [1-^{14}C]-lauric acid,

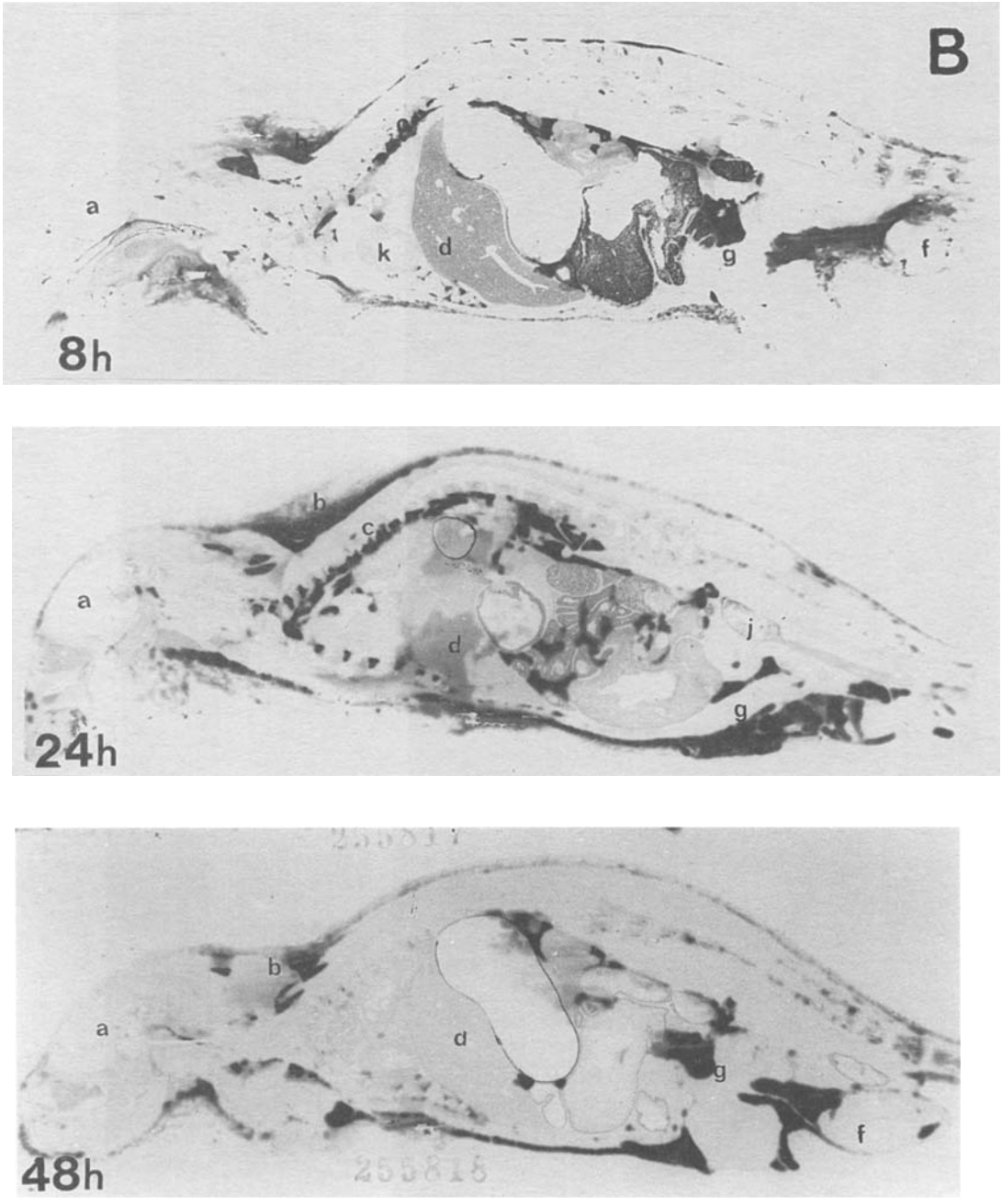

Fig. 1B : Whole body autoradiograms of rats sacrificed 8, 24 and 48 h after an oral administration with the LSESR supplemented with oleic acid (B). Radioactivity appears in black. Key: a, brain; b, brown fat; c, thoracic duct; d, liver; e, prostate; f, testes; g, fat; i, urinary bladder; j, rectum; k, blood.

animals of the second group received LSESR supplemented with [1-^{14}C]-oleic acid; animals of the third group received LSESR supplemented with [4-^{14}C]-β-sitosterol.

In all cases, animals were sacrificed by a deep ether anesthesia 0.5, 2, 4, 8, 24 or 48 h after dosage and prepared for a whole-body autoradiographic study

In the second phase, animals of group B were randomized in 2 groups. They were housed in individual stainless steel cages in order to separate feces and urine. They were all administered orally 10 mg/kg of LSESR supplemented with [1-^{14}C]-oleic acid so that

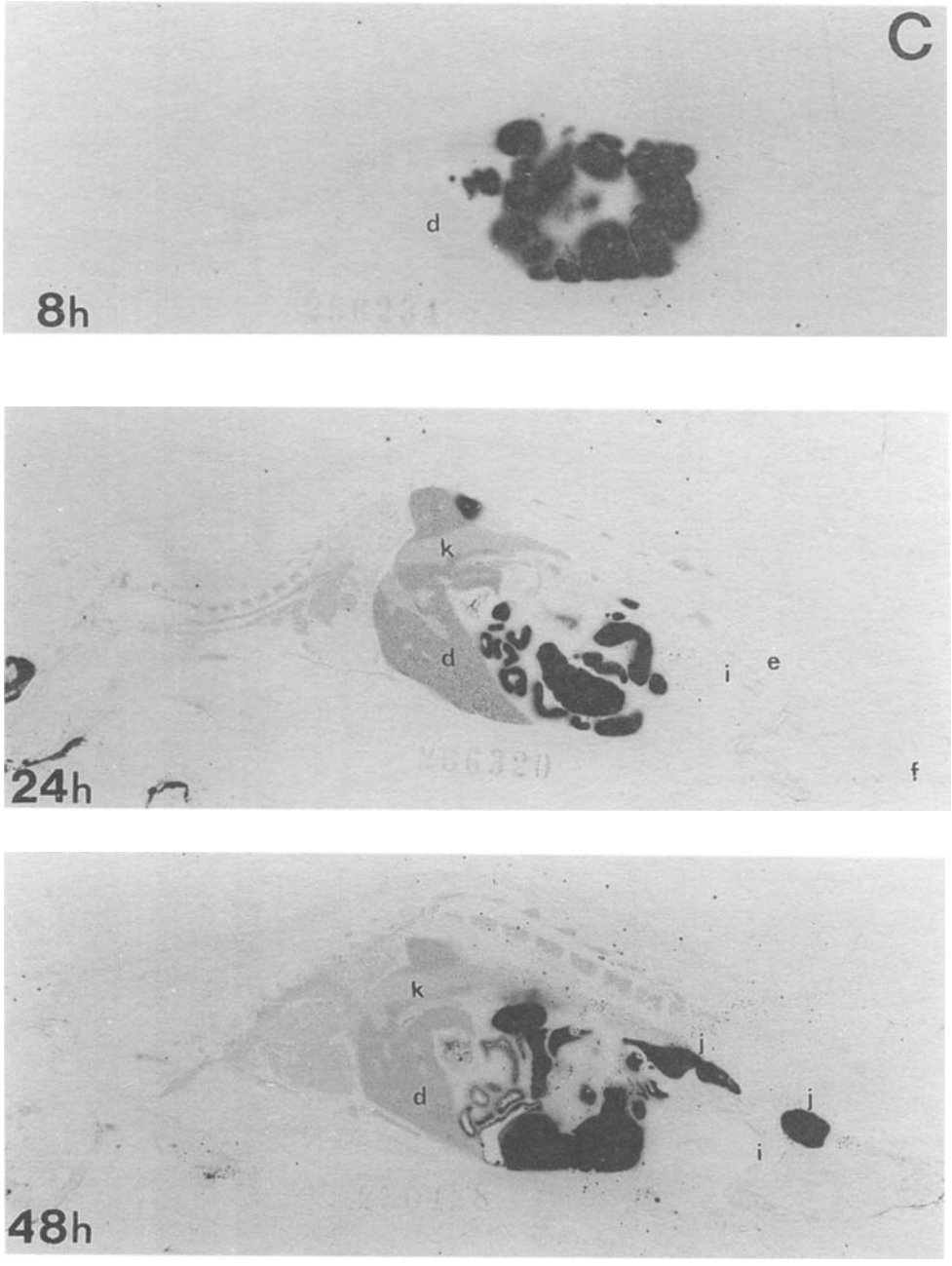

Fig. 1C : Whole body autoradiograms of rats sacrificed 8, 24 and 48 h after an oral administration with the LSESR supplemented with β-sitosterol (C). Radioactivity appears in black. Key: a, brain; b, brown fat; c, thoracic duct; d, liver; e, prostate; f, testes; g, fat; i, urinary bladder; j, rectum; k, blood.

animals received 370 kBq/kg (10 μCi/kg). 7 animals were sacrificed 8 or 24 h after dosage by aortic puncture under ether anesthesia. Blood was collected in heparinized tubes to separate plasma and blood cells. Then, prostate, vesicular glands, urinary bladder, brain, liver, abdominal fat, muscles (biceps femoris) and lumbar skin were collected, rinsed in saline, dried on paper and weighed. They were then minced and frozen (–18°C) until analysis.

In the third phase, animals of group C were dosed orally with 10 mg/kg of LSESR supplemented with [1-^{14}C]-oleic acid so that animals received 3.7 kBq/kg (100 μCi/kg). They were sacrificed by a deep ether anesthesia 8 h after LSESR administration and prepared for a whole-body autoradiographic study.

Instruments and analytical methods

Whole-body autoradiography

This was carried out according to Ullberg's method (29) as described previously (30). Animals were deeply frozen by immersion in a methanol-dry ice mixture so that the average body temperature was –75°C. Then, they were embedded in carboxy-methyl cellulose on a microtome stage which was immersed in the freezing bath. Using a semi-automatic microtome (PMV, Stockholm, Sweden), the surface of the block thus obtained was trimmed in order to reach the desired anatomical level, i.e. the spleen. Then, 20 μm thick sections were collected on an adhesive tape (Scotch, 306).

The sections were dried for 4 days at –25°C inside the cryostat. They were exposed for 6 weeks on Hyperfilm betamax® emulsions (Amersham, France) at low temperature (–18°C).

Radioactivity counting

Two aliquots weighing 100 ± 5 mg of the different samples were prepared in Combustocones® (Packard, France). They were mineralized in a sample Oxidizer 306 (Packard). Radioactive vapours were trapped on 7 ml of Carbosorb® (Packard) and 12 ml of Permafluor V® (Packard) were added as a scintillation cocktail.

The recovery ratio was checked using standard radioactive solutions (SPEC-CHECK®, Packard) with

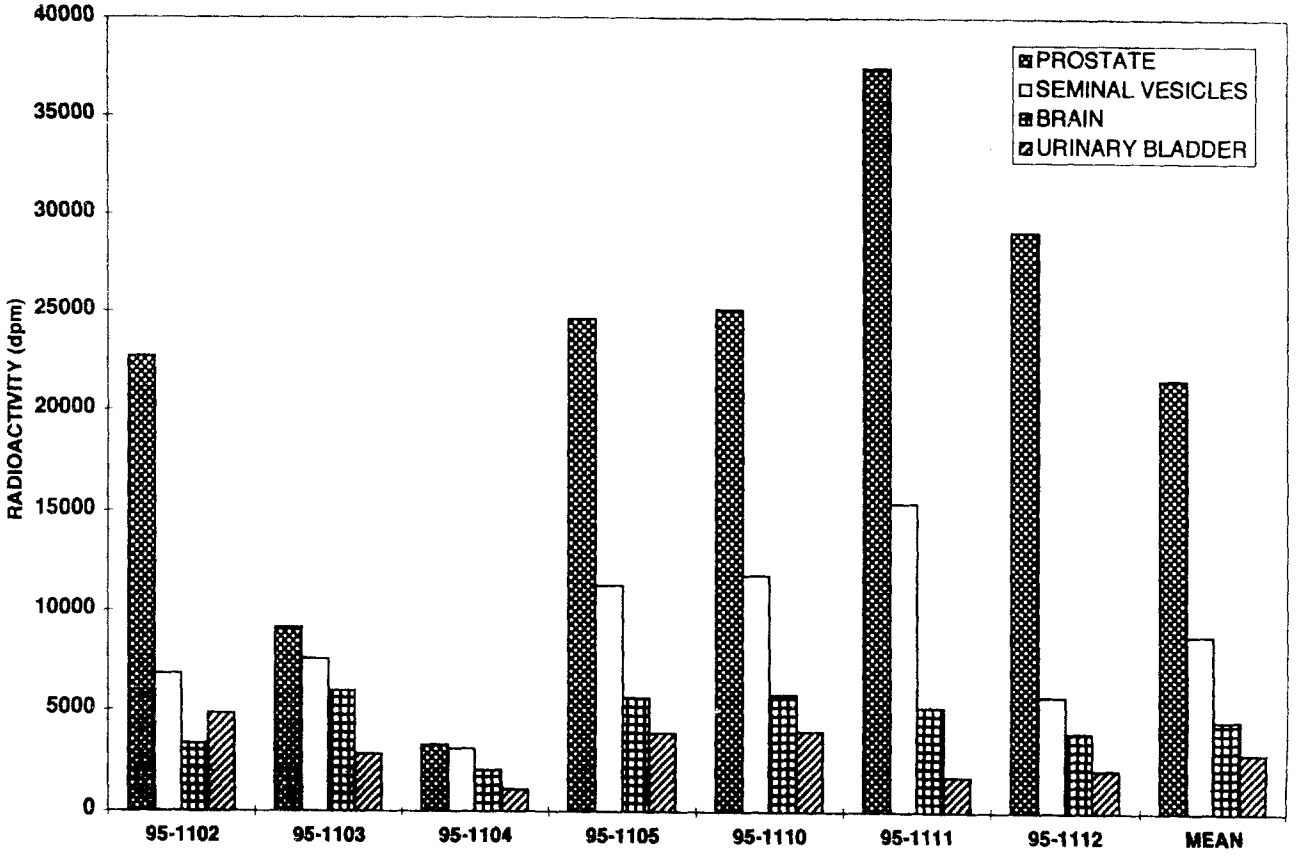

Fig. 2 : Radioactivity concentration in organs of rats sacrificed 8 h after an oral administration of LSESR supplemented with [^{14}C]-oleic acid. Results are expressed as dpm in the whole organ.

a volume of radioactivity of 5000 dpm ± 3%. All combustions were carried out with a recovery ratio of 97%.

All the results are expressed in disintegrations per gram (dpm/g) or disintegrations per millilitre (dpm/ml). They are presented as dpm for the whole organ or as a percentage of the delivered dose.

RESULTS

Distribution of radioactivity in rats after oral administration of LSESR supplemented with [1-^{14}C]-lauric acid, [1-^{14}C]-oleic acid or [4-^{14}C]-β-Sitosterol

Results are presented in Figure 1.

Distribution of radioactivity in rats after oral administration of LSESR supplemented with [1-^{14}C]-lauric acid (Fig. 1A)

In the animal sacrificed 30 min after dosage, highest radioactivity was concentrated in the stomach lumen. Trace amounts of radioactivity were detectable in organs, whereas slight radioactivity was present in the liver and genital organs. In the animal sacrificed 2 h after stomach tubing, highest radioactivity was found in the lumen of stomach and in the small intestine. Liver was the organ in which a rather medium degree of radioactivity was detected. In this organ, the distribution of the isotope was heterogeneous, since it presented a marbled aspect. In the animal sacrificed 4 h later, radioactivity was detected in all organs and it was more intense than in the preceding animal. The liver, abdominal fat and mammary tissues are the organs in which the highest radioactivity was detected. The situation was about the same in the animal sacrificed 8 h after oral administration: lymphatic vessels, abdominal fat were the most labelled organs. In genito-urinary organs, the isotope was present in all the structures, i.e. prostate, seminal vesicles, urinary bladder. In the animal sacrificed 24 h after dosage, all radioactivity present in the lumen of the gastro-intestinal tract has been eliminated, since only trace amounts were still detectable in the lumen of the caecum and of the rectum. Rather low radioactivity was still present in genital organs. No radioactivity was detectable in the animal sacrificed 48 h after treatment. In the

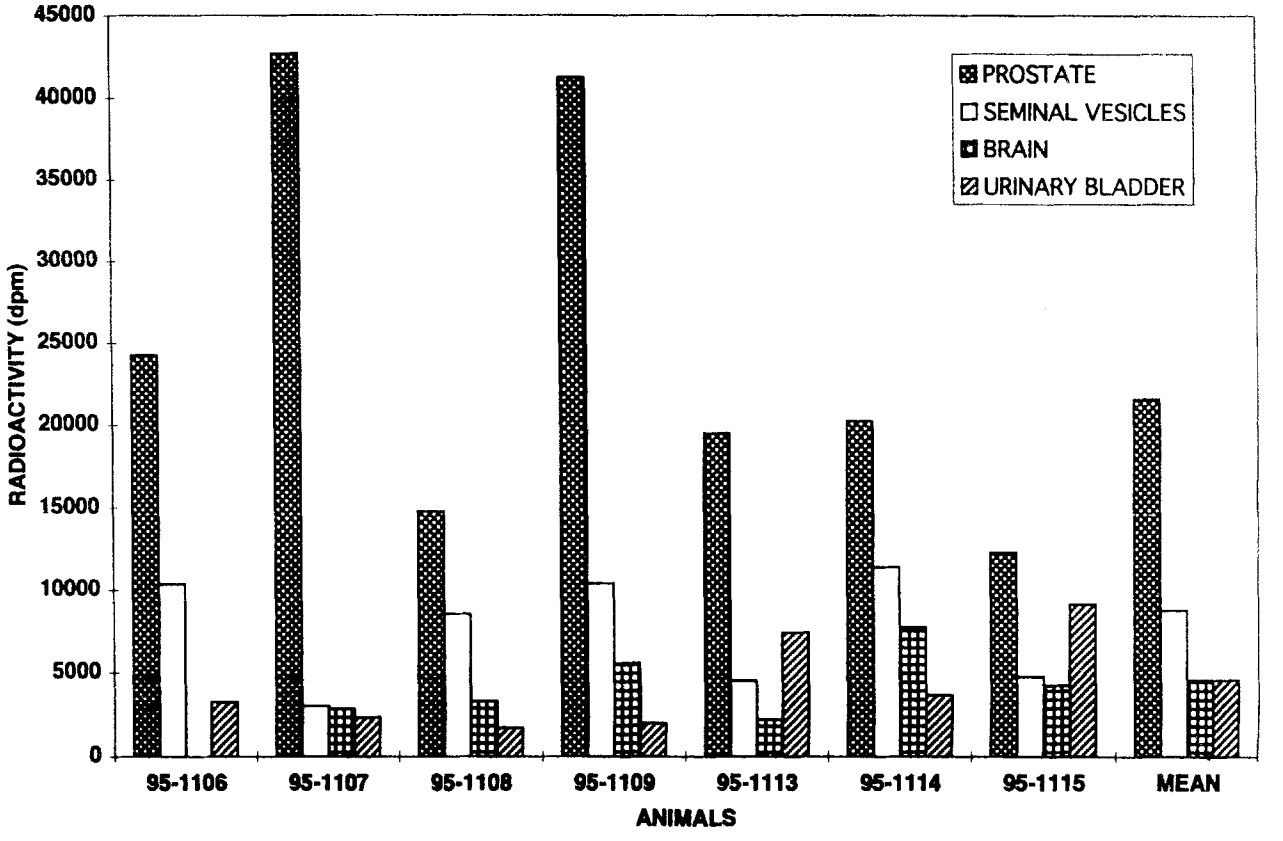

Fig. 3 : Radioactivity concentration in organs of rats sacrificed 24 h after an oral administration of LSESR supplemented with [^{14}C]-oleic acid. Results are expressed in dpm in the whole organ.

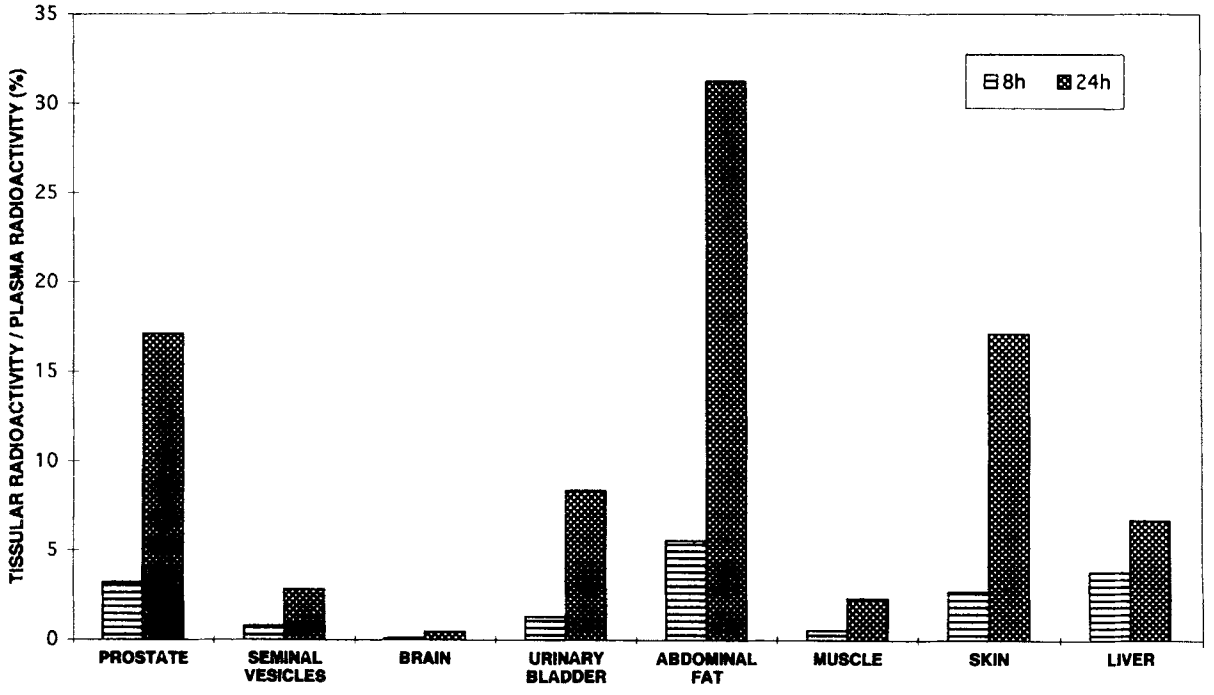

Fig. 4 : Tissue radioactivity concentration in different organs of rats sacrificed 8 and 24 h after an oral gavage with LSESR supplemented with [^{14}C]-oleic acid. Results are expressed as a ratio of the plasma radioactivity (%).

other organs, including genital glands, the isotope was present at low concentrations.

Distribution of radioactivity in rats after oral administration of LSESR supplemented with [1-^{14}C]-oleic acid (Fig. 1B)

If the highest rates of radioactivity were detected in the lumen of the stomach and of the small intestine, the isotope was also present in blood and some organs (liver), but at low concentrations in the animal sacrificed 30 min after dosage. In the animal sacrificed 2 h later, radioactivity was present in the organs. Highest radioactivity was localized in the lumen of the digestive tract and in the thoracic duct and abdominal fat. In this animal, there was a rather important incorporation of the isotope in genital organs, mainly in prostatic tissues. In the animal sacrificed 4 h after dosage, the incorporation of the isotope was greater in most organs than in the preceding animal. In the animal sacrificed 8 h later, an intensification of radioactivity was observed in different organs, including genital glands. In the animal sacrificed 24 h after administration, most of the radioactivity had disappeared in the lumen of the digestive tract, since only trace amounts were still present in the rectum. By contrast, rather high radioactivity was detectable in the liver, the thoracic duct, abdominal fat and in prostatic tissues and seminal vesicles. The pattern of distribution was about the same in the animal sacrificed 48 h after the oral administration of LSESR supplemented with [1-^{14}C]-oleic acid.

Distribution of radioactivity in rats after oral administration of LSESR supplemented with [4-^{14}C]-β-sitosterol (Fig. 1C)

Radioactivity was present only in the lumen of the digestive tract, i.e. the stomach and small intestine, in the animal sacrificed 30 min after administration. In the animal studied 2 h after oral administration, trace amounts of radioactivity were present in the fat and seminal vesicles. In the animal sacrificed 4 h after oral gavage, radioactivity was present in the liver at low concentrations, in the abdominal fat and in the seminal vesicles. In the animal studied 8 h after administration, the isotope was localized at rather low concentrations in blood, in the liver, and in different glands including the prostate and seminal vesicles. The pattern of distribution of radioactivity was almost the same in the animal studied 24 h after oral gavage. Most of the organs contained radioactivity. In the digestive tract, the

isotope was localized in the small intestine's wall. A rather high uptake occurred in the cortical layers of the adrenal glands and in several organs, such as the liver, bone marrow, blood, in which the isotope was detectable at medium concentrations. In the animal studied 48 h after oral gavage with LSESR supplemented with labelled β-sitosterol, the pattern of distribution of radioactivity was almost the same as in the preceding animal, with a low incorporation of radioactivity in genito-urinary tissues.

Quantitative evaluation of radioactivity in rats after oral administration of LSESR supplemented with [1-^{14}C]-oleic acid

The data are presented in Figures 2 and 3. They represent the concentration of radioactivity measured as unchanged [1-^{14}C]-oleic acid in prostate, seminal vesicles, urinary bladder and brain. These data show that 8 h after oral administration of LSESR supplemented with [1-^{14}C]-oleic acid, radioactivity ratios (tissue radioactivity/plasma radioactivity) were between 9.60% in the abdominal fat and 0.05% in the brain, whereas they were between 45.92% in the abdominal fat and 0.32% in the brain of animals sacrificed 24 h after oral administration.

When considering prostatic tissues, these ratios were between 0.49% and 4.68% 8 h after administration and between 5.52% and 30.13% in the animals sacrificed 24 h after oral administration.

As represented in Figure 4, the values obtained for prostatic tissues were much higher than for other genito-urinary tissues, i.e. urinary bladder, seminal vesicles and for other organs, such as the liver.

Quantitative whole-body autoradiographic study of the distribution of radioactivity in rats after oral administration of LSESR supplemented with [1-^{14}C]-oleic acid

The results are presented in Figures 5 and 6 in which are given the details of whole-body autoradiograms carried out on a normal rat and an animal surgically prepared to develop a fibromuscular hypertrophy of the prostate. The comparison of these results first emphasizes the effects of the hormonal treatment on the prostatic tissues, since their surface on the section is very much enlarged. They also demonstrate that the uptake of radioactivity is higher in prostatic tissues than, for example, in seminal vesicles.

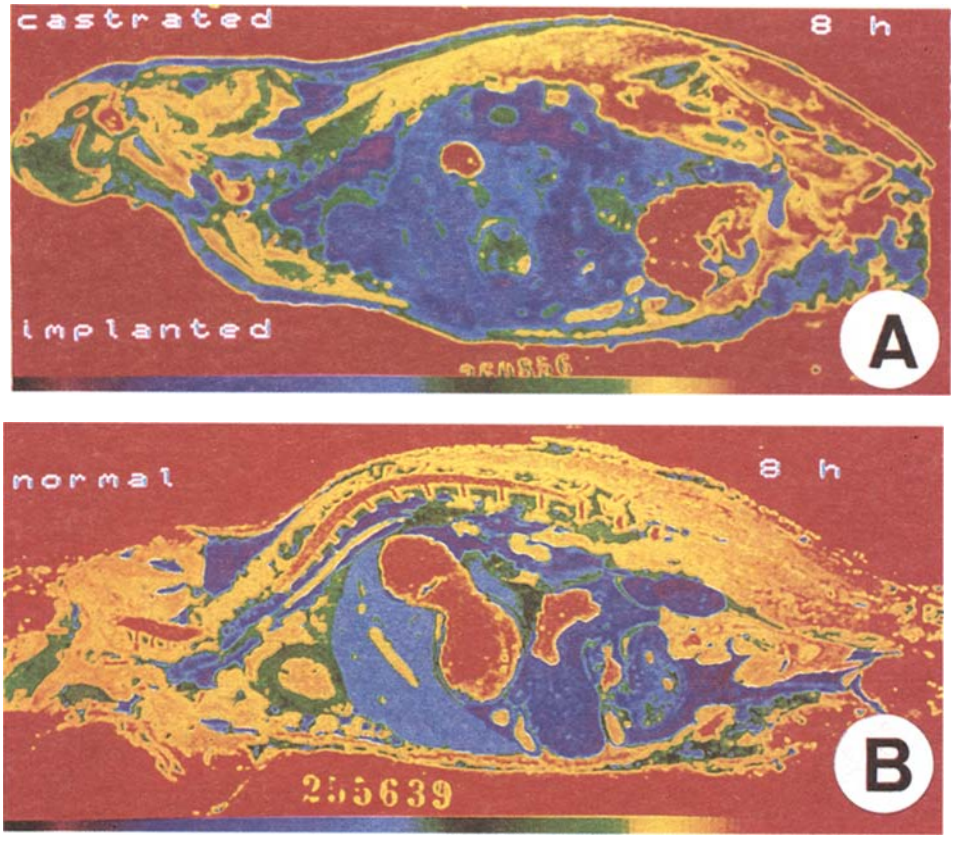

Fig. 5 : Digitalized whole body autoradiograms of rats sacrificed 8 h after an oral administration of LSESR supplemented with oleic acid. Radioactivity is represented in pseudo-colours on a 256 grey level scale represented on the bottom of figures. Red = level 0; black = level 255. (A) Castrated and hormonal implanted rat; (B) control rat.

DISCUSSION

The aim of this study was to provide data concerning the fate of some of the principal chemical constituents present in Permixon®, a drug commercialised for the treatment of benign prostatic hyperplasia.

Since the chemical composition of this extract (LSESR) is rather complex as reported in the introduction, we conducted this study to investigate the fate of LSESR added to the compounds present in high percentage. For this reason, the study was divided into two phases. The first phase consisted of the choice of the compound to be used in the studies. As demonstrated by the whole-body autoradiographic study, the results clearly demonstrate that the highest uptake of radioactivity is found with LSESR added with [1-

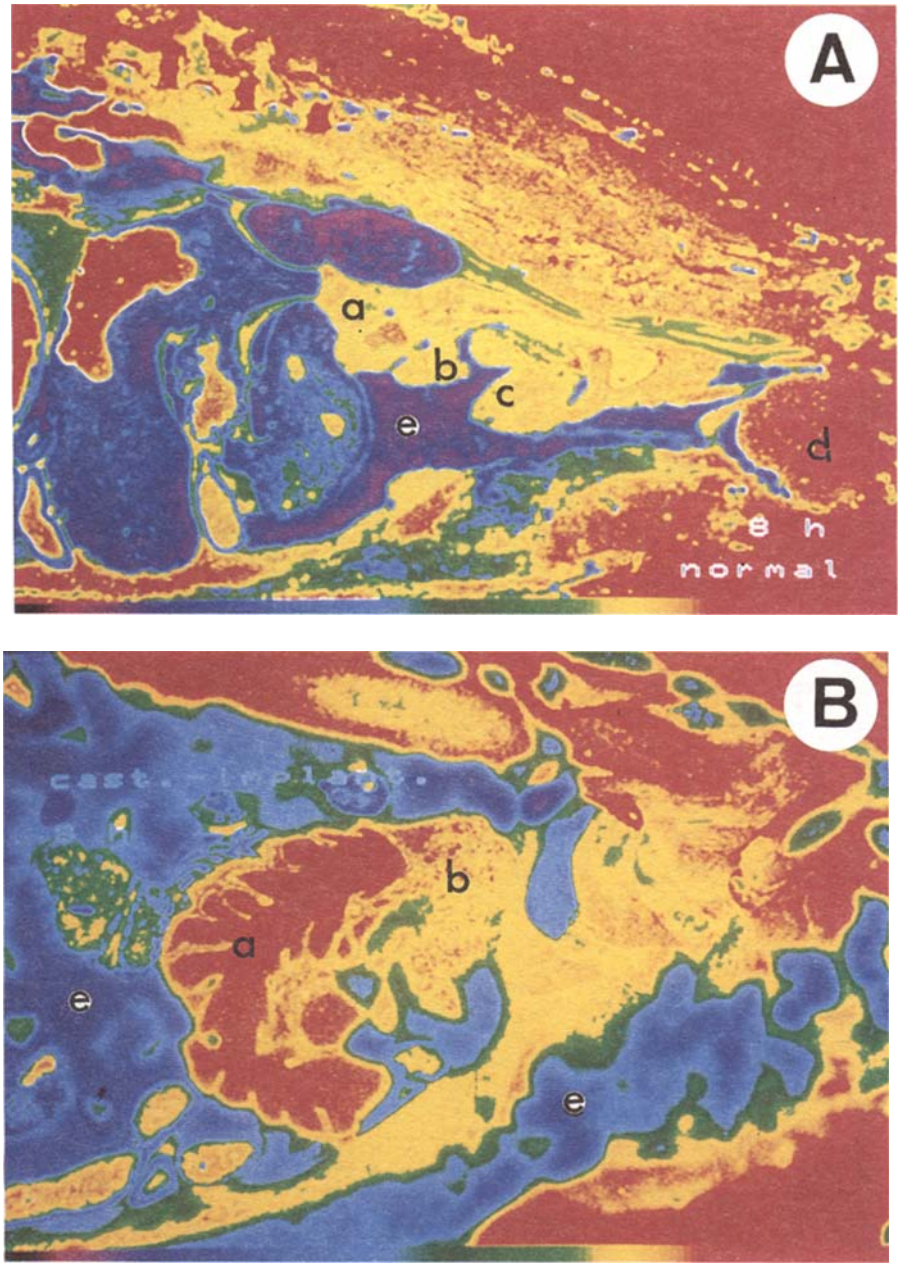

Fig. 6 : Enlargement of digitalized whole body autoradiograms of rats sacrificed 8 h after an oral administration with the LSESR supplemented with oleic acid. Radioactivity is represented in pseudo-colours on a 256 grey level scale represented on the bottom of figures. Red = level 0; black = level 255. (A) Castrated and hormonal implanted rat; (B) control rat. Key: a, seminal vesicles; b, prostatic tissues; c, urinary bladder; d, testes; e, fat.

^{14}C]-oleic acid. Concerning the distribution of fatty acids, some previous papers have reported results obtained with the same methods (31). They are confirmed by our results which clearly demonstrate that the highest uptake occurs in the thoracic duct, the abdominal fat and the liver. No data are available in the literature regarding the fate of β-sitosterol, but our results show that the distribution is very similar to that observed in animals after oral administration of cholesterol (32). In all cases, this preliminary study has demonstrated that the higher uptake of radioactivity is obtained about the 8th hour after oral administration in the animals of LSESR supplemented with [1-^{14}C]-oleic acid.

This conclusion has been confirmed in the second step of the study, which demonstrates that radioactivity uptake expressed as the ratio of total radioactivity measured in a tissue and total radioactivity measured in the plasma is higher in prostatic tissues as compared to other genital glands, urinary bladder and other organs, such as the liver, for example. This latter point appears to be very important in helping to understanding the therapeutic action and the local action of LSESR on the hypertrophic prostate.

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