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Determination of Lufenuron in canine skin layers by radioluminography

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Abstract

In a bioavailability study in dogs we investigated the quantity of [^{14}C]-Lufenuron and its metabolites in the skin by radioluminography. Two Beagle dogs were orally administered 10 mg [^{14}C]-Lufenuron per kg body weight. 21 days later they were euthanized. Skin specimens were taken at 6 different sites. Sections of these skin specimens were exposed on a phosphor imaging plate and the radiolabeled Lufenuron content visualized. Radioluminographical analysis showed that [^{14}C]-Lufenuron appeared in the subcutaneous layer of the skin. We did not discover any activity on the skin surface.

Key words: Lufenuron – radioluminography – skin – dog – program®

Lufenuron-Nachweis in Hautschichten von Hunden mittels Radioluminographie

In einer Bioverfügbarkeitsstudie mit radioaktiv markiertem Lufenuron in Hunden wurden gleichzeitig Hautproben mittels Radioluminographie auf den Gehalt an Lufenuron und seine Stoffwechselprodukte untersucht. Zwei Beagle Hunden wurden je eine orale Dosis von 10 mg [^{14}C]-Lufenuron verabreicht. Die Hunde wurden nach 21 Tagen euthanasiert und je 6 Hautproben an verschiedenen Stellen entnommen und sofort tiefgefroren. Dünnschnitte der Hautproben wurden auf einer Phosphor-Bildplatte exponiert und so der [^{14}C]-Lufenuron-Gehalt visuell dargestellt. Mit der Methode der Radioluminographie konnten wir zeigen, dass sich Lufenuron vorwiegend in den subkutanen Hautregionen ansammelt. Auf der Hautoberfläche konnte hingegen kein [^{14}C]-Lufenuron nachgewiesen werden.

Schlüsselwörter: Lufenuron – Radioluminographie – Haut – Hund – Program®

Introduction

Lufenuron, an insect development inhibitor, is the active ingredient of PROGRAM® (Novartis Animal Health, CH-4002 Basel, Switzerland). PROGRAM® is used in the prevention and control of fleas in dogs and cats. It is administered orally, as tablet or as oral suspension. An injectable formulation for cats has been developed.

Companion animals often live in a close contact with their owners. Topically administered parasiticides represent a high risk of contamination of the owner of a companion animal. Systemic action of Lufenuron is considered advantageous compared to topically administered ectoparasiticides due to its absence on the animal's surface. To verify this assumption we were looking for methods to investigate Lufenuron disposition in cutaneous layers. Such an opportunity was given during a bioavailability study with radiolabeled Lufenuron. Radioluminography was found to be an appropriate method.

Animals, materials and methods

Experimental design

Two Beagles were administered [^{14}C]-Lufenuron orally. Three weeks later the dogs were put to death and tissues taken for further investigations.

Animals and Housing

The 2 Beagles (1 female and 1 castrated male) used in this study weighed approximately 12 kg. The male dog was 44 months and the female 26 months old. The dogs were in good health.

During the study the animals were kept individually in metabolic cages (surface: 1050 mm × 810 mm). The room temperature ranged between 19–20 °C. Relative humidity was within the range of 50–70 %. The room had daylight and additional artificial light from 6:30 a.m. to 6:30 p.m.

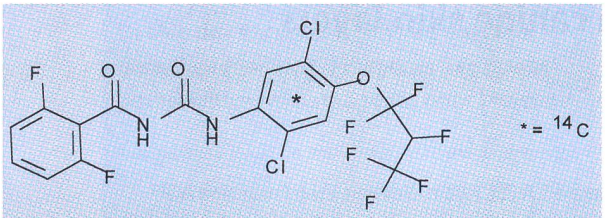

Figure 1: Structural Formula of [14C]-Lufenuron

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Dose and administration of [14C]-Lufenuron

The dogs were given a nominal dose of 10 mg [14C]-Lufenuron / kg body weight. The specific radioactivity was 104 kBq/mg [14C]-Lufenuron. For details on structural formula of Lufenuron see figure 1.

Labeled Lufenuron was dissolved in Polyethylenglycol 300 and thereafter filled in a gelatine capsule (5 ml Torpac "lock ring capsules").

We administered the capsule immediately after feeding by placing it into the dog's pharynx behind the tongue.

Collection of skin specimens

At the end of study, both dogs were put to death by an intravenous injection of 0.5 ml Vetanarcol®/kg bodyweight. (Vetanarcol ® contains 162 mg Pentobarbital sodium per ml.)

Thereafter skin specimens at a size of about 30 mm × 30 mm were carefully taken from the following areas: mandibula, scapula, sternum, sacrum, coxa, and anus.

Immediately after cutting the skin specimens, we pinned them on cardboards. This guaranteed a flat and stretched state of the skin indispensable for later analysis. For instant freezing they were immersed into hacked dry-ice.

Slicing of skin specimens

Skin specimens were placed on a framed specimen stage for embedding. The stage was filled with an aqueous solution of 4–5 % gelatine and submerged in a freezing mixture of n-hexane and dry-ice during 30–60 minutes until complete freezing.

The frozen block was cut in a cryostat microtome, Reichert-Jung *CRYO MACROCAT.* Sections of 40 µm thickness were obtained for radioluminography. The sections were fixed on a transparent adhesive tape, lyophilized for 72 hours in the microtome at –25 °C and then stored at room temperature.

Preparation of the blood standard scales

A series of 9 blood calibration standards with cattle blood were spiked at different concentrations of [14C]-Standards. The concentration covered a range from 0.1 dpm/mg through 10 000 dpm/mg corresponding to a residue level of 0.016 ppm through 1600 ppm Lufenuron equivalents in blood, respectively.

The concentration of radioactivity in each blood calibration standard was determined after combustion in a *Packard* sample oxidizer system 387 (Packard Instrument Company Inc, USA) by liquid scintillation counting (LSC). The concentration of radioactivity in each standard was used to establish a calibration curve for the correlation of PSL (photostimulated luminescence) to DPM (radioactivity). The blood calibration standards were also frozen in a gelatine block and processed the same way as described for skin specimens.

Visualizing and measurement of radioactivity by radioluminography

The sections of skin specimen together with 2 blood standard scales were exposed on a phosphor imaging plate (*FUJI* Photo Film Co. Ltd., Tokio, Japan) in a lead shielded box for 24 hours. During this time a latent image of captured energy of radioactive radiation, based on photostimulated luminescence (PSL), was stored on this ultra sensitive image screen. After exposition the image plate was scanned with a laser beam and the captured radioactivity was visualized on a Bio-Imaging Analyzer, model *BAS 2000* (*FUJI* Photo Film Co. Ltd., Tokyo, Japan). The further processing of the radioluminogram was performed on *TINA* software (*RAY-TEST*, Straubenhardt, Germany).

Calculation of residues

Calculation of radioactivity in skin specimens was based on the correlation of the determined photostimulated luminescence (PSL values) to radioactivity (DPM) as determined with the blood standard scale.

Since the radioactivity is homogeneously distributed within the 40 µm thickness of sections, self-absorption of the ß-radiation is likely to occur. The extent of self-absorption depends on the density and composition of the individual type of specimen. We used a self-absorption ratio of fat to blood (1.43) to calculate quantitative residue data of skin since the subcutis is a very adipose tissue.

For the residue calculation the following equations were used:

$$C_{(dpm/g)} = PSL \cdot CF \cdot SFA$$

$$C_{(ppm)} = \frac{PSL \cdot CF \cdot SFA}{60 \cdot SA}$$

Abbreviations:

C = Concentration of residues (dpm/g) or (ppm Lufenuron equivalents)
PSL = Photostimulated luminescence per area (PSL/mm²)
CF = Calibration factor (dpm·mm²·PSL⁻¹·g⁻¹)
SFA = Self absorption factor, ratio to blood
SA = Specific radioactivity (kBq/mg)

Results

Localization of skin residues

The figure 2 shows the total radioluminogram of the exposed skin sections including the blood standard scale.

For a closer localization of the [^{14}C]-Lufenuron residues in the skin sections the visual images were compared with the determined radioluminograms of the corresponding skin sections. The figures 3 and 4 demonstrate, within the maximum resolution of 100 µm, that the radioactivity was preferably located in the subcutis migrating to some extend into the corium (dermis). A distinct borderline between corium and subcutis could not be seen in the radioluminogram. However, radioactivity in the epidermis could not be observed.

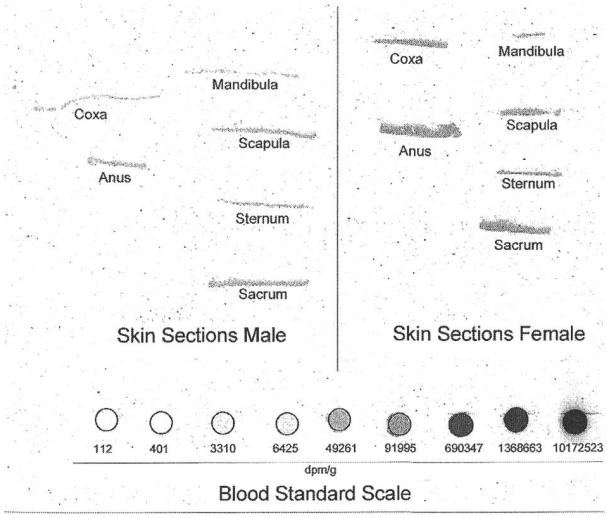

Figure 2: Radioluminogram of the exposed skin sections including blood scale.

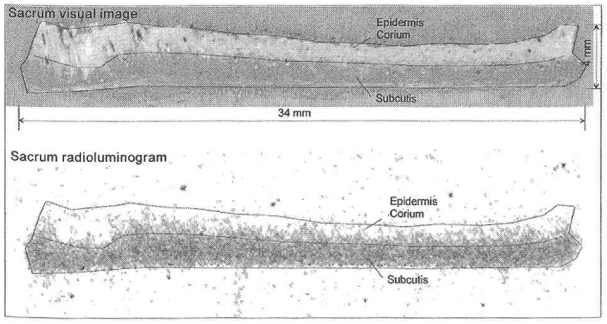

Figure 3: Visual image and radioluminogram of skin section taken at sacrum of the male dog.

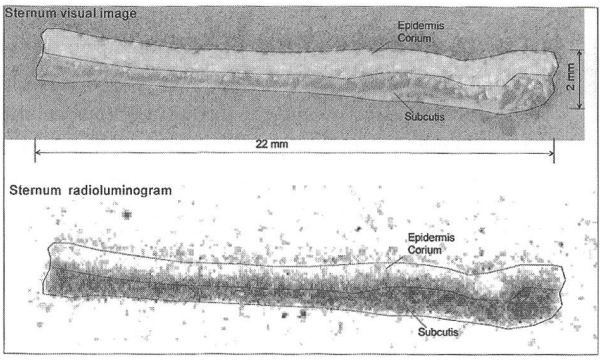

Figure 4: Visual image and radioluminogram of skin section taken at sternum of the female dog.

Quantification of skin residues

The PSL-values of the blood calibration points were plotted as function of the determined concentration of radioactivity. The linear regression ($f_{(x)}=m\cdot x$) revealed a calculated slope (CF) of 4678 with a correlation coefficient ($r^2 > 0.9999$).

The calculated residue levels of [^{14}C]-Lufenuron and its radiolabeled metabolites in skin cross sections of the female dog were about 2 times higher than in the male dog (see table 1). This finding may result from a lower absorption of Lufenuron in the male dog. The residues in all skin cross sections of the different collected areas were located preferably in the subcutis.

Discussion

There exist other methods to visualize radiolabeled compounds in tissues, e.g. autoradiography. However, radioluminography is more sensitive and allows to reduce exposition time to 24 hours instead of weeks as needed for autoradiography. For radioluminography no films have to be developed and the imaging plate can be reused. Digital visualization of captured radioactivity is a further advantage of this method as the pictures can be compared on

Table 1: Skin residues [in dpm/g] and Lufenuron equivalents [in ppm].

	Male dog		Female dog	
Skin sections	dp /g	**ppm**	dpm/g	**ppm**
Anus	40976	**6.6**	92622	**14.8**
Coxa	23080	**3.7**	80647	**12.9**
Mandibula	19735	**3.2**	43350	**6.9**
Sacrum	48870	**7.8**	91017	**14.6**
Scapula	43718	**7.0**	57333	**9.2**
Sternum	34754	**5.6**	61681	**9.8**

screen with digitally scanned tissue specimens.
We did not detect radiolabeled Lufenuron on the surface of the skin and saw migration of it from the subcutaneous layer in the direction of the corium. It could be interesting to know more histological details e.g. on the exact location of Lufenuron and its assumed capacity to bind to lipocytic structures in the subcutaneous layer. Furthermore we recommend the following methodological improvements for future radioluminographical studies:

a) collection of skin specimens should be performed together with muscular tissue for better localization of the tissue layers,
b) evaluation of radioluminograms should be done together with spiked skin specimens.

Conclusions

We successfully used radioluminography to determine the distribution pattern of [^{14}C]-Lufenuron in skin and showed that it is a useful method for analysis of skin residues. We saw a difference of [^{14}C]-Lufenuron content among the different sites of collected skin specimens presumably corresponding to its fat content. The determined [^{14}C]-Lufenuron was preferably located in the subcutis migrating to some extent into the corium. But no activity of [^{14}C]-Lufenuron was discovered on the surface of the skin.

Teneur de Lufenuron dans la peau du chien par radioluminographie

Lors d'une étude de biodisponibilité, des échantillons de peau ont été prélevés afin de quantifier, par radioluminographie, la teneur en Lufenuron et ses métabolites.
10 mg de [^{14}C]-Lufenuron/kg ont été administrés à deux chiens de la race Beagle. Après 21 jours nous avons prélevé à six endroits différents des échantillons de peau des chiens ayant été euthanasiés. Ces échantillons ont été immédiatement congelés.
Les coupes de peau effectuées au microtome ont été exposées sur une plaque munie d'une couche phosphorée. Il a été possible de montrer la présence de ([^{14}C]-Lufenuron au niveau de l'hypoderme. Aucune activité n'a par contre été constatée au niveau de l'épiderme supérieur.

Tenore in Lufenuron di pelle dei cani per radioluminografia

Durante uno studio di biodisponibilità al Lufenuron radioattivo, dei campioni di pelle sono stati prelevati al fine di quantificare, per radioluminografia, il tenore in Lufenuron e dei metaboliti derivati.
Dieci mg di [^{14}C]-Lufenuron/kg sono stati amministrati a due Beagles. Dopo 21 giorni, dei campioni di pelle dei cani eutanasiati, sono stati prelevati in sei punti differenti e immediatamente congelati.
Le sezioni di pelle fatte al microtomo sono state esposte su una placca avente uno strato fosforeo. Con la radioluminescenza, é stato possibile osservare la presenza del [^{14}C]-Lufenuron a livello dell' ipoderma; nessun'attività è stata invece costatata a livello dell'epidermide superiore.

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