Whole-body Autoradiography of Bumetanide-¹⁴C in Dogs by Round Saw Method

—A Combined Method of Autoradiography and Tissue Radioassay—

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The distribution of a new potent diuretic, Bumetanide, in dogs was studied by means of a combined method of whole-body autoradiography and tissue radioassay, after oral administration of Bumetanide-¹⁴C to five young beagle dogs (ca. 1.2 kg). A round saw method developed by Kalberer was applied to obtain frozen slices of dog whole-body with a thickness of 3 mm. After exposure to X-ray films from both sides of the slices at -60°C, frozen samples of the main tissues as well as the blood, urine, bile and intestinal contents were obtained to be assayed for radioactivity and to be analyzed for metabolites by TLC.

The advantages of the round saw method (thick slices) in larger animals as dogs and monkeys as compared to the conventional microtome method (thin sections) were pointed out and discussed.

Key Words: wholebody autoradiography, round saw method, dog, tissue radioassay, combined method, carbon-14-bumetanide

1. Introduction

Whole-body autoradiographic method using thin freeze-dried sections developed by Ullberg¹⁾ has been widely used in small animals as mice and rats. A larger animal as dog or monkey has been also used in the same method, but another method using thicker sections or slices cut by a round saw instead of microtome developed by Kalberer²⁾ has been attracted little attention.

Bumetanide (Lunetoron®) is a new diuretic³⁾ which has a weaker toxicity and is 40 times more potent than Furosemide.⁴⁾ It has been shown that⁵⁾ bumetanide is active in dog and human, while does not exert its diuretic activity in rat because of its extensive metabolism.⁶⁾ In the present study, therefore, the distribution and metabolism of bumetanide-¹⁴C were investigated in dogs as a suitable model for men. Kalberer's method was applied to obtain whole-body autoradiograms and, after the exposure, the main tissues as well as the bile, blood, urine and intestinal contents were removed from the

frozen sections to be assayed for radioactivity and analyzed for metabolites by thin-layer chromatography. The advantages of this method will be discussed.

Bumetanide (*site of labeling)

2. Material and Method

2.1 Labeled compound

Bumetanide-¹⁴C labeled at the phenoxy moiety (3-*n*-butylamino-4-phenoxy-¹⁴C-5-sulfanylbenzoic acid) was prepared from phenol-¹⁴C (G). The specific activity was 13.5 μ Ci/mg (4.9 mCi/mmole) and the radiochemical purity was over 99% by thin-layer chromatography.

2.2 Whole-body autoradiography

Six male young beagle dogs weighing about 1.2 kg were used after fasting for 16 hr. Bumetanide-¹⁴C was dispersed in 0.2% carboxymethylcellulose solution at a concentration of 7.43 mg/

mL by applying an ultrasonic treatment for 10 min. One mL aliquot of the solution was administered orally to each dog with a stomach tube, the dose being about 6.2 mg/80 μ Ci/kg.

Thirty min and 1, 3, 6, 24 and 72 hr after administration, the animals were anesthetized with Ketalar[®] (30 mg/kg, im) and frozen in hexane-dry ice mixture at -76° for about 15 min. The frozen animal was embedded in methylcellulose gel with a plastic frame and was frozen again. After placing and setting on the instrument, which was purchased from MASIBA A.G., Basle, Switzerland, the sagittal whole-body slices of 3 mm thickness were cut by means of a rotary saw (300 mm diameter, 96 teeth) in a freezing room at -15° , according to Kalberer.²¹ In this way, six to eight pieces of the slices were obtained from each animal. After the surface was cleaned carefully to remove the tissue dusts, the slices were brought to contact with Sakura Type N X-ray films from the both sides of the slice and pressed in a modified X-ray film cassette for medical use, in a dark freezing room at -15° . The cassettes were kept at -60° in a deep freezer to be exposed for 4 weeks.

2.3 Determination of tissue concentration of radioactivity

After the exposure was finished, 200 to 300 mg samples of the main tissues, blood, bile and urine were removed from the slices in a frozen state. The liver samples were removed from three different parts which showed a homogeneous blackening on the autoradiogram, in order to avoid a contamination from the biliary ducts. The intestinal contents were removed from the upper, middle and lower parts of intestine based on the visual shape of the tissue and the color of the contents. Each about 200 mg samples were removed carefully from the brain, lung, skeletal muscle (neck), spleen and the cortex and medulla of the kidney. After being weighed, they were dissolved by warming in 0.5 mL of 30% KOH at 60° overnight, in counting vials. The blood samples were decolorized with 0.1 mL of 30% H_2O_2 . Then, 1 g of BBS-III solubilizer was added and, after being neutralized with glacial acetic acid, 15 mL of

methylcellosolve-toluene scintillator was added. The radioactivity was counted in a Beckman LS-250 liquid scintillation spectrometer and the counting efficiencies were determined by ^{137}Cs external standard method.

2.4 Separation of metabolites in urine, bile and intestinal contents

The urine and bile samples were separated by cellulose TLC with the solvent system of butanol : methanol : formic acid (6:2:1). A part of the bile sample was incubated with glucuronidase at 37° for 17 hr and developed. The intestinal contents (about 500 mg) were extracted with 5 mL of a mixture of 4% HClO_4 and methanol (3:1) under homogenization. The extraction was repeated twice and the combined extracts were adjusted the pH to 6.0 with 1N KOH and allowed to stand overnight. After the precipitates were filtered off, the solution was concentrated and the residue was developed. The radioactive spots were detected by autoradiography and counted by liquid scintillation spectrometer after being transferred into the vials by scraping from the plate.

3. Results

3.1 Whole-body autoradiography of bumetanide- ^{14}C after oral administration to dogs

The autoradiograms obtained from dogs 1 and 3 hr after oral administration of Bumetanide- ^{14}C are exemplified in Fig. 1 and 2, respectively. Thirty min after administration, the highest radioactivity was observed in the gall and urinary bladders and kidney cortex as well as in the contents of stomach and upper small intestine, indicating a rapid absorption and elimination. A high concentration was observed also in the liver, the radioactivity being distributed as a spotted pattern. The radioactive concentrations in other organs and tissues were very low. No radioactivity was detected in the parenchymal tissues of the central nervous system and skeletal muscle.

The blood and tissue concentrations appeared to be the highest in the period from 1 to 3 hr after the administration. The highest concentration continued in the gall and urinary blad-

ders, intestinal contents, renal cortex and liver. An appreciable concentration continued in the circulating blood and lung. After 3 hr, the radioactivity in the intestinal lumen appeared to be spread over a wider region of the intestine, suggesting that the radioactive substances excreted *via* the bile moved down to the lower part of intestine gradually. No radioactive uptake was detected in the adrenal, spleen, thymus, bone marrow, pancreas and testis as well as in the central nervous system and skeletal muscle.

After 6 hr, the concentration in the blood declined to an undetectable level and that in the liver also declined to a low level. A high radioactivity still continued in the gall bladder and intestinal lumen, while the concentration in the urinary bladder decreased considerably, indicating a high and long participation of the biliary excretion. After 24 hr, the most of radioactivity disappeared from the body. A prominent radioactivity was, however, still observed in the gall bladder and intestinal contents, while the concentration in the urinary bladder declined to a low level. After 72 hr, a faint radioactivity remained only in the gall bladder.

Enlarged autoradiograms of the kidney and its vicinity are compared in Fig. 3, which demonstrated a high resolution of the autoradiograms by this method. The radioactivity was

Fig. 1 Whole-body autoradiogram from dog 1 hr after oral administration of bumetanide- ^{14}C .

Fig. 2 Whole body autoradiogram from dog 3 hr after oral administration of bumetanide- ^{14}C .

Fig. 3 Enlargement of the autoradiograms of the kidney and its vicinity from dogs 30 min (A), 1 (B), 3 (C) and 24 (D) hr after oral administration of bumetanide- ^{14}C .

found in the specific part of renal cortex. The concentration reached the maximum at about 60 min after administration, decreased considerably after 6 hrs and an appreciable concentration continued even after 24 hrs. The localiza-

Table 1 The concentration of radioactivity in main tissues and body fluids after oral administration of bumetanide- ^{14}C to beagle dogs

Time (hr)	Concentration ($\mu\text{g/g}$ or mL)*									
	Bile	Urine	Blood	Renal cortex	Renal medulla	Liver	Lung	Skeletal muscle	Brain	Spleen
0.5	39.1	4.16	2.04	16.7	3.43	12.6	2.02	0.63	0.12	—**
1	144.0	17.9	2.94	10.6	7.87	7.93	2.71	0.73	0.16	0.96
3	—	—	3.73	11.1	7.32	6.99	1.97	0.50	0.19	0.72
6	372.1	6.32	0.28	3.04	1.29	1.66	0.26	0.10	0.05	—
24	466.8	1.46	0.07	0.31	0.26	0.21	0.07	0.03	n.d.***	—
72	66.7	—	—	—	—	—	—	—	—	—

* μg equivalent to bumetanide; Dose=7.4 mg/kg. Each value represents the mean from two experiments.

** —: The sample was not or could not be obtained.

*** n.d.: not detected.

Fig. 4 Blood and tissue concentrations of radioactivity after oral administration of bumetanide- ^{14}C (7.3 mg/kg) to dogs.

tion of radioactivity appears to correspond to the medullary rays of the renal tubules extended into the cortex.

3.2 Concentration of radioactivity in main tissues and body fluids

In Table 1 and Fig. 4 are shown the results of counting of radioactivity in the main tissues as well as the urine, bile and blood. The

highest concentration was shown in the bladder-bile at all times, followed by the urine, in consistent with the autoradiographic results. The concentration in the bile increased with time up to 24 hr after administration, but appeared to reach the maximum sometime between 6 and 24 hrs after administration, since the concentration declined to a very low level after 72 hrs.

The concentration of radioactivity in the blood and tissues corresponded well to the autoradiographic results and the time course revealed a smooth decline after being reached the maximum between 1 and 3 hr after administration, in spite of the result obtained from each one animal, suggesting that experimental errors or radioactive contaminations accompanied with the incision might be minimized in the present method.

3.3 Metabolites of bumetanide- ^{14}C in bile, urine and intestinal contents

In the urine, only unchanged bumetanide was detected on the TLC autoradiograms at R_f 0.9, while in the bladder bile the main radioactive spot was detected at R_f 0.7 in addition to a weak spot of unchanged drug at R_f 0.9. The spot at R_f 0.7 which accounted for 83% of the total radioactivity in the bile decreased to about 8.5% by glucuronide hydrolysis, while the most part moved to R_f 0.9, as shown in Table 2. The results thus indicated that bumetanide is excreted into the urine mostly as the unchanged drug, while mostly as a conjugate form of bumetanide into the bile.

Table 2 Bumetanide and its metabolite in intestinal contents 6 hr after oral administration of bumetanide- ^{14}C to dogs

Metabolite	Rf**	% to Total radioactivity*				
		Bladder bile		Intestinal contents		
		No treatment	+ Glusulase	Upper	Middle	Lower
Bumetanide	0.9	19.1	91.5	31.7	87.7	90.4
Conjugate	0.7	80.9	8.5	68.3	12.3	9.6

* Each value represents the mean from two experiments.

** Cellulose TLC; BuOH: MeOH: HCOOH=6:2:1

The contents from the upper part of intestine showed the main spot at Rf 0.7 in consistent with that of the bladder bile, while the amount of unchanged bumetanide increased considerably in the middle part of intestine (Table 2). In the contents from the lower intestine, over 90% of the radioactivity was unchanged bumetanide. Therefore, it is assumed that the bumetanide conjugate excreted into the duodenal lumen *via* the bile is gradually hydrolyzed in the intestinal lumen during its downward movement through the intestine, affording free bumetanide.

4. Discussion

As was demonstrated in the present investigation, Kalberer's method²⁾ has the following advantages as compared to the usual method of Ullberg¹⁾: i) Preparation of whole-body sections from a larger animal as dog and monkey is quite easy even for the hard tissues as the skull. ii) The thickness of the slices (3 to 5 mm) becomes to be infinitive for track-length of β -rays from ^{14}C and other nuclides with a comparative energy. Therefore, no uniformity in the specimen thickness is required in contrast to thin sections (20-50 μm) wherein unevenness of the specimen thickness results in ununiform blackening of the film. iii) Any cracks in the soft tissues of the sections and a possible shrinkage of the sections due to the freeze-drying procedure can be completely avoided. This method affords an uniform autoradiograms for such organs as the liver and lung and even for blood and urine. iv) The slices can be used for counting and metabolites separation experiments after the autoradiographic exposure. Thus, both the autoradiographic and quantita-

tive, and also the metabolic, data can be obtained from the same single slice.

It was ascertained from a comparison of the weight of frozen slices before and after the exposure to X-ray films that a weight loss during the exposure time at -60° was negligible. Therefore, quantitative informations can be obtained as the radioactive concentration per wet weight. It might be also assumed that a secondary change of the labeled drug and its metabolites during the exposure period must be minimized at -60° .

The obvious disadvantages of this method might be: i) This method is not suitable for small animals as rats and mice, because the number of slices obtained from each animal is limited resulting in a loss of small organs on the autoradiograms. ii) It is rather a tedious work to bring the slices to contact on X-ray films under a frozen state. iii) The sections can not be stained or stored and it is not easy to compare in detail the autoradiograms with the corresponding sections.

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要 旨

回転円鋸法による Bumetanide- ^{14}C のイヌ全身オートラジオグラフィ ——組織計数との組合わせ法——

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新利尿剤 Bumetanide のイヌ体内分布を, ^{14}C 標識体経口投与後 (約1.2 kg ビーグル小犬) の全身オートラジオグラフィならびに組織計数法によって調べた。このさい, 回転円鋸法を応用し, 厚さ3 mm の全身凍結スライスとし, 両側からX線フィルムに露出 (-60°C) してオートラジオグラムを得た。露出後のスライスから血液, 尿, 胆汁, 腸内容物 (上, 中, 下部) ならびに主臓器, 組織試料を凍結状態で摘出し, 放射能の計数ならびに薄層クロマトグラフィによる代謝物の分離定量を行った。

この方法は中型動物の切片作成が容易で, 厚さの不均一性が問題とならず (無限大厚), 乾燥に伴う軟組織の収縮, 亀裂がなく, 露出後の同一切片から十分量の組織, 体液試料を採取できる, などの利点をもっている。