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AN ESSENTIAL RADIOTRACER TECHNIQUE RADIOLUMINOGRAPHY FOR PHARMACOKINETICS AND METABOLIC STUDIES IN THE SAFETY EVALUATION OF NEW DRUGS IN ANIMALS.

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INTRODUCTION

A workshop of "Drug Metabolism and pharmacokinetics: regulatory guidelines for the registration of pharmaceutical products--theory and practice" was organized by the ISSX committee on regulatory affairs involved in the 3rd international ISSX meeting held at Amsterdam in 1991. This will contribute greatly to the progress of the international harmonization for the registration of pharmaceutical products. In the discussion of the workshop, the focus is to differentiate carefully between regulatory "requirements", "guidelines" and "expectations", and in order to harmonize the contents of the studies, not only test items but also qualitative and quantitative definition of analytical techniques must be discussed as stated by Dr. Shindo, Sankyo Co., Japan.

It is well known that the accuracy and lowest limitation of the analytical measurement has made great raising by use of radiotracer techniques. A benefit of radiotracer techniques is to be able to measure the minute amount of the drug and its metabolites in the experimental animal after administration, omitting all of procedures for separation of the target compounds from the biological samples.

Actual life science must be tetradimensional science. Every constituent is situated 3 dimensionally on a given location of the living structure and momentarily moved on its location or changed into its related metabolites.

The radiotracer techniques are one part of the most suitable tools to study on such a tetradimensional science. The radiotracer techniques are going to make a great progress with timely requirement to the drug metabolism, pharmacokinetics and its disposition. Here, we would like to present a new technique "Radioluminography" one of the tracer techniques established in Japan in 1991. Radioluminography is a sort of the quantitative imaging techniques in place of macroautoradiography. Evidence was presented to elucidate whether or not quantitative analysis of autoradiographs obtained with a ^{14}C-labelled compound by the use of a new type radiosensor, so called "Imaging Plate (IP)". The IP is one of a specially designed photo stimulable phosphor comprised of Ba FX;Eu^{2+} (X=Cl, Br or I) crystals. The linearity was given in a relatively wide range between 10^1 to 10^5 dpm orders

of radioactivity. About 100 times higher sensitivity of the IP than any X-ray film was demonstrated by the use of not only ^{14}C-radioactive standard sources but also an experimentally provided ^{14}C-radioactive spots developed on a TLC plate and macro autoradiographs (MARG).

GUIDELINES FOR PHARMACOKINETICS STUDIES

"An example of protocol" presented by Dr. Benakis in the 2nd ISSX held at Kobe, Japan, in 1988. This is a good sample following the guideline by EC governments as a model of protocol for drug metabolic and pharmacokinetic studies in animals and humans as shown in Figure 1. In this report, the main purposes are to introduce the current in pharmacokinetics and metabolic studies in the safety evaluation of new drugs in animals and humans in the world. The following citations must be very helpful for understanding of the principle of "the guidelines for nonclinical phamacokinetic studies in Japan."

The objective of nonclinical pharmacokinetic studies is to examine the absorption, distribution, metabolism and excretion of a test substance given to test animals to clarify its biolgical fate. Data on the pharmacokinetics of the test substance are not only useful for designing the toxicological and pharmacological studies in animals but also essential, by their assessment and understanding, to establish the appropriate dose regimen in man to secure safety and efficacy.

In performing these studies, it is acceptable to choose test methods appropriate to the properties of the test substance, or to elaborate novel test procedures, based on the following principles, in order to achieve the objective of the studies.

Test Methods

1) Test Substance: Drug substance, isotope-labelled drug (Note 1) and anticipated dosage form, when necessary, should be employed.

2) Animal Species: An appropiate animal species should be chosen, considering the toxicological, pharmacological and clinical studies.

3) Route of administration: The anticipated clinical route of administration should normally be employed. Additional routes of administration should also be employed, when necessary (Note 2).

4) Dose levels: Appropriate dose levels should be employed, considering toxicological, pharmacological and clinical studies.

5) Intervals and duration of administration: Both single-and repeated-dose administration should be employed. In the case of repeated-dose administration, the intervals and durating of administration should be adopted to assess the steady state, accumulation and effects on drug-metabolizing enzymes of the test substance in animal and clinical studies.

6) Assay methodology: The assay method and its sensitivity, precision, specificity, etc. should be clearly defined.

Parameters To Be Determined

The pharmacokinetics of the test substance should be examined. The pharma-

PROTOCOL FOR DRUG METABOLIC AND PHARMACOKINETIC STUDIES IN ANIMALS AND HUMANS (EC)

		RAT	DOG	HUMAN
BLOOD LEVEL	:(total), values according to time, dose, administration route (oral/i.v. or i.p., other routes), single or repeated administration	●	●	●
PLASMA LEVEL	:(unchanged product and /or metabolites);same condition as above	●	●	●
ERYTHROCYTES etc		●	●	●
PLASMA PROTEIN BINDING	:*in vitro, in vivo* (equilibrium dialysis, dynamic dialysis, ultrafiltration, ultracentrifugation, circular dichroism)	●	●	●
BILIARY LEVEL	:unchanged product and metabolites according to time (biliary fistula, extracorporal circuit)	●	●	○
$^{14}CO^2/^3H_2O$	:according to time after administration of radioactive compounds	●	●	○
AUTORADIOGRAPHY	:according to time and administration route	●	□	□
QUANTIFICATION	:at level of organs and tissues, ratio between administered and recovered radioactivity	●	○	□
URINARY, FECAL ELIMINATION	:according to time, administration routes, sex, dose	●	●	●
METABOLITES	:separation and quantification according to time at the plasmatic, urinary, fecal, tissue, etc. levels	●	●	●
CHEMICAL STRUCTURE OF METABOLITES	:determination by TLC, GLC, HPLC, MS, NMR etc.	●	●	●
PATHOLOGICAL CONDITIONS	:metabolic studies in :renal insufficiency, hepatic insufficiency, polyarthritis, etc.	●	●	○

● Regulatory performed ○ Occasionally performed□ Unfeasible

Figure 1. Protocol for drug metabolic and pharmacokinetic studies in animals and humans, proposed by Dr. A. Benakis (1989): Workshop of International Harmonization for drug metabolic and pharmacokinetic studies in animals and humans, the 2nd International Symposium for Study of Xenobiotics, held at Kobe in 1988.

cokinetic parameters of the test substance such as elimination half-life (or other substituted parameters such as elimination rate constant, mean residence time, etc.), clearance, distribution volume, bioavailability, etc. should be examined as much as possible. The linearity in the pharmacokinetics of the test substance should be also determined. The pharmacokinetics of the metabolites should be examined, if necessary.

Absorption is performed in order to clarify the extent and rate of absorption of the test substance. These can be determined from the blood concentration vs. time curve or the cumulative excretion curve (Notes 3 and 4). Factors capable of affecting drug absorption should also be studied, when necessary (Note 5).

Distribution is performed in order to examine the distribution in various organs and tissues, its variation with time and accumulation of the test substance (Note 6). The following items should be examined: 1) concentrations in organs and tissues (Note 7), 2) distribution by whole body autoradiography, 3) transfer into the placenta and fetuses, 4) binding to plasma proteins and distribution in blood cells.

Metabolism is performed in order to identify and quantify the test substance and its main metabolites, and to determine the route, and the extent and rate of its metabolism. It is important in this study to clarify the similarities and differences in the metabolism of the test substance between human and animals. This study is usually performed by isolating and quantifying the unchanged form and metabolite(s) of the test substance in biological speciments such as blood, urine, bile and feces (Note 8). Factors capable of affecting the metabolism of the test substance should also be examined, when necessary (Note 9).

Excretion is performed to determine the route, and the extent and rate of excretion of the test substance and its main metabolites (Note 10). It is also recommended to examine factors capable of affecting the excretion of the test substance, when necessary (Note 11). Excretion by the following routes should be examined: 1) urine, feces, expiration (Note 12), 2) bile (Note 13), 3) milk.

Other Items To Be Examined

Effects of the test substance on drug-metabolizing enzymes and when necessary, drug interaction and first-pass effect, etc. should be investigated and discussed (Note 14).

If the test substance is a recemate, it is desirable to examine the pharmacokinetics of each optical isomer.

Note 1. The following information on isotope-labelled compound should be clearly defined: supplier, method of synthesis, purity, isotope used, labelled position, specific radioactivity, stability, etc.

Note 2. Studies with intravenous or other routes of administration, in which the process of absorption can be eliminated, provide fundamental data for understanding the pharmacokinetics of the test substance.

Note 3. Calculation from data on blood (serum, plasma or whole blood) concentrations. The extent and rate of absorption can be determined from the maximum blood concentration (Cmax) after dosing, the time required for the blood concentration to reach its maximum (Tmax), the area under the blood concentration vs. time curve (AUC), etc. Comparison in those parameters between the emplo-

yed route of administration and intravenous or other standard routes of administration will improve the precision of estimation of the extent and rate of drug absorption.

Note 4. Calculation from data on excretion. Excretion into the urine, feces, bile, expiration, etc. should can lead to good index of the extent of drug absorption.

Note 5. The following factors can affect drug absorption: 1) solubility of the test substance in the gastrointestinal tract, 2) dissolution characteristics of the preparation, 3) absorption site, 4) metabolism and stability in the gastrointestinal tract (metabolism and stability at the administration site in case of a dosage form to be administered into a site other than the gastrointestinal tract), 5) food and pH in the gastrointestinal tract.

Note 6. It is recommended that the determination of the test substance is undertaken at several time points which will properly reflect its pharmacokinetics.

Note 7. This study should be performed after single- and repeated-dose administration. It is recommended to determine chemical species in those organs and tissues in which high concentrations or accumulation is found and in those which can be target of the toxicity or pharmacological effect of the test substance. It is desirable to evaluate the accumulation of the test substance, relative to blood levels.

Note 8. *In vitro* studies with specimens such as tissue homogenates, cell suspensions and cell fractions of organs responsible for the metabolism of the test substance are useful for clarifying its metabolism, in addition to *in vivo* studies.

Note 9. Metabolic processes can vary according to animal species, age, sex, disease state, etc. They can also vary with enzyme induction or inhibition due to the repeated-dose administration of the test substance or of concurrent administered drug(s). The metabolic processes may become nonlinear, depending on the dose level and rate of administration of the test substance. Both the nonlinearity of metabolism of the test substance and dependency on the route of administration can affect the ratio between the unchanged form and its metabolites as well as the pattern of its metabolism.

Note 10. The ratio of the cumulative amount excreted to the dose administered can serve not only as an index of accumulation but also as an index of the reliability of the study results.

Note 11. The following factors can affect drug excretion: such as renal function, urinary pH, etc.

Note 12. After a single-dose administration of a radioisotope-labelled compound, it is recommended that at least 95% of the radioactivity should be recovered or the recovery test should be performed for a 7 day period, whichever is the shorter.

Note 13. If the test substance is excreted mainly into the bile, its enterohepatic circulation should also be examined.

Note 14. If the test substance affects drug-metabolizing enzymes or it strongly binds to plasma proteins, drug interactions are likely to take place. If the test substance is subject to metabolism in the liver or digestive tract and to excretion into the bile, an significant first-pass effect is likely.

CONCEPTS OF PHARMACOKINETICS AND WHOLE BODY METABOLISM

Common and different points in radiotracer techniques between "Pharmacokinetics" and "Whole Body Metabolism" were shown in Table 1. In pharmacokinetics and metabolic studies, radiotracer techniques are essential. First, the test compound expected as a new drug will be labeled with a suitable radioactive nuclide such as ^{14}C, ^{3}H, ^{35}S, ^{32}P, etc.

Specific radioactivity of the labeled compound must be a good indicator for measuring the minute amount of the label compound and its metabolites in biological specimens after administration of the label to animals.

However, it is not necessary to follow up radioactivity if the radioactive atoms would be transferred from metabolites of the drug into any of endogenous compounds distributed in tissues or organs by passing through metabolic processes such as reutilization. When a given amount of the native compound such as sugars, amino acids, lipids or proteins was administered to the animal passing the administered route, it must be exogenous until it would be incorporated into the endogenous substrate pool in the animal. In order to clear the fate of a given endogenous

Table 1. Common and different points between "Pharmacokinetics" and "Whole Body Metabolism" researches with regard to use of radiotracer techniques.

1) Common subjects

	Whole Body Metabolism	Pharmacokinetics and disposition
Instruments	MS, NMR, RI detector, ARG	same as the left description
Procedures	Grinding, Extracting, Separating, Purifying (PAGE, TLC, HPLC, GC, etc.)	same as the left description

2) Different subjects

Subjects	Fate of Endogeneous substances	Fate of Exogeneous substances
Necessary devices	Subtraction of the fate of a given atom or endogenous substance from the dynamics of the administered radioactive substance	Subtraction of the fate of radioactive atoms or metabolites after incorporation of radioactive atom-labeled compounds into endogenous metabolites.

Table 2. Concept of "Whole Body Metabolism" in place of "Biochemistry".

A term, "Whole Body Metabolism" is:

1) ***"Not to Systematize Knowledgements"*** of physico-chemical properties of substances extracted from organisms.
2) ***"To Systematize inclusively and organismically knowledgements"*** elucidated by observation, study, and experimentation carried in order to determine the fate of respective atom of a given compound in the ceaseless state such as transferring, dispersing, absorbing, steady state keeping, metabolizing, reserving, and excreting in a given organism.

compound, the subtraction of the exogenous fate of the administered label will be required from the total dynamics of the administered radioactive compound. On the other hand, in order to clear the fate of a given exogenous compound, the subtraction of the fate of all radioactive components will be required after incorporation of the administered radioactive atoms into endogenous metabolites. No difference of analytical procedures and instruments was found between pharmacokinetics and whole body metabolism researches.

The term of whole body metabolism is shown in Table 2. Consequently, it appears that such a new scientific term as whole body metabolism is not to systematize knowledgements of physico-chemical properties of substances extracted from organisms, and it is to systematize inclusively and organismically knowledgements elucidated by observation, study, and experimentation carried in order to determine the fate of respective atom of a given compound in the ceaseless state such as transferring, dispersing, absorbing, steady state keeping, metabolizing, reserving, and excreting in a given organism.

In order to follow up the fate of a radioactivity labeled group or moiety in the drug or substrate molecules in the experimental animal, the high sensitive and quantitative measuring technique will be necessary. And it is preferable to keep the sensitivity detectable to very minute radioactivity less than 10 dpm in each metabolite of the tissue specimen, using a TLC-autoradiography or TLC prior to radioactivity counting.

Recently, very high sensitive radiosensor was developed by Fuji Photo Film Co. And many investigators, working in the pharmacokinetics and metabolic studies in Japan, want to have a reliable data regarding to the quantification of the radiosensor to the soft β emitters such as ^{14}C and ^{3}H.

PRINCIPLE OF RADIOLUMINOGRAPHY IN PLACE OF X RAY FILM PHOTOGRAPHY

The imaging plate is a flexible image sensor in which bunches of very small crystals (grain size: about 7 μm) of photo-stimulable phosphor of barium fluorobromide containing a trace amount of bivalent europium as a luminescence center, formulated as BaFBr: Eu^{2+}, are uniformly coated 150 to 300 μm thick on a polyester support film. The composite structure of the imaging plate is shown in Figure 2. In addition to this, a trial IP which was specially designed for our experiment, was provided. This is a non-protectable sensor.

Exposure of samples to the imaging plate is performed in a manner similar to that of photo-film. The exposed imaging plate is scanned with a He-Ne laser beam of red light (633 nm) while the plate is being conveyed with high accuracy in a phosphor reader as shown in Figure 3. Depending on the purpose, the reading density may be selected from 25 to 100 pixels/mm^2. The reading sensitivity and sensitivity range can also be selected according to the purpose. A blush purple (400 nm) PSL, released upon laser excitation, is collected through the light collection guide to the photo multiplier tube (PMT), and converted there to analog electric signals in chronological order. Subsequently, these are converted to digital signals of 8 to 12 bits, again depending on the intended purpose. The reserving ene-

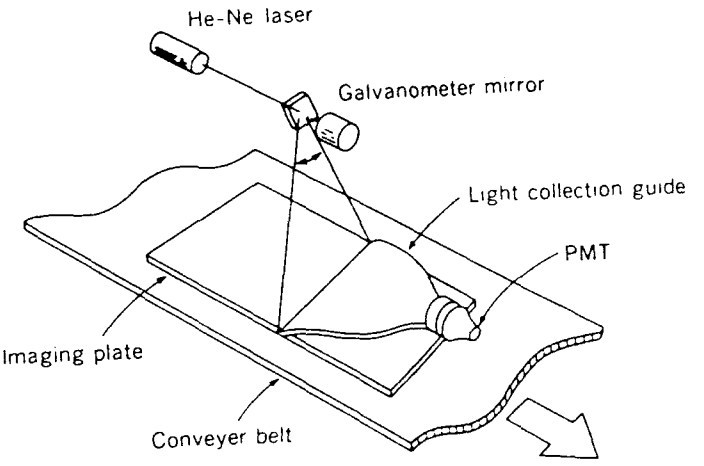

Figure 2. Composite structure of the imaging plate.

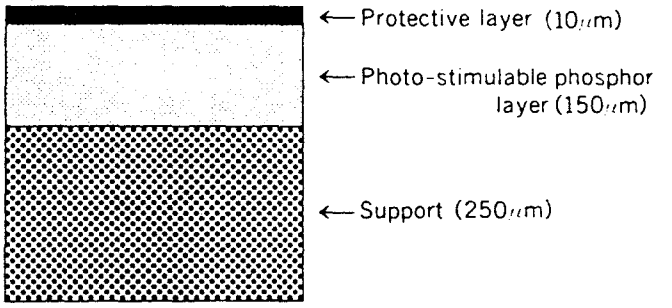

Figure 3. Principle of reading the radiation image from the imaging plate. The exposed imaging plate, while being conveyed, is scanned with a focused He-Ne laser beam. The PSL released upon the laser is collected into the photomultiplier tube (PMT) through the light collection guide and is converted to electric signals.

rgy is kept over 72 hours with no reduction in intensity of imaging.

A TYPICAL EXAMPLES SHOWING THE QUANTIFICATION OF A NEW RADIOSENSOR, IMAGING PLATE (IP)

By contacting of the radioactive specimen with IP in a dark box, the radioactive distribution of the specimen will be memoried on respective point of IP for a relatively short exposure time. After a suitable exposure, the IP will be taken from the dark box and inserted in the Bioimaging Analyzer, BAS2000, to display the visible and computed image. All procedures of autoradiographically processing, recording and imaging analysis is also quantitative with a given gradation displayed as a white and black or color hard copy as shown in Figure 4 and 5.

The sensitivity and quantitativeness of IP and a commercial X-ray film (SCRE; non screen type X-ray film, Konica Co. Ltd. Japan) were shown in Figure 6. In Figure 6 a designated volume (1 μl) of [1-^{14}C] palmitate solution containing respe-

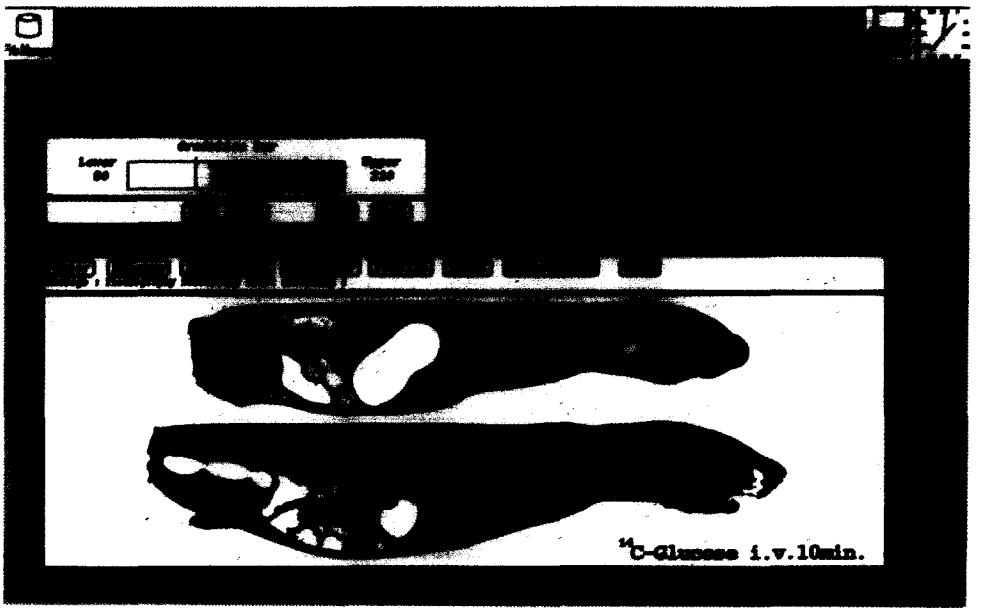

Figure 4. Macroautoradiograph showing ^{14}C-distribution in the rat section. The photo was given by a hard copy of Bioimaging Analyzer(BAS 2000, Fuji Photo Film Co.). The biological section was prepared by a freeze drying technique for macroautoradiography. The radioactive specimen, [U-^{14}C]-glucose of 10 μCi in an aqueous solution, was injected into a male rat, Wistar, and 10 min after the injection, the rat was sacrificed and immediately chilled with LN_2. The contact period of IP with the rat section were 48 hours.

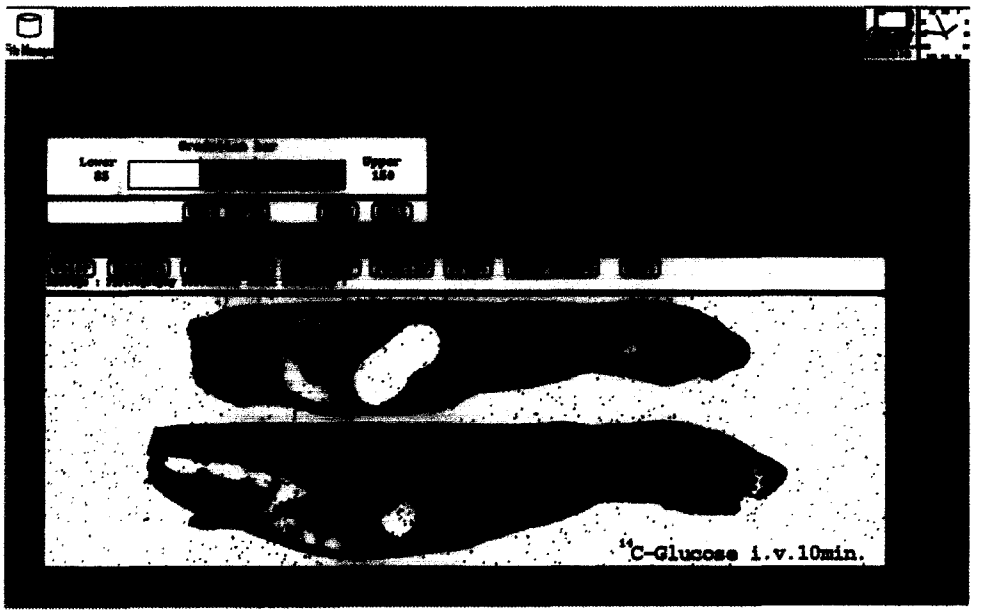

Figure 5. Macroautoradiograph displayed by a color copy showing ^{14}C-distribution in the rat section as same as that shown in Figure 4.

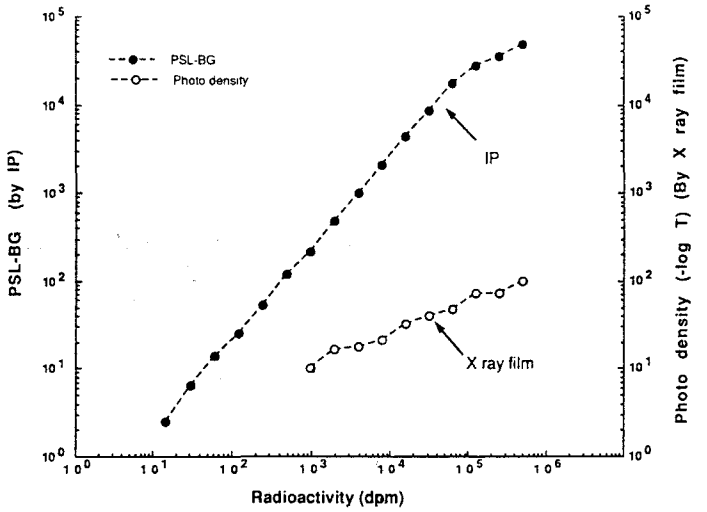

Figure 6. The sensitivity and quantitativeness of IP and a commercial X-ray film.
Radioactive specimen: [1−^{14}C] palmitic acid, TLC plate: Silica gel 60 F_{254} Merck 5715, Solvent: petrolumether: diethylether: acetic acid=50 : 50 : 1.
After developing, the TLC plate was contacted with IP for 3 hr. and then removed it, and the TLC plate was contacted with the high sensitive X-ray Film (SCRE, Konica Co.) for 1 week.

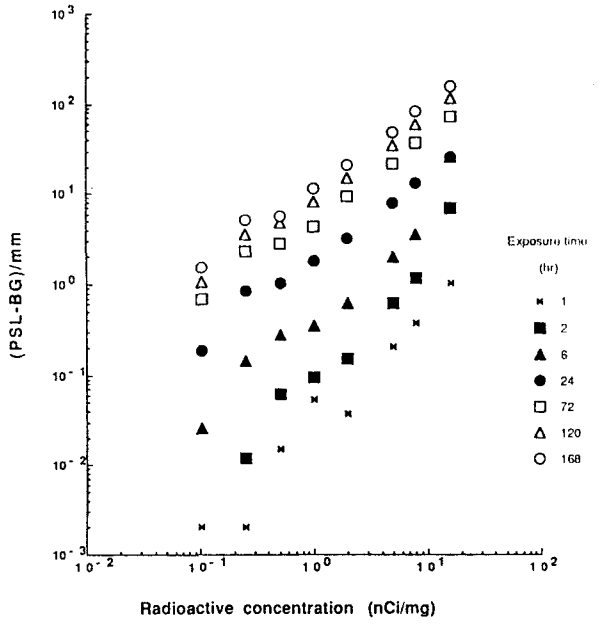

Figure 7. Relationship between radioactive concentration (nCi/mg) of ^{3}H standard sources and relative intensity expressed by photo stimulated luminescence (PSL-BG)/mm^2.

ctive different radioactivities was spotted on a TLC plate and each radioactive specimen was developed with a organic solvent mixture.

The quantitativeness was shown as a radioactivity relative intensity curve. In

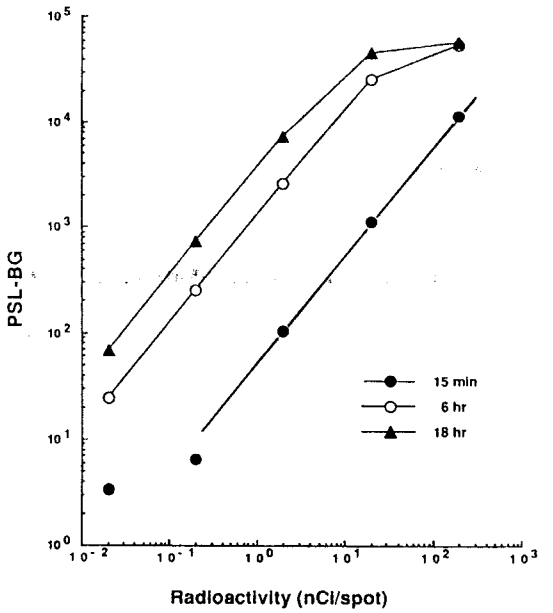

Figure 8. Relationship between ^{14}C-radioactivity on the TLC plate and relative intensity of (PSL-BG).

case of IP, radioactivity emitted -energy was converted into visual light by stimulation of laser light at each pixel. Therefore, the photo stimulated luminescence, (PSL-BG), will be expressed as relative intensity to radioactivity. In case of the X-ray film, photo density, -Log T, is usually used for the relative intensity. In Figure 6, the relative intensity of IP was in direct proportion to radioactivity of each spot on the developed TLC plate. In the wide range of radioactivity from 10 dpm to 10^5 dpm, the radioactivity -relative intensity graph was linear, though the linear graph was limited in a small range from 1×10^3 to 2×10^3 dpm in case of the X-ray film.

Figure 7 shows a relationship between radioactive concentration of ^3H standard sources and relative intensity. The maximum energy of ^3H is 10 keV and the bright range of about 2 μm in average. Results noticed that a space between radioactive specimen and IP gives great effect to receive β energy on the surface of IP. In all cases of the space in thickness of 0, 1.3 and 2 μm the linear relationship between radioactive concentration and relative intensity was obtained and the inclination slop of the lines are paralled each other, the line of no space was about 100 times higher than that of the 2 μm thickness at the same radioactive concentration.

The relationship between ^{14}C-radioactivity on the TLC plate and TLC plate and relative intensity of (PSL-BG) was shown in Figure 8. Results noticed that a linear relationship between ^{14}C-radioactivity and PSI intensity was given under the condition that radioactivity was 20 to 2×10^4 pCi for 18 h. and 2×10^3 to 2×10^5 pCi for 0.25 h exposure. The self absorption of ^{14}C-β rays was reached over the thickness of about 100 μm as an infinite thickness.

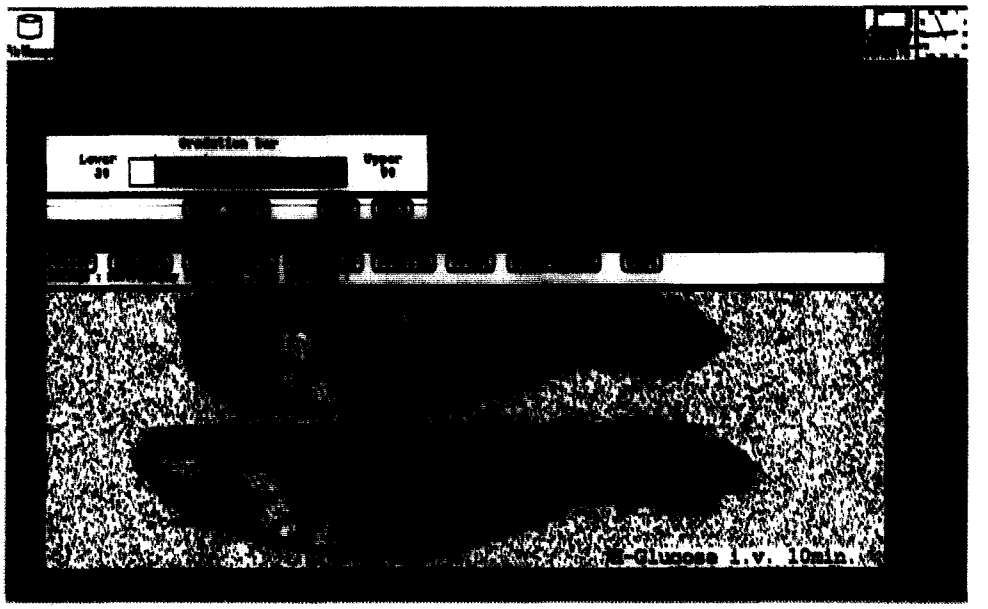

Figure 9. High quality macroautoradiograph of [5,6-^{3}H] glucose 10 min after intravenously injected in a rat.
Radioactive dose: 100 μCi, Exposure for 24 h. under the freezing condition (−25℃).

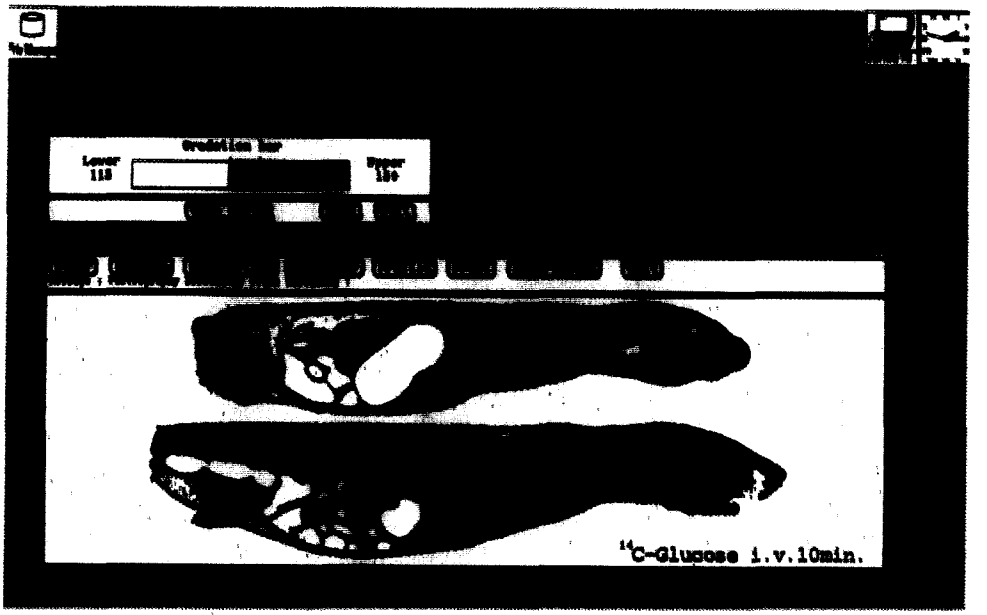

Figure 10. High quality macroautoradiograph of [U-^{14}C] glucose 10 min after intravenously injected in a rat. Radioactive dose: 10 μCi, Exposure for 24 h. after freeze drying preparation.

APPLICATION OF IP TO WHOLE BODY MACROAUTORADIOGRAPHY

The combination technique of IP and BAS2000 Bioimaging Analyzer gave one of the most excellent results in whole body macroautoradiography.

As well known, it has been very difficult to obtain any reliable image of ^{3}H autoradiographs by use of any commercial X-ray film. Figure 9 shows a high quality macroautoradiograph of [5,6-^{3}H] glucose 10 min after intravenously injected in a rat. As to the radioactive distribution, the brain uptake of [5,6-^{3}H] glucose, was the most of all tissues. A relatively high uptake label into the liver, kidney, pancreas and digestive mucous membrane were observed than other organs and tissues such as skeletal muscle, thymus, and lung. As shown in Figure 10, a similar autoradiograph was obtained in the case of [U-^{14}C] glucose 10 min after i.v. injected in a rat.

The strong pints of IP are as follows: 1) to shorter the exposure period because of the 100 times higher sensitivity of IP than that of X-ray film, 2) to give a high quality and quantitative autoradiogrpahic image of not only ^{14}C but also ^{3}H, if an equivalent section thickness has been given as a radioactive specimen such as TLC plate or PhastGel plate for polyacrylamido gel electrophoresis by Pharmacia Co.

CONCLUSION

It is the most honor for me to present one of our recent works to the annual meeting of Korean Society of Toxicology. In this paper, a nobel radiotracer technique is introduced and it become to be essential in not only pharmacokinetics but also toxicokinetics and their metabolic studies. The conclusion of this paper is summarized as follows:

1) Studies of drug metabolism, pharmacokinetics, and disposition become to require internationally for developing and marketing of new drugs.

2) Radiotracer techniques are essential for studying on either drug metabolism, pharmacokinetics and disposition, or whole body metabolism of endogenous substrates.

3) Definition of both terms; pharmacokinetics and whole body metabolism was proposed.

4) A new technique, "Radioluminography" by combination of a new sensor, "Imaging Plate" and a computed imaging analyzer is essential for quantitative and ultramicroscale analysis of the radioactive metabolites moving from second to second in a whole body.

5) A new visualizing sensor, "Imaging Plate" is the most sensitive, quantitative and reproducible among all of conventional sensors for receiving not only ^{14}C-but also ^{3}H-emitters in biological samples.

6) The X ray film will be still useful by means of its high resolution for relatively high radioactivity in samples.

7) An outline of basic and contract researches of "Institute of Whole Body Metabolism" was presented.

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