

# Quantitative Radioluminography of $^{125}\text{I}$ Iodine Whole-Body Autoradiograms

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**Radioluminography (RLG) was applied for the quantitation of  $^{125}\text{I}$  iodine ( $^{125}\text{I}$ ) autoradiograms using the bioimaging analyzer BAS 2000. RLG was performed on 50- $\mu\text{m}$  sections of sagittal whole-body sections of rats and  $^{125}\text{I}$ -radiolabeled erythrocyte calibration scales. Linearity, sensitivity, accuracy, and reproducibility were investigated in comparison to direct  $^{125}\text{I}$  radioactivity measurement. RLG is demonstrated to be a simple, reproducible, and precise analytical tool. However, the spatial resolution of quantitative  $^{125}\text{I}$  RLG is limited by the relatively high average free path of  $\gamma$ -radiation and X rays.** © 2000 Academic Press

## INTRODUCTION

Since its introduction by Ullberg (1954), whole-body autoradiography (WBA) has been widely used to investigate the distribution pattern of radiolabeled compounds and their metabolites in animal tissues and body fluids. WBA has been refined by densitometry of X ray- and  $^3\text{H}$  films in order to quantify tissue radiolabel concentrations (Zane *et al.*, 1997). In densitometry, darkening of tissues is compared with that of a calibration scale containing defined concentrations of the same radiolabel. In order to allow calculation of concentrations of radiolabeled compounds in tissues, the tissues and the calibration scale should absorb radiation to a similar extent. For autoradiography of  $^{14}\text{C}$ -radiolabeled compounds, blood was shown to be most suited for the preparation of calibration scales, because its self-absorption is not different from that of most other tissues, except for bone, eye, and fat (Schweitzer *et al.*, 1987). Film densitometry, however, has two major disadvantages: it needs time-consuming long film exposure periods of up to several months and it offers a low dynamic range of the film material used. Usually, several exposures of the same section are required in order to quantify organs containing high and low radiolabel concentrations.

The introduction of RLG associated with the bioimaging analyzer BAS 2000 promises a major improve-

ment in quantitative WBA. The new technology of phosphor imaging plates (IP) overcomes the disadvantages of film densitometry: IP have a wider linear dynamic range and are more sensitive to radioactivity than X-ray films (Miyahara, 1989) allowing considerably shorter exposure periods and reducing the need of multiple exposures. RLG was shown to be a useful tool in quantitative WBA of  $^{14}\text{C}$ -labeled substances (Tanaka, 1994; Motoji *et al.*, 1995). We investigated the applicability of RLG for quantitative WBA of  $^{125}\text{I}$ -labeled drugs such as iopromide or proteins. The  $^{125}\text{I}$ -labeled contrast agent iopromide, used for angiography and urography, was chosen as a model compound. Drug dehalogenation was investigated prior to the WBA, in order to avoid misinterpretation of the autoradiographic images due to the formation of free  $^{125}\text{I}$ , which would accumulate mainly in the thyroid gland.

## MATERIAL AND METHODS

### Radioactive Substance

$\text{Na}^{125}\text{I}$  was purchased from Amersham-Buchler (Braunschweig, Germany).  $^{125}\text{I}$ -labeled *N,N'*-bis(2,3-dihydroxypropyl)2,4,6-triiodo-5-(2-methoxyacetamido)-*N*-methyl-isophthalamide (CAS-No. 73334-07-3, iopromide) was synthesized at Schering AG. The test preparation contained 50 mg  $^{125}\text{I}$  (444 kBq) per milliliter, corresponding to a specific activity of 8.88 kBq/mg iodine.  $^{125}\text{I}$  emits  $\gamma$ -radiation with an energy of 35.5 keV and X-rays with energies between 27 and 31 keV.

### Preparation of a Calibration Scale

A calibration scale consisting of nine different concentrations of  $^{125}\text{I}$  radioactivity was prepared in the range of ca. 1.3–16136 Bq/ml by addition of defined amounts of [ $^{125}\text{I}$ ]iopromide to an erythrocyte concentrate obtained from the DRK blood donation service (Berlin, Germany). Three radioactivity concentrations (48, 462, and 4814 Bq/ml) were used as quality control samples.

For validation experiments, pores of about 1 cm di-

ameter were drilled into a frozen ( $-20^{\circ}\text{C}$ ) carboxymethyl cellulose block. Pores were filled with precooled  $^{125}\text{I}$ -radiolabeled erythrocyte calibration scales (four-fold) and quality controls (ninefold). Sections of  $50\ \mu\text{m}$  thickness were prepared using a Cryo-Polycut S (Reichert-Jung, Germany) cryomicrotome. Cutting was performed at about  $-30^{\circ}\text{C}$  and sections were taken up by adhesive tape (Scotch tape, type 800, 3M Corp., U.S.A.). Sections were dried within the cryostat for about 3 to 6 days and analyzed by RLG.

For the comparison of calibration scales generated from plasma and erythrocytes,  $\text{Na}^{125}\text{I}$  was used instead of  $^{125}\text{I}$ iodipromide. Seven concentrations between 0.02 and 17 kBq/ml were prepared and processed as described above.

#### Direct Radioactivity Measurement

$^{125}\text{I}$  radioactivity in calibration, quality control, and tissue samples was directly measured by means of a 1272 Clinigamma-counter (LKB-Wallac, Finland). Calibration scales were analyzed 10-fold. All samples were measured for 10 min each.

#### Whole-Body Autoradiography

For WBA, two groups of Wistar rats (strain Wistar-Han SPF, breeder Schering AG) received a single intravenous bolus injection of 250 mg (2.22 MBq)  $^{125}\text{I}$ iodipromide/kg into the tail vein. Group A consisted of four pregnant female animals (body weight 268–297 g, 18th day p.c.) and group B consisted of three male animals (body weight 130–166 g). At defined time points (group A, 15 min, 1 h, 4 h, and 1 day; and group B, 1 h, 3 days, and 7 days after administration) rats were sacrificed in deep diethylether anesthesia by shock-freezing in a mixture of hexane/dry ice for 3 min. Animals were stored at  $-20^{\circ}\text{C}$  until sectioning. WBA was performed according to a routine method (Curtis *et al.*, 1981). Briefly, animals were mounted in a carboxymethyl cellulose block and were frozen at  $-20^{\circ}\text{C}$ . Three different levels of quality controls were also embedded in triplicate. Whole-body sagittal sections of  $50\ \mu\text{m}$  thickness were prepared as described above and subjected to RLG.

#### Instrumentation

The bioimaging analyzer BAS 2000 (Fuji, Tokyo, Japan) was used for quantitative RLG employing an ultrasensitive BAS III imaging plate (Fuji), which accumulates and stores radioactive energy. The IP were exposed to WBA sections of rats and calibration sections at room temperature for 3–6 days in a specially designed shielding box (Raytest, Straubenhardt, Germany), in order to reduce the background signal due to ubiquitous cosmic radiation. After exposure, the IP is inserted into the image-reading unit BAS 2000 and is

scanned with a laser beam. Following excitation, the IP emits luminescence in proportion to the intensity of the stored radiation. The luminescence is amplified by a photomultiplier tube. This signal is processed further with an analysis software (Fuji) running on a computer system (SOLIDsparc station, Sparc computer, Menlo Park, CA). After completion of reading, the IP can be erased using the BAS 2000 IP eraser. More comprehensive descriptions of the principles of measurement have been published previously (Miyahara, 1989).

#### Evaluation

The results of the BAS 2000 system are expressed in photostimulated luminescence units corrected for background per area ((PSL-BG)/ $\text{mm}^2$ ). Areas of 50–60  $\text{mm}^2$  were evaluated in calibration scales and control samples. For evaluation of tissue samples, the area was adjusted to the organ size.

All results of RLG and direct radioactivity measurements ( $N(t)$ ) were recalculated to one reference date ( $N(t_0)$ ) according to Eq. (1), in order to account for the radiation half-life ( $t_{1/2}$ ) of  $^{125}\text{I}$  of 59.6 days.

$$N(t_0) = N(t) * e^{t \cdot \ln 2 / t_{1/2}} \quad (1)$$

In RLG, concentrations of calibration scales and quality controls were calculated for the beginning of the exposure period ( $t$ ). The date of preparation of calibration scales was used as reference date ( $t_0$ ).

Regression analysis is based on log-transformed (PSL-BG)/ $\text{mm}^2$  values and area doses of  $^{125}\text{I}$  radioactivity. The log transformation is needed to equalize variances thus avoiding predominant weighting of high values.

Log-transformed  $^{125}\text{I}$  tissue concentrations obtained by direct radioactivity measurement and RLG were also used to describe the average deviation between both methods.

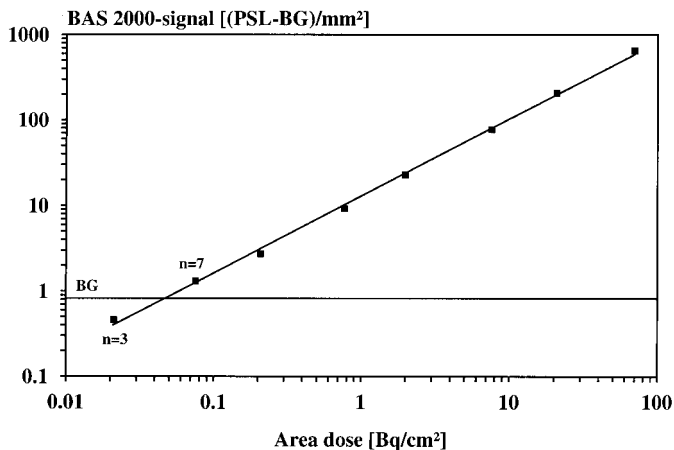
## RESULTS AND DISCUSSION

#### Linearity

Nine different sections each containing the same calibration scale in quadruplicate were analyzed by RLG. The background signal of the BAS 2000 system following 3 days of IP exposure was below 1 PSL/ $\text{mm}^2$ . There was a strong linear correlation between the log-transformed signal of the BAS 2000 system and the log-transformed area dose of  $^{125}\text{I}$  within the range of 0.02–70 Bq/ $\text{cm}^2$  (Fig. 1), which can be described by

$$y = -2.606 \pm 0.093 + 0.924 \pm 0.012x, \quad (2)$$

where  $y$  and  $x$  were expressed in  $1 + \log((\text{PSL-BG})/$



**FIG. 1.** Photostimulated luminescence signal in <sup>125</sup>I RLG as a function of the radioactive area dose. The mean value of nine sections is given. IPs were exposed for 3 days in a shielding box. The lowest concentrations were not detectable in all sections. (BG, background)

$$y = -2.606 \pm 0.093 + 0.924 \pm 0.012x; \quad r = 0.9995 \pm 0.0004.$$

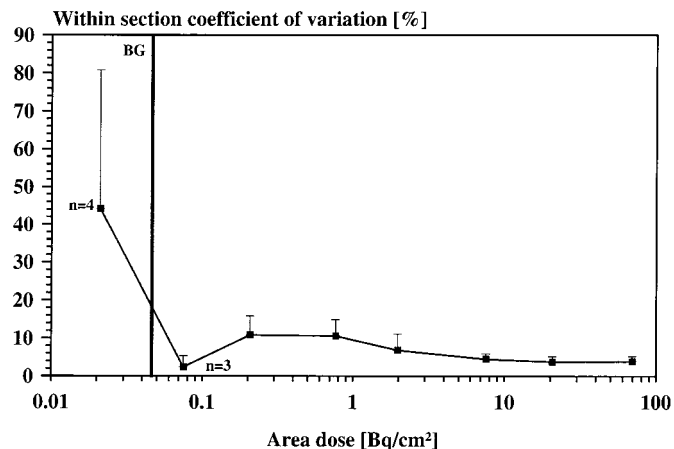
x and y values are expressed as  $1 + \log(\text{dpm/ml})$  and  $1 + \log((\text{PSL-BG})/\text{mm}^2)$ , respectively.

$\text{mm}^2$ ) and  $1 + \log(\text{dpm/ml})$ , respectively. The correlation coefficient was  $0.9995 \pm 0.0004$ .

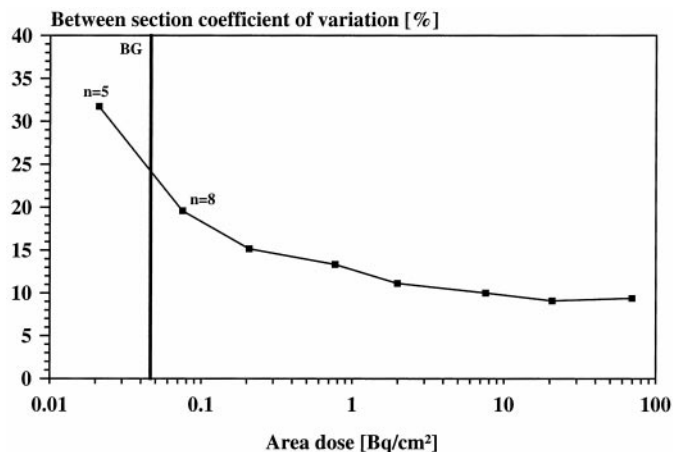
*Reproducibility*

Descriptive statistics (mean, SD, CV) were calculated for each individual concentration-dependent precision profile. The average precision profile of all sections as a function of the radioactive area dose is given in Fig. 2. The mean within section variance was  $11 \pm 5\%$  for  $0.2 \text{ Bq/cm}^2$  and decreased to average values of about  $5\%$  at area doses greater than  $7 \text{ Bq/cm}^2$ .

The between section coefficient of variation was also



**FIG. 2.** Within section coefficient of variation of <sup>125</sup>I RLG as a function of the radioactive area dose. Means + standard deviations of nine sections are given. The lowest concentrations were not detectable in all sections (BG, background).

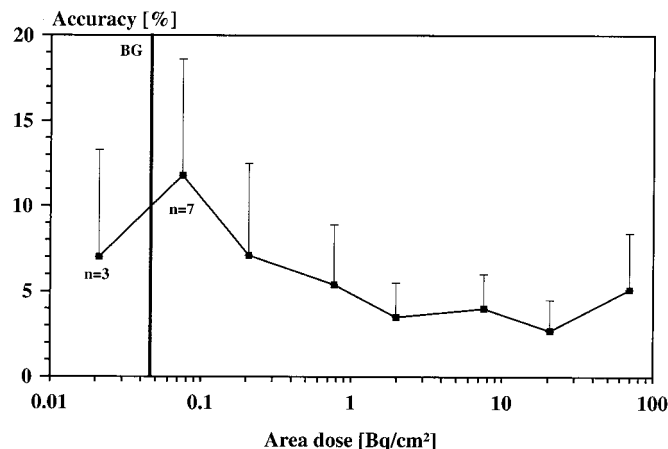


**FIG. 3.** Between section coefficient of variation of <sup>125</sup>I RLG as a function of the radioactive area dose. The lowest concentrations were not detectable in all sections (BG, background).

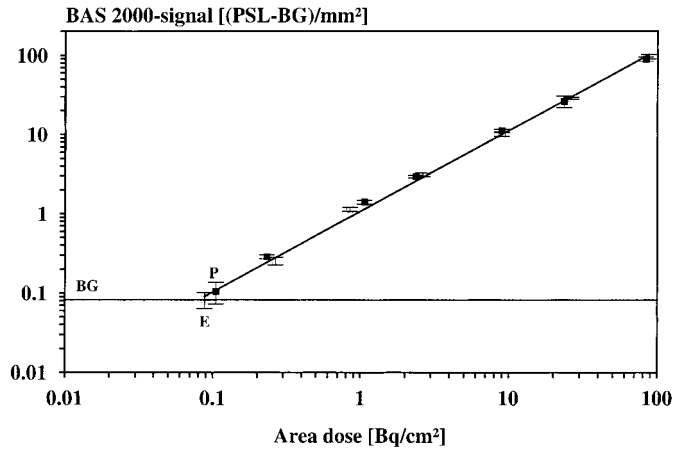
calculated from the same sections (Fig. 3). When the signal exceeded the background by at least twofold, the between section variance was below  $20\%$ . The between section variance decreased monotonously with increasing radioactive area dose and was below  $10\%$ , when the area dose was greater than  $7 \text{ Bq/cm}^2$ . For the total range investigated, the geometric mean variance of <sup>125</sup>I RLG was calculated to  $11.2\%$ .

*Accuracy*

The accuracy of the RLG was defined as the average section specific deviation of the BAS 2000 signal [(PSL-BG)/ $\text{mm}^2$ ] from the respective mean value of all sections. The accuracy profile as a function of the radioactive area dose is shown in Fig. 4. Exceeding the



**FIG. 4.** Accuracy of <sup>125</sup>I RLG as a function of the radioactive area dose. Mean values and standard deviations of nine sections are given. The lowest concentrations were not identifiable in all sections (BG, background).



**FIG. 5.** Photostimulated luminescence signal in  $^{125}\text{I}$  RLG as a function of the radioactive area dose: comparison of calibration scales generated from human plasma (P) and erythrocytes (E). Mean values  $\pm$  standard deviations of three sections are given, each containing the calibration scales in triplicate per matrix (BG, background).

$$\text{E: } y = 0.0025 + 1.0162x, \quad r = 0.9990$$

$$\text{P: } y = 0.1315 + 0.9759x, \quad r = 0.9979.$$

$x$  and  $y$  values are expressed as  $1 + \log(\text{Bq}/\text{cm}^2)$  and  $1 + \log((\text{PSL-BG})/\text{mm}^2)$ , respectively.

background signal by a factor of 3, the mean accuracy is in the range between 3 and 10%.

### Tissue Self-Absorption

Absorption of low-energy  $\gamma$ -radiation, such as emitted from  $^{125}\text{I}$ , occurs mainly via the photo effect and by the generation of secondary electrons. The absorption is dependent on the mass absorption coefficient of the radiated material.

In order to measure whether  $^{125}\text{I}$  is tissue specifically absorbed, 50- $\mu\text{m}$  sections of nonradioactivity exposed rats were placed between a homogeneously  $^{125}\text{I}$ -labeled polymer standard ( $17 \times 15 \times 0.5$  cm, 0.4 MBq) and the IP and exposed for up to 5 days. In contrast to experiments using  $^{14}\text{C}$  as a  $\beta$ -radiation source (Klein *et al.*, 2000), no tissue-specific self-absorption was observed.

In a second experiment, sections of calibration scales generated from human plasma and erythrocytes containing similar  $\text{Na } ^{125}\text{I}$  concentrations were compared by RLG. No relevant differences were observed between both tissues (Fig. 5), indicating that the extent of organ-specific blood supply and iron content should not impair quantitative RLG. Therefore, plasma or erythrocytes can be used for the preparation of control samples and calibration scales for quantitative  $^{125}\text{I}$  RLG.

### Spatial Resolution

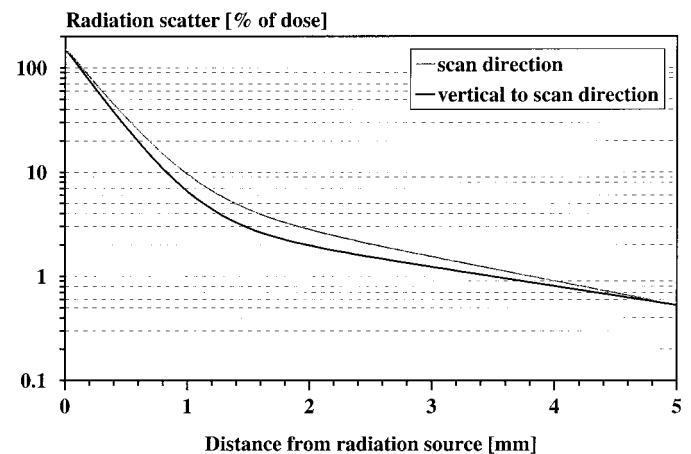
In RLG, spatial resolution of an autoradiographic image is influenced by two parameters: optical resolu-

tion by the equipment (pixel size) and radiation scatter. The minimum pixel size of the BAS 2000 image analyzing system is 0.1 mm.

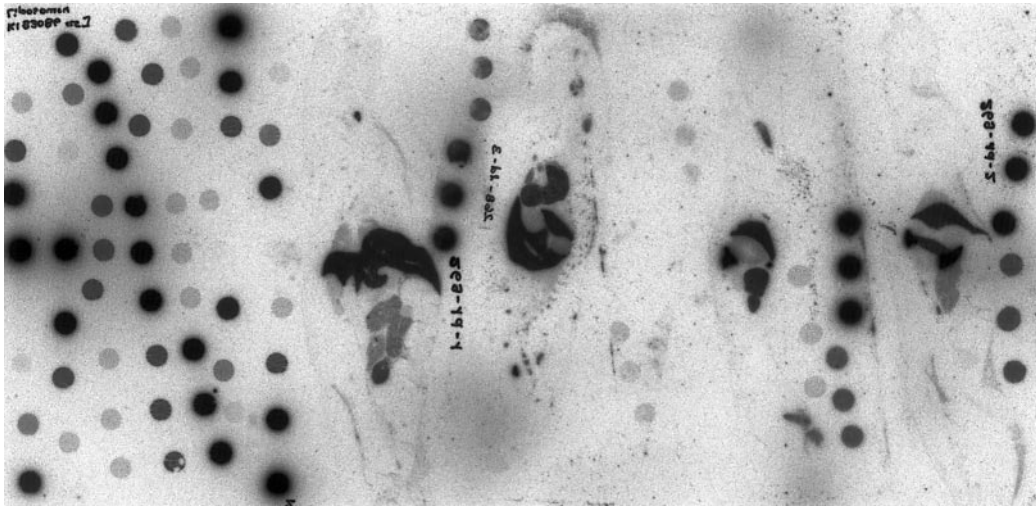
To quantitate the effect of the radiation scatter, the decrease of the photostimulated luminescence was evaluated as a function of increasing distance of a uniform circular radiation source, i.e., the highest  $^{125}\text{I}$  concentration of the erythrocyte calibration scale. Beginning at the edge of the radiation source, (PSL-BG)/ $\text{mm}^2$  values were measured in single pixels of 0.2 mm, both in scanning direction and perpendicular to the scanning direction.

Results were fitted (Fig. 6) by nonlinear regression based on a two-compartment model using the TOPFIT computer program (Boehringer Ingelheim Pharma KG, Biberach, Germany). There was no notable difference in the decay of the radiation scatter for both directions. The radiation scatter decreased biexponentially with the radial distance to the radiation source with half-values of ca. 0.2 and 1.5 mm. The greater value might be attributed to low energy photo radiation emitted by  $^{125}\text{I}$ , whereas the lower half-value might refer to secondary electrons produced by the absorption of photo-radiation in the radiated matrix. In comparison to  $\beta$ -radiation,  $\gamma$ -radiation has a longer average free path in any absorbing material.

At a distance of 1, 4, and 13 mm from the  $^{125}\text{I}$  radiation source, 10, 1, and 0.1%, respectively, of the radioactivity concentration were still detectable. In contrast to carbon-14 (Ahr and Steinke, 1994), in  $^{125}\text{I}$  RLG radiation scatter is the crucial limiting factor for the spatial resolution. If small animals were used for autoradiography, e.g., mice and rats, high radiolabel concentrations in specific body regions, e.g., liver and kidney or the content of stomach and intestine, will impair



**FIG. 6.** Photostimulated luminescence as a function of the increasing radial distance from a  $^{125}\text{I}$  radiation source. (The edge of the  $^{125}\text{I}$  source was set to 100%. Due to the nonlinear regression analysis, in the graph the first data point is somewhat higher than 100%. Five measurements per millimeter. Scan direction,  $r = 0.973$ ; vertical to scan direction,  $r = 0.975$ ).



**FIG. 7.** Quantitative evaluation of RLG. Rat sections containing erythrocyte standards with known radioactivity were exposed together with sections of erythrocyte calibration scales on one IP.

the quantitative analysis of surrounding tissues exhibiting low radiolabel concentration, e.g., gastric and intestinal mucosa. The radiation scatter is characteristic to the nature of the radiation emitted by <sup>125</sup>I and therefore limits not only RLG but also conventional film densitometry.

#### *Limit of Detection and Limit of Quantitation*

In RLG, background and tissue-induced signals were dependent on the exposure conditions such as the individual IP used, the exposure time, the temperature, and the use of specially designed shielding boxes. Therefore, the lower limit of detection (LLD) and the lower limit of quantitation (LLQ) can only be defined for one specific experimental setup. Furthermore the LLQ of a specific region of interest (ROI) is influenced by the radiation scatter originated from its surrounding.

With respect to the precision and accuracy profiles, the general LLQ in PSL/mm<sup>2</sup> should be defined as the threefold background value obtained under defined exposure conditions. If a specific ROI is surrounded by tissues containing a similar or higher radioactivity concentration, the radiation scatter originated from the surrounding of this specific ROI should be estimated according to Fig. 6. The authors recommend that if the radiation scatter contributes to more than 20% of the total signal, a site specific value ( $x$ ) for the LLQ should be defined for this tissue. Respective results cannot be quantified and should be expressed as below the LLQ of  $x$ .

#### *Comparison of Radioluminography with Direct Radioactivity Measurement*

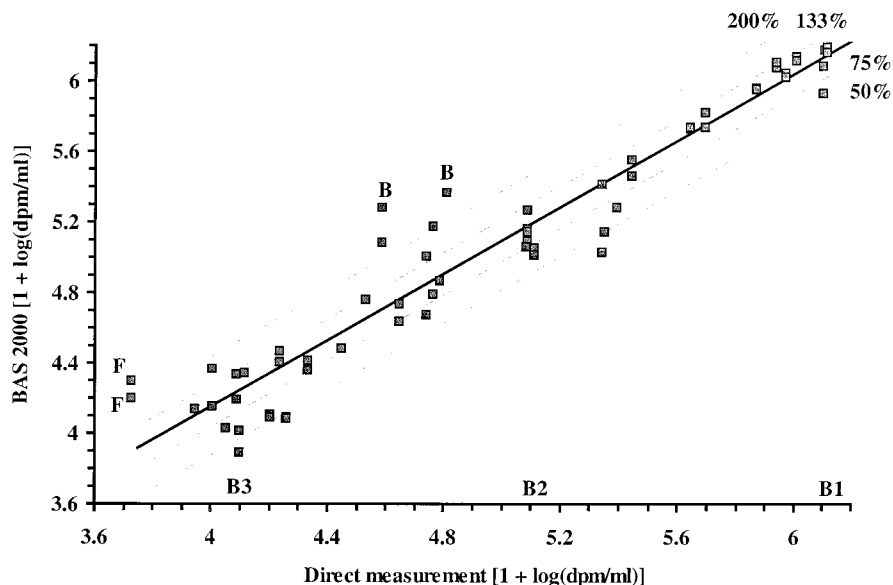
RLG and direct radioactivity measurement were compared in the same animal. One half of the rat was

sectioned for autoradiography. From the other half of the rat, tissue samples were obtained, weighed, and subjected to direct radioactivity measurement.

For quantitative evaluation of RLG, rat sections containing erythrocyte standards with known radioactivity were exposed on IP together with sections of erythrocyte calibration scales (Fig. 7).

Tissue concentrations obtained by RLG and direct radioactivity measurement (Fig. 8) are highly correlated ( $r = 0.966$ ). The average ratio of the methods, i.e., the tissue concentration according to RLG over the tissue concentration determined by direct measurement, was estimated to 1.37, resulting in a mean difference between both methods of 37%. About 7% of this average difference could be attributed to the method of direct radioactivity counting and the procedure of tissue sampling from the frozen animals. The remaining 30% of the difference seems to be due to the total experimental procedure of sectioning and quantitative RLG.

In the experiment, the greatest differences of a factor of approximately 2–3 were observed for bone/bone marrow and fetuses. For these specific tissues however, results were flawed for two reasons. Only small pieces of bone/bone marrow could be obtained for direct measurement (<20 mg), resulting in incorrect numbers due to the inhomogenous nature of bone tissue. In the fetus, a high radioactivity was detected by RLG, but almost no radioactivity was detected by direct measurement. A more detailed analysis of the radioluminographic image showed that the luminescence detected in the fetus was completely explained by the radiation scatter originated in the surrounding organs, the liver and the spleen. Thus, respective results for bone/bone marrow and fetuses are not valid and should have been excluded from the method comparison.



**FIG. 8.** Comparison of tissue concentrations of radiolabeled substances in the same animal, obtained by RLG and direct radioactivity measurement. F, fetus; B, bone/bone marrow; B1-3, quality control erythrocyte samples.

$$y = 0.387 + 0.942x; \quad r = 0.966.$$

## CONCLUSION

Radioluminography is a new reproducible and accurate method for quantitative organ distribution studies of  $^{125}\text{I}$ -radiolabeled compounds.

In contrast to  $^{14}\text{C}$ , spatial resolution is the limiting factor in quantitative  $^{125}\text{I}$  RLG. The spatial resolution is determined by the radiation scatter originating in tissues containing a very high concentration of  $^{125}\text{I}$ , making quantitation of radioactivity in the surrounding tissues which contain a considerably lower concentration of  $^{125}\text{I}$  impossible. The radiation scatter is characteristic to the nature of the radiation emitted by  $^{125}\text{I}$  and therefore limits not only RLG but also conventional film densitometric analysis of autoradiograms.

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