

Quantitative Distribution Studies in Animals: Cross-Validation of Radioluminography versus Liquid-Scintillation Measurement

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Received February 10, 2000

The results of a cross-validation of the radioluminography (RLG) and liquid scintillation counting (LSC) methods are presented. The methods for the determination of radioactivity concentrations were compared in 16 organs, after administration of ¹⁴C-labeled substances to rats. LSC measurements of two kinds were used as reference methods for RLG: (1) quantitative determination of radioactivity after conventional dissection (interindividual comparison) and (2) quantitative determination of radioactivity in tissue punches taken from the whole-body sections after they had undergone RLG measurement (intraindividual comparison). Blood standards containing known concentrations were used for calibration. For statistical evaluation log-linear regression analysis of paired concentration values and organ-specific 95% confidence intervals of the log-transformed RLG/LSC concentration quotients were compared. For most organs, the slopes of the regression lines and the means of the concentration quotients were within the defined equivalence range of 0.80–1.25. Deviations were distinctly smaller in the intraindividual comparison. For some organs, however, it became clear that found concentrations were affected by self-absorption (RLG) and by differences in sample preparation (LSC). In conclusion, quantification with RLG is a reliable and reproducible method with comparable measurement precision and greater accuracy in respect of tissue localization, compared to LSC (dissection). © 2000

Academic Press

INTRODUCTION

Distribution studies with radiolabeled test substances in animals are an important part of preclinical drug development. The results of these studies form

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the principal basis for the assessment of exposure and the elimination of residues in human organs and tissues, in which direct determination is of course impossible; direct determinations of exposure in man are generally limited to measurements in blood or plasma, which is readily accessible. Distribution patterns are determined in animals, usually rats, instead. Radiolabeled test substance is administered—for reasons connected with the measurement technique, but also, as far as possible, to ensure determination of the whole of the parent compound and its metabolites. The results provide pharmacokinetic data on the distribution of the total radioactivity, information on any accumulation or specific affinities of the test substance or its metabolites, and evidence for interpretations regarding potential toxicological and pharmacological target organs. For these and other reasons, distribution studies in animals will continue to play a fundamental role in the assessment of the safety of pharmaceutical active ingredients in man.

The traditional routine methods used for distribution studies are whole-body autoradiography with detection of the radioactivity in whole-body sections on X-ray film (Ullberg, 1954) and liquid scintillation measurement (LSC) in homogenized organs and tissues after dissection. The results of the two methods complement one another. Whole-body autoradiography is a qualitative detection method with a very high local resolution which includes all organs and many small substructures, whereas measurement after dissection yields quantitative concentration data for a limited, preselected number of organs.

Radioluminography (RLG) is a new method of radiation detection based on the phosphorus imaging technique, whose scientific and technical basis has been described by Sonoda *et al.* (1983), Miyahara (1989), and Hamaoka (1990), among others. RLG is now employed successfully and extensively as a detection method in whole-body autoradiography, offering the particular



benefits of being much more sensitive than the X-ray film technique and having a much wider linear measurement range. Because of the latter property in particular, RLG allows one to quantify organ concentrations in whole-body sections.

The present study is part of an extensive, intercompany validation program aiming to pave the way for combining the conventional qualitative and quantitative methods, i.e., film autoradiography and liquid scintillation after dissection, into a single RLG procedure which will supersede them. A European working group on the validation of RLG as a quantitative autoradiographic technique has been set up by the users for reasons connected with the division of work, method harmonization, the feasibility of cross-validations, and the exchange of information.

The results presented here focus specifically on cross-validation of RLG versus LSC. The comparison is based on data from six pharmaceutical companies. LSC is used as the reference method. Intraindividual measurements (same animal, same sample) and interindividual measurements (different animals, same sample) are compared. On the basis of these data the reliability, accuracy, and reproducibility of RLG are discussed, together with its merits and limitations.

METHODS

Since the results of different measurement techniques are to be compared, a detailed description of the methods must be provided. A precise knowledge of methodological differences, particularly in relation to the preparation and handling of samples, will help one to understand differences in results and to identify the limitations of the individual methods. LSC measurements of two different kinds were used as reference measurements for RLG:

1. Quantitative determination of radioactivity after conventional dissection (interindividual comparison) and
2. Quantitative determination of radioactivity in tissue punches taken from the whole-body sections already used for RLG measurement (intraindividual comparison).

This evaluation is based on comparative measurements from six autoradiography laboratories. The methods were compared in 16 organs/tissues, i.e., on the basis of 517 interindividual comparisons and 192 intraindividual comparisons. RLG values are based on one to five individual measurements, depending on the number of sections per animal and organ size. Pairs of individual values were obtained under the same experimental conditions, i.e., with identical test substances, administration routes, and doses and in animals of the same strain and sex. Small, company-specific differ-

ences in sampling and measurement were unavoidable.

Isotope

Only ^{14}C -labeled substances were used.

Animal Experiment

The radiolabeled test substances were administered to male and female albino rats or pigmented rats by the oral or intravenous route. The doses of radioactivity ranged from 4 to 70 MBq/kg body wt. The rats were sacrificed between 5 min and 7 days after administration.

Preparation of Whole-Body Sections

Defined routine methods were used (Ullberg, 1954; Curtis *et al.*, 1981). Whole-body frozen sections were prepared with a cryomicrotome and freeze-dried. The following parameters were used, depending on the test laboratory: cutting temperature -20 to -25°C , section thickness 25–75 μm , freeze-drying time 1–3 days. Three to six sections per animal were measured with RLG.

Radioluminography

The method of measurement is based on that described by Hamaoka (1990). The BAS 2000 or BAS 1500 system (BAS-III imaging plates, laser reader, detector) (all Fuji Photo Film Ltd., Tokyo, Japan) was used. The imaging plates (IP) were exposed to the dry whole-body sections for 2 h to 7 days, either in a refrigerator at 4°C or in a lead shielding box at room temperature. For calibration, the frozen sections were measured together with blood calibration series containing known concentrations of radioactivity (see below). The IP were then read immediately with the above-mentioned laser systems and the result was recorded as photostimulated luminescence (PSL). Image Analysis (Fuji Photo Film Ltd.) or TINA (Raytest, Straubenhardt, Germany) software was used for the determination and evaluation. The calibration lines were fitted and the radioactivity concentrations (Bq/ml) were calculated with TINA software or the Excel or Lotus 1-2-3 tabular calculation programs.

Internal and External Calibration Standards for RLG

Radioactive (^{14}C) blood standards for calibration accompanied each measurement in order to exclude the influence of differing measurement conditions, such as duration of exposure, temperature, shielding, and laser scanning (Maas *et al.*, 2000). Blood was chosen as a matrix because it shows average self-absorption of ^{14}C -radioactivity in relation to other organs (Klein *et al.*, 2000). The blood standards were made by preparing

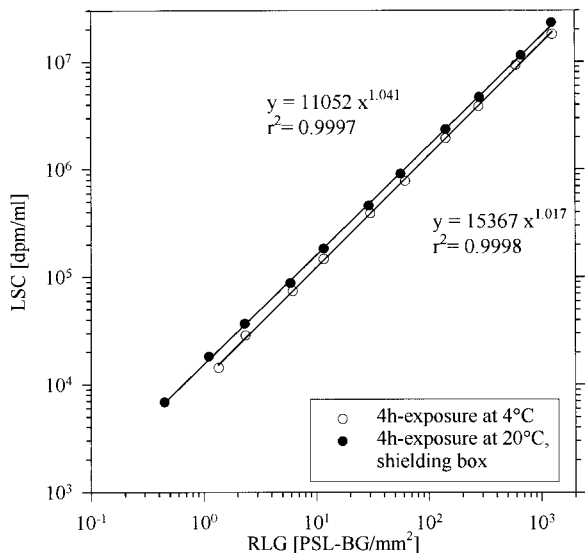


FIG. 1. Examples of RLG calibration curves as obtained by blood standards spiked with ¹⁴C. The same blood standard scale was exposed to imaging plates for 4 h, either at 4°C in a refrigerator (open circles) or at room temperature in a lead shielding box. Both methods were used for calibration. Linear regression was performed after log transformation (PSL, photostimulated luminescence; BG, background).

dilution series with a known content of radioactivity (0.2 to 370 KBq/ml) from dog or rat blood. Figure 1 shows typical calibration lines obtained under different measurement conditions. A distinction is made between external and internal standard samples, according to the mode of preparation. For internal calibration series, the blood samples were introduced into holes bored into the deep-frozen CMC block containing the embedded animal and were cut together with the whole-body section. External standards were prepared separately from the whole-body sections, but under identical conditions as separate frozen sections. The external standards have the advantage of being reusable. Since there were no significant differences of the sort that can arise through variations in section thickness for example (Fig. 2), both types were usually used for calibration—in the form of a mixed calibration curve. Five to 16 calibration points were used to calculate the calibration line. The equation for the optimal line was calculated either by linear regression after log transformation of the calibration values or by linear regression and constraining the line to pass through the origin. The concentrations were related to (wet) tissue volume that was derived from the measured area and the section thickness.

LSC of Tissue Punches

After selected whole-body sections had been measured by RLG, the organ regions used for quantitation were precisely marked out on the sections and then

removed (together with the supporting tape) with the aid of circular punches. After the punch samples had been combusted, LSC was performed to measure the radioactivity content (referred to the punch volume of wet tissue).

LSC after Dissection

The organ samples were collected by dissection of the exsanguinated animals. The organ samples were homogenized and freeze-dried. The radioactivity concentration was then measured with LSC after combustion or treatment with tissue solubilizers. The radioactivity content of solid tissue samples was referred to the tissue weight.

Statistical Analysis

The results of the two methods were compared using two different statistical methods. The concentrations measured with LSC were used as a reference in both cases:

1. Linear regression analysis was performed after logarithmic transformation of the concentration data ($y =$ concentration (RLG) and $x =$ concentration (LSC)), using the model:

$$\log y = c \cdot \log x + \log b$$

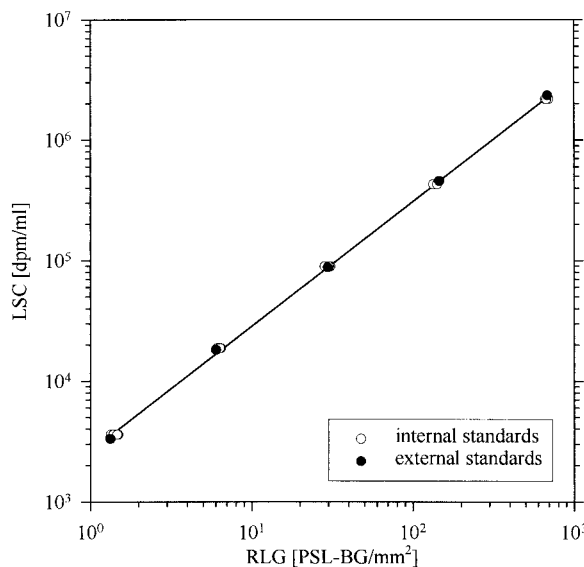


FIG. 2. Comparison of external and internal blood standard curves. The internal blood scales were embedded with the rat and cut together with the whole-body sections. The external blood scales were prepared separately, but under the same conditions as the whole-body sections, and exposed to the imaging plates together with the sections. Both methods were used for calibration. Regression was performed after log transformation.

or

$$y = b \cdot x^c,$$

respectively.

Assuming that the two methods (RLG and LSC) lead to identical concentrations yields $c = 1$ and $b = 1$ ($\log b = 0$). In order to check this assumption, 95% confidence intervals were calculated for the y -axis intercept and the slope.

2. Organ/tissue specific 95% confidence intervals for the logarithmic transformed RLG/LSC concentration quotients were calculated according to

$$[\mu \pm \text{SEM} \cdot t_{n-1,0.025}],$$

where SEM refers to the standard error of the mean μ and $t_{n-1,0.025}$ is the 2.5% quantile of Student's t distribution with $n - 1$ degrees of freedom. The borderline values were retransformed to the original scale. Equivalence of both methods was assumed when the resulting confidence interval was completely embedded in the interval (0.8, 1.25) (80% up to 125%). Furthermore, the RLG/LSC ratios were analyzed regarding laboratory and organ/tissue differences as well as for interactions using analysis of variance (ANOVA). As a result, all effects showed to be statistically significant ($P < 0.01$), concerning the dissection method.

RESULTS

Regression Analysis

The individual values for the comparison of the RLG and LSC methods are presented in Figs. 3.1 to 3.15. A distinction is made between interindividual (LSC after dissection) and intraindividual (LSC in tissue punches) concentration data. The ideal line with slope 1 (diagonal) is plotted in each case for comparison and to visualize the correlation. The pairs of values are scattered closely about the diagonal for virtually all organs, this being particularly evident for blood, liver, muscle, salivary glands, adrenals, spleen, thymus, and testes. It is striking that in the intraindividual comparison of the methods the deviations from the diagonal are in general particularly small. In the interindividual comparison of RLG versus LSC (dissection), on the other hand, the scatter of individual values is relatively large, especially for the kidneys and brain (Figs. 3.3 and 3.7). For the lungs, white adipose tissue, and skin, the concentrations measured with RLG tended to be lower than those measured with LSC; this was particularly evident for the lungs and adipose tissue in the interindividual comparison (Figs. 3.4 and 3.14). For the thyroid and bone marrow the number of individual values is too small to deduce any trend (Fig. 3.12).

The results of the regression analysis are shown in

Tables 1 and 2. The high R^2 statistics confirm the linear correlation between the results of the two methods. For the interindividual comparison (dissection) they range from 0.905 for adipose tissue to 0.982 for skeletal muscle, lower R^2 statistics being found only for the brain and thyroid (0.84 and 0.86, respectively) (Table 1). The intraindividual comparison gave distinctly higher R^2 statistics (Table 2), ranging from 0.949 for adipose tissue to 0.995 for the liver and salivary glands. The two methods show a high linear correlation, even for brain concentrations.

On the basis of the 95% confidence intervals the y -axis intercepts ($\log b$) and the preexponential factors b do not differ from zero and 1, respectively, for the majority of organs (Tables 1 and 2). Only the thyroid in the interindividual comparison and the lungs and brain in the intraindividual comparison do not meet this criterion.

The slope c of the log-linear regression line is a measure of the proportionality of the results obtained with the two methods (Tables 1 and 2). Values which do not differ significantly from 1 indicate the equivalence of both methods, provided that the regression line passes through the origin. Except in the case of the thyroid, values of between 0.905 (brain) and 1.224 (spleen) were obtained for the slope in the interindividual comparison of concentrations. In the intraindividual comparison the range of slope values was distinctly smaller: 0.921 (spleen) to 1.113 (adrenals). On the basis of the 95% confidence intervals the log-linear slope of the regression line in the intraindividual comparison does not differ significantly from 1 in the blood, liver, kidneys, skeletal muscle, salivary glands, adrenals, spleen, testes, skin, and white adipose tissue; in the interindividual comparison, one can add the heart and possibly the bone marrow and brain (Tables 1 and 2). Defining an acceptable range for the slope by 0.8–1.25, for all the investigated organs except the thyroid the observed slopes fall within this range (Fig. 4).

Equivalence Analysis

The mean values of the concentration quotients (RLG/LSC) were compared separately for interindividual and intraindividual data (Tables 3 and 4). The results of the regression analysis were largely confirmed. In the comparison of RLG and LSC the degree of scatter was relatively high for dissection, but small for punches. For the majority of organs the optimal value of 100% was covered by the corresponding 95% confidence intervals in at least one of the comparisons (Tables 3 and 4, Fig. 5). In the intraindividual comparison all the investigated concentration quotients are within the predefined equivalence range of 80–125%; the exceptions are skin and white adipose tissue, in which, on average 78 and 91%, respectively, of the reference concentration were found with RLG. In the

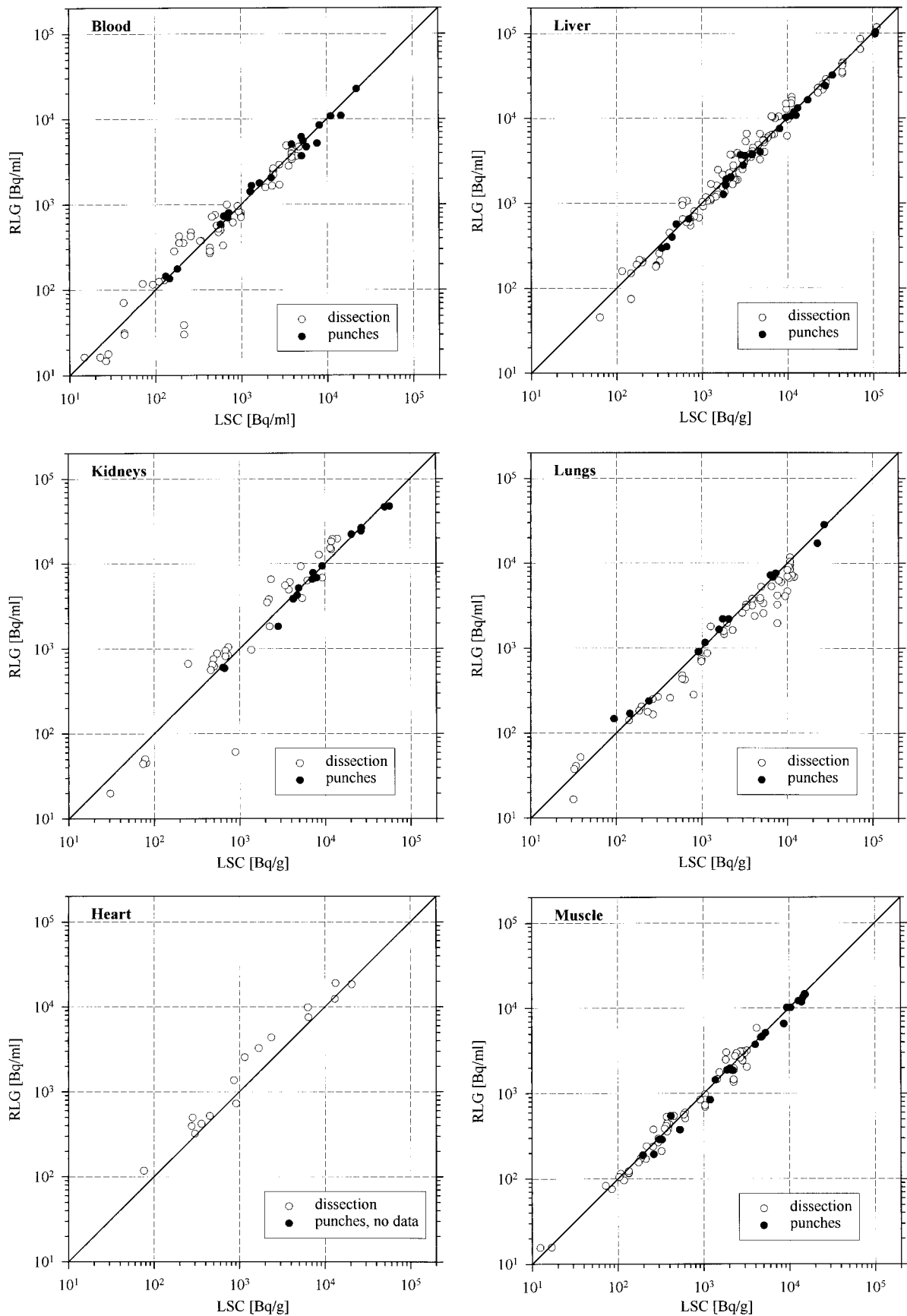


FIG. 3.1-3.15. Comparison of radioactivity concentrations in Bq/ml or Bq/g as determined by RLG and LSC in 16 organs and tissues of rats. The given diagonal line reflects the optimum relation. LSC data were obtained either after dissection of identically treated rats (open circles, interindividual comparison) or in tissue punches of the whole-body sections which previously were subjected to RLG determination (closed circles, intraindividual comparison).

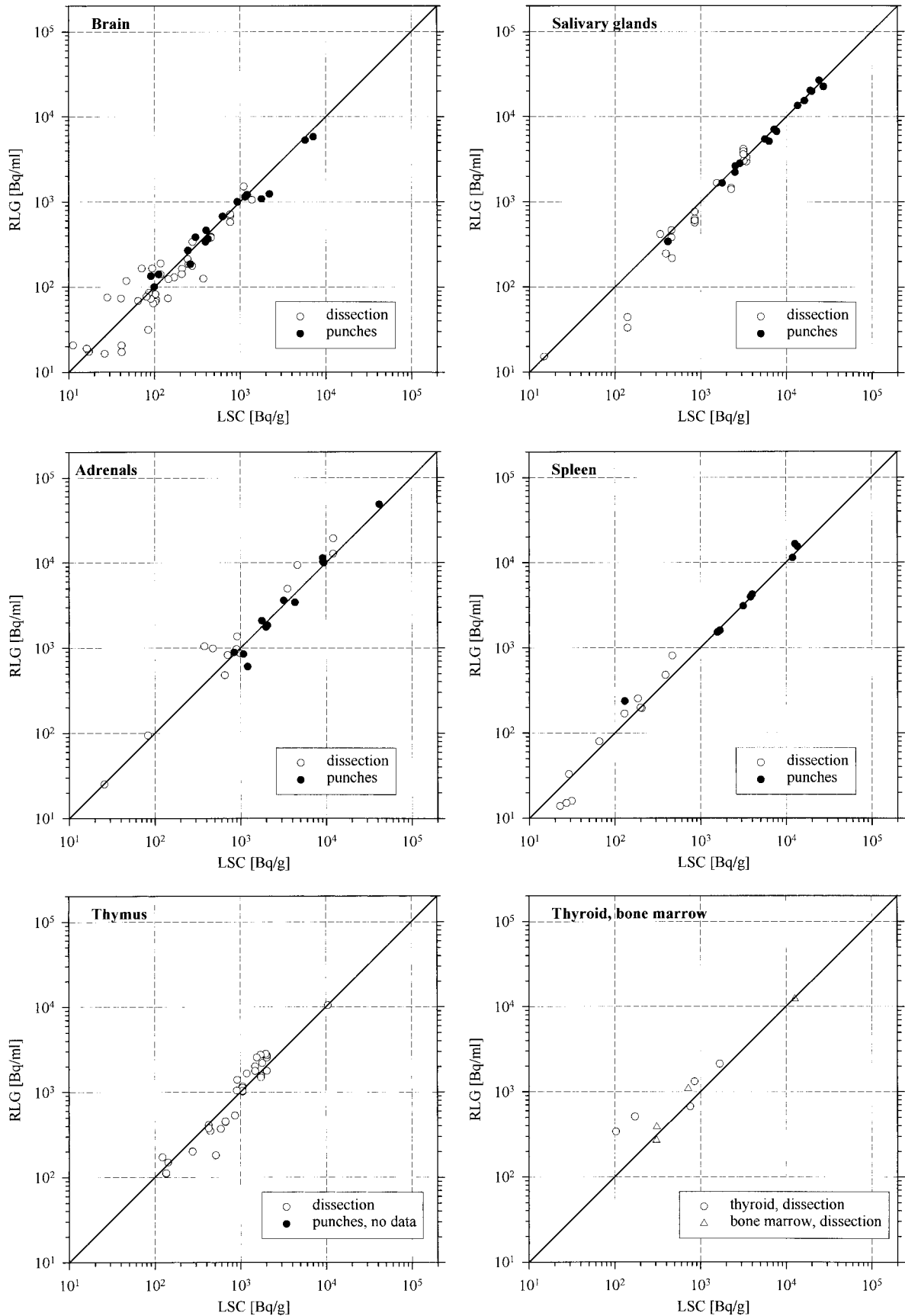


FIG. 3.1-3.15—Continued

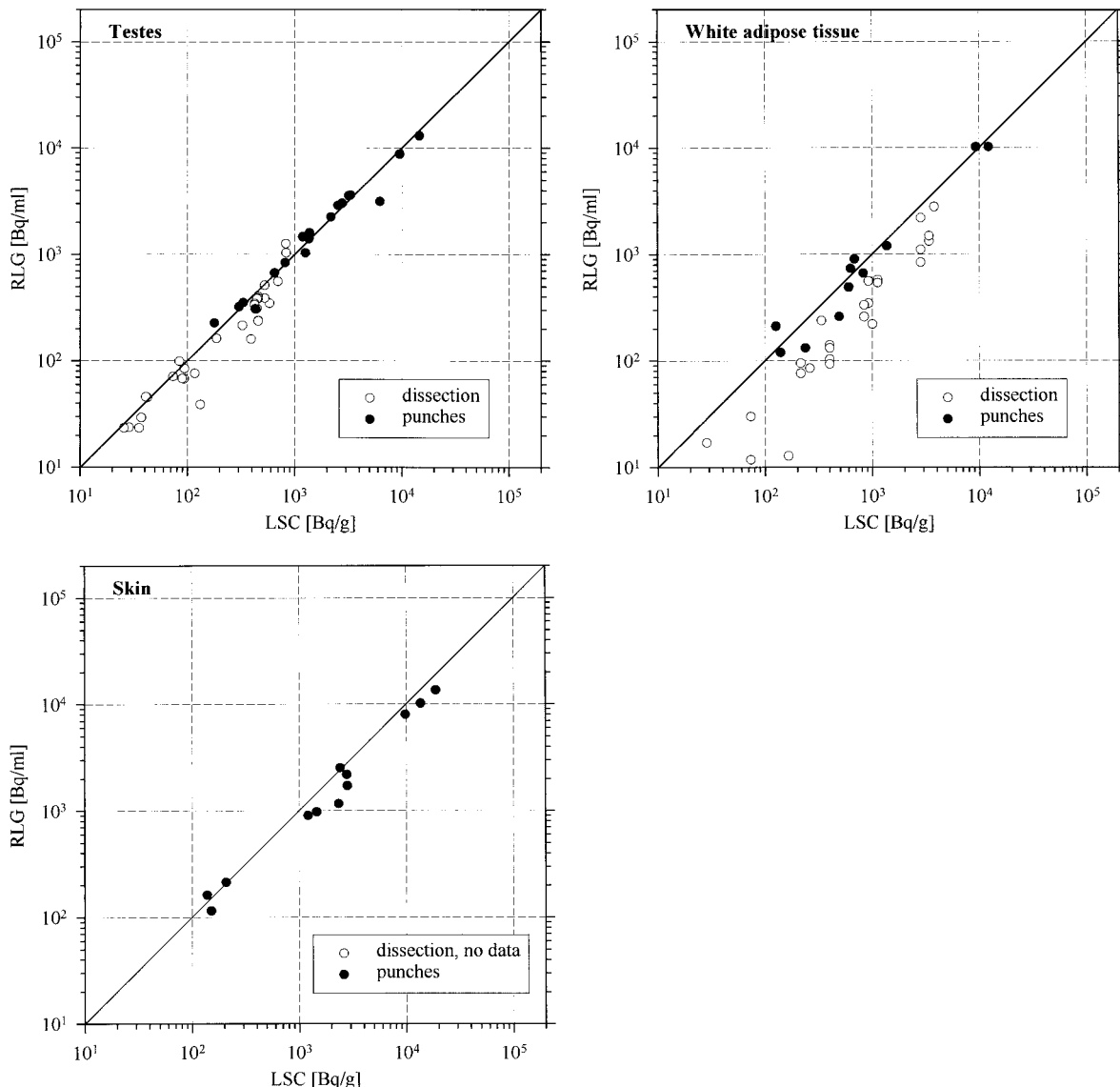


FIG. 3.1-3.15—Continued

interindividual comparison the equivalence criterion is achieved only for some organs, i.e., blood, liver, muscle, and thymus (Fig. 5). In adipose tissue the concentration measured with RLG was on average only 37% of that measured with LSC (dissection); the corresponding intraindividual comparison did not confirm this finding, however.

DISCUSSION

In most of the comparisons there is excellent agreement between the two methods of quantification. First, however, the possible reasons for a number of deviant and inconsistent findings will be discussed. Examination of the two reference procedures for radiolumino-graphic determination shows much greater agreement

in the intraindividual comparison than in the interindividual comparison. In the latter the differences in sample preparation played a major role (i.e., animals were exsanguinated and the dissected organs and tissues were homogenized), as did the biological variation as a result of having different animals. Given a highly heterogeneous distribution of radioactivity within organs—as is often observed in the kidneys, adrenals, and brain—an average concentration will be the result of homogenization. Depending on the blood content and the blood concentration, this too will distort the organ concentrations. Although quantification by RLG was in some cases also done regardless of heterogeneous distribution patterns in the organs, the sample areas used in RLG and the punches were practically identical, so that this could not have influenced the intraindividual

TABLE 1
Linear Regression Analysis of the Log-Transformed Paired Concentration Values
RLG versus LSC (Dissection)

Organ/tissue	N	Slope <i>c</i>	Log-intercept log <i>b</i>	<i>R</i> ²	95% confidence boundaries			
					Slope <i>c</i>		Log <i>b</i>	
					Lower	Upper	Lower	Upper
Blood	56	0.998	-0.025	0.911	0.913	1.083	-0.261	0.211
Liver	109	1.027	-0.092	0.976	0.996	1.058	-0.202	0.017
Kidneys	35	1.131	-0.378	0.917	1.013	1.250	-0.760	0.002
Lungs	53	0.938	0.080	0.966	0.889	0.987	-0.085	0.246
Myocardium	16	0.961	0.253	0.969	0.869	1.053	-0.043	0.548
Skeletal muscle	60	1.002	-0.027	0.982	0.966	1.038	-0.127	0.073
Brain	40	0.905	0.154	0.836	0.775	1.035	-0.125	0.432
Salivary gland	22	1.135	-0.496	0.938	1.005	1.265	-0.886	-0.106
Adrenal gland	13	1.035	0.017	0.960	0.908	1.162	-0.370	0.404
Spleen	12	1.224	-0.442	0.972	1.093	1.356	-0.702	-0.183
Thymus	36	1.115	-0.349	0.927	1.008	1.223	-0.664	-0.034
Thyroid	5	0.582	1.356	0.860	0.310	0.854	0.623	2.088
Bone marrow	4	0.969	0.145	0.981	0.778	1.159	-0.439	0.728
Testes	31	0.978	-0.059	0.921	0.871	1.084	-0.310	0.191
White adipose tissue	25	1.117	-0.759	0.905	0.966	1.267	-1.185	-0.333

Note. Interindividual comparison.

comparison. This means that in the validation of measurement techniques intraindividual comparison should be given greater importance than comparison based on concentrations from traditional dissection studies.

Although the sample volumes were sometimes extremely small, even small amounts of radioactivity were reliably determined with RLG. Nevertheless, the large deviations from the theoretical values in the case of the thyroid may be due not only to the small number of measurements (five) but also to the small size of the

areas measured. The relationship between the area measured and the lower limit of quantitation and measurement accuracy has been shown by Kolbe *et al.* (2000). Binder and Archimbaud (2000) recommended that the minimum area should not be smaller than 3.5 mm². This would of course exclude very small tissue structures from determination. In the case of the present results, only the single background measure was used as the limit of quantitation in order to be able to follow the linearity of the measurement to the detection limit, i.e., to around 10¹ Bq/ml. In some in-

TABLE 2
Linear Regression Analysis of the Log-Transformed Paired Concentration Values RLG versus LSC (Punches)

Organ/tissue	N	Slope <i>c</i>	Log-intercept log <i>b</i>	<i>R</i> ²	95% confidence boundaries			
					Slope <i>c</i>		Log <i>b</i>	
					Lower	Upper	Lower	Upper
Blood	21	0.963	0.118	0.987	0.913	1.014	-0.052	0.289
Liver	25	1.005	-0.044	0.995	0.974	1.036	-0.160	0.071
Kidneys	15	1.016	-0.100	0.992	0.965	1.068	-0.302	0.102
Lungs	13	0.945	0.213	0.994	0.902	0.988	0.069	0.357
Skeletal muscle	24	1.008	-0.064	0.991	0.968	1.048	-0.203	0.076
Brain	17	0.891	0.284	0.967	0.806	0.975	0.044	0.524
Salivary gland	15	1.029	-0.135	0.995	0.988	1.070	-0.293	0.023
Adrenal gland	12	1.113	-0.420	0.971	0.992	1.234	-0.855	0.014
Spleen	9	0.921	0.321	0.983	0.829	1.012	-0.005	0.642
Testes	18	0.941	0.183	0.968	0.855	1.027	-0.094	0.460
White adipose tissues	11	0.996	-0.032	0.949	0.842	1.150	-0.488	0.423
Skin	12	0.939	0.089	0.981	0.857	1.021	-0.183	0.361

Note. Intraindividual comparison.

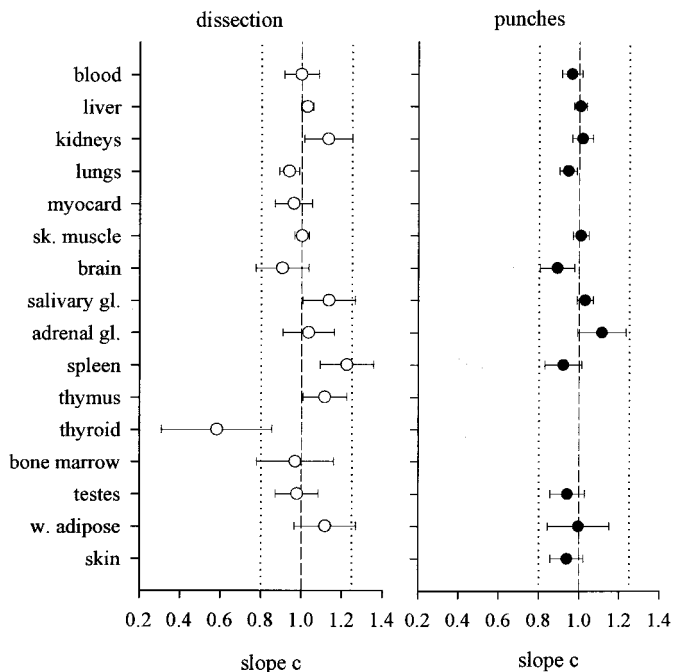


FIG. 4. Slopes and corresponding 95% confidence boundaries based on linear regression analysis of the log-transformed paired concentration values (RLG versus LSC). Interindividual comparison (left, open circles) and intraindividual comparison (right, closed circles). The dotted lines mark the predefined equivalence region from 0.80 to 1.25.

stances (e.g., kidneys, brain) this had an adverse effect on method comparison.

The higher deviations in the interindividual comparison of the methods may also be due to the equation of

weight with volume, i.e., to disregarding the specific weight of the tissues. Thus radioactivity concentrations in solid tissues are usually determined in relation to weight in LSC (dissection), whereas in RLG and LSC (punches) they are determined in relation to volume, as section area or section volume is measured.

Transmission experiments have shown that most organs and tissues exhibit broadly blood-equivalent self-absorption of radioactivity (Klein *et al.*, 2000). The RLG concentration data were therefore calculated with radioactive blood standards. However, in the organs and tissues where self-absorption differed significantly from that of blood—such as adipose tissue, skin, and lung tissue—the deviations from the reference measurements may in part be due to this fact. Thus, adipose tissue and skin, which contains a high proportion of adipose tissue, showed relatively high quench effects in the transmission experiments performed by Klein *et al.* (2000). This undoubtedly led to underestimation of the RLG concentrations in tissues that contain relevant amounts of fat.

The accuracy and precision of quantitative RLG determination can thus be further improved by greater standardization of sample preparation, sample treatment, and measurement; by taking more precisely self-absorption effects into account in calibration; by avoiding measurement of heterogeneous distribution patterns in organs; by taking account of very small areas of measurement and very low radioactivity concentrations when establishing limits of quantitation, etc.

Comparable results have been obtained by other authors, which support the presented results and find-

TABLE 3
Sample Statistics for Concentration Ratios RLG/LSC (Dissection)

Organ/tissue	N	Mean (%)	Min (%)	Median (%)	Max. (%)	95% confidence boundaries	
						Lower (%)	Upper (%)
Blood	56	93	14	96	224	82	107
Liver	109	100	51	99	196	95	106
Kidneys	35	107	7	131	280	85	135
Lungs	53	75	26	79	142	68	83
Myocardium	16	135	79	143	222	115	159
Skel. muscle	60	95	60	94	164	89	101
Brain	40	90	34	84	269	77	106
Salivary gland	22	80	23	89	133	65	97
Adrenal gland	13	132	73	117	276	105	166
Spleen	12	96	51	106	173	75	124
Thymus	36	97	35	97	164	86	109
Thyroid	5	176	88	155	329	87	354
Bone marrow	4	113	88	110	151	76	166
Testes	31	78	30	80	152	69	88
White adipose tissues	25	37	8	39	77	29	46

Note. Interindividual comparison.

TABLE 4
Sample Statistics for Concentration Ratios RLG/LSC (Punches)

Organ/tissue	N	Mean (%)	Min (%)	Median (%)	Max. (%)	95% confidence boundaries	
						Lower (%)	Upper (%)
Blood	21	100	67	102	130	91	108
Liver	25	94	71	93	131	89	99
Kidneys	15	92	64	92	108	85	99
Lungs	13	108	76	105	157	97	119
Skeletal muscle	24	92	71	95	131	86	98
Brain	17	96	56	100	148	83	110
Salivary gland	15	95	82	97	111	89	100
Adrenal gland	12	96	50	106	124	81	113
Spleen	9	110	95	103	181	93	130
Testes	18	99	50	105	127	88	111
White adipose tissue	11	91	53	86	167	72	115
Skin	12	78	50	75	117	66	91

Note. Intraindividual comparison.

ings though they relate solely to interindividual comparison (Potchoiba *et al.*, 1995, 1998, Shigematsu *et al.*, 1995; Motoji *et al.*, 1995; Ahr and Steinke, 1994; Tanaka, 1994). In these studies too there was usually a high degree of equivalence between the results obtained with the two quantitative methods.

CONCLUSIONS

In conclusion, quantification of radioactivity concentrations in organs and tissues with RLG yielded reliable, reproducible results with a high level of accuracy and precision, with a few reservations. Although the comparison data were obtained under rather unfavorable conditions, i.e., the involvement of various laboratories and different sample preparation techniques, the organ concentrations obtained with the two methods of quantification were virtually identical. This is especially true in the case of the intraindividual comparison. It is our opinion that RLG can therefore be regarded as a reliable, adequately validated tool for quantitative autoradiography studies. Compared with distribution studies, which involve animal dissection and homogenization of the organs, RLG can be expected to yield much more accurate results regarding tissue and substructure localization. RLG thus combines the advantages of whole-body autoradiography with the possibilities of traditional quantitative distribution studies.

ACKNOWLEDGMENTS

The authors thank all persons at various companies who actively contributed to this assessment. In particular, we thank Claudia Crummenerl, Nicola Grunwald, Kerstin Maschke, Bettina Sohnius-Lüpertz, Jasmin Percher, Frauke Schiffbauer, Dagmar Trummel, and Heike Weigelt for their technical assistance in assembling and evaluating the data pool.

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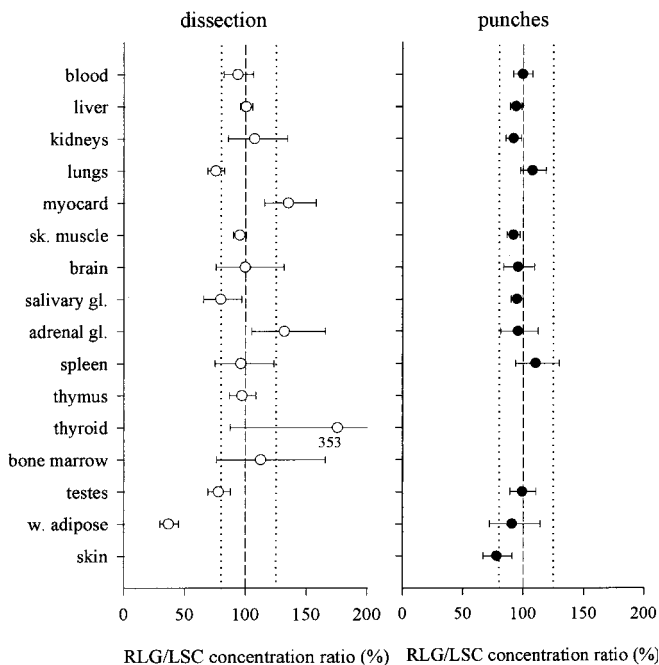


FIG. 5. Mean concentration ratios (RLG/LSC) and 95% confidence boundaries ($P < 0.01$). Interindividual comparison (left, open circles) and intraindividual comparison (right, closed circles). The dotted lines mark the equivalence interval ranging from 0.80 to 1.25.

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