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Original Article

Precision and Accuracy of In Vivo Bone Mineral Measurement in Rats Using Dual-Energy X-Ray Absorptiometry

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Abstract. The aim of this study was to evaluate the precision and accuracy of dual-energy X-ray absorptiometry (DXA) for measuring bone mineral content at different sites of the skeleton in rats. In vitro the reproducibility error was very small (<1%), but in vivo the intra-observer variability ranged from 0.9% to 6.0%. Several factors have been shown to affect in vivo reproducibility: the reproducibility was better when the results were expressed as bone mineral density (BMD) rather than bone mineral content (BMC), intra-observer variability was better than the inter-observer variability, and a higher error was observed for the tibia compared with that for vertebrae and femur. The accuracy of measurement at the femur and tibia was assessed by comparing the values with ash weight and with biochemically determined calcium content. The correlation coefficients (R) between the in vitro BMC and the dry weight or the calcium content were higher than 0.99 for both the femur and the tibia. SEE ranged between 0.0 g (ash weight) and 2.0 mg (Ca content). Using in vitro BMC, ash weight could be estimated with an accuracy error close to 0 and calcium content with an error ranging between 0.82% and 6.80%. The R values obtained between the in vivo and in vitro BMC were 0.98 and 0.97 respectively for femur and tibia, with SEE of 0.04 and 0.02 g respectively. In conclusion, the in vivo precision of the technique was found to be too low. To be of practical use it is important in the design of experimentation to try to reduce the measurement error. This can be achieved by performing measurements in the same position, by repeating measurements several times and by using the mean values of several BMD calculations performed by the same observer on each BMD measurement. Furthermore, better reproducibility can be obtained on the vertebra or the femur than on the tibia.

Keywords: Accuracy; Bone mineral content; DXA; Osteoporosis; Precision

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Introduction

The evaluation of therapeutic strategies for the prevention and treatment of osteoporosis requires the recruitment of a large number of subjects and a long period of follow-up, since modifications of the bone mineral content (BMC) occur very slowly [1,2]. When the method employed has a coefficient of variation (CV) of 3%, a sample size of 75 is needed to detect a 1% change with a significance of 5% [2]. In small animals, bone changes occur more rapidly [3–8]. Preliminary experimental studies in animals are therefore particularly useful. Various techniques have been used for this purpose, including determination of calcium content, bone histomorphometry and the assessment of biomechanical properties [3–8]. These methods, however, are hampered by the need for bone specimens to be analyzed in vitro. Therefore, comparison can only be performed between groups, and a large number of animals is often required to detect time-related differences.

The recent development of dual-energy X-ray absorptiometry (DXA) for the investigation of small animals offers an attractive alternative method, since it

allows non-invasive BMC measurements in vivo [9]. A smaller number of animals may therefore be required as individual variations can be assessed during longitudinal follow-up. To be useful, however, the method should be sufficiently precise and accurate to measure bone mineral mass. In this respect, the data in the literature are rather contradictory, the variability ranging between 0.5% and 4.4% [9–12].

Given the importance of knowing the exact precision of the method before starting an experimental study of osteopenia in rats, we evaluated the in vitro and in vivo precision of DXA at different sites of the skeleton and under different conditions of BMC measurement. The accuracy of the measurements was also assessed by comparing the values with ash weight and with biochemically determined calcium content.

Materials and Methods

Animals

Fifteen female rats were used for the following experiment: 5 Wistar rats aged 50–60 days (weight 127–168 g), 5 Wistar rats aged 123–142 days (weight 214–247 g) and 5 Sprague–Dawley rats aged 190–200 days (weight 318–398 g).

Bone Mineral Measurements

Bone mineral measurements were performed using a Hologic QDR-1000 DXA equipped with commercially available software (V4.47) and collimator (diameter 0.9 cm) for small animals. Ultrahigh-resolution mode was used for scanning (line spacing 0.0254 cm, point resolution 0.0127 cm). The scanning time is set automatically so that 1 cm^2 is scanned in 70 s.

After receiving anaesthesia (0.2 ml phenobarbital given intraperitoneally), the rat was placed in the ventral position with the posterior legs maintained in external rotation secured by tapes. The hip, knee and ankle articulations were flexed at 90°. BMC was measured successively at the level of L2–5 vertebrae, right femur and right tibia. For each measurement the bone of interest was placed in the centre and in the long axis of the scanning area. The rat was then placed in the dorsal position with the posterior legs in external rotation with the hip, knee and ankle in 90° flexion, and the BMC at the same sites again measured. The above measurements (ventral and dorsal) were repeated twice to evaluate the reproducibility error due to repositioning. Finally, with the animal still in the supine position, BMC of the left femur and tibia were also measured once.

Rats were then killed with ether. The right and left femur and tibia were dissected and freed of all soft tissue. The isolated bones were submerged in 2.5 cm of water and measured using DXA and the same software as employed for the in vivo study. In vitro BMC measurements were also performed twice. For the second measurement the bone was repositioned differently in order to modify the area of projection. This allowed us to evaluate the in vitro reproducibility error of BMC due to repositioning.

BMC calculation, both in vivo and in vitro, was performed by two independent observers using *ad hoc* software provided by the manufacturer. The observers had standardized their method of analysis. The evaluation was performed twice by each observer to evaluate intra- and inter-observer variability. The observers had previously been instructed not to remember previous results of the same bone. Bone mineral density (BMD) was also calculated, by dividing BMC by the projection area of the bone. One of the observers also calculated the BMC for individual L2–5 vertebrae and different parts of the femur (by dividing this bone into four equal parts). This was done twice to assess intra-observer variability for smaller regions of interest.

Ash Weight and Calcium Content Determinations

After in vitro measurements, the dissected bones were placed in a muffle furnace at 500 °C for 3 h, followed by 2 h at 800 °C. The ashes of the bones were collected and weighed. The ashed bones were solubilized in concentrated nitric acid, first at room temperature then at 60 °C for 8 h. After dilution, calcium content was determined by flame atomic absorption (Perkin-Elmer 2100) in the presence of lanthanum nitrate using a calibration curve, constructed in a similar matrix with calcium Titrisol (Merck).

Precision and Accuracy

To evaluate intra- and inter-observer variabilities, the differences between two BMC and BMD results were calculated. These differences were expressed as a percentage of the mean value of the two original results. A paired *t*-test was used to assess bias and the SD of the differences was used to evaluate reproducibility error.

The accuracy of both in vivo and in vitro measurements was evaluated by regression analyses between the BMC results and ash weight and calcium content respectively. The comparison of in vivo and in vitro results was also assessed by correlation analysis.

Results

Fig. 1 shows examples of bone images obtained in our study. In all cases the quality of images was satisfactory. Individual vertebrae could easily be identified and the femur was clearly separated from the tibia. However, delineation of the upper part of the femur and the lower

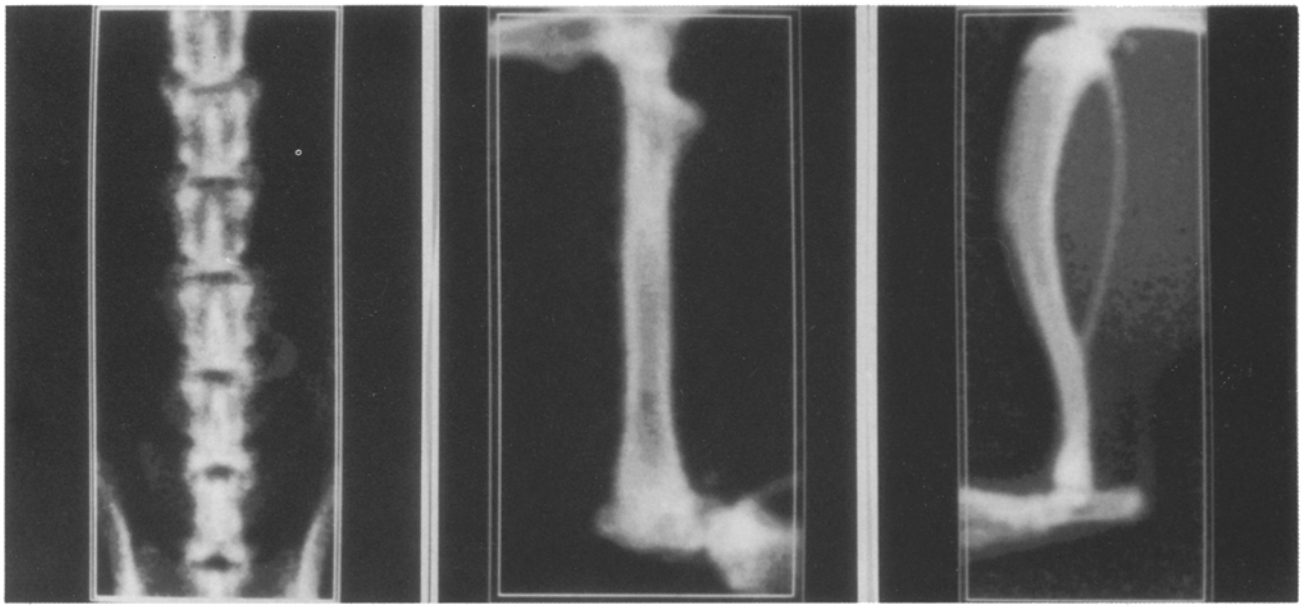

Fig. 1. Examples of DXA images of in vivo rat vertebrae, femur and tibia.

part of the tibia was often difficult because of superimposition of hip and foot respectively.

Precision of Calculated BMC and BMD

Intra-observer Variability. The results of intra-observer variability are presented in Table 1. No systematic bias was observed between first and second calculation. Some differences were observed when comparing the intra-observer variability in ventral and dorsal positions, but these were not systematically in the same direction.

Inter-observer Variability. As with intra-observer variability, the error was smaller when the results were expressed as BMD than when given as BMC (Table 1). Also, smaller errors were observed for the vertebral and femoral sites than for the tibia. However, the results obtained by two independent observers showed a higher variability compared with those obtained by the same observer. Depending on the site studied, inter-observer variability ranged from 1.2% to 7.6%. Some systematic bias was also observed between the results of the two observers (Table 1).

Variability due to Repositioning. Repositioning was often associated with an increase in intra-observer variability error (Table 2). The SD of the differences ranged from 2.3% to 7.9%. The intra-observer variability due to repositioning by the second observer was of the same magnitude as that due to repositioning by the first observer.

In Vitro Measurements. For both femur and tibia there was only a small reproducibility error when the results were expressed as BMC (<1%); when expressed as BMD the error was much higher. This is not surprising because between the two in vitro measurements the

Table 1. Intra- and inter-observer variability without repositioning

	Intra-observer				Inter-observer			
	Ventral		Dorsal		Ventral		Dorsal	
	BMC	BMD	BMC	BMD	BMC	BMD	BMC	BMD
Tibia	4.4	2.5	6.0	4.8	4.1*	3.8	7.6	7.2
Femur	2.7	1.3	4.2	0.9	4.0**	2.6	4.3***	1.2**
L2–5	2.6	0.9	1.6	1.1	4.3	1.7	4.2	1.8

Variability: the standard deviation of the difference between two measurements (in %). Systematic bias tested by paired *t*-test: *$p<0.05$; **$p<0.01$; ***$p=0.001$.

Table 2. Intra- and inter-observer variability after repositioning

	Intra-observer				Inter-observer			
	Ventral		Dorsal		Ventral		Dorsal	
	BMC	BMD	BMC	BMD	BMC	BMD	BMC	BMD
Tibia	5.1	2.3	3.6	6.0	5.2	4.2	7.1	7.9
Femur	3.9	3.1	3.4	3.8	4.3	2.6	3.1	4.3
L2–5	6.2	2.3	6.8	3.6	6.2	2.8	6.6	3.4

Variability: the standard deviation of the difference between two measurements (in %).

bone was voluntarily repositioned in order to modify the projection area on the scan. It illustrates, however, the relationship of the result to the position of the bone during measurement.

Intra-observer Variability of Partial Regions of Interest after Repositioning. When an observer tried to calculate BMD for individual vertebrae (L2, L3, L4 and L5), the intra-observer variability for separate vertebrae was found to be significantly higher (SD 6.0%–7.9%) than for the total L2–5 segment (2.2%–2.3%; *F*-test, $p<0.01$); this was also observed at the femur site (SD 3.5%–4.5% for the smaller segments v 2.9%–3.1% for the total segment).

Variability in Relation to BMC Value and Rat Weight. There was no significant correlation between the different variability coefficients and BMC value or rat weight.

Accuracy. Ash weight ranged between 0.085 and 0.492 g per bone. The correlation coefficients (R) obtained by regression analyses between the in vitro measurements of BMC and the dry weight were higher than 0.99 for both the femur and tibia. For both femur and tibia the standard errors of estimates (SEE) were very low (Fig. 2). Using in vitro BMC, therefore, ash weight could be estimated with an error close to 0.

Calcium content ranged between 29.5 and 198.0 mg per bone. The R values obtained from regression equations between in vitro measurements of BMC and the calcium content were also higher than 0.99. The SEE were 2.0 and 1.63 for the femur and tibia respectively (Fig. 2). Using in vitro BMC, calcium content can be estimated with an accuracy ranging between 0.82% and 6.80%.

The R values obtained by regression analyses between the in vivo BMC and the in vitro BMC were 0.98 and 0.97 respectively for femur and tibia (Fig. 3).

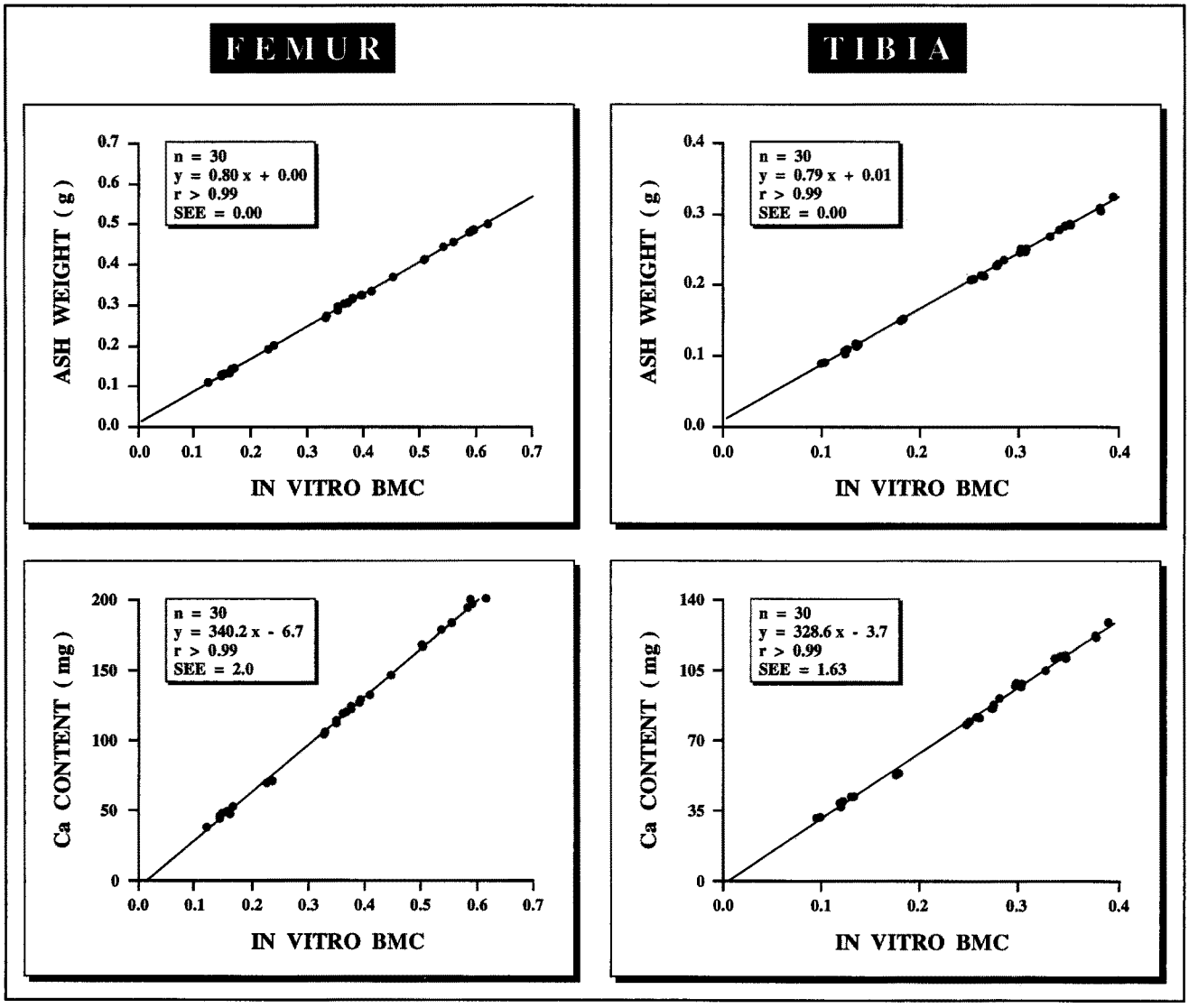

Fig. 2. Regression equations between in vitro BMC and ash weight or calcium content for the femur and tibia respectively.

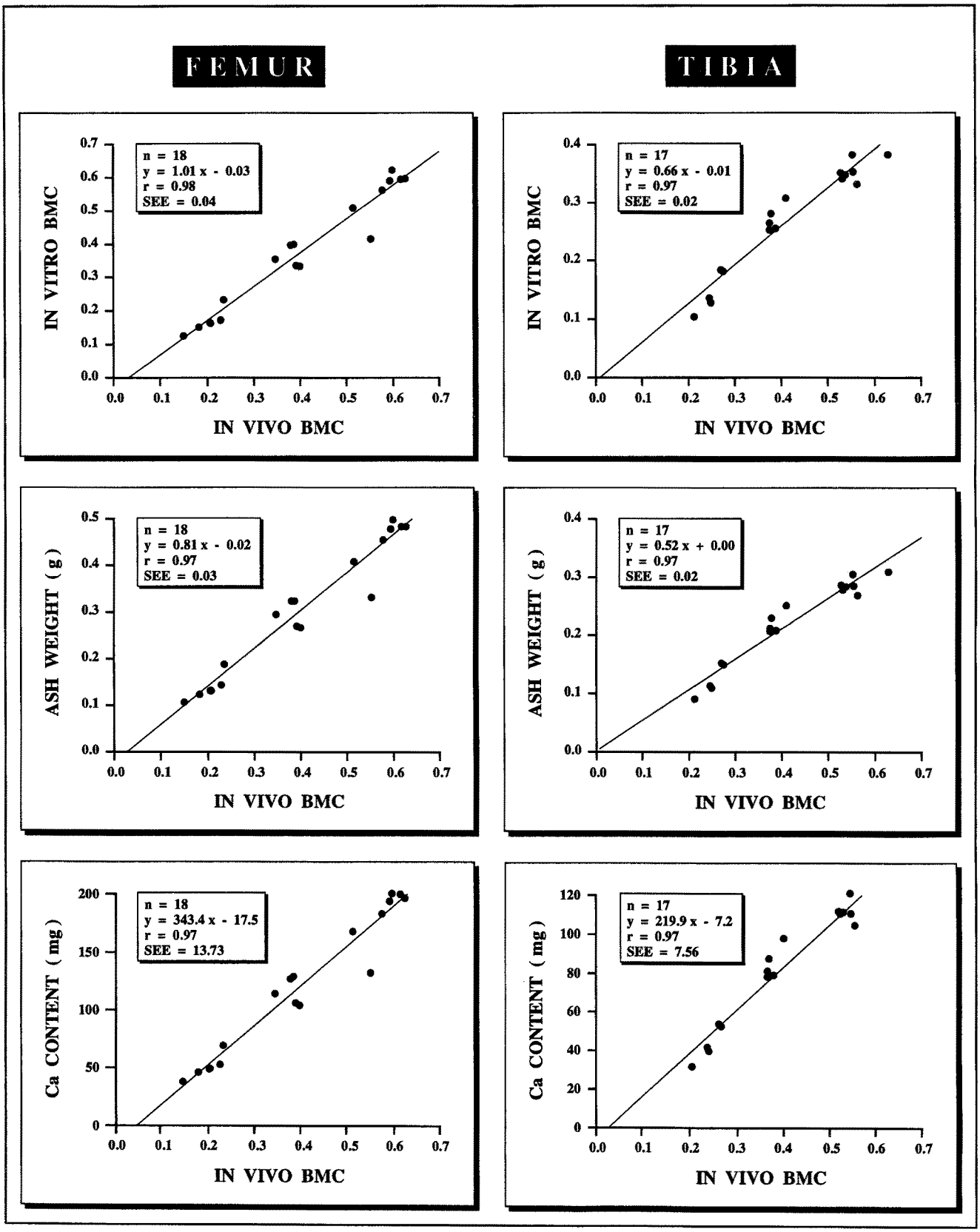

Fig. 3. Regression equations between in vivo BMC and in vitro BMC, and ash weight or calcium content for the femur and tibia respectively.

The SEE was 0.04 for the femur and 0.02 for the tibia. While these correlations are quite high, a significant difference was observed in absolute values as indicated by the regression lines which was, at the tibia site, significantly different from the line of identity.

As would be expected from the above results, in vivo BMC is linearly correlated with the ash weight or the calcium content. The *R* values obtained by regression analyses between the in vivo BMC and ash weight were 0.97 for both femur and tibia, with a SEE of 0.03 and 0.02 for the femur and tibia respectively (Fig. 3). *R* was 0.97 between in vivo BMC and the biochemically determined calcium content. The SEE were 13.73 and 7.56 for femur and tibia, respectively. While these correlations are quite high, differences between the slopes of these regression equations are observed at the femur and tibia sites respectively (Fig. 3).

Discussion

Animal studies are often used to assess drug efficacy before clinical studies are started. Several methods can be used for this purpose but they are all hampered by the need for bone specimens to be analyzed in vitro.

Recently, specific software for DXA has been developed for investigating the bone mineral content of small animals. This technique is particularly attractive for the evaluation of experimental osteopenia as it allows in vivo measurement of BMC non-invasively, so that individual BMC modifications can be assessed during treatment [9]. There are, however, some discrepancies in the literature concerning the precision of this method [9–12]. At the vertebral site the CV has been reported as 0.5% according to Juhn et al. [10], 1.2% according to Griffin et al. [12], 1.4% according to Ammann et al. [9] and 4.4% according to Ongphiphadhanakul et al. [11]. These differences may be due in part to the number of scans which were performed, the number of rats which were used by the investigators and the way the reproducibility error was calculated. Such discrepancies in CV may entail opposite conclusions about the usefulness of DXA in experimental studies on osteopenia.

The precision of DXA measurement of BMC depends on several factors including the position of the animal, the definition of the region of interest (ROI) and the intrinsic precision of the equipment.

Our results indicate that the reproducibility of in vitro measurement of BMC obtained by this method is quite good (around 1%). This is probably the limit of the precision of the equipment in the setting, as neither the position of the animal nor the intervention of the observer were found to play a role in BMC evaluation. In the in vivo results, however, the position of the animal and also the subjective role of the observer in determining the ROI influence the reproducibility of the results.

The effect of ROI is illustrated by the observations that the reproducibility was better when the results were expressed as BMD rather than BMC and that variability was higher between measurements by two observers than one observer [13–14]. Furthermore, this effect is illustrated by the much lower reproducibility obtained for individual vertebrae or for segments of femur than for the total vertebral or femoral segment. Our study also confirms that the error of ROI definition may be site dependent [9,15,16], since analyses of measurements of vertebrae (1%) and at the femur site (1.3%) had a lower precision error than measurements at the tibia site (3.5%). These differences may be explained by the different degrees of difficulty in defining ROI: the vertebral site presents almost no difficulty for ROI definition, while at the tibia ROI definition is most difficult essentially due to superpositioning of the foot and the exclusion of the fibula.

In this study, repositioning was associated with a further increase in precision error of about 1%–3%. The results suggest that the error is lower in the ventral than in the dorsal position. In the ventral position the vertebral site had a lower error (2.3%) than either the femur site (3.1%) or the tibia (4.3%).

The fact that the in vivo results were not identical to those obtained in vitro suggests a limitation in the accuracy of DXA for measuring bone mineral mass [17–19]. The existence of such a limitation is supported by the difference in regression equations obtained for the femur and tibia sites as shown in Figs 1 and 2. This limitation may be explained by factors such as differences in soft tissue content at the tibia and femur. However, despite this limitation, our study indicates that a close linear relationship exists between the two measurements. Furthermore, in vivo and in vitro measurements were also closely and linearly related with ash weight and calcium content.

Evaluation of therapeutic strategies in experimental osteoporosis requires a method of measurement with a high precision and the parameter used should be linearly related to the bone mass modification. Our study indicates that BMC measured in vivo in rats using DXA is closely and linearly correlated with ash weight and calcium content. The precision of the technique, which is about 1%–8% depending on the sites studied, appears rather too low. To be of practical use it is important in the design of experimentation to try to reduce the measurement error. This can be achieved by performing measurements in the same position, by repeating measurements several times and by using the mean values of several BMD calculations performed by the same observer on each BMD measurement. Furthermore, better reproducibility can be obtained on the vertebra or the femur than on the tibia.

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