Acta Ortopédica Mexicana

Volumen *Volume* **18** **Suplemento** *Supplement* **1** **Julio-Diciembre** *July-December* **2004**

Artículo:

Measuring the bone mineral contents in lumbar L2-L4 vertebrae of Long Evans female rats by dual beam X-ray absorptiometry and chemical analysis

Acta Ortopédica Mexicana 2004; 18(Supl. 1): Jul.-Dec: S2-S6

Original article

Measuring the bone mineral contents in lumbar L2-L4 vertebrae of Long Evans female rats by dual beam X-ray absorptiometry and chemical analysis‡

Alejandra Quintana-Armenta,* Lidia Ruiz-Rosano,* René Valdez-Mijares,** María del Pilar Diez-García,*** Fernando Isoard-Acosta,**** Bertha Herrero-Bervera,***** Rebecca Elizabeth Franco-Bourland******

National Rehabilitation Center
"Salvador Zubirán" National Medical Science and Nutrition Center

SUMMARY. Dual beam X-ray absorptiometry is a non invasive method to learn about the status of bone mass in rats. Using a Hologic QDR-2000 densitometer programmed and calibrated to study lumbar L2-L4 vertebrae in rats, we assessed the precision and accuracy of mineral bone density (MBD) and bone mineral contents (BMC) measured in a group of 14 Long Evans female rats, 22 weeks old, weighing 290 to 330 grams. Three consecutive measures were made under anesthesia with rat repositioning. MBD and BMC were (mean ± SD): 175.20 ± 2.00 mg/cm^2 and 450.60 ± 18-00 mg respectively with intra-assay group variance coefficients of 1.5 ± 0.20% and 4.12 ± 1.95% (mean ± SD) respectively showing good precision. Spectrophotometry analysis of calcium, magnesium and phosphorus contents in the HNO_3 digestion product of vertebrae L2-L4 of the same rats were (in mg, mean ± SD): 131.21 ± 6.93, 2.22 ± 0.17 and 55.77 ± 3.45 respectively with linear correlation coefficients (r) against their BMC of 0.52, 0.33 and 0.47 (p > 0.05) (accuracy measure) suggesting that BMC measures are approximations and do not replace chemical analysis.

Key words: Long Evans rats, vertebrae, lumbar, chemical analysis, dual beam X-ray absorptiometry.

RESUMEN. Un método no invasivo para conocer el estado de la masa ósea de ratas es la absorciometría de rayos X de doble energía. Empleando un densitómetro Hologic QDR-2000 programado y calibrado para el estudio de las vértebras lumbares L2-L4 de ratas, evaluamos la precisión y la exactitud de las mediciones de densidad mineral ósea (DMO) y contenido mineral óseo (CMO) de un grupo de 14 ratas hembras Long Evans de 22 semanas de edad y de 290-320 g de peso corporal. Bajo anestesia, se realizaron tres mediciones consecutivas con reposicionamiento por rata. La DMO y el CMO fueron (media ± DE): 175.20 ± 2.00 mg/cm^2 y 450.60 ± 18.00 mg, respectivamente, con coeficientes de variación de grupo (CV) intraensayo de 1.15 ± 0.20% y 4.12 ± 1.95% (media ± DE), respectivamente, demostrando una buena precisión. El análisis espectrofotométrico del contenido de calcio, magnesio y fósforo en el producto de digestión con HNO_3 de las vértebras L2-L4 de las mismas ratas fueron (en mg, media ± DE): 131.21 ± 6.93, 2.22 ± 0.17 y 55.77 ± 3.45, respectivamente, con coeficientes de correlación lineal (r) contra su CMO de 0.52, 0.33 y 0.47 (p > 0.05) (medida de exactitud), lo que sugiere que las mediciones de CMO son aproximadas y no sustituyen al análisis químico.

Palabras clave: ratas Long Evans, vértebras, lumbares, análisis químico, absorciometría de rayos X de doble energía.

* Assistant Researcher, Biochemistry-O, National Rehabilitation Center.
** Associate Researcher, Biochemistry-O, National Rehabilitation Center.
*** Head of Osteoarticular Rehabilitation and Bone Densitometry, National Rehabilitation Center.
**** Chemist A, Nutrition Physiology, "Salvador Zubirán" National Medical Science and Nutrition Center.
***** Chemist, Nephrology and Mineral Metabolism, "Salvador Zubirán" National Medical Science and Nutrition Center.
****** Head of Biochemistry-O, National Rehabilitation Center.
‡ Awarded First Prize in Posters at the 22nd National Orthopedic Journey, Acapulco, Guerrero, Mexico, May 2003.

Mailing address:
Dra. Rebecca Elizabeth Franco-Bourland. Calz. México-Xochimilco 289, Co. Arenal de Guadalupe, Del. Tlalpan. CP 14389 México, D. F., México
Tel: 5999 1000 Ext. 19508; Fax: 5573 5545

Introduction

Over the last few years, non invasive techniques have been developed to evaluate *in vivo* changes in mineral bone density (MBD) as well as bone mineral contents in humans and small laboratory animals. Today, these procedures are more readily available both in clinical practice and in research. They include systems based on attenuating a light beam of γ rays, X rays or ultrasound, in radiology, in single beam photon absorptiometry, and in dual beam X-ray and dual photon absorptiometry (AXD).

AXD in particular is one of the widest used techniques in detecting and monitoring bone disease in humans due to their high reproducibility, low cost and low exposure to radiation.[2] Furthermore, programmed with special software to study small laboratory animals, AXD equipment is more and more used in research work conducted in rat model studies on osteopenia and osteoporosis.[6]

In this study, we assessed the accuracy and precision of BMD and BMC measures of lumbar L2-L4 vertebrae, *in vivo* with AXD equipment, on a group of Long Evans rats. To determine the accuracy of the procedure, we compared the BMC to spectrophotometry measures of calcium, magnesium and phosphorus in an acid extract of the same vertebrae.

Material and methods

Animals. Female, Long Evans rats (National Rehabilitation Center Biological Nursery), 22 weeks old and weighing between 290 to 320 grams. All rats were housed in acrylic cages (three or four to a cage), at a 22 $\pm$ 2 ° C and 12 hour light-darkness cycles. Rats had free access to a standard commercial diet for rats (Purina 5001) and purified water. Animals were handled following Mexican Official Standard NOM 062 A001999 for laboratory animals, to the letter.

Densitometry precision. BMD and BMC were measured in a group of 14 rats. Densitometry determination was performed by using a high performance, dual beam X- ray, phantom calibrated absorptiometer (Hologic DR.2000) with version 5.73 of the software (designed only for lumbar L2, L3, L4 vertebrae in rats) to study small animals.

Deep anesthesia with ketamine (50 mg/kg) mixed with xylacine (10 mg/kg) was given intraperioteneally. Under anesthesia, animals were placed in a supine decubitus position with full limb abduction over an acrylic platform and an acetate grid to make reproducibility easier and be able to position and reposition the rats on the densitometer.

Three consecutive densitometry measures were made for each rat. Every measurement lasted 12 to 14 minutes depending on the rat size. All measurements were made within no more than one month to establish the precision of BMD and BMC AXD short term measures.[5]

Densitometry accuracy. After the BMD and BMC AXD measures were made, all 14 rats were killed with carbon dioxide. Immediately thereafter, lumbar L2-L4 vertebrae were dissected from each rat and placed in a 50% ethanol solution with deionized water. The entire soft tissue was removed by hand and vertebrae were placed at constant weight in a 75° C stove for 24 hours. Next, they were digested in 7 ml of HNO in a digestor (microwave oven NC 28105, CEM, MDS 2000) at a 200 PSI pressure for one hour and 15 minutes. The digestion product of all three vertebrae in each rat was added to 25 ml of deionized water. 1 ml aliquots of this solution were diluted to 1:2000, 1:1000, and 1:50 to determine calcium, magnesium and phosphorus contents respectively. Calcium and magnesium were measured in an atomic absorption spectrophotometer (Perkin-Elmer 2380) and phosphorus was measured by the Fiske and Subbarow method[3] as an unreduced phosphomolibdate complex by absorbance at 340 nm using a Technicon RA-1000 spectrophotometer.

Statistical analysis. Precision of densitometry measures in the group of rats studied was derived by calculating the mean $\pm$ SD of intra-assay variance coefficients (VC) of three consecutive BMD and BMC measures in each rat.

Linear regression correlation coefficients (r) between the dry weight of each group of three lumbar L2-L4 vertebrae and their calcium, magnesium and phosphorus contents were determined for accuracy of chemical analysis on acid digestion products of those vertebrae.

The accuracy of densitometry determination in BMC of lumbar L2-L4 vertebrae by AXD was set by calculating the linear regression correlation coefficients (r) between the total calcium, magnesium and phosphorus contents in every group of three L2-L4 vertebrae of all 14 rats and their corresponding BMC.

Statistical GraphPad Prism software (GraphPad Software, Inc.) was used in all cases.

Results

Densitometry analysis. Figure 1A shows the rats placed on the acrylic platform and over the grid. With the grid the purpose is to assure the proper positioning of rats in each densitometry measure. *Figure 1B* shows the R1 region (lumbar L2-L4 vertebrae) of the spine in rats analyzed by AXD.

Table 1 summarizes the average values of BMD and BMC in lumbar L2-L4 vertebrae of 14 Long Evans rats by AXD. The linear regression correlation coefficient in BMD and BMC densitometer readings was: $r = 0.79$, $p < 0.05$.

Densitometry precision. Intra-assay group VC for densitometry BMD and BMC measures of all 14 rats were (mean $\pm$ SD) 1.15 $\pm$ 0.20% and 4.12 $\pm$ 1.95% respectively).

Chemical analysis. Figure 2 shows the skeleton of one rat illustrating lumbar L2-L4 vertebrae which were first analyzed by densitometry and then dissected, stripped from soft tissue, and digested with HNO_3 for spectrophotometry analysis of their calcium, magnesium and phosphorus contents.

Table 1 summarizes these values for all rats. Of those, mean calcium, magnesium and phosphorus concentrations may be inferred by mg of body weight in every three L2-

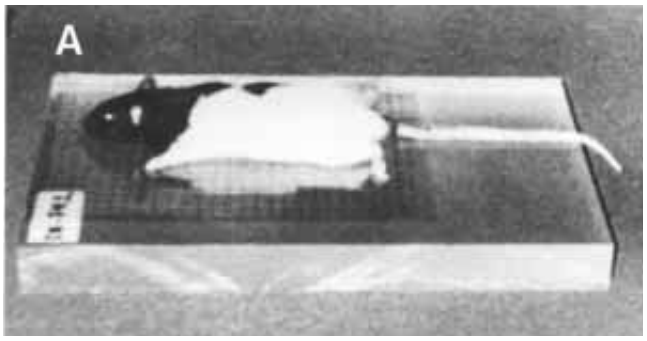

Figure 1A.

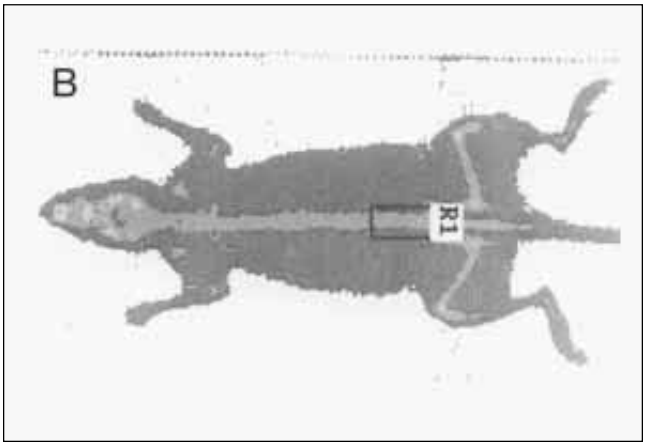

Figure 1B.

Figure 1. Dual beam X ray absorptiometry of lumbar L2-L4 vertebrae of Long Evans rats. In (A) rats are shown on the Hologic QDR-2000 densitometer over an acrylic platform and a grid (used to assure the rat positioning and repositioning). In (B), region R-1 of the spine is shown. This corresponds to lumbar L2-L4 vertebrae analyzed by densitometry.

L4 vertebrae: 232.69 µg/mg, 3.95 + 0.18 µg/mg , 98.87 + 1.72 µg/mg.

Charts 1A, 1B and 1C illustrate linear regressions and their corresponding correlation coefficients (r) derived between the total calcium, magnesium and phosphorus contents in lumbar L2-L4 vertebrae of 14 Long Evans rats and their dry weight. An association between both parameters and the validity of the determinations was seen.

Densitometry accuracy. Charts 1E and 1F show the linear regressions and their corresponding correlation coefficients (r) derived from the calcium, magnesium and phosphorus contents in lumbar L2-L4 vertebrae of 14 rats studied and their BMC. A poor correlation was seen between these two parameters.

Table 2 shows the similarity of relationships between the mean calcium, magnesium and phosphorus contents in lumbar L2-L4 vertebrae of all 14 Long Evans rats and the mean dry weights and bone mineral contents.

Discussion

We assessed the accuracy and precision of short term BMD and BMC densitometry measures in a group of 14 female Long Evans rats with a Hologic QDR-2000 densitometer programmed and calibrated to study lumbar L2-L4 vertebrae by performing 3 consecutive measures repositioning each rat over a period no longer than one minute.

The precision of an analytical method is defined as the capability of such system to always derive the same results for repeated, serial results provided no actual or biological variations have occurred in the same study subject. The precision of parameters analyzed is reported as the variation coefficient, a percentage measure (VC, %).

For precision of bone mass AXD densitometry measures in animals (and consequently their analytical value), precision

Table 1. Densitometry measures and mineral analysis of lumbar L2-L4 vertebrae of all 14 Long Evans rats.

Rat	Body weight, g	BMD[a], mg/cm^2	BMC[a] mg	L2-L4 dry weight, mg	Calcium[b], mg	Mg[b],mg	Ph[b], mg
1	290	174.63	417.97	524.50	125.31	1.97	53.56
2	300	161.93	416.43	507.00	116.63	1.95	50.25
3	300	163.56	436.73	561.00	132.75	2.20	56.56
4	310	163.93	450.13	539.50	125.93	2.20	52.50
5	290	180.80	446.03	586.00	134.62	2.40	58.37
6	300	183.73	458.97	594.30	136.48	2.45	59.56
7	300	164.53	396.43	546.40	127.17	2.09	53.81
8	320	184.13	492.77	619.20	140.20	2.34	61.06
9	300	170.96	453.70	612.10	140.82	2.39	58.81
10	300	190.23	489.40	603.30	137.72	2.25	59.81
11	290	182.43	468.73	549.80	130.89	2.14	55.25
12	300	188.26	492.27	525.00	122.83	2.03	51.37
13	300	169.33	449.80	582.20	132.30	2.39	57.06
14	300	174.53	439.10	550.00	133.37	2.39	52.93
Mean ± SD	300 ± 7.85	175.2 ± 2.00	450.69 ± 18	564.31 ± 35.39	131.21 ± 6.93	2.22 ± 0.17	55.77 ± 3.45

BMD = Bone mineral density; BMC = bone mineral contents; L2-L4, lumbar vertebrae; a = mean of three readings; b = mean of two readings.

will depend on the precise placement of the animal over the densitometer, the accurate definition of the skeletal region in question, and the equipment features.[7] The 1.15 ± 0.20% group variance coefficient we got for the AXD BMD determination reflects good precision of our measures in rats and is comparable to the precision in BMD *in vivo* measured in lumbar spine reported by other authors[1,4,10] whose VC values range between 0.62 and 1.77%. In spite of having derived less precision for BMC findings with a 4.12 ± 1.95% VC, the r correlation coefficient = 0.79 and $p < 0.05$ between these two densitometry parameters reinforce the validity of this procedure for following up the changes in the bone mass of rats subject to some experimental manipulation that might alter the integrity of their bone mass.

Accuracy of a procedure is made by comparing the values for some parameters derived through two or more different methods as the comparison between AXD to measure the status of bone integrity against histomorphometric measures, chemical analysis, and so forth.[8,9] Here we have correlated the BMC values of lumbar L2-L4 vertebrae *in vivo* of a group with 14 rats derived with the AXD method for calcium, magnesium and phosphorus values from the chemical analysis of the pressure acid digestion product of those same vertebrae. In spite of the chemical determinations showing a good correlation between each other (mineral vs. dry weight of vertebrae L2-L4 with r = 0.95, 0.81 and 0.96 for calcium, magnesium and phosphorus respectively, $p < 0.05$), we have found a poor correlation between densitometry BMC measures and mineral measurements derived by spectrophotometry (r = 0.52, 0.47 and 0.33, $p > 0.05$) for calcium, magnesium and phosphorus measures respectively against the corresponding BMC. Our determinations are in contrast with those of other authors re-

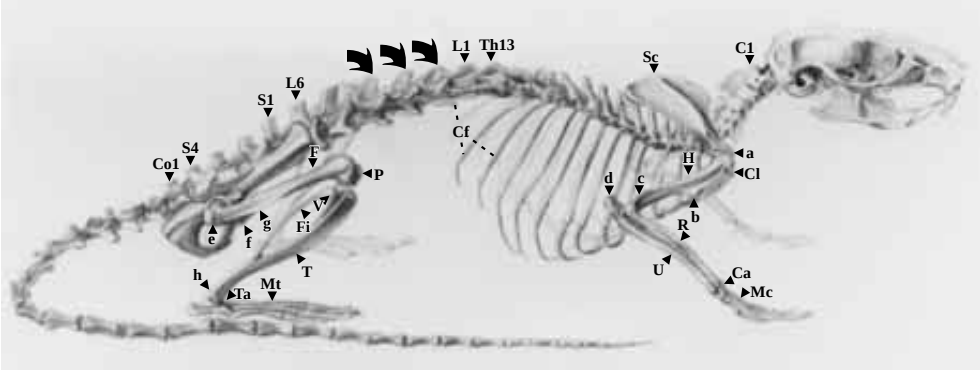

Figure 2. Rat skeleton. Lumbar L2-L4 vertebrae, analyzed by densitometry are shown (arrows). They were then dissected for chemical analysis of calcium, magnesium and phosphorus contents because of their spectrophotometry, upon prior HNO_3 digestion in a microwave oven at 200 PSI. Modified from: Hebel R. Stromberg MW: Anatomy of the Laboratory Rat. Osteology, Baltimore. The Williams and Wilkins Co., 1976: 1-17

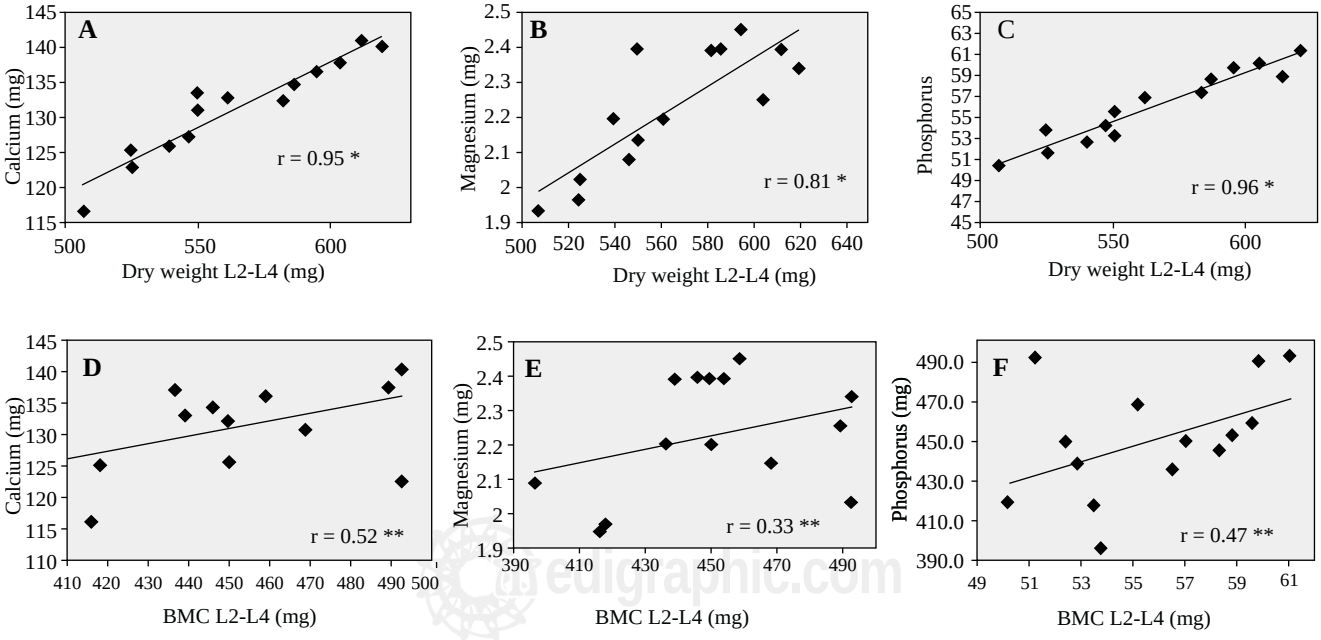

Chart 1. Linear regressions and linear correlation coefficients. (A), (B) and (C) illustrate linear regressions and correlation coefficients corresponding the calcium, magnesium and phosphorus contents in lumbar L2-L4 vertebrae of 14 Long Evans rats against their dry weight (mg). (D), (E) and (F) illustrate the linear regression and correlation coefficients which correspond to the same mineral contents values regarding their bone mineral contents (BMC, mg, densitometry measure). r, linear correlation coefficient. * $p < 0.05$; ** $p > 0.05$.

Table 2. Relationship between the mean calcium, magnesium and phosphorus contents of lumbar L2-L4 vertebrae of 14 Long Evans rats and the mean dry weight and bone mineral contents.

Mineral	L2-L4 vertebrae mineral/ weight, %	Mineral/BMC, %
Calcium	23.27 ± 0.48	29.19 ± 1.77
Magnesium	0.39 ± 0.02	0.50 ± 0.04
Phosphorus	9.87 ± 0.17	12.41 ± 0.79

BMC: bone mineral contents, L2-L4: lumbar vertebrae

porting a good correlation between both methods where r = 0.99, 0.93.[2,10] Today, we are trying to find out the reason for the discrepancy between our densitometry measures (correlating well to each other) and our chemical determinations (also correlating well to each other).

Bibliography

1. Ammann P, Rizzoli R, Slosman D, Bonjour JP: Sequential and precise *in vivo* measurement of bone density in rats dual-energy X-ray absorptiometry. *J Bone Miner Res* 1992; 7: 311-316.
2. Cazez JP, Muehlbauer RC, Lippuner K, Kelly T, Fleisch H, Jaeger P: Dual-energy X-ray absorptiometry for measuring total bone mineral content in the rat: Study of accuracy and precision. *Bone Miner* 1994; 26(1): 61-68.
3. Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. *J Biol Chem* 1925; 66: 375.
4. Gala Paniagua J, Díaz-Curiel M, de la Piedra Gordo C, Castilla Reparaz C, Torralbo García M: Bone mass assessment in rats by dual energy X-ray absorptiometry. *Br J Radiol* 1998; 71(847): 754-8.
5. Gluer CC, Blake G, Lu Y, Blunt BA, Jergas M, Genant HK: Accurate assessment of precision errors: How to measure the reproducibility of bone densitometry techniques. *Osteoporos Int* 1995; 5(4): 262-270.
6. Grier SJ, Turner AS, Alvis Mr: The use of dual-energy X-ray absorptiometry in animals. *Invest Radiol* 1996; 31(1): 50-62.
7. Griffin MG, Kimble R, Hopfer W, Pacifici R: Dual energy X-ray absorptiometry of the rat: Accuracy, precision and measurement of bone loss. *J Bone Miner Res* 1993; 8(7): 795-800.
8. Kastl S, Sommer T, Klein P, Hohenberger W, Engelke K: Accuracy and precision of bone mineral density and bone mineral content in excised rat humeri using fan beam dual-energy X-ray absorptiometry. *Bone* 2002; 30(1): 243-246.
9. Rozenberg S, Vandromme J, Neve J, Aguilera A, Muregancuro A, Peretz A, Kinthaert J, Ham H: Precision and accuracy of *in vivo* bone mineral measurement in rats using dual-energy X-ray absorptiometry. *Osteoporosis Int* 1995; 5(1): 47-53.
10. Yamauchi H, Kushida K, Yamazaki K, Inoue T: Assessment of spine bone mineral density in ovariectomized rats using DXA. *J Bone Miner Res* 1995; 10(7): 1033-1039.