J Bone Miner Metab
DOI 10.1007/s00774-013-0557-3

ORIGINAL ARTICLE

Methodology developed for the simultaneous measurement of bone formation and bone resorption in rats based on the pharmacokinetics of fluoride

Maela Lupo · Maria Lorena Brance · Brenda Lorena Fina · Lucas Ricardo Brun · Alfredo Rigalli

Received: 11 September 2013 / Accepted: 28 November 2013

Abstract This paper describes a novel methodology for the simultaneous estimation of bone formation (BF) and resorption (BR) in rats using fluoride as a nonradioactive bone-seeker ion. The pharmacokinetics of flouride have been extensively studied in rats; its constants have all been characterized. This knowledge was the cornerstone for the underlying mathematical model that we used to measure bone fluoride uptake and elimination rate after a dose of fluoride. Bone resorption and formation were estimated by bone fluoride uptake and elimination rate, respectively. ROC analysis showed that sensitivity, specificity and area under the ROC curve were not different from deoxypiridinoline and bone alkaline phosphatase, well-known bone markers. Sprague–Dawley rats with modified bone remodelling (ovariectomy, hyper, and hypocalcic diet, antiresorptive treatment) were used to validate the values obtained with this methodology. The results of BF and BR obtained with this technique were as expected for each biological model. Although the method should be performed under general anesthesia, it has several advantages: simultaneous measurement of BR and BF, low cost, and the use of compounds with no expiration date.

Present Address:
M. Lupo (✉) · M. L. Brance · B. L. Fina · L. R. Brun · A. Rigalli
Bone Biology Laboratory, School of Medicine, Rosario National University, Santa Fe 3100, Rosario, Santa Fe, Argentina
e-mail: maela_lupo@hotmail.com

M. Lupo · M. L. Brance · B. L. Fina · L. R. Brun · A. Rigalli
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina and Rosario National University Research Council, Rosario National University, Maipu 1065, Rosario, Santa Fe, Argentina

Keywords Bone remodeling · Mathematical model · Fluoride pharmacokinetics · Bone marker · Rat

Introduction

Fluoride is a drug used in the treatment of bone loss that might also be used as a diagnostic drug for bone remodeling measurement. In our laboratory, we developed a technique for the purpose of using it as such in human beings [1, 2]. Bone loss is initiated by an alteration in the bone remodeling process; therefore, knowledge of this process might allow us to predict bone mass modifications, something necessary in order to decide what treatment to follow. Measurement of bone remodeling in rats is not as easy and as common as it is in humans. Firstly, due to the impossibility of obtaining enough samples and, secondly, because there are fewer kits and techniques designed to be used with rats. The cost of commercial kits is another obstacle, especially in emerging countries.

Fluoride pharmacokinetics have been studied in humans and rats, and the equation for plasma fluoride concentration has been obtained [3]. This knowledge was used to develop a mathematical model that can measure the bone fluoride uptake after a dose of fluoride [4]. From these works, we created a mathematical model that allowed us to study bone remodeling in rats.

This paper describes a novel technique for the simultaneous measurement of bone formation and resorption after the injection of a fluoride dose; we also present the results of an ROC analysis showing that this technique is as sensitive and specific as the commercially available methods.

Published online: 21 January 2014

Mathematical model formulation

Pharmacokinetic model

The mathematical model adapted to the measurement of bone remodeling consists of two parts: One, before the intravenous injection of a minimal dose of fluoride, and two, after fluoride injection.

First part: before fluoride injection In this circumstance, plasma fluoride levels have a constant value ($[F]_b$) that results from a balance between bone fluoride uptake and elimination rate. The bone fluoride uptake rate is proportional to the bone formation rate (BF: nmole/min) and the bone fluoride elimination rate is proportional to the bone resorption rate (BR: nmole/min).

In addition, urinary fluoride excretion (Uv) contributes to this stationary state. This state is depicted in Fig. 1.

The pharmacokinetic model has been previously described [3, 4]; here, it was adapted for intravenous injection of NaF. The mathematical model equations are developed next:

Bone formation rate (BF) is proportional to the basal fluoride concentration, $[F]_b$.

$$\mathrm{BF} = \mathrm{kf}.[F]_b. \tag{1}$$

On the other hand, bone resorption rate (BR) is proportional to the quantity of fluoride previously incorporated in bone matter ($[F]_{bone}$):

$$\mathrm{BR} = \mathrm{kr}.[F]_{bone}. \tag{2}$$

Finally, urinary fluoride excretion rate (Uv) is proportional to plasma fluoride concentration:

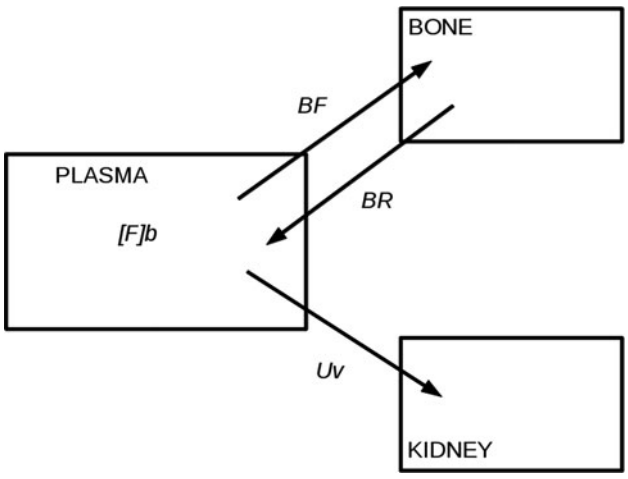

Fig. 1 Pharmacokinetic model in the stationary state, before fluoride injection

$$\mathrm{Uv} = \mathrm{ku}[F]_b, \tag{3}$$

and the constants are as follows:

kf is the fluoride bone uptake rate constant, kr is the fluoride bone elimination rate constant, ku is the fluoride renal excretion rate constant, and $[F]_b$ is the basal fluoride concentration in plasma.

As stated in previous works, fluoride plasma variation is proportional to plasma fluoride concentration:

$$\frac{\mathrm{dF}}{\mathrm{d}t} = \mathrm{ke}.[F] \tag{4}$$

ke is the fluoride plasma elimination rate constant, and it is the sum of the fluoride bone uptake and fluoride renal excretion rate constants:

$$\mathrm{ke} = \mathrm{ku} + \mathrm{kf}. \tag{5}$$

Fluoride plasma variation can also be written as a function of BR, BF, and Uv, and due to the lack of fluoride dose a stationary state is proposed:

$$\frac{\mathrm{dF}}{\mathrm{d}t} = \mathrm{BR} - \mathrm{BF} - \mathrm{Uv} = 0. \tag{6}$$

$$\mathrm{Uv} = \mathrm{BR} - \mathrm{BF}. \tag{7}$$

From Eqs. 3 and 6:

$$\frac{\mathrm{dF}}{\mathrm{d}t} = \mathrm{BR} - \mathrm{BF} - \mathrm{ku}.[F]_b \tag{8}$$

$$\mathrm{BR} - \mathrm{BF} = \mathrm{ku}[F]_b. \tag{9}$$

In the next equations, the fluoride urinary variations, where U is the amount of fluoride eliminated in urine, are developed:

$$\frac{\mathrm{dU}}{\mathrm{d}t} = \mathrm{ku}[F]_b, \tag{10}$$

$$\mathrm{d}U = \mathrm{ku}[F]_b\mathrm{dt}, \tag{11}$$

$$U = \mathrm{ku}[F]_b\mathrm{t}, \tag{12}$$

where the slope of urinary fluoride excretion (U) as a function of time is: kb, fluoride basal renal excretion rate constant.

$$\mathrm{kb} = \mathrm{ku}[F]_b. \tag{13}$$

Equation 12 can be written as

$$U = \mathrm{kb}.t \tag{14}$$

Therefore, from Eqs. 9 and 13, it is deduced that

$$\mathrm{kb} = \mathrm{BR} - \mathrm{BF} \tag{15}$$

and

$$\mathrm{ku} = \frac{\mathrm{kb}}{[F]_b}. \tag{16}$$

In this part of the experiment, urinary fluoride excretion (U) as a function of time, and one value of plasma fluoride level are measured. Using these values and kb, the ku parameter is obtained.

Second part: after fluoride intravenous injection In the equation that expresses fluoride variation in plasma:

$$\frac{\mathrm{dF}}{\mathrm{d}t} = \mathrm{BR} - \mathrm{BF} - \mathrm{Uv}. \tag{17}$$

BR is considered negligible because after a fluoride dose it is assumed that the fluoride concentration in plasma is high, therefore the fluoride bone uptake process is much higher than the release process.

$$\frac{\mathrm{dF}}{\mathrm{d}t} = -\mathrm{kf}[F] - \mathrm{ku}[F]. \tag{18}$$

$$\frac{\mathrm{dF}}{\mathrm{d}t} = -(\mathrm{kf} + \mathrm{ku})[F]. \tag{19}$$

$$\mathrm{ke} = \mathrm{kf} + \mathrm{ku}. \tag{20}$$

$$\frac{\mathrm{dF}}{\mathrm{d}t} = -\mathrm{ke}[F]. \tag{21}$$

$$\frac{\mathrm{dF}}{\mathrm{d}t} = -\mathrm{ke}\frac{F}{\mathrm{Dv}}. \tag{22}$$

Dv is the distribution volume of fluoride in the organism.

$$\mathrm{dF} = -\mathrm{ke}\frac{F}{\mathrm{Dv}}\mathrm{d}t. \tag{23}$$

Integrating Eq. 23 between 0 and t, and between the amount of fluoride injected (Do) and the amount of plasma fluoride at time t (F):

$$\int_{\mathrm{Do}}^{F}\frac{\mathrm{dF}}{F} = -\frac{\mathrm{ke}}{\mathrm{Dv}}\int_{0}^{t}\mathrm{d}t.$$

With this, the next equation is reached:

$$\ln\frac{F}{\mathrm{Do}} = e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}\mathrm{d}t. \tag{24}$$

Do is the initial dose of fluoride, and considering $F/\mathrm{Dv} = [F]$, the next equation is obtained:

$$[F] = \frac{\mathrm{Do}}{\mathrm{Dv}}e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}. \tag{25}$$

From the urinary variation as a function of time:

$$\frac{\mathrm{dU}}{\mathrm{d}t} = \mathrm{ku}[F], \tag{26}$$

and by replacing Eq. 26 with Eq. 25, the next equation is acquired:

$$\frac{\mathrm{dU}}{\mathrm{d}t} = \frac{\mathrm{kuDo}}{\mathrm{Dv}}e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}; \tag{27}$$

integrating:

$$\int_{0}^{U}\mathrm{d}U = \frac{\mathrm{kuDo}}{\mathrm{Dv}}\int_{0}^{t}e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}\mathrm{d}t, \tag{28}$$

the equation of the fluoride renal excretion after the dose of fluoride is obtained:

$$U = \frac{\mathrm{kuDo}}{\mathrm{ke}}(1 - e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}). \tag{29}$$

The total fluoride renal excretion is the sum of before and after the dose fluoride excretion.

$$U = \frac{\mathrm{kuDo}}{\mathrm{ke}}(1 - e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}) + \mathrm{kb}.t. \tag{30}$$

Calculating the first derivative

$$\mathrm{d}U = \frac{\mathrm{kuDo}}{\mathrm{ke}}(0 - e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}}(-\frac{\mathrm{ke}}{\mathrm{Dv}}) + \mathrm{kb} \tag{31}$$

$$\mathrm{d}U = \frac{\mathrm{kuDo}}{\mathrm{Dv}}.e^{-\mathrm{ke}\frac{t}{\mathrm{Dv}}} + \mathrm{kb}, \tag{32}$$

the first derivative at $t = 0$ is:

$$\mathrm{d}U_{(0)} = \frac{\mathrm{kuDo}}{\mathrm{Dv}} + \mathrm{kb}. \tag{33}$$

As ku, Do, and kb are known and already calculated values, and dU can be easily approximated by fluoride urinary excretion 10 min after the dose divided by the urine recollection time, Dv can thus be obtained.

Procedure to obtain BR and BF

Before intravenous fluoride injection, urinary fluoride excretion ($U_{(t)}$) is adjusted by a linear function of time; the slope of this function is the kb parameter (Eq. 12). The value of the fluoride renal excretion rate constant (ku) is obtained from Eq. 16 using the value of plasma fluoride basal concentration ($[F]_{\mathrm{b}}$) and kb parameter.

After the intravenous injection of fluoride, the distribution volume (Dv) of fluoride is obtained, and since we know ku, kb, and the dose of fluoride (Do), we can calculate the amount of fluoride excreted in the10 min following fluoride injection from the urinary fluoride excretion rate (dU(o)) using Eq. 33. d$U(o)$. The fluoride bone uptake rate constant (kf) is obtained from the nonlinear adjustment of urinary excretion (U) as a function of time (Eq. 29). The values of ***BF*** and ***BR*** are then obtained with Eqs. 1 and 7, respectively.

Materials and methods

Mathematical model validation

Bone formation (BF) and bone resorption (BR) were estimated with the technique described in this paper. These

measurements were made in Sprague–Dawley rats in a wide range of biological states of bone remodeling: ovariectomy, ovariectomy with hypercalcic diet, ovariectomy with hypocalcic diet, and ovariectomy with zoledronic acid treatment, all of which are depicted in the next sections. In order to compare the values of bone remodeling obtained with the technique described, bone formation and bone resorption were measured with plasma bone alkaline phosphatase (bAP) and urinary deoxypydirinoline (Dpd), respectively. All the experiments were carried out according to the international rules of animal care [5].

Methodology to obtain BF and BR values

The measurement was carried out on anesthetised Sprague–Dawley rats with different states of bone remodeling [6]. In order to have adequate hydration throughout the experiment, a constant 8 ml/h saline solution was administered by a rectal catheter. A 24 G × ¾ urethral catheter was used to collect urinary samples. At the beginning of the experiment, a 50-µl blood sample was obtained from the vein of the tail. Urinary samples were collected every 5 min for 60 min. After this time, 67 µl/100 g b.w. of 15 mmol/l NaF was intravenously injected in the vein of the tail [7]. Urine was collected for 4 h after fluoride injection.

Measurement of fluoride concentration

Urinary fluoride concentration was measured with an ion-selective electrode ORION 94-09 with a reference electrode Ag/AgCl connected to an analogical-digital converter. The measurement is based on the linear relation between the mV developed by the electrode and the logarithm of the fluoride concentration of the standards: 10^{-3} mol/l–10^{-6} mol/l of NaF [8]. We then added 10 % buffer acetic acid/sodium acetate 2 mol/l to samples and standards in order to adjust the ionic strength and the pH to 5.5.

Plasma fluoride concentration was measured using the same electrode described before, but after isothermal distillation.

Urinary deoxypyridinoline measurement (Dpd, nmole/mmole creatinine)

We measure urinary deoxypyridinoline by radioimmunoassay (Dpd RIA, immunodiagnostic systems, Boldon, UK, with a solid scintillator, Alfanuclear, Buenos Aires, Argentina). Then, 24-h creatininuria was spectrophotometrically measured with a commercial kit (Creatinina cinética AA líquida, Wiener Lab, Rosario, Argentina).

Plasma bone alkaline phosphatase activity

Plasma bone alkaline phosphatase activity (bAP) (U/l) was spectrophotometrically measured with a kinetic method (ALP 405, Wiener Lab, Rosario, Argentina) using p-nitrophenylphosphate (pNPP) as a substrate. We estimated bAP activity as the difference between total and remnant alkaline phosphatase activity after wheat germ lectin treatment [9].

Biological models studied

Different biological models were used in order to obtain a wide range of bone remodeling in rats. These models allowed us to test rats with low, normal or high bone remodeling. For the biological models, Sprague–Dawley rats were used to create the following study groups ($n = 12$, for each group):

- OVX: ovariectomized rats with normocalcic diet for 150 days (1 % calcium)
- OVX + Hypo: ovariectomized rats with hypocalcic diet for 150 days (0.2 % calcium)
- OVX + Hyper: ovariectomized rats with hypercalcic diet for 150 days (2 % calcium)
- OVX + Z: ovariectomized rats treated with zoledronic acid and normocalcic diet for 150 days
- Control group: rats that were subjected to simulated surgery

Ovariectomized rats (OVX)

Ovariectomy produces estrogen deficiency and alterations similar to those produced in post-menopausal women. It is an accepted model of post menopausal osteoporosis and is also a model with high bone remodeling. Bilateral ovariectomy was performed in 9-week-old female Sprague–Dawley rats. The success of the ovariectomy was verified at the end of the experiment by comparing the decrease in weight of the uteri to that of the controls [10].

OVX with antiresorptive treatment (OVX + Z)

Zoledronic acid is a drug that decreases osteoclast activity. It is considered a model of low bone remodeling. Ovariectomized rats received a weekly subcutaneous injection of zoledronic acid, 0.15 µg/100 g of body weight.

OVX with hypocalcic diet (OVX + Hypo)

The administration of a hypocalcic diet (0.2 % calcium) to ovariectomized rats further increases the high bone

remodeling. As a consequence, it is considered a model of high bone remodeling.

OVX with hypercalcic diet (OVX + Hyper)

The hypercalcic diet (2 % calcium) decreases the parathyroid function and induces a low bone remodeling state.

Confirmation of bone remodeling state

The state of bone remodeling was assigned to each rat according to the biological model (see above). However, a histomorphometric study of bone remodeling was performed on each rat in order to confirm it. After euthanasia, tibias were quickly removed and the proximal epiphysis were fixed in 10 % phosphate-buffered formaldehyde, decalcified in 10 % EDTA, dehydrated through ascending ethanol, and embedded in paraffin. Paraffin blocks were cut using a microtome (Leitz, Wetzlar, Germany) in 5-μm longitudinal sections stained with haematoxylin and eosin [11]. Permanent slides were examined using a light microscope (Leitz Wetzlab, Germany). Digital images of proximal epiphysis of the tibia were obtained at 4× magnification (Olympus SP-350, China) in order to assess the trabecular bone. A 2-mm^2 region of interest (ROI) at 1 mm from growth plate-metaphyseal junction was selected. Four micrographs were randomly taken at 400× magnification from the selected ROI in order to measure (1) bone surface covered by osteoblast, Ob.S (μm), (2) eroded surface, ES (μm), and (3) osteoclast number, N.Oc. With these values, the following variables were calculated: (1) the surface area covered by osteoblast: the percentage of trabecular bone surface (BS) covered with osteoblasts, Ob.S/BS (%) [Ob.S × 100/BS]; (2) eroded surface: the percentage of trabecular bone surface covered with eroded surface, including active lacunae with osteoclasts and lacunae in reversal phase, ES/BS (%) [ES × 100/BS]; and (3) osteoclast number: the number of osteoclasts in the total area, N.Oc/mm^2 (1/mm^2) [N.Oc/TA]. The state of bone remodeling was confirmed for each animal by comparing the values of the above variables with the values obtained in the control group.

Statistical analysis

Package pROC [12] was used to perform receiver operating characteristic (ROC) analysis. This analysis was carried out in order to obtain the area under the curve (AUC), sensitivity, specificity, threshold, positive predictive value (PPV), negative predicted value (NPV), true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) values. ROC curves of Dpd and BR as markers of bone resorption, and bAP and BR as markers of bone formation were compared with the ROC.test of the same package; differences were considered significant if $p < 0.05$. The comparison of the ROC curves was done for dependent data, since the measurements were done in the same rats. Nonlinear adjustments of functions were carried out with GraphPad Prism 2.0, San Diego, California, USA.

Results

Comparison of this technique with other accepted techniques for the measurement of bone formation and resorption

Receiver operating characteristic analysis was performed in order to obtain the specificity and sensitivity of this technique and to compare it with the usual techniques for the measurement of bone remodeling. ROC analysis of BR was performed and compared with Dpd, a usual bone marker of resorption. In the case of BF, the ROC analysis was compared with bAP. Table 1 shows the values of the area under the curve (AUC), threshold, sensitivity, specificity, positive predictive values (PPV) and negative predictive value (NPV) of each test. The threshold displayed in Table 1 is the value that produces the best separation between rats with low and high states of bone remodeling.

The test for the comparison of ROC curves showed that no differences exist between Dpd and BR as markers of the bone resorption process (ROC.test $p = 0.1827$). Similarly, no differences were found between bAP and BF as bone formation markers ($p = 0.79$).

Validation of the new methodology in biological models with modified bone remodeling

Values of BR and BF for each group are shown in Table 2; they coincide with the expected values for each model.

Table 1 Values of the AUC, threshold, sensitivity, specificity, PPV, NPV, TP, TN, FP and FN of the different techniques (Dpd, BR, bAP and BF) are shown

	Dpd	BR	bAP	BF
AUC	0.79	0.87	0.92	0.82
Sensitivity	0.85	0.89	0.89	0.85
Specificity	0.67	0.88	0.85	0.82
Threshold	155 (nmole/mmole creatinine)	14 (nmole/min)	48.5 (U/l)	14 (nmole/min)
PPV	0.68	0.86	0.83	0.80
NPV	0.85	0.91	0.90	0.87
TP	23	24	24	23
TN	22	29	28	27
FP	11	4	5	6
FN	4	3	3	4

Table 2 BF, BR, Dpd, and bAP values for each biological model analyzed

Groups	BF (nmole/min) ± SEM	BR (nmole/min) ± SEM	Dpd (nmole/mmole creatinine)	bAP (U/l)
Controls	16.25 ± 4.41	13.12 ± 3.40	403.8 ± 177.0	84.5 ± 12.5
OVX	29.65 ± 19.19	30.64 ± 19.19	391.8 ± 175.7	110.9 ± 13.6
OVX + Hyper	17.52 ± 11.99	18.07 ± 12.14	34.0 ± 7.3	28.0 ± 4.6
OVX + Z	11.04 ± 4.14	12.37 ± 4.50	166.1 ± 70.2	23.3 ± 6.8
OVX + Hypo	31.15 ± 30.05	44.10 ± 18.70	613.6 ± 352.2	143.3 ± 21.2

Data are expressed as mean ± SEM

Also in Table 2, Dpd and bAP are shown. As expected, the average values of BR and BF in OVX rats are higher than those of controls. Coinciding with this model, BR is higher than BF in OVX rats. Consistently, OVX + Z, OVX + Hyper and OVX + Hypo have expected BF and BR values compared to OVX rats.

Discussion

In conclusion, BF and BR results obtained with this technique were what was expected for each biological model. In addition, the ROC analysis demonstrated that BF is as specific and sensitive a marker of bone formation as bAP is. Similarly, BR is as specific and sensitive as Dpd.

Bone remodeling measurement is an invaluable tool for making a decision about which treatment should be applied in order to improve bone mineral density, bone microarchitecture and bone resistance to fracture. Commonly used bone markers have several disadvantages, such as cost, having to measure the two processes of bone remodeling separately, and the high amount of blood needed to perform the assay. The study of bone remodeling in laboratory animals is even more expensive and has less application potential. There has been a constant search for a methodology that allows us to distinguish between both bone processes in less time and with more sensitivity. In this work, the development of a new methodology to measure bone remodeling in rats is presented, one that uses a combination of the pharmacokinetic processes of fluoride with mathematical modeling. The technique we developed allowed us to calculate the pharmacokinetic values of fluoride after an intravenous injection in the rat. The procedure requires the measurement of fluoride concentrations in urinary samples and only one small blood sample. Pharmacokinetic parameters allow the simultaneous measurement of bone formation and resorption processes. BF and BR values calculated from the different modified bone remodeling biological models that we studied coincided with the values expected for each model.

Receiver operating characteristic analysis was used to determine the values of BF and BR for establishing states of low and high remodeling, as well as the specificity and sensitivity of the technique. It was also used to compare the new technique with accepted bone markers, such as Dpd for bone resorption and bAP for bone formation. The ROC analysis showed that BR is a bone resorption marker as good as Dpd. The same result was obtained when comparing BF and bAP. It is worth pointing out that the values of Dpd were not coincident with the expected increased values of bone resorption in OVX rats.

Another advantage of this methodology is the low cost, as it requires only an initial investment in the fluoride electrode; after that, the costs are negligible. The fluoride electrode can function for 20 years. Its value is comparable to 100 determinations using the usual bone markers.

In addition, the technique allows for the measurement of bone formation and bone resorption simultaneously, whereas biochemical bone markers are specific for each process. Drugs with an expiration date are not needed, such as antibodies that have to be used within a fixed period of time. Moreover, this methodology requires a lesser volume of blood than the volume required by normal methodologies. As with other markers, the value of bone resorption or formation can be obtained in 1 day. The dose of fluoride needed is 40 times lower than the dose used for osteoporosis treatment [13], and it is similar to the fluoride intake from water with 1 ppm of NaF, a concentration accepted by WHO [14].

The disadvantage is that the measurement needs 4–5 h of anesthesia in order to perform the urethral catheterism and the injection in the vein of the tail. This is a disadvantage when the handling of the anesthetics is not optimal. Dpd or bAP need only blood or urine samples.

It is important to highlight that the method is not applicable in its current state of development for the measurement of bone remodeling in patients. However, this methodology could be applied to humans when anesthesia and intravenous administration of fluoride can be avoided. The mathematical model should be modified considering the mentioned disadvantages, and especially with the collection of a unique urine sample rather than a serial sampling as in rats. The research to overcome the need for anesthesia and intravenous injection is in progress,

and the experience gained from the application to animals would result in benefits for the development of a human-equivalent methodology. Although avoiding the mentioned disadvantages is a challenge, one invaluable advantage is that the pharmacokinetic process underlying the mathematical model in rats is not different from that in human beings.

Although BR is not different from Dpd as a bone resorption marker, it is worth noting that BR has slightly higher PPVs and NPVs than Dpd. This indicates that this test slightly differentiates more positive results when there really is a modification of bone remodeling. On the other hand, bAP has slightly higher values of AUC, PPV, and NPV than BF. However, both markers estimate bone formation without any difference among the techniques.

Acknowledgments The authors thank Barbara Garcia and Hilda Moreno for technical assistance. This work was founded by Consejo Nacional de Investigaciones Científicas y Tecnologicas (PIP 112-200801-00462).

Conflict of interest All authors declare that they have no conflicts of interest.

References

1. Puche RC, Rigalli A, Bocanera R, Tozzini R, Poudes G, Roberti A (1990) A Clinical test for calcium absorption in climacteric women. A two traces, single blood sample procedure with corrections for body size and skeletal turnover. Medicina 50:16–20
2. Puche RC, Rigalli A, Trumper L, Dip O, Pereyra JL, Poudes G, Roberti A, Bocanera R, Tozzini R (1991) Estimation of bone turnover in climacteric women by the whole body retention of fluoride. Maturitas 14:57–64
3. Rigalli A, Ballina JC, Beinlich A, Alloatti R, Puche RC (1994) Pharmacokinetics differences between sodium fluoride and sodium monofluorphosphate and comparative bone mass increasing activity of both compounds, in the rat. Arzneimittelforschung 44:762–766
4. Lupo M, Buzalaf MAR, Rigalli A (2011) Effect of fluoridated water on plasma insulin levels and glucose homeostasis in rats with renal deficiency. Biol Trace Elem Res 140:198–207
5. Olfert ED, Cross BM, McWilliam AA (1993) Canadian Council on animal care Guidelines. Guide to the care and use of experimental animal. http://www.ccac.ca
6. De Candia F, Rigalli A, Di Loreto V (2009) Anesthesia and analgesia. In: Rigalli A, Di Loreto V (eds) Experimental surgical models in the laboratory rat. Taylor and Francis Group, CRC Press, Boca Ratón, pp 21–30
7. Lupo M, Di Loreto V, Rigalli A (2009) Substances administration. In: Rigalli A, Di Loreto V (eds) Experimental surgical models in the laboratory rat. Taylor and Francis Group, CRC Press, Boca Ratón, pp 55–61
8. Rigalli A, Alloatti R, Puche RC (1999) Measurement of total and diffusible serum fluoride. J Clin Lab Anal 13:151–157
9. Farley JR, Chesnut CJ, Baylink DJ (1981) Improved method for quantitative determination in serum alkaline phosphatase of skeletal origin. Clin Chem 27:2002–2007
10. Di Loreto V, Pera L, Rigalli A (2009) Ovariectomy. In: Rigalli A, Di Loreto V (eds) Experimental Surgical Models in the Laboratory rat. Taylor and Francis Group, CRC Press, Boca Ratón, pp 149–153
11. Brance ML, Brun LR (2009) Histological procedures. In: Rigalli A, Di Loreto V (eds) Experimental surgical models in the laboratory rat. Taylor and Francis Group, CRC Press, Boca Ratón, pp 221–227
12. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, Müller M (2011) pROC: an open-source package for R and S + to analyze and compare ROC curves. BMC Bioinformatics 12:77. doi:10.1186/1471-2105-12-77
13. Brun L, Pera L, Rigalli A (2010) Bone morphometry and differences in bone fluorine containing compounds in rats treated with NaF and MFP. Biomed Pharmacother 64:1–6
14. Fawell J, Bailey K, Chilton J, Dahi E, Fewtrell L, Magara Y (2006) Fluoride in drinking-water. World Health Organization, IWA Publishing, London