Use of histological samples for assessing skeletal calcium

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SUMMARY Bone samples were decalcified using 5% formic acid. Their calcium content was then quantified by analysing the calcium extracted in the decalcification solution. This was correlated with matched samples digested in hydrochloric acid. No significant differences were found. This indicates that bone biopsy specimens taken for histological analysis can be used for the direct assessment of skeletal calcium. The method can therefore simplify investigations into metabolic bone diseases and provide a direct correlation between skeletal calcium and bone histology.

When investigating metabolic bone diseases, it is advantageous to monitor changes in bone histology and for these to be related to changes in calcium homeostasis by detailed calcium balance studies, photon absorptiometry, or computed tomography. These metabolic methods are time consuming and expensive, and there may be poor correlation between bone loss at different parts of the skeleton.¹

The aim of the present study was to develop a technique for quantifying the calcium extracted during the decalcification of bone samples taken for histological analysis. This would give a direct comparison between bone histology and calcium state at the same sample site.

Material and methods

The method entailed the use of paired embryonic avian long bones and homogenised sheep bone, both decalcified in formic acid; the method was validated against matched material dissolved in hydrochloric acid.

Thirty six paired intact and cleaned embryonic chick femurs were prepared. These were taken from three similar size groups of 14, 17, and 20 day old embryos. The wet weights of these femurs ranged from about 40–250 mg and their calcium content from 0.025–5.00 mg per bone. They were randomly allocated to new 10 ml plain "Labco" blood collecting tubes containing 7 ml of either 6N hydrochloric acid or 5% formic acid in a 10% solution of formalin.² The tubes were capped and then left for a total of four

days. Those containing the hydrochloric acid were heated in a heating block at 105°C for 12 hours to extract any calcium present. The method was repeated using five known weights of powdered dried or ashed adult sheep femur.

Calcium was then determined on the supernatants of all solutions. Duplicate aliquots were sampled on a Micro-Medic dispenser and dilutor and diluted with 0.25% lanthanum chloride in distilled water. The diluted solutions were dispensed into disposable 4 ml autoanalyser cups to give a final volume of 2 ml and an assay range from 0–5 mg/l. The dilution of the original 7 ml samples ranged from 10 to 200 times.

Calcium was determined with a Perkin Elmer Model 370 atomic absorption (AA) spectrophotometer, using recommended operating conditions and assay methods.³ The standard curve was 0 to 15 samples determined in a single assay, with the matched samples assayed concurrently to minimise any effects of machine drift. BDH Analar grade reagents and distilled water were used throughout. The calcium content of all solutions was checked against BDH Analar grade water.

As histological samples are fixed in buffered formalin before decalcification any possible decalcifying effect of the buffered formalin was monitored. The calcium released into the supernatant of 10 tubes, each containing four 20 day embryo femurs in 7 ml of buffered formalin, was determined after one, two, five, and 20 days. The solution was changed at each sample interval.

To determine if residues of the buffered formalin carried over into the decalcification solution would affect the results the protocol was repeated on five sets of powdered sheep bone extracted in the formic or

hydrochloric acid but spiked with 5% buffered formalin—that is, a percentage larger than any likely to be present.

Results

The mass of calcium (mg) present in the volume of solution used was as follows: buffered formalin (0.0017), 6N hydrochloric acid (0.0126), 5% formic acid (0.0070), Autoanalyser cup (below 0.0002). These levels of contamination were negligible compared with the calcium content of bone.

POSSIBLE DECALCIFICATION BY THE BUFFERED FORMALIN

A mean mass of 0.046 mg of calcium was detected at day one in the first volume of buffered formalin used, but none was detected in the subsequent solutions. The mass found on day one was equivalent to less than 0.1% of the total bone calcium present. It was assumed that this initial extraction was due to blood present in the tissue and not to decalcification.

CORRELATION BETWEEN TWO METHODS OF DECALCIFICATION

Paired embryonic femurs

The figure shows the correlation between the calcium content of matched femurs extracted by the two meth-

Table Mean (1 SD) calcium content as percentage of dry weight of dried and ashed sheep bone

	Dried bone		Ashed bone	
	Mean	1 SD	Mean	1 SD
Hydrochloric acid only	28.72	0.88	42.34	0.81
Formic acid only	29.26	1.16	44.28	0.77
Hydrochloric acid + 5% formalin	29.28	0.91	42.40	1.08
Formic acid + 5% formalin	30.12	0.55	44.02	1.21

Using Student's *t* test these differences were not significant at the 1% level.

ods. The correlation coefficient was 0.9974 with the regression of the line $Y = 0.0407 + 0.9732 X$, where *Y* is formic acid and *X* is hydrochloric acid—that is, formic acid decalcification correlates to within 2% of the mass extracted into hydrochloric acid for bones containing 1.00 mg or more of calcium.

Dried or ashed adult sheep bone

The table shows the mean percentage of calcium extracted from five weighed samples of either ashed or dried sheep bone, with or without 5% added buffered formalin.

These results matched those seen with the chick femurs, with no significant differences between the two acids. They also showed that the results were unaffected by any buffered formalin residues.

Discussion

These results indicate that calcium can be extracted quantitatively from bone during decalcification with formic acid, and these results closely match those obtained with hydrochloric acid digestion. Using standard hospital consumables there were no significant blank problems; consequently the method can be used with the minimum of sample and tube preparation. As buffered formalin did not decalcify the samples this determination can be carried out any time after fixation—that is, on old stored histological samples. Although the present study used biopsy sized samples, the method could easily be scaled up for the examination of larger bone samples so long as they were decalcified in separate containers.

Other groups have determined the calcium present in decalcification solutions using aqueous samples⁴ and organic edetic acid.⁵ Both groups, however, used their method to determine the end point of decalcification, with the samples decalcified as a group, and not as an assay for bone calcium.

We suggest that, with the technique described, skeletal calcium can be determined on bone biopsy specimens without compromising their histological analysis in any way. Additional information can therefore be obtained on the patient's calcium state at the site

Figure Calcium content (mg) per bone of matched femurs extracted in formic acid or hydrochloric acid.

of interest, with the minimum of distress to the patient, inconvenience, and assay cost.

The method can also be used in experimental investigations of bone growth and repair. In laboratory investigations we showed that the method can be used to obtain direct comparison between bone growth and calcium state and incidentally reduce the numbers of experimental animals needed.

By decalcifying mineralised tissue in separate tubes their calcium content can be determined easily and economically. This method is applicable to samples previously stored in buffered formalin. This combined approach could simplify and improve the investigation of metabolic bone diseases and experimental orthopaedics.

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