Simple estimation of blood content in bone homogenates for the determination of the real amount of drugs in bone tissue

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Summary. The content of blood in bone homogenates can be determined quantitatively by estimation of the haemin in the homogenate and in a sample of peripheral blood taken at the same time. Haem is completely split from the haemoglobin molecule by reaction with hydrochloric acid-acetone and converted to haemin, which can be analyzed spectrophotometrically. The blood content can be calculated from the amount of haemin in the homogenate in relation to that of the peripheral blood. This method is useful to determine the real amount of drugs in bone tissue.

There is a growing interest in the distribution of drugs in the tissues. Although it is usually possible to estimate the total concentration of a drug in the tissues by appropriate methods, the accuracy of such determinations is often impaired by residual blood in the tissue samples or, in the case of samples removed surgically, for instance, by adherent blood, which is often difficult to remove. More exact data on the concentration of a drug in a given tissue could be obtained if one could determine the amount of blood present in the tissue sample and then the amount of drug present in the same volume of blood, which is calculable from the concentration in the peripheral blood.

Our concern with these questions arose in the course of determinations of the quantity of cephacetrile present in the cancellous bone of the head of the human femur, which still contained blood. For measurements in bone homogenates, the method described by Meijer[2] of estimating the blood content on the basis of the haemoglobin content of the homogenized tissue, proved to have the drawback that only about half of the haemoglobin in bone homogenate could be recovered from the supernatant after centrifugation. Its inadequacy in assays of bone homogenate caused us to seek an alternative method.

As it was not possible to separate the entire haemoglobin molecule from the bone homogenate, an attempt was made to use only the prosthetic group in determining the concentrations. For this purpose, we utilized a method employed in molecular biology and devised by Winterhalter and Huehns[3] for isolating globin. With the aid of this method, the haemoglobin molecule can be split quantitatively into the 2 constituents apoprotein and haem, the prosthetic group being forcibly removed from the hydrophobic pocket between the E and F helices of the protein by a quick reaction with hydrochloric acid-acetone. The haem thus liberated can be used in the form of haemin for quantitative determinations of the blood content.

Materials and methods. Principle. Simultaneous removal of bone sample (head of femur) and a sample of peripheral blood (heparinized syringe or vacutainer). Liberation of haemin in the homogenate of the bone sample and in the blood sample by reaction with hydrochloric acid-acetone, spectrophotometric determination of haemin and calculation of blood volume.

Bone homogenate. Samples of bone, as removed surgically, are frozen, and, if large enough (e.g. head of femur), sawn into discs weighing about 5 g; the cortical layer is chiselled away and the cancellous bone weighed, wrapped in waxed paper and deep-frozen in dry ice. A bone mortar and pestle are precooled for about 30 min in a mixture of dry ice and isopropanol (approximately −77 °C). The bone mortar is a cylindrical pot of 10 mm-thick stainless steel (outer diameter: 8 cm; height: 6 cm); the 'pestle' is a close fitting, stainless steel cylinder about 12 cm long with 3 equidistantly spaced airvent slots approximately 1 mm wide, 0.5 mm deep and 6.5 cm long running from the base along the surface. All other implements used, such as spatulae, centrifuge tubes and a 2-kg hammer are likewise cooled for about 30 min in dry ice. The mortar is then placed on a metal plate and the disc of bone introduced. The pestle is inserted and rotated by gloved hand between vigorous blows of the hammer, until the bone is crushed. The bone powder is transferred with a spatula to the centrifuge tube and cold phosphate buffer pH 6.0 (one half volume by weight of the bone) added. The mixture is then homogenized for about 10 min in a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland).

Determination of blood volume

Reaction mixture 1:	0.25 ml	0.07 M phosphate buffer pH 6.0
(for calibration curve)	2.0 ml	1N HCl
	6.0 ml	acetone
	20 µl	blood[a] (peripheral sample)
	[a] or 10.0 µl	blood made up to 20 µl with 0.9% NaCl solution
	12.0 µl	
	15.0 µl	
Reaction mixture 2:	750 mg	bone homogenate[b]
	2.0 ml	1N HCl
	6.0 ml	acetone
	20 µl	0.9% NaCl solution
	b = 500 mg	bone
	+ 0.25 ml	0.07 M phophate buffer pH 6.0

To release haemin, the mixture is shaken until the white apoprotein has completely precipitated, homogenized (Polytron homogenizer) and centrifuged for 2 min at 1000 × g. The supernatant is pipetted off and centrifuged for a further 2 min. The particles floating on the supernatant are removed by filtration through filter paper. The absorbances of the haemin hydrochloride are measured in the supernatant, or filtrate thereof, at 510 nm and 700 nm in 1-cm cuvettes in an UV-spectrophotometer against corresponding blank values. The instrument used was a Pye Unicam Spectrophotometer, SP 1800, connected to an Unicam AR 25 Linear Recorder (Pye Unicam Ltd, Cambridge, England). Blank values for the photometric measurement of reaction mixture 1 or 2 are obtained using mixture 1 but with 20 µl 0.9% NaCl solution instead of blood.

3 or 4 analyses are made of mixture 1, with varying quantities of blood; from the resultant differences between A_{510} and A_{700}, plotted on the ordinate against the blood volumes (µl) on the abscissa, a calibration curve can be constructed or the linear regression calculated (see discussion and figure 2), from which the blood content of 500 mg of bone (mixture 2, i.e. 750 mg bone homogenate = $^2/_3$ bone and $^1/_3$ buffer) can be read off or calculated. (The quantities of bone homogenate can be selected according to the blood content, e.g. 300–750 mg corresponding to 200–500 mg of bone, due account being taken of the volume of buffer (0.1–0.25 ml) used in mixture 1.)

Results and discussion. Since these estimations of the blood content are based on determinations of the haemin concentration, the identity of the 'haemin' liberated by the method

Table 1. Recovery of human blood added to human bone homogenate

	Blood added[a]	A_{510}	A_{700}	Blood found	
	µl			µl	% of blood added
Calibration curve[b]	10	0.128	0.006	–	–
	15	0.182	0.007	–	–
	20	0.239	0.008	–	–
Bone homogenate 450 mg[c]	0	0.090	0.012	6.01	residual blood of bone homogenate
	10	0.203	0.015	16.10	101
	15	0.263	0.017	21.42	103
	20	0.313	0.017	26.19	101

[a] Blood made up to 20 µl with 0.9% NaCl solution; [b] see reaction mixture 1; [c] see reaction mixture 2.

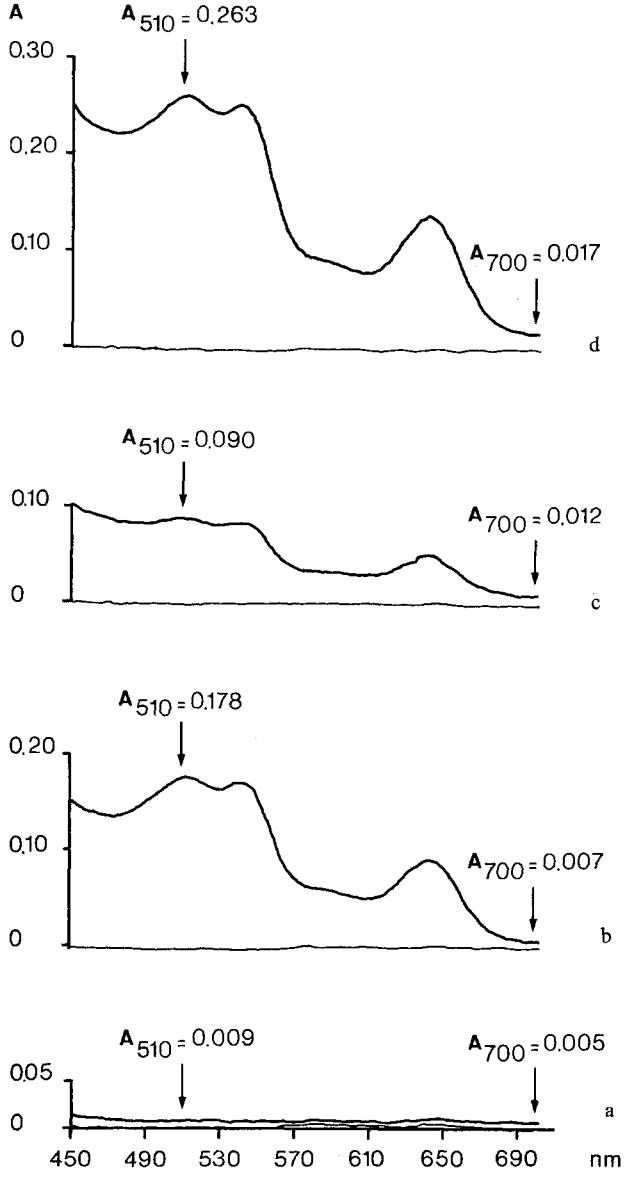

Fig. 1. *a* Absorption spectrum (AS) in centrifugation supernatant (CS) of exsanguinated bovine bone homogenate (⅔ spongiosa of caput femoris, ⅓ phosphate buffer pH 6: mixture 2). *b* AS of haemin from 15 µl human blood, mixture 1. Peaks: 510, 540 and 642 nm. *c* AS of CS from homogenate of human caput femoris containing blood, mixture 2. *d* AS of CS of bone homogenate as in c after addition of 15 µl human blood as in b. A_{510} and A_{700} of b and c are summed.

Table 2. Blood content of cancellous bone of head of femur in 6 patients with coxarthrosis

Patient No.	Age (years)	Sex	Blood volume ml/g spongiosa
1	59	♂	0.0336
2	66	♂	0.0174
3	58	♂	0.0205
4	62	♂	0.0249
5	77	♀	0.0052[a]
6	63	♂	0.0178

[a] Bone poorly perfused with blood.

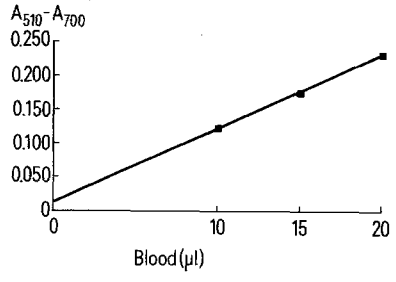

Fig. 2. Calibration curve for the determination of the blood volume in bone homogenate (see table 1).

described had to be verified with pure haemin. Comparison with pure substance prepared according to the method of Labbe and Nishida[4] showed no difference in the absorption spectrum. The native bivalent haem is rapidly oxidized after separation from globin to trivalent haemin. To make certain that all the haem was oxidized, potassium ferricyanide was added as an oxidizing agent; the absence of any shift in the absorption spectrum of the liberated 'haemin' proved that only haemin was present.

The selection of wave-lengths for measurement and correction was based on the spectra between 450 and 700 nm (Pye Unicam Spectrophotometer. The haemin spectrum obtained from 15 µl human blood with mixture 1 (figure 1, b) has 3 peaks: at 510, 540 and 642 nm. The highest peak (510 nm) was taken as the best wave-length for analysis. The only human bone preparation available consisted of blood containing cancellous bone (head of femur). As a model for the estimation of the photometric absorption due to the bone substance (blank value) we therefore used almost completely exsanguinated bovine bone (head of femur). The spectrum of the supernatant of mixture 2, prepared with bovine bone homogenate (figure 1, a), showed roughly constant absorbances throughout the bandwidth. Corresponding determinations with human bone

gave the haemin spectrum because of the presence of blood in human bone (figure 1,c); but since both species show roughly the same absorbance values at 700 nm, where haemin absorbs least, it can be assumed that the blank value of human bone, like that of bovine bone, is low and remains approximately constant throughout the bandwidth. Owing to the low absorbance of haemin at 700 nm and the fact that absorption due to the bone substance (blank value) is most readily detectable at this wave-length, 700 nm was selected as the best wave-length for correction of the haemin read-out. The addition of 15 µl of human blood (same blood as in figure 1,b) to the bone homogenate, of which the spectrum is shown in figure 1,c, gave absorbances in the haemin sprectrum at 510 and 700 nm equal to the sum of the 2 individual values (figure 1,d).

The suitability of the method described was demonstrated by analyses of bone homogenates to which blood had been added (table 1). The regression line $y=a+bx$, where y denotes the $A_{510}-A_{700}$ differences for the 3 analyses of mixture 1 and x the blood volumes of these mixtures (10, 15 and 20 µl) (values from table 1), gives an intercept on the y axis at $a=0.0125$, a slope of $b=0.0109$ and a correlation coefficient of 0.999.

$$\text{From the equation } x=\frac{y-a}{b}=\frac{(A_{510}-A_{700})-0.0125}{0.0109}$$

the recovered blood volumes listed in table 1 are obtained for the bone homogenate samples.

Heads of femora removed at surgery from 6 patients requiring prosthetic joints were available to us for analysis. The blood contents of the cancellous bone determined by the haemin method of analysis are shown in table 2.

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Organotin complexes of pyridine-2-carbothioamide

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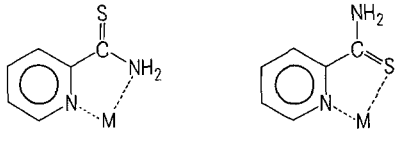
Summary. Complexes of pyridine-2-carbothioamide with diethyltin dichloride, dibenzyltin dichloride and phenyltin trichloride are described. In each case, the chelating agent is bound to the tin(IV) atom by the pyridine nitrogen and the carbothioamide sulphur, giving support to proposals that certain organotin compounds cause enzyme deactivation via the formation of tin-sulphur bonds.

Pyridine-2-carbothioamide could act as a bidentate chelating agent through the pyridine nitrogen and either the nitrogen or the sulphur of the carbothioamide group.

In previous investigations[2–5], the mode of coordination of the carbothioamide group has been inferred from infrared evidence. Bonds in the infrared spectrum of the free base have been variously assigned to N-H stretching and bending modes, to C-N stretching modes and to C=S stretching modes[6,7]. The infrared spectra of the metal complexes reportedly fall into 2 categories, those in which the absorptions due to N-H stretching and bending modes shift to lower energies while the C=S absorption remains unaltered from the values observed for the free base, and those in which the absorptions due to N-H deformations remain unaltered, the C-N absorptions are shifted to higher wavenumbers and the C=S absorptions are shifted to lower wave numbers. The metal complexes with infrared spectra in the 1st category are considered to involve the nitrogen-bonded carbothioamide group, while those in the 2nd category are considered to involve the sulphur-bonded carbothioamide group. The different modes of coordination of the ligand in the various metal complexes appear to correlate with the class A or B character[8,9] of the central metal ion, the class A metals being nitrogen-bonded and the class B metals being sulphur-bonded.

Reaction between pyridine-2-carbothioamide and the mono- and di-alkyltin halides used gave 1:1 complexes in each instance. Infrared data for the free base and the complexes are listed in table 1. For each of the complexes, the N-H stretching and bending modes occur at almost the same frequency as in the free base, the C-N frequency is raised, while the C=S stretching mode has been shifted to lower wave numbers. Thus these complexes fit into the second of the 2 categories described above, and are assigned 6-coordinate structures involving sulphur-bonding to the metal ion by the carbothioamide group.

Tin(IV) is a class A metal ion, and its complexes with pyridine-2-carbothioamide would be expected to be nitrogen-bonded. The unexpected preference by the tin(IV) ion in these complexes for coordination via the sulphur rather than the nitrogen of the carbothioamide group provides some support for the proposed mode of toxic action of dibutyltin diacetate in rats and mice[10], which is considered



Possible modes of coordination of pyridine-2-carbothioamide.

Table 1. Infrared spectral data (cm^{-1})

Compound	ν(NH)	δ(NH)	ν(CN)	ν(CS)
Pyridine-2-carbothioamide (L)	3350	1580	1405	800
$(C_2H_5)_2SnCl_2 \cdot L$	3350	1595	1415	790
$(C_6H_5CH_2)_2SnCl_2 \cdot L$	3340	1590	1420	785
$(C_6H_5)_3SnCl \cdot L$	3350	1590	1430	775