Proton Induced X-ray Emission
for Measurement of Bromine
in Micro Quantities of Human Blood Plasma

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Abstract

Studies have been made with proton induced X-ray emission to measure bromine in human blood plasma, and thereby estimate the extracellular fluid volume which is a determinant of biological importance. With careful attention to sample preparation, the method is precise. Since only very small amounts (less than 0·1 ml) of material are needed, the technique is applicable to the newborn and to small mammals.

1. Introduction

Extracellular fluid (ECF) surrounds the cells of the metabolically active tissue conveying nutrients and removing waste products of metabolism. For many years the halogens Cl and Br have been employed to measure ECF volume (see Cheek 1961 for a review). These halogens are distributed through the body in a very similar manner and allowance is made, for example, for the 10% halogen present in red blood cells, gastric and renal epithelium, and for that not present within the ECF as defined biochemically or anatomically (Cheek *et al.* 1957). The extracellular volume (ECV) can therefore be found by measuring the Br concentration in blood plasma three hours after oral or intravenous administration of NaBr so as to elevate the plasma bromine concentration from a resting level of $0·06$ mmol l^{-1} up to $1–2$ mmol l^{-1} (a sedative level is about 5 mmol l^{-1} and 'bromism' occurs at $15–20$ mmol l^{-1}).

The purpose of the present paper is to draw attention to the fact that very small quantities of fluid—as low as 4 μl—can be analysed for bromine by proton induced X-ray emission (PIXE) using strontium as an internal standard. Conventional methods of analysis require much larger quantities, about 1 ml.

The principle of PIXE as applied to biological materials has been described and reviewed in detail by Cheek *et al.* (1979), Mangelson and Hill (1981) and Deconninck (1981). A proton beam excites atoms such that each emits the characteristic X-rays K_α and K_β in constant proportions. The energy emitted is related to the atomic number, and hence the peaks in an energy spectrum produced by various elements separate and the potential exists to measure multiple trace elements simultaneously.

During a previous study (Cheek *et al.* 1979) of Zn, Cu, Fe and Cr in plasma it was noticed that samples of plasma which contained about 1 mmol l^{-1} of added Br showed strong Br X-ray peaks which were well isolated from other X-ray peaks. Thus, PIXE appeared suitable for the quantitative measurement of Br. However, the initial results consistently underestimated the amount of Br, and they had an

unacceptably large variability. The use of Sr as an internal reference overcame these problems, and it also showed that they arose from difficulties in converting the plasma into a form which met the rather stringent specifications we had set for quantitative PIXE.

In the present paper a method which has been developed for making suitable targets is described. Estimates of Br by PIXE are compared with determinations made by two other established procedures. It is shown how a detailed examination of the X-ray spectra can provide a check on the quality of the results, and also give clues concerning the nature and likely origins of difficulties encountered with target manufacture. Finally, some measurements of the ECF volume in children are presented.*

2. Experimental Methods

The equipment and methods used for PIXE have been described by Newton and Hay (1980). Experience has shown that it is straightforward, but time-consuming, to maintain stability of the proton accelerator, the X-ray detector and associated electronics and to keep them calibrated to within 2%.

Target holders which are exposed in vacuo to 2 MeV protons consist of a mylar film 4 μm thick stretched over a 15 mm diameter ring made of aluminium. The ring is pressed into a holder which in turn fits into a wheel so that 40 targets can be consecutively presented to the proton beam for about 10 minutes each.

The preparation of thin and uniformly spread samples of plasma on an inert backing film and the removal of excess protein is a complicated procedure. The layer needs to be thin since X-rays are absorbed in the target, the degree of absorption depending on their energy and the thickness of material that must be penetrated before reaching the detector. The normal amount of protein in plasma makes the targets thicker than desirable, not only increasing the loss of X-rays by absorption, but also increasing unwanted background radiation due to electrons which are generated by the passage of energetic protons. Additionally, when whole plasma is used, the dried protein tends to curl and become detached from the backing. However, if all the protein is removed the residual salt crystals do not adhere to the backing.

The difficulties can be largely overcome by the addition of an internal standard which is not normally present in the body or is present only in minute quantities. For Br determination such an element is Sr, since its X-ray emission lines are at energies not susceptible to confusion with other elements such as Na, S, Cl, K, Ca, Fe, Cu and Zn.

Plasma targets were prepared as follows:

(1) Mix one part of plasma with 19 parts of 85% MeOH; for example, 50 μl of plasma mixed with 950 μl of MeOH.

(2) Centrifuge at 2000 r.p.m. for 15 minutes.

(3) Take off 800 μl of supernatant and dry under nitrogen.

(4) Resuspend in 100 μl of 2 mmol l^{-1} of $SrCl_2$.

* Blood samples were taken by 'heel stick' from newborn, premature or dysmature infants with the permission of the Ethics Committee of The Queen Victoria Maternity Hospital. Blood samples were taken also from aboriginal children in various settlements with the permission of the Department of Health, Western Australia, the permission of the Aboriginal Councils and according to the principles of the Declaration of Helsinki.
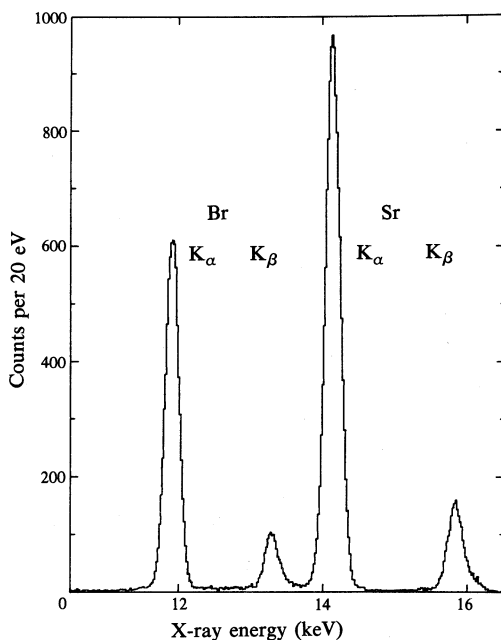
Fig. 1. An X-ray spectrum from a plasma target exposed for 10 minutes to 100 μC of 2 MeV protons. Note the separated energy peaks K_α and K_β for Br and Sr. The X-rays are counted and their energies measured by a Si(Li) detector.
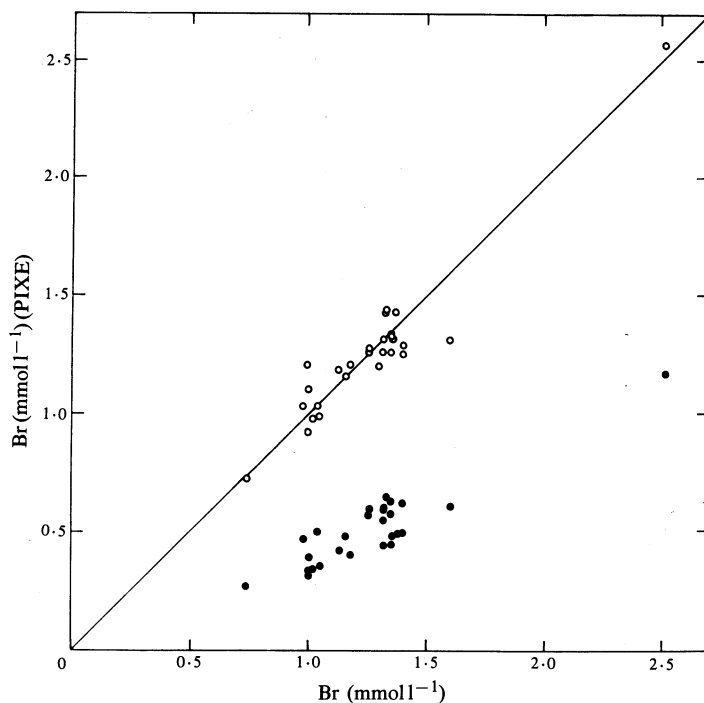

Fig. 2. The results of an analysis for Br of 27 samples of plasma. The abscissa is the average of results obtained by the microdiffusion (Graystone and Ortgies 1968) and the colorimetric (Goodwin 1971) methods. The ordinate records the present PIXE results, both with (open circles) and without (solid circles) an internal reference standard.

(5) Deposit 10 μl of the solution on mylar film over a 5 mm diameter and dry in a dessicator.

Step 1 precipitates 80% of the protein and the Br is readily taken up by 85% MeOH. The protein which remains assists uniform spreading and adhesion to the mylar at step 5.

Four targets were made from each plasma sample, since it is not possible to recover all of the solution by micropipette. At the time of deposition each target contained the same standard amount of Sr and also the Br contained originally within 4 μl of plasma. We show in Section 4 how analysis in quadruplicate permits one to distinguish between irregularities in X-ray yields due to unpredictable occurrences such as variations in the handling of the wet plasma extract, loss of dried material before bombardment by the proton beam, and changes in accelerator and spectrum collection conditions.

3. Results

A typical X-ray spectrum of the lines from Br and Sr is shown in Fig. 1. There are four peaks superimposed on a low background. The numbers of counts in the peaks depend on the number of atoms of Br and Sr in the target. The peak counts are extracted by a computer program which locates the peaks, finds where the base of each peak meets the background, sums the counts between these limits and subtracts background by interpolation between those portions of the spectrum which reside outside the peaks (Hay 1969, 1985). Since X-ray production is a random process described by Poisson statistics, the standard deviation expected for each peak, and for the ratios of peaks, can be calculated.

The number of atoms of an element in a target can be deduced from the number of X-rays it produces by utilising the known X-ray production probability by 2 MeV protons (Johansson and Johansson 1976), the geometry of the target–detector arrangement, and the efficiency of detecting and recording the spectrum. Fig. 2 illustrates a comparison of Br determined in this way for 27 samples of plasma, whose Br concentration was also measured by two other independent methods: by microdiffusion (Graystone and Ortgies 1968) and by a colorimetric method (Goodwin 1971). The PIXE results (solid circles) are consistently too small by a factor of 2·5.

Table 1. **ECV data measured by the present PIXE and the Goodwin (1971) methods**
Units are ml per kg of body weight

Subject	Goodwin	PIXE	Subject	Goodwin	PIXE
1	313	302	8	252	259
2	272	272	9	249	249
3	259	242	10	259	255
4	256	265	11	260	278
5	262	244	12	334	325
6	255	238			
7	269	275	Mean ± SD	270 ± 26	267 ± 26

From the amounts of plasma (4 μl) and Sr (10 μl of a 2 mmol l^{-1} solution) used to make the targets, and the ratio of the X-ray production cross sections [i.e. 8·11 and 3·90 b (Johansson and Johansson 1976)], it was expected that the Br : Sr yield ratio for an infinitely thin target would be 0·416 ± 0·008 per mmol l^{-1} of Br. Fig. 2 shows

(open circles) that application of this factor to the PIXE results brings them to within 1%, on average, of the other measurements. In addition, the standard deviation of the individual points from the regression line has reduced to 8%, from the 14% observed when only the Br yields were used. The reasons for this improvement are discussed in the next section.

The ECV data for 12 children from an aboriginal settlement are given in Table 1. The data, based on Br measurements by the present PIXE and by the Goodwin (1971) methods, do not differ statistically by paired analysis. They represent the first 12 analyses taken at random from the plasma of 30 aboriginal children.

4. Discussion

The results of Fig. 2 and Table 1 show that, with the use of Sr as a reference standard, it is possible to obtain quantitatively acceptable estimates of Br with the correct average values. However, detailed study of data from individual samples of plasma shows a variation beyond that expected from the X-ray statistics alone.

Table 2. X-ray measurements from (a) a 'typical' set and (b) a 'worst' set of four targets

Standard deviations (in parentheses) are given as percentages and are calculated for individual targets assuming Poisson statistics

$Sr\,K_\alpha$	$Br\,K_\alpha$	$Sr(\beta/\alpha)$	$Br(\beta/\alpha)$	Br_α/Sr_α
		(a) Counts from 'typical' set		
13114(0·9)	7267(1·2)	0·183(2·2)	0·164(3·6)	0·554(1·5)
12061(0·9)	7338(1·2)	0·178(2·2)	0·167(3·6)	0·608(1·5)
11305(0·9)	6887(1·2)	0·183(2·2)	0·177(3·6)	0·609(1·5)
11388(0·9)	6897(1·2)	0·182(2·2)	0·163(3·6)	0·606(1·5)
		Mean (SD) from four targets		
11967(7·0)	7097(3·4)	0·182(1·4)	0·168(3·8)	0·594(4·5)
		(b) Counts from 'worst' set		
31437(0·6)	5153(1·4)	0·178(1·5)	0·147(5·3)	0·164(1·5)
28994(0·6)	5092(1·4)	0·187(1·5)	0·151(5·3)	0·176(1·5)
10143(1·0)	5112(1·4)	0·186(2·6)	0·147(5·3)	0·504(1·5)
13398(1·0)	5096(1·4)	0·178(2·6)	0·173(5·3)	0·380(1·5)
		Mean (SD) from four targets		
20993(51·0)	5113(0·5)	0·183(2·6)	0·154(7·7)	0·306(54·0)

Table 2 gives the results from sets of four targets made from two different plasma samples. Table 2a is 'typical', while Table 2b is the 'worst' case we have encountered. The following effects are worth noting:

(a)   Column 1 of Table 2a shows the numbers of Sr counts recorded. The four observations have a standard deviation of 7%, about eight times larger than expected from counting statistics, whilst in column 2 the Br counts have a scatter about three times greater.

(b)   Column 1 of Table 2b shows that in some samples there is considerable variability in the data. The Sr yields in two targets vary by a factor of nearly 3. (In no case have we recorded more counts than the 34 000 expected from the amount of Sr intended to be in each target.)

(c)   In Table 2a, columns 3 and 4, the β/α ratios are satisfactorily close to the 'free atom' values, i.e. 0·183 for Sr and 0·168 for Br (Scofield 1974), and the scatter in the four observations is also acceptable.   However, in Table 2b, the β/α ratios differ by several standard deviations from the batch means, and that for Br departs significantly from 0·168.

It is therefore necessary to 'prune' the data.   Whilst variations of type (a) must be accepted, those such as in Table 2b must be rejected on the grounds of (b) or (c) or both.   A suitable criterion is to reject the data when the Sr counts differ by more than 10 times the standard deviation predicted for Poisson statistics, or when a β/α ratio differs by more than three standard deviations from the mean for all the targets made in a batch from several plasma samples.   We now consider possible reasons for the non-statistical scatter discussed above.

Systematic errors may arise from incorrect calibration of the accelerator and placement of the detector relative to the target.   For example, extreme errors such as 5% in proton energy and 5% in geometry change the X-ray yields by 15% and 10% respectively.   These are small compared with the factor 2·5 shortfall which has been found for the bromine yields.   The actual calibration errors were considerably smaller than 5%; Fig. 2 includes two sets of data for which the bromine factors are within 1% of each other.   The data were recorded a year apart, during which time the equipment was dismantled and used for other purposes.   Further, the effects of calibration errors are virtually eliminated by the use of Sr since the cross-section ratio changes by much less than 0·5% and the geometrical factors are identical for all X-rays.

There are three aspects of target making which may contribute to the observed variations.   They were identified from a variety of target making methods which were tried before the procedure described in Section 2 was adopted.

(1)   Only 10 μl of solution is spread as uniformly as possible over an approximately 5 mm diameter region of the plastic supporting foil.   This is achieved by delivering directly from the micropipette.   Occasionally an amount not equal to 10 μl may be placed on the target.

(2)   The targets do not all dry in the same way.   The most common pattern is a reasonably even thickness 'scab' broken by three to six radial cracks; if any pieces were to become detached during subsequent handling, then the X-ray yields would diminish in proportion [cf. (a) above and Fig. 2].   In a small number of cases a target can dry unevenly; either the central portion or the edges may be quite thick. By scanning across several such targets with a narrow (0·5 mm diameter) beam of protons we found that the Br and Sr distributions are irregular and dissimilar; hence loss of part of a target could change the relative yields of the two elements quite erratically, and differential absorption, which depends on the X-ray energy, could affect the β/α ratio as well [cf. (c) above].   The greater variability in Sr than in Br [cf. (a) above] suggests that their salts do not dry with identical distributions in the residual protein matrix.   This may be because the Br enters the plasma cells *in vivo*, whereas the Sr is added *in vitro*.   In fact, studies of targets made with 'blank' plasma to which both NaBr and $SrCl_2$ had been added *in vitro* did show increased variation in the Br yields, comparable with the usual variability for Sr.

(3)   The impact of the proton beam produces intense localised heating in the targets.   Usually the target becomes more brittle and darker in colour, whilst under

prolonged bombardment (e.g. six times the normal 10 minutes) the X-ray yield may decrease slowly (e.g. 1–2% each 10 minutes). Occasionally, however, the initial impact of the beam causes all, or a large part, of the target material to peel off the backing—this shows up as a sudden drop in counting rate. These effects of bombardment can reduce the total counts recorded, but the Br and Sr may not behave in similar ways [cf. point (2) above].

Therefore, not all of the Br and Sr in the original 10 μl of solution may be exposed to the protons. However, apart from a few cases which can be identified, the addition of Sr enables the Br measurements to be corrected for this loss and reliable (±8%) values for the Br concentration to be obtained.

In practice, the technique for making targets which has been developed produces targets with a high degree of uniformity in their Br : Sr ratios. Among over 500 targets fewer than 5% showed anomalies significant enough for them to be discarded; in most cases this still left three targets from the sample of plasma.

In summary, methods for recognising and rejecting 'bad' data, and some reasons for their origin, have been given. In Table 2b, application of our criteria rejects the first three targets and leaves only the fourth which has a Br : Sr ratio of 0·38; the Goodwin (1971) method for this sample gives 0·37. For Table 2a, the criteria do not lead to the rejection of any target. One might reject the first since its Br : Sr ratio differs from the others by five times the expected standard deviation. If this is done, the mean ratio changes by less than 3% from 0·59 to 0·61; for this sample the Goodwin value is 0·57.

5. Conclusions

Use of proton induced X-ray emission appears to be the most precise and most economical (in terms of plasma volume) method for the measurement of plasma bromine. Overall, the agreement with other methods is excellent when Sr is used as an internal standard of reference. Analyses in quadruplicate using PIXE are highly desirable since one can distinguish between variations in results due to inconsistencies in target making procedures, loss of material during transport to the location of the accelerator (in this case from Adelaide to Canberra) or during evacuation of the target chamber, and changes in target composition due to the proton bombardment itself. Moreover, they provide continuous monitoring of the accelerator conditions, the performance of the X-ray detection system and the line-shape analysis program.

The use of PIXE for the estimation of extracellular fluid volume by the use of bromine has been demonstrated with very small amounts of biological fluid, 5 to 50 μl. The simultaneous measurement of total body water using D_2O would allow the calculation of cell water and of cell mass, a parameter with highly important biological significance. The technique is applicable to the newborn and to small mammals.

PIXE represents a very powerful tool by which nanogram amounts of elements can be detected and measured precisely. Accuracy could be improved still further by improving sample preparation. Success for plasma has been obtained, by reduction of the protein content and deposition on a thin mylar film, and further attention to these approaches should make possible the simultaneous measurement of trace metals and other elements present in biological fluids.

Acknowledgments

References

Cheek, D. B. (1961). *J. Pediatr.* **58**, 103–25.
Cheek, D. B., Hay, H. J., and Newton, C. S. (1979). *Aust. Phys. Sci. Med.* **2**, 85–93.
Cheek, D. B., West, C. D., and Golden, C. C. (1957). *J. Clin. Invest.* **36**, 340–51.
Deconninck, G. (1981). *Nucl. Instrum. Methods* **191**, 543–50.
Goodwin, J. F. (1971). *Clin. Chem.* **17**, 544–8.
Graystone, J. E., and Ortgies, A. M. (1968). *In* 'Human Growth' (Ed. D. B. Cheek), p. 656 (Lea and Febeger: Philadelphia).
Hay, H. J. (1969). *In* 'Least Squares Methods in Data Analysis' (Eds R. S. Anderson and M. R. Osborne), p. 70 (Aust. Nat. Univ. Press).
Hay, H. J. (1985). *Nucl. Instrum. Methods* B **10/11**, 624–30.
Johansson, S. A. E., and Johansson, T. B. (1976). *Nucl. Instrum. Methods* **137**, 473–80.
Mangelson, N. F., and Hill, M. W. (1981). *Nucl. Instrum. Methods* **181**, 243–8.
Newton, C. S., and Hay, H. J. (1980). *Aust. J. Phys.* **33**, 549–59.
Scofield, J. H. (1974). *Phys. Rev.* A **9**, 1041–53.