Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Some Techniques in the Separation of Inorganic Ions in Biological Material

Taft Y. Toribara[a]
[a] Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York

To cite this Article Toribara, Taft Y.(1967) 'Some Techniques in the Separation of Inorganic Ions in Biological Material', Separation Science and Technology, 2: 3, 283 — 292
To link to this Article: DOI: 10.1080/01496396708049702
URL: http://dx.doi.org/10.1080/01496396708049702

SEPARATION SCIENCE, 2(3), 283–292 (1967)

Some Techniques in the Separation of Inorganic Ions in Biological Material*

TAFT Y. TORIBARA

DEPARTMENT OF RADIATION BIOLOGY AND BIOPHYSICS
UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY
ROCHESTER, NEW YORK

Summary

There are many studies of the effect of inorganic ions in biological systems in which trace amounts are of great importance. Unequivocal estimations of these ions often involve a separation, either partial or complete, of the ion of interest prior to final measurement. Three procedures—precipitation, absorption on an ion-exchange resin, and liquid-liquid solvent extraction—are described in some detail. The three procedures have been treated from the standpoint of the distribution of the ion between two immiscible phases. Examples of these techniques from the author's own experience have been described.

In studies such as the toxicological effects of inorganic ions or the effects of hormones and other substances on electrolyte balance, it is necessary to measure the levels of inorganic ions in various tissues and fluids. The ion of interest is usually at the trace level and interfering substances are present to prevent direct, accurate measurement. When it is not possible to remove or inactivate the interfering substances, the ion must be isolated. Very often extremely low concentrations require the treatment of a large amount of sample to obtain a sufficient amount for measurement, and the separation scheme also serves as a concentration process. Since very few chemical methods for the quantitative measurement

* This paper is based on work performed under contract with the U.S. Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N.Y.

of inorganic ions are specific, an unequivocal measurement is best made on the isolated ion.

The ever-present phosphate ion is almost always a major source of interference, for it often prevents separations by liquid-liquid extraction and ion-exchange resins as well as quantitative formation of colored or fluorescent compounds. The separation of trace levels of cations in bone is usually the most difficult problem. Here some preliminary separation in which the bulk interfering material is removed to a large extent is the simplest procedure.

PRELIMINARY SEPARATION

In the case of inorganic ions stable to heat, the most common method of disposing of a large amount of extraneous material is to ash the sample (either wet or dry). Since a large part of biological samples consist of organic matter, ashing results in the production of carbon dioxide and water, both of which are readily volatile. Dry ashing, which begins with a low-temperature treatment, also removes water, which forms an appreciable part of samples such as serum.

Wet ashing is used when the higher temperatures of dry ashing may render the desired ion insoluble or cause losses due to volatility. In the case of wet ashing, it is desirable to use all volatile reagents so that no extraneous material is added to the sample. Some preferred reagents are:

Acids:
1. Strong: HNO_3, HCl
2. Weak: Acetic, sulfurous

Base: Ammonium hydroxide

Oxidizing agents:
1. Hydrogen peroxide
2. Concentrated nitric acid

The oxidizing agents used in wet ashing are usually effective only under concentrated conditions (nitric acid) or at elevated temperatures (addition of hydrogen peroxide to a hot concentrated sulfuric acid solution), and the strong acids must be removed at the end either by volatilization or neutralization.

When large numbers of samples are to be handled, dry ashing is the preferred method. Greater care must be rendered in wet ashing in many cases to prevent losses by bumping or spattering.

Many biological samples contain protein, which may interfere with the measurement of the desired ion. A simple preliminary separation often employed is the removal of the protein by precipitation. Fortunately, proteins are capable of little cationic binding at pH's below 5(*1*). One of the most generally used reagents for protein precipitation is trichloroacetic acid, which simultaneously lowers the pH and causes the protein to precipitate.

QUANTITATIVE SEPARATIONS

Basically any separation step involves a distribution of a substance between two phases. The controlling factor is the distribution coefficient, which may be written

$$D = a_1/a_2 \qquad (1)$$

where a represents the activity of a particular substance and the subscript represents the phase. More commonly concentrations are used instead of activities because the latter are difficult to determine in phases such as organic solvents. By maintaining conditions such as temperature, ionic strength, and acidity constant, the following relationship holds:

$$D' = c_1/c_2 \qquad (2)$$

The techniques found most useful in the present work are:

1. Precipitation—phases are solid and solution.
2. Ion-exchange resin—phases are solid and solution.
3. Solvent extraction, liquid-liquid,—phases are two immiscible liquids.

The following sections will show how the distribution coefficient may be used in establishing limitations of the usefulness of the method.

Precipitation

In biological systems, precipitation is probably the least desirable of all the methods for the separation of micro quantities of a substance because of certain inherent difficulties. Here the desired material is made to form a very insoluble precipitate—a term which is only relative. In applying the distribution coeffiicent, the solid phase is the pure precipitate, which has a constant activity, and the solubility product is applicable (2). Increasing the concentration of the precipitant should decrease the amount of ion in solution, but

there are practical limits to this decrease (such as the solubility of the precipitant, the total volume, and other vague factors). The solubility loss may be minimized but not eliminated, and the magnitude of this loss sets the lower limit of the quantity which may be measured.

Ion-Exchange Separation

The preparation of synthetic ion-exchange resins introduced a very valuable tool for the analytical chemist in the separation of various ions. Here the solid phase is the ion-exchange resin, and the ion is distributed between the solid and the solution. Tompkins and Mayer (3) expressed the distribution coefficient as

$$K_D = (M_s/M_l)(v/m) \tag{3}$$

where M_s and M_l are, respectively, the fractions of the ion M in m grams of the resin phase and v milliliters of solution. Very often the distribution coefficient is not very favorable, and a batch process in which a quantity of resin is added to the solution much in the manner of a collecting precipitate cannot be used. However, a column technique which operates similarly to a fractionating column for distillation may be used. The length of the column necessary depends upon the distribution coefficient K_D—the larger the value of K_D the shorter the column.

Ion-exchange resin columns have been used extensively for chromatographic purposes, but that is an extensive subject in itself. In the present discussion the ion-exchange column will be considered as a means for removing an ion completely from the solution with or without other ions. The ion is then stripped from the column and a final separation is made by some other method when necessary.

Ion-exchange resins are prepared with widely varying properties in addition to the two general classifications of anionic and cationic resins. This permits a more varied choice of conditions in special cases. However, one of the most useful techniques in sharp separations utilizes the simple process of changing an element of interest from a cation to an anion, and then to employ an anion-exchange resin to separate it from all cations.

Solvent Extraction (Liquid-Liquid Extraction)

Of all the methods for separation of micro quantities, the distribution of a substance between two immiscible liquid phases is

probably the most versatile. Since the distribution coefficient D is not dependent on the quantity of a material present, it is possible to separate extremely small quantities with the same completeness as larger quantities. A great advantage of the method is that a wide latitude of conditions is permitted with the consequence that specificity may be increased.

By the choice of the organic phase with the appropriate density, the substance may be extracted into the top or bottom layer. When a single extraction suffices, the desired substance should be in an upper organic phase, because aqueous washing may be carried out without transfer to another vessel. If multiple extractions are required because of an unfavorable distribution coefficient, the substance to be extracted should move into a lower organic phase, which can be withdrawn easily.

Morrison and Freiser (*4*) use a more practical form of the distribution equation to express the per cent extracted, % E, as follows:

$$\% E = 100D/D + (V_w/V_o) \quad (4)$$

For the equal-volume case, they show graphically the relation between the per cent extracted and the distribution coefficient. For quantitative purposes a large D is desirable, but it is possible to use a single extraction as a method for the quantitative measurement of a substance even when the distribution ratio is not too favorable. Careful standardizations of conditions such as pH and ionic strength may be obtained easily by using buffers, and the volume ratio of the phases can be controlled by using pipets or other volumetric equipment. Since the % E will then be constant, a standard curve may be constructed by extracting varying but known quantities. The above expression shows that the precision to which the volume ratio must be controlled depends upon the magnitude of D and the accuracy demanded.

EXAMPLES

Calcium

The activity of the parathyroid hormone is measured by the increase in the serum level of calcium, and studies with the hormone require as unequivocal a determination of calcium as possible. The quantitative measurements were made by either flame photometry or EDTA titration. Interferences to flame photometry were caused by the presence of alkali metals and phosphate (as well as the pro-

tein), which seemed to counteract the effect of the phosphate (5). The EDTA titration gave erratic results in the presence of phosphate. The interferences were eliminated by separation of the calcium as the oxalate which was converted to the carbonate and dissolved in hydrochloric acid. The final measurement was then made on an essentially pure calcium chloride solution.

Incomplete precipitation of the calcium oxalate occurred on some aged serum samples (6) and those from patients who had been treated with EDTA. By first acidifying the sample with acetic acid, adding the ammonium oxalate, and the ammonia solution until the pH was 4.7 (bromocresol green indicator will just turn blue-green), essentially complete precipitation of the calcium was obtained even in the presence of equimolar amounts of EDTA (7). A practical lower limit to the loss by solubility was about 0.6 microgram, and a correction could be made by running known standards along with the samples.

Beryllium

In studying the toxicity of beryllium a preliminary distribution and excretion pattern had to be established, and this investigation required the quantitative measurement in a variety of biological samples. Bone proved to be the most difficult sample, inasmuch as relatively large samples were necessary to yield measurable quantities. The fluorescence of a beryllium-morin compound at pH above 11 was selected as the method of measurement, because of its sensitivity and the absence of interference from aluminum. However, a multistep separation was necessary and consisted of the following on a sample of bone ashed at 600° (8).

1. Solution of the bone in hydrochloric acid and precipitation of calcium sulfate by the addition of sulfuric acid.
2. Dilution of filtrate from previous step and passage through a cation-exchange column to remove all divalent ions.
3. Removal of beryllium with most other cations by 5 *N* hydrochloric acid.
4. Evaporation of solution to dryness and electrolysis of a perchloric acid solution with a mercury cathode.
5. Extraction of beryllium with acetylacetone in benzene at pH 4.5.
6. Back-extraction of beryllium into 5 *N* hydrochloric acid and evaporation to dryness.

Steps 1 and 2 are for separation from the large amount of phosphate. The bulk of the calcium was removed first so that a reasonable-sized ion-exchange column could be used. Steps 5 and 6 are a classical example of the effect of pH on the formation of extractable chelates, as discussed by Morrison and Freiser (*4*).

Plutonium

The study of the fate of inhaled plutonium involved the analysis of a variety of samples. Since plutonium is entirely radioactive, its measurement should be simple, but the usual isotope, plutonium-239, is an alpha emitter. It was found that the activity could be measured by liquid scintillation counting with essentially 100% efficiency in pure solutions or in the presence of limited amounts of certain ions (*9*). The separation of plutonium to an extent suitable for efficient counting was relatively simple, but to do so the sample had to be entirely in solution. The most difficult sample was fecal ash because a large amount of acid-insoluble residue remained, and acid treatment did not dissolve the plutonium. It was found that plutonium seemed to accompany a precipitate and to remain with the insoluble portion (*10*). To solubilize all the plutonium, it was necessary to use a sodium–potassium carbonate fusion. Table 1

TABLE 1

Distribution of Plutonium in Fecal Ash

Fraction	% of Pu
2 *N* HNO_3 extract	3.6
Water extract of carbonate fusion	14.5
HCl extract of carbonate fusion	82.3
Bisulfate fusion of residue	1.2

shows the distribution of the plutonium activity in a 2 *N* acid extract of the insoluble ash and in the several fractions of the carbonate-fused sample. These fractions could not be mixed because an insoluble precipitate would again result and most of the plutonium activity would again become unavailable. To collect all the plutonium from the various fractions into one sample for measurement, an anion-exchange column was used as a combination collecting and separating step. The various fractions were each made up to

about 8 *N* hydrochloric acid, and the solutions were passed successively through the same ion-exchange column. The plutonium was stripped from the column with sulfurous acid and measured by liquid-scintillation counting (9). The small amount of iron which accompanied the plutonium during the separation scheme did not interfere with the final measurement.

In this case it was possible to process four different solutions rather quickly because a very favorable distribution coefficient (*11*) permitted the use of a very short column through which a rapid flow rate could be maintained. The separation was relatively simple because most of the cations in biological samples do not form anionic complexes in strong hydrochloric acid solution, whereas plutonium forms one which is strongly retained on an anion-exchange column.

Thorium

The two principal sites of deposition of thorium in the body are the bone and liver. Because thorium phosphate is one of the few acid-insoluble phosphates, it would be expected that bone would present the most difficulty (*12*). To separate thorium from the bulk of the phosphate, a collecting precipitate of a ferric salt was used. In the case of bone, a second precipitation was necessary because the first carried along sufficient calcium phosphate to interfere with the subsequent step. The final isolation of the thorium was accomplished by its retention on a cation-exchange column from 6 *N* hydrochloric acid solution. None of the usual cations found in biological samples would be retained on the column under such strongly acid conditions, and the iron added in the collection step was converted to an anionic complex under these conditions. The thorium was eluted from the column with an ammonium carbonate solution, and evaporation to dryness completes the isolation.

Magnesium

As one of the major cations in biological systems, the importance of magnesium in both mineral metabolism and cellular function is the subject of much study. In the search for a simple, sensitive, and relatively rapid method for determining magnesium in serum, a liquid-liquid extraction procedure which separated the element in

a form suitable for quantitative measurement was developed (*13*). The major cations present are sodium, potassium, and calcium, with quantities of iron and copper sufficient to cause interference in certain samples. Because the presence of protein caused emulsions, serum samples were first deproteinized with trichloroacetic acid.

Luke and Campbell (*14*) found that a mixed solvent of chloroform and butoxyethanol would dissolve the water-insoluble magnesium 8-quinolinolate, and Jankowski and Freiser (*15*) made a more detailed study of the extraction of the compound in chloroform with other additives. The reagent 8-quinolinol forms colored extractable compounds with many metals, but biological systems do not contain many of them. Calcium, which by itself does not form an extractable complex, does seem to accompany any magnesium extracted (*16*).

For the biological samples, the addition of tartrate and cyanide to the buffer solution prevented the extraction of calcium, ferrous iron, and copper in quantities much larger than those expected to be in any sample. Using the isotope magnesium-28, the distribution coefficient was found to be 14.9, and a single extraction transferred 93% of the element to the organic phase, which consisted of butoxyethanol or isoamyl alcohol in 1,1,2-trichloroethane. The latter solvent is more suitable for quantitative work because it is much less volatile than chloroform. With a distribution coefficient of this magnitude, it was possible to use a standard curve and obtain results with a standard deviation of less than 1%.

REFERENCES

1. C. W. Carr, *Arch. Biochem. Biophys.*, **43**, 147 (1952).
2. S. Glasstone and D. Lewis, *Elements of Physical Chemistry*, 2nd ed., Van Nostrand, Princeton, N.J., 1960, p. 516.
3. E. R. Tompkins and S. W. Mayer, *J. Am. Chem. Soc.*, **69**, 2859 (1947).
4. G. H. Morrison and H. Freiser, *Solvent Extraction in Analytical Chemistry*, Wiley, New York, 1957, p. 12.
5. P. S. Chem, Jr., and T. Y. Toribara, *Anal. Chem.*, **25**, 1642 (1953).
6. T. Y. Toribara and L. Koval, *Talanta*, **7**, 248 (1961).
7. T. Y. Toribara and L. Koval, *J. Lab. Clin. Med.*, **57**, 630 (1961).
8. T. Y. Toribara and R. Sherman, *Anal. Chem.*, **25**, 1594 (1953).
9. T. Y. Toribara, D. A. Morken, and C. Predmore, *Talanta*, **10**, 205 (1963).
10. T. Y. Toribara, C. Predmore, and P. A. Hargrave, *Talanta*, **10**, 211 (1963).
11. L. Wish and M. Rowell, U.S.N.R.D.L. Rept. TR-117, Oct. 1956.
12. T. Y. Toribara and L. Koval, *Talanta*, **14**, 403 (1967).

13. T. Y. Toribara, L. Koval, and J. F. P. Olive, *Talanta,* **10,** 1277 (1963).
14. C. L. Luke and M. Campbell, *Anal. Chem.,* **26,** 1778 (1954).
15. S. J. Jankowski and H. Freiser, *Anal. Chem.,* **33,** 776 (1961).
16. C. L. Luke, *Anal. Chem.,* **28,** 1443 (1956).

Received by editor December 12, 1966
Submitted for publication February 16, 1967