KATTIAPPURATHU A. VALSALAN. Recent advances in analytical methodology of some clinically important compounds. (1976) Directed by: Dr. Harvey B. Herman

Here we have endeavored to document the recent advances, developments, and modifications of the analytical methodology of some of the clinically important compounds. Selectivity has been exercised to limit the survey and evaluation only to a very few compounds, specifically vitamin B_1, vitamin B_6, carbonates, and phosphates. They have been chosen by virtue of their importance in clinical or pharmaceutical chemistry.

First of all, the analytical methods for the compounds in question are not highly accurate, as the methods quite often are cumbersome and time consuming. Secondly, it is our hope that this would prove to be an excellent guide for future scientists who are interested in doing further study in this field.

We have discussed the chemical basis of the analytical methods. Instrumental and procedural details have been kept to a minimum. It is difficult to draw a demarcation line between the old and the recent developments. But emphasis has been given to the publications that have appeared during the last decade and a half and up to December, 1975, and at the same time we have selected earlier references whenever it was pertinent.

RECENT ADVANCES IN THE ANALYTICAL METHODOLOGY OF SOME CLINICALLY IMPORTANT COMPOUNDS

by

Kattiappurathu A. Valsalan

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
May, 1976

Approved by

Harvey B. Herman

Harvey B. Herman

APPROVAL SHEET

This thesis has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Thesis Adviser — Harry B. Herman

Oral Examination Committee Members — Walter D. Patterson

T. A. Nile

May 6, 1976
Date of Examination

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Professor Harvey B. Herman for his guidance and constructive criticism as a thesis advisor of this research. I am also thankful to the remaining members of the faculty for their help and encouragement.

I gratefully acknowledge the financial support in the form of a teaching assistantship from the University of North Carolina at Greensboro during the course of my studies and research. Thanks are also due to Mr. Joseph J. Guanci, Jr. for valuable discussions.

TABLE OF CONTENTS

VITAMIN B_1 AND B_6 ANALYSIS

INTRODUCTION

This survey includes analytical methodology of selected vitamins B_1 and B_6, that have appeared mainly during the last fifteen years and up to December, 1975. The emphasis is given to clinical and pharmaceutical methods of analysis; nevertheless, mention has been made to the vitamin assay of foods and nutrients, from time to time. The survey indicates a substantial number of publications have appeared in the sixties and the present flux of publications are in foreign journals. Optical methods of analysis continue to be popular, while the recent developments in electrochemical techniques have been fastly implemented as the analytical method of choice. Many of them are on a trial stage. It is worth mentioning that the introduction of ion-selective electrodes in clinical chemistry is gaining a tremendous momentum.

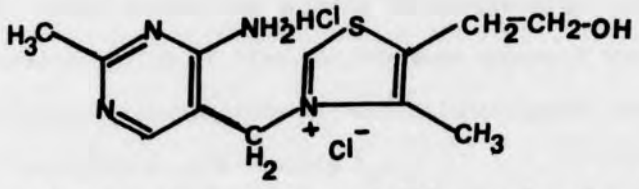
Chromatographic and ion exchange methods are also widely used depending upon the source of the sample, degree, and types of impurities present. The colorimetric method usually requires pure samples.

There is a difference between results obtained by chemical and microbiological methods and the latter gives higher results probably due to the measurement of non-thiamine materials in the latter procedures or the incomplete extraction or recovery of vitamin in the former procedures.

Mention also has been made to some uncommon methods like titrimetric, gravimetric, and refractometric methods, etc. They are not of prime importance as they are time consuming and require pure samples and such methods are not of much use in a clinical chemistry laboratory.

PRELIMINARY CONSIDERATIONS[1,2] VITAMIN B_1

First breakthrough came in 1890 when it was discovered that Beri-Beri was caused by the deficiency of vitamin B_1. This discovery led to the isolation and characterization of vitamin B_1, which was later named thiamine. Thiamine exists either in the free or combined form and is present in natural foods and biological materials. Thiamine hydrochloride is more popularly known as vitamin B_1 and has the structural formulas:

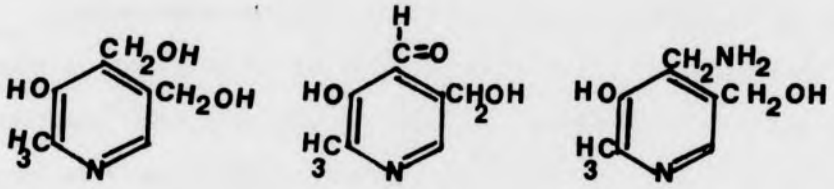


Thiamine is a white crystalline powder when it is pure. It has a yeasty odor, and a slightly nutlike taste. Thiamine can be purified by crystallization from alcoholic aqueous solutions. The crystals formed are hemihydrate and monoclinic in shape.

PRELIMINARY CONSIDERATIONS VITAMIN B_6[3]

Vitamin B_6 exists in three forms and is a composite of a group of vitamins popularly known as vitamin B complex. The structural

formulas of the three vitamin B_6 are given below:



Pyridoxine Pyridoxal Pyridoxamine

Pyridoxine was the first to be isolated. Evidence for the presence of growth promoting factors surpassing pyridoxine led to the discovery of two other vitamins viz pyridoxal and pyridoxamine. It has been recognized that only certain types of plants and animals need vitamin B_6, while others are capable of synthesizing by itself. From a chemical point of view, it has been observed that, certain acids like sulphuric, phosphotungstic and silicotungstic are capable of forming a precipitate with vitamin B_6.

The division of analytical methodology has been done well in accordance with the principles underlying the procedures. In some cases, it may be questionable as to whether a certain type of classification is very appropriate, nonetheless the best has been done.

METHODS AVAILABLE

Electrochemical Methods

Electrochemistry involves the electron transaction or proton transfer, resulting in the flow of electricity. Electrochemical techniques[4] have been used in analytical chemistry over a long period of time, perhaps over one hundred years, but with limited application in clinical chemistry. But recently with the modifications of the various types of electrodes, clinical application of electrochemistry gained great momentum. With the advent and introduction of ion-selective electrodes in clinical chemistry, a whole new area of research has been opened up. Also other electrochemical methods like potentiometry, amperometry and voltametry have also been introduced to support and supplement some of the existing analytical methods. Coulometry which involves the electron requirements of the redox reactions has come to the attention of clinical chemists as a useful analytical tool.

Probably the latest development in electrometric methods, for clinical applications, is the ion-selective electrodes. Ion-selective electrodes have been developed for the determination of vitamin B_1 and B_6. The liquid membrane electrodes developed by Nobyko[5] exhibit appropriate Nerstian responses to vitamin B_1 and B_6 down to 10^{-5} M in concentration. High selectivities for vitamin B_1 and B_6 over Na^+, NH_4^+ and K^+ were also reported. But below a concentration of 10^{-5}, deviation from Nerstian behavior has been observed. The reason is

not well understood but could be due to the elution of membrane solution to the adjacent aqueous solution. Deviation at a higher concentration may be due to not taking into account the activity coefficient. The same authors also published their work on the development of ion-selective electrodes[6], selective to organic compounds other than vitamin B_1 and B_6. The selectivity of the membrane mainly depends on the membrane solvent. Different types of ion exchange sites were used in the membrane.

Oscillopolarographic[7] and polarographic methods also have been employed for vitamin analysis. In a polarographic method, the waveheight is proportional to the concentration of electroactive species. Kale and Fahr[8] determined thiamine by polarographic method. They explained the best potential range and the indifferent electrolyte used. The results were comparable to those which were obtained by traditional methods.

Another method worth mentioning is the determination of thiamine in pharmaceutical preparations by making use of catalytic current[9] of Cobalt (II) thiamine disulphide system. Thiamine was determined polarographically with a DME Cathode and a SCE reference in a supporting electrolyte of Borax-NaOH at pH 10.3 in presence of Co^{+2}. The cobalt catalytic pre-wave is not affected by the presence of other water soluble vitamins. The limit of detection was in the range of 0.1 μg/ml. and relative error was less than 3.5 percent.

Fluorometric Method

Fluorometric method of analysis is highly sensitive. The principle involves the determination of characteristics and amount of fluorescence as a result of the return of the excited electron to the ground state.

Many molecules have the property of absorbing radiation at a specific portion of the electromagnetic spectrum and many molecules do possess the property of reemitting part of the radiation, which appears as luminiscence.

Luminiscence can be broadly classified into two groups, depending upon the time taken for the reemission of radiation. Of course, there are other methods of fluorescence, of which we are not concerned here. When a molecule absorbs radiant energy, ground state electrons are raised to the excited state. When the electrons return to the ground state, with reemission of energy (from singlet to singlet) the phenomena is known as fluorescence and the time taken is 10^{-8} seconds or less. On the other hand if the electron passes through a triplet state and returns to ground state, the time taken is usually longer and the process is referred to as phosphorescence. Electron transition from singlet to triplet and back is not very common, as it is forbidden by selection rules, nevertheless it happens very rarely.

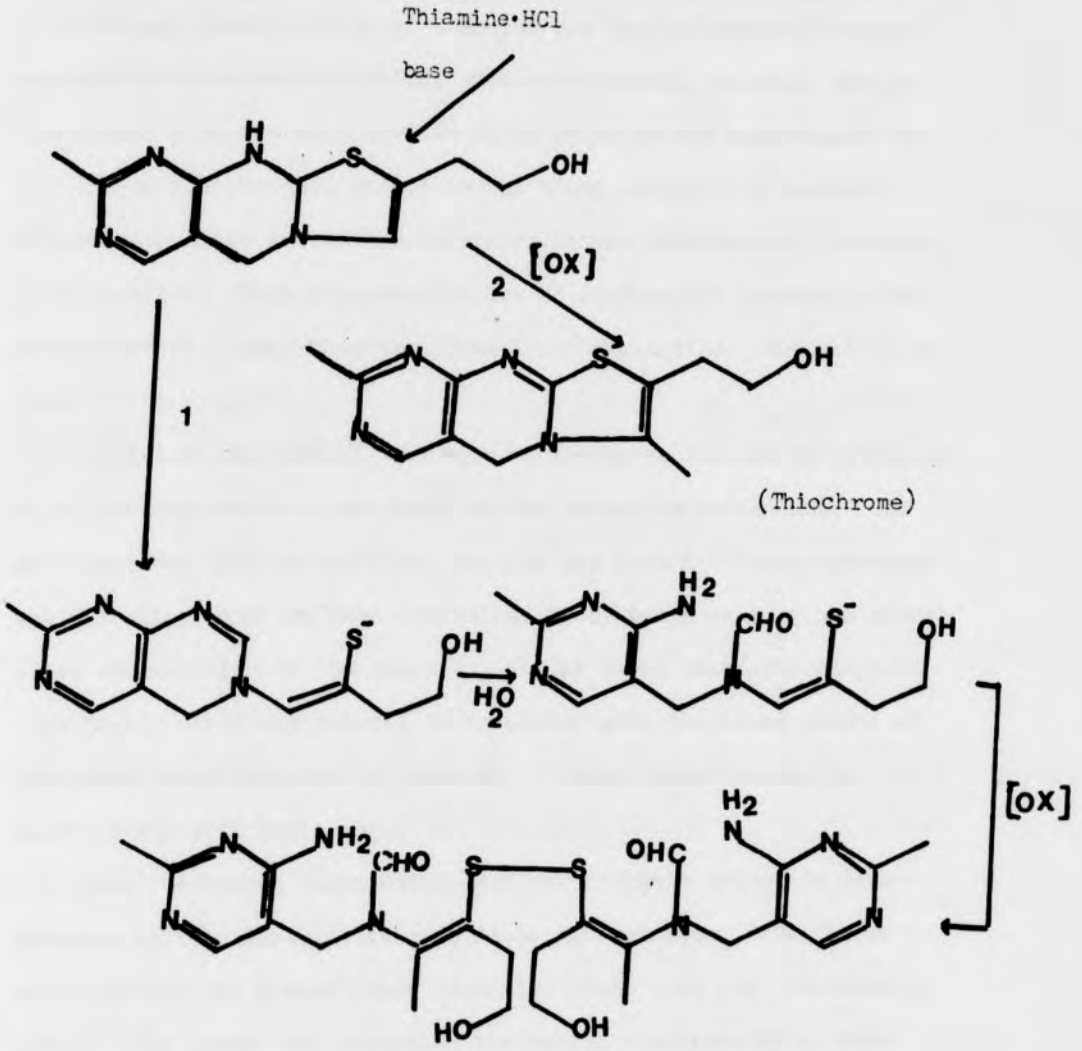
It would be of considerable interest to know what the compounds are that would possibly fluoresce. The important factor is that the molecule should have a structure in which the electrons could conveniently be excited at the least expense of energy. Examples are many, and they include a conjugated system of double bonds. There is a very good

likelihood that aromatic molecules would also fluoresce, especially if structural ramifications and properties enhance the delocalization of electrons. Hence, substituents in the aromatic ring system can either increase or decrease the fluorescent properties of aromatic compounds. This fact gives us an insight into the idea that weakly or non-florescent compounds could be made to fluoresce as a result of structural transformations. In fact, this is the basis of a large number of fluorescent determinations and a chemical reaction is the process by which many such structural changes have been brought about.

It would be equally interesting and important to note a term in luminescence terminology. "Quenching" is used to denote when fluorescent intensity is reduced to less than the theoretical value irrespective of the cause. It is necessary to know the compounds and substances that bring about such an effect, before an experiment is carried out.

Mechanism of Thiamine Oxidation[10]

Fluorometric method of thiamine determination has been in use since the early part of the century. But the exact mechanism of thiamine oxidation is still controversial. The mechanism proposed by Rissinger and Pell is as follows:



It has been observed that the oxidation products of thiamine were dependent on the pH of the medium. Solvents of high dielectric constants favored reaction course (1) as it would stabilize the ionic intermediates, whereas formation of thiochrome is favored by solvents of low dielectric constant, like methanol and ethanol, resulting in enhanced fluorescence.

Muirun, Romsos and Dirk[11] carried out the analysis of urinary thiamine by an automated method, without isolating thiamine. Two of the sample aliquots were treated alike prior to the measurement of thiochrome fluorescene, except in the blank addition of benzene sulphonylcholride and sodium hydroxide blocks oxidation of thiamine to thiochrome. Thus fluorescence due to nonspecific substances can be subtracted. Quenching was found to be negligible. The detection limit was 0.05 μg/ml.

Pelletein and Madre[12] did work on the determination of pyridoxine by chloroimide action, and modified the method by using DEPA. The main problems with chloroimide reaction was that the color developed was transitory and the measurement had to be taken exactly one minute after the addition of the reagent. It was found that ascorbic acid interfered, which was removed by oxidizing with increased amount of potassium ferric cyanide and removal of other interferences by decolorizing with Boric acid.

Park[13] compared fluorometric and colorimetric method of determination of thiamine by a discreet sampling technique. The fluorometric method has a much lower detection limit than the colorimetric method. The author has discussed the merits and demerits of each method.

A very good colorimetric method was developed by Das Gupta, details of which are given elsewhere in this article. The fluorometric method is used in the microgram range and the colorimetric method is used when the thiamine content is high. The colorimetric

method is simple and faster. The analysis rate is about forty samples per hour for the colorimetric method, whereas the fluorometric method takes twice as much time for the analysis of the same number of samples.

The thiochrome[14] method is widely used in food analysis for the determination of thiamine. The main problem of thiamine analysis in food is the isolation and purification process. Decalso[15, 16] has been widely used for the purpose of purification. Pippan and Porter[17] made necessary modifications of the above method and improved the recovery of thiamine. In former processes, thiamine was not recovered completely or the quantity of unrecovered thiamine was enough to cause substantial error in the analytical procedure. Hence, the above method is tested for various variables that would affect the total or maximum recovery of thiamine. Temperature and volume of the eluant was studied. Also the thiamine Decalso ratio and regeneration of Decalso was studied to find the percentage of recovery. It has been found by the authors that temperature control does effect a better recovery and does it by using an optimum volume of eluant. With the optimum temperature and eluant volume, the recovery went up by a total of eleven percent.

Papova and Papova[18] analyzed the drug preparation containing tetracyclines and vitamins. For tetracycline was removed by extraction at 8.5-9.0 pH. The error was found to be less than 0.5 percent. A 250 mg tetracycline sample was used.

Olivo, Giarrun and Fasella[19] carried out the analysis of thiamine and its phosphoric acid esters. The phosphoric acid esters of thiamine

were separated chromatographically and estimated fluorometrically.

Contractor and Shane[20] conducted studies on the amount of vitamin B_6 present in human blood and urine at different times of a woman's menstrual cycle. Cyanohydrin and lactone derivatives were made and the B_6 vitamin was determined fluorometrically. After preliminary processing of urine, the samples were chromatographed.

Yasihiro, Koko and Yoka[21] used the product of the reduction of thiolized thiamine with Cu^{++} for determining thiamine. The method was used as a spot test. The limit of detection was 5 $\mu g/ml$. sulfur and riboflavin interferred. Cobb and Williams[22] (North Carolina State University at Raleigh) used the procedure from official methods and determined thiamine. The detection limit was down to less than 0.004 μg (different enzyme preparations were used).

Duke and Youka[23] made diphenyl iodine derivatives and separated on silica gel plates. The derivatives were eluted with NaOEt into the correspondingly intense flourescent isobryofurane sulphonyl derivatives which was fluorometrically determined.

A spectrofluorometric method without separating thiamine from other substances was described in an article by Thorne and Jose.[24]

Spectrophotometric Method

A plethora of publications are available on spectrophotometric analysis of vitamin B_1 and B_6. In most of the colorimetric methods

adopted, the sample is purified first to the best of purity possible, by the purification methods available hitherto. In comparison with fluorometric method, the colorimetric method does not give as good a low detection limit as the fluorometric method.

Spectrophotometric methods are widely used in clinical chemistry for various types of analysis. If the substance is not colored, it can be, in most cases, converted to a colored derivative which in turn can be used for colorimetric analysis. As the instrument is fairly easy to operate, most technicians could be easily trained to use it in a matter of hours. The principle of colorimetric methods is fairly simple. At a particular wavelength, the colored solution absorbs electromagnetic radiation. The light absorbed is proportional to the concentration of the solution in question. The more the absorbance, the more concentrated the solution is. Hence, absorbance and concentration are related.

As the colorimetric method requires pure sample the main difficulty encountered in a colorimetric method is the purification procedure. A number of color developing reagents are available for both vitamins B_1 and B_6 analysis.

Quite often, the colorimetric method is used in conjunction with many other analytical techniques and it is the terminal analysis.

Almost all of the automated methods for vitamin B_1 and B_6 analysis are based on colorimetric or fluorimetric methods, as the color developing of the analyte with the reagent is very fast and it is easy to automate such a method. A simple detailed account of the

automated system of analysis is given in a manual by Technicon.

Probably the best colorimetric method available is the one reported by Das Gupta and Cadwallader.[25] They used Bromothymol blue as a suitable dye for a color developing agent with thiamine. The method is very specific and there was no interference from other vitamins. In another publication, Herman,[26] et al, studied the effect of optimum pH range and dye concentration on extraction of thiamine with an organic solvent.

A photodensitometric method of determining vitamin B_1 and B_6 was published by Vincent.[27]

Chromatographic Methods

Chromatography, first introduced by Tswett, now encompasses a variety of techniques. It is one of the few analytical techniques which has been so extensively used in all areas of chemistry. Chromatography has its importance as a method of purification and separation.

The underlying principles of chromatography are selective adsorption or distribution ratio, depending upon the techniques employed. A brief account of various types of chromatography is given below.

Chromatographic techniques are composed of mainly an adsorbing medium and a mobile phase. If the adsorbing phase is solid and the mobile phase is either gas or liquid, the technique is called adsorption chromatography. If the stationary phase is a liquid supported in a solid, then the technique is termed as partition chromatography.

What the chemists call molecular sieves, the biochemists call gel chromatography[28] which is in fact a type of adsorption chromatography. The molecular sieves are made up of polymeric materials of carbohydrates or acrylanides, that have an open network. These molecular sieves are capable of adsorbing water, as a result of which it swells. Swelling causes the holes to open up. The size of the holes depends upon the degree of cross linking.

The molecules that are separated are in equilibrium with the solvent and they are eluted in the same manner as in other chromatography. The smallest molecules enter the holes of the gel and thereby kept behind. The molecular size can vary greatly depending on the gel. What is known as the "exclusion limit" in gel chromatography is the molecular weight of the smallest molecule that will permeate the gel and be retained.

Ion exchange chromatography is another well known separation technique, used for the separation of both anion and cations. The stationary phase looks like beads. They are made by cross-linking polystyrene molecules with divinyl benzene. The crosslinked polymer is known as resins. They have free phenyl groups attached to the chain which can add ionic functional groups. There are different types of ion exchange resins in use via strong acid, weak acid, strong base and weak base.

Gas chromatography is another technique which has extensive use in separation and purification. The mobile phase, as it is apparent from the name, is a gas phase and the stationary phase a

solid one. Other common chromatographic techniques are TLC and paper chromatography which have further modifications. The modification of chromatography since its discovery has been tremendous.

Electrophoretic separations are based on the relative mobility of ions under the influence of an electric field. There are several types of electrophoretic techniques, like particle electrophoreses, microscopic electrophoreses, etc.

For the separation and determination of vitamin B_1 and B_6 various types of chromatographic techniques have been employed. In a majority of chromatographic methods, the vitamins were separated by chromatography and then finally determined by optical methods.

Thielman[29] separated and determined the vitamin B complex by TLC using silufol as the adsorbing medium. A high performance ion exchange method was described by Calmer and Davis[30] They determined vitamin B_1 and B_6 using high performance ion exchange chromatography.[31] They also studied the various factors that affect an effective separation, like temperature, pH concentration of sample, etc. Williams, Baker and Schmit[32] separated vitamin B_1 and B_6 by high performance ion exchange chromatography using pellicular resins. The advantage of the method included a detection limit of fifty nanograms, fast analysis time and minimum clean up time required. Ismiel and Yassa[33] determined thiamine by TLC in the presence of decomposition products. An iodine solution was used as a spraying agent on TLC. The method is not applicable when riboflavin, tetracycline or oxytetracycline is present. Another chromatographic determination

was done by Ashby[34] and Deavin. They analyzed vitamin B_1 and nicotinamide without derivatization. The vitamins were separated at different temperatures.

Microbiological Methods

It is based on the fact that certain micro-organisms require specific vitamins for their growth. Using a suitable medium (based), growth responses were compared in standard and unknown solutions.

The most popular microbiological method is by using sacharomyces carlsbergenesis.[35, 36] Only a limited number of publications are available about it. Usually the method is used for food analysis. Davis, Smith and Currow[37] analyzed serum pyridoxal by an automated microbiological method. The test organism was chlorampinicol resistant.

Miscellaneous Methods

In addition to the above methods there are other methods which are not usually employed. They are refractometric, gravimetric, non-aqueous titrimetric methods, etc. These are not used frequently due to the obvious problems of sensitivity, prior purification, and rapidity of the method.

CONCLUSION

The review evaluates various analytical methods available for vitamin B_1 and B_6 analysis. None of the methods listed and explained are excellent. At present, the majority of vitamin (B_1 and B_6) analyses in clinical chemistry laboratories involves fluorometric

or colorimetric methods. Biological assay methods are rarely used in clinical chemistry, which is apparent from the number of publications that have appeared. For the vitamin analysis of foods, chromatographic methods are extensively used due to its rapidity and the ease with which the method could be automated. From one of the chromatographic methods, it has been found that, especially in the case of 'Decalso' as an adsorbing medium, the recovery of thiamine is not complete.

It is hard to say which of the above methods is the best. The best methods are chosen, based on quite a few factors like the source, type and degree of impurities present, accuracy required, rapidity of the method, etc. It is still a challenge to the analytical chemist to develop a suitable and accurate method for vitamin analysis.

REFERENCES

1. Myer Freed, "Methods of Vitamin Assay," Interscience Publishers, New York, 1966, Chpts. 6 and 7.

2. A. Cantarow and M. Trumper, "Clinical Biochemistry," W. B. Saunders Company, 1962, Chpt. 23.

3. Ibid.

4. R. Cannon, et al, "Clinical Chemistry," Harper and Row Publishers, New York, 1974, Chpt. 4.

5. Ishibashi Nobyko, et al, Chem. Lett., 119 (1973).

6. Kina Kenya, et al, Bull. Chem. Soc. of Japan, 46, 2772-73 (1973).

7. M. M. Evstifeev, et al, Zh. Khim., 2, 89-90 (1973).

8. F. Fahr, et al, Pharmazie, 29 (10-11), 726-27 (1974).

9. J. C. Tutor, et al, An. Quim., 69 (4), 455-61 (1973).

10. Sidney Undenfriend, "Fluorescence Assay in Biology and Medicine," V of II, Academic Press, 1969.

11. Kathaleen L. Muirui, et al, The Am. J. of Clin. Nutri., 27, 837-39 (1974).

12. O. Pelletier, et al, Can. J. Pharm. Sci., 9 (4), 99 (1974).

13. John Y. Park, Anal. Chem., 47 (3), 452 (1975).

14. William Horwitz, Methods of Analysis of the Association of Official Agricultural Chemists.

15. O. Pelletier, et al, J. Food Sci., 40, 376 (1975).

16. O. Pelletier, et al, Clin. Chem., 18 (9), 937 (1972).

17. Eldon Pippen, et al, J. Agric. Food Chem., 23 (3), 253 (1975).

18. Y. Papova, et al, Vkusova Prom Plodiv, 14 (pt. 1), 261-4 (1967). Chem. Abstr., 72, 28957 (1970).

19. F. Olivo, et al, Giorn. Biochem., 11, 122-25 (1962). Chem. Abstr., 57, 16993 (1962).

20. S. F. Contractor, Clin. Chim. Acta., 21, 71-7 (1968).

21. Montomiya Koko, Bitamin., 36, 205-8 (1967).
Chem. Abstr., 67, 97550 (1967).

22. William Cobb, et al, J. Ass. Offi. Anal. Chem., 54 (1), 211-12 (1971).

23. Irene Durko, et al, Clin. Chem. Acta., 49 (3), 407-14 (1973).

24. L. Jose, et al, Affinidad, 30 (308), 635-9 (1975).

25. D. E. Cadwallader and V. Das Gupta, J. Pharm. Sci., 57 (1), 112 (1968).

26. H. B. Herman and V. Das Gupta, J. Pharm. Sci., 62 (2), 311 (1973).

27. M. Vincent, Fran. Soc. Pharm., 29 (1), 35-42 (1969).
Chem. Abstr., 71, 105248 (1969).

28. Gary D. Christian, "Analytical Chemistry," Xerox College Publishing, Waltham, Mass., 1971.

29. H. Thielemann, Sci. Pharm., 42, 94-6 (1974).

30. K. Calmer, et al, Chromat., 7 (11), 644 (1974).

31. R. C. Williams, et al, J. Chromat. Sci., 11, 618 (1973).

32. R. C. Williams, et al, J. Chromat. Sci., 11, 618 (1973).

33. A. Yassa, et al, Analyst, 98, 5-8 (1973).

34. J. D. Ashby, et al, J. Pharm. Pharmaco., 21, Suppl., 525-545 (1969).

35. William Horwitz, "Methods of Analysis of the Association of Official Agricultural Chemists."

36. Myer Freed, "Methods of Vitamin Assay," Interscience Publishers, New York, 1966.

37. R. E. Davis, et al, J. Clin. Path, 26, 871-74 (1973).

CARBONATE ANALYSIS

INTRODUCTION

Almost all the organisms require a congenial and constant environment for their survival. For the proper functioning of the biological systems variables like temperature, osmotic pressure, and pH should be controlled effectively. Within the biological systems, the pH control or acid-base balance has been accomplished by a variety of buffering systems and in humans the pH control is effected mainly by carbonate-bicarbonate buffering system.

Carbonic acid is formed as a result of the dissolution of CO_2 in water. The concentration of CO_2 in water is affected greatly by slight variations in temperature and pressure. Hence, it is very complicated to make an accurate carbon dioxide measurement in blood. Moreover, as the renal and pulmonary mechanisms control the acid base balance the amount of oxygen level in blood is also an important factor, because the respiratory process not only involves removal of carbon dioxide but the intake of oxygen, too.[1]

Analysis of carbonate-bicarbonate is important to several disciplines of science. From geologists to biologists are interested in the rapid and accurate method of carbonate analysis. But our survey has been limited to clinical, pharmaceutical and environmental analysis. The right pH is a matter of life and death to living organisms, and that itself explains why it is so important for us to have an excellent analytical methodology for man and his environment.

THE BLOOD BUFFERING SYSTEM[2]

It has been emphasized how important is the buffering systems in biological systems. Human blood contains more than one system of buffers; but the carbonate-bicarbonate system is by far the most important. The bicarbonate system carries out most of the buffering activity in the plasma while hemoglobin does it in blood cells.

The carbon dioxide passes into the blood from the tissues. From the blood plasma CO_2 gets into the red blood cells where it combines with water to form carbonic acid. The reaction is catalyzed by an enzyme, carbonic anhydrase.

$$CO_2 + H_2O \underset{\text{catalyzed}}{\overset{\text{enzyme}}{\rightleftharpoons}} H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

The relationship between pH, pCO_2, HCO_3 is expressed by using the Henderson-Hasselbalch equation.

$$K_a = \frac{[H^+][HCO_3^-]}{[H_2CO_3]}$$

$$[H^+] = K_a \frac{[H_2CO_3]}{[HCO_3^-]}$$

$$\log [H^+] = \log K_a + \log \frac{[HCO_3^-]}{[HCO_3^-]}$$

$$pH = pK_a + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

$[H_2CO_3]$ is proportional to the dissolved CO_2. Hence

$$pH = pK_a + \log \frac{[HCO_3^-]}{k[CO_2 \text{ dissolved}]}.$$

The dissolved carbon dioxide is proportional to the partial pressure of Carbon dioxide in lungs. The Carbon dioxide in lungs is in equilibrium with the plasma. CO_2 dissolved = $a' \cdot pCO_2$. Where a' is a constant.

$$pH = pK + \log \frac{[HCO_3^-]}{a \cdot pCO_2} \quad \text{and } a = a'k$$

The common expression used to show the concentration of carbonic acid is CO_2 or pCO_2.

CLINICAL SIGNIFICANCE OF CARBONATE ANALYSIS[3, 4]

It has already been mentioned that the acid-base balance is controlled by the renal and pulmonary mechanisms or in simpler words, the bicarbonate concentration is controlled by the kidneys and carbonic acid by the lungs. A vegetarian diet makes the blood slightly acid. Not only dietary mode changes the acidity and alkalinity of the blood, but metabolic disorders also can bring about imbalances in the body pH. Loss of CO_2 causes increase in pH which is known as alkalosis. When such an imbalance takes place in the body fluids renal and pulmonary mechanisms operate to counteract the changes.

Uncompensated acidosis within a limited range is not highly symptomatic whereas uncompensated alkalosis shows hyperventilation as specific symptoms. Hyperventilation has been observed in many diseases, like fever, encephalitis, and at high altitudes due to oxygen deficiency. Hence, acid-base imbalance can be used as a diagnostic basis, in case

of metabolic disorder or a pathogenic condition. There are four types of acid base imbalances.

Respiratory Acidosis

This is caused as a result of hypoventilation. The plasma level of pCO_2 goes up, lowering the pH.

Respiratory Alkalosis

This is caused by hyperventilations or due to excessive loss of alveolar CO_2, resulting in a decrease in plasma level pCO_2 and in turn a higher pH.

Non-Respiratory Acidosis (Metabolic)

This is caused by renal tubular dysfunction or in diabetics and like disorders. Actually the loss of bicarbonate or gain of acid in ECF fluid causes it.

Non-Respiratory Alkalosis (Metabolic)

As a result of the loss of strong acid from the body by vomiting or due to excessive intake of bicarbonate.

SOME USEFUL DEFINITIONS[5, 6]

The arterial blood is slightly more alkaline pH 7.40 than venous blood pH 7.37. Deviation from normalcy has been explained using different terms.

Acidosis: Refers to excess of acid or acid intoxication.

Alkalosis: Refers to excess of base or bicarbonate or of acid deficit.

Alkalis Reserve: The bicarbonate present when all non-volatile acids have been neutralized and ready for neutralization of further acid. This is also known as the alkali reserve of the body or CO_2 combining power.

CO_2 Content or Total CO_2: CO_2 derived from carbonic acid, dissolved Carbon dioxide, bicarbonic acid and carbonious CO_2 from anaerobically drawn blood.

Buffer base: It is the buffer anions present.

Base excess: For fully oxygenated blood at pCO_2 of 40 mm Hg at 38°C and at pH 7.40 the base excess (ΔBB) is taken as zero, positive ABB indicates alkalosis and negative value refers to acidosis.

Nomograms[7]: It is a graphical representation of numerical relations. The value of a dependent variable is read from the graph when the values of two independent variables are given.

METHODS AVAILABLE

Electrometric Methods

More than any other analytical methods, electrometric methods are gaining importance. Major strides have taken place in the development of ion-selective electrodes.[8] Electrometric titrimetric methods are also employed for the carbonate analysis. Weller[9] has come out with a method of clinical analysis using the simple principles of electrochemistry. Principles of potentiometry are explained with

special reference to the determination of CO_2 and standard HCO_3^- content from pH measurements. Also the paper described nonogramphs and glass capillary microelectrodes. Dreux[10] and Boign determined the alkali reserve in a plasma sample by potentiometric method by measuring the pH. Makay[11] et al used an electrical method for the determination of serum bicarbonate. The paper explains the electrical circuitry used and the manipulations in electrical circuitry. The major advantage is that the analysis time is about 60 seconds.

Potentiometric method was employed by Weunch[12] for the determination of alkali reserve and carbonate. The end point detection has been improved by using a glass electrode. The excess of HCl was back titrated with NaOH in the above method. The blood plasma was used as the sample.

A conductometric method of determination of bicarbonate and carbonate was used by Pasovskya[13] et al. The results were comparable with those that were obtained by HCl titration with methyl orange. Microglass electrodes were used by Plathe[14] et al. The blood was equilibrated with CO_2 and O_2 and the pH was measured. The bicarbonate was determined by using standard curves for interpolation.

An electrometric titration was employed by Mass[15] to determine the bicarbonate in the cerebrospinal fluid and plasma or serum. Constant ionic strength and temperature were maintained during the titration to obtain better accuracy. An important problem of bicarbonate determination in plasma is the temperature at which blood should be centrifuged to obtain plasma. But the above authors

have proved experimentally, there is not much difference between the bicarbonate present when the centrifuging was done at 20°C or 38°C. Mass[16] in another publication explained the use of an isotonic sodium chloride bridge to minimize the liquid junction potential. If the blood is the test solution, KCl may coagulate plasma protein. On the other hand, KCl diffusion into the blood during continuous pH measurements is not desired either.

Dahms[17] invented reagents for the bicarbonate and chloride analysis of serum. Also, according to another patent[18], he developed instruments to analyze carbonate and bicarbonate in serum.

A coulometric determination of bicarbonate in duodenal juice was done by Novek, Jamicek and Rumbs[19]. In vitro determination of pH and bicarbonate concentration were re-evaluated by Puschett and Zurbach.[20]

The behavior or various types of microelectrodes such as glass, lead and quinhydrone were evaluated. An electrode system was developed for the analysis of small samples of blood by Macphee and Mowcary[21]; Malcato and Tomio[22] studied the possibility of utilizing a pCO_2 electrode for the determination of carbonates. The response of the electrode was relatively fast, 90-120 seconds.

Herman and Rechnitz[23] used a carbonate ion-selective membrane electrode. The electrode responded in the 10^{-2} to 10^{-6} M range. The advantage with this electrode was that there was a high selectivity of carbonate over chloride, phosphate and sulphate ions. Smith[24] and Hahn studied the electrode stability memory and S plots of a Severinghaus pCO_2 electrode. The electrode was found to have a

good memory effect depending on the last sample of CO_2 to which the electrode has been exposed to. When the reference gas was of low concentration the electrode showed high reproducibility and stability. High concentration of carbon dioxide caused unusual electrode behavior. Smith,[25] et al in another paper carried out the CO_2 measurements using a single control analysis. A single calibration point--single control analysis was used. Such an analysis was found to give very accurate and reproducible results over a limited cone range around the calibration point.

In another paper, Herman and Rechnitz,[26] determined the serum carbon dioxide content using an ion-selective membrane electrode. The method brought about good precision and accuracy. The electrode was used in a continuous flow system and was easy to be automated. In comparison with other classical methods the results obtained by the electrode correlated well. The electrode does not involve high initial installation cost.

An elaborate explanation of the preparation and properties of an ion-selective membrane electrode also was published by Herman and Rechnitz[27]. A semi-automatic system was described by Lynstarev[28] et al. The analysis of sea water was performed using a high sensitivity pH meter.

A miniature probe of multi-functional electrochemical electrodes for the analysis of CO_2, O_2, H^+, K^+, or Ca^{2+} was patented by Brown[29]. Other patents for CO_2 and pH sensor were obtained by Le Blanc.[30]

Titrimetric Methods

Titrimetric methods are of different types, electrometric, volumetric, photometric, etc. Here only the volumetric method, where endpoint detection is made by an indicator is dealt with. Other titrimetric methods are grouped under appropriate titles whenever applicable.

The majority of the titrimetric techniques are electrometric. Pure classical titrimetric methods are encountered sometimes where the determination is less intricate or complicated. Moreover, on a routine basis they are time consuming. A volumetric method of determination of natural waters is given by Dumitra, et al.[31]

A simultaneous titrimetric determination of bicarbonate and titrable acid of urine has been done by Gregory and Edwards.[32] The accuracy and pression of the method is the same as that of the Van Slyke manometric method. The advantage of Van Slyke's method is that it combines into a single operation to determine urinary bicarbonate and titratable acid or titrable base when the pH of the urine can be titrated without titrating H_2CO_3. The main source of error which can occur is the slow reaction between HCl acid and bicarbonate in the presence of buffer. This error can be avoided by eliminating CO_2 formed by making use of vacuum line technique. for about twenty minutes or using a vacuum while stirring the acid. This method considerably reduces reaction time.

Gasometric Measurements

Under this section measurements made by monitoring pressure or volume of a gas are investigated.

Chinderri[33] made use of the pressure and volume measurements and the procedural and instrumental details are explained. Robinson,[34] et al made use of an ingenious method by measuring the volume of CO_2 evolved when the gas was collected as a single bubble.

Claude[35] determined pH, pCO_2 and bicarbonate by Van Slyke's gasometric method. The results were comparable to that obtained by the method of Astrup.

Larson,[36] et al. determined CO_2 in biological materials by a simple vacuum line technique. The CO_2 evolved out of bone and muscle tissue was determined. This method comprises the utility and versatality of a vacuum line technique for the determination of CO_2.

Optical Methods

All kinds of spectral techniques employed for carbonate analysis are included in this section. Usually the carbon dioxide evolved is dissolved in a solution and the resulting pH changes alter the color of a pH sensitive indicator. IR spectral methods are also employed. Golden,[37] et al. carried out studies leading to the selection of right analytical lines for the long path IR spectrometry in the analysis of air pollutants. Another IR method was given by Peterson.[38] The CO_2 was evolved on acidification. The evolved CO_2 was collected in an IR cell and determined. This method is comparatively rapid. The absorption signal is integrated to provide a direct reading of sample concentrations on a digital voltmeter.

Hyanek[39] determined CO_2 colorimetrically using phenolphthalein as an indicator. Several mixed or screened indicators have been prepared by Cheoux.

Another publication on the analysis of CO_2 by IR is given by Thomas,[40] et al.

An atomic absorption method of determining CO_3^{--} in inorganic substances was given by Yale[41] and Donovan.[42] Myers measured the tissue gas levels with a Mass Spectrometer.

Determination of carbon dioxide in air was done by Brounihlein[43] according to the IR absorption spectrum of the solar radiation.

An enzymatic colorimetric method was developed by Norris[44] and a photoelectric detection of equivalent point in colorimetric titration was devised by Dahms.[45]

Chromatographic Methods

There are very limited publications available for chromatographic methods for the determination of carbonates.

Pennington[46] employed gas chromatographic methods for the analysis of carbonates and bicarbonates. Dissolved carbon dioxide in water was estimated by suganocunio. Helium was used as a carrier gas. The column was packed with activated charcoal and detection was made by a conductivity measurement.

An ingenious cell for the chemical amplification of small amounts of carbon dioxide was devised by Levin,[47] et al. The amount of carbon dioxide was proportionately increased by a redox method and the final product of CO_2 was analyzed by chromatographic techniques. Multi-column[48] chromatographic systems were discussed by Hepter, et al.

Radiometric Methods

A few radiometric methods are also reported. Johanneson[49] used the modified substoichiometric isotopic dilution technique to determine carbonate. The carbon dioxide evolved was precipitated as $BaCO_3$. From the amount of labeled carbon and the degree of dilution, the amount of carbonate in the original sample was determined. Also carbonate was estimated by liquid scintillation counting of the radioactive carbonate. This was discussed in a short communication by Adrian and Aghdash.[50] The difficulty of the insolubility of inorganic carbonate salts was overcome by using gelling or emulsifying agents.

Miscellaneous Methods

These methods are only being mentioned. These techniques vary in principles are procedures. Among them include complexometry, olfactory detection, interferometry, gravimetric, turbidometric, enzymatic determinations, etc. Only extremely limited publications have appeared on these analytical techniques.

Autoanalyzers

Autoanalyzer methods are being used extensively these days. They reduce time and cost of analysis. Above all they increase precision and accuracy too. Most of the autoanalyzer systems are based on electrometric or opticometric measurements as such methods could be conveniently automated.

CONCLUSION

Clinical analysis of carbonate is very intricate. Carbonic acid is formed as a result of the dissolution of the gas in water. As it has been mentioned in the earlier part of this article, several factors affect the CO_2 content in blood. Exposure of serum in plasma sample to air results in the loss of CO_2. This is a major drawback in automated systems.

Henry and Winkelman[1] in "Principles of Clinical Chemistry" deal in depth and detail fairly well, about the analytical methods of choice and the current analytical methods. Manometric and titrimetric methods are explained well. Procedural and instrumental details are also given.

Carbonate analysis is mainly required in clinical chemistry. Geologists and soil chemists also encounter the necessity of carbonate analysis quite often. In pharmaceutical chemistry, except in certain preparations carbonate analysis is seldom required.

In order to define the acid base status completely, the values for three variables are necessary. They are, the respiratory component, the metabolic component, and the blood pH. Knowledge of any of the two will enable us to compute the third. The respiratory component can be represented by pCO_2 and metabolic component by the concentration of plasma bicarbonate. Due to the complexities involved in clinical carbonate analysis, the ion-selective electrode would be the best choice. The latest publication in this field is by Herman and Rechnitz.[23]

REFERENCES

1. R. J. Henry and James Winkelman, "Clinical Chemistry," Principles and Techniques, Harper & Row Publishers, New York, 1974.

2. Ibid.

3. S. Raymond Gambino, "Acid Base Balance," published by Albot Laboratories. 1974.

4. D. N. Baron, A Short Textbook of Clinical Biochemistry, J. B. Lippincott Co., Philadelphia. 1975.

5. D. D. Van Slyke, G. E. Cullen, J. Biol. Chem., 30, 290 (1917).

6. R. J. Henry and James Winkelman, "Clinical Chemistry," Principles and Techniques, Harper & Row Publishers, New York, 1974.

7. Websters Third International Dictionary.

8. David F. Boltz, CRC Critical Review in Analytical Chemistry, 36, 147-99 (1973).

9. H. Weller, Med Lab, 17 (5), 99-105, 136-143 (1964).
Chem. Abstr., 61, 16432 (1964).

10. Cl. Dreux and J. M. Boigne, Ann. Biol. Clin. (Fr.), 21, 417-26. (1963).
Chem. Abstr., 64, 2034 (1964).

11. D. M. Makay, et al, Clin. Chim. Acta., 12 (1), 75-79 (1965).

12. L. Weunch, Collect. of Czech. Chem. Comm., 29 (9), 2252-9 (1964), (Ger.).
Chem. Abstr., 62, 3053 (1965).

13. G. B. Pasovskya, et al, Khim. Geol. Newk., (4), 112-114 (1961).

14. O. Muller Plathe, et al, Aerztt Lab., 12 (11), 323-8 (1966).
Chem. Abstr., 66, 73134 (1967).

15. A. J. Mass, Clin. Chim. Acta., 29, 567-574 (1970).

16. A. J. Mass, J. Apply. Physio., 30 (2) (1971).

17. Harold Dahms, U. S. Patent, 3, 551, 356 (1970).

18. Harold Dahms, U. S. Patent, 3, 551, 109 (1970).

19. K. Novak and A. Jamicek, J. Biochem. Clin. Bohemoslev, 1 (1), (1972), 46 (53) (1972).
Chem. Abstr., 78, 120949 (1973).

20. Peter E. Zurbach, Kidney Internat., 6 (2), 81-91 (1974).

21. John Macphee, U. S. Patent, 3, 763, 422 (1973).

22. Noshiro Makoto, et al, Nippos Kagaku Kaishi, (11), 2099-104.
Chem. Abstr., 82, 67702 (1975).

23. H. B. Herman and G. A. Rechnitz, Anal. Lett., 8 (2), 147-159 (1975).

24. Crampton A. Smith, et al, Br. J. Anaesth., 47 (5), 553-87 (1975).

25. Crampton A. Smith, et al, Br. J. Anaesth., 47 (5), 559-69 (1975).

26. H. B. Herman and G. A. Rechnitz, Science, 184, 1074-75 (1974).

27. H. B. Herman and G. A. Rechnitz, Anal. Chim. Acta., 76, 155-164 (1975).

28. S. V. Lynstarev, et al, Konf. Khim. Morya., 133-138 (1973).

29. John F. Brown, Jr., U. S. Patent, 3, 900, 382 (1975).

30. H. Le Blank, Jr., U. S. Patent, 3, 898, 147 (1974).

31. Ceavses Dumitra, A Pelor Meteorol, 1968, 13 (3), 147-9.
Chem. Abstr., 69, 30027 (1968).

32. A. Z. Gregory, et al, J. of Exptl. Biol. Med. Sci., 45 (2), 141-8 (1967).

33. Ascen Chinderri, Bull. Lab. Chim. Prov. (Bologoni), 17 (1), 120-31 (1966), (21st).
Chem. Abstr., 66, 34469 (1967).

34. C. Robinson, et al, Caries Res., 2 (1), 1-9 (1968).
Chem. Abstr., 69, 94192 (1968).

35. Montgrain Claude, Med. Lab., 21 (3), 67-71 (1968).
Chem. Abstr., 69, 25034 (1968).

36. Lynn Larsen, et al, J. Apply Physio., 35 (5), (1973).

37. Bruce M. Golden, et al, Anal. Chem., 47 (13), (1970).

38. John Peterson, Clin. Chem., 16 (2), 144-6 (1970).

39. J. Hyanek, et al, 103 (44), 1222-5 (1964).
Chem. Abstr., 63, 4644 (1965).

40. Anna Thomas, et al, Can. J. Bot., 40 (4), 481-5 (1965).

41. John Yale, et al, At. Absorption Newsletter, 8 (2), 30-3 (1969).

42. William E. Donovan and Bert Myers, Adv. Exp. Med. Bid., 37A, 67-72 (1973).
Chem. Abstr., 82, 53642 (1972).

43. A. M. Brounihlein, et al, Tr. Bl. Briofis. Obs., 324, 6-13 (1974).
Chem. Abstr., 83, 187943 (1975).

44. Kenneth A. Norris, Clin. Chem., Winston-Salem (1975).
Chem. Abstr., 83, 110730 (1975).

45. Harold Dahms, U. S. Patent, 2, 218, 055.

46. Sam M. Pennington, Trans. Mo. Acad. Sci., 3, 51-2 (1969).
Chem. Abstr., 79, 71357 (1971).

47. N. V. Levin, et al, Zh. Anal. Khim., 1975, 30 (5), 976-80 (1975).
Chem. Abstr., 83, 187777 (1975).

48. Jascek Hepter, et al, Zesz. Neuk. Inst. Cerkiej. Synt. Org. Blachowin Slak, 6 (28), 67-81 (1974).
Chem. Abstr., 83, 90257 (1975).

49. J. K. Johanneson, Analyst, 92, 766-768 (1967).

50. Kuyper Adrian and M. Aghdashi, Anal. Biochem., 45 (1), 41-43 (1971).

PHOSPHATE ANALYSIS

INTRODUCTION

For the alchemist, the name phosphorous meant "carrier of light;" but today for the modern biochemist, phosphorous is the "energy carrier" in living organisms. Practically all energy exchanges in living organisms are carried out by phosphorous compounds. Most of the hard parts of animals, like bones and teeth, are composed largely of phosphates.

Phosphorous compounds occupy a unique position due to their biological functions and industrial applications.[1] In our everyday life, we come across the use of numerous phosphorous compounds, like toothpaste, polishing agents, plasticizers, detergents, fertilizers, pesticides, and many others. Due to its importance in industry and living organisms, the analytical chemistry of phosphorous compounds is very important. The accumulation of phosphorous compounds in the environment is critical, as an excessive amount can cause eutrophication of natural water,[2] thereby reducing the oxygen content which is detrimental to the animal life in water.

Clinical determination of phosphate is used in the diagnosis of bone dysfunction, renal problems and disorder of parathyroid gland. A higher level of acid phosphatase in blood is an indication of a prostastic infection. Phosphorous is present as inorganic as well as organic phosphate. In the determination of inorganic phosphate, organic phosphate usually interferes which contributes complexities to the analytical procedure. The phosphate determinations in a

clinical laboratory is carried out by automated systems.

The most recent advances are the introduction of ion-selective electrodes. It is the same case with other compounds with which we are dealing. Most of the analyses in clinical laboratories are based on optical methods.

TERMINOLOGY

Orthophosphates

The prefixes ortho-, pyro-, and meta- are used to distinguish among orthophosphoric acid and their salts. The most hydroxylated species is ortho. Pyro acid is formed by the removal of one molecule of water from two molecules of ortho acid. The acid formed by the removal of one molecule of water from one molecule of ortho acid is meta acid.

Superphosphates

This is a commercial name and consists of calcium sulfate ($CaSO_4 \cdot 2H_2O$) and monocalcium phosphate, $Ca(H_2PO_4)_2 \cdot H_2O$. It is used as a fertilizer.

Condensed Phosphates

When secondary sodium orthophosphate is fused the product is known as sodium pyrophosphate. $2Na_2HPO_4 \rightarrow Na_4 P_2O_7 + H_2O$.

METHODS AVAILABLE

Halmann's[3] Analytical Chemistry of Phosphorous Compounds gives a detailed account of the analytical methodology of phosphorous and

its compounds from various sources. Also, the book explains the various methods by which the different phosphate compounds could be converted to orthophosphate by oxidation with nitric acid or any other suitable oxidizing agents, as analysis is carried out on orthophosphates.

Most of the clinical analyses are automated and almost all are optical methods. Methods other than optical methods are rarely employed in a clinical laboratory at present.

Optical Methods

One of the earliest analytical methods of phosphates was the optical method, first introduced by Fisk[4] and Subbarow. It is also the analytical method of choice in a clinical laboratory. The principle involves the formation of phosphomolybdate which is reduced with a variety of reducing agents to give molybdenum blue.

Orthophosphate ---------→ Ammonium Phosphomolybdate $\xrightarrow{\text{reduction}}$ Molybdenum Blue (Composition uncertain)

There are several procedures available for the determination of phosphates colorimetrically. Itaya,[5] et al, used malachite green to determine phosphate. Malachite green forms a complex with phosphomolybdate which is used colorimetrically. Baginiski[6] and coworkers digested the phosphate with nitric acid-Ca mixture to convert to orthophosphate. The acid destroys the organic material. The calcium prevents the loss of phosphates. After the formation of orthophosphate, the color developing reagent is added and phosphate

is determined. This method is suitable for biological materials. In a method given by Knox,[7] et al, phosphate was determined by atomic absorption. The phosphomolybdate complex was extracted into an organic phase and determined by atomic absorption.

Guirgis[8] and co-workers used metamizol as a reducing agent for the determination of inorganic phosphate in blood. The reducing agent gave good results even after three-five months when kept at $4^{o}C$ and the results were comparable to that of a freshly prepared solution.

Hynie, et al,[9] found that a stable alkaline, violet lake was formed when methyl violet was mixed with phosphotungstomolybdic acid. This method could be used to detect phosphates. Smith and Meun devised a microphosphate method by making use of aminonapthol-sulphonic acid (ANS) of Fiske and Subbarow and strong acid conditions to prevent the non-specific heteropoly blue color.

Goodwin[10] devised a direct method where serum inorganic phosphorous and phosphatase could be determined directly without the preliminary precipitation of protein with trichloroacetic acid. Here ferrous sulphate is used as a reductant.

London and Marymount[11] analyzed serum inorganic phosphate from heat coagulated serum since it eliminates the use of TCA; and monomethyl-p-amino-sulphate is used as a reducing agent. It is commercially known as "Elon." The main disadvantage is that it takes around forty-five minutes to develop the color. Morin and Prox[12] used o-phenlenediamine as a reducing agent in the determination of serum phospherous, and it is perhaps the most sensitive phosphate procedure reported.

A rapid micro-determination of phosphate in biological materials was done by Jaenicke.[13] The sample is ashed with perchloric acid and the phosphate is determined by molybdenum blue method. The method is suitable for automation. Wimmer[14] and Wallner determined inorganic phosphate in serum using malachite green.

Parek and Young[15] used p-phenylenediamine dihydrochloride as a reducing agent for the colorimetric determination of phosphorous in serum. Drewes[16] determined inorganic phosphate in serum by using p-methylaminophenol sulphate as a reducing agent which was further alkalinized with monoethanolamine resulting in a blue color. The method is less time consuming and the reagents can be easily prepared. Labile organic phosphorous usually interfers in the study of inorganic phosphorous. Zak,[17] et al, carried out the study of the interference of labile organic phosphorous. Interference was prevented by the addition of citrate arsenite solution which would complex with the excess molybdenum present. Hence, if more phosphorous is formed, it will not complex with molybdenum.

West and Narayanaswamy[18] used Rhodamine B which formed an ion-association complex with phosphomolybdate. The excess of dye reagent was extracted into chloroform and removed. The Rhodamine B molybdate is extracted into chloroform butanol mixture (4:1 $^{v}/_{v}$) and intensity of fluorescence in this solvent was measured at 575nm with excitation at 350 nm. They also investigated the interference of about 37 ions and found that the method is very selective.

Electrometric Methods

As it is, electrochemical methods are not employed in clinical laboratories. Serduyukowa[19] determined phosphates amperometrically and Petrov-Spiridenov[20] determined it polarographically.

Christian, et al,[21] determined phosphates by direct argentometric titration. The end point was determined potentiometrically. Khudayakowa, et al,[22] analyzed phosphates in fertilizers and industrial products by chronoconductometric method. Muller, et al,[23] used a high sensitivity direct recording linear recording conductometric titrator. All the electronic circuiteries and its complexities are explained in this paper. Tutendize, et al,[24] determined phosphate by coulometric bismethometry. Phosphate was also determined by Walter[25] with an ion-selective lead electrode. Another ion-selective electrode with liquid-liquid ion-exchanger was used by Guilbault.[26] The phosphate heteropoly compounds were studied as ion-exchangers. Rechnitz, et al,[27] evaluated phosphate and sulphate sensitive membrane electrodes.

In an indirect determination, Duca, et al,[28] converted the phosphates to heteropoly molybdates which was determined. Mishra[29] and Choudry devised a rapid polarographic method for the determination of phosphates. An oscillometric end point detection was used by Tesy-Van-Dorfty, et al,[30] in the titration of orthophosphates with ferric chloride. Kreshkov, et al,[31] analyzed phosphates in natural waters by cathodic stripping voltametry. Nagelberg, et al,[32] in a short communication described use of a divalent phosphate ion

electrode with a liquid ion exchange membrane system. The organic ion exchanger consists of an amine hydrochloride membrane. The sensitivity was as low as 10^{-5} M for divalent phosphate. A micro determination of arsenates and phosphates, by ion-selective electrodes, was given by Walter.[33] Guilbault,[34] et al, studied various inorganic phosphate salts embedded in silicon rubber as indicating electrodes for phosphates. $AlPO_6$, $CrPO_6$, $FePO_4$, $Co_3(PO_4)_2$ were some of the salts used for the study. The study proved that these salts cannot be used as they are not selective. Guilbault[35] made further investigation by making use of the polyphenyl-onium bases and other materials for phosphate ion-selective electrodes. The study proved that the compounds used are not satisfactory. High frequency titration of phosphoric acid and other acids were carried out by Chakravarty, et al.[36]

Gravimetric Methods

Gravimetric determinations are not usually employed in a clinical laboratory. But in environmental and industrial analysis it could be used, but it is time consuming. Sequi,[37] et al, determined phosphate gravimetrically after precipitating it out as ammonium phosphomolybdate. Campen,[38] et al, determined phosphate in fertilizers as quinoline phosphomolybdate which is used as a referee method in international trade. In presence of varying amounts of vanadium, phosphate was determined gravimetrically by Thomas,[39] et al.

Titrimetric Methods

Titrimetric methods are not extensively used in clinical laboratories. Acidimetric and iodometric determinations of phosphate were done by Vigni,[40] et al. Spitzer[41] and co-workers titrated phosphate in ammonical solution by using $MgCl_2$ and Eriochrome black T as indicator. Also, phosphate was estimated in presence of Fe^{3+}, H^+, F^-, titrimetric method using thymolthalien as indicator. Another titrimetric method was given by Das Gupta,[42] et al. The phosphate was precipitated out by the addition of excess of Zr. The excess of Zr was determined by EDTA titration.

In an indirect titrimetric method, West,[43] et al, determined phosphate by the addition of thorium nitrate and the excess of thorium was determined by EDTA titration at pH 3. Birinboin[44] estimated phosphates by the addition of excess $Bi(NO_3)_3$ to a hot HNO_3 solution of the phosphates and the excess was back titrated with EDTA. A review of the chelatometric analysis in clinical chemistry including that of phosphates was given by Holasek.[45]

Copello,[46] et al, determined phosphate by precipitation with lead acetate solution and the excess of lead acetate was determined by EDTA titration. De Sousa[47] determined phosphates by an indirect complexometric titrimetric method as proposed by Flaska. The phosphate was precipitated as silver phosphate which was dissolved in an ammonical solution of potassium tetracyanonicklate. The liberated nickel was estimated by EDTA.

Chromatographic Methods

Chromatographic methods are rarely used in clinical laboratories. But for the identification of phosphates in biological materials, the method could be conveniently employed. As a matter of fact, the chromatographic method is used as a method of separation and the terminal analysis is done by using a suitable color developing method.

Chess and Binhart[48] separated condensed phosphates by chromatographic method. The quantitative evaluation is done by the color development and the color is measured by a densitometer. Keller,[49] et al, identified phosphates as multispot chromatograms by neutron activation analysis. Rothwell[50] and co-workers used anion exchange chromatography for the determination of phosphates by an autoanalyzer method. The polyphosphates were treated with 6.6 N sulphuric acid and converted to orthophosphates. The stream thus coming out contains orthophosphates. It was further treated with ammonium molybdate and hydrazine sulphate to form molybdenum blue. The color produced was measured with a colorimeter.

In a short note, Kankane[51] and Suovaneimi explained a simple method to determine phosphates by using TLC plates. The samples were separated, the phosphate spots were scraped off and dissolved in 10 N H_2SO_4, and determined colorimetrically with suitable color developing reagents. In another short note by Davidson[52] and Drew, phosphates were separated on TLC and determined by the usual colorimetric method.

Autoanalyzer Methods

Automated methods are extensively used in clinical laboratories. Nibet,[53] et al, used SMA 12/60 methodology for the analysis of phosphate and some other clinically important compounds in urine. Young[54] improvised the automatic analysis of serum inorganic phosphate. The inorganic phosphate is removed from protein by dialysis. The separated phosphate is made to react with ammonium molybdate at pH 6. The heteropoly complex is then further reduced by metol (p-methylaminophenol sulfate). The blue complex formed is determined colorimetrically. Klein,[55] Kauman and Issacs used stannous chloride hydrazine solution for the molybdenum blue reaction. The analysis was carried out in an automated system. Ehrlich,[56] et al, determined the low concentration of inorganic phosphorous by an automated method. The main advantage was the non-interference from ATP. In the measurements, the inorganic phosphate produced by ATP hydrolysis is extracted by isobutanol and then converted to phosphormolybdate by ammonium molybdate. The excess molybdate is complexed by sodium citrate. The phosphomolybdate concentration is measured in the organic phase at 310 nm.

Yee[57] used ferrous sulfate and thiourea as the reductant for the determination of inorganic phosphate in serum and urine. The paper also explains various reducing agents currently in use. In the determination of total phosphorous, Bide[58] used a mixture of sulfuric acid and perchloric acid in the wet digestion, thereby eliminating the use of catalysts. Hence, it is possible to determine

not only phosphates but other similar materials, like selenium. Miyada,[59] et al, investigated the interference from bilirubin, hemoglobin and lipids on the Teepol SMA 12/60 method. The study proved that there was no interference. Hirsch, Kupfer, and Orenska[60] analyzed low concentration of inorganic phosphate by an automated method. Stannous chloride was used as a reducing agent.

Miscellaneous Methods

Here mention is made about some of the methods which are not commonly employed in clinical or environmental analysis. They are radiometric, microscopic, enzymic, chemilumniscence and ring oven methods. Perhaps the most important of all of them is the radiometric method. Some of them are used only for identification purposes. In the presence of elements whose atomic weights are around thirty-two like sulfur and chlorine, the radiometric method does not give good results.

CONCLUSION

Analysis of phosphates can be complex, as well as simple, depending upon the source of the sample. The common materials which interfere in the phosphate analysis are arsenites, arsenates, and silicates. When analysis of inorganic phosphate is carried out in the presence of labile organic phosphorous compounds, erroneous results could be obtained as a result of hydrolysis of organic phosphorous compounds.

Application of phosphate analysis is mainly encountered in industrial environmental and clinical laboratories. We have seen in the earlier part of this article that there are different types of phosphate compounds. If it is not an orthophosphate, the phosphorous compound has to be converted to orthophosphate, in most cases, before analysis could be carried out.

The most popular methods are the colorimetric methods, as they are easy to automate. The chromatographic method is used only as a method of separation usually, and the terminal analysis is carried out by colorimetric method.

Analysis of phosphates is very important by virtue of its application in industry and unique position in biological materials. Research is being carried out in the field of ion-selective electrodes which would revolutionize the analytical methodology of phosphorous.

REFERENCES

1. M. Hallmann, Analytical Chemistry of Phosphorous Compounds, Wiley, New York, 1972.

2. Ibid.

3. Ibid.

4. Cyrus H. Fiske and Yellapragada Subbarow, J. of Biol. Chem., 66, 375 (1925).

5. Michi Itaya and Micho W., Clin. Chim. Acta, 14, 361-76 (1966).

6. E. S. Baginski, P. P. Foa, and B. Zak, Clin. Chem., 13 (4), 326 (1967).

7. W. S. Zaugg and R. J. Knox, Anal. Biochem., 20, 282-293 (1967).

8. Faye K. Guirgis and Yahia A. Habib, Clin. Chem., 17 (2), 78 (1971).

9. I. Hynie, J. Stepan, and B. Vecerels, J. Chromate, 24, 298-299 (1966).

10. J. F. Goodwin, Clin. Chem., 16 (9), 776 (1970).

11. Morris London and J. H. Marymerrent, J. Clin. Chem., 10 (4), 417 (1964).

12. L. G. Morin and J. Prox, Clin. Chim. Acta., 46, 113-117 (1973).

13. L. Jaenicke, Anal. Biochem., 61, 623-627 (1974).

14. W. Hohen Wallner and E. Wimmer, Clin. Chim. Acta., 45, 169-175 (1973).

15. D. H. Jung and A. C. Parek, J. Clin. Path., 25, 263-65 (1972).

16. P. A. Drews, Clin. Chim. Acta., 39, 80 (1972).

17. E. S. Baginski, P. P. Foa, and B. Zak, Clin. Chim. Acta., 15, 155-58 (1967).

18. G. F. Kirkbright, R. Narayanaswamy and T. S. West, Anal. Chem., 43 (11), 143 (1971).

19. O. K. Serduyukowa, Khim. Tecknol., 29 (1), 95-99 (1955). Chem. Abstr., 51, 10311 (1957).

20. A. E. Petrov-Spiridenov, Udobrinie i Urothai, 2 (11), 44-6 (1957).
Chem. Abstr., 52, 9843 (1958).

21. Gary D. Christian, E. C. Knoblock and William C. Purdy, Anal. Chem., 35 (12), 1869-71 (1963).

22. T. A. Khudayakowa and V. M. Voskov and A. P. Kveshkov, Khim V. Selisk, 3 (8), 14-21 (1965).
Chem. Abstr., 64, 7358 (1966).

23. T. R. Muller, R. W. Stelzner, D. J. Fisher and H. C. Jones, Anal. (1), 13-29 (1965).

24. Pants S. Tutendize and Drinka J. Stojkovic, Microchem. Acta. (4-5), 666-75 (1966).
Chem. Abstr., 66, 8101 (1969).

25. Selig Walter, Mikrochem. Acta., 564-7 (1970).
Chem. Abstr., 73, 116074 (1970).

26. G. C. Guilbault and P. J. Brignec, Anal. Chim. Acta., 56 (1), 139-42 (1971).
Chem. Abstr., 75, 104491 (1971).

27. G. A. Rechnitz, J. F. Lin and S. B. Zamochnick, Anal. Lett., 1 (1), 29-33 (1967).
Chem. Abstr., 68, 45887 (1968).

28. A. Duca, Ch. Moroi and M. Caremlau, Bul. Inst. Politeh Tasi, 15 (1-2), 1-9 (1969.
Chem. Abstr., 75, 58377 (1971).

29. A. K. Roy, B. K. Banerjie and M. B. Mishra, Tech., 5 (4), 308-10 (1968).

30. Tesy-Van-Dorfty and Farunce Domolki L. Erdey, Acta. Chim. (Budapest), 60 (3), 217-23 (1969).

31. A. P. Kreshkov and L. B. Kunzentsova, Zh. Anal. Khim., 22 (7), 1046-50 (1967).
Chem. Abstr., 67, 113483 (1967).

32. I. Nagelberg, L. Bradock and G. Barbero, Science, 166 (3911), 1403-4 (1969).

33. Selig Walter, Mikrochim. Acta., (3), 349-59 (1973).
Chem. Abstr., 78, 143486 (1973).

34. G. C. Guilbault and P. G. Brignec., Anal. Chem., 41 (8), 1136-38 (1969).

35. G. C. Guilbault, Anal. Chim. Acta., 77, 19-27 (1975).

36. J. N. Chakravarty and K. Sengupta, Ind. J. of Chem., 8 (8), 754-5 (1970).
Chem. Abstr., 73, 116069 (1970).

37. A. Sequi and A. Marchesini, Chimice (Milan), 41 (2-31), 65-6 (1965).
Chem. Abstr., 63, 17147 (1965).

38. W. A. C. Campen and A. M. J. Sledsur, Analyst, 86, 467-71 (1961).

39. G. Thomas, Fresinnius Z., Anal. Chem., 230 (2), 99-106 (1967).
Chem. Abstr., 67, 113446 (1967).

40. R. Vigni, Boll. Chim. Farm., 97, 738-141 (1958).
Chem. Abstr., 51, 7859 (1957).

41. A. Spitzer, Farmacie (Bucharest), 10, 751-4 (1962).
Chem. Abstr., 58, 13714 (1963).

42. B. C. Sinks, S. Das Gupta and S. Kumar, Analyst, 93 (1107). 609-12 (1968).

43. G. F. Kirkbright, A. M. Smith and T. S. West, Analyst, 94, 321-323 (1969).

44. S. N. Birinboin, Farmetsuet Zh. (Kiev), 19 (5), 41-4 (1963).
Chem. Abstr., 60, 6704 (1964).

45. A. Holasek, Z. Anal. Chem., 212 (1), 90-8 (1965).
Chem. Abstr., 63, 11989 (1965).

46. M. A. Copello, E. A. Dorfman and J. L. Amorous, Safybi, 7 (19), 10-14 (1967).
Chem. Abstr., 67, 104897 (1967).

47. A. De Sousa, Anal. Chim. Acta., 67, 234-5 (1973).

48. O. N. Benfort and W. B. Chess, Anal. Chem., 31 (6), 1026 (1959).

49. M. A. Rommel, R. A. Keller, J. Chromat., 18, 349-358 (1965).

50. F. H. Pollard, G. Nickless, D. E. Rogers and M. T. Tothwell, J. Chromato., 17, 157-167 (1965).

51. P. Kankane and O. Suovaneimi, J. Chromato., 62, 485-486 (1971).

52. I. W. F. Davidson and W. A. Drew, J. Chromato, 21, 319-323 (1966).

53. M. Baines, A. Jarvis and J. A. Nisbet, Clin. Chim. Acta., 53, 331-338 (1974).

54. D. S. Young, J. Clin. Path., 19, 397 (1966).

55. B. Klein, J. H. Kaufman and J. Issacs, Clin. Chem., 13 (12), 1072 (1967).

56. E. Ehrlich, R. Coelho and F. Guilloin, Anal. Biochem., 50, 503-8 (1972).

57. H. Y. Yee, Clin. Chem., 14 (9), 898 (1968).

58. R. W. Bide, Anal. Biochem., 29, 393-404 (1969).

59. D. S. Miyada, J. Elder and R. M. Nalcamurs, Clin. Biochem., 8, 62-68 (1975).

60. C. Hirsch, S. Kupfer and I. Onenska, Clin. Chem. Acta., 18 (2), (1967).

GENERAL CONCLUSIONS

In the preceding pages we have presented the various analytical methodologies and advancements for the analysis of vitamin B_1 and B_6, carbonates and phosphates. Not only has a critical evaluation been made but at the same time many recent, relevant publications are included, which differ slightly only in their analytical methodologies. This way, the interested analytical chemist can find the necessary material without further exploration of the literature especially from 1960 onwards.

Perhaps the most significant progress is made by the advent of ion-selective electrodes for the analysis of the compounds we are dealing with; and for many other anions and cations. The ion-selective electrodes are easy to handle with low detection limits.

Every analytical method has its merits and demerits and we have discussed them whenever possible. Here we leave it for the analytical chemist to choose an appropriate method and make necessary modifications to suit the analysis of a particular sample. Choosing the right analytical method is probably the most significant decision the analytical chemist makes in his laboratory.

APPENDIX

The survey includes publications that have appeared from 1960 onwards and up to December, 1975. Few earlier relevant references are also included. In making the literature research the following pattern has been observed.

Vitamin B_1 (Thiamine) and B_6

Determination of vitamin B_1 or B_6
Analysis of vitamin B_1 or B_6

--in blood
--in urine
--in CSF
--in water
--in pharmaceutical preparations
--in biological materials
--in food
--by spectrophotometry
--by fluorometry
--by electrometry (coulometry, amperometry, etc.)
--by chromatography (gas, TLC, column, etc.)
--by gravimetric methods
--by titrimetric methods
--by microbiological methods

--(by any other relevant analytical method)

Whenever the Chemical Abstract indicated, a new analytical methodology has been referred to. Also, standard books under the title "Clinical Chemistry," "Clinical BioChemistry" and "Vitamins" were also researched.

For carbonate and phosphate analysis the research was done in a similar manner. Publications were researched for the analysis of carbonates or phosphates in blood, urine, water and sometimes in biological materials, if relevant. Above all, in all these cases, where a new analytical methodology has been employed, that publication has always been included.