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Review Article

The measurement of ionised calcium in blood plasma

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Plasma calcium fractions

Calcium in plasma is distributed between ionised and bound fractions (Fig. 1). (Throughout this paper, the term 'plasma' is synonymous with the plasma phase of whole blood, undiluted separated blood plasma, or undiluted serum). In normal subjects about 50% is present as free hydrated calcium ions (hereafter called ionised calcium; Ca^{2+}). Bound calcium comprises several species which are usually distinguished on the basis of molecular size as protein-bound and complex-bound calcium; albumin accounts for about 75% of the calcium bound to protein.[1, 2]

Calcium can bind to albumin at about 30 binding sites which have various association constants,[3] although the overall effect can be described by a more simplistic model with 12 sites having an overall apparent binding constant at pH 7·4 of 95 L/mol.[4, 5] The association of Ca^{2+} ions is pH-dependent and the presence of fatty acids bound to albumin enhances Ca^{2+} association.[6] The binding of calcium to protein is also temperature dependent, being greater as temperature increases.[2, 7] As pH is also influenced by temperature, the overall effect is that plasma Ca^{2+} concentration at 37°C is about 6% higher than at 20°C and that of whole blood, about 3% higher.[2, 4, 7] Bicarbonate and, to a lesser extent, lactate and phosphate are the most important of the low molecular weight calcium ligands *in vivo*[2, 4, 8, 9] and in total they bind about 10% of all calcium in normal plasma. However, in some circumstances exogenous anions, such as citrate, may bind substantial additional amounts of calcium.[10, 11] The distribution of calcium between all of these fractions is subject to the law of mass action and, therefore, depends on calcium concentration, on pH and on the concentrations of plasma proteins and low molecular weight ligand anions.

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PHYSIOLOGICALLY ACTIVE CALCIUM

The electrochemical activity of free Ca^{2+} ions influences their diffusion equilibria and determines their reactivity and it must thus be the principal determinant of their physiological activity. The foundations of our understanding

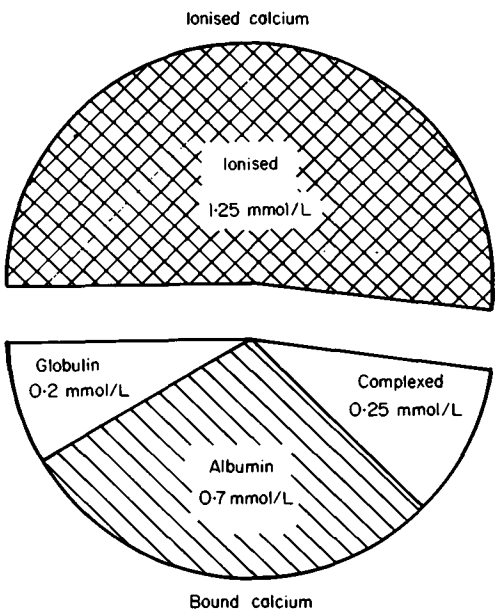

FIG. 1. The distribution of calcium fractions in blood plasma.

of the physiology of Ca^{2+} were laid by McLean and Hastings in the 1930s when, in a series of remarkable experiments, they demonstrated that the contraction of isolated frog heart ventricles varied with changes in the Ca^{2+} concentration of the perfusate.[12-14]

Since then it has been shown that Ca^{2+} ions, rather than any other fraction, determine the intestinal absorption of calcium,[15] its renal tubular reabsorption,[16] parathormone secretion,[17, 18] calcitonin secretion,[19] the action of calcium in modulating hormone action and secretion,[20] cardiac contractility,[21-24] neuromuscular excitability,[25, 26] sperm motility,[27] and the exchange of calcium between extracellular fluid and bone.[28]

Some workers have suggested that calcium-protein complexes[29] and 'ultrafiltrable calcium', i.e. its complexes with low molecular weight anions[30] may be directly active in physiological processes in a manner not mediated by Ca^{2+}, although plausible mechanisms have not been suggested for this.

Non-potentiometric methods of Ca^{2+} measurement

BIOASSAY

Ca^{2+} concentration was first estimated in serum by comparison of the amplitude of contraction of isolated frog heart ventricles when perfused with serum to their contraction when perfused with standard calcium saline solutions.[12] The authors recognised that the technique was not suitable for routine application and provided a nomogram for the calculation of Ca^{2+} from total Ca concentration in clinical practice. Later bioassays were based on measurement of the calcification of rachitic rat cartilage[31] and on blood coagulation.[32]

Bioassay methods are now only of historical interest. They are cumbersome, slow, prone to error, and are particularly sensitive to interference by other components of plasma such as potassium and phosphate. That the McLean and Hastings method[12] was for many years regarded as the reference method for Ca^{2+} estimation was an indictment of the alternative techniques then available.

ADSORPTION METHODS

Ion-exchange strips have been used in an attempt to measure plasma concentrations of Ca^{2+} and ionised magnesium.[33] They were immersed in anaerobic whole blood at 37°C, followed by elution and measurement of calcium and magnesium. This technique is limited by the need to use either very large sample volumes or exquisitely sensitive measurement techniques so that the quantity of resin used will cause minimal disturbance to the equilibria between calcium fractions in plasma. Similar problems affect an analogous method employing charcoal coated with albumin.[34] Neither technique has been widely used in practice.

PHOTOMETRIC METHODS

A number of compounds which form coloured complexes with calcium are in use for calcium measurement. o-Cresolphthalein is well known; others include alizarin, methyl thymol blue and glyoxal-bis-(2-hydroxyanil). These compounds are reviewed in detail elsewhere.[35] As they all bind calcium strongly and strip calcium from its complexes in plasma they are unsuitable for use in determining Ca^{2+}.

The metallochromic indicator murexide and its derivatives are potentially more useful.[36] The formation of metal complexes by these highly optically-active dyes results in a spectral shift in the visible region. They bind less than about 1% of Ca^{2+} ions in plasma and thus have only a small influence on their equilibrium distribution.[37-39] However, murexide also binds to albumin[40] and is pH-sensitive.[34] Although these problems are overcome to some extent by the use of its derivative tetramethyl murexide (TMM),[41, 42] sodium interferes with this technique, and thus results are inaccurate in the presence of hypo- or hypernatraemia.[43] Another limitation is the need to ultrafilter or dialyse plasma prior to adding murexide or TMM to optimise results.

Other Ca^{2+}-sensitive dyes such as Arsenazo III and Antipyrylazo III have been used as intracellular probes, but have not been used in plasma Ca^{2+} measurement.[44] Metallochrome Ca^{2+} indicators have been comprehensively reviewed.[45]

A number of derivatives of EGTA, such as quin2 and fura-2, are fluorescent when they complex with calcium,[46, 47] but they have mainly been applied to the attempted measurement of intracellular Ca^{2+} concentration and have not found application in the analysis of plasma samples.

The bioluminescent photoproteins aequorin from the jellyfish *Aequorea* and obelin from the hydroid *Obelia* emit blue light in the presence of calcium ions, the amplitude of the intensity of the light pulse being a non-linear function of

the calcium ion concentration.[44, 48] As the light pulse emitted lasts only about 5–10 ms, rapid mixing of sample and reagent is required. The proteins are scarce and expensive and are changed irreversibly in their interaction with Ca^{2+}. Affinity for calcium is high, thus the photoproteins disturb plasma calcium equilibria. Light emission is also influenced by H^+, Na^+ and Mg^{2+} concentrations[44] and hence these compounds are unsuitable for use in routine clinical chemistry.

Potentiometric methods of Ca^{2+} measurement

EARLY INSTRUMENTS

Potentiometric measurement of Ca^{2+} in plasma has the fundamental advantage that introduction of a Ca^{2+}-selective electrode into the sample does not disturb the equilibria between free and bound forms of calcium. In responding to the electrochemical potential of calcium ions, the technique probably best reflects their physiological activity.

Since Luther in 1898 described the first, unsuccessful, calcium ion electrode,[49] attempts have been made for almost 90 years to measure calcium ions potentiometrically in aqueous solution. Just 2 years after McLean and Hastings had reported their frog heart bioassay,[12] a calcium fluoride electrode was described in a paper with the optimistic title 'A new and easy method for the potentiometric determination of calcium concentrations in solutions'.[50] This was soon followed by the description of a calcium amalgam electrode.[51] Sulphonated polystyrene-collodion,[52] multilayer[53] and paraffin membrane[54] electrodes were described some years later. However, none of these devices were capable of operating successfully in biological fluids.

The first ion selective electrode (ISE) which could do this was developed by Ross[55] in 1966, based on a membrane impregnated with calcium di-n-decyl phosphate, a hydrophobic liquid ion exchanger (Fig. 2a), dissolved in a non-polar solvent di-n-octylphenyl phosphonate (Fig. 2b). This electrode was used in the first (1967) commercially available Ca^{2+} analyser, the Orion 92–20 (Orion Research Inc, Cambridge, Mass, USA) and in several later instruments from this and other companies from the late 1960s onwards.

However, the early Ca^{2+} analysers were plagued by unreliability, poor electrode selectivity and non-Nernstian response. Measurements were susceptible to error due to factors such as

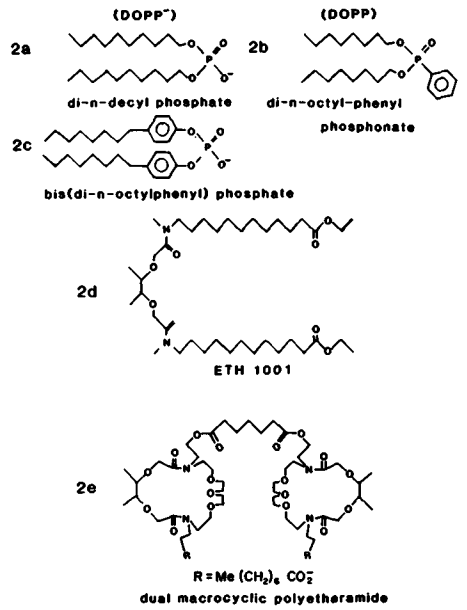

FIG. 2. Structures of electroactive Ca^{2+}-ISE membrane constituents.

the presence of erythrocytes at the reference electrode liquid junction[56, 57] and the clinical use of Ca^{2+}-selective electrode analyers was effectively restricted to enthusiasts.

The literature on Ca^{2+} in blood plasma up to the late 1970s should be read with caution. Apart from the instrumentation difficulties many workers were unaware of the necessity for care in anticoagulation and for anaerobic processing of specimens. Furthermore, little was known about the appropriate composition of calibration solutions, resulting in reference ranges for plasma concentrations of Ca^{2+} which differ substantially from those established with modern instruments.[58]

MODERN INSTRUMENTS

Modern instruments which permit the reliable measurement of Ca^{2+} in blood plasma[58] were made possible by the almost simultaneous development of two different kinds of Ca^{2+}-selective membrane. In 1972, Simon *et al.*[59, 60] described a Ca^{2+}-selective membrane based on ETH 1001, a so-called neutral carrier (Fig. 2d), and this material is now widely used in clinical chemistry (Table 1). In 1973, Ruzicka and colleagues[61] reported that the selectivity of the Ross-type of ion-exchange electrode was improved by using calcium-bis (di-n-

TABLE 1. Comparison of design differences between some commercial ionised calcium analysers

Manufacturer & model	Calcium ionophore	Reference electrode	Salt bridge	Liquid junction	Calibrant matrix
AVL 980	ETH1001	Calomel	1·2M KCl	Open static	Triethanolamine
Corning 634	ETH1001	Ag/AgCl	Sat KCl	Dialysis	MOPS + BES
Kone Microlyte	ETH1001	Ag/AgCl	3M KCl	Porous plug	Tris acetate
Nova 8	ETH1001	Ag/AgCl	2M KCl	Open flowing	HEPES
Radiometer ICA1	DOPP in DOPP	Calomel	4·6M CHOONa	Open static	TES/BES

BES: N,N-bis (2-hydroxyethyl)-2-aminoethanesulphonic acid
DOPP : calcium bis(di-n-octylphenyl) phosphate
DOPP: calcium bis(di-n-octylphenyl) phosphonate
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
MOPS: 4-morpholinepropanesulphonic acid
TES: 2-[tris(hydroxymethyl)-methylamino]-1-ethanesulphonic acid

octylphenyl) phosphate (Fig. 2c) instead of the di-n-decyl phosphate salt (Fig. 2a) and this type of electrode is also used extensively (Table 1). These materials allowed the development of the modern generation of Ca^{2+} analysers and this review will concentrate on developments in the potentiometric measurement of Ca^{2+} which have occurred since robust and reliable instruments became available in the early 1980s.

Calcium ion-selective electrode (ISE) analysers are constructed around a cell which comprises a Ca^{2+}-selective electrode and a reference electrode in electrochemical contact with the sample (Fig. 3). The electromotive force (emf) of the ISE is compared with the constant emf of the reference electrode by a high impedance millivoltmeter. The potential difference across the cell reflects the activity of Ca^{2+} ions in the sample according to the Nernst Equation:

$$E_{Ca} = E_o \pm \frac{RT}{zF} \ln (a_{Ca})$$

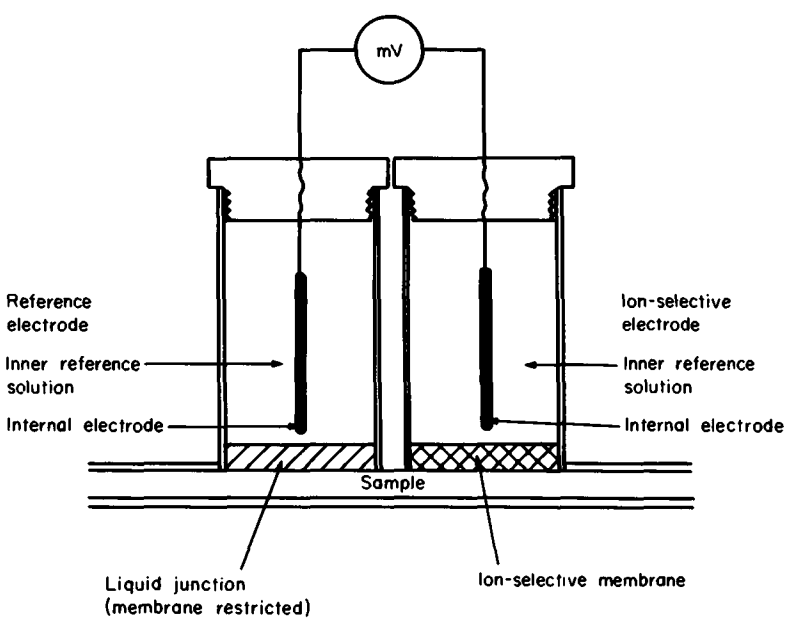

FIG. 3. Schematic diagram of a typical flow-through ISE-reference electrode measuring cell.

where: E_{Ca} is the emf of the calcium ISE, E_o is a constant which represents a composite of constant potentials within the ISE such as the emf of the internal reference electrode and membrane surface potentials, R is the gas constant, T is the temperature (in °K), z the ion charge (in this case +2) and a_{Ca} the electrochemical activity of Ca^{2+}.

The basic theory of ISEs is extensively reviewed elsewhere.[62–66]

Typical analysers employ a system of pumps and valves under microprocessor control to transport calibration solutions, samples and wash solutions through the measuring cell, and are equipped with software to facilitate calibration and the reporting of results. Most analysers report results as Ca^{2+} concentration, which is related to the measured activity by the equation:

$$a_{Ca} = \gamma_{Ca} c_{Ca}$$

where a_{Ca} is calcium activity, γ_{Ca} is the activity coefficient and c_{Ca} is Ca^{2+} concentration.

Despite the fact that all modern Ca^{2+}-selective electrodes behave in a Nernstian manner, ie they produce virtually identical responses in emf for given changes in Ca^{2+} activity, their final reports of Ca^{2+} concentration for the same plasma sample may differ significantly from one design to another. This is mainly due to differences in calibration solution composition, reference electrode design and geometry, and (to a lesser extent) sensor electrode design. Some instruments at present available or currently in use are listed in Table 1, which shows the main points of difference in design. The principal components of a Ca^{2+} ISE system will be described in detail below.

Ca^{2+} ION SELECTIVE ELECTRODES

A typical Ca^{2+} ion selective electrode (Fig. 3) consists of an assembly in which a Ca^{2+}-selective membrane encloses an inner reference solution of $CaCl_2$ held within a stem of plastic or glass.[67] An internal reference electrode, usually Ag/AgCl, is immersed in the inner reference solution. In its simplest form this consists of a standard aqueous solution of $CaCl_2$, usually saturated with AgCl to prevent dissolution of the AgCl from the internal reference electrode, and containing NaCl and KCl in physiological concentrations.

Modern calcium ISEs in routine use in clinical chemistry are based on so-called liquid membranes in which an ion-selective electroactive substance or 'ionophore' is dissolved in an organic liquid phase which is trapped within a polymeric matrix, usually polyvinyl chloride (PVC). Two categories of ionophore are in commercial use at present.

In neutral carrier membranes, an electrically uncharged Ca^{2+}-selective complexing agent such as ETH 1001 (Fig. 2d) is dissolved in the plasticiser, for instance o-nitrophenyl octyl ether or bis (2-ethylhexyl) sebacate, in a PVC membrane.[59–66] The ionophore and plasticiser must be as hydrophobic as possible to avoid being leached out of the membrane by samples. ETH 1001 and related ionophores are selective for Ca^{2+} because they provide an environment of conformation and atomic interaction into which the ion reversibly 'fits', rather as a substrate fits the active site of an enzyme. The mobility of the ionophore in the liquid membrane allows the ions to permeate across under the influence of the prevailing electrochemical gradient.[68] Recently, a highly calcium-selective bis-(crown) neutral carrier based on a macrocyclic polyetheramide with two polyether rings has been described[69] (Fig. 2d) and it is likely that further crown ether compounds will be designed, possibly with linked heterogeneous crown rings, to further improve ion selectivity.

So-called ion-exchanger or negatively charged carrier membranes consist of ionic or ionogenic compounds dissolved in an organic water-immiscible solvent which is trapped in PVC or other suitable polymer matrices. Formerly, cellulose acetate membranes and porous plugs soaked in the electroactive solution were used instead of PVC.[70] At present, calcium-bis (di-n-octylphenyl) phosphate (Fig. 2c) is the preferred ion exchanger, dissolved in di-n-octylphenyl phosphonate (Fig. 2b), which in turn is held within a PVC matrix.[2, 8, 61] The interactions between Ca^{2+} and this kind of membrane comprise both ion binding by the ion exchanger and solution interaction between the ion and the membrane solvent. The degree of ion selectivity achieved depends on careful matching of the ion exchanger with its solvent in the membrane.[66, 71]

Membrane performance is usually optimised by the presence of further additives which may serve a number of functions. For example, potassium tetrakis (p-chlorophenyl) borate is frequently added to lower the electrical impedance of neutral carrier membranes and its large lipophilic anion serves to prevent entry of sample anions into the membrane. However,

care must be taken with the use of such additives as their anion may itself serve as an ion exchanger and, if present in sufficiently high concentration (>80% on a molar basis relative to the calcium ionophore), can dominate in the membrane with its own cation selectivity.[72]

In designing Ca^{2+}ISE membranes for clinical chemistry, their selectivity must be sufficient to show no detectable interference from Na^+, K^+, H^+, Mg^{2+}, or from anions such as Cl^- and bicarbonate in concentrations encountered in pathological plasma specimens. Selectivity is determined by properties both of the ionophore and of the solvent in which it is dissolved within the PVC matrix. The inclusion of lipophilic anions such as tetrakis (p-chlorophenyl) borate in the membrane helps to minimise anion interference.

The required membrane selectivity can be calculated using the following relationship, derived[66] from the Nikolskii-Eisenman expansion of the Nernst equation:

$$K^{Pot}_{Ca,M\ max} = \frac{a_{Ca,\ min}}{(a_{M,\ max})^2} \times \frac{P_{CaM}}{100}$$

where:

$K^{Pot}_{Ca,M\ max}$ is the maximum allowable value of the selectivity factor for electrode use in plasma

$a_{Ca,min}$ is the lowest encountered molar activity of calcium ion in plasma

$a_{M,max}$ is the highest encountered molar activity of the interfering ion M in plasma

P_{CaM} is the highest allowable percentage error in the molar activity a_{Ca} due to interference by a_M

The selectivity required of Ca^{2+} ISEs for use in blood plasma and the reported selectivity constants for a number of Ca^{2+} electrodes are shown in Table 2. The selectivities of these modern neutral carrier and ion exchange membranes are more than adequate for use in plasma, since naturally occurring cations at extremely high pathological concentrations cause immeasurably small errors even in gross hypocalcaemia (Table 3).

THE REFERENCE ELECTRODE

The reference electrode acts as a 'half-cell' of constant emf against which the emf of the Ca^{2+} electrode in various samples can be compared.[73] Reference electrodes used in Ca^{2+}

TABLE 2. Ca^{2+} electrodes in plasma: required and reported values for the selectivity factor log $K^{Pot}_{Ca,M\ max}$

Interfering ion (M)	H^+	Na^+	K^+	Mg^{2+}
Maximum ionised conc. encountered in plasma	160 (nmol/L)	180 (mmol/L)	8 (mmol/L)	1 (mmol/L)
Required selectivities (log $K^{Pot}_{Ca,M\ max}$)	7·8	−4·0	−1·3	−2·3
Reported selectivities (log $K^{Pot}_{Ca,M\ max}$)				
$DOPP^-$ in DOPP	4·2*	−5·2**	−5·7**	−3·6**
ETH1001	0·0***	−5·0***	−5·2***	−5·1***
bis-(crown)	n.a.	−5·0	4·5	−5·0

* Measured by fixed primary ion method[61]
** Measured by separate solution method[67]
*** Measured by fixed interference method[69]

TABLE 3. Predicted interference by pathologically high concentrations of cations on different calcium ISEs at 0·5 mmol/L Ca^{2+} concentration

Interfering cation	H^+	Na^+	K^+	Mg^{2+}
Concentration	160 (nmol/L)	180 (mmol/L)	8 (mmol/L)	1 (mmol/L
Interference (percent):				
DOPP in DOPP	$2{\cdot}10^{-4}$%	$6{\cdot}10^{-2}$%	$4{\cdot}10^{-5}$%	$5{\cdot}10^{-2}$%
ETH1001	$2{\cdot}10^{-8}$%	$8{\cdot}10^{-2}$%	$2{\cdot}10^{-4}$%	$1{\cdot}10^{-3}$%
bis-(crown)	n.a.	$8{\cdot}10^{-2}$%	$6{\cdot}10^{-4}$%	$2{\cdot}10^{-3}$%

ISE analysers are very similar in general design to those used in pH meters, but more closely resemble those in instruments such as blood gas and sodium-potassium ISE analysers. They are invariably based on calomel or Ag/AgCl electrodes which make electrochemical contact with the sample via a bridge, usually a solution of KCl of concentration greater than 2 mol/L. Sometimes other bridge solutions, eg sodium formate 4·6 mol/kg are used.[74] The properties and designs of reference electrodes are discussed in detail in several monographs.[74–76]

The interface between the bridge solution and the sample is called the liquid-liquid junction. Because ions diffuse across their electrochemical gradients from the sample into the bridge and vice versa, and electrochemical potential is developed across the junction, called the liquid junction potential. If very high concentrations of an unreactive salt are used, whose anion and cation ideally move rapidly and at the same rate from bridge to sample (e.g. KCl), the liquid junction potential is predominantly a function of the concentration gradient of this salt across the junction. However, it is also influenced by the ionic strength of the sample and by the presence of some anions, notably compounds such as tris and zwitterionic buffers which may be used in calibration solutions. Thus, while the reference electrode itself maintains a constant emf, the liquid junction potential can be influenced by the sample matrix so that the overall emf produced by the reference 'half-cell' may not be constant.[77] Thus, the liquid-liquid junction is prone to interference by a number of important variables in sample composition, as will be described in more detail below.

The difference between the liquid junction potential of the calibrant solution and that of a sample is called the residual liquid junction potential.

Other electrochemical devices for determining plasma Ca^{2+}

ISFETS

Ca^{2+}-selective field-effect transistors (ISFETs) have been described[78–81] which have been used to measure plasma Ca^{2+} concentration continuously, both intravascularly in dogs[78] and extracorporeally[79] in man. In these devices, an ion-selective carrier material containing an ionophore such as ETH 1001 takes the place of the metal gate of a transistor. This barrier material is in contact with the SiO_2/Si_3N_4 dielectric (Fig. 4). The potential generated by the selected ion at the gate affects current flow from source to drain. If appropriate circuitry is employed to maintain a constant drain current, the final output of the device is a voltage which is related to the activity of the sensed ion by the Nernst Equation. A reference electrode completes the cell.

Because ion diffusion occurs over a very short distance, these devices have the advantage of very short response times. They are very small, and many different ISFETs can be sited on the same 2·5×2·5 mm chip along with their associated control and amplification circuitry.[78, 80, 81] A number of problems have

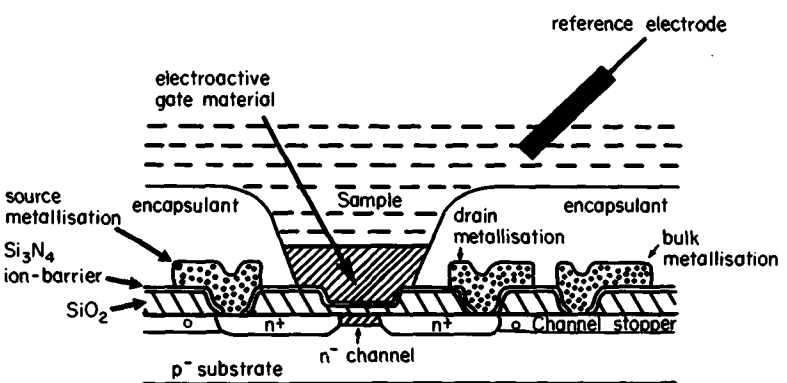

FIG. 4. Schematic diagram of depletion-mode ion selective field effect transistor (ISFET) incorporating an n^- ion-implanted channel. (After Covington AK, Sibbald A,[80] with permission).

prevented the widespread use of ISFETs, particularly the difficulty of accurate deposition of the electroactive barrier in the gate region and the selective insulation of the semiconductor chip and its ciruitry from the sample while leaving the electroactive gate region exposed to the analyte.

Although ISFETs might be thought to be ideal for intravascular deployment inside catheter tips, they are very difficult to sterilise, tend to promote clot formation on their surface, interfere with the electrical isolation of the patient, and their calibration *in situ* cannot be continuously assured. Thus, Covington has proposed[80] that they are still best used *ex vivo*, blood being continously drawn out of the appropriate site in the patient and passed over the ISFETs.

Even if the problems of fabrication of ISFETs are overcome, there remains the fact that useful reference electrodes have not yet been devised in ISFET form, so that much of the advantage of miniaturisation is lost at present by the need to use conventional reference electrodes. It is still not certain therefore that ISFETs will eventually replace conventional ISEs.

COATED WIRE ELECTRODES

The presence of an internal filling solution does not appear to be fundamental to the operation of ion-sensing potentiometric electrodes. Coated wire electrodes (CWEs) can be made very simply by dipping copper wire into a solution of PVC containing an ionophore, and allowing this to dry as a film on the wire. Properly made, they respond in a Nernstian manner in simple solutions, but they are not yet sufficiently refined for use in plasma Ca^{2+} determination. The construction and behaviour of CWEs has been reviewed by Freiser.[82]

ISES FOR MEASURING TOTAL CALCIUM CONCENTRATION

A number of instruments are available which measure total plasma calcium concentration potentiometrically using Ca^{2+} ISEs.[83–85] These avail of the high selectivity of neutral carrier membranes against H^+ ions (Table 2), measuring Ca^{2+} concentration in plasma which has been acidified to about pH 3·5 to release all protein-bound Ca^{2+}. An alternative approach has been proposed[86] using sample dilution and Ca^{2+} displacement from protein binding with Zn^{2+}. The accuracy and precision of these devices compare very favourably with atomic absorption spectrometry. They may well prove superior to colorimetric techniques of total calcium estimation and may find application in high-throughput multi-parameter clinical analysers in the future.

Standardisation of ionised calcium analysis

Routine calibration of the present generation of Ca^{2+} ISE analysers entails the use of two solutions of different Ca^{2+} concentration, usually with the same near-physiological ionic strength, made up with NaCl. A buffer is included to stabilise pH and often is designed to allow calibration of an integral pH electrode. At present, most Ca^{2+} analysers also measure other ions (e.g. Na^+, K^+, H^+) and Ca^{2+} electrodes are increasingly being used in blood gas analysers. Thus, their calibration solutions must be multifunctional, capable of use in calibration of the other ions as well. Manufacturers employ a variety of quite different calibration solutions which are each matched to the electrochemical characteristics of the instrument design (Table 1). The effects of different buffers and other factors on liquid junction potential and Ca^{2+} binding are accounted for in the instrument software; therefore, it is not usually possible to calibrate one instrument with calibration solutions meant for that of another manufacturer.[87] Futhermore, there is as yet no way in which all Ca^{2+} analysers can be standarised to give the same results on the same samples and thus eliminate variations between instruments from different manufacturers.

Ideally, routine analytical methods in clinical chemistry are standardised by means which are traceable to a primary reference material, generally an ultrapure form of the analyte, through definitive and reference methods. Thus, the accuracy of routine methods should be founded on that of the definitive method. The primary reference material for total plasma calcium determination is $CaCO_3$; NBS/SRM 913, which can be used to prepare solutions in which the total calcium concentration is known with considerable accuracy. The definitive method for total calcium is isotope dilution mass spectrometry, and the reference method is based on flame atomic absorption spectrometry. A lyophilised human serum, NBS/SRM 909, may be used as a secondary reference material.[88]

Unfortunately, it is not possible to base the accuracy of electrometric methods of ion activity measurement on this framework. Ca^{2+} ions in aqueous solution are hydrated. They also interact with nearby ions of the same or different species, for instance repelling cations and attracting anions. In physiological solutions, which are of relatively high ionic strength, these interactions are appreciable, particularly since calcium ions are divalent. In solutions which resemble in ionic strength and composition the aqueous phase of plasma, Ca^{2+} ions are so 'preoccupied' with interactions with their neighbours that their activity is only about one third of their concentration, i.e. their activity coefficient is about[89] 0·34.

At present there is no way by which the exact activity of a single ion can be measured in solutions which resemble the aqueous phase of plasma. Activity coefficients which are used for calculating ion activity are themselves calculated from mean ionic activities of ion combinations (i.e. anion and cation) which are split, using a convention, into the contributions of the single ions. The assignment of activity values to ions in a particular solution therefore derives from calculations which are themselves based on models of presumed ion behaviour and on conventions.[89, 90] There are several such models, each providing activity coefficients which differ from each other to a greater or lesser extent.[89, 90] Although the differences are not large, the activity coefficients range from about 0·304–0·344,[89, 90] they prevent the unequivocally accurate assignment of activity values to ions in solution.

At present, it seems unlikely that a definitive method will be developed for measurement of Ca^{2+} activity. There is no equivalent to the Harned cell with hydrogen gas electrode used[91] as the definitive method for pH and there is little encouragement in the literature for the use of calcium amalgam electrodes in this role. Thus, standardisation of methods for the measurement of Ca^{2+} must for the present rely on a series of conventions which assign values for the concentration and activity of Ca^{2+} to defined primary reference solutions.

Three such solutions have been proposed[92] by the European Working Group on Ion Selective Electrodes of the International Federation of Clinical Chemistry. There are based on NBS/SRM 913 $CaCO_3$ at final Ca^{2+} concentrations of 0·250, 1·250 and 2·500 mmol/L in unbuffered carbonate/bicarbonate-free aqueous solution, brought to a final ionic strength of 160 mmol/kg with NaCl. The concentrations of free hydrated Ca^{2+} ions in these solutions are known with a high degree of accuracy because they are based on a primary reference material. Their Ca^{2+} activities can be calculated with a reasonable degree of assurance and assigned to them by convention.[89]

Because plasma contains many more constituent species than these relatively simple solutions, the liquid junction potential produced with them differs from that with plasma[93] by about 6%. It has been proposed[93, 94] that inclusion of 110 mmol/L HEPES buffer in the primary standards would abolish this residual liquid junction potential, though this can only be achieved at the expense of binding Ca^{2+}. As the concentration of free Ca^{2+} ions would then have to be assigned by calculation, such buffered solutions could not then be described as truly 'primary'.

Once agreement has been reached on the nature and compositions of primary reference solutions, they can be used to calibrate a reference method. A reference method based on a rigidly defined design of Ca^{2+} ISE cell, very similar to the reference method for pH[95] has been proposed.[92] On the adoption of a reference method, the development of secondary reference materials will be possible, allowing verification of the accuracy of Ca^{2+} analysers in routine use.

Manufacturers have developed and marketed instruments before the long process of international standardisation can be completed. Thus, most have designed their instruments to give results which produce a reference range, based on theoretical considerations, for Ca^{2+} concentration in normal adult blood plasma of about 1·18–1·30 mmol/L.[2, 9, 87, 96–98] As a result, most of the present generation of Ca^{2+} analysers produce results on plasma which are closely similar to each other.[58] Although this reference range has a reasonably sound basis, the approach adopted is purely pragmatic. Nevertheless, it is likely that agreement on reference solutions and a reference method will not radically alter the reference range.

UNITS OF MEASUREMENT

Ca^{2+} analysers report results in terms of substance concentration with the units mmol/L. However, it is not widely realised that the denominator refers to litres of plasma water and not to the whole volume of plasma.[92] This is because the instruments are calibrated using colloid-free aqueous solutions and results are

not adjusted for the fraction of ion-inaccessible plasma volume occupied by protein. The results thus approximate more to molal than to the molar concentration. This, of course, reflects better the physiological role of Ca^{2+} ions than would attempts to report in terms of molar concentration. Such a system is practicable for Ca^{2+}, unlike sodium and potassium, because clinicians are unencumbered by gravimetrically-determined reference ranges for Ca^{2+} concentrations in plasma.

Plasma Ca^{2+} measurement in practice

Microprocessors have greatly simplified instrument operation in recent years. Measurement of Ca^{2+} concentration in a sample involves its introduction without pre-dilution into an appropriately calibrated ISE analyser. Typically, the fluid transport path and the electrode cuvette will already have been flushed with a solution resembling the set-point calibrant, which may also contain a proteolytic enzyme or a surfactant. The sample is then pumped to the electrode cuvette and the liquid junction established either by pumping or diffusion of the bridge solution. The instrument's microprocessor seeks a stable potential difference between the ISE and the reference electrode, and calculates the sample concentration from the previous calibration data. The result is usually available within a few minutes. The sample is then pumped to waste and the entire fluid path is flushed in preparation for the next sample. Because Ca^{2+} ISEs sense the activity of Ca^{2+} ions in the aqueous phase, they should give the same result whether used in whole blood, its separated plasma or its serum.

PRE-ANALYTICAL ERRORS

The reference range for Ca^{2+} concentration in plasma (about 1·18–1·30 mmol/L) is very narrow, having a span of only about 10% of its median concentration. Thus, relatively small errors in measurement may have a substantial effect on interpretation.

The determination of plasma Ca^{2+} concentration is susceptible to a number of specific pre-analytical factors, each of which may cause errors in the final result which can simulate or mask true pathological changes. Pre-analytical errors may be induced during collection of the blood specimen, by its anticoagulation, or by pH changes which occur in the specimen prior to analysis.

SPECIMEN COLLECTION

The posture of the subject has only a small effect on plasma Ca^{2+} concentration,[2, 99] a change from upright to supine decreasing Ca^{2+} by less than 2%, a much smaller change than seen in total Ca concentration.

Arterial, capillary or venous blood samples may be used for Ca^{2+} determination, though the Ca^{2+} concentration is influenced by blood pH at the sampling site. Blood pH influences the binding equilibrium between Ca^{2+} and plasma protein (see below). Thus, exercise by the subject[100] may cause a significant increase in Ca^{2+} concentration, the extent of this rise being related to the amount of lactic acid formed. Venous stasis for up to 10 min without forearm exercise causes minimal changes in Ca^{2+} concentration but vigorous hand 'pumping' increases this in proportion to the amount of lactic acid formed and to the fall in blood pH.[2, 101] Clearly, activities such as hyperventilation which change blood pH[9, 25, 26] will alter Ca^{2+} concentration and should be avoided.

ANTICOAGULATION OF THE SPECIMEN

Compounds such as citrate, oxalate and EDTA which are commonly used *in vitro* as anticoagulants and which strongly bind calcium should be avoided when Ca^{2+} is to be measured. It is less widely appreciated that Ca^{2+} is also bound by heparin.[1, 2, 56] If 50 IU heparin is added per mL of whole blood, a decrease in Ca^{2+} of about 6% is observed, although this figure may vary with the source and purity of the heparin. Use of 15 IU heparin per mL of whole blood usually causes an insignificant change[100] (less than 2%) in Ca^{2+}, but does not guarantee anticoagulation, especially of capillary samples. Therefore, preparations of calcium-titrated heparinate are commercially available in which the final concentration of Ca^{2+} is about 1·25 mmol/L, i.e. about the midpoint of the plasma concentration reference range. In general, adequate anticoagulation is achieved and changes in Ca^{2+} concentration in the specimen are minimal when up to 50 IU of this preparation are used per mL whole blood, unless the true value of Ca^{2+} concentration is very abnormal.[2] However, capillary blood samples from neonates may require higher heparin levels for adequate anticoagulation.

Use of liquid preparations of heparin in the dead space of the syringe hub may cause a significant dilution error when syringes less than 2 mL are used or when bigger syringes are incompletely filled.[102, 103] It is thus perferable

to use syringes and capillaries containing dry or lyophilised calcium-titrated heparin for routine Ca^{2+} measurement in whole blood or plasma.[104] Lyophilisation is preferable to drying as it creates a more open heparin particle structure which is more rapidly dissolved in the blood specimen and obviates the need for 'flea'-mixing, avoiding the possiblity of haemolysis.

HAEMOLYSIS

Haemolysis of blood causes errors in the determination of Ca^{2+} both through Ca^{2+} binding and dilution by the intracellular contents.[105] Complete haemolysis of blood with an original haematocrit of 45% decreases Ca^{2+} concentration to about 40% of the original level in plasma. In addition, pH change due to intracellular acid release may occur. Thus, care should be taken to avoid haemolysis, whether arising from specimen collection or from instrument design.

CHANGE IN PH IN THE SPECIMEN

The theory of the complex relationship between pH and Ca^{2+} concentration has been described in detail.[9] Loss of CO_2 from the specimen between sampling and analysis increases the pH of the specimen and decreases Ca^{2+} concentration. When pH change *in vitro* is due solely to loss of CO_2, log Ca^{2+} is linearly related to pH in the interval pH 7·0–7·7 with a slope of about −0·24 for plasma and −0·22 for whole blood.[2] Algorithms based on this relationship are used in a number of commercial Ca^{2+} analysers[2, 58] to adjust Ca^{2+} concentration to that predicted as obtaining in the sample at pH 7·40 or at the actual arterial pH of the patient, thereby attempting to compensate for CO_2 loss during specimen processing.[2, 95] Lactic acid formed by glycolysis in whole blood because of delay in analysis increases Ca^{2+} concentration, though the slope of the relationship is less than that expected with CO_2 loss because of some Ca^{2+} complexation with lactate.[2] The slope of the relationship also depends on the albumin and globulin concentrations in the specimen.

In view of these factors, blood specimens for Ca^{2+} determinations should ideally be handled anaerobically to prevent CO_2 loss, and undue delay in analysis should be avoided to minimise lactic acid generation. If blood specimens are kept in sealed tubes, Ca^{2+} concentrations are stable[2, 98, 104, 106, 107] for over an hour at 37°C and for over 5 h at 4°C.

It is difficult to prepare serum or plasma specimens without allowing alteration in specimen pH,[2, 96] particulary if centrifugation occurs at temperatures other than 37°C. Therefore, it has been recommended[2, 96, 98, 103, 104] that, when Ca^{2+} measurements are made on specimens of plasma or serum, the results should be adjusted to pH 7·40 or, preferably, to the actual pH of the patient.[26, 108]

Analytical errors

CARRYOVER EFFECTS

Carryover from one sample to the next is most unlikely in modern Ca^{2+} analysers as the fluid paths are extensively flushed between analyses. However, a rather more subtle carryover may occur between the flush solution and the samples or the calibrants.[109, 110] As flush solutions usually contain Ca^{2+} concentrations of about 1·25 mmol/L, this may result in a positive bias in very hypocalcaemic samples or a negative bias in very hypercalcaemic samples. Carryover usually occurs at the leading edge of the sample bolus, which picks up residues from the flush solution deposited by surface tension on the walls of the fluid path. Manufacturers attempt to minimise carryover by purging with air between flush solution and sample and by using the leading edge of the sample to clean the fluid path, although this inevitably sets a lower limit on the sample volume required for measurement. In practice, modern instruments only show significant carryover effects at extreme sample concentrations.

ERYTHROCYTE EFFECTS

It has been known for many years that erythrocytes may cause a significant bias compared with plasma alone at the liquid-liquid junction when concentrated KCl is used as the reference electrode bridge solution.[77, 111, 112] The bias may be observed irrespective of the ion being measured. This so-called 'suspension effect' has been attributed to crenation, precipitation and haemolysis of erythrocytes at the junction,[77] to effects of erythrocyte surface charge[113] and to different mobilities of ions between junction and cells.[114] Its true nature is still controversial.[115]

The effect caused serious difficulties[56] with older designs of instruments, as the extent of the bias varied directly with haematocrit: for instance whole blood samples with a haemoglobin concentration of 15 g/dL had Ca^{2+} concentrations apparently 4·6% higher than their plasma.

The suspension effect may be virtually eliminated by careful design of junction geometry in addition to such measures as the use of sodium formate instead of KCl as bridge solution,[74, 98] by flowing the sample past the junction during measurement,[113] by use of a dialysis membrane at the junction, by use of mixed salt bridge solutions,[116] or by electronic surveillance of the changes in potential which occur immediately after the junction is established.[2] Users should establish that their own instrument does not show plasma-whole blood bias of this kind.[97]

INTERFERENCE BY OTHER IONS

The high selectivity of modern Ca^{2+} ISEs (Tables 2 and 3) means that other cations, in the concentrations encountered in plasma, do not penetrate the selective membrane to interfere directly with Ca^{2+} measurement. However, there are mechanisms whereby cations may interfere indirectly with measurement.

If protein is gradually deposited in the fluid transport path of an analyser, high Mg^{2+} concentrations in samples will appear to cause an increasing positive interference on Ca^{2+} measurement as these deposits accumulate. This effect may be erroneously interpreted as a deterioration in the selectivity of the ISE, but will not be abolished by renewal of the ISE. The effect is due to the deposited protein acting as an exchanger of divalent cations,[5] the Mg^{2+} in the sample displacing Ca^{2+} from the deposited protein and increasing the Ca^{2+} concentration in the sample when it reaches the electrode chain.[117] This problem can be overcome by careful instrument maintenance with regular removal of protein from all the lines.

Plasma ionic strength is mainly determined by Na^+ and is changed when plasma Na^+ concentration deviates from normal. Consequently, the activity coefficient of Ca^{2+} is changed. As Ca^{2+} analysers are usually calibrated with solutions of ionic strength 0·16 mol/kg, a hyponatraemic sample will appear to have a lower Ca^{2+} concentration than is actually true, since the activity coefficient (v) in the sample is less than that in the calibrant because of its lower ionic strength. The effect of ionic strength changes on the activity coefficient (γ) of Ca^{2+} is largely counterbalanced by an effect of opposite sign on the residual liquid junction potential. The overall effect is such that a plasma sample with an ionic strength of 0·15 mol/kg will have a measured value of Ca^{2+} concentration about 1·2% lower than its true value.[2] However, this apparent bias is largely academic, as it correctly reflects the change in true Ca^{2+} activity which in any case is the physiologically relevant parameter.[2, 118] It may nevertheless lead the naive user into wrongly believing that, for instance, the increasing Ca^{2+} concentration observed in plasma if it is spiked with solid NaCl represents a lack of selectivity.

INTERFERENCE BY PROTEIN

When using ISEs based on ion exchange membranes in solutions containing protein, the membrane tends to bind protein, causing a negative shift of electrode potential of up to −2 mV. Deposition of even a monolayer of protein on the membrane changes the physical properties of the interface. The effect is most marked with the first sample presented to the electrode as the protein layer forms. The protein may gradually be washed off the membrane, with restoration of the original electrode potential.[8, 96] In addition, protein may increase the extraction of membrane components if these are not sufficiently lipophilic[119] and careful design of membrane composition is needed to counteract this effect which is usually noted as a drift in potential.[66] These effects are probably more marked with ion exchange than with neutral carrier membranes. Interactions of ISE membranes with proteins are said to be eliminated in one make of analyser by the use of a cuprophan dialysis membrane between the ISE membrane and the sample, at the expense of increasing the electrode 95% response time from about 1 s to about 15 s.[2] It is contended that the uneven Donnan distribution of Ca^{2+} across the cuprophan, because of the dialysis effect, is reflected in a Donnan potential, the total electrochemical potential of Ca^{2+} sensed by the electrode being identical in the plasma sample and in the dialysate layer next to the ISE membrane.[8, 96]

It is a consistent finding that, if Ca^{2+} is measured using ISEs in plasma following ultrafiltration, Ca^{2+} concentration in the retentate exceeds that in the filtrate. Furthermore, there is a linear relationship between protein and Ca^{2+} concentration in the retentate.[26, 56, 120, 121] Similar effects have been described following dialysis of plasma *in vitro*[121–123] and, by venestasis, *in vitro*.[124] The phenomenon has been interpreted as showing that protein exerts a positive interference on Ca^{2+} measurement with a bias in concentration of about + 0·02 mmol/L for every 10 g/L increase in protein concentration.[26, 56, 121, 122]

This interpretation has been challenged however, on theoretical grounds[125, 126] and by balance studies[108] as being an inevitable consequence of Donnan equilibration of ions across semipermeable membranes. Hence, interpretation of experiments which use dialysis or ultrafiltration to prepare samples with different protein concentrations is complicated by a circular argument about the validity or otherwise of calculations of Donnan equilibria. Reports of a correlation between albumin and Ca^{2+} concentrations *in vitro*[115, 127] are also subject to this problem of interpretation. Studies using comparative cells which effectively eliminate liquid junctions, a technique which avoids possible Donnan effects, have been interpreted as showing that protein does not interfere with Ca^{2+} measurement.[128] The issue, therefore, has yet to be resolved.

Reporting results

For reasons which should be apparent from the foregoing, Ca^{2+} and pH should always be measured together on any sample.[92, 104] Measurements made on whole blood handled anaerobically should be reported as the actual Ca^{2+} concentration with the actual pH of the sample. Where the sample has lost CO_2 before measurement, the result may be adjusted to allow for this by extrapolation to pH 7·40 or, better still, to the actual pH of the patient if this is known. It is emphasised that this adjustment is purely a compensation for pre-analytical CO_2 loss by the sample and the Ca^{2+} concentration adjusted to pH 7·40 does not of itself have any fundamental physiological meaning. For instance, the Ca^{2+} at pH 7·40 of a patient with an acute respiratory alkalosis may be entirely normal even though the patient is grossly tetanic.[26]

The correction of Ca^{2+} for pH is, in any case, an approximation as it assumes normal plasma protein concentrations. The slope of the line relating log Ca^{2+} to pH depends on the concentration of albumin and other Ca^{2+}-binding proteins in plasma, and values usually given in the literature and incorporated in instrument software relate only to normal plasma. Thus, measurement of Ca^{2+} in patients should be made using anaerobic techniques with whole blood wherever possible. As a further consequence of the dependance of pH-corrected Ca^{2+} values on protein concentration, the standard correction algorithms are relevant only to plasma *in vitro*—as the rapid equilibrium of Ca^{2+} between plasma and interstitial fluid *in vivo* makes the relationship behave in a manner approximating a model containing only half the albumin concentration of plasma,[2] with a slope of about −0·1.

Quality control

The nature of the liquid-liquid junction makes ISE analysis particularly susceptible to influence by the matrix of the sample. Changes in reference electrode and liquid-liquid junction performance, which alter the response to plasma samples, may not be reliably detected by the use of simple protein-free solutions for quality control.[58, 129–131] For instance, the presence of added buffers or of abnormal concentrations of other ions may evoke changing instrument responses with time, and these changes may be instrument-specific. Therefore, the ideal material for use in the quality control of Ca^{2+} determinations would be human plasma equilibrated with CO_2.[92] Where human serum pools are used they should comprise a collection of serum samples which have individually been shown to be free of HIV and hepatitis infection. Material suitable for use in precision control can be made from non-human serum pools[129–131] or identified by trial and error from commercially available sera.[108, 132] Experiences with the use of a number of materials based on human and animal serum have been reported.[108, 130–134] Ideally, quality control materials would have the properties outlined in Table 4. At present, few commercially available materials fulfil these specifications in all respects.

Pending international agreement on a reference method for Ca^{2+} determination, there is no standard material available for the assessment of accuracy.

Analytical performance of modern Ca^{2+} analysers

A recent comparison[58] of four modern Ca^{2+} analysers has reported excellent day-to-day precision of instruments from four different manufacturers. All had CVs of less than 2% with pooled human serum at a Ca^{2+} concentration of 1·20 mmol/L. This standard of performance is typical of that reported extensively in the literature[96–98] and contrasts with experiences with older designs of instrument where imprecision and unreliability were major obstacles to routine use.[56, 135]

TABLE 4. Required properties of quality control materials for plasma ionised calcium analysis

(1) Stable Ca^{2+} concentrations at intervals between 0·5–2·0 mmol/L.
(2) Stable pH levels at intervals between 6·8–7·8.
(3) Stable ionic strength at around 0·160 mol/kg.
(4) Other ions present at levels encountered clinically in plasma.
(5) Buffer matrix causing minimal residual liquid junction potential.
(6) No xenobiotic ions (e.g. Cs^+, NH_4^-, NO_3^-) present.
(7) No compounds present (e.g. ethylene glycol) which might interact with the ISE membrane matrix.
(8) No therapeutically-used antibiotics present (e.g. gentamycin, chloramphenicol), whose use with instruments in clinical areas might give rise to resistent bacterial strains.

Two multicentre surveys of inter-laboratory performance have been reported,[131, 136] which investigated up to 24 different laboratories all using the same make of Ca^{2+} analyser. Using a variety of test materials, routine-condition CVs of between 0·7–3·1% were obtained. Despite the problems in primary standardisation, these results illustrate that modern Ca^{2+} analysers are now sufficiently precise and reliable for general use in clinical chemistry.

The role of ionised calcium measurement

The measurement of total calcium concentration in plasma is firmly established as a routine test in clinical chemistry. Since the advent of continuous flow analysers it has been cheap, rapid and easy to perform on large numbers of specimens which have required few special precautions in handling. In contrast, Ca^{2+} measurement has in the past been difficult, requiring relatively expensive, slow and unreliable equipment and entailing special precautions in specimen handling. In recent years, however, there have been major improvements in Ca^{2+} analysers which are now inexpensive, rapid (up to 60 samples per hour) and robust. Their analytical performance is at least as good as that of total calcium methods and far superior to the combined imprecision and inaccuracy of total calcium and albumin methods, especially at low levels of albumin.

It is now recognised that total calcium concentration in plasma may be a poor indicator of the physiological state of calcium in a number of situations, particularly when abnormal concentrations of calcium ligands are present.[58, 137]

The necessity for measuring Ca^{2+} in these situations is uncontested. However, the question of replacement of total calcium by Ca^{2+} measurement is more controversial.

Although the presence of protein in the intravascular compartment may cause plasma Ca^{2+} concentration to be a few per cent higher than in the extracellular fluid,[127] Ca^{2+} concentration more closely reflects the physiological state of extracellular calcium. It would thus seem logical that it should be the quantity to be measured. Nevertheless, the number of measurements of total calcium performed exceeds by several orders of magnitude that of $Ca.^{2+}$ This can be attributed to a number of factors. Firstly, there is considerable residual suspicion about the reliability of $Ca,^{2+}$ and indeed ISE measurements in general. The principles of ISE measurement are poorly understood by workers in clinical chemistry, many of whom suspect some sleight of hand on the part of manufacturers in manipulating the results. Secondly, most laboratories have large capital investments in instrumentation which measures total calcium; while they have this capability they do not accord Ca^{2+} measurement a sufficiently high priority for purchase. Manufacturers of large multi-test instruments thus do not perceive a demand for Ca^{2+} to replace total calcium on their instruments. Thirdly, the procedure for specimen collection and processing employed by many laboratories entails CO_2 loss from the specimen, making it less suitable for Ca^{2+} analysis. However, the increasing use of sealed centrifugible evacuated blood collection tubes which may be placed directly on multi-test analysers will minimise this problem.

Unfortunately, these considerations all relate to logistics and to the convenience of laboratory management rather than to physiology and diagnosis. That logistical problems can be overcome has been shown by Bowers *et al.*[58] who perform some 31 000 Ca^{2+} assays per annum in the clinical chemistry department of a large general hospital in the USA. There is no fundamental reason that Ca^{2+} analysis should not be performed on board large multiparameter analysers. The increasing availability of Ca^{2+}

analysis using small near-patient instruments and particularly the installation of Ca^{2+} in blood gas analysers is likely to stimulate the interest of clinicians in Ca^{2+} measurement. It is thus timely that clinical chemists should critically review their approaches to this important analyte.

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