

# Measurement of the limiting equivalent conductivities and mobilities of the most prevalent ionic species of EGTA (EGTA<sup>2-</sup> and EGTA<sup>3-</sup>) for use in electrophysiological experiments

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## Abstract

In many experimental biological situations, chelating agents like EGTA (ethylene glycol-bis-( $\beta$ -amino-ethyl ether) *N,N,N',N'*-tetra-acetic acid) are commonly used to control or suppress the concentration of divalent ions like Ca<sup>2+</sup>. The evaluation of liquid junction potentials in electrophysiological measurements, and particularly in patch-clamp situations, requires information about the ions within the solution. Where there is a significant concentration of EGTA present, it is necessary to know the values of the relative mobility of at least the most predominant ionic species of EGTA in order to complete these calculations. EGTA, with four negative charges with different pK<sub>a</sub>s, can therefore exist as four differently charged ions in solution (EGTA<sup>-</sup>, EGTA<sup>2-</sup>, EGTA<sup>3-</sup> and EGTA<sup>4-</sup>) or as uncharged, although between pH 5.5 and 8 it is almost exclusively EGTA<sup>2-</sup>. We have measured limiting equivalent conductivities of the most common ionic forms of EGTA (EGTA<sup>2-</sup> and EGTA<sup>3-</sup>) encountered at physiological pHs. These were  $35.9 \pm 0.7$  and  $56 \pm 2.5$  S cm<sup>2</sup> equiv<sup>-1</sup> respectively. Their mobilities relative to K<sup>+</sup> were  $0.24 \pm 0.01$  for EGTA<sup>2-</sup> and  $0.25 \pm 0.01$  for EGTA<sup>3-</sup>. Thus for typical electrophysiological solutions, the contribution of EGTA to the liquid junction potential should be small (e.g.  $\sim 0.4$  mV). © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Ion mobilities; Conductivities; Liquid junction potentials; Patch-clamp; EGTA

## 1. Introduction

Liquid junction potentials arise whenever two solutions of different ionic composition or concentration are in contact and can significantly contribute errors to the measurements of membrane potential, unless appropriate corrections for them are applied. In patch-clamp and bilayer situations especially, liquid junction potential corrections are often up to 10 mV or even more (see discussion in Barry and Lynch (1991), Neher (1992) and Neher (1994) and Ng and Barry (1995)). These junction potential corrections can either be estimated by measurement (Neher, 1992, 1994), although even these measurements need additional corrections (Barry and Diamond,

1970), or they can simply be calculated directly from a knowledge of (or at least estimates of) the ionic mobilities of the ions present in the solutions at any significant concentration. These mobilities can be determined from limiting equivalent conductivity data (e.g. see data in Robinson and Stokes (1965), Dean (1992), Vanysek (1995)). Relative mobilities of the more common ions have been published (Zuidema et al., 1985; Barry and Lynch, 1991) and more recently the mobilities of some less common, but now frequently used, organic ions have also been measured (Ng and Barry, 1995). These values have now been incorporated into a program, JPCalc, that has been developed (Barry, 1994) together with a full Windows version, JPCalcW, produced in conjunction with Axon Instruments, Foster City, CA, USA) to calculate junction potentials for a full range of patch-clamp and other electrophysiological situations using the generalised Henderson equation (Morf, 1981; Barry and Lynch, 1991; Ng and Barry, 1995).

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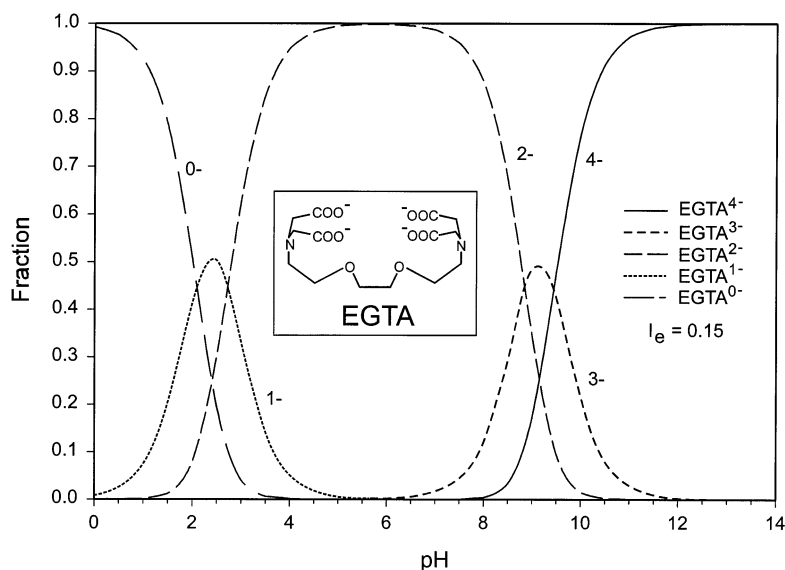


Fig. 1. Plot of fraction of EGTA species in solution vs. pH for a solution with an ionic equivalent of 0.15 equiv  $l^{-1}$  and a temperature of 22°C (see also Table 1 for values from pH 7–8). Inset is the structural formula of an  $EGTA^{4-}$  molecule ( $C_{14}H_{24}N_2O_{10}$ ; redrawn from Fig. 2 of Bers et al. (1994)).

of their parameter ‘*b*’ in their Eq. (12), which should have been “...*b* is a constant (= 0.25·*A*). *A* is a constant...” (confirmed by Donald Bers; personal communication), the *K*s could be evaluated under the appropriate conditions. The relative proportions of each ionic species could then be deduced from each of the terms in Eq. (5).

Typical fractions of each ionic species of EGTA over a range of pH values from pH 4.5 to 9 and an ionic equivalent of 0.15 equiv  $l^{-1}$  (equivalent to a 150 mmol  $l^{-1}$  NaCl solution) and a temperature of 22°C, are given in Fig. 1 (with some values also in Table 1). It can be seen that at physiological pH the predominant ionic species is  $EGTA^{2-}$ , however, at other pHs, often encountered experimentally, other species become significant. At the above ionic equivalents,  $EGTA^{3-}$  becomes significant above pH 7.5 and  $EGTA^{1-}$  becomes significant below pH 4; it should be noted, however, that these values of ionic fractions can be very sensitive to ionic equivalents. For example, at a pH of 8.8, decreasing the ionic equivalents from 0.15 to 0.02 increases the fraction of  $EGTA^{2-}$  from 0.48 to 0.69.

### 3. Experimental methods

#### 3.1. Preparation of solutions

$CaCl_2$  was obtained from UNIVAR-AJAX (Auburn, NSW, Australia) and NaCl and NaOH from BDH (Kilsyth, VIC, Australia). EGTA was obtained from Sigma (St. Louis, MO, USA). All reagents were at least of analytical grade and the solutions were prepared using double demineralised water as the solvent.

The 10 mM EGTA solution was prepared and pH adjusted with a stock solution of 1 mol  $l^{-1}$  NaOH. For EGTA solutions at a pH of 6, dilutions of 1, 3 and 5 mmol  $kg^{-1}$   $H_2O$  were prepared using the 10 mmol  $kg^{-1}$   $H_2O$  EGTA as a stock solution. For the pH 8.8 measurements, the proportions of  $EGTA^{2-}$  and  $EGTA^{3-}$ , in the limit as  $[EGTA]$  tends to zero, are 0.831 and 0.164, respectively (see Table 1) and vary considerably with pH. The pH of the solution, therefore, needed to be controlled much more precisely and this was achieved by adding very small and precise amounts of 1 mol  $l^{-1}$  NaOH to each solution after dilution.

#### 3.2. Measurement of $\Lambda^m$ values

Each experiment involved the measurement of three samples of each of the 1, 3, 5 and 10 mmol  $kg^{-1}$   $H_2O$  concentrations. The values of  $\Lambda^m$  in each experiment were fitted to Eq. (1) by linear regression. Experiments for the NaCl, EGTA (pH 6) and EGTA (pH 8.80) were repeated four to six times until we had four experiments whose data points fitted the linear regression with  $r^2 > 0.95$ . All conductivity measurements were corrected for the conductivity of the double de-ionized water used for the solutions (from 0.8 to 1.0  $\mu S cm^{-1}$ ) as well as a small adjustment which was needed to calibrate the conductivity meter (Radiometer CDM 83, Copenhagen) to the values published for the limiting equivalent conductivity of NaCl (see Table 2). The conductivity experiments were performed at room temperatures of  $22.5 \pm 1^\circ C$  and the reference temperature to which all measurements were referred was 25°C.

Table 2  
Limiting conductivity and mobility data for NaCl, Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup> and EGTA<sup>2-</sup> and EGTA<sup>3-</sup> ionic components<sup>a</sup>

Solution and ions	pH	Limiting molal conductivity ( $\Lambda^{om}$ )	S.E.M.	Fraction of predominant EGTA species	Limiting equivalent conductivity ( $\Lambda^o$ )	S.E.M.	Other published values <sup>c</sup> of $\Lambda^o$	Ion mobility (relative to K <sup>+</sup> )	S.E.M.
NaCl		126.45 <sup>b</sup>	0.23				126.45		
Na <sup>+</sup>							50.10		
K <sup>+</sup>							73.50		
Cl <sup>-</sup>							76.35		
NaEGTA	~6	170.1	0.5	0.998 × EGTA <sup>2-</sup>	35.9	0.7		0.24	0.01
	8.80	195.7	0.9	0.831 × EGTA <sup>2-</sup>					
				0.164 × EGTA <sup>3-</sup>	55.8	4.3		0.25	0.02

<sup>a</sup> Units for limiting molal conductivity are S cm<sup>2</sup> mol<sup>-1</sup>. Units for limiting equivalent conductivity are S cm<sup>2</sup> equiv<sup>-1</sup>. Reference temperature for all values is 25°C.

<sup>b</sup> The conductivity meter was calibrated to give a limiting equivalent conductivity for NaCl of this value.

<sup>c</sup> Robinson and Stokes (1965).

Temperature compensation was achieved with the built-in conductivity meter function (Ng and Barry 1995).

### 3.3. Measurement of EGTA junction potentials

The apparatus and protocol used to measure junction potentials was the same as that described in Ng and Barry (1995). Initially the voltmeter was zeroed with identical control solutions in both the bath and pipette.

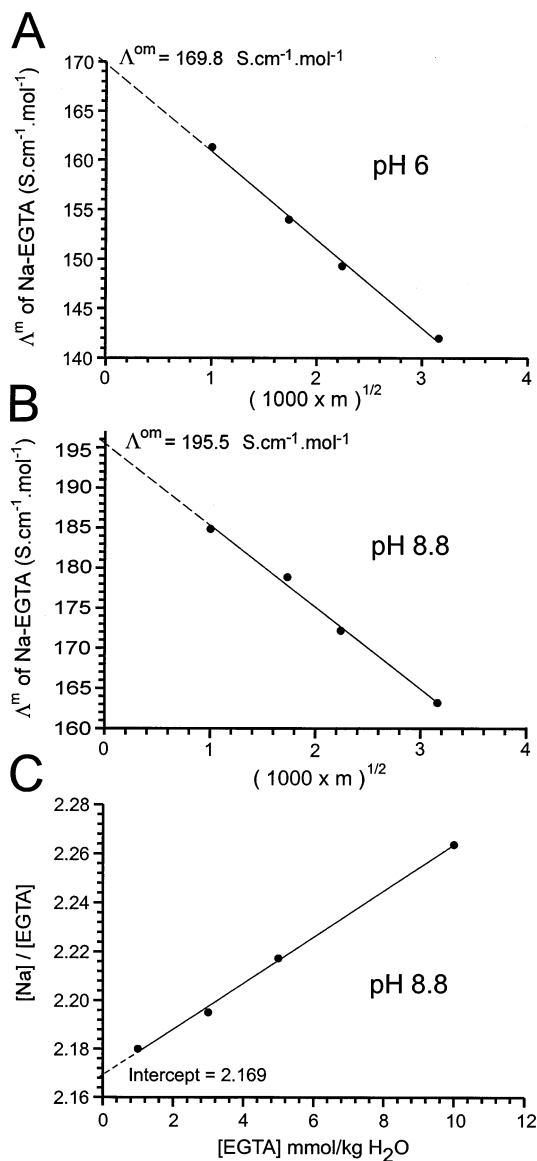


Fig. 2. Solution measurements used to determine the limiting molal conductivity of NaEGTA. Panel A shows a set of measurements at pH 6. Panel B shows a typical set of solution measurements at pH 8.8. Each data point represents the average of three repeat measurements within the same experiment. In panels A and B the line was fitted by linear regression to the function  $\Lambda^m = \Lambda^{om} + s\sqrt{m}$ , where the intercept on the ordinate represents the limiting molal conductivity ( $\Lambda^{om}$ ) and  $m$  is the molal concentration (in mol EGTA ( $\text{kg H}_2\text{O}$ )<sup>-1</sup>). Panel C shows an example of a typical plot used to determine the limiting ratio of  $[\text{Na}^+]/[\text{EGTA}]$  as  $[\text{EGTA}] \rightarrow 0$  for a set of EGTA solutions each precisely titrated to a pH of 8.80.

The bath solution was then changed to the appropriate test solution and the liquid junction potential measured. Each experiment consisted of a control–test pair and each of the two sets of experiments were repeated four times. Each time a test solution was measured it was bracketed by control measurements. In most cases 200 ml of the final solution was placed in a shallow crystallizing dish (bath) used for recording. For test solutions containing 2 mmol kg<sup>-1</sup> H<sub>2</sub>O Ca<sup>2+</sup>, CaCl<sub>2</sub> was added directly to the bath and well stirred. The new liquid junction potential was then measured and compared with the previous value in the absence of CaCl<sub>2</sub>. The experimental apparatus was enclosed in a grounded Faraday cage. The reference pipettes were also grounded. All experiments were conducted at an ambient room temperature of  $22 \pm 1^\circ\text{C}$ .

## 4. Results and calculations

As already mentioned, the procedure was to initially measure limiting molal conductivities for the NaEGTA solutions at two pH values: at a pH of about 6 which was almost exclusively only contributed to by EGTA<sup>2-</sup> and precisely at pH 8.80 at which there was a known fraction of EGTA<sup>2-</sup> and EGTA<sup>3-</sup> and virtually no other EGTA ionic species. From these measurements, the limiting equivalent conductivity of Na<sup>+</sup> would first be subtracted, the limiting molal conductivity of EGTA determined and finally the limiting equivalent conductivity of the two EGTA species evaluated.

### 4.1. Calculation of limiting equivalent conductivities of EGTA<sup>2-</sup> and EGTA<sup>3-</sup>

These values were determined from measurements at various pHs. A typical example of the relationship between molal conductivity (conductivity/molal concentration of EGTA) and the square root of the molal concentration of EGTA at pH 6 is given in Fig. 2A. In this example, the limiting molal conductivity ( $\Lambda^{om}$ ) was 169.8 S cm<sup>2</sup> (mol of EGTA)<sup>-1</sup>, obtained by extrapolating  $[\text{EGTA}]$  to zero. The average value from four plots of molal conductivity ( $\Lambda^{om}$ ) of NaEGTA was  $170.1 \pm 0.45$  S cm<sup>2</sup> (mol of EGTA)<sup>-1</sup>. As EGTA<sup>2-</sup> was essentially the only ionic species present (see Table 2), then  $\Lambda^{om}$  of NaEGTA becomes essentially  $\Lambda^{om}$  of Na<sup>+</sup> + EGTA<sup>2-</sup>. The ratio of  $[\text{Na}^+]/[\text{EGTA}]$  averaged over all the stock solutions made was equal to  $1.963 \pm 0.018$  ( $n = 4$ ). Using the value for the limiting equivalent conductivity of Na<sup>+</sup> ( $\Lambda_{\text{Na}^+}^o$ , published) = 50.10 S cm<sup>2</sup> equiv<sup>-1</sup>, the limiting molal conductivity of EGTA<sup>2-</sup> alone could be estimated as  $\Lambda_{\text{EGTA}^{2-}}^{om} = 170.1 - (1.963 \times 50.10) = 71.8$  S cm<sup>2</sup> mol<sup>-1</sup>. The limiting equivalent conductivity of the EGTA<sup>2-</sup> species ( $\Lambda_{\text{EGTA}^{2-}}^o$ ) was then calculated as:

Table 3

A comparison of experimental and theoretical liquid junction potentials (with S.E.M.) for some simple solutions containing EGTA<sup>a</sup>

Solutions (mmol l <sup>-1</sup> )			pH	Liquid junction potential (mV)		
	Control	Test		Experimental	S.E.M.	Theoretical
1	20 NaCl	10 NaEGTA	6.7	12.0	0.8	14.1
2	20 NaCl	10 NaEGTA, 2 CaCl <sub>2</sub>	4.3	10.4	1.2	11
3	150 NaCl	130 NaCl, 10 NaEGTA	6.5	1.5	0.0	1.6
4	150 NaCl	130 NaCl, 10 NaEGTA, 2 CaCl <sub>2</sub>	4.3	1.4	0.0	1.3

<sup>a</sup> The potentials in each case represent the potential of the test solution with respect to the control solution.

$$\Lambda_{\text{EGTA}^{2-}}^o = \Lambda_{\text{EGTA}^{2-}}^{om} / |z| \quad (6)$$

$\Lambda_{\text{EGTA}^{2-}}^o = 71.8/2 \text{ S cm}^2 \text{ mol}^{-1}$ , which allowing for the S.E.M. above becomes,  $\Lambda_{\text{EGTA}^{2-}}^o = 35.9 \pm 0.7 \text{ S cm}^2 \text{ equiv}^{-1}$ .

Since the generalised ionic mobility ( $u$ ), which is required for junction potential calculations, is given by:

$$u = \Lambda^o / |z| F \quad (7)$$

and, since for K<sup>+</sup>,  $\Lambda_{\text{K}^+}^o = 73.50$  (Robinson and Stokes, 1965), then  $u_{\text{EGTA}^{2-}}/u_{\text{K}^+} = (35.9/2)/73.50$ . Thus  $u_{\text{EGTA}^{2-}}/u_{\text{K}^+} = 0.244 \pm 0.005$ , or better  $0.24 \pm 0.01$ .

Similarly, from measurements at pH 8.8, where a significant fraction of the EGTA is in the form of EGTA<sup>3-</sup>, it is shown in the Appendix A that  $\Lambda_{\text{EGTA}^{3-}}^o = 55.8 \pm 4.3 \text{ S cm}^2 \text{ mol}^{-1} \text{ equiv}^{-1}$  and that  $u_{\text{EGTA}^{3-}}/u_{\text{K}^+} = 0.25 \pm 0.02$ .

#### 4.2. Measurement of liquid junction potentials and comparison with calculated values

In order to confirm these mobility values and to further indicate their contribution to the liquid junction potentials in the different solution combinations, these junction potentials were directly measured for some simplified patch-clamp type solutions containing EGTA and compared to theoretically predicted values using the above ionic mobilities. The theoretical calculations were done using the full MS Windows version of the junction potential program JPCalc (Barry, 1994), JPCalcW. The results are shown in Table 3 and, as can be seen, the measured junction potentials generally agreed well with the theoretically predicted values.

#### 4.3. Practical implications of correcting for EGTA mobility

At pH 7.4, it can be assumed that EGTA is almost exclusively in the EGTA<sup>2-</sup> form. With the use of a typical pipette solution (based on 150 mmol l<sup>-1</sup> KCl or CsCl, and containing 2 mmol l<sup>-1</sup> CaCl<sub>2</sub> and 10 mmol l<sup>-1</sup> EGTA) and a bath solution (based on 150 mmol l<sup>-1</sup> NaCl, and also containing 2 mmol l<sup>-1</sup> CaCl<sub>2</sub>), the difference in calculated liquid junction potentials, be-

tween ignoring the presence of EGTA and allowing for it with its measured mobility, is only 0.4 mV. Obviously, in more unusual situations where the proportion of EGTA is much greater (see Table 3), its contribution can become more significant.

## 5. Discussion

This paper has reported measurements of the limiting equivalent conductivities and relative mobilities of the most prevalent ionic species of EGTA, EGTA<sup>2-</sup> and EGTA<sup>3-</sup>, in the typical physiological pH range. It has also clearly demonstrated that such measurements for pH-dependent polyvalent ions like EGTA are by no means trivial exercises and require considerable care and theoretical underpinning. It should be noted that for a chelating agent like EDTA, the experimental measurement of its mobility would be further complicated by its binding of Na<sup>+</sup> ions. However, since EDTA has a molecular weight of 292 and lies between the molecular weight of EGTA (mol. wt. 380, mobility 0.24) and HEPES (mol. wt. 238, mobility 0.30; Ng and Barry (1995)) and, for large organic ions, relative mobility is determined in large measure (but not solely) by molecular weight, it seems likely that the relative mobility of EDTA would lie between 0.24 and 0.30.

It is interesting that although there was a considerable difference between limiting equivalent conductivities of the two ionic species ( $35.9 \pm 0.7 \text{ S cm}^2 \text{ equiv}^{-1}$  for EGTA<sup>2-</sup> and  $55.8 \pm 4.3 \text{ S cm}^2 \text{ equiv}^{-1}$  for EGTA<sup>3-</sup>), the generalised mobilities of the two ionic species were equal within our errors of measurement ( $0.24 \pm 0.01$  for EGTA<sup>2-</sup> and  $0.25 \pm 0.02$  for EGTA<sup>3-</sup>). Given the large size of these ions (mol. wt. 380.4), it is not surprising that this mobility for a generalised force appears to be less dependent on ionic charge, presumably because the amount of hydration of such a large molecule would not be significantly altered by the amount of its protonation. By inference then, the mobilities of both EGTA<sup>1-</sup> and EGTA<sup>4-</sup> should also be about  $0.24 \pm 0.02$ , which would in turn imply limiting equivalent conductivities of about  $18 \pm 2$  and  $71 \pm 6 \text{ S cm}^2 \text{ equiv}^{-1}$  for EGTA<sup>1-</sup> and EGTA<sup>4-</sup>, respectively.

In conclusion, although EGTA is a complex polyvalent ion, it is still possible to determine the concentration and relative mobility of its ionic species. These EGTA ions can, even under common electrophysiological conditions make a small but significant contribution to liquid junction potentials, but with the information that is now presented in this paper, those contributions can now be calculated.

### Acknowledgements

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### Appendix A. Calculation of limiting equivalent conductivity of EGTA<sup>3-</sup> at pH 8.8

The limiting molal conductivity of EGTA was measured at pH 8.80, where there was a reasonable fraction of EGTA<sup>3-</sup> also present together with the EGTA<sup>2-</sup> (Table 1). A typical example of the molal conductivity plots for one set of solutions is given in Fig. 2B, where  $\Lambda^{om}$  for this particular experiment was 195.5 S cm<sup>2</sup> (mol of EGTA)<sup>-1</sup>. The average limiting molal conductivity ( $\Lambda^{om}$ ) of Na<sup>+</sup> plus EGTA<sup>2-</sup> and EGTA<sup>3-</sup> over all these experiments was 195.7 ± 0.9 S cm<sup>2</sup> (mol of EGTA)<sup>-1</sup>. Now it was found that the ratio of [Na<sup>+</sup>]/[EGTA] needed to keep the pH at 8.8 varied with the concentration of EGTA (see Fig. 2C). Fitting a line to this data and extrapolating it, the limiting ratio of [Na<sup>+</sup>]/[EGTA], as [EGTA] tends to 0, = 2.169 ± 0.000 ( $n = 3$ ). Hence the component of the conductivity of EGTA<sup>2-</sup> and EGTA<sup>3-</sup>,  $\Lambda_{EGTA^{2-} \& EGTA^{3-}}^{om}$ , on subtracting the contribution of the Na<sup>+</sup> ions, was = 195.7 - (2.169 × 50.10) = 87.1 ± 1.0 S cm<sup>2</sup> mol<sup>-1</sup>.

In general, it will be true that the total conductivity of the solution will be the sum of all the appropriate fractions of each EGTA ionic species, together with the molal conductivity of Na<sup>+</sup>. It is known that in the limit

as [EGTA] tends to 0 at pH 8.8, the fraction of EGTA<sup>2-</sup> is 0.831 and the fraction of EGTA<sup>3-</sup> is 0.164. Thus, making use of the value of  $\Lambda_{EGTA^{2-}}^{om}$ , as previously determined, the value of  $\Lambda_{EGTA^{3-}}^{om} = (87.1 - (0.831 \times 71.8))/(0.164)$  S cm<sup>2</sup> mol<sup>-1</sup>. Thus, the limiting molal conductivity,  $\Lambda_{EGTA^{3-}}^{om} = 167$  S cm<sup>2</sup> mol<sup>-1</sup>. Hence, again taking into account the S.E.M.s, the limiting equivalent conductivity of EGTA<sup>3-</sup>,  $\Lambda_{EGTA^{3-}}^o = 167/3$  S cm<sup>2</sup> mol<sup>-1</sup> = 55.8 ± 4.3 S cm<sup>2</sup> mol<sup>-1</sup> equiv<sup>-1</sup>. Therefore, the generalised ionic mobility, relative to K<sup>+</sup>,  $u_{EGTA^{3-}}/u_K = (55.8/3)/73.5$ . Thus,  $u_{EGTA^{3-}}/u_K = 0.25 \pm 0.02$ .

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