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Stability constant determinations of alitame with H(I) and Cu(II) under physiological conditions using potentiometric measurements

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Abstract

Stability constant determinations of the new artificial sweetener alitame (L-α-aspartyl-*N*-(2,2,4,4-tetramethyl-3-thietanyl)-D-alaninamide), an HL type ligand (L^- = the deprotonated form of the ligand), was carried out with H(I) and Cu(II) under physiological conditions ($I = 0.15$(NaCl) in water, 37°C) using potentiometric titration. The molar concentration stability constants of H(I) and Cu(II) complexes with alitame, together with a distribution diagram of the Cu(II)-alitame complex species as a function of pH, are obtained. Cu(II) is able to form binary complex species (CuL^+, CuL_2) with alitame in solution. Those species are further deprotonated to form the corresponding $CuLH_{-1}$, $CuLH^{-}_{-2}$ and $CuL_2H^{-}_{-1}$ species. The neutral Cu(II)-alitame complex, CuL_2, is the major species between pH = 6.5 and 7.5 followed by $CuLH_{-1}$. A comparison with the less-stable artificial sweetener, aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester), is considered. Preliminary stability tests for alitame and Cu(II)-alitame solutions, using pH and stability constant methods, are reported.

Keywords: Alitame; Artificial sweetener; Stability constants; Equilibrium study; Copper complexes

1. Introduction

Alitame (L-α-aspartyl-*N*-(2,2,4,4-tetramethyl-3-thietanyl)-D-alaninamide) is a dipeptide of the complex series L-α-aspartyl-D-alaninamide. It was synthesized in 1979 by Pfizer laboratory researchers and possesses an intense sugared taste due to the terminal amide (2,2,4,4-tetramethylthietanylamine, TTA) (Moyal, 1992). A request of authorization for use was presented to the FDA (USA) in 1986 and other countries. It is classified as a non-nutritive sweetener that is approximately 2000 times sweeter than sucrose (Budavari, 1989).

The structure of the alitame molecule (Moyal, 1992; Budavari, 1989) given in Scheme 1 resembles the well-known artificial sweetener aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) in the amino acid moiety of aspartic acid. The L-phenylalanine was replaced by D-alanine and the methoxy group of the ester part was replaced by the TTA group in alitame. Alitame owes its increased stability to the terminal amide TTA, forming a second peptide link in the molecule, in comparison with aspartame's methyl ester group.

The stability constant values of this new sweetener with H(I) and Cu(II) are now obtained under physiological conditions (ionic strength $I = 0.15$(NaCl) in water, 37°C). They are compared with the previously found values of aspartame (Kholeif and Anderegg, 1997). Stability tests for alitame and Cu(II)-alitame solutions, retained at room temperature and given pH, are followed by potentiometric pH measurements and stability constant determinations.

The determination of stability constants allows the evaluation of all the pH-dependent charged and uncharged complex species of alitame and Cu(II)-alitame in solution, which shows a possible behaviour of alitame, when present in food, towards metal ions and particularly Cu(II). This is extended to specifically understand the speciation of alitame in the presence of Cu(II) at the duodenal pH between 6.5 and 7.5. The above information may also assist in a proper formulation and application of an end-user artificial sweetener product of alitame.

The potentiometric pH titration of alitame solution is useful in controlling the purity and consistency of the

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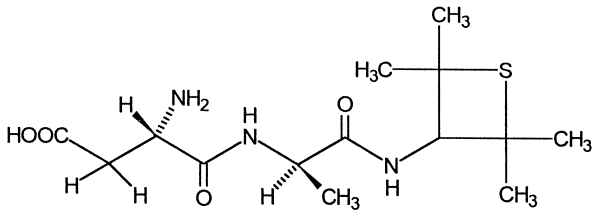

Scheme 1. Alitame structure.

product by determining the stability constants and concentration at given experimental conditions. The stability constants and equilibrium information may be further used to design other methods of analysis.

2. Materials and methods

Alitame (L-α-aspartyl-*N*-(2,2,4,4-tetramethyl-3-thietanyl)-D-alaninamide) of molecular formula $C_{14}H_{25}N_3O_4S{\cdot}2.5H_2O$ and molecular weight 376.47 was obtained from Pfizer UK and used as supplied. $CuCl_2{\cdot}2H_2O$, analysis grade, was obtained from Merck and standardized against 0.100 mol/l standard EDTA solution prepared from concentrated titriplex-III titrisol Merck ampoule. The standard 0.100 mol/l EDTA was also checked by titration against standard $ZnCl_2$ solution prepared from 99.999% Zn metal supplied by Fluka. NaCl salt, analysis grade >99.5%, was obtained from Merck. The 0.100 mol/l standard NaOH titrant solution was prepared from concentrated titrisol Merck ampoule and made up to a total ionic strength $I=0.15$ with NaCl. It was checked by titration against three standard solutions of potassium hydrogen phthalate Merck, analysis grade (99.95–100.05%) and 0.100 mol/l standard HCl solution prepared from concentrated titrisol Merck ampoule.

The calibration solution for the potentiometric system was composed of 4×10^{-3} mol/l HCl and made up to a total ionic strength $I=0.15$ using NaCl. The cell calibration was carried out in concentration scale by titrating 5.00 ml of the calibration solution against the standard NaOH delivered from a 1.000 ml Metrohm 665 Dosimat motor burette. The cell potential was read in mV using an Orion SA720 potentiometer and the whole titration was monitored using an IBM AT computer and the TIT212 data acquisition-titration control software. The computation of the calibration curve parameters (the cell apparent electrode potential $E^{\circ\prime}$ and the negative logarithm of the ion-product of water, pK_w) was performed using CAL program taking into consideration the correction for the liquid junction potential in acid ($E_j=61.54\times\log(1+14.28\ [H^+])$ mV) and alkaline media ($E_j=61.54\times\log(1+6.14\ [OH^-])$ mV) (Anderegg, 1993). The temperature of the titration and reference cells was maintained at 37.0°C ±0.1 using a Lauda MS M3 thermostat. Other experimental details may be found elsewhere (Anderegg and Kholeif, 1995).

Table 1
Total concentration in mol/l of metal M, ligand L, proton H and the pH range of the computation for the system of alitame and Cu(II)-alitame in aqueous solution at $I=0.15$(NaCl), 37°C

M	L	$[M]_t\times10^3$	$[L]_t\times10^3$	$[H]_t\times10^3$	pH range
	Alitame		2.996	6.004	2.86–8.89[a]
			2.996	7.016	2.68–8.11[a]
			3.002	6.994	2.68–8.47[a]
Cu(II)	Alitame	3.023	3.039	3.039	3.59–6.27
		1.512	3.047	3.047	3.77–10.50
		1.512	3.028	3.028	3.77–10.50
		1.008	3.039	3.039	3.87–10.34
		1.008	3.078	3.078	3.87–10.34

The subscript 't' stands for total concentration.
[a] The titration was conducted up to pH = 10.60 (see Fig. 1).

The solutions of alitame and Cu(II)-alitame were all prepared so that the ionic strength $I=0.15$(NaCl) and the titration curves were obtained by titrating 5.00 ml of these solutions against the standard NaOH.

3. Results and discussion

The total ligand, Cu(II) and proton concentrations of the solutions titrated together with the pH range of the computation are all given in Table 1.

The titration curves of alitame ligand in the presence of 4×10^{-3} mol/l HCl or in the presence of Cu(II) chloride in the molar ratios metal to ligand (1:1), (1:2) and (1:3) are all given in Fig. 1. A calculated titration curve for a strong acid at the same total H(I) concentration of alitame is also included in Fig. 1 for comparison.

The computation of the molar concentration stability constants at $I=0.15$(NaCl), 37°C was performed using SuperQuad program (Gans et al., 1985) from a total of 372 titration points in the case of alitame ligand, 483 titration points in the case of Cu(II)-alitame in one computational run for each case and the results are given in Table 2.

The free deprotonated alitame ligand (L^-) did not react with the hydroxide up to pH = 10.60 during the time of the titration. This can be observed from its titration curve and that of the strong acid given in Fig. 1. The potential reading was stable during the whole titration and controlled using the TIT212 software.

The presence of the terminal amide TTA in the alitame ligand has increased its stability, especially at high pH up to 10.60 and in comparison with the methoxy group in the aspartame ligand that was only titratable up to pH = 8.3 (Kholeif and Anderegg, 1997).

The stability constants of both alitame and aspartame ($\log\beta_{0,1,1}=7.39$ and $\log K_{0,1,2}=3.00$) (Kholeif and Anderegg, 1997) are almost identical. The small differences may be considered within the experimental errors.

Table 2
Molar concentration stability constants $\beta_{m,n,p}$ of alitame and Cu(II)-alitame valid in aqueous solution at $I=0.15$(NaCl), 37°C

M	L	m	n	p	log $\beta_{m,n,p}$[a]	$K_{m,n,p}$	log $K_{m,n,p}$
	Alitame	0	1	1	7.357(3) (0.01)	[HL]/[H][L]	7.357(3) (0.01)
		0	1	2	10.319(3) (0.01)	$[H_2L]/[HL][H]$	2.962(3) (0.01)
Cu(II)	Alitame	1	1	0	5.946(2) (0.005)	[ML]/[M][L]	5.946(2) (0.005)
		1	2	0	10.708(4) (0.01)	$[ML_2]/[ML][L]$	4.762(4)[b] (0.01)
		1	1	−1	−0.517(3) (0.009)	$[ML]/[MLH_{-1}][H]$	6.462(3)[b] (0.01)
		1	1	−2	−9.480(4) (0.01)	$[MLH_{-1}]/[MLH_{-2}][H]$	8.964(5)[b] (0.02)
		1	2	−1	2.584(8) (0.02)	$[ML_2]/[ML_2H_{-1}][H]$	8.124(9)[b] (0.03)
	OH^-	0	0	1	13.349(6) (0.01)	[H][OH]	−13.349(6) (0.01)

The values in parentheses are the least-squares unbiased estimates and those beneath them are their corresponding errors at 95% confidence level.
[a] $mM+nL+pH \rightleftharpoons M_mL_nH_p$; $\beta_{m,n,p}=\frac{[M_mL_nH_p]}{[M]^m[L]^n[H]^p}$
[b] Errors propagated.

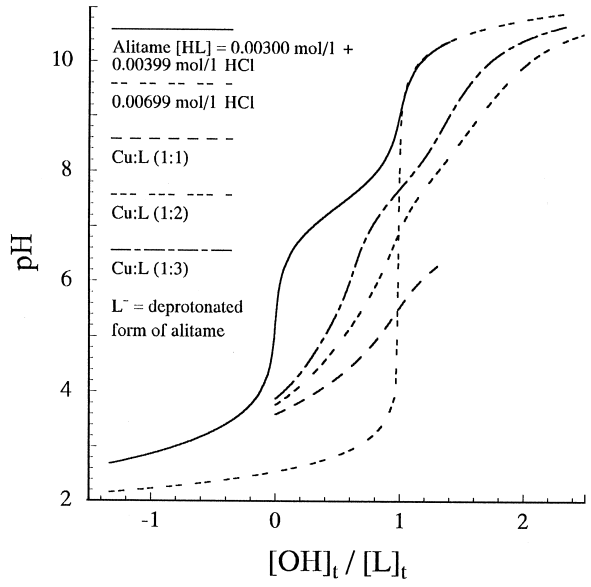

Fig. 1. Alkalimetric titration curves of alitame (HL) alone and in the presence of Cu(II) in 0.15(NaCl), 37°C.

Thus the existence of new moieties in alitame did not affect the proton equilibria of the aspartic acid moiety to any extent. Knowing the experimental stability constants of alitame and aspartame, the calculated isoelectric points for alitame and aspartame are at pH = 5.16 and 5.20, respectively (aqueous solution $I=0.15$(NaCl), 37°C).

The titration of Cu(II) alitame in the molar ratio (1:1) was conducted up to pH = 6.3 only due to precipitation of some free Cu^{2+} at higher pH resulting from the slight metal disproportionation and formation of the binary complexes CuL_2 and $CuLH_{-1}$. The titration curves of the molar ratios (1:2) and (1:3), conducted up to pH = 10.5 against a pH value of only 5.5 and 7.4 for the (1:2) and (1:3), respectively, in the case of aspartame, show the possible formation of Cu(II)-alitame species associated with H_{-1} in accordance with the stability constants found and listed in Table 2. Those species stabilize the Cu(II) against the formation of the insoluble $Cu(OH)_2$ (Kholeif and Anderegg, 1997) and are formed at higher pH values (Fig. 2) than those encountered with aspartame (pH $\geq$4.5) leading to the remarkable stability of alitame compared with aspartame. Moreover, the species $H_{-1}L$ was not detected with the free ligand during the time of the titration.

The value of log $\beta_{1,1,0}$ for the Cu(II)-alitame complex is lower than that of the Cu(II)-aspartame complex by 0.15 log unit whereas the log $K_{1,2,0}$ is within the experimental errors in both cases. The difference between the values of the stability constants of log $\beta_{1,1,0}$ is totally attributed to differences in structures since the basicities of both ligands are almost identical. The values of log $K_{1,1,-1}$ and log $K_{1,2,-1}$, corresponding to the species $CuLH_{-1}$ and $CuLH_{-2}$, respectively, when L^- = alitame, are higher by 0.80 and 0.53 log units, respectively, than the case when L^- = aspartame. Accordingly, those species, in the case of alitame, have a higher tendency to protonate giving the corresponding binary complexes CuL^+ and CuL_2, which is in harmony with the extra stability found in the alitame molecule. The species $CuLH_{-2}$ exists only in the case of alitame at high pH (above 7.5) and predominates at pH > 9 as shown in Fig. 2. This species was not possible to identify with

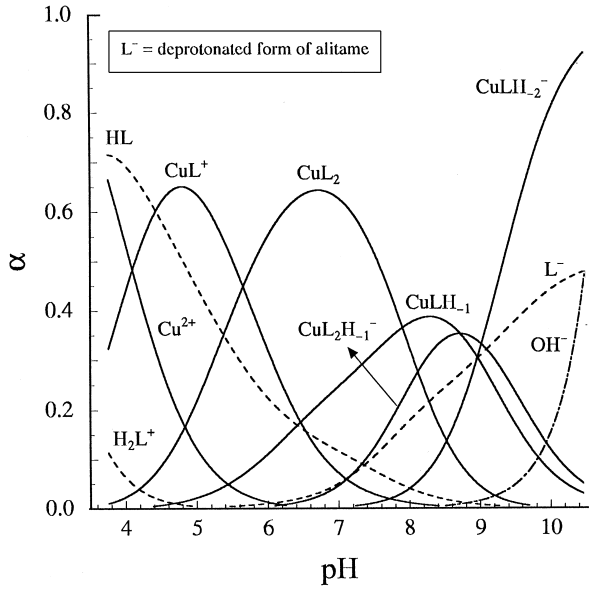

Fig. 2. Distribution diagram of Cu(II)-alitame complexes in molar ratio (1:2) as a function of pH in 0.15(NaCl), 37°C. α = [metal species]/$[M]_t$ or [ligand species]/$[L]_t$. The subscript 't' denotes total concentration.

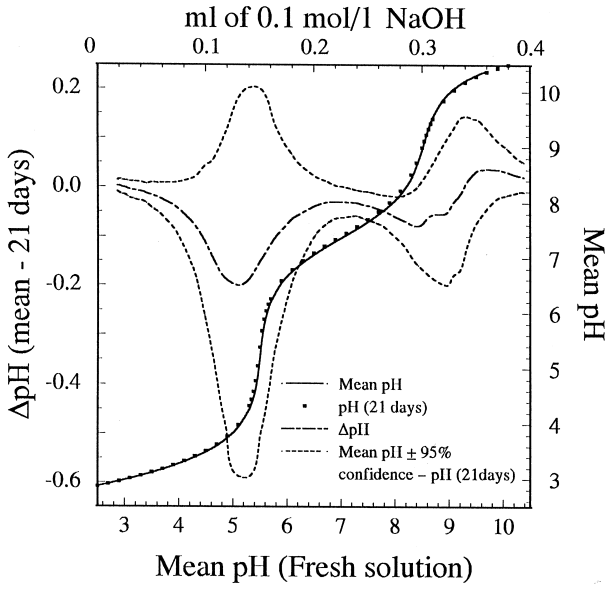

Fig. 3. pH stability test of alitame solution at pH = 2.9. The mean pH titration curve and that after 21 days belong to the upper *x*-scale and right *y*-scale. All others belong to the lower and left *x*- and *y*-scales.

aspartame due to the presence of a kinetic reaction at high pH values in such a way that the potential reading was unstable.

Alitame forms complexes with Cu(II) through the carboxylate group and the N atom of the $-NH_2$ group in the aspartic acid moiety much like the β-alanine ligand. This was previously found with aspartame (Kholeif and Anderegg, 1997). Both alitame and aspartame have lower complexing ability toward Cu(II) in comparison with β-alanine and have much lower basicities (for β-alanine, log $\beta_{0,1,1} = 9.706(2)$, log $K_{0,1,2} = 3.495(3)$ $I = 0.15$(NaCl), 37°C (Maeda et al., 1990) log $\beta_{1,1,0} = 6.93(5)$, log $K_{1,2,0} = 5.3(1)$ at $I = 0.1$, 37°C) (Smith and Martell, 1993). If we assume that alitame is stable against the variation in pH, the enzymatic cleavage of the dipeptide bonds in the intestines is expected to produce its major constituents much like the case of aspartame (Oppermann et al., 1973). These constituents are considered to be stronger complexing agents than alitame itself (for L-aspartic acid and Cu(II), log $\beta_{1,1,0} = 8.745(2)$, log $\beta_{1,2,0} = 15.509(4)$ (Kholeif & Anderegg, 1997); for D-alanine and Cu(II), log $\beta_{1,1,0} = 7.94(6)$, log $\beta_{1,2,0} = 14.5(2)$ (Smith and Martell, 1993) all at $I = 0.15$, 37°C). This is in favour of the formation of a variety of ternary complexes between the enzymatic degradation products of alitame, alitame and Cu(II) that are much stronger complexes than the individual binary complexes (Kholeif and Anderegg, 1997). However, the sequestering of the degradation products of alitame with Cu(II) is almost similar to those obtained from aspartame.

A preliminary pH stability test was carried out with an alitame solution of pH 2.9. The solution was titrated in three replicates against standard NaOH and the remaining part was left at room temperature for 21 days where it was once again titrated against standard NaOH. Fig. 3 shows the difference in pH units (ΔpH) between the mean pH values of the fresh and 21 day-old solutions. The large negative values are only noticed in the regions of the deflection in the titration curve where dpH/dml is normally very high. Apart from these regions, the ΔpH value does not exceed ±0.06 pH unit. However, the ΔpH values found are still within the 95% confidence limit so that no serious degradation can be proven at this pH (= 2.9) and during the time the test was performed. The stability constants of alitame were also calculated from the data of the titration curve measured after 21 days. A difference of −0.02 log unit (fresh−21 days) for log $\beta_{0,1,1}$ was found that is within the experimental errors for this measurement but a negligible difference was found for log $K_{0,1,2}$. A similar treatment was carried out for alitame in the presence of Cu(II) (in the molar ratio metal to ligand 1:2). The test solution was allowed to remain for only 6 days at room temperature and the maximum ΔpH found was −0.03 pH unit when the initial pH of the solution was 3.80. The ΔpH values found are within the 95% confidence limit and converge with increasing pH. The stability constants for the 6 days solutions were also calculated and differences of +0.05, +0.03, +0.03, −0.05, −0.03 for log $\beta_{1,1,0}$, log $K_{1,2,0}$, log $K_{1,1,-1}$, log $K_{1,1,-2}$ and log $K_{1,2,-1}$, respectively, were found. These differences are slightly outside the 95% confidence interval, established from the calculation of the fresh solutions, by ±0.02 to

± 0.04 log unit. The differences of the stability constants given above are not dramatic but indicate the possible variation in the composition of the alitame molecule with time and particularly in the presence of metal ions.

4. Conclusion

The stability constants and equilibrium information presented are important for further analysis and formulation of the the new low calorie artificial sweetener alitame. Moreover, they are essential for speciation studies in biological fluids.

The increased stability of solutions of alitame over aspartame in the absence of metal ions is an important step. However, and in the presence of Cu^{2+}, degradation products similar to those already known with aspartame (Prudel et al., 1986) are not excluded in this case but are highly minimized due to the presence of the extra stabilizing TTA group in comparison with the methoxy group of aspartame. The TTA group is expected to strictly hinder the cyclization of alitame to produce the diketopiperazine found in aspartame (Prudel et al., 1986) where the methoxy group is easily cleaved to produce methanol.

Before any enzymatic reactions, and at the duodenal pH between 6.5 and 7.5, Cu(II) is mainly present as the uncharged CuL_2 ($L^- =$ alitame) as the major product followed by the $CuLH_{-1}$ complex. The presence of other complexing agents at this pH will lead to the formation of ternary complexes as well (Kholeif and Anderegg, 1997).

Metabolic studies are important, especially for the TTA or the alaninamide moiety. As described in case of aspartame, the presence of alitame in food can influence the equilibria between metal ions and complexes, particularly in the case of Cu(II). This can occur directly or through the ligands obtained as products of the enzymatic decomposition of alitame (aspartic acid, alanine or alaninamide) at the duodenal pH in the small intestines.

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