

## Research Article

# A New Strategy to Stabilize Oxytocin in Aqueous Solutions: I. The Effects of Divalent Metal Ions and Citrate Buffer

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**Abstract.** In the current study, the effect of metal ions in combination with buffers (citrate, acetate, pH 4.5) on the stability of aqueous solutions of oxytocin was investigated. Both monovalent metal ions ( $\text{Na}^+$  and  $\text{K}^+$ ) and divalent metal ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$ ) were tested all as chloride salts. The effect of combinations of buffers and metal ions on the stability of aqueous oxytocin solutions was determined by RP-HPLC and HP-SEC after 4 weeks of storage at either 4°C or 55°C. Addition of sodium or potassium ions to acetate- or citrate-buffered solutions did not increase stability, nor did the addition of divalent metal ions to acetate buffer. However, the stability of aqueous oxytocin in aqueous formulations was improved in the presence of 5 and 10 mM citrate buffer in combination with at least 2 mM  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , or  $\text{ZnCl}_2$  and depended on the divalent metal ion concentration. Isothermal titration calorimetric measurements were predictive for the stabilization effects observed during the stability study. Formulations in citrate buffer that had an improved stability displayed a strong interaction between oxytocin and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Zn}^{2+}$ , while formulations in acetate buffer did not. In conclusion, our study shows that divalent metal ions in combination with citrate buffer strongly improved the stability of oxytocin in aqueous solutions.

**KEY WORDS:** citrate buffer; divalent metal ions; improved stability; oxytocin.

## INTRODUCTION

According to the World Health Organization, half a million of women in Africa, Asia, and Latin America die each year due to problems during pregnancy and childbirth. At least 25% of those deaths can be attributed to bleeding after child birth (post-partum hemorrhage), mainly caused by failure of the uterus to contract adequately after child birth (atonicity) (1).

The preferred drug to prevent post-partum hemorrhage is oxytocin. Oxytocin is a cyclic nonapeptide hormone [sequence: cyclo (Cys<sup>1</sup>-Tyr<sup>2</sup>-Ile<sup>3</sup>-Gln<sup>4</sup>-Asn<sup>5</sup>-Cys<sup>6</sup>, -Pro<sup>7</sup>-Leu<sup>8</sup>-Gly<sup>9</sup>-NH<sub>2</sub>)], which is naturally produced in the hypothalamus. It is involved primarily in uterine contraction and stimulation of milk release from the mammary tissue (2). Oxytocin, which is currently available in synthetic form (3),

has been widely used for indications such as induction of labor, augmentation of labor, post-partum hemorrhage, or uterine atony, and also for other indications such as diabetes insipidus and vasodilatory shock. Reported additional functions for oxytocin include an antidiuretic effect and blood vessel contraction (2,4,5).

Unfortunately, oxytocin preparations are highly unstable at elevated temperatures, which is an issue particularly in tropical countries (6). Stability studies conducted by Groot *et al.* (6) have shown that injectable oxytocin formulations are rapidly degraded as the storage temperature rises to 30°C or higher. Oxytocic tablets for oral administration (ergometrine, ethylergometrine, oxytocin, and desamino-oxytocin) are also not stable under simulated tropical conditions. Because of its poor stability at elevated temperatures, the use of oxytocin in many developing countries is limited. Thus, there is a clear need for a heat-stable oxytocin formulation, preferably an aqueous injectable solution, with improved thermal stability.

One way to effectively improve stability of several peptides in aqueous solution is using metal salts in combination with a suitable buffer (7). To investigate the effect of metal ions in buffered solutions on the stability of oxytocin, we screened various combinations of unbuffered and buffered solutions with monovalent or divalent metal ions. Hawe *et al.* (8) observed that the degradation of oxytocin strongly depends on the pH of the formulation, with the highest stability at pH 4.5. Therefore, all formulations will be set to pH 4.5. The purpose of this study is to investigate whether

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specific combinations of buffer and metal ions can stabilize oxytocin.

## MATERIALS AND METHOD

### Materials

The following materials were used in this study: oxytocin monoacetate powder (Diosynth, Oss, The Netherlands), citric acid, calcium chloride (Riedel-de Haen, Seelze, Germany), acetic acid, magnesium chloride, zinc chloride (Fluka, Steinheim, Germany), sodium hydroxide, sodium chloride, potassium chloride, sodium dihydrogen phosphate dihydrate, acetonitrile, formic acid (Merck, Darmstadt, Germany) and Baxter Viavlo Ringer's lactate solution for intravenous infusion (Baxter, Utrecht, The Netherlands).

### Formulation and Stability Study

Oxytocin was formulated at a concentration of 0.1 mg/ml in citrate (5 or 10 mM) or acetate (10 mM) buffer at pH 4.5 (pH adjusted with sodium hydroxide) with different additions of metal ions. pH samples were controlled and remained within  $\pm 0.1$  pH units during the stability study. The initial concentration of oxytocin was determined using UV spectrophotometry (9) at 280 nm with an extinction coefficient of  $1.52 \text{ ml mg}^{-1} \text{ cm}^{-1}$ . All metal ion solutions were prepared using their chloride salts at concentrations of 2, 5, 10, and 50 mM. Control solutions were formulated in water (pH  $6.9 \pm 0.2$ ) and Ringers's lactate ( $6.4 \pm 0.2$ ). Ringers's lactate solution consists of 131 mM sodium, 5 mM potassium, 2 mM calcium, 111 mM chloride, and 29 mM bicarbonate (as lactate). In this report, the following codes were used: First character(s) refer to the type of buffer or water; CB (citrate), AC (acetate), RL (Ringer's lactate), and W (water). Following digit(s) refer to buffer concentration in mM, following character(s) to the type of metal ion, and last digit(s) to metal ion concentration in mM. Thus, *e.g.*, CB10Mg10 means 10 mM citrate buffer (pH 4.5) and 10 mM  $\text{MgCl}_2$ . After preparation, the solutions were stored in 6R glass type 1 vials for 4 weeks at either  $4^\circ\text{C}$  or  $55^\circ\text{C}$ , and protected from light.

Based on the results of the screening study, oxytocin formulations in 10 mM citrate buffer pH 4.5 with 10 or 50 mM divalent metal salts were selected for a longer period of stability study for 6 months at  $40^\circ\text{C}$  according to ICH guidelines for long-term and accelerated stability study for climatic zone III and IV (10).

### Reversed-Phase High-Performance Liquid Chromatography

The recovery of oxytocin (remaining oxytocin as percentage of initial amount) was determined by RP-HPLC. RP-HPLC was performed according to the procedure described by Hawe *et al.* (8) An Alltima C-18 RP column with 5  $\mu\text{m}$  particle size, inner diameter of 4.6 mm, and length of 150 mm (Alltech, Ridderkerk, Netherlands), a Waters (Millipore) 680 Automated Gradient Controller, two Waters 510 HPLC pumps, a Waters 717 Plus Autosampler, and a Waters 486

Tunable Absorbance UV Detector were used. Samples of 20  $\mu\text{l}$  were injected and the separation was carried out at a flow rate of 1.0 ml/min and UV detection at 220 nm. Samples were eluted using 15% (v/v) acetonitrile in 65 mM phosphate buffer pH 5.0 as solvent A and 60% (v/v) acetonitrile in 65 mM phosphate buffer pH 5.0 as solvent B. The acetonitrile concentration was linearly increased from 15% at the beginning, to 20% at 10 min, to 30% at 20 min, and finally to 60% at 25 min.

### Size Exclusion HPLC

The fraction of monomeric oxytocin (percentage of total remaining oxytocin) was assessed by Size Exclusion HPLC (HP-SEC). HP-SEC was carried out using a Superdex peptide 10/300 GL column (GE Healthcare Inc., Brussels, Belgium) on an isocratic HPLC system, according to the method previously reported by Hawe *et al.* (8). A Waters 510 pump, a Waters 717 plus auto sampler, a Waters 474 Scanning Fluorescence Detector and Waters 484 Tunable Absorbance Detector (Waters, Milford Massachusetts, USA) were used. Samples of 50  $\mu\text{l}$  were injected, and separation was performed at a flow rate of 1 ml/min. Peaks were detected by UV absorption at 274 nm, as well as fluorescence detection at excitation wavelength of 274 nm and emission wavelength of 310 nm. The mobile phase consisted of 30% acetonitrile and 70% 0.04 M formic acid.

### Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was used to investigate the interaction between oxytocin and divalent metal ions in the presence of citrate buffer and acetate buffer. Microcalorimetric titrations of divalent metal ions to oxytocin were conducted by using a MicroCal ITC 200 Microcalorimeter (Northampton, MA 01060 USA). A solution of 300  $\mu\text{L}$  of 5 mM oxytocin in 10 mM of either citrate or acetate pH 4.5 was placed in the sample cell, while 30  $\mu\text{L}$  of 125 mM divalent metal chloride either calcium, magnesium, or zinc in 10 mM citrate or acetate buffer pH 4.5 was placed in the syringe. The reference cell contained 300  $\mu\text{L}$  of the corresponding buffer. Experiments were performed at  $55^\circ\text{C}$ . Automated titrations were conducted up to a divalent metal ion/oxytocin molar ratio of 5:1. The effective heat of the peptide-metal ion interaction upon each titration step was corrected for dilution and mixing effects, as measured by titrating the divalent metal ion solution into buffer and by titrating buffer into oxytocin solution. To investigate the possibility of oxytocin or metal ion binding to the buffer components, control experiments were performed in water. The heats of bimolecular interactions were obtained by integrating the peak following each injection. All measurements were performed in triplicate.

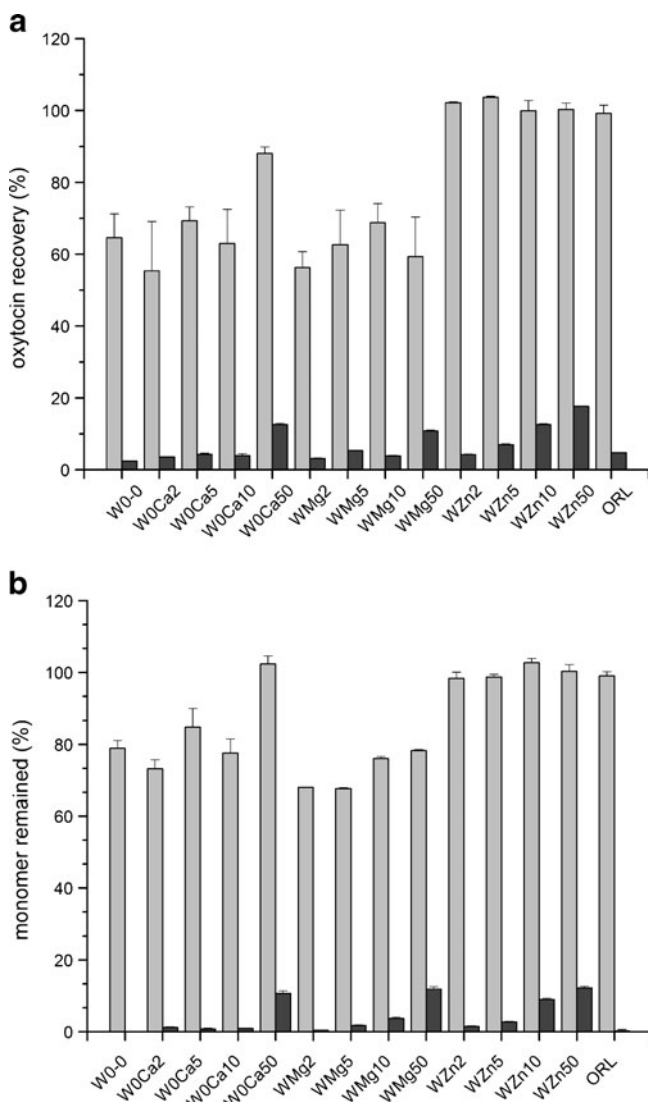
ITC data were analyzed by using the ITC non-linear curve fitting functions for one or two binding sites from MicroCal Origin 7.0 software (MicroCal Software, Inc.). The calculated curve was determined by the best-fit parameter, which was used to determine the molar enthalpy change for binding and the corresponding association constant,  $K_a$ . The molar free energy of binding  $\Delta G^\circ$  and the molar entropy

change  $\Delta S^\circ$  were derived from the fundamental equations of thermodynamics  $\Delta G^\circ = -RT \ln K_a$  and  $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ .

## RESULTS

### Influence of Divalent Metal Ions on Oxytocin Stability in Unbuffered Solutions

First, the effect of divalent metal ions on the stability of oxytocin in water without any buffer salt was investigated. RP-HPLC results (Fig. 1a) showed that after 4 weeks of storage at 4°C, oxytocin recovery was almost 100% in the presence of 2–50 mM zinc or 50 mM calcium ions. No stabilizing effect was observed from the presence of magnesium (2–50 mM) and calcium (2–10 mM), where the recovery was reduced to about



**Fig. 1.** Recovery of oxytocin in the presence of divalent metal ions in non-buffered, pure water stored for 4 weeks at pH 4.5 and a temperature of 4°C (light gray bars) or 55°C (dark gray bars). The divalent metal ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$ ) were used in concentrations of 2, 5, 10, and 50 mM. **a** recovery determined by RP-HPLC. **b** oxytocin monomer recovery determined by HP-SEC. The results are depicted as averages of three independent measurements  $\pm$ SD

65%, similar to levels found for oxytocin solutions in water. HP-SEC results (Fig. 1b) showed a similar trend in the recovery of monomeric oxytocin. However, when the solutions were stored at 55°C, both RP-HPLC and HP-SEC measurements showed substantial degradation of oxytocin after 4 weeks. These results demonstrate that divalent metal ions in non-buffered aqueous oxytocin formulations have only a limited stabilizing effect at elevated temperature.

### Oxytocin Stability in Buffered Solutions

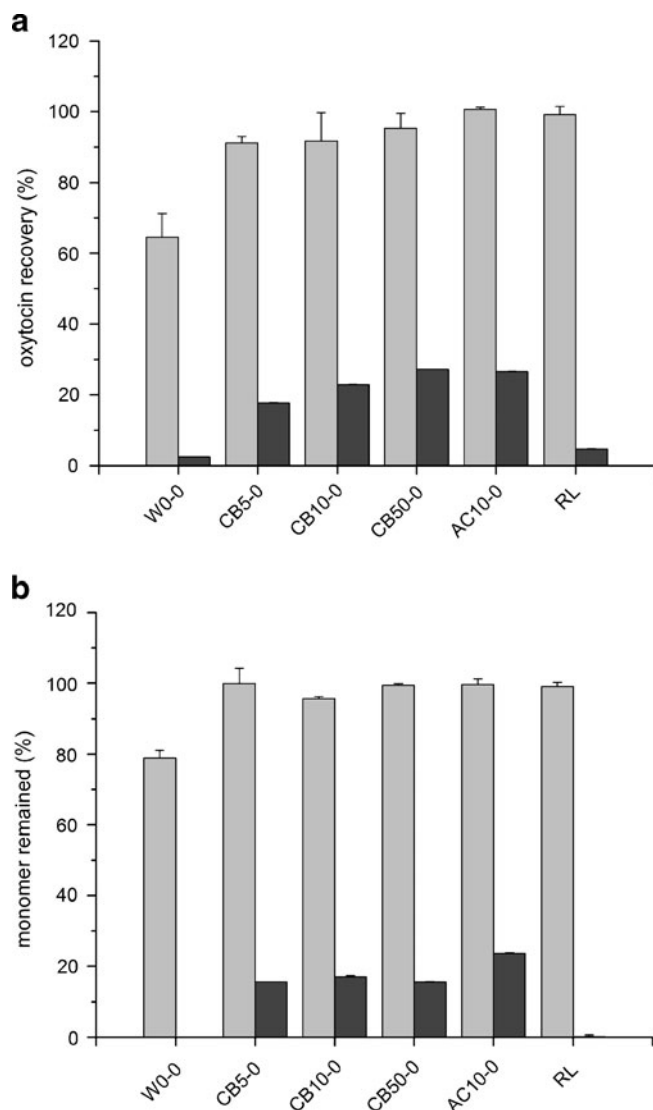
To determine the effect of buffer on stability, oxytocin was formulated in 5 and 10 mM citrate buffer and 10 mM acetate buffer. As a reference, the stability of oxytocin in pure water and in Ringer's lactate buffer was investigated. Figure 2a shows the oxytocin recovery in RP-HPLC after 4 weeks of storage either at 4°C or 55°C in the buffered solutions. Compared to pure water, the stability of oxytocin was substantially increased at 4°C in the presence of the buffer salts. After storage at 55°C, the recovery of oxytocin after 4 weeks in citrate and acetate buffer was much higher as compared to water or Ringer's lactate solution. However, the recovery of oxytocin was still poor. In addition, only about 20% of oxytocin remained in its monomeric form after storage (Fig. 2b).

### Oxytocin Stability in Buffered Solutions Containing Monovalent Metal Ions

To investigate the stability of oxytocin in the presence of a combination of buffer and monovalent metal ions, citrate and acetate buffer were used at a concentration of 10 mM, in combination with the monovalent metal ions, sodium and potassium, added at a concentration of 10 and 20 mM (excluding sodium from the buffer component). The presence of monovalent metal ions had only a minor effect on the stability of oxytocin (stored at 55°C). Although the oxytocin recovery was slightly improved compared to oxytocin in the presence of buffer alone, the maximum recovery of oxytocin was only 35% in 10 mM acetate buffer with 10 mM sodium chloride. In addition, only about 30% of oxytocin in this formulation remained monomeric (data not shown). These results clearly indicate that the presence of a combination of buffer and monovalent metal ions is not sufficient to substantially stabilize oxytocin in aqueous solution.

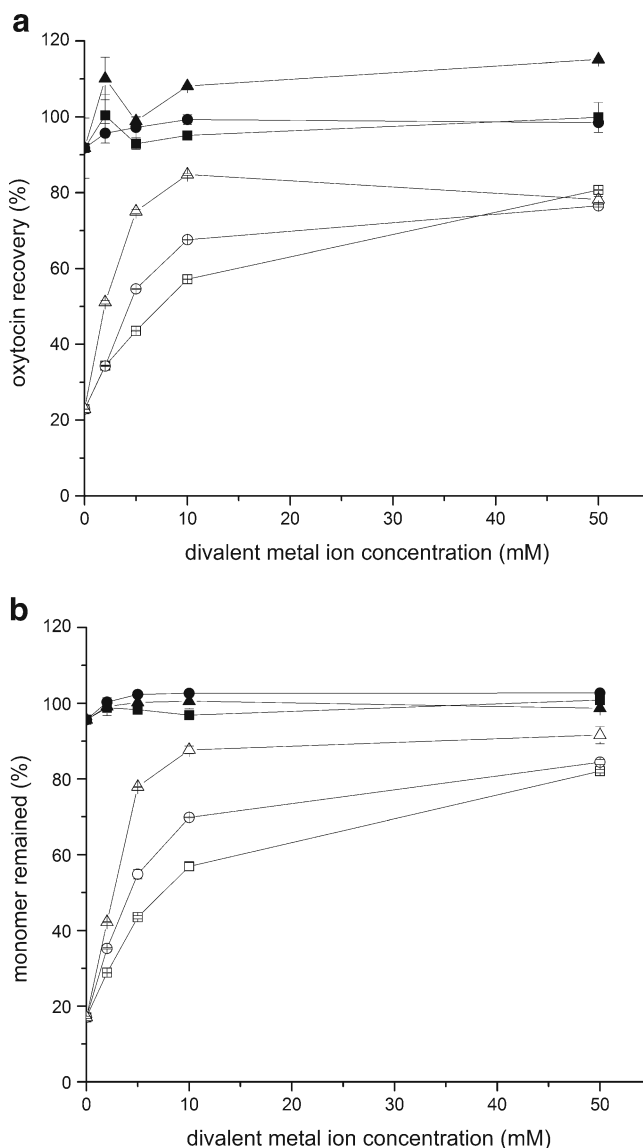
### Oxytocin Stability in Citrate-Buffered Solutions Containing Divalent Metal Ions

To study the stability of oxytocin in the presence of citrate buffers and divalent metal ions, formulations containing 5 and 10 mM citrate buffer in combination with divalent metal ions (calcium, magnesium, and zinc) added at concentrations of 2, 5, 10, or 50 mM were used. The results of RP-HPLC and HP-SEC of formulations in 10 mM citrate buffer are presented in Fig. 3a and b. The stability of oxytocin solutions was clearly improved when formulating them with citrate buffer in combination with calcium ions. The oxytocin stability increased with increasing calcium ion concentrations. The recovery of oxytocin and the remaining percentage of oxytocin monomers after 4 weeks of storage at 55°C were increased up to almost 80% in the presence of 50 mM calcium.



**Fig. 2.** Recovery of oxytocin in pure water, with or without a buffer. Citrate buffer at a concentration of 5, 10, or 50 mM, acetate buffer at a concentration of 10 mM, or Ringer's lactate solution were used. The formulations contained no metal ions and were stored for 4 weeks at pH 4.5 or 6.4 for Ringer's lactate solution at a temperature of 4°C (light gray bars) or 55°C (dark gray bars). **a** recovery determined by RP-HPLC. **b** oxytocin monomer recovery determined by HP-SEC. The results are depicted as averages of three independent measurements  $\pm$ SD

Similar results were obtained for the combination of citrate with magnesium. The degradation of oxytocin in citrate buffer at 5 and 10 mM decreased with an increasing concentration of magnesium ions. Formulations with zinc ions in citrate buffer also preserved oxytocin during storage. These combinations exert even a stronger effect on oxytocin stability than combinations of citrate buffer and calcium or magnesium ions. Stability was strongly improved at zinc concentrations as low as 2 or 5 mM. Both oxytocin recovery (RP-HPLC) and the monomeric oxytocin fraction (HP-SEC) were substantially higher (up to 90%) in the presence of 10 mM zinc ions (CB5Zn10) after storage for 4 weeks at 55°C. When citrate buffer was used at a concentration of 5 mM, similar results were found (data not shown).



**Fig. 3.** Effect of Ca<sup>2+</sup> (squares), Mg<sup>2+</sup> (circles), and Zn<sup>2+</sup> (triangles) concentration on the recovery of oxytocin in citrate buffer at the concentration of 10 mM after 4 weeks of storage at either 4°C or 55°C and pH 4.5. Solid symbols denoted 4°C storage, while open symbols correspond to 55°C. **a** recovery determined by RP-HPLC. **b** oxytocin monomer recovery determined by HP-SEC. The results are depicted as averages of three independent measurements  $\pm$ SD

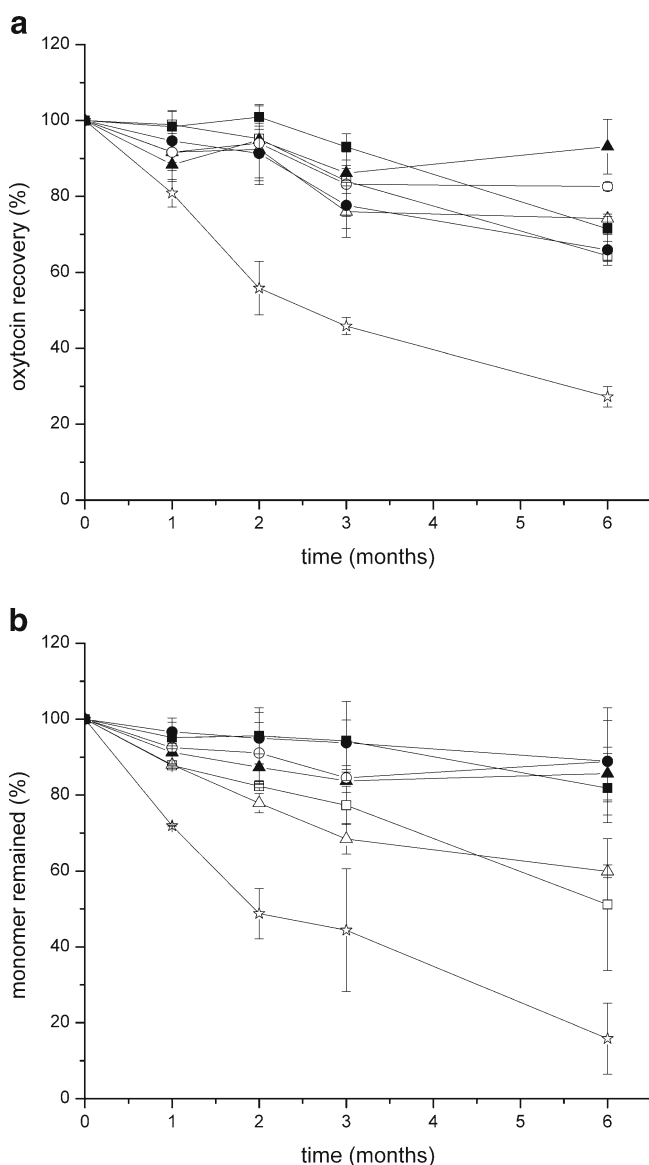
Beside citrate, we also carried out several further experiments to investigate whether divalent metal ions affect the stability in the presence of acetate buffer. Acetate buffer at a concentration of 10 mM was used with and without calcium, magnesium, and zinc ions at various concentrations (2, 5, 10, 50 mM). The combination of these ions with acetate buffer was found to be less efficient in stabilizing oxytocin (data not shown).

#### Long-term Stability of Oxytocin in Selected Formulations Containing Citrate and Divalent Metal Ions

For the combination of citrate with Ca<sup>2+</sup>, Zn<sup>2+</sup>, and Mg<sup>2+</sup>, a long-term stability study for 6 months at 40°C was conducted. A

temperature of 40°C was chosen to simulate tropical conditions (10,11). The long-term stability study at 40°C clearly demonstrates the synergistic stabilizing effect of citrate buffer and divalent metal ions. Even though the oxytocin recovery decreased gradually with time, the recovery of oxytocin (Fig. 4a) and the remaining percentage of oxytocin monomers (Fig. 4b) after 6 months storage at 40°C were increased up to 80% in the presence of 50 mM calcium, and even higher (up to 90%) in the presence of 50 mM magnesium.

Formulations with 10 mM zinc ions in citrate buffer exerted the same effect on oxytocin stability as combinations of citrate buffer and 50 mM magnesium ions. This shows that zinc ions at lower concentrations have already a higher impact on increasing oxytocin stability compared with calcium or magnesium ions.



**Fig. 4.** Oxytocin recovery over time storage at 40°C and pH 4.5 in the presence of 10 mM citrate buffer, without (*star*) and with divalent metal ions. Ca<sup>2+</sup> (*square*), Mg<sup>2+</sup> (*triangle*), and Zn<sup>2+</sup> (*circle*) were used in concentrations of 10 mM (*open symbols*), and 50 mM (*solid symbols*). **a** recovery determined by RP-HPLC. **b** oxytocin monomer recovery determined by HP-SEC. The results are depicted as averages of three independent measurements ±SD

This result also confirms the short-term stability study that showed up to 90% remaining oxytocin in the presence of 10 mM zinc ions after storage for 4 weeks at 55°C.

### ITC to Study the Interaction Between Oxytocin and Divalent Metal Ions

To examine the interaction between oxytocin and the metal ions, ITC experiments were carried out that are summarized in Table I. The titration of calcium ions into an oxytocin solution in citrate buffer resulted in an exothermic reaction (Fig. 5a) with a  $K_a$  value of about 400 M<sup>-1</sup> and an apparent ion/oxytocin stoichiometry close to 4:1 (Table I). Remarkably, when titrating magnesium into oxytocin we observed heat absorption (Fig. 5a) and this endothermic reaction occurred with an identical apparent stoichiometry (4:1) and a similar  $K_a$  value of about 200 M<sup>-1</sup> as with calcium ions. However, with magnesium ions the ITC trace was complex and showed an exothermic phase at magnesium concentrations below 5 mM. Due to the rapid saturation of this early phase—typically, within three injections—and the low enthalpy of the ion/oxytocin interaction it was not possible to quantitatively analyze the exothermic stage. To analyze the following endothermic phase we omitted the first 4-titration steps and fitted the remaining data using a single site model. A similar dual-phase response was observed for the zinc:oxytocin interaction (Fig. 5a, **open triangles**), but the exothermic and endothermic stages were well-resolved and suitable for the analysis using a two sites model (Table I). In contrast, in the presence of acetate buffer there was no measurable interaction between oxytocin and calcium, magnesium, or zinc ion (Fig. 5b).

### DISCUSSION

Our study clearly demonstrates that the stability of oxytocin in aqueous formulations is greatly increased in citrate buffer in combination with divalent metal ions. The improved stability was found to be dependent on the divalent metal ions concentration.

The WHO reported that there is no loss of potency of oxytocin in injection preparation after 12 months refrigerated storage (2–8°C). However, oxytocin lost 14% of its potency after 1 year at 30°C (12). In another study, the oxytocin concentration in Ringer's lactate solution was reduced by about 10% after 35 days storage at room temperature (near 23°C) (13). From our observation Ringer's lactate solution was able to stabilize oxytocin in aqueous solution at low temperature (4°C). However at a higher temperature (55°C), the stability of Ringer's lactate solution was poorer than in the presence of citrate or acetate buffers. This result can be attributed to the pH and/or the amount of metal ions in solution. Oxytocin in Ringer's lactate solution has a pH of 6.4 and contains less than 2 mM of divalent metal ions. Hawe *et al.* (8) observed that the degradation of oxytocin strongly depends on the pH of the formulation, with the highest stability at pH 4.5. Therefore, a pH of 6.4 might have caused an increased rate of decomposition. However, formulating oxytocin in acetate buffer at pH 4.5 only maintain approximately 30% oxytocin recovered after 1 month storage at 55°C.

**Table I.** Thermodynamics of Divalent Metal binding to Oxytocin as Determined by Isothermal Titration Calorimetry in 10 mM Citrate Buffer

Metal	Phase	<i>N</i> (sites)	<i>K<sub>a</sub></i> (M <sup>-1</sup> )	$\Delta H^\circ$ (cal mol <sup>-1</sup> )	$\Delta S^\circ$ (cal/mol/deg)
Ca <sup>2+</sup>	1	0.26	400	-2,100	5.6
Mg <sup>2+</sup>	1	n.d.	n.d.	<0	n.d.
	2	0.23	220	4,400	24
Zn <sup>2+</sup>	1	0.78	2,700	-800	13
	2	1.48	800	610	15

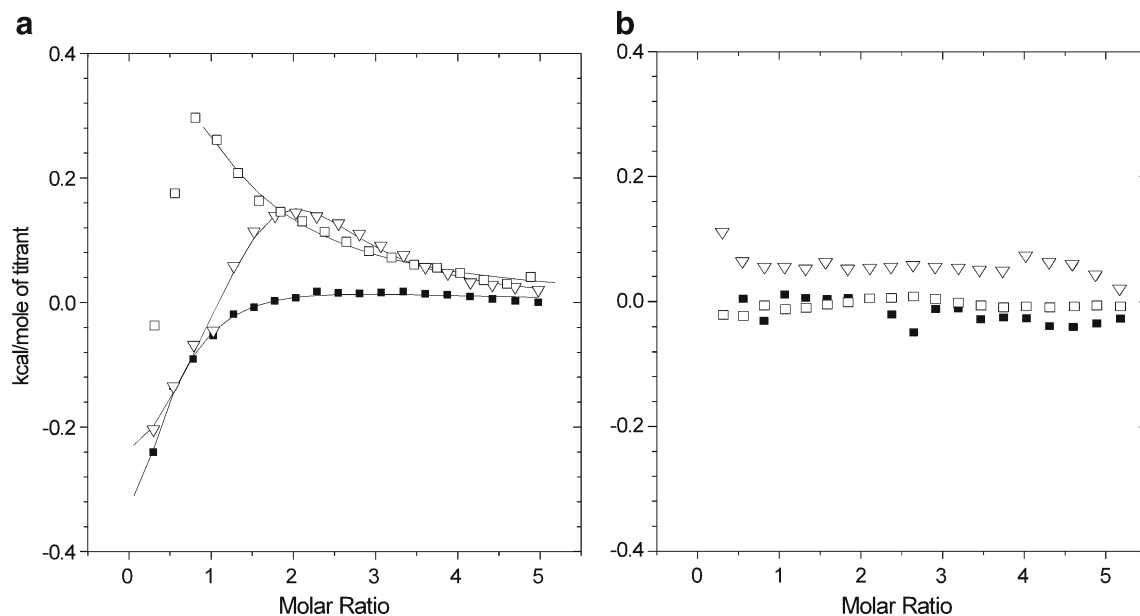
The results are depicted as averages of three independent measurements with relative standard deviations below 10%  
*n.d.* not determined

The decomposition of oxytocin is mainly caused by deamidation, oxidation, hydrolysis, and dimerization (7,8). Deamidation is likely to occur in Gln<sup>4</sup> (14), Asn<sup>5</sup> (15), and Gly<sup>9</sup> (7,16). Under acidic condition (pH below 3), deamidation of Asn<sup>5</sup> and Gln<sup>4</sup> occur by direct hydrolysis (7). Oxidation might occur at Tyr<sup>2</sup> (17) and Cys<sup>1,6</sup> (18), whereas dimerization might occur due to thiol exchange in Cys<sup>1,6</sup> (19).

Although the specific mechanism has not been elucidated yet, the presence of sufficient amounts of divalent metal ions at pH 4.5 in citrate buffer, however, greatly improved the stability of oxytocin in aqueous solution. In previous studies, the interaction of oxytocin with calcium (20) and zinc (21) ions was investigated using NMR and nano-electrospray mass spectrometry (MS). Those studies which were also supported with molecular modeling, showed that Ca<sup>2+</sup> is coordinated by seven carbonyl oxygen atoms (O-Tyr<sup>2</sup>, O-Ile<sup>3</sup>, O-Gln<sup>4</sup>, O-Asp<sup>5</sup>, O-Cys<sup>6</sup>, O-Leu<sup>8</sup>, and O-Gly<sup>9</sup>) which formed a more compact structure for the oxytocin-Ca<sup>2+</sup> complex compared to free oxytocin (20). Whereas zinc ions formed an octahedral complex with six of the backbone carbonyl oxygen atoms (O-Tyr<sup>2</sup>, O-Ile<sup>3</sup>, O-Gln<sup>4</sup>, O-Cys<sup>6</sup>, O-Leu<sup>8</sup>, and O-Gly<sup>9</sup>) (21). It was suggested that in the presence of such divalent ions, the hydrophobic

groups are situated inside the peptide keeping them away from water molecules. These conformational changes can increase the stability of oxytocin in aqueous medium as they will prevent dimerization and further aggregation by hydrophobic interactions among oxytocin molecules. Metal salts are often used to stabilize peptides or proteins by chelation or ionic interactions (22). Wang *et al.* examined the peptide (P66) stability in the presence of ZnCl<sub>2</sub>, MgCl<sub>2</sub>, and CaCl<sub>2</sub> in non aqueous solution, and found that in the presence of 1 mM ZnCl<sub>2</sub>, P66 was significantly stabilized. However in the aqueous solution (pure water), these ions did not show any stabilizing effect (22). In our experiments, the addition of calcium, magnesium, and zinc ions in combination with citrate buffer had a large impact on oxytocin stability in contrast to similar experiments in pure water or acetate buffer. This study suggests that there is a synergistic effect between citrate buffer and the divalent metal ions, possibly due to the protection of the disulfide bridge by complex formation of divalent metal ion and citrate with oxytocin which suppressed intermolecular reaction leading to tri/tetrasulfide formation as well as dimerization (unpublished data).

ITC is a sensitive method for studying the thermodynamics of binding events and quantifying binding reactions. When



**Fig. 5.** Least squares fit of the data from calorimetric titration profiles of aliquots of 125 mM divalent metal ions: Ca<sup>2+</sup> (solid square), Mg<sup>2+</sup> (open square), and Zn<sup>2+</sup> (open triangle) into 5 mM oxytocin in 10 mM **a** citrate buffer and **b** acetate buffer pH 4.5. The heat absorbed per mol of titrant is plotted versus the ratio of the total concentration of divalent metal ions to the total concentration of oxytocin

divalent metal ion are added to oxytocin, the ITC data indicate an interaction between oxytocin and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Zn}^{2+}$  ions in the presence of citrate buffer. Both  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  ions demonstrated complex, dual-phase interaction profile, while a single phase was observed for  $\text{Ca}^{2+}$ . Each interaction was entropy driven, while both exothermic and endothermic reactions were observed. It may be speculated that the solvation effect, *i.e.*, release of structured water molecules plays a key role in binding, while the specific ion–oxytocin interaction further contributes to the complex stability. The latter is also predicted by molecular dynamic simulations (20,21). Remarkably, no interaction between oxytocin and either of the tested ions was detected in the acetate buffer or deionized water. These observations underscore the role of a particular environment in the ion–oxytocin interaction and agree well with our findings on the peptide stability. Isothermal titration calorimetric measurements were predictive for the effects observed during the stability study.

In conclusion, this study shows that with a combination of divalent metal salts and citrate buffer, the stability of oxytocin in aqueous solution can be strongly improved. The increased stability of oxytocin aqueous formulations was achieved in the presence of citrate acid buffer and 2 mM or more of the salts  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , or  $\text{ZnCl}_2$ . The oxytocin stability is further increased with increasing concentration of the divalent metals ions up to 50 mM. In combination with citrate buffer,  $\text{Zn}^{2+}$  has a superior stabilizing effect as compared with  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ .

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