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Capillary electrophoresis separation of the desamino degradation products of oxytocin

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Abstract

Oxytocin is an endogenous and therapeutic hormone necessary for maternal health. It is also the subject of fast growing research in the field of behavioral science. This article describes a rapid capillary electrophoresis method using UV detection at 214 nm for the determination of the deamidation products of oxytocin. Deamidation is the most common degradation pathway of peptides and proteins and can lead to reduced therapeutic efficiency of biopharmaceuticals. To achieve a separation of the seven structurally similar desamino peptides from oxytocin, 11 mM sulfobutyl ether β -cyclodextrin and 10% v/v MeOH were added to a background electrolyte of 50 mM phosphate buffer at pH 6.0. The assay is linear within 5–100 μ M for all species with a total analysis time of 12 min. The method was then applied to monitor the heat-stress degradation of oxytocin at 70°C, where all seven desamino species were observed over a 96 h period.

Keywords

biopharmaceuticals; capillary electrophoresis; deamidation; degradation; oxytocin

1 Introduction

Oxytocin (OT) is a cyclic nonapeptide hormone secreted from the pituitary gland of the hypothalamus. Traditionally, OT is used therapeutically in maternal health to induce and augment labor as well as prevent death from postpartum hemorrhage. However, low concentrations of endogenous OT have also been implicated in behavioral disorders such as depression, anxiety, and autism [1]. As research progresses in this field, new therapeutic uses for OT and its analogs will be discovered. To ensure safety and efficacy, inexpensive and fast analytical methods are needed to determine the integrity and stability of OT.

As a biopharmaceutical drug, OT is susceptible to both chemical and physical degradation during purification, shipping, storage, and delivery. Yet, prior to 2009, there were few reports on the specific degradation pathways of OT. In 1981, Nachtmann et al. compiled the

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then current knowledge of the physical, chemical, and pharmaceutical characteristics of OT, noting the importance of formulation pH on the rate of degradation [2]. In the following years, several methods were published in which LC-UV was used to determine OT quality in pharmaceutical dosage forms [3-6], including the US and EU Pharmacopoeias [7, 8]. In these stability-indicating methods, OT was resolved from its pH-, heat-, light-, and oxidative-stress degradation products. However, none of these reports identified the degradation products formed. Hawe et al. were the first to publish a systematic discussion of the formation of specific degradation products and the degradation kinetics of OT using LC-MS [9].

Deamidation is the most common chemical degradation pathway for proteins and peptides [10]. It is not surprising then that OT undergoes deamidation under both acidic and basic conditions and that the rate of deamidation increases with exposure to heat [9]. Proper functionality of OT at the uterine receptor requires that both the receptor binding region (Ile³, Gln⁴, Pro⁷, and Leu⁸) and the active site (Asn⁵ and Tyr²) be intact [11]. Both of these sites rely on amino acids with amide functional groups (Gln⁴ and Asn⁵). Different combinations of deamidation at those sites, and at the amidated C-terminus, lead to a total of seven possible desamino-OT species (Figure 1). It has also been shown that deamidation at each site significantly diminishes the peptide's biological activity [12-14].

To evaluate the extent of OT deamidation, we employed a CE-based method for analysis. CE has several benefits over LC for peptide analysis. CE requires very little reagent use for the preparation of the run buffer (mL), allowing the use of expensive and exotic additives without significantly increasing the cost-per-test or waste generated by this technique. Additionally, CE is known for high efficiency separations of both proteins and peptides. The size-to-charge based separation is particularly useful for the identification of deamidation products [15, 16]. This is because deamidation yields an ionizable side group from a neutral amide ($R-CONH_2 \rightarrow R-COOH$), which alters the electrophoretic mobility of the peptide depending on the pH of the background electrolyte.

To the best of our knowledge, there is only one report that uses CE to address OT degradation. In that report, the separation of the degradation products was performed with an acidic background electrolyte inside positively charged, noncovalently coated capillaries [17]. At this low pH the desamino side chains are protonated and are, therefore, neutral, producing a +1 charge for all species due to the protonated N-terminus. Because of this, only partial resolution of OT from the desamino species was possible. For identification of the products, the authors relied on MS to collect extracted-ion electropherograms to identify the degradants. Additionally, the authors did not identify which specific site(s) of the peptide had undergone deamidation, instead distinguishing only between mono- and bis-deamidated OT.

In this article, a CE method is described that utilizes a modified charged β -cyclodextrin as pseudo-stationary phase and the organic modifier MeOH in a phosphate buffer (pH 6.0) to achieve a separation of the seven desamino degradation products from OT. All seven of these desamino species are detected after a 96-h heat-stressed degradation study of OT. This

fast and cost-effective analysis method makes it possible to determine the extent of OT deamidation in 12 minutes without requiring the use of expensive, high-resolution MS.

2 Materials and methods

2.1 Chemicals and reagents

OT acetate salt was obtained from Bachem (Torrance, CA, USA). OT desamino standards of mono-desamino at Gln⁴ (Gln⁴), mono-desamino at Asn⁵ (Asn⁵), mono-desamino at Gly⁹-NH₂(Gly⁹), bis-desamino at Gln⁴ and Asn⁵ (Gln⁴Asn⁵), bis-desamino at Gln⁴ and Gly⁹-NH₂(Gln⁴Gly⁹), bis-desamino at Asn⁵ and Gly⁹-NH₂(Asn⁵Gly⁹), and tris-desamio at Gln⁴, Asn⁵, and Gly⁹-NH₂ (Gln⁴Asn⁵Gly⁹) were synthesized by Shanghai Mocell Biotech Co. (Shanghai, China). β -cyclodextrin, *o*-phosphoric acid, monobasic sodium phosphate, dibasic sodium phosphate, HEPES, mesityl oxide (MO), acetonitrile, methanol, dimethyl sulfoxide, concentrated HCl, and NaOH were obtained as analytical grade reagents from Sigma Aldrich (St. Louis, MO, USA). Sulfobutyl ether β -cyclodextrin (SBE) was donated by Cydex Pharmaceuticals (Lenexa, KS, USA).

2.2 Preparation of buffers

2.2.1 Buffer composition optimization—Several background electrolytes were investigated over a pH range of 4.0-9.0; each was prepared at a concentration of 50 mM. The phosphate buffers (pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) were prepared by mixing the appropriate amounts of acid and conjugate base as calculated by the Henderson-Hasselbalch equation, and the pH was adjusted as needed with either 1 M NaOH or 1 M *o*-phosphoric acid. The HEPES buffer (pH 7.0) was prepared by simply adding the correct amount of HEPES to water and adjusting the pH as needed with 1 M NaOH or 1 M HCl. All buffers were prepared with 18 M Ω deionized water (Millipore, Billerica, MA, USA) and filtered through 0.2 μ m pore nylon filters before use (GE Water and Process Technologies, Trevose, PA, USA).

2.2.2 Buffer additive optimization—Phosphate buffer stock (pH 6.0) was prepared at 100 mM and diluted daily to 50 mM with DI water and selected buffer additives to prepare the background electrolyte (BGE). β -cyclodextrin stock was prepared at 10 mM in 50 mM pH 6.0 phosphate buffer and diluted with buffer to 1, 5, and 10 mM. SBE stock was prepared at 100 mM in water and was added to BGE at 1-12 mM (in increments of 1 mM). The organic solvents ACN and MeOH were added to the BGE at concentrations 1-20% v/v (in increments of 5%), while DMSO was evaluated at 1 and 5% v/v. Any pH change due to the addition of organic solvents was not corrected for (ie. 10% MeOH can increase the pH up to 0.2 units). All measurements of the EOF were done with a 0.3% v/v solution of MO into BGE.

2.3 Standard preparation

For all BGE optimization and spiking studies, standard solutions of OT and the desamino peptides were made daily by dissolving a measured amount of the lyophilized peptide into 50 mM phosphate buffer, pH 2.0. 50 mM phosphate buffer was prepared at pH 2.0 by

combining the appropriate amounts of *o*-phosphoric acid (15 M) and monobasic sodium phosphate.

2.4 Equipment conditions

Separations were performed on a Beckman Coulter P/ACE MDQ (Brea, CA, USA) with UV detection (214 nm). The system was controlled using 32-Karat software. Subsequent data analysis was performed using this software and Origin 8.6 (Northampton, MA, USA). A 50 cm fused silica capillary (50 mm id × 360 mm od) from Polymicro Technologies (Phoenix, AZ, USA) was used for the separation, and a small window was burned into the polyimide coating 10 cm from the capillary end for detection. The capillary was conditioned by rinsing for 5 min with 0.1 M HCl, water, methanol, water, 0.1 N NaOH, water, and for 10 min with BGE daily just prior to use. To expedite equilibration of the buffer within the capillary a 20 kV potential was applied across the capillary for 25 min following the conditioning. To maintain separation efficiency, the capillary was rinsed for 3 min with 0.1 M HCl, water, 0.1 N NaOH, water, and BGE between every run. Pressure injections were performed at 1 psi for 5.0 s. A separation voltage of 20–30 kV was applied.

2.5 Acid-catalyzed deamidation of oxytocin

Lyophilized OT acetate was dissolved at 0.1 mg/mL in 50 mM phosphate buffer (pH 2.0) and sealed in borosilicate glass vials with TFE-lined caps (Fisher Scientific, Fairlawn, NJ, USA). To track the degradation, the sealed vials were placed in a water bath at 70°C, and aliquots were removed at regular intervals. The extent of degradation was monitored by measuring the OT peak height using CE-UV. The pH of the blank (50 mM phosphate buffer, pH 2.0) was monitored at the start and end of every experiment and was found to fluctuate within ±0.2 pH units.

3 Results and discussion

3.1 Separation optimization

3.1.1 Background electrolyte selection—Initially, phosphate buffer was investigated over a pH range of 4–9 as a potential background electrolyte (BGE) for the analysis of OT and its desamino degradation products. To evaluate the usefulness of phosphate as a BGE, migration time reproducibility, separation current, and separation efficiency were determined for triplicate runs of the OT standard. At the low pH (4–5) the EOF was significantly reduced causing increased migration times and poor separation efficiency. The reproducibility increased with the pH from 6–7 with minimal improvements past pH 7, while the efficiency of the separation improved continually from pH 6–9. However, the separation current also increased significantly over this pH range.

To avoid high currents which can lead to band broadening HEPES, a low conductivity buffer, was also tested. However, this BGE resulted in large system peaks, possible due to difference in conductivity between the BGE and the sample. For this reason HEPES was excluded from further buffer studies. For the best separation efficiency while maintaining a relatively low current it was concluded that 50 mM phosphate buffer at pH 6.0 provided the and was implemented for further optimization.

With 50 mM phosphate buffer at pH 6.0 as the BGE, the initial separation of OT and the seven desamino-OT standards gave four distinct peaks. From spiking studies, the peaks were identified as OT, the three mono-desamino OT species, the three bis-desamino OT species, and finally the tris-desamino OT species. Given the similar size of the analytes, it is not surprising that species migrated based on their overall charge (+1, 0, -1, and -2, respectively). Additional optimization of buffer additives was performed to increase the resolution of these species.

3.1.2 Cyclodextrin additives—Under the free zone electrophoresis conditions mentioned above, it was not possible to resolve the multiple mono- and bi-desamino species from one another due to their identical size-to-charge ratios. In order to improve the separation, cyclodextrins were investigated due to their ability to act as a pseudo-stationary phase. The cyclodextrins form inclusion complexes with hydrophobic peptide residues, such as the Tyr² residue of OT, thereby increasing the apparent size of the peptide and changing its mobility. However, the addition of neutral β -cyclodextrin to the BGE yielded no noticeable improvement in resolution because, based on hydrophobicity alone, the selectivity for peptide inclusion would be identical for each species.

To introduce more selectivity for the separation, sulfobutyl ether-modified β -cyclodextrin (SBE) was evaluated. When the peptide associates with the SBE, both its size and overall negative charge increase, significantly reducing the electrophoretic mobility. Positively charged OT has the highest affinity for the SBE and, as the concentration of SBE in the BGE was increased, the migration time of OT increased. This is illustrated in the plot of the migration time of the analytes, relative to the EOF maker MO, versus SBE concentration shown in Figure 2a.

The mono-desamino species have a weaker interaction with the SBE than OT does. The additional negative charge from the deamidated side group creates an electrostatic repulsion with the negatively charged cyclodextrin, lowering the binding constant. However, a significant improvement in selectivity was introduced by the addition of the SBE. Figure 2 shows that by increasing the concentration of SBE from 0 to 11 mM it was possible to completely separate all three mono-desamino species (peaks 1, 2, and 6). This is extremely useful since they are neutral species at this pH and, without the negatively charged SBE, they would be impossible to separate with only free zone electrophoresis. The enhanced selectivity most likely comes from the relative position of the mono-desamino site to the Tyr² residue.

As OT deamidation progresses, increasing the peptide's overall negative charge, a less dramatic shift in relative migration time was observed with the bis-desamino species due to decreased interaction with the SBE. At 11 mM SBE, Gln⁴Asn⁵ (peak 3) is resolved from Asn⁵Gly⁹ and Gln⁴Gly⁹ which are still co-migrating (peak 4/5). However, when all the standards were run together, Gln⁴Asn⁵ was found to co-migrate with the mono-desamino Gln⁴ (peak 2/3) as shown in Figure 2b.

At all concentrations of SBE, Gln⁴Asn⁵Gly⁹ migrates last (peak 8), showing a small difference in migration time with respect to the EOF. This indicates a minimal interaction with the SBE due to electrostatic repulsion from the three deamidated sites.

3.1.3 Organic modifiers—To further resolve the co-migrating species (Gln⁴/Gln⁴Asn⁵ and Asn⁵Gly⁹/Gln⁴Gly⁹), the organic solvents DMSO, ACN, and MeOH were evaluated as modifiers to the BGE. The organic solvent in the BGE alters the hydrophobic affinity of the cyclodextrin for the peptide, changing the binding constant and therefore affecting the relative electrophoretic mobilities. Neither DMSO nor ACN exhibited an appreciable benefit to the separation at any concentration. ACN increased the EOF, which in turn increased the separation efficiency; however, the resolution of all species was lost as a result. DMSO at 1% v/v did not change the separation in any way; yet increasing the concentration to 5% v/v resulted in an electropherogram with zero peaks, probably due to a significant decrease in the EOF. MeOH not only changed the polarity of the BGE, it also slightly decreased the EOF, increasing the resolution. With 10% v/v MeOH, it was possible to achieve a complete separation of all seven desamino species from OT. Figure 3a demonstrates the change in migration time (relative to the EOF) versus the concentration of MeOH.

3.1.4 Separation Voltage—The effect of voltage on the separation efficiency was investigated by increasing the separation voltage from 20–30 kV, in increments of 2 kV, and calculating the theoretical plates and resolution of the peptides in the resulting electropherograms. Based on these experiments, 22 kV was chosen for the greatest separation efficiency without loss of resolution due to Joule heating. An electropherogram demonstrating the fully optimized separation of all eight species is shown in Figure 3b.

3.2 Separation parameters

The linear range of this assay is 2.5–100 μ M for all analytes, except Gln⁴, which exhibited a linear range of 5–100 μ M. For all peptide calibration curves the R^2 value was greater than 0.9995 ($n = 3$ for five concentrations). The LOD ($S/N = 3$) and LOQ ($S/N = 10$) are provided in Table 1. It should be noted that a common concentration for OT in maternal health therapeutics is 10 IU/mL (approximately 20 μ M). While this concentration falls well within the linear range of the assay, the LOQ for OT on the current system is not ideal for research or quality control settings. However, it would be possible to improve the low sensitivity with improved CE-UV technology such as a bubble cell or Z-shaped capillaries [18].

Migration time can be a helpful tool for peak identification. However, CE is notorious for irreproducible migration times due to changes in the EOF over time and slight differences in BGE additive concentration. To improve the %RSD for the migration times of all species, the desamino peak migration times were taken relative to the OT peak. Table 1 provides the %RSD values for the migration time and relative migration times of the deamidation peaks.

3.3 Tracking degradation in heat-stressed oxytocin samples

The heat-stressed degradation of OT was monitored using the optimized separation described above. OT was prepared at 0.1 mg/mL in acidic conditions (pH 2.0) to ensure that

the primary degradation pathway was acid-catalyzed deamidation. Three samples were degraded concurrently at 70°C over the course of 5 days, sampling every 12 hours.

The degradation of OT was determined by monitoring the peak heights of all species as a function of time. Using the method described in this paper, it was possible to monitor the appearance and disappearance of OT and all seven desamino species over the five-day period. In Figure 4a, electropherograms from five different time points (time points 10, 34, and 58 h excluded for clarity) are stacked to illustrate the extent of degradation over time. $t = 0$ h shows one peak in the electropherogram corresponding to OT. All subsequent peaks of the degradation products were identified by migration time relative to OT and by spiking studies.

At $t = 24$ h, the OT peak height was diminished slightly, giving rise to the three mono-desamino species. The concentration of both Gln⁴ and Gly⁹ (peaks 2 and 4) increased over time, cresting at the 72 h time point, seen more clearly in Figure 4b. However, the height of the Asn⁵ peak (peak 1) stayed at a fairly constant low concentration from 10-72 h. This could be indicative of the change in relative reactivity between the deamidation sites due to the secondary structure of the peptide. Structural studies of OT have shown hydrogen bond formation between the hydroxyl group of the Tyr² and the carboxamide of Asn⁵, which would decrease the likelihood of deamidation at this site [19].

As the sample continued to degrade at 70°C, the mono-desamino species gave rise to the bis-desamino species. Due to the low reactivity of Asn⁵, both Gln⁴Asn⁵ and Asn⁵Gly⁹ (peaks 3 and 5) were first seen at 34 h and remained in low concentrations over the course of the study. The more reactive sites of Gln⁴ and Gly⁹ led to a steady increase in the presence of Gln⁴Gly⁹ (peak 6) from 24-96 h.

The Gln⁴Asn⁵Gly⁹ species was first observed as a small peak in the $t = 48$ h time point, and its peak height steadily increased until the end of the study. Notably, using the LC-MS method, the appearance of Gln⁴Asn⁵Gly⁹, Asn⁵, and Gln⁴Asn⁵ was either not observed or not at high enough concentrations to be detected by the method [9].

From this degradation study, the kinetics for the disappearance of OT ($n = 3$) were calculated to be pseudo-first order with a k_{obs} of 0.465 days⁻¹ (3.4% RSD) and a $t_{1/2}$ of 1.5 days (3.9% RSD). This half-life is similar to that reported by Hawe et al., 1.1 days with a less than 5% RSD[9]. At each time point, all of the peaks were accounted for by mass balance ($104\% \pm 5$), by adding up the peak heights and comparing them to the initial OT peak. This indicates no sample loss by adsorption on the capillary wall or by precipitation in the sample vial during degradation.

4 Concluding remarks

A CE-UV method to separate the desamino degradation products of OT has been described. Deamidation creates seven desamino-OT species with structures very similar in size and charge, making resolution with free zone electrophoresis a challenge. To resolve the many desamino degradation species with identical size-to-charge ratios, it was necessary to include the pseudo-stationary phase SBE as well as the organic modifier MeOH in the BGE.

Using this approach, the heat-stressed degradation of OT in acidic buffer was monitored over time. All seven desamino degradation products were detected over a 96 h heat-stress period at 70°C in acidic conditions.

For this study, formulation in a pH 2.0 buffer was chosen to force deamidation to be the major degradation pathway of OT. However, as reported by both Hawe et al. and Hasselberg et al., OT degradation is pH dependent and deamidation is not the major pathway at higher pHs [9, 17]. At the pharmaceutically relevant pH 4.5, both papers report minimal deamidation, with the formation of both dimers and tri- and tetrasulfide as the primary degradation species.

Preliminary results from analysis of heat-stressed OT at pH 4.5 with the assay described in this paper, corroborate the previous reports showing little deamidation after 96 h under 70°C heat-stress conditions. Additional work is ongoing to modify the current assay and create a comprehensive CE-UV method for the determination of all degradation species of OT.

Having a low-cost and fast CE-UV method for OT analysis will enhance the field of study by allowing more labs to participate in research without having to invest in expensive LC-MS instrumentation. Additionally, CE has the potential for miniaturization through method transfer to microchip electrophoresis (ME), reducing the cost-per-test and analysis time even further. ME allows portability, which increases the range of the assay to places like developing countries, where traditional laboratory equipment is not readily available.

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List of non-standard abbreviations

OT	oxytocin
Gln⁴	mono-desamino Gln ⁴
Asn⁵	mono-desamino Asn ⁵
Gly⁹	mono-desamino Gly ⁹
Gln⁴Asn⁵	bis-desamino Gln ⁴ Asn ⁵
Gln⁴Gly⁹	bis-desamino Gln ⁴ Gly ⁹
Asn⁵Gly⁹	bis-desamino Asn ⁵ Gly ⁹
Gln⁴Asn⁵Gly⁹	tris-desamio Gln ⁴ Asn ⁵ Gly ⁹
SBE	sulfobutyl ether β -cyclodextrin
MO	mesityl oxide

Figure 1.
(A) Progression of desamino-OT species starting with the individual mono-desamino products and ending with the tris-desamino peptide. (B) Amino acid sequences of oxytocin and seven desamino-oxytocin species with specific sites of deamidation underlined.

Figure 2.

(A) Effect of SBE concentration on relative migration time. BGE: 50 mM phosphate buffer, pH 6.0 with varying concentrations of SBE. Migration times are based on the average of triplicate runs, relative to the neutral marker MO. (B) Separation of peptides with optimized SBE. BGE: 50 mM phosphate buffer, pH 6.0 and 11 mM SBE. Peptides are 0.05 mg/mL in 50 mM phosphate buffer, pH 2.0. Peaks: 1 Asn⁵; 2 Gln⁴; 3 Gln⁴Asn⁵; 4 Asn⁵Gly⁹; 5 Gln⁴Gly⁹; 6 Gly⁹; 7 OT; 8 Gln⁴Asn⁵Gly⁹.

Figure 3.

(A) Effect of MeOH concentration on relative migration time. BGE: 50 mM phosphate buffer, pH 6.0, 11 mM SBE with varying concentrations of MeOH. Migration times are based on the average of triplicate runs, relative to the neutral marker MO. (B) Optimized separation of peptides. BGE: 50 mM phosphate buffer, pH 6.0, 11 mM SBE, 10% v/v MeOH. Peptides are 0.05 mg/mL in 50 mM phosphate buffer, pH 2.0. Peaks: 1 Asn⁵; 2 Gln⁴; 3 Gln⁴Asn⁵; 4 Gly⁹; 5 Asn⁵Gly⁹; 6 Gln⁴Gly⁹; 7 OT; 8 Gln⁴Asn⁵Gly⁹.

Figure 4.

(A) Degradation time points of heat-stressed oxytocin. BGE: 50 mM phosphate buffer, pH 6.0, 11 mM SBE, 10% v/v MeOH. Peaks: 1 Asn⁵(■); 2 Gln⁴(▲); 3 Gln⁴Asn⁵(□); 4 Gly⁹(●); 5 Asn⁵Gly⁹(○); 6 Gln⁴Gly⁹(); 7 OT; 8 Gln⁴Asn⁵Gly⁹(x). (B) Formation of desamino-OT products over time at 70°C. Initial OT concentration was 0.1 mg/mL in 50 mM phosphate buffer, pH 2.0.

Table 1
Separation parameters of OT and the desamino-OT standards (*n* = 9)

Peptide	LOD (μM)	S/N = 3	LOQ (μM)	S/N = 10	Theoretical plates	(plates/m)	Ave. migration time min.	(%RSD)	Ave. rel. migration time min.	(%RSD)
Asn ⁵	0.83			2.5	240 000		7.92	(2.5)	0.840	(0.46)
Gln ⁴	2.6			8.0	240 000		8.16	(2.5)	0.866	(0.38)
Gln ⁴ Asn ⁵	3.7			11.0	260 000		8.73	(2.5)	0.916	(0.14)
Gly ⁹	4.0			12.0	220 000		8.84	(2.7)	0.937	(0.20)
Asn ⁵ Gly ⁹	8.6			26.0	190 000		9.11	(2.6)	0.955	(0.10)
Gln ⁴ Gly ⁹	2.0			5.9	240 000		9.23	(2.6)	0.972	(0.20)
OT	3.0			8.9	150 000		9.35	(3.6)	1.000	
Gln ⁴ Asn ⁵ Gly ⁹	1.5			4.4	230 000		11.42	(3.9)	1.200	(0.95)