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Liquid chromatographic analysis of a potential polymeric-pendant drug delivery system for peptides

Application of high-performance size-exclusion chromatography, reversed-phase high-performance liquid chromatography and ion chromatography to the evaluation of biodegradable poly[(chloromethoxytrialanine methyl ester) phosphazenes]

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ABSTRACT

A novel water-soluble polymer, poly[(chloromethoxytrialanine methyl ester)phosphazene] (poly-Tame), was characterized and evaluated using high-performance size-exclusion chromatography, gradient reversed-phase high-performance liquid chromatography and ion chromatography. These novel liquid chromatographic methods were validated for application to *in vitro* biodegradation experiments of poly-Tame in aqueous solutions. Results from method validation experiments are presented.

INTRODUCTION

Water-soluble macromolecular conjugates are becoming increasingly important as drug delivery platforms for therapeutic agents [1,2]. The structural complexity and diversity of these polymeric molecules requires a sophisticated array of analytical methodologies to evaluate their performance as drug delivery systems. Liquid chromatographic methods have been used by Larsen *et al.* [3] to measure the disappearance of dextran-metronidazole ester conjugates and the release of metronidazole from the hydrolyzed polymeric pro-drug *in vitro*. Kurtzhals *et al.* [4] applied high-performance size-exclusion chromatography, (HPSEC) to *in vitro* and *in vivo* evaluations of dextran–fluoresceinyl isothiocyanate (FITC) conjugates.

Similar liquid chromatographic methods are needed for polyphosphazenes, a class of inorganic polymers proposed as macromolecular conjugates for drug delivery

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of a wide variety of covalently attached and biologically active side groups [5]. Grollemand and co-workers [6,7] measured the *in vitro* and *in vivo* release of naproxen with a lysine spacer conjugated to an implantable poly(glycine ethyl ester)phosphazene device using reversed-phase high-performance liquid chromatography (RP-HPLC) and determined the molecular weight (MW) of the polymers using gel permeation chromatography (GPC). Goedemoed and De Groot [8] used RP-HPLC with pre-column amino acid derivatization to measure the release of amino acid from a bioerodible poly(amino acid ester) phosphazene device and monitored the diffusion of melphalan from the polymer matrix using RP-HPLC.

The biodegradation of the poly[(peptide ester)phosphazene] (Fig. 1), will ultimately lead to the formation of ammonia, phosphate, alanine, methanol and hydrochloric acid. However, the release of free tripeptide, trialanine methyl ester (Tame) or trialanine (Tala), used here as model peptides, could occur during intermediate degradation processes under physiological conditions. Furthermore, the degradation mechanism for poly[(amino and amino acid ester)phosphazenes] have been postulated based on spectroscopic and qualitative chemical analyses of hydrolyzed cyclic and polymeric phosphazenes in aqueous solutions [9,10]. However, these techniques are incapable of measuring changes in MW and are not sensitive or selective enough to measure pendant release at low concentrations. The interdependent kinetics of side chain release with polymeric degradation is a relationship best determined using HPSEC and RP-HPLC.

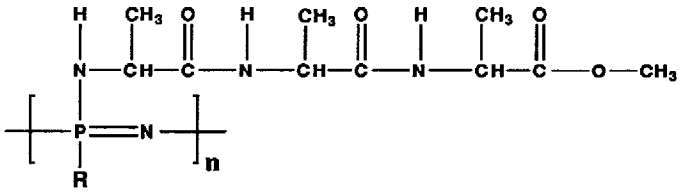

Fig. 1. Structure of polyTame. R = Tame, OCH_3 or Cl.

Evaluating the hydrolytic stability and determining the degradation mechanism for polyTame in aqueous solutions is predicated on the development of validated analytical procedures for degradation intermediates. These methods should identify and quantitate polyTame and its potential hydrolysis products and probably would utilize both spectroscopic and chromatographic techniques. The components necessary for quantitation and characterization of this polymer with liquid chromatographic methods include MW calculations of number-average MW ($\bar{M}_n$), weight-average MW ($\bar{M}_w$), MW distribution (MWD) and polyTame concentrations using HPSEC. The degree of substitution of Tame and release of both Tame and Tala from the polyphosaphazene backbone will be characterized using RP-HPLC. The amount of unreacted chloride and the amount bound to the polymeric backbone as the hydrochloride salt will be determined by inorganic anion analysis using ion chromatography (IC). Monitoring the formation of phosphate and release of unreacted chloride during polyTame hydrolysis will be achieved using the same IC method.

EXPERIMENTAL

Materials

The HPLC grade reagents used were methanol, acetonitrile and ammonium acetate from J.T. Baker (Phillipsburg, NJ, U.S.A.), trifluoroacetic acid (TFA) and triethylamine (TEA) from Pierce (Rockford, IL, U.S.A.) and octanesulfonic acid sodium salt from Kodak (Rochester, NY, U.S.A.). HPLC-grade water was obtained from a Milli-Q Plus water system (Millipore, Milford, MA, U.S.A.).

The narrow molecular weight standards used for external calibrations of the HPSEC were poly(ethylene glycol)(PEG) (Polymer Lab., U.K.), poly(ethylene oxide) (PEO) (Polymer Labs.) and poly(2-vinlypyridine) (PVP) (Pressure Chemical, Pittsburgh, PA, U.S.A.).

Ala–Ala–Ala–CH_3 (Tame), Ala–Ala–Ala (Tala) and other peptides and amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). Gold label phthalic acid and 4-hydroxybenzoic acid were purchased from Aldrich (Milwaukee, WI, U.S.A.). Chloride and phosphate standards were prepared from a standardized 1.00 *M* hydrochloric acid volumetric solutions and reagent grade sodium phosphate dibasic, respectively (J.T. Baker).

The poly [(chloromethoxytrialanine methyl ester) phosphazene] polymer (poly-Tame) was synthesized in-house (Sterling Research Group, Malvern, PA, U.S.A.) as described elsewhere [11].

Instrumentation and chromatographic conditions

The chromatographic system included a Waters 840 multisystem controller (Waters Chromatography Division/Millipore, Milford, MA, U.S.A.) for control of three independent liquid chromatographs (version 6.21A software). Raw data were automatically transferred to a Waters 860 software system (version 2.1) with LC/GPC software capability located on a VAX 8250 (Digital Equipment, Maynard, U.S.A.) for subsequent analyses and reduction.

The HPSEC system consisted of a Waters 712 autosampler with cooling unit maintained at 20°C, a Waters Model 510 pump with pulse dampener and high sensitivity noise filter and a Waters 490E UV–VIS detector set a 225 nm connected in series with a Waters 410 differential refractometer (sensitivity = 128, internal temperature = 40°C). Chromatographic size exclusion was achieved by employing a mobile phase of 0.1 *M* ammonium acetate in methanol at a flow-rate of 2.0 ml/min through Zorbax GF-250 and GF-450 (25 cm × 9.4 mm I.D., 5μm) HPSEC columns (DuPont, Wilmington, DE, U.S.A.) maintained at 50°C in a Waters column oven. Integration of size-exclusion chromatograms was determined by peak slicing at 20 slices/min from the beginning of the peak to the end of the peak with baselines projected horizontally from a stable baseline point before the exclusion limit (usually at 6 min) to a stable baseline point after the void volume (usually at 17 min).

The RP-HPLC gradient system consisted of a Waters 712 autosampler with cooling unit maintained at 20°C, two Waters 510 pumps and a Spectroflow 783 UV–VIS detector (Applied Biosystems, Ramsey, NJ, U.S.A.) with the wavelength set at 210 nm. The analytical column was a Zorbax C_8 reliance cartridge (4 cm × 6 mm I.D., 3 μm) with Zorbax C_8 guard cartridge (1 cm × 3.2 mm I.D., 5 μm) (DuPont). A linear gradient profile of 100 to 40% A over 8 min at a flow-rate of 2.0 ml/min

through was used with a 3-min re-equilibration period between injections. The mobile phases employed for gradient reversed-phase separation of Tame and potential hydrolysis products were: (A) 5.0 m*M* octanesulfonic acid sodium salt and 0.05% (v/v) TFA in water, and (B) 0.05% (v/v) TFA in acetonitrile–water (60:40, v/v).

THe IC system consisted of a Waters 712 autosampler with cooling unit maintained at 20°C, a Waters Model 510 pump and a Waters 484 UV–VIS detector set at 270 nm connected in series with a Waters 430 conductivity detector. The mobile phase was prepared by dissolving 1.0 mmol of phthalic acid in 50 ml of methanol and adding 950 ml of water and 300 μl of TEA. The pH of this solution was adjusted to pH 6.3–6.5 with 50 m*M* 4-hydroxybenzoic acid dissolved in methanol. The mobile phase was prepared fresh daily. Separation and analysis of free chloride and phosphate from hydrolyzed polyphosphazene polymers was achieved with a flow-rate of 3.0 ml/min through a Vydac 300 IC anion exchange column (5 cm × 4.6 mm I.D., 5 μm) with Vydac 300 IC guard cartridge (2.5 cm × 4.6 mm I.D., 5 μm), (The Separations Group, Hesperia, CA, U.S.A.).

RESULTS AND DISCUSSION

Development and validation of HPSEC method

PolyTame is a cationic polymer capable of strong ionic, adsorptive and hydrophobic interactions with chromatographic supports. Thus the development of a rugged and precise HPSEC method requires that all interactions between the polymer and stationary phase are minimized so that MW separation is based on pore diffusion only. Attempts to chromatograph polyTame using standard aqueous buffered mobile phases and silica columns were unsuccessful. In addition, polyTame could not be chromatographed with strong organic mobile phases and standard resin-based GPC columns. In both cases, polyTame failed to elute from the columns presumably due to

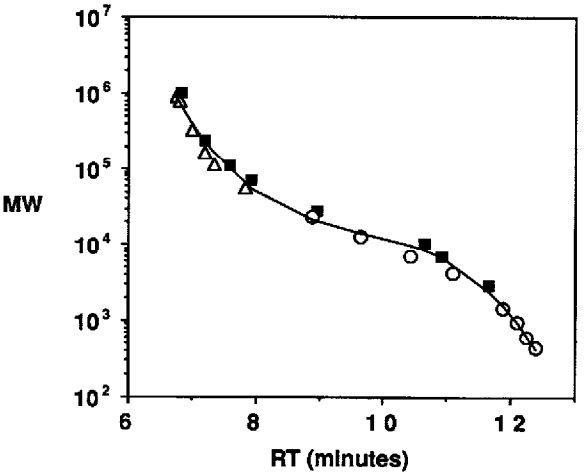

Fig. 2. HPSEC narrow standard calibration curve for MW determination of polyTame. △ = PEO standards; ■ = PVP standards; ○ = PEG standards. Curve fit to third order polynominal: $MW = 50.76 \cdot RT^3 - 14.08 \cdot RT^2 + 1.44 \cdot RT - 0.05$ ($R^2 = 0.992$); RT = Retention time (min). Conditions are described in the Experimental section.

strong interactions with the stationary phase. These interactions were minimized when a mobile phase of methanol, an excellent solvent for polyTame, and a hydrophilic diol stationary phase on a silica support were used. Because of the polymer's cationic nature and the unknown stability of the 'protected' diol stationary phase, ammonium acetate was added in the mobile phase to suppress potential ionic interactions with any free silanol groups.

The HPSEC method was calibrated using narrow MW neutral and cationic polymers that were methanol soluble. The PEO, PEG and PVP standards from 440 to $1 \cdot 10^6$ dalton were dissolved in methanol at about 1 mg/ml and 25μl injected into the HPSEC. The results illustrated in Fig. 2 indicate a good correlation between retention time and MW for these three classes of polymers over the MW range. The calibration was fit to a third order polynomial ($R^2 = 0.992$) with a mean residual error of 17% between calculated and actual MWs. Although values for PVP appear to deviate slightly from PEO and PEG, this is not surprising considering the difference in chemical structure. PVP is a linear, short branched, cationic polymer with physico-chemical characteristics more similar to polyTame than the other linear, neutral polymers, PEO and PEG. Therefore, in the absence of a 'universal' calibration, it is believed that the use of all three sets of standards represents the best 'average' external calibration for polyTame. Absolute MW determinations using this procedure can only be considered to be approximate. However, HPSEC time course evaluations measuring polymeric degradation of polyTame will not be significantly affected by the choice of external calibration since only differences in MW are important.

Separation of standards over the entire MW range demonstrates the high efficiency of this system (Fig. 3). The reproducibility of the method was determined by

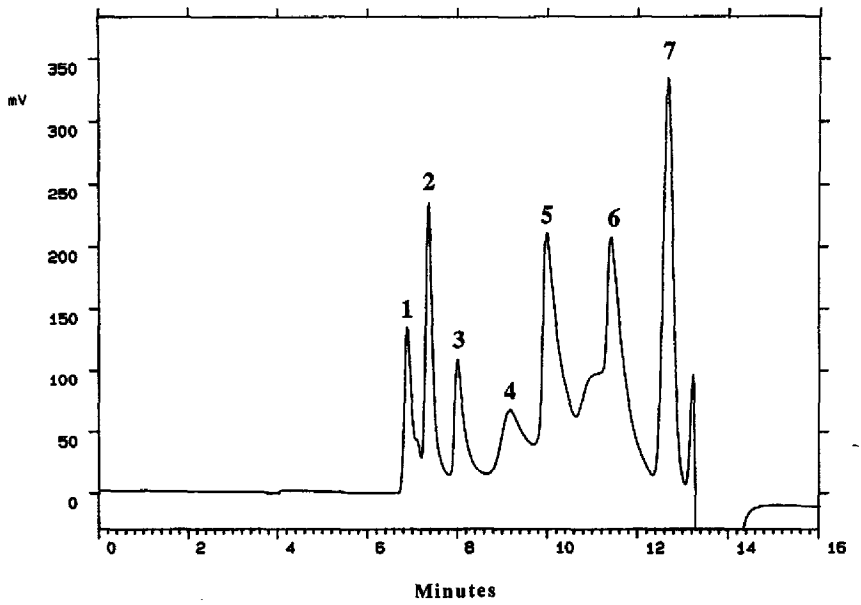

Fig. 3. HPSEC separation of narrow MW standards in methanol solution, 25 μl per injection. Peaks: 1 = PEO, MW = 900 000, 0.75 mg/ml; 2 = PVP, MW = 240 000, 0.75 mg/ml; 3 = PEO, MW = 56 300, 1.25 mg/ml; 4 = PVP, MW = 28 000, 0.5 mg/ml; 5 = PEG, MW = 12 600, 3.0 mg/ml; 6 = PEG, MW = 4250, 1.7 mg/ml; 7 = PEG, MW = 440, 1.7 mg/ml. Conditions are described in the Experimental section.

analyzing MW, peak area and peak height following repetitive injections of a polyTame-in-methanol solution. The precision of the method was better than 5% coefficient of variation (C.V.) ($n = 15$) over 3 days (Table I) for all variables monitored. The application of this method for studying the hydrolysis of polyTame is illustrated in Fig. 4. In a 0.8 *M* hydrochloric acid solution, polyTame undergoes rapid MW decline, a significant change in the MWD and a high disappearance of polymer over a 14-h period at 20°C.

TABLE I

HPSEC PRECISION OF POLYTAME ANALYSES

Analyses of 100 μg/ml polyTame in methanol, 25 μl per injection. Conditions as described in the Experimental section. $\bar{M}_p$ is peak MW.

Time (days)	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_p$	Peak height	Peak area
0.02	33 416	58 017	120 867	23 592	1 967 650
0.11	32 632	58 362	120 867	23 210	1 817 827
0.21	32 795	57 297	120 867	23 336	1 879 397
0.30	31 498	55 414	109 668	23 574	1 957 417
0.40	33 238	59 075	120 867	23 758	1 938 014
0.49	32 806	55 988	120 867	23 369	1 947 826
0.59	32 580	56 578	120 867	23 699	1 927 924
0.69	32 505	55 937	109 668	23 670	1 915 044
0.78	31 644	56 329	109 688	23 247	2 050 452
1.44	30 723	55 173	120 867	23 210	1 897 355
1.68	33 092	58 812	120 867	23 198	1 900 700
1.76	32 940	58 075	120 867	24 022	2 122 675
1.84	29 968	52 834	109 668	23 096	1 997 348
2.96	32 105	55 318	109 668	22 295	1 853 547
3.04	33 792	57 810	109 668	24 093	2 041 558
Mean ($n = 15$)	32 382	56 735	116 388	23 425	1 947 649
C.V. (%)	3.2	3.0	4.9	1.9	4.1

Development and validation of RP-HPLC method

Traditional methods for the separation of peptides by RP-HPLC based on a gradient of TFA in water and acetonitrile lacked the selectivity necessary for the separation of Tame from its major hydrolysis product, Tala. However, when a more hydrophobic ion-pairing agent such as octanesulfonic acid was incorporated into the aqueous mobile phase, the selectivity was improved such that Tame and Tala were easily resolved. In addition, the use of short 4 cm, 3-μm particle size C_8 columns at relatively high flow-rates greatly reduced the run time without compromising the efficiency compared to standard dimension columns. This approach has also been applied to the rapid separation of much higher MW vasopressin peptides from intestinal metabolites [12].

Separation of Tame from its major hydrolysis product, Tala, and other low-MW peptides and amino acids illustrated in Fig. 5 demonstrates the selectivity of this

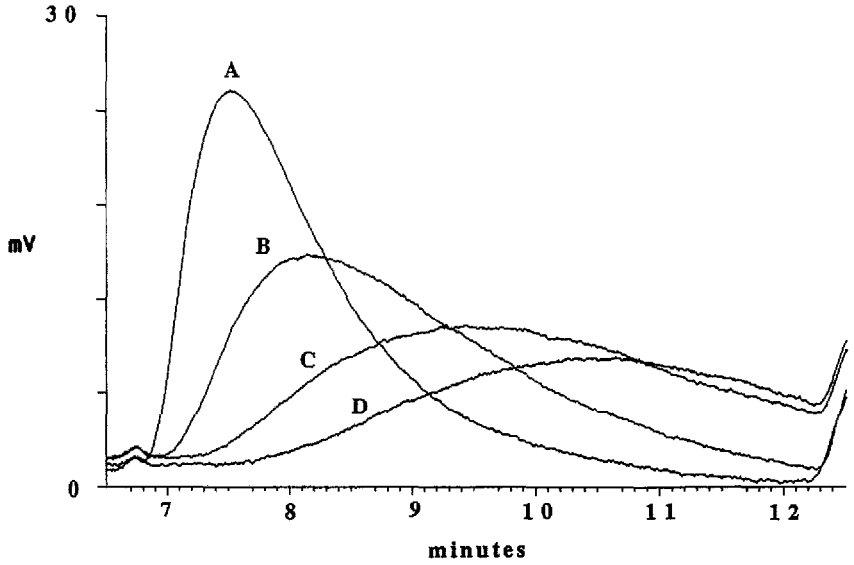

Fig. 4. HPSEC chromatograms of a polyTame sample in a 0.8-*M* hydrochloric acid solution at 20°C: (A) after 0.5 h, $(\bar{M}_n\bar{M}_w)^{1/2}$ = 39 542; (B) after 7.4 h, $(\bar{M}_n\bar{M}_w)^{1/2}$ = 25 626; (C) after 12.0 h, $(\bar{M}_n\bar{M}_w)^{1/2}$ = 18 818; (D) after 14.3 h, $(\bar{M}_n\bar{M}_w)^{1/2}$ = 14 137. The small peaks before the exclusion limit at a retention time of 6.85 min are 'unknown system peaks' and have not been included in MW calculations. Conditions are described in the Experimental section.

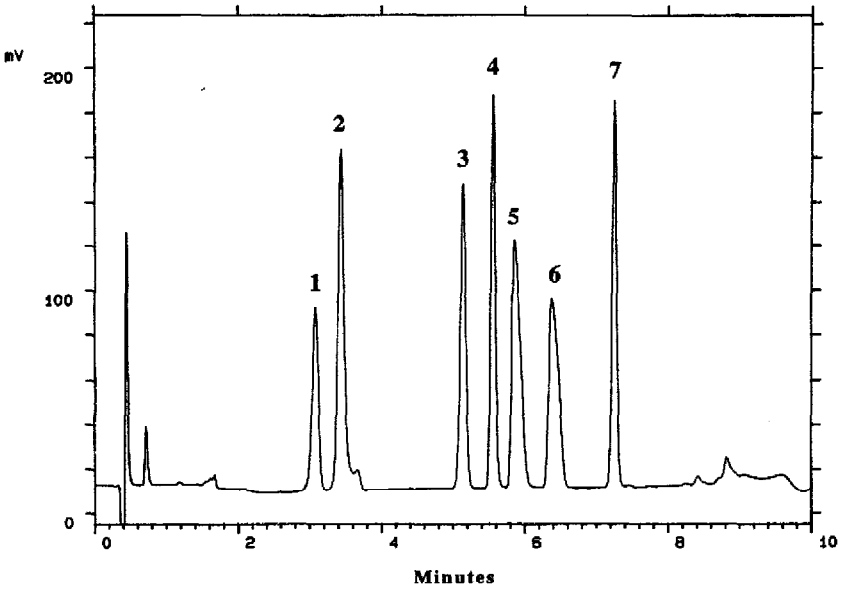

Fig. 5. RP-HPLC separation of Ala–Ala–Ala–CH_3 (Tame) from its hydrolysis product Ala–Ala–Ala (Tala) and similar dipeptides and amino acids. Peaks: 1 = Gly–Gly–NH_2, 1.5 m*M*; 2 = Gly–Gly, 3.7 m*M*; 3 = Ala–Ala, 2.0 m*M*; 4 = Tala, 1.0 m*M*, capacity factor (k') = 12.7; 5 = Ala–CH_3, 46 m*M*; 6 = Gly–CH_2–CH_3, 48 m*M*; 7 = Tame, 1.0 m*M*, k' = 17.0. The resolution between Tala and Tame is 11. Conditions are described in the Experimental section.

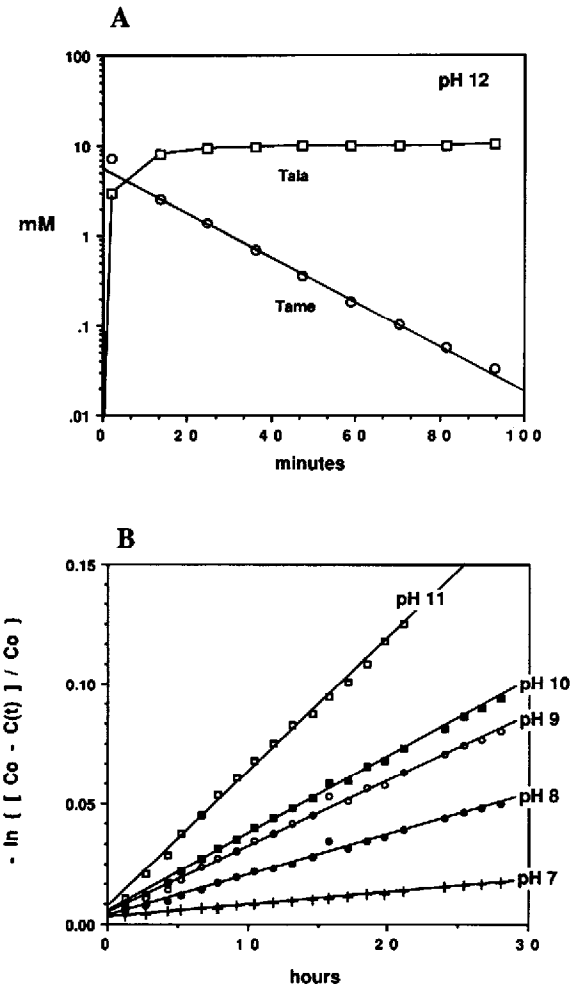

Fig. 6. Effect of pH on the hydrolysis of Tame to Tala at 20°C. Solutions were prepared in 0.05 M K_2PO_4 and adjusted to the appropriate pH with concentrated hydrochloric acid or sodium hydroxide. The initial concentration (Co) is 10 mM Tame. (A) Disappearance of Tame (○) and appearance of Tala (□) at pH 12, first order rate constant, $k = 3.485\ h^{-1}$, $t_{1/2} = 0.20$ h. (B) First order initial rate plot of Tame disappearance based on appearance of Tala, C(t); pH 11, $k = 5.59 \cdot 10^{-3}\ h^{-1}$, $t_{1/2} = 124$ h; pH 10, $k = 3.20 \cdot 10^{-3}\ h^{-1}$, $t_{1/2} = 216$ h; pH 9, $k = 2.76 \cdot 10^{-3}\ h^{-1}$, $t_{1/2} = 252$ h; pH 8, $k = 1.70 \cdot 10^{-3}\ h^{-1}$, $t_{1/2} = 408$ h; pH 7, $k = 5.06 \cdot 10^{-4}\ h^{-1}$, $t_{1/2} = 1370$ h.

method. Standard solutions of Tame and Tala were prepared in 0.05% (v/v) acetic acid–water and 20 μl injected for analyses. The detector response was linear ($R^2 = 0.999$) over the concentration range from 0.1 to 5.0 mM for both Tame and Tala based on peak area. The intra-day precision over this concentration range was 0.6% C.V. ($n = 4$). The inter-day reproducibility of the method was determined by repetitive analyses over 23 days ($n = 27$) of a 3.65-mM Tame solution in water during the course of hydrolysis experiments and was better than 1.0% C.V. The minimum quantifiable

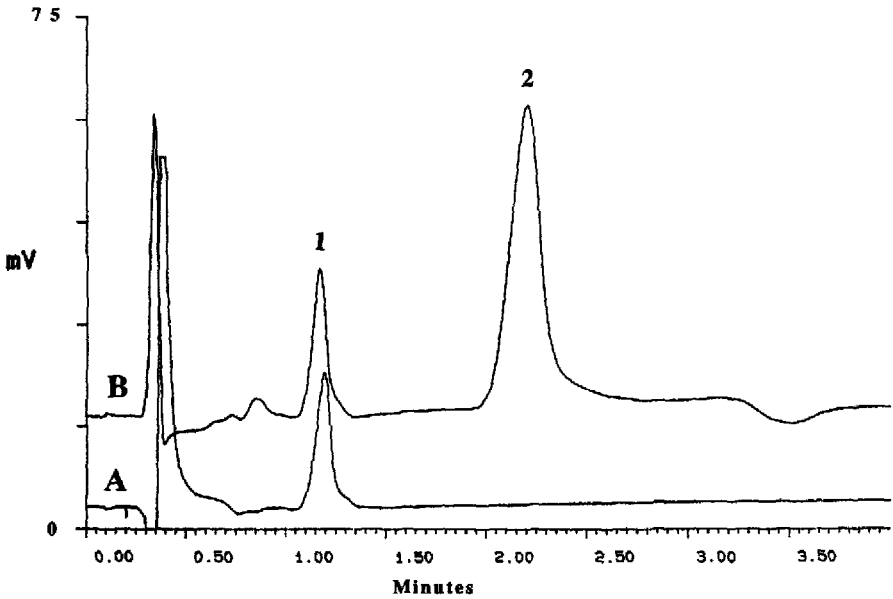

Fig. 7. IC chromatograms of hydrolyzed polyTame in water after (A) 3 days at 20°C, 1.49 m*M* chloride, capacity factor (k') = 2.9 and (B) 4 days at 160°C, 1.53 m*M* chloride, k' = 3.0, and 5.92 m*M* phosphate, k' = 6.4. Peaks: 1 = chloride; 2 = phosphate. The resolution between chloride and phosphate is 4.2. Conditions are described in the Experimental section.

concentration is 0.01 m*M* with a signal-to-noise ratio of 3:1. This method was applied to the base hydrolysis of Tame in aqueous solutions. The results shown in Fig. 6 indicate that Tame hydrolysis occurs mainly at the C-terminal ester, follows first order disappearance kinetics and is strongly pH dependent. Application of this method for the determination of Tame/Tala N-terminal hydrolysis from the polyTame backbone is presented elsewhere [11].

Development and validation of IC method

The IC method was a modification of the procedure supplied by the column manufacturer for inorganic anions. The chromatographic conditions were optimized for the separation of chloride and phosphate with indirect UV detection. Electronically suppressed conductivity detection could not be used because of a poor signal-to-noise ratio for phosphate.

The method was calibrated by injecting 20 μl of chloride and phosphate standard solutions prepared in water from 0.1 to 3 m*M* chloride and 0.05 to 2.5 m*M* phosphate. The indirect UV detector response is linear for chloride (R^2 = 0.999) and phosphate (R^2 = 0.998) based on peak area. The standards were injected in triplicate and the precision over the concentration range was better than 3% C.V. (n=12) for chloride and 6% C.V. (n=18) for phosphate. Because of the inter-day variability in column retention, (*e.g.* note change in retention for chloride in Fig. 7), the IC system was calibrated immediately prior to sample analysis. Minimum quantifiable concentrations based on a signal-to-noise ratio of 3:1 for chloride and phosphate are 0.01

and 0.025 m*M*, respectively. Sample chromatograms of polyTame samples hydrolyzed in water at 20 and 160°C illustrate the application of the IC method to *in vitro* degradation experiments (Fig. 7).

REFERENCES

1 J. Drobnik, *Adv. Drug Delivery Rev.*, 3 (1989) 229.
2 H. Sezaki, Y. Takakura and H. Hashida, *Adv. Drug Delivery Rev.*, 3 (1989) 247.
3 C. Larsen, P. Kurtzhals and M. Johansen, *Acta Pharm. Suec.*, 25 (1988) 1.
4 P. Kurtzhals, C. Larsen and M. Johansen, *J. Chromatogr.*, 491 (1989) 117.
5 H. R. Allcock, *ACS Symp. Ser.*, 232 (1983) 49–67.
6 C. W. J. Grolleman, A. C. de Visser, J. G. C. Wolke, H. van der Goot and H. Timmerman, *J. Controlled Release*, 4 (1986) 119.
7 C. W. J. Grolleman, A. C. de Visser, J. G. C. Wolke, C. P. A. T. Klein, H. van der Groot and H. Timmerman, *J. Controlled Release*, 4 (1986) 133.
8 J. H. Goedemoed and K. de Groot, *Makromol. Chem. Macromol. Symp.*, 19 (1988) 341.
9 H. R. Allcock, T. J. Fuller, D. P. Mack, K. Matsumura and K. M. Smeltz, *Macromolecules*, 10 (1977) 824.
10 H. R. Allcock, T. J. Fuller and K. Matsumura, *Inorg. Chem.*, 21 (1982) 515.
11 W. M. Eickhoff, G. G. Liversidge and R. Mutharasan, *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, Vol. 17, Controlled Release Society, 1990, p. 160.
12 W. M. Eickhoff, G. G. Liversidge and K. C. Cundy, *Pharm. Res.*, 6 (1989) S-114.