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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FERTIRELIN ACETATE AND RELATED COMPOUNDS

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SUMMARY

Separation of fertirelin acetate (FA) from process impurities, potential degradation products and related peptides including luteinizing hormone releasing hormone has been achieved by reversed-phase high-performance liquid chromatography (HPLC). A number of chromatographic conditions (column type, mobile phase composition, isocratic/gradient elution) and detection systems have been utilized to examine the bulk drug and formulation of FA. Examples of separations designed for potency and impurity determinations are described. Complete recovery of FA is obtained with an isocratic HPLC system. An external standard method is used to determine potency with a precision of <1% R.S.D. A gradient HPLC system is used to determine impurities with a precision of *ca.* 5–10% R.S..D. at the 1–2% impurity level. As little as *ca.* 0.1% (area%) of related peptides are detected at 214 nm.

INTRODUCTION

Fertirelin acetate [FA, also referred to as TAP-031 (Takeda), U-69,689E (Upjohn) and 38324-21- 8 (*Chemical Abstracts*)] is a synthetic analogue[1] of luteinizing hormone releasing hormone (LHRH)[2] (See Table I). It is prepared by either solution-phase[3] or solid-phase methods[4] and it is formulated as a sterile solution[5] for use in the treatment of ovarian cysts in dairy cows[6–8].

Several widely used reversed-phase high-performance liquid chromatography (HPLC) systems for peptide separation were evaluated for selectivity, ruggedness and quantitation of FA and potential process impurities and degradation products in FA bulk drug and formulation. The goal of the study was to validate HPLC methods for determination of the purity/potency and impurities levels in FA bulk drug and formulation.

EXPERIMENTAL

Materials

FA and related peptides were obtained from Takeda Chemical Industries (Osaka, Japan), Sigma (St. Louis, MO, U.S.A.) and Serva (Westbury, NY, U.S.A.).

TABLE I

ABBREVIATIONS AND STRUCTURES OF THE COMPOUNDS STUDIED

All amino acids are L unless designated as D.

Abbreviation	*Structure*
FA	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro ethylamide (acetate salt of fertirelin)
1–6	pGlu-His-Trp-Ser-Tyr-Gly
Z-LAP	benzoxycarbonyl-Leu-(MBS)Arg-Pro ethylamide
MBS-F	p-methoxybenzenesulfonamide of F on Arg
(D)Ser-4	pGlu-His-Trp-(D)Ser-Tyr-Gly-Leu-Arg-Pro ethylamide
Glu-1	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro ethylamide
1–3	pGlu-His-Trp
2–3	His-Trp
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
4–10	Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
3–10	Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
pGlu	pyroglutamic acid

Reagents of analytical grade were obtained from Mallinckrodt (St. Louis, MO, U.S.A.) or J. T. Baker (Phillipsburg, NJ, U.S.A.).

Instruments

Modular HPLC systems utilized various combinations of the following: Ultrachrom GTi gradient 2150 pump and 2152 controller (LKB Instruments, Gaithersburg, MD, U.S.A.), Model 783 or 773 variable-wavelength detector (Kratos, Ramsey, NJ, U.S.A.), ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.), 840 data station (Millipore-Waters, Milford, MA, U.S.A.) and Upjohn VAX-based data acquisition system (Digital Equipment Corporation, Merrimack, NH, U.S.A.). Columns studied included: 250 × 4.6 mm LiChrosorb Hibar C_{18} (5 μm) (E. Merck, Darmstadt, F.R.G.), 250 × 4.6 m Bakerbond WP-C_4 (J. T. Baker Chemical), 250 × 4.6 mm PLRP-S 300 Å (8 μm) (Polymer Labs, Amherst, MA, U.S.A.), 250 × 4.6 mm Vydac C_{18} (Phase Separations, Norwalk, CT, U.S.A.) and 80 × 6.2 mm Poly F and 80 × 6.2 mm PEP-RP1 (DuPont, Wilmington, DE, U.S.A.). Mobile phases were prepared with HPLC-grade acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.) as the stronger solvent. The aqueous portions of the mobile phase were prepared using Milli-Q water (Millipore, Bedford, MA, U.S.A.) with 0.1% trifluoroacetic acid (TFA) (pH 2.2) (Pierce, Rockford, IL, U.S.A.), triethylammonium phosphate (TEAP) (pH 3.5) prepared from triethylamine (TEA) (Fisher Scientific, Fairlawn, NJ, U.S.A.) and concentrated phosphoric acid (Mallinckrodt), sodium phosphate (pH 4.7), ammonium acetate or sodium phosphate (pH 7.0) or ammonium bicarbonate (pH 7.8). Linear gradient segments were used to elute FA and related compounds as described in Fig. 1. Sample concentrations of 2–50 μg/ml were prepared using mobile phase as the solvent and 200 μl aliquots were injected.

An isocratic HPLC system developed by researchers at Takeda was used for the potency/purity determinations. It utilized a LiChrosorb C_{18} column, a mobile phase consisting of acetonitrile–0.01 *M* ammonium acetate (pH 7.0) (24:76, v/v), a flow-rate of 1.0 ml/min and detection at 280 nm (0.1 a.u.f.s.). Samples and standards

were accurately prepared with concentrations of *ca.* 50 μg FA/ml and 200 μl of these were injected.

Compounds studied

FA was prepared by solution synthesis from the N-terminal hexapeptide and a N-blocked and Arg-protected tripeptide. The scheme involves coupling protected fragments, deblocking the protected fertirelin and titrating the free base (F) as follows (see Table I for structures):

(1) Z-LAP is N-deblocked and coupled to 1–6 → MBS-F
(2) MBS-F is treated to remove MBS → F
(3) F is titrated with acetic acid → FA

F may undergo several degradation reactions[9–13] which include (see Table I for structures):

(1) inversion of Ser-4 → (D) Ser-4
(2) cleavage of peptide chain → 1–3 + 2–3 + others
(3) ring opening of Z → Glu-1

RESULTS AND DISCUSSION

Survery of HPLC systems to determine their selectivity

Mixtures of process impurities, potential degradation products and selected LHRH-related compounds (Table I) were separated with the gradient HPLC systems described in Figs. 1–3. In each of these systems the compounds studied were resolved from fertirelin and from each other. While there is some shifting in relative peak positions with changes in mobile phase composition the order of elution of the compounds studied remained constant. It is believed that the large peak observed at the position for an unretained compound (about 2 min in Figs. 1–3) is due at least in part to the elution of acetic acid present in the samples. The (D) Ser-4 isomer was the most difficult peptide to resolve from fertirelin (peak E in Figs. 2 and 3) in all of the systems studied. It also is the largest impurity observed in the FA bulk drug and formulation samples studied. Small fragments, such as 1–3 and 2–3 showed very low retention compared to F (peaks H and I in Fig. 3), while the hexapeptide fragment (peak B in Fig. 1) and LHRH fragments (peaks L and M in Fig. 3) elute between F and the small fragments. The protected process intermediates Z-LAP and MBS-F (peaks C and in Fig. 1) showed considerably higher retention than F on all systems as expected due to the presence of the hydrophobic blocking groups. LHRH (peak K in Fig. 3) elutes between the Glu-1 analogue and the (D) Ser-4 isomer.

Bulk drug and formulation assays

Bulk drug purity and formulation potency were determined with the isocratic system (see Experimental) using an external standard for calibration. Isocratic elution was utilized to simplify the instrumental requirements and to reduce analysis time in the quality control testing laboratories. All compounds described in Table I are also resolved by the isocratic system. The process intermediates Z-LAP and MBS-F do

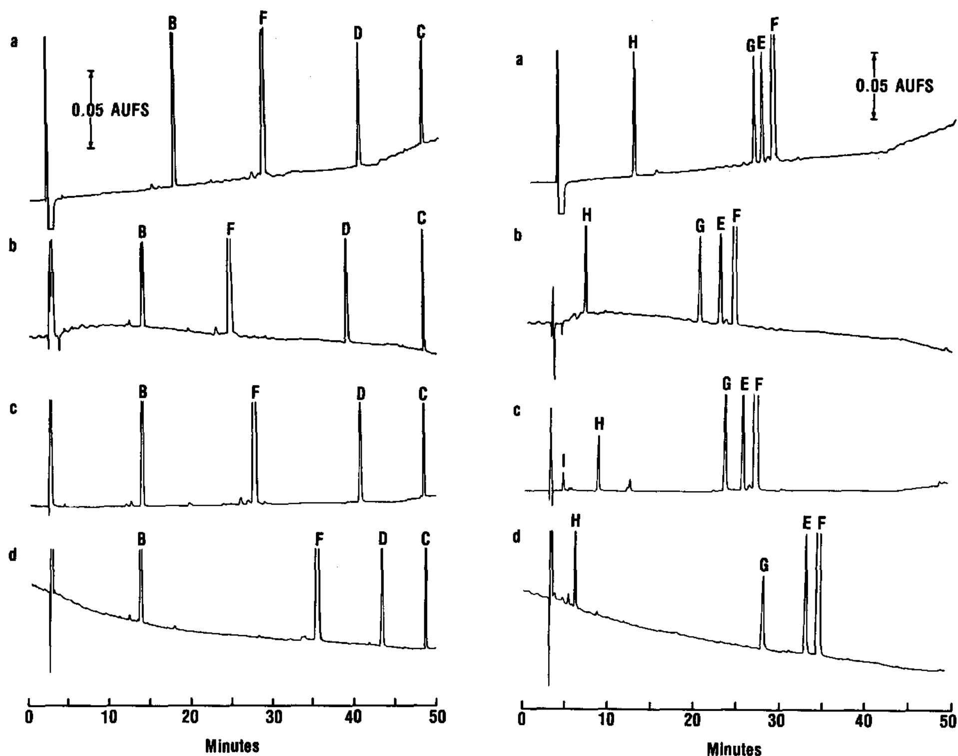

Fig. 1. Gradient HPLC of process impurities on Bakerbond WP-C_4 column. Gradient elution with linear segments, 5–25% B in 40 min, followed by 25–45% B in 10 min (B = acetonitrile). A portion of the mobile phase consisted of (a) 0.1% TFA (pH 2.2), (b) 0.1 *M* TEAP (pH 3.7), (c) (0.1 *M* sodium phosphate (pH 4.7), (d) 0.1 *M* ammonium bicarbonate (pH 7.8). Detection at 214 nm 0.1 a.u.f.s. Flow-rate, 1.5 ml/min. Peaks: B = 1–6, C = Z-LAP, D = MBS-F.

Fig. 2. Gradient HPLC of degradation products on Bakerbond WP-C_4. Conditions as in Fig. 1. Peaks: E = (D) Ser-4, G = Glu-1, H = 1–3, I = 2–3.

not elute for all practical purposes under isocratic elution conditions however (k' > 50). Retention of F and the other peptides is strongly dependent upon the acetonitrile concentration in the mobile phase. A change from 23 to 26% acetonitrile (v/v) reduced the capacity factor of F from 7.2 to 3.7 for instance. Recovery of fertirelin from sterile solution formulation was determined by a spiked addition study. In cases where the external standard was dissolved and injected in water without an amount of salt equivalent to that contributed by the formulation recovery was about 98%. Preparation of the external standard in a solution containing an amount of salt equivalent to that contributed by the formulation gave apparent recovery of 100%. The isocratic elution method yielded precision of < 1% R.S.D. for multiple assays of bulk drug purity and formulation potency.

The gradient HPLC conditions described in Fig. 1c were detemined to be the preferred ones for study of the stability of bulk drug and sterile solution formulation, because they provide a rugged and sensitive method for determining low levels of

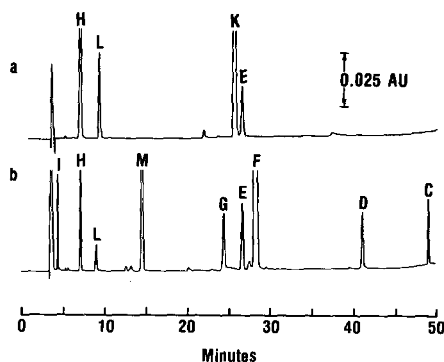

Fig. 3. Gradient HPLC of FA and LHRH-related peptides on Bakerbond WP-C_4 column. Conditions as in Fig. 1c except 0.05 a.u.f.s. Peaks: K = LHRH, L = 4–10, M = 3–10, other as in Figs. 1 and 2.

peptides related to FA. This system yields sharp peaks for both slightly retained compounds, such as the dipeptide and tripeptide fragments, and for the highly retained compounds, such as the blocked synthetic intermediates. This allows their detection in proportions of *ca.* 0.1% (area%) with a precision of *ca.* 5–10% for peptides detected at the 1–2% (area%) level. Response factors are assumed to be one for these calculations due to the lack of suitable reference samples of the potential impurities. The baseline obtained at 214 nm with the sodium phosphate mobile phase is considerably less noisy than with TFA,TEAP, acetate or bicarbonate buffers.

Fig. 4 shows representative chromatograms obtained with several different columns and the gradient elution conditions of Fig. 1c for sterile solution formulation samples stored at 40°C for three years. Under these storage conditions FA undergoes some apparent degradation as evidenced by the observation of several small peaks not observed in chromatograms of freshly prepared formulation samples (not shown in the figures). Note that some of the minor degradation products are not resolved

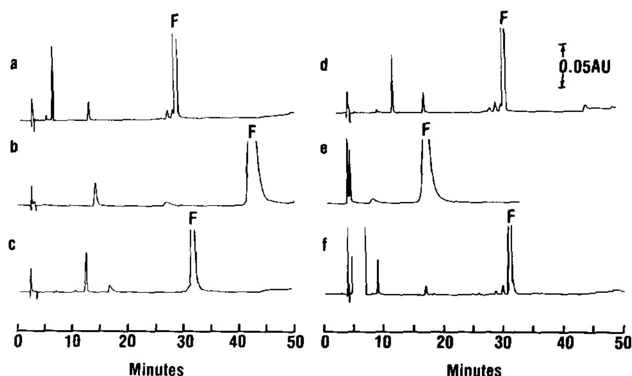

Fig. 4. Gradient HPLC of sterile solution samples stored at 40°C for 3 years. Conditions as in Fig. 1c except columns were: (a) Bakerbond WP-C_4; (b) LiChrosorb RP-18; (c) PLRP-S: (d) Vydac C_{18}; (e) Poly F; (f) PEP-RP1. Peaks as in Figs. 1–3.

from F under these gradient conditions on the LiChrosorb, PLRP-S, PEP-RP1 or Poly-F columns, while the Bakerbond and Vydac columns do resolve them. Efforts to characterize these minor degradation products continue.

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