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Investigation of the chemical stability of (D-Phe^6, Gln^8) GnRH (1–9)-ethylamide (Folligen) by high-performance liquid chromatography

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ABSTRACT

The stability of a new, superactive gonadotropin hormone-releasing hormone (GnRH) analogue (D-Phe^6, Gln^8) GnRH (1–9)-ethylamide [Folligen], which may be a new drug in the "GnRH family" was investigated during storage at different temperatures, both in solid (lyophilized) form and in aqueous solution. Samples stored for various periods of time were analysed for Folligen content and for degradation products using two validated reversed-phase high-performance liquid chromatographic (HPLC) methods. The HPLC technique and the calculation method applied were found to be applicable to monitoring the chemical stability and the possible degradation products of Folligen. It is concluded that under the given experimental conditions Folligen is stable (1) in lyophilized form kept at ambient temperature at least for 1 year, (2) in frozen aqueous solution kept at −20°C for at least 11 months, (3) in sterile aqueous solution kept in a refrigerator (5–8°C) for 5 months, (4) in sterile aqueous solution kept at ambient temperature (20–25°C) for 5 weeks and (5) in sterile aqueous solution kept at 37°C for 6 days.

INTRODUCTION

On the basis of previous studies on different species-specific gonadotropin hormone-releasing hormones (GnRHs), a novel GnRH analogue has been developed in our laboratory. It was synthesized by a classical large-scale liquid-phase method and has been patented as Folligen [1–4].

Folligen stimulates reproductive functions, induces follicular maturation and ovulation in various kinds of fish and mammals [1,5,6] and stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) release in rat pituitary cells [1,2,6]. It is effective in accomplishing the artificial propagation of fish both during and out of the spawning season [1,7,8] and in treating cows with various sexual disorders (anestrous, inactive ovaries, acycling, discycling etc.) [2,3,5,6,9,10]. It has a unique anti-tumour activity in dimethylbenzanthracene-induced mammary carcinomas in rats, it causes almost 100% tumour remission without blocking ovarian functions and it has a direct inhibitory effect on MDAMB 230 human breast cancer cells [11,12].

All these results are very promising for the development of a new drug in the

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"GnRH family" which may have the above-mentioned advantages compared with known peptides. In the very complex process of developing a new pharmaceutical, a step of paramount importance is to study the stability of the given compound.

The objective of this study was to establish stability data for (D-Phe^6, Gln^8) GnRH (1–9)-ethylamide (Folligen) under different storage conditions using a high-performance liquid chromatographic (HPLC) technique for monitoring possible degradation products.

EXPERIMENTAL

Folligen

The structure is (D-Phe^6,Gln^8) GnRH (1–9)-ethylamide · CH_3COOH, pyro-Glu–His–Trp–Ser–Tyr–D-Phe–Leu-Gln–Pro–NHC_2H_5 · CH_3COOH, molecular weight 1274.38 and purity 99.1% (calculated on the basis of the peak area of HPLC traces recorded at 215 nm). Folligen was synthesized in our laboratory.

For solid samples, a solution of 0.5 mg/ml Folligen in distilled water was prepared and dispensed into amber-glass vials (1 ml each). The vials were lyophilized, stored as described below and reconstituted in 1 ml of distilled water just before analysis.

For solution samples, a solution of 0.5 mg/ml Folligen in distilled water was prepared and sterilized by passing it through a 0.2-μm Millipore filter unit. This solution was dispensed into sterile glass microtubes (150 μl each) and sealed first by PTFE tape and then Parafilm under sterile conditions.

Storage

Solid samples were stored in amber-glass vials in a desiccator containing potassium hydroxide pellets. The desiccator was evacuated by a vacuum pump and kept at ambient temperature.

Solution samples were kept in clear-glass microtubes, either at 37°C, at ambient temperature (20–25°C), in a refrigerator (5–8°C) or in a freezer (−20–25°C).

Chemicals

Acetic acid and trifluoroacetic acid were purchased from Fluka (Buchs, Switzerland), ammonia solution from of Reanal (Budapest, Hungary) and acetonitrile from Pierce (Rockford, IL, USA).

Analysis

The Folligen content of the samples and the formation of degradation products were followed by HPLC. A pair of injections using the full-loop filling method were made from duplicate samples and analyzed by two different validated HPLC methods. This resulted in eight data points per incubation time at each incubation temperature.

HPLC conditions

The flow-rate was 1 ml/min [ISCO (Lincoln, NE, USA) Model 2350 pumps]; detection, UV absorbance at 215 nm; sensitivity, 0.5 a.u.f.s. (channel 1) and 0.1 a.u.f.s. (channel 2); ISCO V4 UV detector; injection, 25 μl of sample into a 10-μl loop

(= 5 μg of Folligen). Two methods were used, as follows. Method A: column, Vydac 218TP5 ODS, 5 μm (250 × 4.6 mm I.D.) [Separations Group (Vydac), Hesperia, CA, USA]; eluent, 0.1% trifluoroacetic acid in acetonitrile–water (28:72, v/v). Method B: column, Shandon (Astmoor, Cheshire, UK) ODS-Hypersil, 5 μm (250 × 4 mm I.D.); eluent: acetonitrile–0.02 *M* ammonium acetate (pH 5.0) (33:67, v/v).

Calculation method

Digital data collected from HPCL runs were analysed by ISCO's ChemResearch chromatography software using an IBM XT compatible computer (data collection sampling rate 4 s^{-1}). Integrated peak areas of Folligen and those of impurities derived from four chromatograms per time point were averaged and standard deviations were calculated. The amount of degradation products was calculated by subtracting the average total area of non-Folligen peaks in control runs from that in chromatograms of stored samples.

RESULTS

The average peak areas and standard deviations for Folligen and the degradation products calculated from four chromatograms per time point are shown as a function of storage time in Tables I–V. "Not detectable" or "0" is given for the amount of degradation products in these tables whenever one of the folowing two criteria was fulfilled, respectively: (1) no extra peaks or increase in the amount of existing impurities compared with control (0 day) runs could be visually observed on the more sensitive recorder trace; or (2) the amount of degradation products calculated as described under Experimental was less than 0.1% of the intact Folligen peak area.

Fig. 1 shows the chromatograms of Folligen and its degradation products obtained by the chromatographic methods A and B (see *HPLC conditions*).

TABLE I

STABILITY OF FOLLIGEN IN SOLID (LYOPHILIZED) FORM

Storage time (months)	Peak area			
	Folligen		Degradation products	
	Mean S.D.[a]	%	Mean S.D.[a]	%
Method A				
0	38833 ± 999	100.0	0.0	0.0
4	40001 ± 806	103.0	Not detectable	
8	37893 ± 847	97.6	Not detectable	
12	38487 ± 1287	99.1	Not detectable	
Method B				
0	37864 ± 733	100.0	0.0	0.0
4	38944 ± 122	100.3	Not detectable	
8	37790 ± 662	97.3	Not detectable	
12	37551 ± 813	96.7	Not detectable	

[a] $n = 4$.

TABLE II

STABILITY OF FOLLIGEN IN AQUEOUS SOLUTION AT 37°C

Storage time (days)	Peak area			
	Folligen		Degradation products	
	Mean S.D.[a]	%	Mean S.D.[a]	%
Method A				
0	35812 ± 436	100.0	0.0	0.0
3	35999 ± 793	100.5	Not detectable	
6	36001 ± 474	100.5	Not detectable	
10	35203 ± 676	98.3	5 ± 160	0.0
14[b]	34976 ± 676	97.7	38 ± 75	0.1
21[b]	37052 ± 72	103.5	193 ± 203	0.5
28[b]	34000 ± 209	94.9	310 ± 196	0.9
Method B				
0	35159 ± 611	100.0	0.0	0.0
3	36677 ± 495	104.3	Not detectable	
6	36294 ± 1101	103.2	Not detectable	
10	35593 ± 1210	101.2	83 ± 110	0.2
14[b]	35530 ± 125	101.1	65 ± 102	0.2
21[b]	37674 ± 79	107.2	146 ± 54	0.4
28[b]	34203 ± 362	97.3	157 ± 71	0.4

[a] $n = 4$.
[b] $n = 2$.

Solid (lyophilized) samples

As can be seen from Table I, no degradation of Folligen could be detected by either method during the investigated period of 1 year.

Aqueous solutions kept at 37°C

The first signs of degradation were observed on the tenth day of incubation (see Table II). Since the last preceding analysis was carried out on the sixth day, we can claim that Folligen is stable in sterile aqueous solution at 37°C for 6 days. On days 14, 21 and 28, the number of analysable samples was reduced because of evaporation.

Aqueous solutions kept at ambient temperature (20–25°C)

No breakdown of Folligen was observed for 35 days of storage in solutions kept on a laboratory bench (Table III). During this period, room temperature fluctuated between 20 and 25°C. Unfortunately, digitized data for 260-day runs by Method B were lost owing to disk failure, although analogue chromatograms showed a similar extent of degradation to that found by Method A.

Aqueous solutions kept in a refrigerator (5–8°C)

The first appearance of degradation products was visually observed on the 260-day chromatograms. This was supported by integration results of runs with Method A (see Table IV). As the last preceding analyses were performed on the 150th day of storage, Folligen is considered to be stable in sterile aqueous solution at 5–8°C for 150 days.

TABLE III

STABILITY OF FOLLIGEN IN AQUEOUS SOLUTION AT AMBIENT TEMPERATURE

Storage time (days)	Peak area			
	Folligen		Degradation products	
	Mean S.D.[a]	%	Mean S.D.[a]	%
Method A				
0	35812 ± 436	100.0	0.0	0.0
3	35109 ± 113	98.0	Not detectable	
7	34791 ± 191	97.1	Not detectable	
14	34839 ± 316	97.3	Not detectable	
21	35988 ± 178	100.5	Not detectable	
28	35643 ± 459	99.5	Not detectable	
35	35145 ± 452	98.1	Not detectable	
50	36456 ± 498	101.8	243 ± 139	0.7
260	34978 ± 78	97.7	540 ± 162	1.5
306[b]	33543 ± 238	93.7	723 ± 133	2.0
Method B				
0	35159 ± 611	100.0	0.0	0.0
3	35006 ± 627	99.6	Not detectable	
7	34698 ± 464	98.7	Not detectable	
14	34756 ± 367	98.9	Not detectable	
21	36601 ± 390	104.1	Not detectable	
28	34780 ± 203	98.9	Not detectable	
35	35490 ± 463	100.9	Not detectable	
50	36228 ± 759	103.0	73 ± 117	0.2
260	Digital data lost because of disk failure			
306[b]	34057 ± 888	96.9	718 ± 140	2.0

[a] $n = 4$.
[b] $n = 2$.

Frozen aqueous solutions kept at −20°C

In frozen aqueous solutions kept at −20°C, Folligen remained intact during the whole of the investigated period of 326 days (see Table V). It should be noted that we have no data regarding how repeated thawing and freezing would affect the stability of the peptide.

Summary of results

Folligen in sterile aqueous solution (pH 5.5–6.0) was found to be stable for 6 days at 37°C, for 35 days at ambient temperature, for 5 months when kept in a refrigerator and for at least 11 months when fozen at −20°C.

It is not very suprising that no degradation was found with solid samples during the investigated period of 1 year, as peptides are usually stable for years in lyophilized form. It should not be disregarded, however, that this result was obtained with the exclusion of humidity (in a vacuum desiccator) and light (in amber-glass vials), and we have no data about the influence of these factors on the stability of Folligen.

TABLE IV

STABILITY OF FOLLIGEN IN AQUEOUS SOLUTION KEPT IN A REFRIGERATOR (5–8°C)

Storage time (days)	Peak area			
	Folligen		Degradation products	
	Mean S.D.[a]	%	Mean S.D.[a]	%
Method A				
0	35812 ± 436	100.0	0.0	0.0
10	34884 ± 212	97.4	Not detectable	
20	35567 ± 217	99.3	Not detectable	
30	34655 ± 192	96.8	Not detectable	
42	35425 ± 395	98.9	Not detectable	
60	35392 ± 417	98.8	Not detectable	
150	35178 ± 123	98.2	Not detectable	
260	35492 ± 376	99.1	55 ± 132	0.2
326	34678 ± 627	96.8	0 ± 128	0.0
Method B				
0	35159 ± 611	100.0	0.0	0.0
10	35180 ± 399	100.1	Not detectable	
20	34921 ± 278	99.3	Not detectable	
30	34765 ± 438	98.9	Not detectable	
42	35210 ± 151	100.1	Not detectable	
60	35499 ± 369	101.0	Not detectable	
150	34761 ± 430	98.9	Not detectable	
260	35004 ± 382	99.6	0 ± 24	0.0
326	34759 ± 651	98.9	88 ± 70	0.3

[a] $n = 4$.

DISCUSSION

The stability of a nonapeptide GnRH analog, Folligen, was investigated both in solid (lyophilized) form and in aqueous solution during storage at different temperatures. Samples stored for various time periods were analysed for Folligen content and for degradation products by HPLC.

There are two possibilities for investigating the stability of a compound: (1) to determine the decrease in the peak area of the investigated parent compound (parent peak area method); or (2) to determine the sum of the peak areas of the degradation products (degradation products peak area method). In our opinion, the most accurate and most reliable information can be obtained by using the two methods simultaneously.

In our experiments, the sample-to-sample reproducibility was not better than 5% (evaporation of the sample and losses during the lyophilization are among the reasons). As a consequence, the peak area of the undegraded parent compound (Folligen) is not a sensitive indicator of its degradation, because low levels of degradation (lower in percentage than the reproducibility) could not be statistically verified.

With a compound of low degradability, this parent peak area method (method

TABLE V

STABILITY OF FOLLIGEN IN FROZEN AQUEOUS SOLUTION AT −20°C

Storage time (days)	Peak area			
	Folligen		Degradation products	
	Mean S.D.[a]	%	Mean S.D.[a]	%
Method A				
0	35812 ± 436	100.0	0.0	0.0
20	35530 ± 359	99.2	Not detectable	
42	35448 ± 1065	99.0	Not detectable	
60	34997 ± 524	97.7	Not detectable	
161	34629 ± 857	96.7	Not detectable	
260	36278 ± 1155	101.3	Not detectable	
326	35363 ± 305	98.7	Not detectable	
Method B				
0	35159 ± 611	100.0	0.0	0.0
20	35533 ± 330	101.1	Not detectable	
42	35702 ± 128	101.5	Not detectable	
60	34658 ± 154	98.6	Not detectable	
161	36177 ± 1871	102.9	Not detectable	
260	35950 ± 208	102.3	Not detectable	
326	36098 ± 300	102.7	Not detectable	

[a] $n = 4$.

1) is ineffective. Although these difficulties could have been overcome by using an internal standard, an added compound might have influenced the stability of the investigated compound and this method was therefore avoided. In this instance it is more favourable to characterize the degradability by the sum of the peak areas of the degradation products (method 2), in spite of the fact that the relative responses are

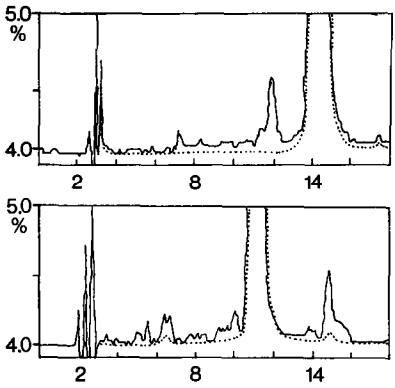

Fig. 1. Chromatograms of Folligen (mean peak on both chromatograms) and its degradation products obtained by chromatographic methods A (top) and B (bottom). For chromatographic parameters, see Experimental. Abscissa: time in minutes; ordinate: relative detector response as a percentage. Dotted lines show the chromatograms of pure (undegraded) Folligen.

not known. In product qualification and in stability investigations (where the degradation products generated by UV irradiation, heating, freezing, etc., are not identified and as a consequence their molar responses relative to the parent compound are not known), characterization of the product by the chromatographic purity is generally accepted. In these instances the concentrations of the parent compound and those of the degradation products or contaminants are given as percentages of the sum of the peak areas. The concentrations specified in this way may differ from the absolute concentrations of the components. This is why the term "chromatographic purity" always must be applied.

This method of calculation implicitly involves the assumption that the value of all the relative molar responses of the individual peaks is 1, which may not be taken for granted, of course. However, considering an even worse case (when the relative responses of the degradation products are not 1 but, *e.g.*, 3) the highest degree of degradation is not 2% but 6% (Table III, day 306), which hardly exceeds the reproducibility mentioned before. This low degree of degradation would be undetectable or hardly detectable by the parent peak area method.

In our opinion, the most reliable method is to characterize the degree of degradation by both methods and to use the worst value (the higher degree of degradation) in each instance. In Table I–V we give the results of both methods for comparison.

In the stability investigation, two HPLC methods with different selectivities were used to minimize the possibility that a degradation product is not detected because of its coelution with the Folligen peak. Both methods were previously examined according to pharmaceutical industry standards and proved to be suitable for separating impurities and degradation products from Folligen.

For sample introduction, the full-loop-filling method was used in order to increase the precision and reproducibility. Two injections of duplicate samples were analysed by the two different methods, yielding eight data points for each incubation time. UV absorption detection was used at 215 nm, a wavelength at which any peptide fragment formed by degradation could be detected. Detector signals were recorded at two different sensitivities by an analogue recorder. One setting was chosen to give *ca.* 50% deflection of full-scale for the Folligen peak, and the other setting was five times more sensitive in order to enable us to detect by visual inspection peaks as small as 0.1% of the peak height of Folligen. In addition to analogue recording, detector signals were also digitized and data were stored on floppy disk for later quantitative analysis by computer. In this way, even peaks of 0.1% of the total peak area could be integrated.

Folligen was considered not to be degraded as long as no signs of degradation could be visually observed on the more sensitive recorder trace or the combined area of non-Folligen peaks did not exceed that in control runs (0 day) by more than 0.1% of the Folligen peak area. Therefore, the stability data obtained should be interpreted such that during these periods less than 0.1% degradation product is formed from Folligen under the indicated conditions.

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