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Comprehensive determination of the cyclic FEE peptide chemical stability in solution

R. Rotival[a], M. Bernard[a,*], T. Henriet[a], M. Fourgeaud[a], J.R. Fabreguettes[a], E. Surget[a], F. Guyon[a,b], B. Do[a,b]

[a] Department of Quality Control and Analytical Development, General Equipments and Health Products Agency, Assistance Publique – Hôpitaux de Paris, Paris, France
[b] Department of Physical Chemistry of Drug, Faculty of Pharmaceutical and Biological Sciences, Paris-Descartes University, Paris, France

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ABSTRACT

The FEE cyclic hexapeptide (cFEE) is an investigational new drug added to the insemination medium in order to improve the *in vitro* fertilization rate. The pharmacological activity of small peptides is highly dependent on the conservation of the amino acid sequence and of the structural conformation of the active site. To enhance the scientific knowledge required for the clinical use of cFEE, a comprehensive determination of its chemical stability in solution was realized in accelerated conditions. Degradation products have been detected and identified by liquid chromatography/Qtrap® mass spectrometry. The main degradation products highlighted during the product shelf life were produced by hydrolysis and only certain sites were involved. In most cases, the cyclic conformation was lost and regarding the major degradation pathway, the sequence representing the active site was affected.

1. Introduction

The product of interest, named "cFEE", is a sequence of 6 amino acids (Cys-Ser-Phe-Glu-Glu-Cys), cyclized through a disulphide bond between two cysteine residues (Fig. 1).

This cyclic hexapeptide has the ability to increase the fusiogenic capacity of human oocytes [1]. Binding of cFEE to the human oocyte membrane simulates spermatozoon contact and induces displacement of adhesion proteins to the oocyte surface, which facilitates the sperm–egg fusion event.cFEE peptide is tested to supplement the culture media used for *in vitro* fertilization (IVF), in order to improve the fertilization rate of IVF performed for idiopathic infertility or sperm deficiency. This use may lower the need for microinjection techniques and improve embryo quality [1].

The biological effect of cFEE has been explained by both specific amino acid composition and cyclic structure whereas similar peptides have been shown to induce inhibitory effects [2–7]. The active site responsible of the peptide binding property has been identified as the Phe-Glu-Glu (FEE) sequence in the cyclic structural constraint (Fig. 1). Depending on their nature, chemical changes affecting peptides constitutional amino acids may result in various consequences on biological activity [1].

The understanding of the chemical reactivity and the characterization of the impurities related to cFEE are thus crucial to assess the drug activity, toxicity and to control its stability.

The present work aimed to identify degradation impurities produced during drug storage in standard and accelerated conditions, by monitoring their relative contents and studying their fragmentation pathways by LC–MS/MS.

In the present case, it is worth to mention that stress test studies were hardly controllable and gave rise to countless numbers of HPLC peaks at almost equal importance with an important loss of cFEE. These outcomes were part of our preliminary studies but are not detailed in the present manuscript. To avoid misinterpretations tied to the presence of secondary degradation products, we have opted for real time studies whose conditions were accelerated by use of two influencing factors, temperature and stability in solution.

Abbreviations: CID, collision-induced dissociation; cFEE, cyclic protein with a sequence of 6 amino acids (Cys-Ser-Phe-Glu-Glu-Cys); HPLC, high performance liquid chromatography; MS, mass spectrometry.

* Corresponding author at: Department of Quality Control and Analytical Development, Agence Générale des Équipements et des Produits de Santé, Assistance Publique – Hôpitaux de Paris, 7 rue du fer à moulin, 75005 Paris, France.
Tel.: +33 146691490; fax: +33 146691492.
E-mail address: melisande.bernard@eps.aphp.fr (M. Bernard).

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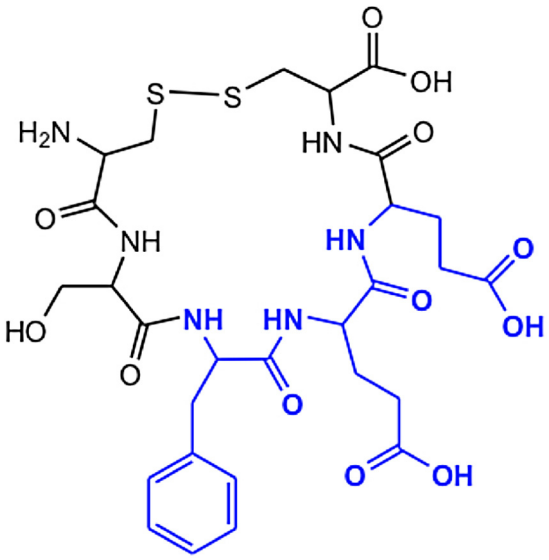

Fig. 1. Chemical structure of cFEE peptide (-Cys-Ser-Phe-Glu-Glu-Cys-) and active site drawn in blue-bold. (for interpretation of the references to color in the artwork, the reader is referred to the web version of the article).

2. Materials and methods

2.1. Reagents and materials

Analytical-reagent-grade acetonitrile was obtained from Sigma–Aldrich®, nitrogen from ALS® and trifluoroacetic acid from VWR®. All solutions were prepared using water from a Milli-Q synthesis system from Millipore®. cFEE was produced by Synprosis SA laboratory® (Fuveau, France).

2.2. Stability study of the drug substance

cFEE dosage form (7.22 mg/ml) in aqueous solution was packaged in type-I glass bottles, closed by chlorobutyl caps.

This section presents a six-month stability study of cFEE dosage form, stored at 2–8 °C and at 25 ± 2 °C in stability chambers. The latter was defined as its accelerated-storage conditions. Different stability results were compared to the starting point data.

2.3. Liquid chromatography/UV/mass spectrometry system

The HPLC method involved a Kinetex™ C18 column (100 mm × 2.1 mm i.d., 2.6 μm) thermostatically set at 40 °C and a mobile phase flowing at 0.5 ml/min. To get acceptable separation between the drug and its degradation products, a combination of 0.1%, v/v trifluoroacetic acid/acetonitrile was used in gradient program (95:5 → 88:12, v/v in 34 min). UV detection was performed by diode array (Dionex, Les Ulis, France) with a wavelength-scanning range of 200–300 nm.

The mass spectrometer was a 4000 QTrap® system (AB Sciex, Les Ulis, France) with an electrospray source, used in positive ionization mode. Analyses of cFEE and impurities were achieved by scanning a mass range from 200 to 1000u (m/z). Under those conditions the LOD was determined at 275 ng/ml in scan mode using signal/noise ratio. The concentration of injected cFEE samples was set at 550 μg/ml to detect 0.05% of degradation products, based on the ICH Q3 guidelines [8], which set thresholds for the identification, reporting and qualification of related impurities in active substances manufactured by chemical synthesis.

Fragmentation of cFEE and its degradation products was carried out in low-energy collision-induced dissociation (CID) in the Q2 quadrupole collision cell with nitrogen as collision gas.

The softwares for data processing were Analyst®, AB Sciex and MS Manager®, ACD Labs (Advanced Chemistry Development, Inc.), version 10.0. Analysis in CODA (Component Detection Algorithm) mode allowed extracting ions that significantly contribute to the total ion current.

3. Results and discussion

3.1. Analysis of degradation profiles by HPLC-UV-MS/MS

A representative HPLC-UV-MS/MS chromatogram of cFEE solutions at T0 and stored after 6 months (M6) in accelerated conditions are shown in Fig. 2(a) and (b), respectively. In comparison with initial samples, several extra chromatographic peaks were detected in addition to cFEE in M6 samples stored at 25 °C. All of them exhibited similar UV spectra with a maximum absorption at 257 nm, suggesting the conservation of a phenylalanine residue in the molecule. Assuming equivalent UV response factors, their relative contents have been calculated using area normalization. Five of them were detected at relative concentrations greater than 0.05% and were analyzed by MS/MS experiments. The compounds eluting at a RRT (relative retention time) of 0.22 (0.13%), 0.32 (0.11%), 0.39 (1.18%), 0.51 (0.11%) and 0.85 (0.26%) gave rise to a series of MS isobaric precursors at m/z 733 by electrospray MS (Fig. 2(c)). Such a trend was not noticeable in the M6 and M12 samples stored at 2–8 °C (Fig. 2(d)).

Thereafter, "DPn" designates degradation products with "n" corresponding to their elution order.

3.2. Characterization of degradation products by MS/MS

3.2.1. Fragmentation pattern of cFEE

In order to facilitate further structural investigations, fragmentation pathways of cFEE were analyzed following MS/MS experiments employing low-energy collision-induced dissociation (CID). The amount of energy transferred to the molecule defines the type of the resulting fragmentation. In this dissociation process, the weakest bonds in the molecules are preferentially cleaved. High-energy CID often carries more information [9,10], as the high internal energy of the resulting excited ions leads to a wealth of backbone and side-chain fragmentations (yielding N-terminal (d-) and C-terminal (v- and w-) fragment ions). But in the present case, as the molecular structure of the cyclic peptide is known and the degradation products were assumed to derive from cFEE as precursor, information obtained with low-energy CID was sufficient and rendered the interpretation of spectra easier. Fragment species were labelled using the nomenclature commonly applied in tandem mass spectrometry [11]. Fig. 3 illustrates that scheme.

MS/MS fragmentation of the protonated peptide results in charge retention on the N-terminus (denoted as a, b, and c ions) and on the C-terminus (denoted as x, y and z ions). Apart from fragmentations occurring along the peptide's backbone, cleavage of the side-chain may yield N-terminal (d-) and C-terminal (v- and w-) fragment ions. This nomenclature was employed in the following mass spectra.

Fig. 4 displays a mass spectrum obtained from cFEE in the range that exhibited significant mass peaks. The spectrum shows the presence of singly protonated cFEE molecule at m/z 715. Neither doubly nor triply protonated molecules were obviously detected. Most of the peaks at lower masses have been clearly assigned to the