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RESEARCH ARTICLE

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Spectrofluorimetric determination of eptifibatide in human plasma and dosage form

Eman Saad Elzanfaly[1] | Enas Abdel Hakim Amer[2] | Sara Abdel Basset Galal[2] | Hala Elsayed Zaazaa[1]

[1] Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El- Aini Street, Cairo, Egypt

[2] National Organization for Drug Control and Research (NODCAR), 51 Wezerat El- Zeraa Street, Agouza, Giza, Egypt

Correspondence
Sara Abdel basset Galal, National Organization for Drug Control and Research (NODCAR), 51 Wezerat El- Zeraa Street, Agouza, Giza, 12553, Egypt.
Email: sara_galal@live.com

Abstract

A spectrofluorimetric method for the determination of eptifibatide is presented based on its native fluorescence. The type of solvent and the wavelength of maximum excitation and emission were carefully selected to optimize the experimental conditions. Under the specified experimental conditions, the linearities obtained between the emission intensity and the corresponding concentrations of eptifibatide were in the range 0.1–2.5 μg/ml for the calibration curve constructed for direct determination of eptifibatide in dosage form and 0.05–2.2 μg/ml for the calibration curve constructed in spiked human plasma with a good correlation coefficient (r > 0.99). The lower limit of quantification for the calibration curve constructed in human plasma was 0.05 μg/ml. Recovery results for eptifibatide in spiked plasma samples and in dosage form, represented as mean ± % RSD, were 95.17 ± 1.94 and 100.29 ± 1.33 respectively. The suggested procedures were validated according to the International Conference on Harmonization (ICH) guidelines for the direct determination of eptifibatide in its pure form and dosage form and United States Food and Drug Administration (US FDA) Guidance for Industry, Bioanalytical Method Validation for the assay of eptifibatide in human plasma.

KEYWORDS

dosage form, eptifibatide, plasma, spectrofluorimetry

1 | INTRODUCTION

Eptifibatide (EPT) (Figure 1) is N6-(aminoiminomethyl)-N2-(3-mercapto-1-oxopropyl)-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic(1–6)-disulfide.[1] It has a cyclic heptapeptide structure, used as an antiplatelet drug and classified as a glycoprotein IIb/IIIa inhibitor.[1] EPT stability over pH range 4.25–6.25 under accelerated temperature conditions was studied by Zhao and Yalkowsky.[2] They created a semi-aqueous formulation to improve drug stability during storage and transportation. Two liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods were suggested for the analysis of EPT in plasma.[3,4] EPT and its related peptide were determined in EPT injection by Yao and Chun-Lai using a high pressure liquid chromatography ultraviolet (HPLC-UV) method.[5] Patil and colleagues proposed two UV spectrophotometric methods for EPT analysis in its bulk and pharmaceutical formulation.[6]

Savadkouhi and colleagues used reverse phase (RP)-HPLC for EPT analysis in its bulk and pharmaceutical formulation.[7] An RP-HPLC-MALDI-TOF MS/MS system was used for the analysis and structure elucidation of new impurity detected in research sample chromatograms of EPT manufactured by a new technique and formulated into a drug product.[8] Chen and colleagues discussed the effect of retaining the integrity of the intra-loop structure on increasing the

Abbreviations used: CV, coefficient of variation; EPT, eptifibatide; HPLC, high pressure liquid chromatography; ICH, International Conference on Harmonization; LC, liquid chromatography; LLOQ, lower limit of quantification; MALDI-TOF, matrix assisted laser desorption/ionization–time of flight; MS, mass spectrometry; QC, quality control; US FDA, United States Food and Drug Administration; USP, United States Pharmacopeia; UV, ultraviolet; ULOQ, upper limit of quantitation

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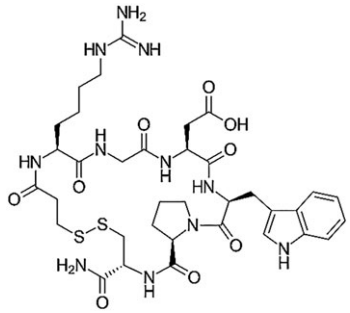

FIGURE 1 Chemical structure of eptifibatide

relative abundance of formation of immonium ion fragments to facilitate the analysis and quantitation of peptides containing intra-chain disulfide bonds such as EPT in triple quadrupole MS.[9] Rocheleau and colleagues reported impurity profiling of EPT using a sub-2 μm stationary phase column and compared it with conventional LC columns.[10] Enhancement of fluorescence emitted by functionalized ZnS nanocrystals by the addition of various EPT concentrations was found to be proportional to the added amounts of EPT and was utilized for its quantitation.[11] Spectrofluorimetric analysis was used due to its high sensitivity, simplicity and relatively low cost compared with other methods (HPLC and gas chromatography (GC)). Chromatographic methods are limited by the number of samples that can be processed in 1 working day in contrast with spectrofluorimetric methods, in which a large number of samples can be prepared and processed in a shorter time within 1 working day. The determination of substances by spectrofluorimetry depends on measuring their native or intrinsic fluorescence upon excitation by a certain wavelength or the reaction of the substance with certain reagent to induce the production of fluorescent product proportional to the original substance of interest.[12,13] Literature surveys revealed that no spectrofluorimetric method had been developed to determine EPT neither in dosage form nor in plasma. The aim of the present work was to develop and validate accurate, sensitive and precise methods for the determination of EPT in human plasma and its pharmaceutical formulation based on its native fluorescence.

2 | EXPERIMENTAL

2.1 | Instruments

- Cary Eclipse fluorescence spectrophotometer Agilent Technologies; equipped with a 150 W xenon arc lamp. The excitation and emission slit width was 10 nm, operated with Cary Eclipse scan application software version 1.2 (147).
- Sartorius electric balance, Germany.
- Basic pH bench-top meter HANNA instruments, USA.
- Benchmark Vortex Mixer, USA.

2.2 | Materials and reagents

The primary reference standard for EPT was kindly supplied by Merck MSD, USA and its purity was found to be 99.98% according to reported method.[5] Acetonitrile (Scharlau, Barcelona, Spain), methanol (Macron Fine Chemicals, Poland), acetic acid (Adwic Co., Egypt, 1% aqueous solution), sodium hydroxide (LOBA Chemicals, India, 0.1 N aqueous solution), concentrated sulphuric acid (Sigma Aldrich, USA, 0.1 N aqueous solution), and water for injection, United States Pharmacopeia (USP). An Integrilin® vial was purchased from the local market, labeled to contain 2 mg/ml EPT (B.N 2SBHA01015). Plasma samples were obtained from Suez Canal University Hospital, Ismailia, Egypt and were kept frozen until use after gentle thawing.

2.3 | Eptifibatide standard stock solution (50 μg/ml)

A stock standard solution of EPT (50 μg/ml) was prepared by accurately weighing 5 mg EPT in a 100 ml volumetric flask and dissolving in water. A working standard solution of EPT (2.5 μg/ml) for the direct determination of EPT in dosage form was prepared by transferring 5 ml from the EPT stock standard solution (50 μg/ml) into a 100-ml volumetric flask and then diluting to volume with methanol.

2.4 | Procedures

2.4.1 | Study the effect of different solvents on fluorescence intensity

Aliquots equivalent to 15 μg of EPT were separately transferred from the EPT standard stock solution (50 μg/ml) into 10-ml volumetric flasks and completed to volume with different solvents (water/acetonitrile/methanol / 0.1 N NaOH/0.1 N H_2SO_4/1% glacial acetic acid).

2.4.2 | Spectral characteristics

The native fluorescence behavior was first studied in a pre-scan mode to determine the excitation and emission wavelengths for maximum intensity in each solvent. Then by fixing the wavelength of maximum excitation intensity and measuring the emission intensity at maximum λ.

2.4.3 | Construction of calibration curves

i. For direct determination of EPT in its pure form and dosage form

Aliquots equivalent to 1–25 μg were accurately transferred from the EPT working standard solution into 10-ml volumetric flasks, and completed to volume with methanol to yield final respective concentrations of 0.1, 0.25, 0.75, 1.5, 2, 2.5 μg/ml of EPT. The native fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 223 nm/335 nm.

ii. For spiked human plasma preparations

Transfer of 0.1 ml aliquots of drug-free human plasma into a series of centrifugation tubes was carried out. Spike samples with different quantities of EPT (1.25–55 μg) from the stock standard solution of EPT (50 μg/ml) were made and vortexed for 30 sec. The volume was completed to 5 ml with acetonitrile, the mixture was vortexed

again for 1 min to completely precipitate plasma proteins and centrifuge for 30 min in a cooling centrifuge at 6000 g_x. From the resultant clear supernatant, 1 ml of each concentration was diluted to 5 ml with methanol to yield final respective concentrations of 0.05, 0.1, 0.2, 0.5, 1, 1.4, 1.8, 2.2 μg/ml of EPT in human plasma based on drug concentration. The fluorescence intensity was measured at λ_{ex}/λ_{em} = 223 nm/335 nm. A blank solution was prepared in a similar manner using drug-free human plasma.

For both procedures (i) and (ii), the fluorescence intensity after subtracting blank reading was plotted versus the final concentration of the drug (μg/ml) for each concentration to get the calibration graphs and the regression equations were computed.

2.4.4 | Assay of drug product

2.5 ml from Integrilin® vial (2 mg/ml) were accurately transferred into a 100-ml volumetric flask and completed to volume with methanol to reach a final concentration of (50 μg/ml). Suitable dilutions were performed in methanol, then the fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 223 nm/335 nm.

3 | RESULTS AND DISCUSSION

None of the reported methods utilized the intrinsic fluorescence of EPT for its direct assay in its pure form, pharmaceutical formulation or in human plasma. The aim of this study was to develop a simple and cheap spectrofluorimetric method to analyse the investigated drug in its pure form, pharmaceutical formulation and spiked human plasma, based on the affordability of fluorescence spectrophotometers in many laboratories compared with more expensive and sophisticated reported tandem mass spectrometry devices.[3,4] The developed method was based on measuring the native fluorescence of EPT upon excitation with a suitable wavelength of maximum excitation intensity.

3.1 | Effect of different solvents on emission intensity

The effect of different solvents on the fluorescence intensity was studied. Dilution with different solvents such as methanol, water, acetonitrile, 0.1 N NaOH, 0.1 N H_2SO_4 and 1% glacial acetic acid revealed that methanol was the best solvent to use, as it gave the highest relative fluorescence intensities and the lowest blank readings with reproducible results (Figure 2). In contrast, 1% glacial acetic acid quenched the fluorescence completely. Therefore, methanol was used throughout this study with maximum excitation and emission intensities at wavelengths 223 and 335 nm respectively. Figure 3 shows a typical excitation and emission spectra of EPT in methanol.

3.2 | Removal of endogenous plasma proteins interference

On the other hand, to determine EPT in human plasma, protein precipitation provides a quick and simple technique to remove the interference due to endogenous proteins in plasma, many precipitating

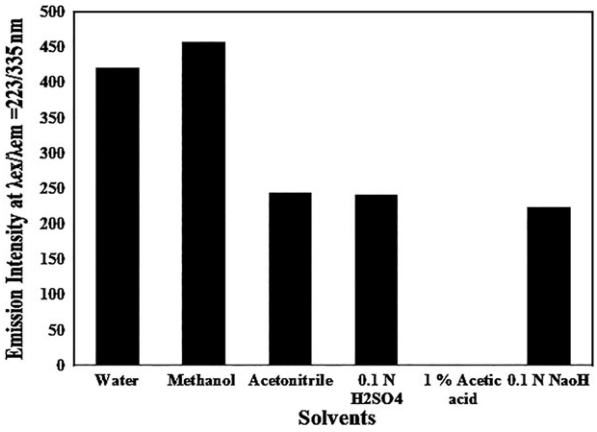

FIGURE 2 Effect of different solvents on EPT emission intensity

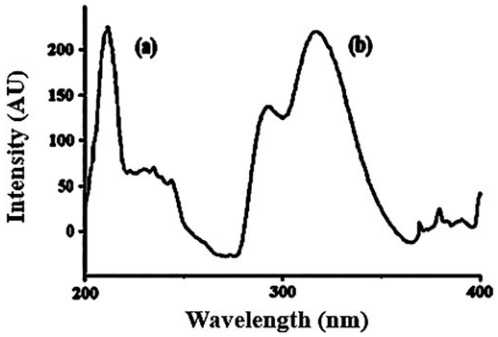

FIGURE 3 Fluorescence spectra of EPT (1.5 μg/ml). (a) Excitation spectrum. (b) Emission spectrum

solvents including methanol – methanol and acetonitrile mixture in 1:2, 1:1, 2:1 v/v ratios – and acetonitrile alone were tried to achieve complete precipitation of plasma proteins in spiked plasma samples. It was found that only acetonitrile achieved complete precipitation of plasma proteins with a clear supernatant.

3.3 | Method validation

The suggested procedures were subjected to the validation scheme according to the International Conference on Harmonization (ICH) guidelines[14] for the direct determination of EPT in its pure form and dosage form and FDA Guidance for Industry, Bioanalytical Method Validation[15] for the assay of EPT in human plasma.

3.3.1 | Linearity and range

Under the specified experimental conditions, a linear relationship was obtained between the emission intensities and the corresponding EPT concentrations in the range 0.1–2.5 μg/ml for a calibration curve constructed in methanol and 0.05–2.2 μg/ml for a calibration curve constructed from spiked human plasma preparations.

The acceptance criteria for the standard curve constructed in spiked human plasma preparations were that at least 75% of non-zero standards should not deviate by more than 15% nominal concentrations, except at lower limit of quantification (LLOQ) where the standard/calibrator should not deviate by more than 20%.

3.3.2 | Lower limit of quantification

The lowest standard on the calibration curve constructed in human plasma was accepted as the LLOQ. The acceptance criteria were that the back-calculated concentration should have precision that did not exceed 20% of the coefficient of variation (CV) and accuracy within 20% of the nominal concentration. The LLOQ was 0.05 μg/ml with CV% 2.82 and it was established using five samples.

$$CV\% = (\text{SD of the five determinations}/\text{their mean}) \times 100$$

3.3.3 | Accuracy and precision

Accuracy and precision of the calibration curve constructed in human plasma were determined by replicate analysis of quality control (QC) samples containing known amounts of EPT using five determinations per concentration on 1 single day to assess intra-day accuracy and precision and on different days to assess inter-day accuracy and precision.

The acceptance criteria were that a mean value of maximum 15% of the nominal value should be fulfilled, except at LLOQ where it should not deviate by more than 20%. The precision determined at each concentration level should not exceed 15% CV except for the LLOQ, where it should not exceed 20% CV. Results for accuracy and precision are shown in Table 1.

For the calibration curve constructed in methanol, accuracy was studied by applying the suggested procedures to known added concentrations of EPT in samples and calculating the percentage recovery. Also, % RSD was taken as a measure of the intra-day precision of the applied method. The same procedures were also applied to three replicates of three added EPT concentrations over different days to study the inter-day precision. Results for accuracy and precision are shown in Table 2

$$\%\text{recovery} = (\text{recovered concentration}/\text{nominal concentration}) \times 100$$

3.3.4 | Recovery of EPT in spiked plasma samples

Recovery of EPT from the extraction procedure was determined as per FDA guidelines[15] by comparing the detector response (emission intensity) obtained from an amount of EPT added to and extracted from plasma with the detector response obtained for the true concentration of analyte in the solvent. Recovery indicates the extraction efficiency of an analytical method within the limits of variability. Recovery need not be 100%, but the extent of recovery of an analyte should be consistent, precise, and reproducible. A recovery experiment was performed by comparing the analytical results for extracted

TABLE 1 Precision and accuracy for determination of eptifibatide in human plasma

Nominal concentration (μg/ml)	Intra-day accuracy and precision[a]		Inter-day accuracy and precision[a]	
	%Recovery	CV%	%Recovery	CV%
0.15 μg/ml	100.16	9.32	101.30	9.54
0.9 μg/ml	94.52	11.67	101.03	10.12
1.7 μg/ml	111.00	2.26	103.15	9.37

[a]Average of five determinations per concentration within single day for intra-day accuracy and precision or five determinations per concentration repeated three times in three different days for inter-day accuracy and precision.

TABLE 2 Validation parameters of the proposed spectrofluorimetric method for the determination of eptifibatide in its pure form, pharmaceutical formulation and spiked plasma samples

Parameter	EPT in its pure form and pharmaceutical formulation	EPT in spiked plasma samples
Excitation wavelength	223 nm	
Emission wavelength	335 nm	
Linearity	0.1–2.5 μg/ml	0.05–2.2 μg/ml
Intercept	7.275	5.786
Slope	300.853	340.232
Correlation coefficient (r)	1.00	0.999
Determination of EPT in Integrilin® vial (Mean ± RSD)	100.29 ± 1.33	—
Precision (% RSD)		
Repeatability[a] Intermediate precision[b]	1.12 1.25	—
LLOQ[c]	—	0.05 μg/ml

[a]The intra-day (n = 9) average of three different concentrations (0.05, 1.75 and 2.25 μg ml^{-1}) repeated three times within 1 day.

[b]The inter-day (n = 9) average of three different concentrations (0.05, 1.75 and 2.25 μg ml^{-1}) repeated three times in 3 successive days.

[c]Calculated as per FDA guidelines[15] as the lowest calibration standard which has precision that does not exceed 20% of the CV and accuracy within 20% of the nominal concentration.

samples at three concentrations (low, medium, and high) with non-extracted standards that represent 100% recovery.[16] Results of the recovery study are shown in Table 3.

3.3.5 | Stability of EPT

EPT stability in human plasma was studied to cover the expected sample handling and storage conditions during the analysis of samples: short-term stability at room temperature, freeze–thaw stability, standard stock solution stability at room temperature and long-term stability after storage at – 20°C for 2 months. Three replicates of two concentrations at low (maximum three times the LLOQ) and high levels were processed and compared against freshly prepared QC samples in all stability studies. All stability sample results were within 15% of nominal concentrations, fulfilling the acceptance criteria for stability studies.

Bench-top stability (short-term stability)

Three replicates of each QC sample were left at room temperature for 6 h to cover the laboratory handling conditions for study samples. After 6 h, stability samples were compared with the freshly prepared equivalent concentrations (Table 4).

Freeze–thaw stability

Stability of EPT was studied in plasma samples subjected to three freeze–thaw cycles of – 20°C to mimic the intended sample handling conditions to be used during sample analysis. Three replicates of each QC sample were stored and frozen in the freezer at the intended temperature and thereafter thawed at room or processing temperature. After complete thawing, samples were refrozen again applying the same conditions. Samples were subjected to three cycles of freeze–thaw operations on 3 consecutive days and then compared with freshly prepared QC samples (Table 4).

Evaluation the stability of standard stock solution at room temperature

The standard stock solution was prepared and left at room temperature for 24 h and then used for spiking plasma samples at two QC concentrations at the low and high end of the range. The concentrations of the drug in the stability samples were compared with the fresh ones at equivalent concentrations (Table 4).

Long-term stability

Three replicates of each QC sample were subjected to freeze storage (−20°C.) for 60 days then were prepared and compared with the mean values of freshly prepared QC samples of the same concentrations (Table 4).

3.3.6 | Dilution effects

The integrity of the dilution was monitored during validation by diluting QC sample above the ULOQ (2.2 μg/ml) with plasma matrix to bring to within quantitation range and the accuracy and precision of these diluted QC samples were demonstrated. The recovered diluted concentrations fulfilled the acceptance criteria for accuracy and precision (Table 5).

TABLE 3 Recovery results for eptifibatide in spiked plasma samples

Concentration	%Recovery (pre- extraction/ post extraction × 100)[a]	% CV
0.15 μg/ml	96.16	5.59
0.9 μg/ml	96.30	4.78
1.7 μg/ml	93.04	6.49
Mean ± RSD		95.17 ± 1.94

[a]Average of three determinations.

TABLE 5 Dilution effect

Tested concentration (2.4 μg/ml)		
Diluted concentration nominal value (μg/ml)	Recovered concentration (μg/ml)	% Recovery
1 μg/ml	0.867582	86.76
1 μg/ml	0.902058	90.21
1 μg/ml	0.975919	97.59
1 μg/ml	0.894872	89.49
1 μg/ml	0.983017	98.30
Mean ± RSD		92.47 ± 5.59

TABLE 4 Results of stability studies of eptifibatide under different conditions

Stability condition	Nominal concentration (μg/ml)	Mean concentration recovered (μg/ml)	%Recovery[a]	%CV
Short-term stability (6 h)	0.15 μg/ml 1.7 μg/ml	0.15068 1.66523	100.45 97.96	6.75 8.36
Freeze and thaw (three cycles)	0.15 μg/ml 1.7 μg/ml	0.16197 1.85756	107.98 109.27	4.88 4.31
Stability of standard stock solution at room temperature for 24 h	0.15 μg/ml 1.7 μg/ml	0.15341 1.58018	102.27 92.95	7.11 8.49
Long-term stability (2 months)	0.15 μg/ml 1.7 μg/ml	0.14760 1.65660	98.40 97.45	5.57 5.74

[a]Average of three determinations.

3.4 | Analysis of marketed formulation (Integrilin® vial)

The suggested method was successfully applied for the determination of EPT in its pharmaceutical formulation (Integrilin® vial). The results were with good agreement with the labeled amount (Table 6). The results were favorably compared with the reported method[5] showing no significant difference (Table 7).

TABLE 6 Determination of eptifibatide in Integrilin® vial by the proposed spectrofluorimetric method

Drug	Claimed concentration taken (μg/ml)	Found concentration (μg/ml)	% Recovery[a]
Eptifibatide	0.5	0.50022	100.04
		0.50802	101.60
		0.50357	100.71
	7.5	7.3816	98.42
		7.3968	98.62
		7.5963	101.28
	1.00	0.98758	98.76
		1.01119	101.12
		0.99946	99.95
	1.75	1.78535	102.02
		1.77443	101.40
		1.78435	101.96
	2.25	2.23022	99.12
		2.21279	98.35
		2.27082	100.93
	Mean ± RSD		100.29 ± 1.33

[a]Average of three determinations.

TABLE 7 Statistical comparison of the results obtained by applying the proposed spectrofluorimetric method and the reported HPLC method for the determination of eptifibatide in its dosage form

Statistical term	Reference method**	Spectrofluorimetric method
Mean[a]	99.98	100.10
SD	0.71	1.37
SE	0.32	0.61
%RSD	0.71	1.37
n	5	5
V	0.5	1.88
t (2.306) *		0.17
F (6.39) *		3.76

*Figures in parentheses are the theoretical t and F values at (p = 0.05).

**HPLC method, mobile phase consisted of acetonitrile-water (0.13% trifluoroacetic acid).[5]

The determination was performed on AichromBond-AQ C18 column and detection wavelength of 220 nm.

[a]The mean for the average determination of five concentrations of dosage form (Integrilin® vial) repeated three times.

4 | CONCLUSION

A simple, fast and economic method was developed and validated for the assay of eptifibatide in human plasma and in the dosage form. The method can be applied to the analysis of eptifibatide in its pharmaceutical formulation in routine QC work without preliminary preparation steps such as conventional chromatographic methods.

ORCID

Sara Abdel Basset Galal http://orcid.org/0000-0001-8105-186X

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