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Highly Sensitive, Selective and Validated Spectrofluorimetric Assay for Novel Oxazolidinone Antibiotic: Tedizolid Phosphate in Pharmaceutical Dosage Form and Human Plasma

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ABSTRACT

Objectives: In this work we presented a highly sensitive, selective and validated method for determination of tedizolid phosphate (TEDP) antibiotic, based on its native fluorescence in aqueous solution. **Methods:** The maximum fluorescence intensity was measured at 408 nm after excitation at 298 nm after optimization of all experimental conditions. **Results:** The measured fluorescence was directly proportional to the concentration of the drug over the range of 2-30 ng/mL with a limit of detection of 0.13 and limit of quantification of 0.44 ng/mL The method succeeded to determine TEDP in its pharmaceutical dosage form and in human plasma with mean % recovery of 100.49 ±1.32 and 99.40 ± 2.18 respectively. **Conclusion:** The developed data was found to be with a good agreement with a valid method. The method was validated according to ICH guidelines for determination of the drug in its pure form and dosage form and according to FDA Guidance for Industry, Bioanalytical Method Validation for determination of TEDP in human plasma.

Keywords: *Tedizolid phosphate; Oxazolidinone antibiotic; Dosage form; Human plasma; Spectrofluorimetry.*

INTRODUCTION

Tedizolid phosphate (TEDP) is one of novel antibiotics which has a chemical name of [(5R)-(3-{3-Fluoro-4-[6-(2-methyl-2H-tetrazol-5-yl) pyridin-3-yl] pheny-2-oxooxazolidin-5-yl] methyl hydrogen phosphate **Figure 1**. It has a chemical formula of $C_{17}H_{16}FN_6O_6P$ and a molecular weight of 450.32 g mole^{-1} [1]. It is non official drug but FDA approved for treatment of acute bacterial skin and skin structure infections (ABSSSI) caused by susceptible isolates. It is microbiologically inactive oxazolidinone prodrug converted in vivo by plasma phosphatases to its active moiety free tedizolid (TED). TED reaches maximum concentration (C_{max}) of 2 µg/mL after 2 hr from oral administration [2]. Since TED is a newly developed drug, the need to develop a new and reliable analytical method for quantitative determination of the studied drug has been raised greatly. To the best of our knowledge, only few methods had been reported related to quantification of TEDP. Those approaches include; Enantioseparation by RP-HPLC[3], chiral separation using capillary electrokinetic chromatography[4] and UPLC-MS/MS assay for identification of its forced degradation products[5]. No spectrofluorimetric assay had been reported for the drug which encouraged us to develop and validate a new,

rapid, simple, and economic method for analysis of the drug in its dosage form and in human plasma with comparable sensitivity to the reported methods.

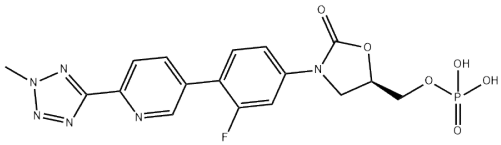

Figure. 1. Chemical structure of tedizolid phosphate ($C_{17}H_{16}FN_6O_6P$).

MATERIALS AND METHODS

Apparatus

- JASCO FP 6200-Spectrofluorimeter with a 150 W xenon lamp and a1 cm quartz cell Excitation and emission slits width were both adjusted to 10 nm. Spectra were evaluated using Spectra Manager FP-6200 Control Driver software, Version1.54.03 [Build 1], JASCO Corporation.
- Bench- top sonicator, USA.
- Precision scientific incubator, USA.
- pH bench-top meter HANNA instruments, USA.
- Vortex mixer, USA.

Materials and reagents

- TEDP reference material purity (99.8%) batch no. (19041107321) was kindly obtained from Hikma pharmaceutical co. (Giza, Egypt.), used as it is.
- Market samples were purchased from local market, Tedimerp film coated tablet (200 mg TEDP / tablet) batch no. (192316) with manufacture date of (5/2019) and expiration date of (5/2021), Hikma Pharmaceutical Co. (Giza, Egypt)
- Sodium dodecyl sulphate (SDS), tween 20 and Cetrimonium chloride (Riedel–deHaen, Germany) 1.0% (W/V) aqueous solutions were prepared.
- β-Cyclodextrin (β-CD) (Merck, Germany), 0.5% (W/V) aqueous solution was prepared.
- 1 x 10^{-2} M phosphate buffer with pH value (2-10), prepared according to USP pharmacopeia.
- Hydrochloric acid (Sigma, Germany) 1.0 M aqueous solution was prepared.
- Fresh frozen human plasma samples were obtained from VACSIRA (Cairo, Egypt) and were kept frozen until use after gentle thawing in room temperature.

All organic solvents used in the method (methanol, ethanol, acetonitrile and ethyl acetate) are of HPLC grade (99.9%). Double Distilled water was used through all the method.

Standard stock solutions

A stock standard solution of 100 µ/mL TEDP was prepared by accurate weighing 25.0 mg of the drug, dissolving in water of 60 ˚C for 5 minutes, cooling and accurately transferred to 250-mL volumetric flask. The volume was adjusted using double distilled water. A serial dilution of the standard stock solution was applied to obtain a working standard solution of 50 ng/mL.

Procedures

Construction of the calibration graph for pure drug

Aliquots of working standard solution equivalent to 20-300 ng TEDP were transferred into 10-mL volumetric flasks, the volume was adjusted using double distilled water to give a final concentration covering the range 2-30 ng/mL. The fluorescence spectra were measured at λ_{em} 408 nm after excitation at 298 nm. Blank experiments were applied in the same manner. The response as fluorescence intensity was plotted against the final drug concentrations (ng/mL) to construct the calibration graph followed by computing the regression equation.

Application of the proposed method for assay of TEDP in film coated tablets

Ten tablets (Tedimerp 200 mg TEDP /tablet) were weighed grinded to a fine powder. The average weight of one tablet was calculated and a weight equivalent to 25.0 mg was dissolved in hot water at 60 ˚C by sonication for 60 minutes, followed by cooling then accurately transferred into 250-mL volumetric flask then volume was adjusted using double distilled water. The solution was filtered using 0.45 µm disposable syringe filter. Aliquots of filtered solution were diluted as previously mentioned for (Construction of the calibration graph for pure drug). The concentration of the dosage was conducted from previously constructed calibration curve or regression equation.

Construction of the calibration graph for TED in spiked human plasma

Aliquots of 500 µL of carefully room temperature-thawed human plasma were transferred into a series of centrifugation tubes, spiked with 0.5 mL of working solution equivalent to 20-350 ng, and then incubated at 37˚C for 2 hr. A 500 µL aliquot of 1.0 M hydrochloric acid were added to stop the reaction of phosphatases [6]. The samples were extracted by mixing with 2 x 2 mL of ethyl acetate for 2 min using vortex, then centrifuged at 6000 rpm for 30 min. The combined ethyl acetate extract were collected in small beakers and evaporated. The residue was reconstituted with distilled water and quantitatively transferred into 10 mL volumetric flask [7]. The fluorescence intensity plotted against final concentration TEDP to construct the

calibration curve and the corresponding regression equation was conducted.

RESULTS AND DISCUSSION

Fluorescence spectral characterization

Since no previously spectrophotometric nor spectrofluoremetric approaches had been reported, spectral characterization for emission and excitation peaks were recorded. It was found that only one characteristic excitation peak at 298 nm was observed as shown in **Figure 2**. At excitation 298 nm; fluorescence spectra of studied drug showed only one characteristic emission peak at 410 nm.

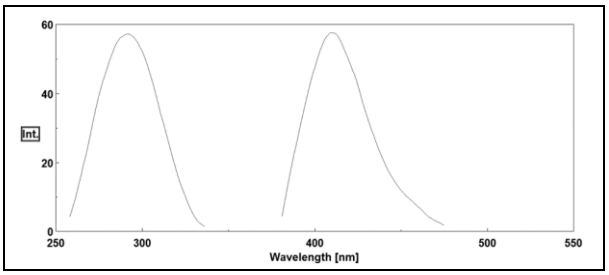

Figure 2. Excitation and emission spectra for 2 ng /mL TEDP in aqueous solution.

Optimization of the Experimental Conditions

At λ_{ex} 298 nm and λ_{em} 408 nm, different experimental factors that affect fluorescence intensity of studied drug were carefully studied and optimized. Those factors include; different diluting solvents, effect of pH (2-10), and effect of surfactants. Each factor was individually changed, while other factors were kept constant. All factors were studied for blank experiments as well as for solutions of studied drug.

Effect of diluting Solvents

Different diluting solvents like water, methanol, ethanol and acetonitrile were tried to determine their effect on the response as represented in **Figure 3**. The FI of the drug in ethanol and acetonitrile showed slight increase in the fluorescence intensity but no increase was observed using methanol in comparison with aqueous solution of studied drug. Despite organic solvents showed higher fluorescence signal, water was the diluting solvent of best choice for detailed spectrofluoremetric study. No one can forget that water is readily available, economic, and non-hazard solvent. This achieves the aim of proposed method to develop a highly sensitive method without using any organic solvents.

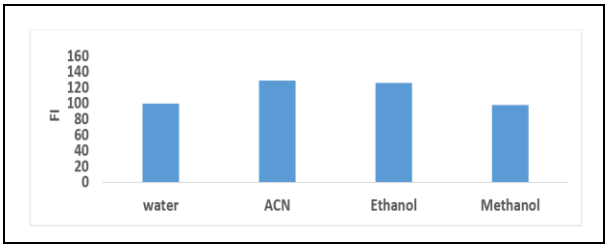

Figure 3. Effect of diluting solvents on the FI of 5ng/mL TEDP solution.

Effect of fluorescence enhancers

It had been reported that several fluorescence enhancers were used to test their effect on the fluorescence signal of many fluorescent substrates, so different types of surfactants were tried; SDS (anionic), tween 20 (aionic) and Cetrimonium chloride (cationic). Also, it had been known that β-CD (solubilizing agent) also used as fluorescence enhancer, so it was tried to determine its effect on the FI of TEDP as shown in **Figure 4**: Cetrimonium chloride caused a significant decrease (59.62%) in the FI while SDS and tween 20 showed no remarkable effect on the FI. On the other hand, β-CD showed remarkable increase (145.99%) in FI. This increase may be attributed to inclusion of studied drug into macromolecule of β-CD. This enhancement in the FI may be resulted from decrease in deactivation energy loss due to molecular collision hence remarkable increase in the signal. Depending on those results, several conditions like: concentration of β-CD, time of mixing and temperature of the solution were tested but it was found that the reaction was not quantitative, also the method achieved fair sensitivity with highly low limit of detection (LOD) and limit of quantification (LOQ) without using it. So aqueous solution without using β-CD was chosen for detailed study.

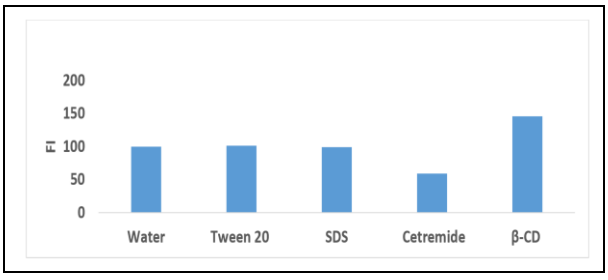

Figure 4. Effect of fluorescence enhancers on the FI for 5ng/mL TEDP aqueous solution.

Effect of pH

1 x 10^{-2} M phosphate buffer with pH range from 2 to 10 was used to determine the effect of pH on the

fluorescence as represented in **Figure 5**. It was found that there is no significant increase in FI at any studied pH value so, there is no need to use a buffer solution to adjust pH of the media.

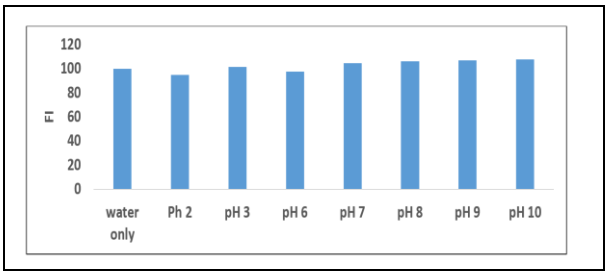

Figure 5. Effect of pH on the FI for 5ng/mL TEDP solution.

Method validation

After optimization of all experimental conditions, the validity of the method was tested according to ICH guidelines for the direct determination of TEDP in its pure form and dosage form [8]. FDA Guidance for Industry, Bioanalytical Method Validation was used for the assay of TED metabolite in human plasma [9].

Linearity and Range

For pure drug TEDP: Under the optimized experimental conditions it was found that there is a linear relationship between TEDP and its FI in aqueous solution over the concentration range of 2-30 as shown in **Figure 6** (**A, B**) at 408 nm after excitation at 289 nm. The regression equation for determination of TEDP in pure form was found to be:

$$FI = 29.81\ C + 8.5529\ (R^2{=}0.9999)$$

Where C is the TEDP concentration in ng/mL in aqueous solution.

The limit of quantitation (LOQ) and the limit of detection (LOD) were calculated according to ICH guidelines for TEDP. Also, the accuracy and precision of the method was evaluated using percent relative error and standard deviation respectively. The results were shown in Table 1. $LOQ = \frac{10\sigma}{S}$, LOD $= \frac{3.3\sigma}{S}$ Where $\boldsymbol{\sigma}$ = the standard deviation of response and $\boldsymbol{S}$ = slope of the calibration curve.

For human plasma: All pharmacokinetic studies demonstrated that tedizolid phosphate is a prodrug converted in vivo into the free drug metabolite tedizolid by the action of plasma phosphatases, which is the active moiety. Free Tedizold was confirmed to be the only measurable metabolite in plasma after oral and intravenous administration [6]. So, we designed a method to determine the drug metabolite in human plasma (calculated as tedizolid phosphate) depending on the action of plasma phosphatase enzymes. FDA Guidance for Industry, Bioanalytical Method Validation was used for the assay of TED metabolite in human plasma [9].

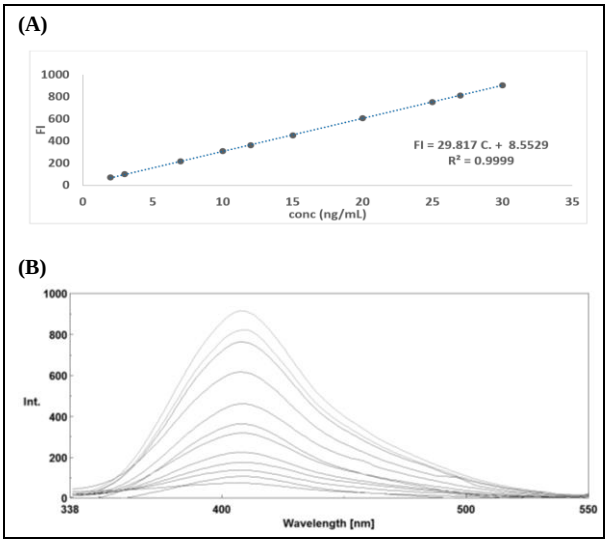

Figure 6. (A) Calibration curve of 2-30 ng /mL aqueous solution of TEDP. (B) Emission spectra of aqueous solution of TEDP 2-30 ng/mL.

The constructed calibration curve met the acceptance criteria specified from FDA guidance for Industry, Bioanalytical Method Validation which states that 75% of non-zero standards shouldn't deviate by more than 15% nominal concentrations except at lower limit of quantification (LLOQ) where the standard shouldn't deviate by more than 20%. The linearity was evaluated using six non-zero samples covering the concentration range 2-35 ng /mL as shown in **Figure 7**. The LLOQ was found 2 ng/mL with coefficient of variation (CV %) 3.07.

$$CV\% = \left(\frac{SD\ of\ the\ six\ determinations}{mean}\right) x\ 100$$

Where *FI* was measured at 408 nm after excitation at 289 nm. As shown in **Figure 7**: the regression equation for determination of TEDP in plasma was found to be: $FI = 12.857C + 133.51$ (R^2=0.9998) Where *C* is the TEDP concentration in ng/mL in spiked human plasma. All regression parameters were listed in **Table 1**.

Accuracy

The results of the assay of TEDP in pure form were compared with previously reported method using RP-HPLC /UV technique [3]. The Statistical comparison of results obtained by the proposed method and those obtained by the reported one revealed accuracy and precision of the method using mean recoveries, students t-test and variance ratio f-test as shown in **Table 2**.

Table 1. Validation parameters of TEDP in aqueous solutions and in human plasma

Parameter	Results of TEDP in pure form	Results of TED in human plasma
Range (ng/mL)	2.0-30.0 ng/mL	2.0-35.0 ng/mL
LOD[a]	0.13	
LOQ[a]	0.44	
Correlation co-efficient	0.9999	.9999
Slope	29.81	12.86
Intercept	8.56	133.51
Standard deviation (S.D)	1.32	2.18
Relative standard deviation (R.S.D)	1.31	2.19
Standard error	0.40	
% Error	0.40	
LLOQ[b]		2.0 ng/mL

[a]calculated as ICH Guidelines. [b]calculated as FDA Guidelines.

Table 2. Statistical comparison of the results of the proposed method and validated report method (RP-HPLC/ UV) for determination of TEDP assay in pure form

Parameter	Proposed method	Comparison method[b]
	% recovery	% recovery
	99.80	101.67
	100.10	99.60
	99.33	100.43
	100.87	99.89
	100.43	101.33
Mean	100.11	100.58
± S.D	0.52	0.80
No. of experiments	5	5
variance	0.34	0.80
F-test	0.43	(6.39)[a]
T-test	0.35	(2.306)[a]

[a]tabulated F and t values at P=0.05. [b] RP-HPLC /UV, Phenomenex Luna, Phenyl-Hexyl, 250x 4.6mm, 5µm HPLC column, mobile phase consisting of mixture of aqueous buffer of ph 7 of disodium hydrogen phosphate with β-CD and triethylamne with acetonitrile, flow rate of 1.0 mL/min and gradient elution, wavelength detection at 300 nm.

Table 3. Repeatability and reproducibility of the proposed method for the determination of TEDP in pure, dosage form

Precision	Conc.(ng/mL)	Pure form (%recovery)	Dosage form (%recovery)
Intra-day	5	101.42 ± 1.30	99.15 ± 3.51
	15	99.33 ± 1.03	99.09 ± 1.75
	30	99.33 ± 0.63	100.18 ± 0.83
Mean ± S.D		100.03 ± 0.99	99.47 ± 0.50
Inter-day	5	100.31 ± 2.11	102.00 ± 2.85
	15	100.13 ± 2.25	102.29 ± 3.00
	30	99.18 ± 1.12	101.12 ± 3.05
Mean ± S.D		99.87 ± 0.61	101.80 ± 0.58

Each result listed in table is the average of three different measurements.

Table 4. Repeatability and reproducibility of the proposed method for the determination of TEDP in spiked human plasma

Precision	Conc.	% Recovery	CV%
Intra-day[a]	6	101.16	1.64
	15	99.65	1.96
	28	101.64	1.93
Inter- day[b]	6	100.95	3.56
	15	100.44	1.38
	28	102.83	2.21

[a]each result is the average of six different measurements per concentration on one day. [b]each result is the average of six different measurement per concentration repeated three times in three different days.

Table 5. Results of the proposed method for the determination of TEDP in its dosage form and results of standard addition technique

Amount taken	Amount of standard added	% Recovery found
(ng/mL)	(ng/mL)	
5	0	98.51
	5	95.91
	10	98.29
Mean		97.57
± R.S.D		1.47

Each result is the average of three different measurements.

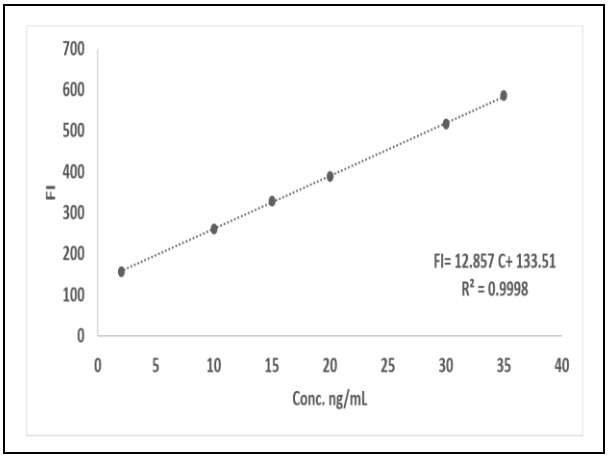

Figure 7. Calibration curve of 2-35 ng /mL TEDP in human plasma.

Repeatability and precision

For pure drug; the repeatability of the method was tested by replicate analysis of the drug in pure form and dosage form using three different concentrations (5, 15, 30) ng/mL on one day to determine intra-day precision and on three different days to determine inter-day precision **Table 3**.

For human plasma: the repeatability was tested by replicate analysis of quality control samples (low, mid, high QC) using 6 determinations for each concentration on one day to determine intra-day precision and on three different days to determine inter-day precision **Table 4**.

Selectivity

The ability of the method to determine TEDP in pharmaceutical preparation without any interference from excipients or preservatives that may be present in the drug formulations was proved by applying the experiment on a placebo sample. **For human plasma**: six Blank plasma samples were analyzed and compared to plasma samples spiked with LLOQ and no peaks found proving the selectivity of the proposed method.

Robustness of the method

The constancy of the Fl using minor changes in the experimental conditions, like wavelength of excitation 298 ± 2 nm revealed the robustness of the method and this minor change did not affect the FI.

Application

Applying the proposed method after sample pre-treatment as discussed before as mentioned in section 2.4.2 was able to determine TEDP in its pharmaceutical dosage form (Tedimerp 200 mg-TEDP-film coated tab) as shown in Table 3. Standard addition technique was applied to determine the ingredients effect and its role in the deviation of the results obtained by the proposed methods. No significant matrix effect was proved as shown in **Table 5**. Also, the method succeeded to determine the drug metabolite in human plasma without any interference detected during analysis of the sample QCs as shown in **Table 4**.

CONCLUSION

The developed method succeeded to introduce first spectrofluoremetric approach for quantification of TEDP. The method is simple, rapid, highly sensitive, accurate and precise technique applied for determination of tedizolid phosphate in its pure, dosage formula and human plasma. Beside that it had the advantage of using aqueous solvent which is economic and non-associated with any environmental hazards over the previously reported method. The results showed the validity of the method.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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