A Recent Validated Synchronous Spectrofluorimetric Method in Comparative to Ratio Derivative One for Determination of Desmopressin Acetate in Dosage Form and Biological Fluids

Khadiga M. Kelani1,2, Ahmed M.Wafaa Nassar2, Wael Talaat1, Samir Morshedy3

1Pharmaceutical Analytical Chemistry Department, Cairo University, Cairo, Egypt; 2Pharmaceutical Analytical Chemistry Department, Modern University for Technology and Information (MTI), Cairo, Egypt; 3Pharmaceutical Analytical Chemistry Department, Damanhour University, Cairo, Egypt

ABSTRACT
In this study green, rapid, specific and highly sensitive methods have been developed and validated for the determination of desmopressin acetate in pharmaceutical formulations and biological fluids (spiked human plasma).

Second derivative synchronous spectrofluorimetric method

Ratio derivative spectrophotometric method: Sensitivity ranges of (0.25–2.25 μg/ml) and (2–14 ug/ml) and LOD and LOQ values were found to be (0.060-0.183 ug/ml), (0.049-0.162 ug/ml), respectively. The developed methods were validated according to the (ICH) guidelines demonstrating good accuracies and precisions. The results of the developed methods were statistically compared with those obtained by the reported methods without any significant difference. Both methods were applied in quality control laboratories and in routine analysis for analyzed the drug in presence of its acidic degradation products (stability indicating assay and in biological fluids).

Keywords: Desmopressin acetate; Second derivative synchronous spectrofluorimetry; Ratio derivative spectrophotometry and acidic degradation product.

Abbreviation: Desmopressin Acetate (DA); Limit of Detection (LOD); Limit of Quantification (LOQ)

INTRODUCTION
Desmopressin acetate (DA) is [acetic acid-(2S)-N-{(2R)-1-{(2-amino-2-oxoethyl)] amino}-5,(diaminomethylideneamino)-1-oxopentan-2-yl}-I[(4R,7S,10S,13S,16S)-7-2-amino-2-oxoethyl]-10-(3-amino-3-oxopropyl)-13-benzyl-16-(4-hydroxyphenyl) methyl]-6,9,12,15,18-penta(oxy)-1,2-dithia-5,8,11,14,17-pentaza-cycloicosane-4-carbonyl|pyrrolidine-2-carboxamide} [1] (Figure 1). This medicament is usually used in treatment of diabetes insipidus, bedwetting, haemophilia A, and elevated levels of urea in the blood [2]. The literature survey shows that many methods for quantitative determination of desmopressin acetate have been carried out; including HPLC methods [3-14], spectrophotometric methods [15] and electrochemical methods [16-21]. The stability study of pharmaceutical formulation affects the safety and efficacy of the drug product [22,23]. Based on that, there is no stability indicating methods have been reported before. As the drug can be forced degraded in acid medium (Figure 2), Consequently, It is important to arise a stability indicating methods. The literature review reveals that there is no chemical stability study performed on DA in acidic degradation product before. Therefore, the aim
of the present work was to establish green, highly economic and sensitive synchronous second derivative spectrofluorimetric and UV ratio derivative spectrophotometric methods for the estimation of desmopressin acetate in presence of its acidic degradation product and to make comparative study between both methods.

Figure 1: Structural formula of Desmopressin acetate.

As the spectrofluorimetric method was show native fluorescence spectra of drug and its acidic degradation product which are overlapped to each other, that need to make synchronous spectra then deriving the second derivative as the first derivative was not suitable, also UV ratio derivative spectrophotometric method was successfully applied, the results of the developed methods were compared and validated according to the (ICH) guidelines, so it is possible to consider it as one of the most effective methods for analysis of desmopressin acetate in pharmaceutical industry.

MATERIALS AND METHODS

Instruments
Jasco FP6200 spectrofluorometer (Japan), equipped with 150 Watt Xenon lamp. Slit widths for both monochromators were set at 10 nm. A 1 cm quartz cell was used. Shimadzu UV-Vis. 1650 Spectrophotometer (Japan). Hot plate (Torrey pines Scientific, USA). Jenway, 3510 ph meter (Jenway, USA). Rotatory evaporator (Scilogex-RE 100-pro, USA). FT-IR, Nicolet IR 200 (Thermo electron corporation, USA). GCMS-QP-1000 EX mass spectrometer (Shimadzu, Tokyo, Japan).

Materials
Reagent: Acetonitrile, ammonia (30%), chloroform, ethanol, methanol, 1-propanol and tetrahydrofuran, all of HPLC grades (Sigma-Aldrich, Germany). Hydrochloric acid and potassium hydroxide (El-Nasr Company, Egypt), Ammonium acetate (El-Nasr Company, Egypt), potassium chloride, potassium biphthalate, sodium acetate, monobasic potassium phosphate, boric acid, glacial acetic acid and sodium hydroxide (El-Nasr Company, Egypt). Acetate buffer of different ph values prepared as prescribed in US pharmacopeia. And the blank plasma samples were obtained from a healthy volunteer. All reagents used were of analytical grade, solvents were of HPLC grade and water used throughout the procedure was freshly distilled.

Pure sample: Pure desmopressin acetate was kindly provided by Omega pharmaceutical industries - El-Mearag City, Zahraa El-Maadi, in Cairo - Egypt with Purity-101.5% according to the official method.

Market sample: Omegapress® tablet labeled to contain 0.1 mg desmopressin acetate per tablet manufactured by Omega pharmaceutical industries - El-Mearag City, Zahraa El-Maadi, in Cairo - Egypt. (batch number 40357), purchased from local market.

Degraded sample: It was prepared by dissolving 100 mg of pure DA powder in 25 ml of 0.1 M hcl in a 100-ml round bottomed flask, the solution was heated at 60 oc under reflux for 6 hours. Finally, the solution was evaporated to dryness under vacuum and the obtained residue was extracted with 20 ml ethanol, filtered into a 100-ml volumetric flask and diluted to volume with water to obtain a stock solution labeled to contain 1 mg/ml acidic degradation product. Working solutions of DA acidic degradation product (10 μg/ml) was obtained by further dilution of the stock solution with water.

Standard Solutions
Stock solution (1 mg/ml): Amount equivalent to 100 mg of DA powder was accurately weighted and transferred into 100 ml volumetric flask, 50 ml water was added, shaken, and diluted to volume with water.

Working solution: Working solution (10 μg/ml) for synchronous second derivative prepared by transferring 1 ml of stock solution into 100 ml volumetric flask and the volume were completed to the mark by water, and for ratio derivative method working solution (100 μg/ml) prepared by transferring 10 ml of stock solution into 100 ml volumetric flask and the volume were completed to the mark by water.

Procedures
General procedure: Synchronous second derivative spectrofluorimetric method.

A) Spectral characteristics data: Desmopressin acetate exhibits native fluorescence and its emission could be measured at 340 nm after excitation at 288 nm. However there was fluorescence for the degradation product which makes interference at 340 nm leads to difficulty in direct determination of desmopressin acetate, the recorded overlapping between the emission spectra of the drug and its acidic degrade, hindered the application of the direct native fluorescence technique for selective determination of desmopressin acetate in the presence of its acidic degradation product. This band overlapping could be resolved by measuring the synchronous fluorescence at Δλ=60 nm, using data points=9. The resulted second derivative synchronous fluorescence spectra of desmopressin acetate were well scanned and separated and at 347 nm without any interference from its acidic degradation product.
B) Construction of Calibration Curve: By taken portions of (0.25-2.25 ml) of DA standard working solution (10 μg/ml) and transferred into a series of 10-ml volumetric flasks in addition of 1 ml of acetate buffer (ph 6). The content of each flask was completed with the water to volume to get a final concentration of [0.25-2.25 μg/ml] of DA. And the calibration curve representing the relation between peak amplitude and the corresponding concentrations was constructed, and the regression equation was derived.

Derivative ratio spectrophotometric method (DD1): A) Spectral characteristics data: The zero order absorption spectra of DA (10 μg/ml) and its acidic degradation product (10 μg/ml) were recorded against methanol as blank over the range of 200 - 400 nm. For the determination of DA in presence of its degradation product, the Smoothed ratio spectra of DA is divided by the spectrum of (10 μg/ml) degradate, Then the first derivative of the ratio spectra (DD1) with Δλ=4 nm and scaling factor 10 was found and the peak amplitude was chosen at 236 nm.

B) Construction of Calibration Curve: Portions of [0.2-1.4 ml] of DA standard working solution [100 μg/ml] were transferred to a series of a _10 ml volumetric flasks and completed with the water to the volume to get a final concentration of [2-14 μg/ml] of DA. And the calibration curve representing the relation between peak amplitude and the corresponding concentrations was constructed, and the regression equation was derived.

Application to laboratory prepared mixtures: Laboratory prepared mixtures containing different ratios (10-90%) of DA and its acidic degradation product were analyzed using the recommended methods, aliquots of DA and its acidic degradation product were mixed to prepare different mixtures and were proceeded as mentioned under each method, the concentrations from the corresponding regression equations were calculated.

Application to pharmaceutical formulation: For both Methods, Ten Omegapress® tablets (0.1 mg/tablet) were weighted and finely powdered. Appropriate weight of powder equivalent to 1 mg of DA was accurately weighted, transferred to 10 ml volumetric flask and the volume was made up to 7.5 ml with water. The solution was shaken vigorously for 15 min then sonicated for 30 min and then filtered. The volume was completed to 10 ml with water to obtain a concentration of 100 μg/ml. Then repeat the general procedure using aliquots covering the working concentration range.

Application to spiked human plasma: Two portions of (0.2 and 0.4 ml) of DA standard working solution (4 μg/ml) were transferred into a 10-ml centrifuging-tube, followed by 1 ml of human plasma and vortexed for 20 seconds. Then 1.5 ml of acetonitrile was added to precipitate the plasma proteins, vortexed for 30 second, followed by addition of 2 ml methanol, vortexed again for 1 min and then centrifuged at 3000 rpm for 5 minutes. The supernatant was evaporated to dryness; the residue was reconstituted with the least amount of methanol, vortexed for 20 seconds and transferred into 10-ml volumetric flasks, the content of each flask was completed with the water to the volume to get a final concentration [2.4 μg/ml] of DA.

RESULTS AND DISCUSSION

The stability study of pharmaceutical formulation is a matter of concern as it affects the safety and efficacy of the drug product, in addition to awareness of the drug’s stability aids to select the correct formulation and package, also to provide the right storage conditions and shelf life, which are needed for regulatory documentation.

DA is liable to acidic hydrolysis where complete degradation was obtained after reflux with 0.1 M hcl for 6 hrs. (Figure 2). The obtained degradant was separated by TLC on silica gel GF254 plates, using Methanol: Water (80: 20 by volume) as developing solvent. The structure of the acidic degradation was clarified by IR and mass spectroscopy.

![Figure 2: Suggested pathway of desmopressin acetate degradation.](image)

Figures 3 and 4 show that the IR peak at of amino group (-NH) at 3340.31 cm⁻¹, while IR spectrum of degradation product, showed disappearance of (-NH) stretch of amino group which indicate the cleavage of structure.

![Figure 3: IR spectra of desmopressin acetate.](image)
The mass spectrum Figure 5 showed the life of a peak at m/z 1069.22 corresponding to DA acidic degradation represented in the same figure. Reviewing the literature in hand shows that there is no analytical method is reported for the determination of DA in presence of its acidic degradant.

Therefore, the aim of this work was to develop and validate stability indicating methods for the determination of DA in pure form and in pharmaceutical form.

Synchronous Second Derivative Spectrofluorimetric Method

Synchronous fluorescence spectroscopy (SFS) involves the simultaneous scan of the excitation and emission monochromators. Depending on the scan rate, three basic types of SFS technique are possible: constant-wavelength, constant-energy and variable-angle. Constant-wavelength SFS is the basic type and the most widely used of all synchronous modes, where a constant wavelength interval was kept between the excitation and emission monochromators. Synchronous fluorescence spectroscopy has several advantages over conventional fluorescence, including; narrowing of spectral band, simplification of emission spectra and contraction of spectral range.

The sharpness and narrowness of the peak of a SFS spectrum, compared to those of conventional spectrum, makes it more selective and useful to analyze multi-component mixtures without pre-separation procedures.

Desmopressin acetate exhibits a native fluorescence and its emission could be measured at 340 nm after excitation at 288 nm (Figure 6). However, in presence of acidic degradates there was fluorescence for the degradation product which makes interference at 340 nm leads to difficulty in direct determination of desmopressin acetate. The recorded overlapping between the emission spectra of the drug and its acidic degraded hindered the application of the direct native fluorescence technique of drug and its degrade (Figure 7). Which could be overcome by measuring the second derivative synchronous fluorescence at 347 nm using Δλ=60 nm and scaling factor=10 (F 8 and 9), as the first derivative was not suitable.
Figure 8: Synchronous fluorescence spectra of (A) desmopressin acetate (2 μg/mL) and (B) desmopressin acetate acidic degradation product (2 μg/mL), in water using Δλ=60 nm.

Figure 9: Second derivative synchronous fluorescence spectra of (A) desmopressin acetate (2 μg/mL) and (B) desmopressin acetate acidic degradation product (2 μg/mL) in water using Δλ=60 nm.

The resulted second derivative synchronous fluorescence spectra of desmopressin acetate and its acidic degradation product were well separated and allow the selective quantification of desmopressin acetate at 347 nm without any interference from its acidic degradation product (Figure 10).

Under the described experimental conditions, the calibration graph for the method was constructed by plotting the amplitudes of the second derivative of the synchronous spectra at 347 nm versus drug concentrations in (μg/ml). And a linear response was obtained, regression equations were found to be:

\[ P_{370 \text{ nm}} = 1.051C + 0.8516 \quad r=0.9998 \]

Where C is the concentration of desmopressin acetate in μg/ml, P is the trough amplitude of the synchronous second derivative spectrofluorimetric spectrum curve at 347 nm, respectively and r is the correlation coefficient. And the limit of detection (LOD) was (0.060 μg/ml) while limit of Quantitation (LOQ) was (0.183 μg/ml).

Finally, this method is very sensitive and can be used as purity test for determination of the DA in biological fluids and concentration of drug in the plasma as the Cmax. Of DA is (4 μg/ml).

**Derivative Ratio Spectrophotometric Method (DD1)**

Spectrophotometric techniques were investigated for this purpose to solve the problem of overlapping absorption spectra of DA and its acidic degradant that found in zero order absorption spectra of DA (10 μg/ml) and its acidic degradation product (10 μg/ml) as shown in Figure 11, by the DD1 method. For the determination of DA in presence of its degradation product, the stored spectra of DA is divided by the spectrum of (10 μg/ml) degradate, smoothed with Δλ=16 nm and scaling factor 10, Then the first derivative of the ratio spectra (DD1) with Δλ=4 nm is obtained.

Figure 10: Second derivative synchronous fluorescence spectra of desmopressin acetate (0.25-2.25 μg/mL) in water using Δλ=60 nm.

Figure 11: Zero-order absorption spectra of intact desmopressin acetate (10 μg/mL) (―) and its degradation product (10 μg/mL) (…..) in methanol.

The amplitude of the first derivative trough of (DA/degradate) is measured at 236 nm (Figure 12), and a linear response was obtained, regression equations were found to be:

\[ P_{236 \text{ nm}} = 0.15657C - 0.00329 \quad r=0.9998 \]
**Table 1**: Determination of desmopressin acetate in mixtures with its degradation product by the proposed second derivative synchronous spectrofluorimetric method and UV derivative ratio spectrophotometric method.

<table>
<thead>
<tr>
<th>Intact (μg/mL)</th>
<th>Degradate (μg/mL)</th>
<th>% Degradate</th>
<th>Intact found (μg/mL)</th>
<th>% Recovery of Intact</th>
<th>Intact (μg/mL)</th>
<th>Degradate (μg/mL)</th>
<th>% Degradate</th>
<th>Intact found (μg/mL)</th>
<th>% Recovery of Intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.25</td>
<td>0.25</td>
<td>13</td>
<td>2.24</td>
<td>99.55</td>
<td>12</td>
<td>2</td>
<td>14.29</td>
<td>11.91</td>
<td>99.28</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>38</td>
<td>1.49</td>
<td>99.33</td>
<td>10</td>
<td>4</td>
<td>28.57</td>
<td>10.1</td>
<td>101.01</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>63</td>
<td>0.99</td>
<td>99</td>
<td>8</td>
<td>6</td>
<td>42.86</td>
<td>7.95</td>
<td>99.4</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>75</td>
<td>0.49</td>
<td>98</td>
<td>6</td>
<td>8</td>
<td>57.14</td>
<td>5.94</td>
<td>98.96</td>
</tr>
<tr>
<td>0.25</td>
<td>2.25</td>
<td>90</td>
<td>1.01</td>
<td>100.92</td>
<td>4</td>
<td>10</td>
<td>71.43</td>
<td>4.02</td>
<td>100.64</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>98.97</td>
<td></td>
<td></td>
<td></td>
<td>99.66</td>
<td></td>
</tr>
<tr>
<td>% RSD</td>
<td></td>
<td></td>
<td></td>
<td>0.455</td>
<td></td>
<td></td>
<td></td>
<td>0.916</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**: Recovery study of desmopressin acetate by adopting standard addition technique using the proposed second derivative synchronous spectrofluorimetric method and UV derivative ratio spectrophotometric method.

<table>
<thead>
<tr>
<th>Found*%</th>
<th>Pure added (μg/mL)</th>
<th>Pure found (μg/mL)</th>
<th>% Recovery</th>
<th>Found*%</th>
<th>Pure added (μg/mL)</th>
<th>Pure found (μg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.48</td>
<td>98.4</td>
<td></td>
<td>2</td>
<td>1.99</td>
<td>99.71</td>
<td></td>
</tr>
<tr>
<td>99.12%</td>
<td>1.5</td>
<td>1.49</td>
<td>99.35</td>
<td>100.02%</td>
<td>4</td>
<td>3.98</td>
<td>99.39</td>
</tr>
<tr>
<td>2</td>
<td>1.95</td>
<td>98.23</td>
<td></td>
<td>6</td>
<td>5.95</td>
<td>99.23</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>98.66</td>
<td></td>
<td></td>
<td></td>
<td>99.44</td>
</tr>
<tr>
<td>% RSD</td>
<td></td>
<td></td>
<td>0.611</td>
<td></td>
<td></td>
<td></td>
<td>0.246</td>
</tr>
</tbody>
</table>

**Pharmaceutical Applications**

All the proposed methods were successfully applied for the determination of DA in pharmaceutical dosage form. The validity of the methods was further assessed by application of the standard addition technique, (Table 2). A statistical comparison of the results achieved by the proposed methods and the reported method [24-25] is shown in Table 3. Calculated (t-and F-values) are less than the tabulated values, which reveals that there is no significant difference with respect to accuracy and precision between the proposed methods and the reported method.

The results of assay validation of the proposed methods show that they are accurate, precise, specific, and rugged according to the RSD values of intraday and interday determinations (Table 4).
Table 3: Determination of desmopressin acetate in Omegapress® tablet by the proposed second derivative synchronous spectrofluorimetric method and the reported method and UV derivative ratio spectrophotometric method.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Second derivative synchronous spectrofluorimetric method</th>
<th>UV Derivative ratio spectrophotometric method</th>
<th>Reported method* [4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of measurements</td>
<td>5</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Mean % recovery of desmopressin acetate</td>
<td>99.12</td>
<td>100.02</td>
<td>100.6</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.929</td>
<td>0.64</td>
<td>0.77</td>
</tr>
<tr>
<td>Student’s t-test**</td>
<td>1.042 (2.306)</td>
<td>0.401 (1.812)</td>
<td>—</td>
</tr>
<tr>
<td>F-value**</td>
<td>2.173 (6.388)</td>
<td>1.230 (6.161)</td>
<td>—</td>
</tr>
</tbody>
</table>

*Reported method is using first derivative spectrophotometric method with zero crossing point at 232.6 nm.
**The values in parenthesis are tabulated values of “t ” and “F ” at (P=0.05).

Table 4: Regression and validation data for the determination of desmopressin acetate by the proposed second derivative synchronous spectrofluorimetric method and UV derivative ratio spectrophotometric method.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Second derivative synchronous spectrofluorimetric method</th>
<th>UV Derivative ratio spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (nm)</td>
<td>347, $\Delta \lambda$ = 60 nm</td>
<td>236 nm</td>
</tr>
<tr>
<td>Linearity range</td>
<td>0.25–2.25 (µg/mL)</td>
<td>2–14 (µg/mL)</td>
</tr>
<tr>
<td>-Slope (b)</td>
<td>1.051</td>
<td>0.1565 ± 0.008</td>
</tr>
<tr>
<td>-Intercept (a)</td>
<td>0.8516</td>
<td>-0.0032 ± 0.037</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>Accuracy (% R)</td>
<td>100.34</td>
<td></td>
</tr>
<tr>
<td>LOD</td>
<td>0.060 (µg/mL)</td>
<td>0.049 (µg/mL)</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.183 (µg/mL)</td>
<td>0.162 (µg/mL)</td>
</tr>
<tr>
<td>Precision (% RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability c</td>
<td>0.455</td>
<td>1.406</td>
</tr>
<tr>
<td>Intermediate precision d</td>
<td>0.724</td>
<td>1.117</td>
</tr>
<tr>
<td>Robustness (% RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- $\Delta \lambda$ (± 1 nm)</td>
<td></td>
<td>0.246</td>
</tr>
<tr>
<td>- pH (± 0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Acetate buffer volume (± 0.1 mL)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The peak amplitude of the second derivative of synchronous fluorescence spectra.
b Concentration in mg/mL.
c The intraday (n=3), average of three concentrations of desmopressin acetate (0.5, 1.5 and 2 µg/mL) repeated three times within the day.
d The interday (n=3), average of three concentrations of desmopressin acetate (0.5, 1.5 and 2 µg/mL) repeated three times in three days.
Biological Applications:

Display that high sensitivity of the proposed methods allowed for determination of DA in biological fluid (spiked plasma) in which, the concentrations of DA (2 and 4 μg/ml) were determined in triplicate and calculating the corresponding concentrations from the regression equation. The results found show that they are accurate, precise, specific and selective method for determination of drug in biological fluids, as shown in (Table 5).

Table 5: Second derivative synchronous spectrofluorimetric proposed method for estimation of desmopressin acetate in spiked human plasma

<table>
<thead>
<tr>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery % ± S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>82.13 ± 1.03</td>
</tr>
<tr>
<td>4</td>
<td>84.04 ± 1.17</td>
</tr>
</tbody>
</table>

*The mean percentage recovery of three separate determinations

CONCLUSION

In the present work, superiority of a synchronous second derivative spectrofluorimetric method was approved compared to UV ratio derivative spectrophotometric method in terms of sensitivity, accuracy and precision. The proposed methods were successfully applied to the determination of DA in presence of its acidic degradation product either in their pure powder form or in their pharmaceutical formulation. The method was validated according to the ICH guidelines and the results of the validation show that the two proposed method have acceptable accuracy and purity of pharmaceutical preparations of desmopressin acetate.

ETHICAL STANDARDS

In case of Funding

This Article study has no funded form anywhere.

Conflict of Interest

Author A: Professor Dr. Khadiga M. Kelani declares that she has no conflict of interest with this study.

Author B: (Corresponding author) Dr. Ahmed Mohamed Wafaa Nassar declares that he has no conflict of interest with this study.

Author C: Dr. Wael Talaat declares that he has no conflict of interest with this study.

Author D: Dr. Samir Morshedy declares that he has no conflict of interest with this study.

In Case Animals were Involved in this Study

Ethical approval: This article includes experiments with human subjects only, but not with animals conducted by any of the contributors.

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