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The Application of Light-Absorbing Photostabilizers for the Determination of Protoporphyrin IX in Human Plasma by LC-MS/MS

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OVERVIEW

Purpose

Inhibit photodegradation of protoporphyrin IX in plasma using a treatment with light-absorbing preservative.

Method

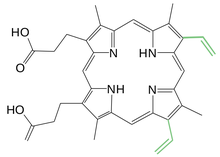
Bench-top stability of protoporphyrin IX in plasma under light was evaluated using various light-absorbing preservatives. A number of lighting conditions, tube colors, and exposure durations were tested.

Results

Crystal violet dye was shown to significantly improve photostability of protoporphyrin IX, which could be used for a validated method as per the BMV Guidance.

INTRODUCTION

Protoporphyrin IX (PPIX) (**Figure 1**) serves as the substrate for ferrochelatase, a critical enzyme in the heme biosynthetic pathway. In human plasma, PPIX is readily oxidized under light. In order to develop a robust bioanalytical method for PPIX, precautionary measures were taken to ensure analyte stability, such as working under yellow light and using amber tubes. Despite these precautions, photodegradation of PPIX in plasma was still observed. Consequently, further measures were evaluated, including the incorporation of colored solution (crystal violet and methylene blue, see **Figure 2**) as a light-absorbing preservative. Herein, the current research demonstrates the novel application of crystal violet dye to plasma to photostabilize PPIX.



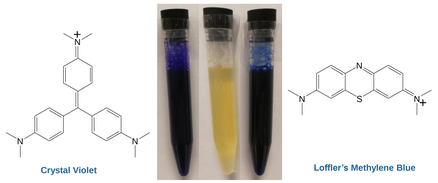


Figure 2. Human plasma with 10% v/v of 1% crystal violet solution (left), human plasma (middle) and human plasma with 10% v/v of Loffler's methylene blue solution (right).

METHOD

Stability Evaluation

The stability of PPIX in human plasma was evaluated at 22 ˚C under different lighting conditions: yellow fluorescent (Shat-R-Shield's Gold lamps), white fluorescent (Philips Light Series 800 32 watt), and white LED (Standard's LED T8). For each lighting condition, crystal violet (10% v/v of a 1% solution) and Loffler's methylene blue (10% v/v) were evaluated using transparent and amber polypropylene tubes from Sarstedt. Samples were exposed to light for 0, 2, and 24 hours.

Crystal violet (Cat. no V5265) and Loffler's methylene blue (Cat. no 1.01287.0500) solutions were purchased from Sigma-Aldrich and used as is.

Sample Preparation

Human plasma (100 µL) fortified with mesoporphyrin IX internal standard (IS) was extracted using a light-protected protein precipitation with acetonitrile.

Precautionary measures were taken to prevent photo-oxidation during extraction by working in a yellow fluorescent light environment, using black 96-well plates throughout the procedure and covering the plates with aluminum foil. In addition, all sample tubes were covered with aluminum foil to effectively shield them from light.

Analysis

Chromatographic separation was achieved on a C18 column under isocratic conditions with a high pH aqueous mobile phase and methanol.

Parent ions formed by +ESI were dissociated and detected at MRM transitions *m/z* 563>445 (PPIX) and 567>449 (IS) using a SCIEX API5000.

Choice of Photostabilizer

In the context of LC-MS/MS bioanalysis, the ideal photostabilizer must have the following properties:

- Shields from wavelengths causing photodegradation of the compound of interest
- Does not cause signal suppression or enhancement
- Readily available as a pre-made solution that can be shipped directly to the preclinical or clinical site
- Adequate shelf life

Another feature to consider is the light environment in which the photostabilizer will be employed. Yellow fluorescent lamps have a wavelength cutoff of 520 nm, light emission is 520 nm and above, which include a mixture of green, yellow, orange, and red light (see **Figure 3**). The photostabilizer to be selected must show strong absorbance over 520 nm.

Molar extinction coefficients spectrum of crystal violet (**Figure 4**) indicate that it strongly absorbs from 520 to 620 nm, making it a good candidate as a photostabilizer when working under yellow light environment. Methylene blue absorbs mostly from 600 to 700 nm, also making it a potential candidate under these conditions.

RESULTS

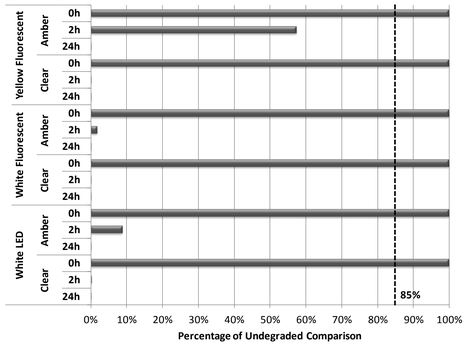


Figure 5. Analyte loss obtained in plasma without photostabilizer in different conditions. Columns on the left side indicate lighting condition, polypropylene tube color, and exposure duration (in hours), respectively.

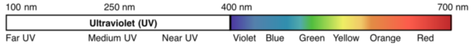


Figure 3. Electromagnetic spectrum (UV and visible). Retrieved on 2019/05/06 from: *https://www.masterorganicchemistry.com/2016/09/16/introduction-to-uv-vis-spectroscopy/*

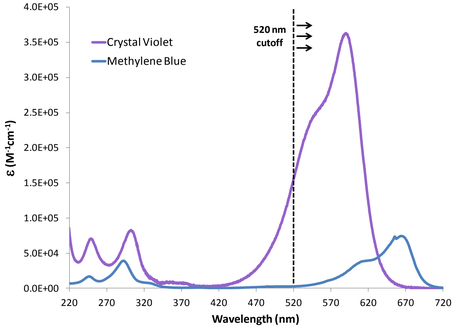


Figure 4. Spectra of molar extinction coefficients for crystal violet and methylene blue in water. The 520 nm cutoff of the yellow fluorescent light is indicated by the yellow line. Reference: *https://omlc.org/*

RESULTS (CONTINUED)

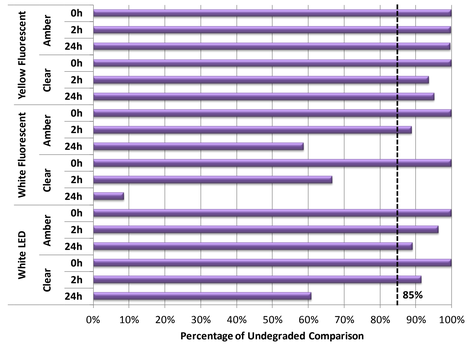


Figure 6. Analyte loss obtained in plasma with crystal violet photostabilizer in different conditions. Columns on the left side indicate lighting condition, polypropylene tube color, and exposure duration (in hours), respectively.

For bioanalytical methods of photosensitive compounds, it is routine protocol to work with amber tubes in a yellow-light environment. PPIX undergoes over 40% photodegradation under these conditions after two hours of exposure (**Figure 5**). Under fluorescent and LED white light, over 90% degradation was noted for the same duration of exposure.

Shielding samples with aluminum foil effectively prevented photodegradation; the process is both tedious and labor intensive. In contrast, adding a colored solution in plasma as a light-absorbing preservative provides a suitable approach, facilitating sample handling. In this work, both crystal violet and methylene blue solutions were evaluated as photostabilizers for PPIX due to their strong molar extinction coefficients in the yellow and red light regions of the visible spectrum.

Both crystal violet (**Figure 6**) and methylene blue (**Figure 7**) aided the photostabilization of PPIX in plasma, without signal suppression or enhancement at either analyte or IS retention time.

Methylene blue (**Figure 7**) provided minimal analyte loss after two hours under yellow fluorescent light in clear tubes. Amber tubes conferred sufficient stability of PPIX after two hours under LED white light, but losses of 58% were observed with clear tubes under these conditions. PPIX degraded by more than 22% after 24 hours for all conditions. Exposure to fluorescent white light resulted in more PPIX losses after two hours than LED white light, for both amber and clear tubes.

RESULTS (CONTINUED)

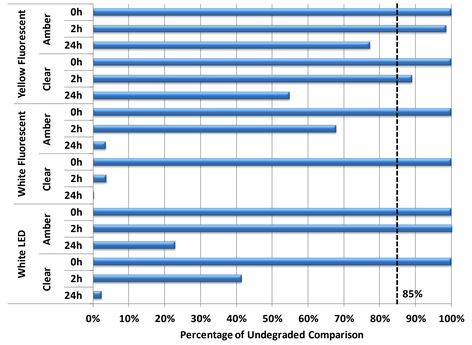


Figure 7. Analyte loss obtained in plasma with methylene blue photostabilizer in different conditions. Columns on the left side indicate lighting condition, polypropylene tube color, and exposure duration (in hours), respectively.

Crystal violet was the most effective photostabilzer inhibiting PPIX degradation under yellow light after 24 hours for both clear and amber tubes (**Figure 6**), a marked contrast to the 100% degradation observed without light-absorbing preservative under identical conditions (**Figure 5**). For white fluorescent and LED light, minimal losses were obtained after two hours using crystal violet in amber tubes. Under the same conditions in clear tubes, respective losses of 36% and 5% were noted for fluorescent and LED white light. After 24 hours in amber tubes, losses of 40% and 8% were noted under fluorescent and LED white light, respectively; the latter being within acceptance criteria for the validation of a bioanalytical method.

CONCLUSION

The research conducted herein demonstrated the application of light-absorbing compounds to biological matrix to confer photostabilization of PPIX.

Future work shall include the evaluation of other photosensitive compounds to gauge the universality of the technique.

ACKNOWLEDGMENTS

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