American Journal of Marine Science, 2021, Vol. 9, No. 1, 12-15
Available online at http://pubs.sciepub.com/marine/9/1/3
Published by Science and Education Publishing
DOI:10.12691/marine-9-1-3

Development of a Simple HPLC Method for the Sea-lamprey Pheromone, 3-ketopetromyzonol Sulfate (3kPZS)

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Received September 27, 2021; Revised October 28, 2021; Accepted November 04, 2021

Abstract The development of a simple HPLC method for detection of the sea lamprey pheromone 3-ketopetromyzonol sulfate (3kPZS) is reported. The ketone moiety of the bile alcohol reacts readily with 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) hydrazine to form the hydrazone. This hydrazone has a strong UV absorbance at 333 nm and visible fluorescence at 518 nm. Solutions containing the dansyl derivatized 3kPZS are readily analyzed by reverse phase HPLC with UV detection. A simple water-methanol gradient elutes the derivatized 3kPZS in just over 10 minutes using a standard 150 mm column. Detector response has high linearity throughout the ppm range, and the detection limit by UV is below 100 ppb. This methodology is useful for high throughput analysis of large numbers of samples in the ppm range.

Keywords: *HPLC, derivatization, bile salts, sea lamprey*

Cite This Article: Kenneth Banks, and James E. Hanson, "Development of a Simple HPLC Method for the Sea-lamprey Pheromone, 3-ketopetromyzonol Sulfate (3kPZS)." *American Journal of Marine Science*, vol. 9, no. 1 (2021): 12-15. doi: 10.12691/marine-9-1-3.

1. Introduction

We have previously reported our development of controlled-release formulations for the sea lamprey pheromone 3-ketopetromyzonol sulfate (3kPZS). [1] This compound is a bile alcohol released by male sea lamprey to attract female lamprey to their nesting grounds. [2,3] The female lamprey are extremely sensitive to this attractant, and respond to concentrations as low as 10^{-13} M. [4] Most of the HPLC methods developed for 3kPZS have employed mass spectrometric detection to achieve high sensitivity. [5] As our development process for the controlled-release "emitters" generated a large number of samples, we wanted an analytical technique that did not require this specialized and expensive equipment. Here we describe the development of a method for the analysis of 3kPZS that uses HPLC with a simple UV detector.

3kPZS (Figure 1) is an oxidized variant of the unique sea lamprey 24 carbon bile alcohol (5α-petromyzonol-24-sulfate, or 3α,7α,12α,24-tetrahydroxy-5α-cholan-24-sulfate) found in lamprey.[6] Apart from the alicyclic skeleton, the structure contains a ketone group, several hydroxyls, and a sulfate. Only the ketone has any UV absorbance, and the molar absorptivity is so low (on the order of 25 L $mol^{-1}cm^{-1}$) that this absorbance does not provide sufficient sensitivity for detection at analytically relevant concentrations. To improve the sensitivity, a derivatization method seemed appropriate. The ketone group can be readily derivatized using substituted hydrazines to form a hydrazone. The Dansyl (short for 1-dimethylaminonaphthyl-5-sulfonyl) hydrazine is commercially available and has an intense UV absorbance and strong fluorescence if even greater sensitivity is needed.

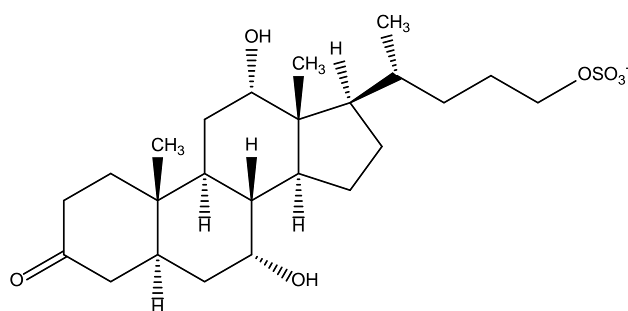

Figure 1. The sea lamprey pheromone 3kPZS

2. Materials and Methods

Materials. 3kPZS was generously provided by the Great Lakes Fisheries Commission. Dansyl hydrazine was purchased from Sigma or TCI and used as received. Ethanol was purchased from Spectrum Chemical. HPLC grade methanol and water were purchased from Alfa/Aesar and used as received. Polyethylene Glycol (MW 6000) (PEG6000) was purchased from Alfa/Aesar and used as received.

Derivatization procedure. The derivatization method was based on literature precedents. [7,8] A 5 mL sample of an aqueous solution of 3kPZS (~1 mg/mL) was treated with 10 mL of a 1 mg/mL aqueous solution of dansyl hydrazine and 2-3 drops of glacial acetic acid. The resulting solution was stirred for 15 minutes prior to performing HPLC analysis. For more concentrated 3kPZS solutions, the concentration of the dansyl hydrazine solution was also increased.

HPLC method. HPLC was performed on a HP/Agilent 1050 HPLC system with auto-injector, quat pump for gradients, and UV-vis detector, using a 150 mm Zorbax C18 column. The mobile phase was a potassium hydrogen phosphate buffer with a standard methanol gradient.

3kPZS Emitters. Emitters were prepared from PEG6000 and 3kPZS. [1] PEG6000 was melted in an oven or water bath held at 70°C. A methanolic solution of 3kPZS (5 mg/mL) was added to the molten PEG6000 such that there were 10 mg of 3kPZS for each 16 mL of total volume. The combined PEG6000/3kPZS mixture was well stirred and allowed to equilibrate while held at 70°C. Each emitter was a filled PVC tube (I.D. 2 cm, length 5 cm, volume ~16 mL). The emitter tubes were sealed to a metal plate with a small amount of pure molten PEG. After the PEG6000/3kPZS mixture had equilibrated for a few hours, the mixture was poured into the PVC tubes and allowed to cool and harden.

Analysis of emitted 3kPZS. Controlled release from the prepared emitters was performed in a well stirred, water filled 1 L Ehrlenmeyer. The Ehrlenmeyer was filled with 1 L of water and cooled to ~5-10°C to model the temperature of the streams that feed into the Great Lakes. An emitter was then placed in the Ehrlenmeyer and the water stirred with an overhead stirrer. The water was changed every 30 minutes, and with each change, a 5 mL aliquot was taken and subjected to the derivatization procedure above. The resulting solution of derivatized 3kPZS was then analyzed by the HPLC method described above.

3. Results

In the preparation of controlled-release polymer emitters for the sea lamprey pheromone 3kPZS, [1] we required a robust and inexpensive analytical method to analyze a large volume of samples. We required this method to use standard HPLC equipment, not HPLC-MS or other expensive or unusual equipment or methodology. The controlled-release emitters were prepared from 6000 MW PEG compounded with 3kPZS and were designed to release ~1 mg of 3kPZS per hour. For laboratory analysis, this would be released into approximately 1-2 L of water, resulting in a concentration of ~1 ppm. (In the field, the volume would be much greater, perhaps 1000 L or more, resulting in low ppb or sub-ppb concentrations.) By molarity, 1 ppm of 3kPZS is approximately 3 x 10^{-6} M. Preliminary results with UV spectroscopy convinced us that direct detection of 3kPZS by UV absorbance would not possess the sensitivity we needed for our analysis: with a molar absorptivity of 25-40, the absorbance of the ketone would be approximately 1 x 10^{-4} Absorbance Units, and injection into the HPLC system would result in further dilution and reduction of absorbance.

Derivatization is a common way to enhance the sensitivity of chromatographic detection. Analytes with little or no absorbance are reacted with a derivatizing reagent that couples the analyte to a molecule that is strongly absorbing (or possesses some other detectable quality). The derivatizing reagent should couple with near 100% efficiency and should be compatible with the chromatographic method. There are two types of functional groups on 3kPZS that might provide a reactive site for derivatization – the hydroxyl groups at C7 and C12, and the C3 ketone. With only a single reactive site, the ketone is a better choice for derivatization. Reaction with hydrazines to form hydrazones is a common method for ketone derivatization, and we chose the dansyl (5-(N,N-*d*imethyl*a*mino)*n*aphthyl -1-*s*ulfon*yl*) hydrazine as the derivatization reagent. [9] This reagent absorbs strongly at 333 nm and fluoresces at 518 nm. The molar absorptivity at 333 nm is about 4300, at least a 100 fold increase in absorbance, with a predicted absorbance of about 1 x 10^{-2} Absorbance Units. While an even greater absorbance may be desirable, this should be sufficient for detection at 1 ppm, and the possibility of fluorescence detection would provide even greater sensitivity and selectivity.

For development of the method, the 3kPZS was derivatized following a standard method derived from the literature. [7,8] Solutions of 3kPZS in water (typically 5 mL) were treated with a twofold excess of dansyl hydrazine (typically 10 mL of a 1 mg/mL solution) and a catalytic amount of glacial acetic acid (~1 drop for each 5 mL). The reaction to form dansyl-3kPZS is shown in Figure 2, and the UV-Vis absorbance spectrum of the resulting product is shown in Figure 3. The expected dansyl absorbance is observed at 333 nm.

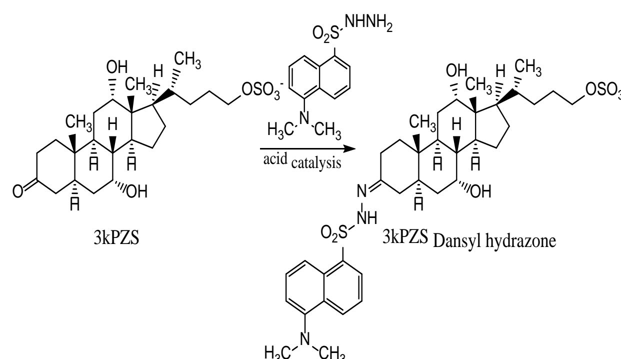

Figure 2. Derivatization of 3kPZS with Dansyl Hydrazine

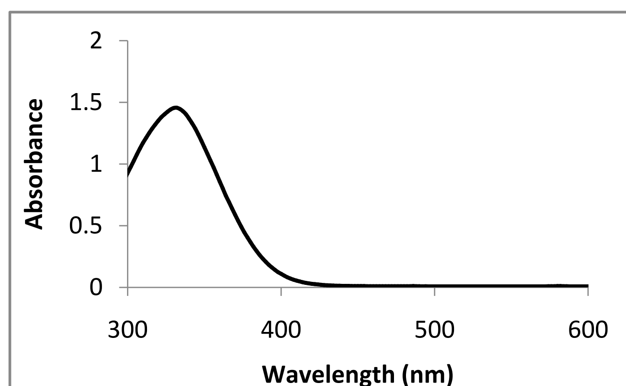

Figure 3. Electronic (UV-Vis) absorbance spectrum of dansyl hydrazone derivatized 3kPZS (3.3 x 10^{-4} M) in KHP buffer

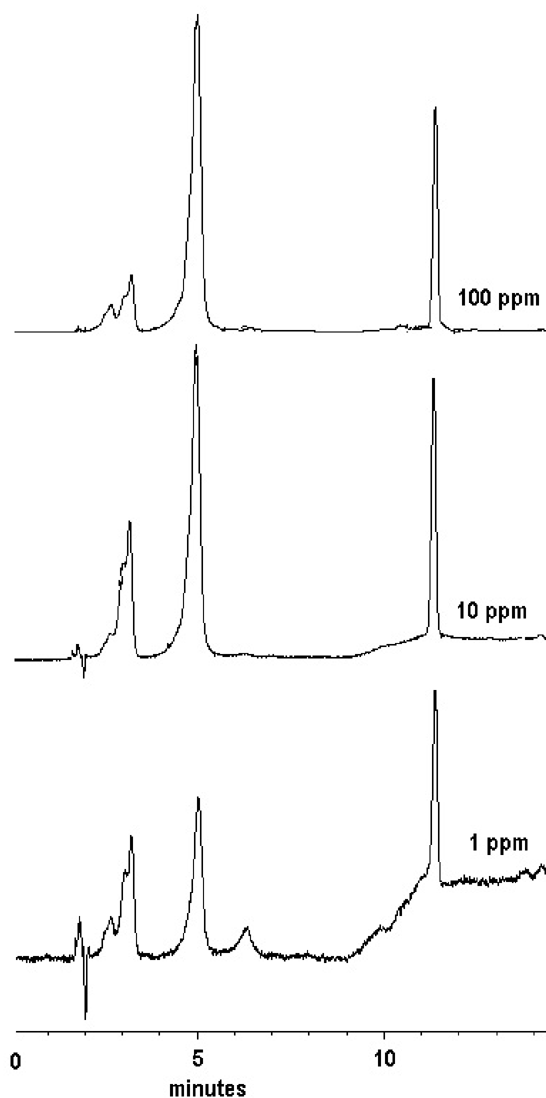

Figure 4. Representative chromatograms for dansyl derivatized 3kPZS at different concentrations

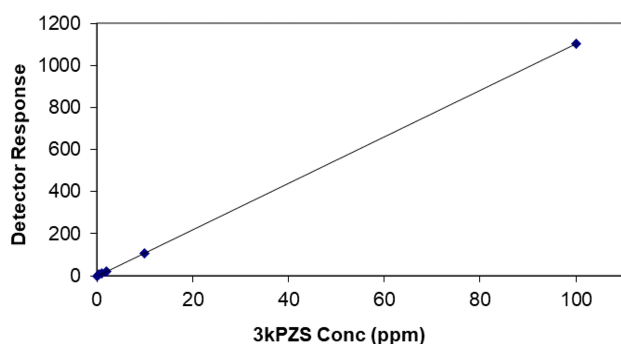

Figure 5. Response linearity of dansyl derivatized 3kPZS, from 0.1 ppm to 100 ppm. The line is a linear regression fit with a correlation coefficient of 1.0

HPLC analysis was performed on a C18 column with UV/Vis detection using a water/methanol gradient. Representative chromatograms at concentrations of 1, 10 and 100 ppm are shown in Figure 4. Excess dansyl hydrazine and some other impurities elute rapidly, at times before 5 minutes, while the dansyl derivatized 3kPZS elutes at 11.3 minutes. The linearity of the response of the UV detection to the dansyl derivatized 3kPZS was established by analysis over a range of concentrations, with linearity established from 0.1 ppm (100 ppb) to 100 ppm. The linearity results are shown in Figure 5. The line on the graph is fitted to the data by linear regression with a correlation coefficient of 1.0. A similar plot from 0.1 to 10 ppm also gave a correlation coefficient of 1.0. [10]

The HPLC method was then applied to 3kPZS released from polymer emitters in the laboratory. These emitters are controlled-release devices designed to be used in a variety of lamprey control settings. [1] They were prepared by adding 3kPZS in methanol to molten PEG6000 to a concentration of 0.625 mg/mL, then using the PEG6000/3kPZs mixtur to fill lengths of PVC tubing. Each emitter contained ~10 mg of 3kPZS. Emitters were placed in a 1 L Ehrlenmeyer filled with 5-10 ^{o}C water and stirred. Every 30 minutes, the water was replaced and a portion was derivatized and analyzed as described in the Materials and Methods. Figure 6 shows the release of 3kPZS from an emitter by HPLC analysis over a 4 hour period. The observed concentration from emitter dissolution in the water is between 1.4 and 1.8 ppm for each 30 minute period.

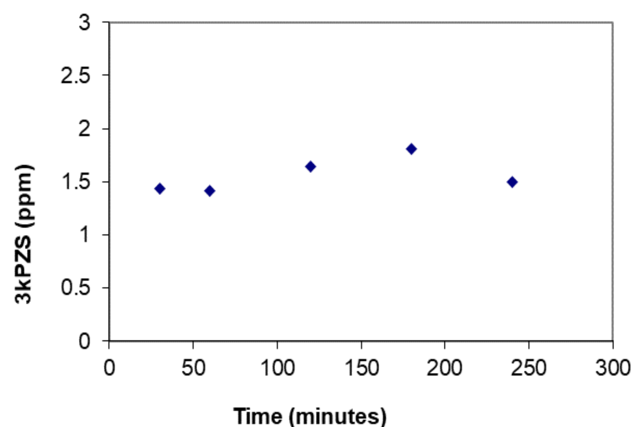

Figure 6. Release of 3kPZS from polymer emitters followed by HPLC using the dansyl derivatization method

4. Discussion

As expected, 3kPZS was readily derivatized with dansyl hydrazine. Dansyl hydrazones have been used for many years as readily detected derivatives of ketones and aldehydes. [9] We have demonstrated here that 3kPZS reacts with dansyl hydrazine in aqueous solution with mild acid catalysis. Critically, the derivatization procedure was simple and did not require evaporation or extraction of the aqueous solution. Excess dansyl hydrazine and related impurities were observed in the chromatograms, but eluted well before the derivatized 3kPZS. Some literature procedures use pyruvate to remove excess dansyl hydrazine, [7,8] and this could be included in the procedure if desired. Since our purpose was quantifying 3kPZS released from polymer emitters, we kept the processing of sample solutions to a minimum.

HPLC analysis of the dansyl derivatized 3kPZS was straightforward as well. A standard water-methanol gradient eluted the dansyl derivative in less than 12 minutes. The sensitivity was sufficient for our needs – the detection limit was somewhere below 100 ppb (~10^{-7} M, 0.1 mg/L), and the signal was strong at 1 ppm (our target

for 3kPZS release in the laboratory). The response to concentration was linear from 100 ppb to 100 ppm. Fluorescence detection is available for dansyl derivatives, and this would be expected to lower the detection limit by several orders of magnitude to the 10^{-10} or 10^{-11} M or ppt range.[7,8] Since female lamprey respond to 3kPZS down to 10^{-13} M, this is still above the detection limit needed for some field applications! Analysis of 3kPZS in water taken from rivers/streams in the Great Lakes basin has revealed 3kPZS concentrations between 2 and 120 ng/L [11,12]. These concentrations would be near the limit of detection for fluorescence detection – although some studies have claimed a limit of detection below 1 ng/L using dansyl derivatization. [13] Field applications of 3kPZS have targeted concentrations of 10^{-12} to 10^{-13} M, [14,15] equivalent to 0.4 to 0.04 ng/L. If fluorescence detection can achieve detection below 1 ng/L, then this method might be applied in the field using portable HPLC equipment as recently described. [16]

Application of the method to evaluation of polymer emitters demonstrated the utility of the method for laboratory evaluations. The method is rapid and simple, and uses relatively common and simple analytical equipment. This allowed us to run numerous samples as we optimized the ratio of polymer and 3kPZS in the emitter preparation to provide a release of 3kPZS of about 1 mg/hour.

Acknowledgements

Funding for this project and donation of 3kPZS material were provided by the Great Lakes Fisheries Commission.

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