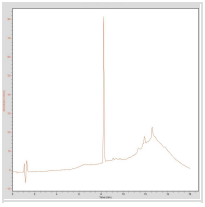

Nov 10, 2021

Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors

Oswald Yedjinnavenan Djihinto[1], Luc S. Djogbenou[1,2,3], Luisa Nardini[4,5], Armorel Van Eyk[6], Lizette L. Koekemoer[4,5]

[1]Tropical Infectious Diseases Research Center (TIDRC), University of Abomey-Calavi, 01 BP 526, Cotonou (Benin);

[2]Regional Institute of Public Health, University of Abomey-Calavi, BP 384, Ouidah, Benin;

[3]Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, United Kingdom;

[4]Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg 2000, South Africa.;

[5]Centre for Emerging, Zoonotic & Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg 2131, South Africa;

[6]Division of Pharmacology, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg 2000, South Africa

dx.doi.org/10.17504/protocols.io.by4cpysw

Tropical Infectious Diseases Research Center (TIDRC)

Oswald Yedjinnavenan Djihinto
Tropical Infectious Diseases Research Center (TIDRC), Univer...

Ecdysteroids are arthropod steroid hormones that are primarily involved in insect moulting. In arthropod vectors, especially in mosquitoes, ecdysteroids of interest include mainly ecdysone (E) and 20-hydroxyecdysone (20E). These two compounds are involved in several important biological processes. Targeting these compounds and their regulatory pathways could lead to the characterisation of novel genetic tools towards implementing new malaria control strategies. To date, there are two main methods for quantifying E and 20E. These methods include an enzymatic method (Enzyme-Linked Immunosorbent Assay (ELISA)) and a chromatographic method (High-Performance Liquid Chromatography (HPLC)). However, for ecdysteroids quantification, the HPLC methods available in literature go from 30 minutes to one hour. Here, we developed a short HPLC gradient method for 20-hydroxyecdysone quantification in the malaria vector *Anopheles gambiae*. This method was developed specifically when sample material is limited as well as to save time and cost.

DOI

dx.doi.org/10.17504/protocols.io.by4cpysw

Citation: Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors https://dx.doi.org/10.17504/protocols.io.by4cpysw

Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer 2021. Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors. **protocols.io** https://dx.doi.org/10.17504/protocols.io.by4cpysw

International Union of Biochemistry and Molecular Biology
Grant ID: 2020 Wood Whelan Research Fellowship awarded to OYD.

The authors would like to acknowledge the Department of Science and Innovation (DSI) and the National Research Foundation (NRF) South African Research Chairs Initiative
Grant ID: UID 64763 to LLK, for partial funding

20-hydroxyecdysone (20E), HPLC, Anopheles gambiae, malaria

protocol ,

Oct 15, 2021

Nov 10, 2021

54116

- A new column has to be equilibrated in the mobile phase for at least 1-2 hours before use and any buffer should be removed from the column daily with 20% methanol or Acetonitrile: 80% HPLC grade water and stored in 80% methanol or Acetonitrile: 20% HPLC grade water.
- The partial loop option should be chosen ("µL-pickup") for sample analysis to inject sample volumes as little as 1µL, resulting in no sample wastage and to avoid large volumes of standard sample preparation.
- All samples and mobile phase buffers should be filtered through a 0.45 um filter prior to being analysed.
- The 20E synthesis in mosquitoes is tightly regulated. To quantify the 20E in non-blood feed mosquitoes, be sure that female mosquitoes have sufficient time for mating to occur.
- All organic mobile phases used for HPLC analysis should be HPLC grade (Sigma Aldridge Inc).
- All other chemicals and standards should be of the highest purity grade (Sigma Aldridge Inc.)

1. **Reagents**

20-hydroxyecdysone **Sigma Aldrich Catalog #H5142-5MG or H5142-10MG**

Methanol HPLC **Fisher Scientific Catalog #9093-03**

Acetonitrile HPLC **fisher Catalog #9012-03**

Acetic acid glacial **Sigma Aldrich Catalog #ARK2183**

Water MilliQ **Contributed by users**

2. **Consumables**

- Eppendorf tubes
- 2 mL white HPLC glass vials
- 250 µL conical glass Micro Inserts
- Membrane Filter 0.45 µm pore size
- 2 mL syringe
- Cones tips
- Micropipettes

3. **Equipments**

Flexar LC
High-Performance Liquid Chrommatography system
Perkin Elmer **N2910402**

Eppendorf Vacufuge Concentrator System
Eppendorf **5301**

Pump pressure should be closely monitored: an increasing pressure toward the limit of the system is an indication of mobile phase leaking (very low pressure) or blockage in the system (very high pressure).

Citation: Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors https://dx.doi.org/10.17504/protocols.io.by4cpysw

- The HPLC system pump should be purged prior to usage to remove air bubbles.
- The autosampler (if available) should be flushed to clean the injection system.
- The column should be equilibrated with the mobile phases (starting conditions) for at least 30 min prior to initiating analyses.

Instrument

1 Analytical HPLC separations were performed on the Flexar LC system (Perkin Elmer) with a UV/Vis Detector and a Phenomenex Kinetex RP C18–5 µm column (4.6 x 150 mm).
The detection wavelength was set at 254 nm.
The flow rate was 1 ml/minute.
The mobile phase included gradient elution with Methanol:Acetonitrile (85-15) and 0.5% acetic acid-Water for 16 minutes (See the chromatographic conditions).
The injection volume was 1 µL.

Chromatographic conditions

2 Column: Phenomenex, Kinetex® 5 µm C18, 100 Å, 150 x 4.6 mm
Column temp: 20°C
Mobile phase A: 85:15:Methanol:acetonitrile (v:v)
Mobile phase B: 0.5% acetic acid: 95% HPLC grade water (v:v)
Flow rate: 1 mL/min
Detector: UV at 254 nm
Injection volume: 1 µL
Run time: 16 min
Gradient profile: (ramps are linear)

A	B	C	D
		% Mobile phases	
Time (min)	**Flow** (ml/min)	**A** (MeOH-ACN)	**B** H2O – 0.5% acetic acid
0.5	1	10	90
10	1	70	30
6	1	10	90

If available on the HPLC system, consider choosing the µL-pickup injection mode.

Mobile phase preparation

3 Mobile phase A: 85:15 Methanol:acetonitrile (v:v)
Mobile phase B: 0.5% acetic acid: water (v:v)

Preparation of mobile phase A

Citation: Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors https://dx.doi.org/10.17504/protocols.io.by4cpysw

Mobile phase A was prepared by mixing methanol and acetonitrile to have a ratio of 85:15.

For example, to prepare **1 L** , combine **850 mL** of methanol, **150 mL** of acetonitrile and mix well.

Preparation of mobile phase B

Mobile phase B was prepared by adding glacial acetic acid to water at a ratio of 0.5% glacial acetic acid.

For example, to prepare **1 L** , combine **5 mL** of glacial acetic acid and **995 mL** of MilliQ water and mix.

The solutions preparation may be scaled up as necessary.

Standard samples preparation

4 The standard samples used were prepared from the 20-hydroxyecdysone (20E) stock solution at (5 µg/L).
All samples were prepared in methanol to a final volume of 500 µL.
Make three injections for each standard sample.

A	B	C	D
Concentration (µg/L)	**Final volume (µL)**	**Volume of 20E solution (5 µg/L) in µL**	**Volume of methanol to be added (µL)**
2.5	500	250	250
1.25	500	250	250
0.625	500	250	250
0.3125	500	250	250
0.156	500	250	250
0.078	500	7.8	492.2
0.0097	500	0.97	499.03

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Example of calculation

Citation: Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors https://dx.doi.org/10.17504/protocols.io.by4cpysw

5

Concentration (µg/L)	Concentration as % of analyte target	Peak Area (mean of three injections)	Peak Area Standard Deviation	Peak Area RSD (%)
0.009765	0.10	24518.79	324.9409	1.33
0.078	0.78	102442.4	2838.319	2.77
0.15625	1.56	213946.2	7341.033	3.43
0.3125	3.13	366212.8	7090.87	1.94
0.625	6.25	790091.8	58677.44	7.43
1.25	12.50	1557900	26350.44	1.69
2.5	25.00	2776984	9269.747	0.33
Linearity range			0.009 to 2.5 µg/L	
Equation for regression line: Y = 1118158*X + 45412			Correlation coefficient (R^2): 0.9964	

Equation for regression line: Y = 1118158*X + 45412		Correlation coefficient (R^2): 0.9964
SE of intercept		32934
SD of intercept	SE of intercept*sqrt(N)	87135.17368
LOQ	10*(SD of intercept/slope)	0.779
LOD	3.3*(SD of intercept/slope)	0.257

In the "SD of intercept" calculation formula, "N" represents the total number of the standard samples.

Total ecdysteroid extraction and 20E quantification

6

20-hydroxyecdysone (20E) was detected in total ecdysteroid extract from only female mosquitoes.

Here, female mosquitoes of *Anopheles gambiae* (COGS strain) were used.

Total ecdysteroid was extracted using methanol according to the method described by McKinney et al., 2017.

McKinney DA, Strand MR, Brown MR (2017). Evaluation of ecdysteroid antisera for a competitive enzyme immunoassay and extraction procedures for the measurement of mosquito ecdysteroids. General and comparative endocrinology. https://doi.org/10.1016/j.ygcen.2017.08.028

1. homogenize 15 non-blood fed adult females (4 days old) in1 mL of methanol 95% in 1.5 mL

Citation: Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors https://dx.doi.org/10.17504/protocols.io.by4cpysw

Eppendorf tubes using plastic pestles.
2. Vortex and centrifuge for 5 min at 13000xg
3. Transfer the supernatant to another tube
4. Homogenize the remaining pellet in 500 µL of methanol 95% and centrifuge
5. Poole the supernatant with the first one.
6. Dry the pooled supernatants in a vacuum centrifuge
7. Resuspend the dried sample in 500 µL of methanol 100% and filter the solution before injection.

6.1 HPLC analysis of the extracts

Inject the sample using the same method as with the standard samples.

For the HPLC analysis, it was observed that with the 1 µL injection volume the interpolated X value (the concentration of the sample) falls outside the output range of X for the fitted curve. We then recommend analysing the mosquito extracts with at least 5 µL injection volume and reporting the calculated concentration to 1 µL injection by dividing the calculated value by a factor of 5.

Citation: Oswald Yedjinnavenan Djihinto, Luc S. Djogbenou, Luisa Nardini, Armorel Van Eyk, Lizette L. Koekemoer Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors https://dx.doi.org/10.17504/protocols.io.by4cpysw