NCL Method PCC-18

Quantitation of APIs in Polymeric Prodrug Formulations

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov
https://ncl.cancer.gov

https://ncl.cancer.gov

This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

Method Written By:

Jeffrey D. Clogston

Yingwen Hu

Please cite this protocol as:

Clogston JD, Hu Y, NCL Method PCC-18: Quantitation of APIs in Polymeric Prodrug

Formulations. https://ncl.cancer.gov/resources/assay-cascade-protocols DOI: 10.17917/JM45-

VA25

1. Introduction

Polymeric prodrugs (polymer-drug conjugates) are unique and can provide various advantages over other nanotechnology platforms. Rather than encapsulation of drug in a nanoparticle shell, polymeric prodrugs use covalent conjugation to bind the drug, or active pharmaceutical ingredient (API), to the polymer backbones. Additionally, the drug loading ratio and subsequent drug release rates can be optimized by tailoring the chemistry used to incorporate the APIs. Unlike the encapsulation of APIs in the other nano-sized drug delivery systems, the quantification of chemically conjugated drugs in polymeric prodrugs is more difficult.

Here, we describe a protocol for drug quantification in polymeric prodrugs using an automated elemental analyzer (PE 2400 Series II CHNS/O, Perkin Elmer). Briefly, materials containing carbon (C), hydrogen (H), nitrogen (N), and/or sulfur (S) are combusted under high temperature and high oxygen conditions into their oxidized gas form, then analyzed by internal gas chromatography (GC) for a quantitative analysis which is reported as a percentage of the original sample weight [1, 2]. This document summarizes the laboratory procedure. For a more descriptive review of the procedure, we refer you to the publication "Total Drug Quantification in Prodrugs Using an Automated Elemental Analyzer" which describes the methods and related analysis to measure the amount of drug loading in a polymeric drug conjugate using poly(L-lysine succinylated) (PLS) and lamivudine (LAM) [3].

2. Reagents and Equipment

2.1 Materials

    2.1.1 Samples: This procedure requires (a) free drug, (b) free polymer backbone and (c) final polymeric prodrug product. It is best to use the free drug and free polymer backbone from the same lot as used to prepare the final conjugated product.

    2.1.2 Standards: Acetanilide, Cat. 02401121, or cysteine, Cat. N2410324, PerkinElmer

    2.1.3 Tin capsules (Precleaned Tin Capsules, 5 mm × 8 mm, Cat. N241-1255, PerkinElmer)

    2.1.4 Tweezers for holding the capsules

2.1.5 CHN mode: CHN combustion tubes (2400 CHN Pre Packed Comb Tube-Silica, Cat. N2410695, PerkinElmer) and reduction tubes (2400 CHN Pre Packed Reduction Tube, Cat. N2410694, PerkinElmer)

2.1.6 CHNS mode: CHNS combustion tubes (2400 CHNS Pre Packed comb/red tube, Cat. N2410693, PerkinElmer) and reduction tubes (CHNS/Oxygen Reduction Furnace, Cat. N2410698, PerkinElmer)

2.2 Equipment

2.1.1 Elemental analyzer (2400 CHNS/O Series II System, PerkinElmer)

2.1.2 Autobalance (AD6000, PerkinElmer)

3. Measurement Principle

The instrument may be operated in either CHN or CHNS mode. Small quantities of sample (typically ~2 mg) are accurately weighed into small tin capsules. At elevated temperatures, in the presence of excess oxygen, organic materials combust to form CO_2, H_2O, various N_xO_y compounds, and SO_2 if sulfur is present. These reactions are facilitated by solid catalysts packed in the combustion tube; other combustion products like HCl are generally removed by silver gauze and other solid materials packed in the tube. The N_xO_y combustion products are reduced by fine copper in the reduction tube to N_2. The excess oxygen used to support combustion is also scrubbed out in the copper reduction tube. The only gases collected for quantitative analysis are:

- CO_2 representative of carbon content,
- H_2O representative of hydrogen content,
- N_2 representative of nitrogen content
- SO_2 representative of sulfur content (only with the CHNS configuration)

The combustion gases are collected automatically in a fixed volume in the instrument, and then dosed over time onto a GC column designed specifically for the quantitative analysis of gases. The GC uses a thermal conductivity detector. Because the gases are dosed over a period of time (as opposed to being instantly injected), discrete, well-separated peaks are not observed. Instead, "plateaus" are observed, and the steady-state values of each plateau are recorded by the instrument at pre-determined times. This is referred to as "frontal chromatography".

In order to convert the detector signals (baseline, nitrogen, carbon dioxide, water, sulfur dioxide) into %C, %H, %N, and %S, it is necessary to:

- Establish steady state conditions in the instrument

- Determine "blank" values

- Run pure standards (known %C, %H, %N, %S) to establish "K-factors"

- Run accurately weighed representative replicates of each sample

4. Experimental Procedure

4.1 Instrument Preparation (specific for PE 2400 CHNS/O Series II System, other instruments may vary slightly in operation (see instrument manual))

 4.1.1 Check gases

  • He >200 psi in the tank; regulator set at 20 psi

  • O_2 >200 psi in the tank; regulator set at 18 psi

  • Air >80 psi

 4.1.2 Verify Instrument Mode

  PARAMETER 6, CHN mode or CHNS mode.

 4.1.3 Verify Run Capacity

  PARAMETER 4, Run counters. All three must have values in excess of expected total number of runs, including conditioners, blanks, calibrants, and samples.

 4.1.4 Check for Leaks

  DIAGNOSTIC 2-1-2, resulting printout should be "pass".

 4.1.5 Optimize Combustion (optional)

  PARAMETER 9, optimize-combustion parameters. For CHN work, choose "constant", verify the settings are 3,5,1,0. To use different values, choose "select" and set appropriate values in one of the four available programs. For CHNS, choose "constant" and verify that the values are 1,1,1,0.

 4.1.6 Purge system

  Purge with He for 300 s and O_2 for 150 s whenever a tube or gas cylinder is replaced or before a run sequence is initiated.

 4.1.7 Stabilize system

- Press the SINGLE RUN key
- Press 1 for BK (blank)
- Enter 4 (minimum) for number of runs. The system will switch to the "STANDBY" mode.
- Load folded tin capsules onto the Auto-Injector. Close lid
- Press START

4.2 Sample and Standard Preparation

4.2.1 Tare the weight of capsule on the autobalance.

4.2.2 Add appropriate amount of sample / standard to capsule.

4.2.3 Check weight; adjust if necessary. When capsule contains correct amount of sample / standard, fold as shown on Operator's Manual.

4.2.4 Re-weigh. Record weight.

4.2.5 Place sample / standard in clean 96 well storage tray and record tray position.

Notes:

- Avoid cross contamination of samples / standards by cleaning all tools between groups
- Minimize exposure of standard to light and air

4.3 Calibration

4.3.1 Blank

- Press the SINGLE RUN key
- Press 1 for BK (blank)
- Enter 1 for number of runs -- the system will switch to the "STANDBY" mode
- Drop blank into funnel in sample drop opening at top of Auto-Injector; close lid
- Press START

4.3.2 K factor

4.3.2.1 CHN mode

- Press the SINGLE RUN key
- Press 2 for KFACT
- Press 1 for Standard S1 (Acetanilide)
- Enter the weight of the standard in mg

- Drop standard into funnel in sample drop opening at top of Auto-Injector; close lid
- Press START

4.3.2.2 CHNS mode

- Press the SINGLE RUN key
- Press 2 for KFACT
- Press 1 for Standard S1 (Cystine)
- Enter the weight of the standard in mg
- Drop standard into funnel in sample drop opening at top of Auto-Injector; close lid
- Press START

Note: Repeat Procedure 4.3.1 and 4.3.2 four times (minimal) to calibrate the system. Follow accept limit chart as follows:

K-Factor Theoretical Values

	% C	% H	% N	% S
Acetanilide	70.99 – 71.28	6.63 – 6.91	10.25 – 10.42	-
Cystine	29.83 - 30.01	4.94 - 5.19	11.52 - 11.76	26.60 - 26.96

4.4 Sample Analysis

Capsule samples can be analyzed in either the Single Run OR Auto Run modes. Procedures for running samples in each of these modes are outlined below.

Single Run Mode:

1) Press the SINGLE RUN key
2) Press 3 for Sample
3) Key in Sample ID up to 12 characters, NO spaces; ENTER
4) Key in Sample Weight in mg; ENTER
5) Drop sample into sample drop opening at top of Auto-Injector; close lid
6) Press START

7) Repeat step 1) – 6) for samples replicates and remaining samples

Auto Run Mode:

1) Press AUTO RUN

2) Press 4

3) Press 1 to clear old run sequence from memory and reset run sequence to # 1

4) Adjust carousel clockwise to line up space 1 with the sample drop port.

5) Press 3 for Sample

6) Enter ID and sample weight in mg

7) Place sample in appropriate position on clean autosampler carousel

8) Record Auto Run # and Autosampler position of sample on separate data sheet

9) Press START

To edit an entry in Auto Run Mode (i.e. weight, ID, run type)

1) Change number to run# to be edited

2) Press Parameters key and make changes

4.5 Shut down

4.5.1. Bleed valves D (4) and E (5)

Press the DIAGNOSTICS key and follow this order:

2	GAS
2	VALVE
4	VALVE 4 - selects valve "D"
1	ON - turns on valve "D"
5	VALVE 5 - selects valve "E"
1	ON - turns on valve "E"

Wait for about fifteen seconds and then press the DIAGNOSTICS key again (automatically closes the valves just opened and exits from the diagnostics mode)

4.5.2. Put the system in Gas Saver Mode:

- Press the PARAMETER key and enter the following:

22	Gas saver valve
1	Select ON

- Enter today's date (dd/mm/yy format) and time (24-hour format) for gas saver mode to turn on.

- Enter the date and time for the gas saver to turn off.

- Press the PARAMETER key to exit to the standby mode

5. Data Analysis

Drug loading using the element of interest (e.g., C, H, N, S) is calculated using the following elemental mass balance equations:

$$\%X_{drug} \times \%Wt_{drug} + \%X_{polymer} \times (1- \%Wt_{drug}) = \%X_{prodrug} \qquad \textbf{Eq. 1}$$

Or more simply, the drug loading, as a weight percent of the total product ($\%Wt_{drug}$), can be calculated as:

$$\%Wt_{drug} = \frac{\%X_{prodrug} - \%X_{polymer}}{\%X_{drug} - \%X_{polymer}} \times 100\% \qquad \qquad \textbf{Eq. 2}$$

For a detailed example of the data analysis we recommend reviewing the manuscript "Total Drug Quantification in Prodrugs Using an Automated Elemental Analyzer" which describes quantitation of drug loading in the polymeric drug conjugate poly(L-lysine succinylated) (PLS) and lamivudine (LAM) [3]. For this study, the drug loading was calculated using nitrogen (%N) and sulfur (%S) mass balance via the CHN and CHNS modes on the instrument, respectively.

By CHN operation mode, the average weight percentages of each element (carbon, hydrogen and nitrogen) of the polymer (PLS), free drug (LAM), and the polymeric prodrug (PLS-LAM) were measured. The %N was used to calculate the drug loading and Eq. 2 was applied to calculate the %Wt_{drug}. The %N for PLS, LAM and PLS-LAM was 12.2± 0.1, 18.4 ± 0.1 and 12.6 ± 0.1, respectively, and the LAM drug loading was calculated as 6.5 ± 0.9%.

By CHNS operation mode, similarly, the average weight percentages of carbon (%C), hydrogen (%H), nitrogen (%N) and sulfur (%S) of each sample were measured. The %S was used to

calculate the drug loading. The %S for PLS, LAM, and PLS-LAM was 0.5 ± 0.5, 14.2 ± 0.6 and 1.4 ± 0.04, respectively, and the LAM drug loading was calculated as $6.6 \pm 0.4\%$.

6. Safety

The CHN/S analyzer operates at very high temperatures. Personal protective equipment (lab coat, safety glasses and gloves) should be equipped at all time of operation.

7. References

1. Culmo RF. The Elemental Analysis of Various Classes of Chemical Compounds Using CHN. PerkinElmer Application Note.
2. http://www.perkinelmer.com/product/2400-chns-o-series-ii-system-100v-n2410650. PerkinElmer Website.
3. Hu YW, Stevens DM, Man S, Crist RM, Clogston JD. Total drug quantification in prodrugs using an automated elemental analyzer. Drug Deliv Transl Re. 2019;9(6):1057-66. doi:10.1007/s13346-019-00649-8.