Summer 2017

Purification and Analysis of A Pteroic Acid Conjugate

Naveen Kumar Ganji
Governors State University

PURIFICATION AND ANALYSIS OF A PTEROIC ACID CONJUGATE

Project Submitted to

Governors State University

By: Naveen Kumar Ganji

In partial fulfillment of the requirement for Master of Science in

Analytical Chemistry

AUGUST 2017

Governors State University,

University Park, Illinois.

DEDICATED TO MY FAMILY

Acknowledgments

I am very grateful to Dr. Walter Henne who gave me guidance and provided essential equipment for this research. Without his help and cooperation, this research wouldn't be successful. My sincere gratitude to Dr. Joong-Won Shin and Dr. K.G. Sanjaya Ranmohotti who are the readers of this paper and for their assistance in analyzing the data of my graduate work. I am very thankful to Dr. John Sowa and Dr. Shelly Kumar for their support throughout my graduation. I would like to thank my undergraduate student assistant, Enas Horeish who worked with me on this project. I would also like to take this opportunity to acknowledge Janith Wazio for her help.

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ABSTRACT

Folate receptor (FR) is overexpressed on the cell membrane in cancer tissues and activated macrophages associated with rheumatoid conditions. However, FR is restricted in normal healthy tissues. Folic acid has shown high affinity to folate receptors and thus can be used in targeting the tumor cells. Folic acid residue, pteroic acid is also used in several studies that are involved in targeting cancer cells. Several examples include technetium -99m radiotracer labelled pteroyl-lys conjugates(Guo H, Xie F, Zhu M, Li Y, Yang Z, Wang X, Lu J. *The synthesis of pteroyl-lys conjugates and its application as Technetium-99m labeled radiotracer for folate receptor-positive tumor targeting.* Bio-org Med Chem Lett. 2011 Apr 1;21(7):2025-9), pteroic acid-conjugated nitroheterocyclic phosphoramidates (Gali Steinberg and Richard F. Borch. *Synthesis and Evaluation of Pteroic Acid-Conjugated Nitroheterocyclic Phosphoramidates as Folate Receptor-Targeted Alkylating Agents. J. Med. Chem.*, 2001, *44* (1), 69–73).

In this project, we were successfully able to conjugate pteroic acid with polyethylene Glycol-Amine to create an elegant building block(pte-PEG-amine) for synthesis of future imaging agents. The compound was purified and analytically tested by HPLC (High Pressure Liquid Chromatography) and MS (Mass spectrometry) supporting successful synthesis and purification.

INTRODUCTION:

Folic acid is a water-soluble B_9 vitamin. It is also known as Pteroylglutamic acid. Folic acid is not physiologically active but when it is converted to 5-MTHF it becomes active. It plays a major role in metabolism of homocysteine and its synthesis, methylation, and repair of DNA (folate is used to synthesize thymine)[1,2,3]**.** Thus, it has a vital role in cell growth and cell proliferation. Insufficiency of folate in the body can result in many health problems. Lack of folate may cause an imbalance in DNA precursors, uracil misincorporation into DNA, and chromosome breakage[4]. Once bound to folate receptors that are present on the cell surface, invagination of the cell membrane occurs and the compound is internalized through the endocytosis process[5] given that FR is a membrane bound, glycosylphosphatidylinositol anchored glycoprotein(GPI)[6]. Folic acid has shown high affinity (K_D, 10^{-10} M) towards surface cell oriented receptor.[7] Thus, folate conjugates can be used in treatment of tumor and rheumatoid arthritis (RA) by attaching payloads to folic acid.

Folic acid is a combination of the pteroic acid (pte) (*Fig-1)* and glutamate (glu) and provides two positions for derivatization, the α- and γ-carboxylic acid (*Fig-2*). The glutamate residue and free carboxylic acid group of folic acid are believed to retain binding affinity to Folate receptor(FR)[7,8,9]. These findings debated that Glu moiety of folic acid is responsible FR binding and folic acid that lack of Glu moiety at the distal end exhibited poor binding of folate receptor. However, further studies had shown that removal of glutamyl carboxylate moiety from folic acid had no inhibitory effect on its cell internalization and these pteroic conjugates could also still selectively bind to FR+ cancer cells. Mark A Green[10] had shown that pteroic acid conjugate efficiently targeted FR as clinically studied Folate conjugate.

The current research involves purification and analysis of a pteroyl conjugate, pteroic-PEG-amine(*Fig-3*). By removing Glutamate moiety, this rapid synthesis allows the building of a conjugatable targeting ligand without the complexity of having a conjugatable free carboxylate group along with the free amine.

EXPERIMENTAL PROCEDURE:

Synthesis of pteroic conjugate:

- 0.0640 g of Universal PEG Nova Tag Resin was placed in syringe and DMF (dimethyl formamide) was added to it. Then it was shaken for 20 min and tip off. 20% piperidine (in water) was added to the syringe and it was shaken for further 5min to get Fmoc (fluorenylmethyloxycarbonyl) off from resin. After shaking, piperidine was squeezed off from the syringe.
- 0.0175g of HATU(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) , a peptide coupling reagent, was added to the syringe that contained resin and washed 2-3 times with 20% piperidine and DMF. This solution was squeezed in pteroic acid container and added 0.022 ml of DIPEA (N,N-diisopropylethylamine) to dissolve this substance. 0.5 mL of dimethyl sulfoxide (DMSO) also added to container to enhance pteroic acid dissolution. This whole solution was taken into the syringe and was put on shaker for 24 hrs.
- After 24 hrs, the solvent inside the syringe was removed and again washed with DMF(2ml), dichloromethane (2ml), Methanol (2ml).
- The leftover present in the syringe was washed with 100µL of distilled water and trifluoroacetic acid(TFA) and dried.

- The dried yield still had MMT (methylcyclopentadienyl manganese tricarbonyl) group on it. 1 mL of distilled water and TFA was added to above leftover. The solution became orange in color and It was shaken for 2hrs. By this reaction, the MMT group and PEG moiety (polyethylene glycol) was cleaved off from the resin.
- Ether was used and placed in a centrifuge tube and kept in ice bath. The above solution was added to ether solvent which finally formed a foggy solution. This mixture was centrifuged for 3 min. TFA helps the compound to be soluble in ether.
- After centrifugation process, the solvent was remained above the sediment in centrifuge tube. This was decanted from the tube.
- Argon gas was used to dry the obtained product in centrifuge tube. 9mL of distilled water and 10% of ammonium hydroxide (in water) was added to centrifuge tube.
- This produced the molecule of interest.

HPLC purification of Pteroic conjugate :

The crude conjugate was dissolved in DMSO and centrifuged for 10 minutes. The HPLC, Hewlett Packard, series 1050 equipped with diode array detector (DAD) and Chemstation software was used in this project. Rigel^{TM} HPLC, C18 column was used for purification of the sample (Reverse phase technique). Size of the of the column was 250 mm ×4.6 mm ×5 □m. Ammonium bicarbonate, 0.45g, in 500 mL of distilled water (10 mM) is used as a buffer (pH 7.5).

Table 1

	SOLVENTS	FLOW RATE	RUN TIME
a) b)	10mM NH_4HCO_3 Acetonitrile (ACN)	1mL/min	60 min

The parameters for solvent gradient was mentioned below.

Table 2

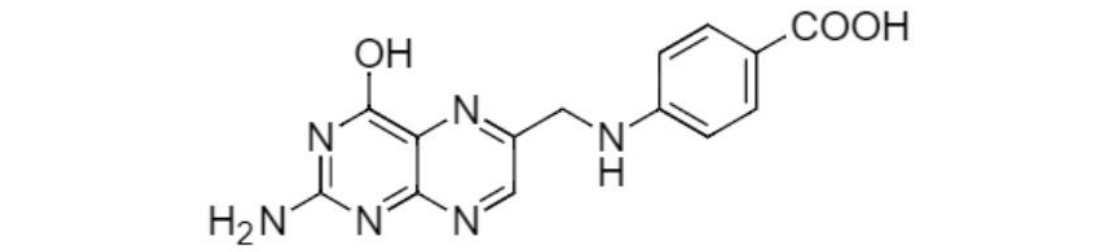
Time (min)	ACN (%)
0	1.0
40	30
50	60
55	80
56	99
65	99

Direct Injection into Agilent 1100 LC/MSD XCT trap

The purified fraction obtained from HPLC was run through LC/MS for further verification of the synthesized compound. This was run in positive ion method to generate the results. The molecular weight of the compound had Exact mass- 514.27 and Mol.Wt was 514.58. The parameters utilized for the methodology are given below.

Table: 3

FLOW RATE	0.5mL/min
SCAN RANGE	300-800 m/z
STOP TIME	10 min
SAMPLE	40 µL
NEBULIZER	8 psi
DRY GAS	8 L/min
TEMPEARTURE	300°C



4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)benzoic acid

Fig 1. Structure of pteroic acid

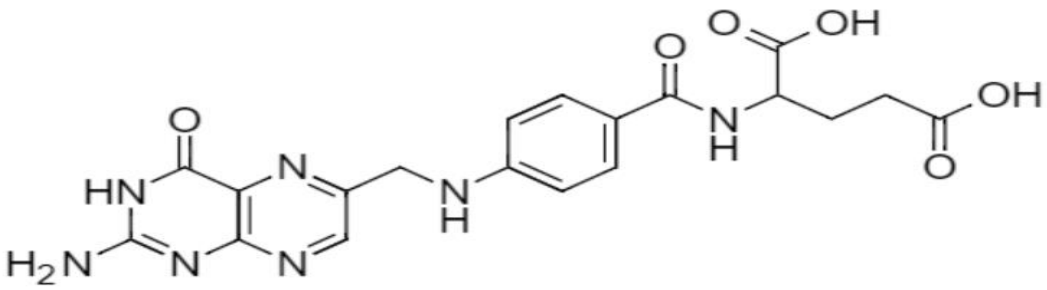
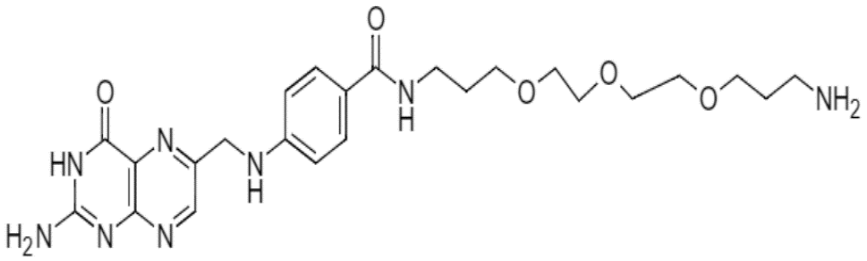


Figure 2. Structure of folic acid



4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)-N-(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)benzamide

Fig: 3. Structure of pte-PEG-amine (Exact mass: 514.27)

RESULTS AND DISCUSSION:

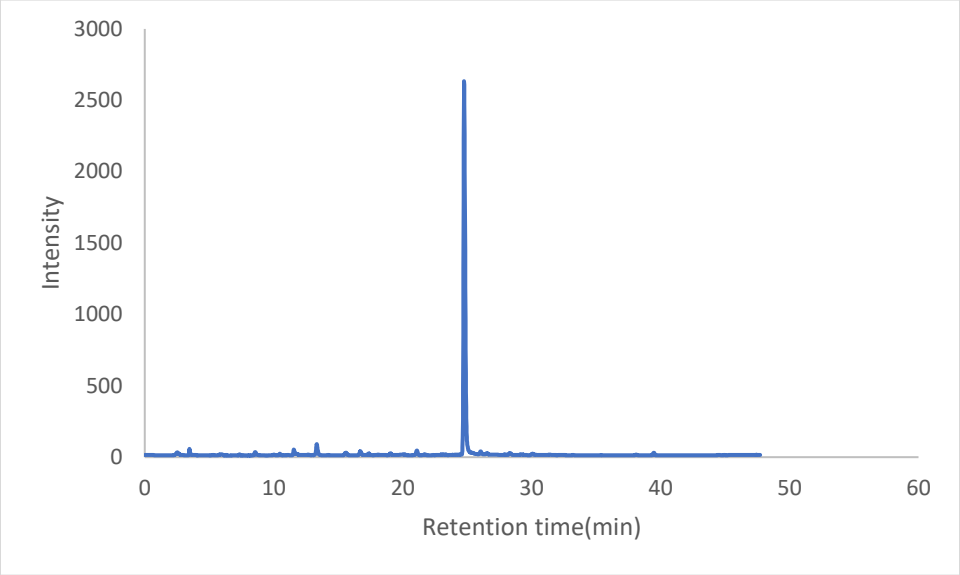


Fig 4: Chromatogram from HPLC purification of pteroic conjugate.

Purification of the synthesized compound by HPLC resulted a major peak at 25 min. As shown in the above figure(*Fig.4*), the obtained peak confirmed further by DAD Spectrum. DAD Spectrum demonstrated pteroic acid character (*Fig.5*) with maximal absorption taken place at 280 nm. The desired peak was isolated and collected at 25 min.

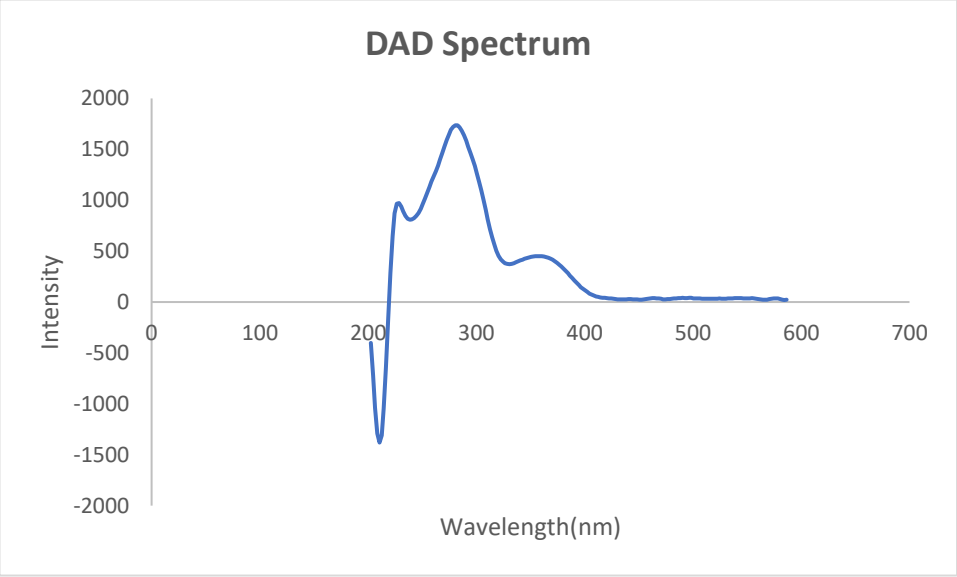


Fig 5: Diode array detector results with Pteroic acid character

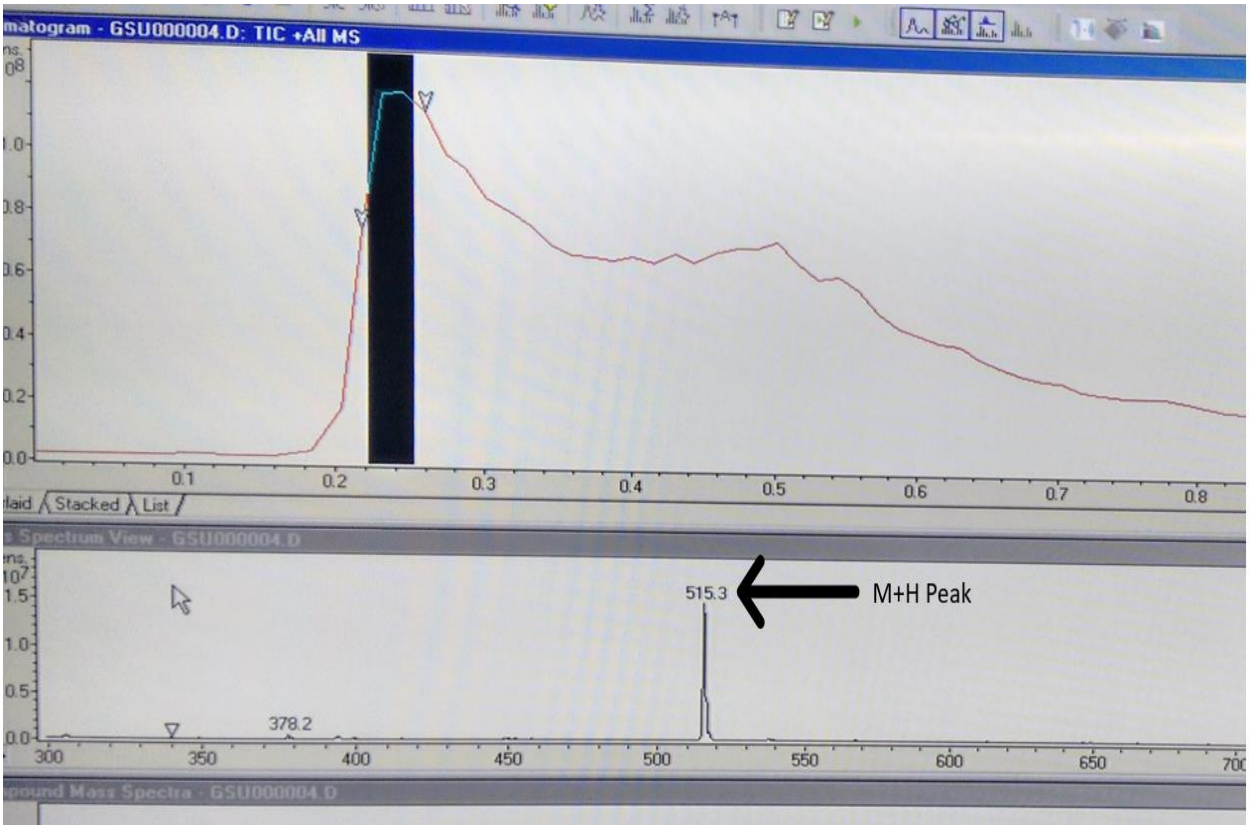


Fig:6. Mass Spectrum of Total Ion Chromatogram at 0.2 min for pteroic conjugate

The collected fraction from HPLC purification process was run through LC/MS for further identification of synthesized molecule. The above figure (*Fig-6*) demonstrated the Total ion chromatogram which shows a base peak of m/z is 515.3 and it was assumed as molecular ion. From the above spectrum, it was understood that proton was associated with the molecule ion that corresponded to the synthesized molecule.

CONCLUSION:

The pteroic conjugate was successfully synthesized in lab by Dr. Henne. Further, the conjugate, pte-PEG-amine was purified and analysed by RP-HPLC (Reverse phase technique). DAD spectrum verified isolated fraction of synthesized compound. Then, this fraction was run through LC/MS. The mass spectrum of this conjugate was demonstrated at 0.2min in total ion chromatogram. It supported the identity of the molecule by showing result in mass spectrum in positive ion mode. This building block can be used to attach further to drug delivery systems or imaging dyes that are used in studies of cancer and RA.

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