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Analyzing Brain Sample via HPLC Method

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Abstract:

Objective: This paper aims to investigate the principle methodology behind extraction and detection of an anti-cancer drug from brain samples via the high performance liquid chromatography (HPLC) method.

Methods: In order to assess the efficiency of the methodology carried out in the Rumbaugh-Goodwin Institute of Cancer Research (RGI), numerous peer-reviewed journals and articles were analyzed over the duration of this internship. They were filtered on the basis of inclusion of keywords, date written, peer-reviewed credibility, and similarity to RGI's research. The literature that has been chosen to be reviewed includes studies that present similar methodologies or test drugs with similar functions.

Main Results: From the comparison of RGI's protocol to protocol from other studies, it was found that some aspects of RGI's procedure could be revised to be even more efficient and accurate. These revisions include considering the addition of purifying substances to our sample, different homogenization techniques, and order of operation.

Conclusion: From this analysis, it can be concluded that the methodologies used at RGI to extract and detect the brain samples with HPLC were effective but could also use some improvements.

Keywords: HPLC, brain sample, cancer, drug, and VEGFR

Introduction:

The HPLC method developed after gas chromatography which, although was the main separation technology in the 1960's, had its limitations. These limitations included not being able to properly analyze large or polar molecules. However HPLC was able to overcome this by being able to analyze a broader range of compounds, making it critical in the exploration of drug discovery. In the 1970's the addition of septum less injectors evolved the apparatus to work at a higher performance, giving its name- high performance liquid chromatography. Once columns could be packed with particles as small as 3 μm , faster separations could be performed in smaller, narrower column. Then chromatographers were able to develop new separation methods and detectors to improve HPLC in the 1980's. Furthermore, the use of high pressures in a narrow column allowed for a more effective separation to be achieved in much less time than was required for previous forms of liquid chromatography. Even now, in the 21st century, HPLC is still experiencing more evolution as time passes.

Because of the advancements of HPLC, RGI is able to experiment with certain drugs in a precise and accurate manner. The drug in particular, which has been studied for the duration of this internship, is called F16. F16, whose chemical formula is *1,3-dioxo-2,3-dihydro-1H-isoindol-5-yl-amide*, was created to use against tumor growth in cancer cells (structure can be seen in Appendix A). It is a drug that is patented by the Executive Director of RGI, Dr. Appu Rathinvaleu, and is currently being tested for its retention period in brain samples taken from mice. In previous studies, the effect of F16 was tested by looking at its impact on tumor angiogenesis in a GI-101A breast cancer xenograft. This was done by seeing if F16, as a VEGFR-2 specific inhibitor, could reduce VEGFR expression on breast cancer cells. The results proved to be successful and indicated that F16 did in fact cause an anti-cancer effect through the

anti-angiogenic mechanism along with the anti-proliferative effect of blocking VEGFR expression.

To confirm that F16 is directly affecting the tumor size and not any other variable, a new project was embarked upon. The research we are currently undergoing is being done to test for the presence and retention period of the drug in the brain sample. This is done by the extraction and detection of an anti-cancer drug in brain sample by HPLC method. Brain sample was specifically chosen for this experiment because brain cancer glioblastomas were formed in the brains of the mice. In order for the drug to be proven effective in reducing the tumor size, it would have had to pass the blood-brain barrier (BBB). High-grade gliomas are only able to be treated with limited options because of the BBB which prevents drug uptake by brain tissue. Therefore a substantial amount of research is focused on circumventing the BBB in a localized fashion to reduce CNS toxicity towards the drugs (Bredlau, et al., 2018). This paper will analyze the methodologies used to test the brain sample and will determine the validity and efficiency of these methodologies. Based on our results, the methods used to extract and detect the brain sample via HPLC is the best fit for the research conducted at RGI.

Methodology:

To conduct this literature review many factors were taken into consideration when selecting sources. Primarily, the databases provided by the Alvin Sherman Library were mainly used. From the databases provided under the Biology subject, two specific databases: Medline (from Proquest) and the Biological Science Collection (from Proquest). Both of these databases contain articles and literature pertaining to medical and scientific research. Because it was important for these sources to contain relevant research that pertained to the current being done in our labs, it seemed best fit to set a date range from 2009-2019, within the last decade. This

area of research is still fairly new because due to the demand for anti-cancer drugs and the development of VEGFR inhibitors within the past couple of years.

Also, to make ensure all the articles are being held to a high standard of validity, it was critical for all the sources to be peer-reviewed. The process of peer-reviewing has experts of the field improvise the work by providing valuable feedback and selecting the most important research findings. The next stipulating factor was the use of certain keywords that were entered into the databases. The key words include: HPLC, brain sample, cancer, drug, and VEGFR. These keywords were essential to our paper and provided several resources that relate to the research conducted at RGI. From these results, the sources that were excluded were sources that didn't relate to cancer therapy, sources that did not HPLC as a main method, and sources that use samples taken from the brain. The sources that were included in this literature review are sources that fit all the previously listed requirements.

Literature Review:

Extraction of the brain sample from the previously-experimented on female athymic NCr-nu/nu nude mice is a crucial step in this research and requires a precise and accurate techniques (control and experimental groups can be seen in Appendix B). The first step is to thaw the frozen brain sample that was previously placed in -80°C temperatures. To thaw and prevent denaturing of the protein via localized heating, the brain sample was placed on top of a styrofoam box filled with ice cubes. A step that was not part of RGI's protocol but was seen in other studies was the exposure of the samples to streams of nitrogen to dry the sample (Jinfeng Hou et al., 2012) (Swales et al., 2015). After it thawed and was equivalent to room temperature, 100 mg of the brain sample was extracted and placed in a centrifuge to prepare for homogenization. After taking the two samples of 100 mg brain and an amount of acetonitrile,

one of the samples had F16 added to it. In the lab, the sample that has F16 added to it is labeled the spiked sample.

Traditional tissue analysis techniques usually require homogenization of the sample prior to analysis via HPLC. Our protocol differs from other studies that stores the brain homogenate in DMSO. This was done in a study conducted in 2003 which compared the HPLC method to the polyacrylamide gel electrophoresis while detecting for the loss of heterozygosity of LOH in glial tumor models (Chernova et.al, 2003). Rather than adding DMSO, the only substance we homogenized the brain sample with (aside from F16) was acetonitrile. The addition of acetone can also be seen in the methodology of a study published in the *Drug Delivery* journal (Bredlau et al, 2018). However, they also added chloroform which was to extract the doxorubicin and idarubicin, internal standards that were added earlier. This is however, irrelevant to our methodology.

In terms of the tool used to homogenize, there is a also a lot of variations. Bredlau et. al used Kontes 2 mL All Glass Dounce Tissue Grinder but at RGI the we used the TPE Rotor-Stator Homogenizer (Bredlau et al, 2018) (image of equipment in Appendix C). There are also many different methods to approach homogenization; including, but not limited to: sonification, freeze-thaw, and blender. Although RGI chose the blender method, other studies like the “Quantitative determination and pharmacokinetic study...” chose to use the freeze thaw method by placing their rat sample with their drug, FLZ, in three freeze cycle of -80 degrees C and ambient temperature (Jinfeng Hou et al., 2012).

After homogenizing the spiked and unspiked samples, the samples are placed in the HPLC machine. At RGI we use the Hitachi LaChrom Elite® 2000 HPLC System which may vary in every study due to the prices and access to these machines (image of system can be seen

in Appendix D). Earlier in the semester, RGI used the Perkins model which was a newer model but due to it being contaminated frequently, the Hitachi was selected to continue experimentation. The entire homogenized samples were transferred to centrifuge tubes. However, in other studies like the article published in the *Journal of Pharmaceutical and Biomedical Analysis*, the supernatant of the homogenized materials were extracted (Jinfeng Hou et al., 2012). The centrifuge tubes were centrifuged at 10,000 rpm for 10 minutes. This step also has a lot of variations in the compared studies. In the previously mentioned study, they centrifuged the components of their sample at $16,654 \times g$ for 10 min (Jinfeng Hou et al., 2012).

Following centrifugation we extracted the supernatant and placed 500 μL into a HPLC vial. At this point there were 4 vials, each containing a different substance: ACN, unspiked sample, spiked sample, and F16 standard. For the brain sample run, the system was set according to Figure 1 in the appendix. Every injection made by the system was 10 microliters which is equivalent to other studies like Swales et al. In terms of the extraction efficiency there was a similar use of HPLC the Atlanta Study. The extraction efficiency (% recovery) of SN-38 in porcine tissues was similar to that of tumors which had more than 90% recovery in all concentrations. Therefore, this extraction and HPLC protocol was applied to determine the amount of SN-38 in tumors. In comparison to RGI's protocol, the Executive Director, Dr. Appu Rathinavelu, also expects a 90% recovery in all concentrations (Rathinavelu et al, 2017).

Limitations:

From this review, it is understandable to see how the extraction and detection of brain sample via the HPLC method can be done in numerous ways. Although each step can be approached in a different matter, there is an overall consensus on the general procedure regarding these steps. From the start, these studies were bound to contain many dissimilarities to

the nature of biomedical and cancer research. For example, all the studies from this literature review are from different places around the world. Because of this, researchers may have limitations in regards to accessing certain models and substances and may prefer one option over the other. Although most equipment and materials are standardized in the research community, this may create slight variations.

Discussion:

In terms of methodology, the protocol researchers follow at RGI seems to follow the general procedure among the range of studies. Although there are details that could be improved to obtain more accurate results, it seems these methods are just as efficient. The most critical differences can be reflected upon with the research team to improvise the methodology for RGI's future experimentation.

One difference that can garner accurate results is the usage of additional substances in purifying the brain sample prior and after homogenizing. Certain substances like DMSO should possibly be added to the sample because most of the other studies included it in their protocol. Another recommendation from this review can be to consider the freeze-thaw method when homogenizing the samples. Although using the homogenizer saves time and is efficient, sometimes the sample can get stuck between the blades and can accidentally be washed away. Au contraire, freeze-thaw will preserve the sample in its entirety but will take a significantly longer amount of time. The similarities between RGI's methodology and the other studies indicates that majority of the steps involved in the protocol are efficient and effective. Another point to consider is that when assessing these differences, it's important to understand the underlying problem that needs to be overcome when doing these steps. For example, the inability of HPLC the technique to distinguish between actual tissue and residual blood contamination can

affect the results significantly. Because of problems like this, the overall protocol should be even more accurate and precise to overcome variables that cannot be controlled.

Furthermore, there are certain restraints to consider when comparing the methodology used at RGI to other studies found through the Medline and Biological Science Collection database. The fact that peer-review articles were specifically requested decreased the number of studies that could be reviewed. This becomes even more difficult because the topic is already quite specific and narrowed down. Another constraint of this review can be found when considering the location of where these studies originally took place. Some studies, e.g. the study conducted by Xie et al., could have been mistranslated because they are international resources. Certain meanings and steps could have been lost in translation which can consequently derail the reliability of this review. As mentioned earlier, the equipment and materials that many of these studies have access to can greatly differ from what RGI has access to. For example, the majority of the studies used samples from rats rather than mice. This could be possibly due to the difference in price. Either way, both animal models are able to produce reliable results that should not significantly affect the validity of the methodology.

Although the topic was quite specific in nature, it required a lot of effort to find sources that fit all the standards for this literature review. This area of research is still fairly new and the HPLC method has been constantly evolving throughout the decade. Although one methodology might have been the standard in 2010, there might be a variation of that method five years down the line. However, because the standards of this review were so specific, the studies included were quite relevant and carried most of the same practices as the research conducted during this internship.

Conclusion:

This literature review was highly effective in determining the validity and efficiency of the methodology for extraction and detection of the brain sample in HPLC. Although there are certain steps that can be improvised based on the reviewed studies, the general protocol is effective in detecting the retention period of the F16 drug. However, this should not dismiss the differences found between the studies. It can only benefit the researchers at RGI to look at these differences as possibly ways to improvise the current methods. If reasonable and feasible, RGI should consider certain elements and intertwine these steps into the current methods.

In the future, when reviewing additional articles, it would be better to include more keywords to broaden the search of this paper. This would allow the search to explore more variations in methodologies and construct an understanding of the most popular ones. This would be helpful in creating a standard to compare the RGI methodology to rather than just comparing the RGI methodology to several individual studies. Overall, the review was successful and was definitely worth conducting. Much more information regarding HPLC protocol was learned that would not have been learned if this review was not completed.

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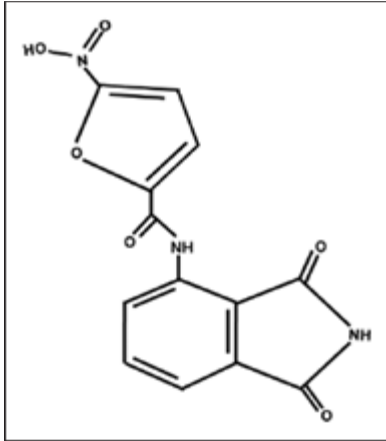
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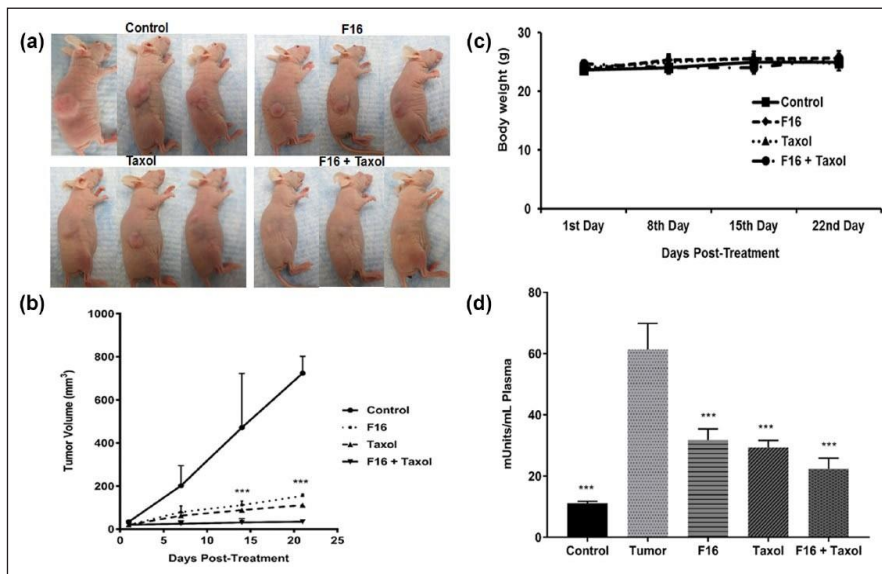
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Appendix A: Image of Chemical Structure of F16**Appendix B:** Image taken from Rathinvalu et al., 2017

From this image you are able to view the mice model used for RGI's research and can also view the correlating plasma values to the control and experimental groups.



Appendix C: Homogenizer tool used for HPLC protocol



Appendix D: Image taken from Rathinvalu et al., 2017

