Proteomic Profiling of Serotonin Receptor Knock-out Agents

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ABSTRACT

The binding of the novel drug $^3$H-LBT 3004, labeled with tritium $^3$H, to serotonin (5-HT) receptors was analysed using one-dimensional SDS-PAGE. Proteins from the mammalian mouse brain were separated using a 1 mm 10% SDS-polyacrylamide gel. Following electrophoresis, the protein bands were detected using Coomassie Brilliant Blue staining technique. Three lanes were used for liquid scintillation counting in $^3$H quantification. The other three lanes were used for Western Blotting onto PVDF membrane that was utilized in direct autoradiography. Results of the liquid scintillation counting produced two peaks of high counts per minute. A calibration plot was also done to confirm the linear relationship between relative mobilities of protein markers and their known molecular masses. It was utilized in the determination of molecular weights of the proteins that produced the two peaks. Direct autoradiography showed no indication of binding between drug and protein, though liquid scintillation counting concluded that the drug bound to proteins other than serotonin receptors but does not conclude that the drug does not bind to serotonin receptors. The failure of detection of binding via autoradiography was accounted for and suggestions for improvement of experiment were given.

INTRODUCTION

Proteomics has a vast field of applications, including the area of drug discovery. The drug receptor of interest here is the serotonin receptor, which has been categorized into as many as seven different classes, of which the 5-HT1, 5-HT2 and 5-HT3 receptor classes are well defined (Martin and Humphrey, 1994). The interest in serotonin has also grown immensely since its discovery (Rapport, 1949) and its effects on the mammalian brain (Amin et al., 1954).

A novel neuroactive drug, $^3$H-LBT 3004, synthesized via the modification of serotonin, was developed by Lynk Biotechnologies (Singapore). The experiment aims to determine if the drug binds to serotonin receptors and other proteins as in specific binding and non-specific binding respectively. Non-specific binding will lead to the determination of the occurrence of side effects. The protein separation process of one-dimensional SDS-PAGE was used and different protein detection methods were used in determining the type of binding displayed by the drug.

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METHODS AND MATERIALS

The dissected mouse brain was washed twice in 2ml cold PBS, with 0.1M EDTA and 0.1M PMSF additives. Homogenization was done in 2ml cold hypotonic lysis buffer with the same additives. Residual tissues on the homogenizer were washed into the homogenate with ~4ml lysis buffer, giving a 5.6ml final volume.

The heavier cell debris was allowed to settle before centrifuging the supernatant at 800rpm at 4°C for 2 min to remove fine debris. The two membrane pellets obtained were washed in 200µl Hank’s II Balanced Solution (HBSS) (Rickwood, 1994) with 0.1M PMSF, and centrifuged at 2000 rpm for 10s before resuspension in 250µl HBSS.

The drug ³H-LBT 3004 (45.5mM) was added to each sample at 1mM. Incubation was at room temperature 25°C for 30 min, with vortexing every 5 min. before centrifuging. The pellets were washed once in 1ml HBSS, centrifuged and resuspended in 50µl HBSS.

Protein assay, using the method of Lowry et al. (1951), was used to find out the protein amounts given in the appendix. The samples were pooled and made up to 75µl using HBSS, with addition of 75µl denaturing loading buffer. One-dimensional SDS-PAGE, using a 1.0mm 10% denaturing polyacrylamide gel, was utilized in the separation of proteins sample. Sample of 30µl was applied into each of the six wells seen in Figure 1. The last well was loaded with 30µl protein markers of known Mr. The gel was washed three times with deionized water after electrophoresis and cut into half. One half was stained with Coomassie Brilliant Blue, and destained before cutting into 2mm wide strips for liquid scintillation counting (Hames, 1982) and the other for Western Blotting. The PVDF membrane was used for direct autoradiography.

![Table of Well/Lane Number](image)

**Figure 1:** The ten wells of the gel showing the lanes used for the protein detection processes. Gray shaded wells indicate wells that were loaded with sample.

RESULTS AND DISCUSSION

**Liquid Scintillation Counting**

A calibration graph of the relative mobilities of protein markers that underwent electrophoresis versus the logarithm of their Mr, was done to obtain the equation
Using Eq. (1), the $M_r$ of the protein detected at a position of 23 mm was 36.5kD while the $M_r$ of the protein detected at position 44.5 mm was 17.0kD. The position parameter indicates the distance of the protein band from the stacking gel-resolving gel interface.

It was observed that the calculated molecular masses do not lie in the range of 50kD to 60kD for serotonin receptors. The drug displayed non-specific binding, where proteins other than serotonin receptors were bound to the drug. It is highly possible that the drug can cause side effects, depending on the cellular processes these proteins are involved in. There is a possibility in one-dimensional SDS-PAGE that two or more polypeptides migrate together as a single band (Sinclair and Rickwood, 1982), leading to the co-elution of these polypeptides from the gel during liquid scintillation counting. Hence the usage of two-dimensional SDS-PAGE prevents this from occurring.

It is still probable that the drug displays specific binding to serotonin receptors. One reason for the failure to detect specific binding is due to the small protein quantity in the final sample prior to SDS-PAGE sample application. The loss of proteins was mainly attributed to the way the protein assay was conducted. The results of the assay are shown in the appendix. A small dilution factor of 2 times was chosen, resulting in volumes as large as 12.5µl and 37.5µl of the two samples being used for the assay, where the samples were destroyed. A larger dilution factor could have been used instead, since a much smaller sample volume will be required and the unnecessary loss of protein-drug complex could be avoided.

**Western Blotting and Direct Autoradiography**

The scanned image showed no detection of the binding of drug $^3$H-LBT 3004 to proteins, even though results were obtained when liquid scintillation counting was used. The optimum exposure time is dependent on the type of isotope used and the amount of radioactivity present in the labeled proteins (Hames, 1982). The exposure time of the film to the PVDF membrane could have been increased since the low-energy beta rays emitted by $^3$H (Overend, 1951) was not sufficient for detection on film.

Two-dimensional SDS-PAGE concentrates the separated proteins into individual spots on the polyacrylamide gel. By this method of concentrating proteins, the proteins are less diffused unlike in the protein bands produced by one-dimensional SDS-PAGE. The level of radioactivity can thus be enhanced within a single spot and facilitates detection by autoradiography.

Another way of enhancing detection via autoradiography is by tagging the drug with a different radioisotope that emits higher energy radiation than $^3$H. A possible candidate for such usage is $^{32}$P, with a half-life of 14.5 days. Its half-life is reasonably long enough to maintain its function throughout the length of the experiment. Moreover, it is a widely used radioisotope in biological studies (Overend, 1951). However, the length of its half-life is also an indication of the storage time of the drug. Hence with $^{32}$P labeling, the labeled drug cannot be kept in storage for long and has to be resynthesized each time the radioisotope decays completely. The molecular mass of $^{32}$P also has to be taken into account when deducing the $M_r$ of bound proteins since protein separation in SDS-PAGE is based on the differences in molecular weights.
REFERENCES


