[BIO43] Development of medium throughput muscarinic receptor binding assay

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Introduction

Muscarinic receptors are members of the large family of G protein-coupled receptors (GPCRs). These receptors are widely distributed throughout the body and are responsible for numerous vital functions in both the brain and autonomic nervous system (Lefkowitz *et al*., 1990). Activation of muscarinic receptors in the periphery causes decrease in heart rate, relaxation of blood vessels, constriction in the airways of the lung, increase secretions and motility of the gastrointestinal tract, and increase secretions of lacrimal and sweat glands. In the brain, muscarinic receptors participate in many important functions such as learning, memory and posture control (Lefkowitz *et al*., 1990).

The majority of drugs currently available that act on muscarinic receptors have limited therapeutic utility due to side effects. Therefore, the discovery of novel agonists and antagonists of muscarinic receptors with fewer side effects is desirable.

Receptor-radioligand binding assays are often employed as preliminary screening techniques in pharmaceutical discovery programmes and are valuable basic research tools with wide application in many disciplines (Gattu et al., 1995). The basic stages involved in the radioligand-receptor binding assay are summarized in Figure 1.

We had developed a 96-well filtration based muscarinic radioligand-receptor binding assay suitable for medium throughput screening. This method allowed rapid screening and identification of potential new muscarinic agonists and antagonists from natural resources.

Materials and Methods

Tissue preparation

Total brain membrane (minus cerebellum) of male Sprague-Dawley rats was prepared according to the protocol described by Gattu *et al.* (1995) with minor modifications. Male Sprague-Dawley rats (250-300 g) were decapitated and the brains removed. The cerebellum was dissected out and the rest of the brain finely chopped with scissors, homogenized in 10 volumes of ice cold 50 mM Tris-HCl, pH 7.4 buffer using Ultra-Turax (2 x 10 seconds) and followed by glass-teflon pestle homogenisation at 800 rpm for 20 strokes.

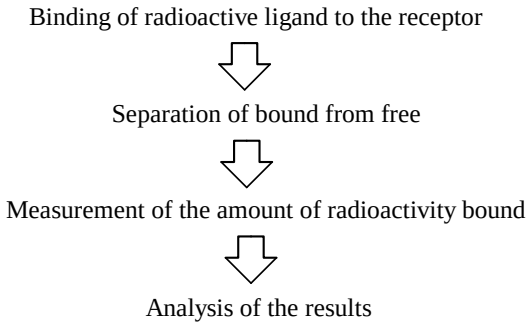

FIGURE 1 Steps involved in radioligand-receptor binding assay.

The homogenate was centrifuged at 40,000 g using ultra high speed centrifuge (Ultra Pro 80, Sorvall, USA), type T-880 rotor at 4°C for 15 min. The pellet was retained and washed twice by resuspending in ice-cold 50 mM Tris-HCl pH 7.4 buffer (centrifuged at 40,000 g, 15 min). The final pellet was suspended in 5 mL ice-cold 50 mM Tris-HCl pH 7.4 buffer, aliquoted and kept at -80°C until use.

Membrane protein concentration was determined by a modified method of Lowry *et al.* (1951) using Sigma Diagnostics Total Protein Reagent with BSA as the standard.

Radioligand-receptor binding assay

All experiments were performed in triplicate in a total volume of 250 µl/well. Six concentrations of membrane protein (0.06-2.20 mg/ml) were used to perform tissue linearity study and to determine the suitable concentration of protein for the saturation and competition assay.

In saturation and optimisation experiments, 200 µl/well membrane suspension was added to 0.01-1.0 nM [^{3}H] *N*-methyl scopolamine ([^{3}H]NMS) to give total binding (TB). Non-specific binding (NSB) was determined by adding 25 µl of atropine (10^{-5} M) to the reaction mixture. TB was subtracted by NSB to give specific binding (SB). The reaction mixture was incubated (temperature: 4, 21 and 37ºC; pH: 7.4, 8.0 and 8.9) for a specified time (0-180 min). After incubation, the reaction mixture was filtered with GF/C filter plate using a cell harvester (FilterMate, PerkinElmer) and washed with 200 µl/well of buffer (2-10 times). The plate was air-dried; bottom seal was then applied and followed by addition of 25 µl/well scintillation cocktail (MicroScint-O). The top side of the plate was then sealed with TopSeal A (PerkinElmer), agitated at 400 rpm for 5 min before scintillation counting for 1 min/well with TopCount NXT (PerkinElmer).

Data analysis

Data analysis was performed with PRISM® Software, version 3.0 (GraphPad, USA). Saturation analyses were made by nonlinear regression. K_i values were calculated according to the equation of Cheng and Prusoff (1973).

Results

Saturation experiments

The linearity of [^{3}H]NMS binding as a function of increasing amount of protein was established for protein concentration up to 0.67 mg/ml (or 133 µg of protein/well). This shows the optimum concentration of membrane tissue is 0.11 – 0.20 mg of protein/ml (22-40 µg of protein/well).

The influence of incubation temperature and pH (7-9) (Figures 2 and 3) on the dissociation constant (K_D) and number of binding sites present in the membrane

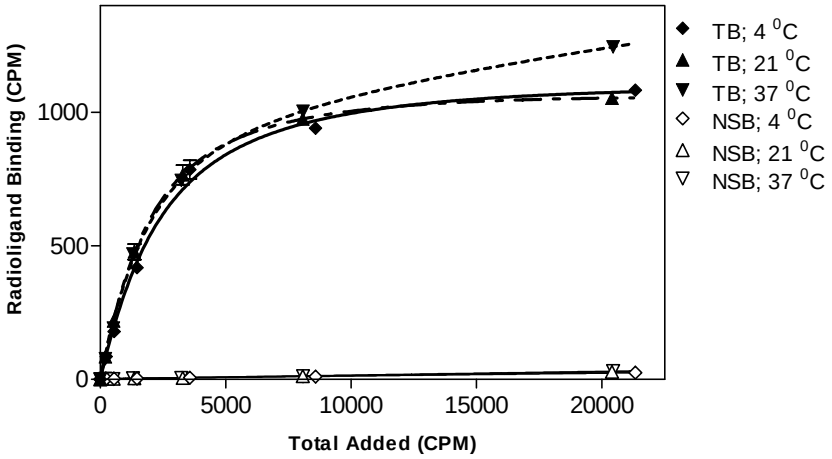

FIGURE 2 The effects of temperature on muscarinic radioligand-receptor binding assay. TB: Total binding; NSB: Non-specific binding

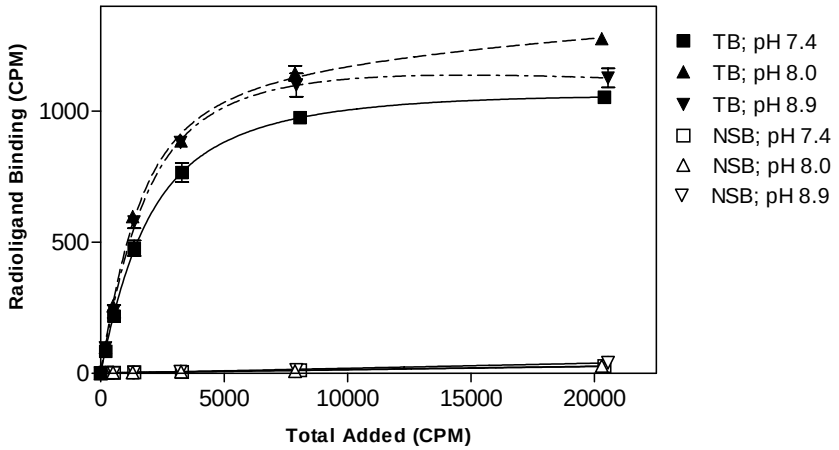

FIGURE 3 The effects of pH on muscarinic radioligand receptor binding assays. TB: Total binding; NSB: Non-specific binding.

receptor (B_{max}) was not significant. Rosenthal plot (Figure 4) indicates that only one binding site existed at the [^{3}H]NMS concentrations used to perform the assay.

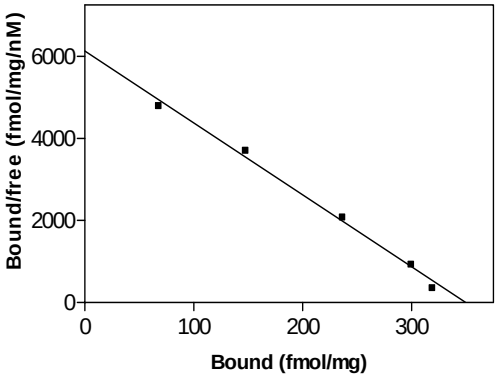

FIGURE 4 The Rosenthal plot of muscarnic receptor binding at pH 7.4, temperature 21 °C.

The steady state (equilibrium) for muscarinic receptor binding assay was reached between 60-180 min after initiation of incubation (Figure 5). Hence, 90 min incubation was used for further experiments to ensure all assays performed have reached the steady state before the assay was terminated.

The optimum number of washings required was determined by calculating the SB/NSB ratio for each washing performed. The ratio of SB/NSB decreased initially, and stabilised after 4 times of washing with minimal reduction in specific binding thereafter. The reason for the decrease is probably a result of partial dissociation of the bound [^{3}H]NMS from muscarinic receptors. Therefore, 4 times of washings was adopted.

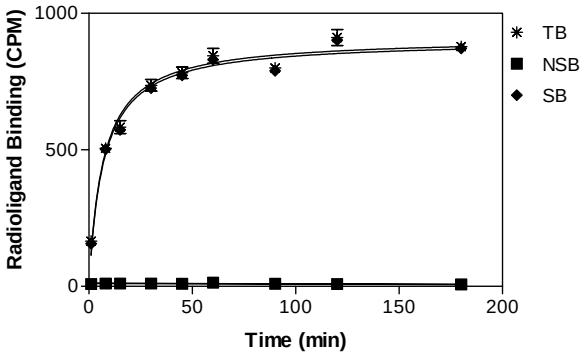

FIGURE 5 Incubation time for muscarnic receptor binding at pH 7.4, temperature 21 °C.

Competition experiments

Eight drugs which include known muscarinic antagonists were prepared in 10 concentrations (10^{-3} – 10^{-12} M) and tested using muscarinic receptor binding competitive assay. Competitive dose response curves for the drugs was shown in Figure 6. K_i and IC_{50} values obtained from the competition experiments with known muscarinic receptor ligands were 0.30 nM (atropine), 0.052 nM (scopolamine) and 1.6 nM (dicyclomine). K_i for atropine (0.30 nM) was 5.6 folds smaller than the reported K_i value (1.68 nM) (Gattu *et al.*, 1995). K_i obtained for non-muscarinic receptor ligands were 5.2 mM (propanolol), 17.2 μM (buspirone), 0.98 mM (serotonin), 9.4 μM (phentolamine) and 4.1 μM (8-OH-DPAT) (Figure 6).

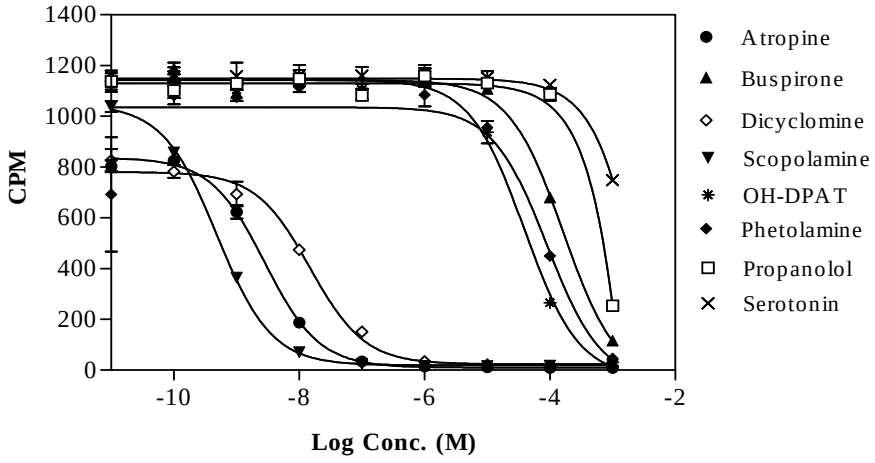

FIGURE 6 Competitive dose response curves for eight drugs.

The effects of DMSO

Due to its physiochemical properties, high solvent power, low chemical reactivity, and relatively low toxicity, DMSO is a solvent of choice for sample storage and handling in the pharmaceutical industry, particularly in primary high-throughput bioscreening. We tested the inhibitory effects of DMSO on the muscarinic radioligand-receptor binding assay (0.5%-10% v/v at final concentration). The results show the inhibition was minimal and insignificantly different compared to the control, up to 8% v/v DMSO. However at 10% v/v DMSO, the inhibition was about 20%. (Figure 7).

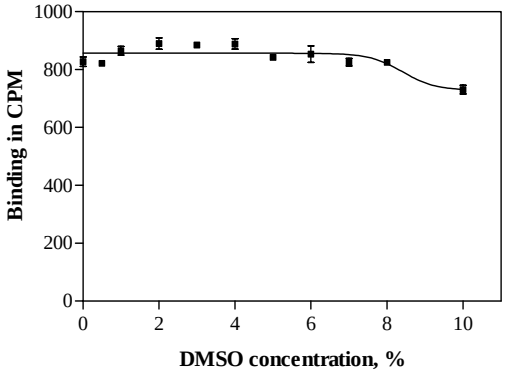

FIGURE 7 The effects of DMSO on the muscarnic radioligand-receptor binding.

Intra-plate and inter-plate validation

To assess the quality of assay, we have adopted statistical analysis using Z'-factor as it is accepted as a more reliable characteristic parameter compared to signal/background ratio (Zhang *et al.*, 1999). The calculated Z' factor is 0.8 (>0.5) for intra-plate. Coefficient variations (CV) for the inter- and intra-plate are 4.9% and 5.6% respectively.

Discussion

In summary, the optimised protocol consisted of 36 µg of protein/well, 0.5 nM [^{3}H] *N*-methylscopolamine ([^{3}H]NMS) and 50 mM Tris-HCl buffer, pH 7.4. The receptor-radioligand equilibration was reached after 90 min incubation at 21ºC.

In this protocol, less than 3 ml of scintillation cocktail is needed for 96 wells as compared to 4 ml/filter in traditional assays (total of 384 ml for 96 wells). This gives a 160 folds reduction of radioactive waste and hence cost saving and reduction in potential hazards.

Saturation analysis of [^{3}H]NMS gave B_{max} of 293 fmol/mg protein and K_D of 0.059 nM. The B_{max} value shows the muscarinic receptor density is high whilst K_D value suggests [^{3}H]NMS has high affinity and selectivity towards the receptor and is a suitable radioligand source. The linear Rosenthal plot suggests single site binding in this receptor-radioligand interaction. The K_i values, low intra-, inter-plate variability (CV, 4.9% and 5.6% respectively) and Z' factor of 0.8 clearly show this assay protocol is robust and reliable as a medium throughput screening assay for the detection of potential muscarinic receptor active compounds in chemical or natural products libraries.

Acknowledgements

The authors wished to thank the Ministry of Science, Technology and the Environment, Malaysia (MOSTE) for the financial support (grant IRPA: 26-02-06-0127) and National Science Fellowship (NSF) awarded to Yap Kim Fah.

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