Recent Research in Science and Technology 2010, 2(1): 78–82
ISSN: 2076-5061
www.recent-science.com

BIOTECHNOLOGY

RHODAMINE-B LABELED PEPTIDE HORMONE EVALUATION BY THIN LAYER CHROMATOGRAPHY

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Abstract
A novel thin layer chromatographic procedure was devised to evaluate the activity of rhodamine-B tagged peptide hormone such as vasopressin & octreotide. Peptide hormones viz. octreotide and vasopressin were added with rhodamine-B (0.2 ml of concentration 2.5mg/ml) and were estimated through thin layer chromatography (TLC) in a concentration and time dependent manner i.e. 25µg, 50µg for 30 & 60 minutes and 4.8µg, 9.6µg for 30 & 60 minutes respectively. The solvent medium used was a mixture of butanol, acetic acid and water in a ratio of 4:1:1. Stationary phase used in this experiment was Silica gel mixed with an inert binder like calcium sulphate and water. Different peptides travel at different rates due to the differences in their attraction to the stationary phase and because of difference in solubility in the solvent. The plates were made to visualize under a UV detector and respective Rf values were calculated. Results showed that rhodamine-B tagged peptides were well seen under UV detector as well as with naked eyes and is an efficacious marker when compared with other developing systems such as ninhydrin, iodine spray etc.

Key Words: TLC, vasopressin, octreotide, Rf, GPCR.

Introduction

G protein coupled receptors (GPCR) binding ligands are extensively studied as GPCRs represent the single largest molecular target of therapeutic drugs currently on the market, but is also the most common target in high-throughput screening (HTS) assays for identifying potential new drug candidates [1]. We chose two candidate hormones i.e vasopressin (9 amino acid) and Octreotide (8 aminoacid) almost similar in size for this study. Both these hormones bind to their respective GPCR and their analogues are being studied for novel therapeutic purposes. Vasopressin is a human hormone that is released when the body is low on water; it causes the kidneys to conserve water, but not salt, by concentrating the urine and reducing urine volume. It also raises blood pressure by inducing moderate vasoconstriction. Vasopressin is a peptide hormone. It is derived from a preprohormone precursor that is synthesized in the hypothalamus, from which it is liberated during transport to the posterior pituitary. Most of it is stored in the posterior part of the pituitary gland to be released into the blood stream; some of it is also released directly into the brain.The vasopressins are peptides consisting of nine amino acids (nonapeptides) [2]. The amino acid sequence of arginine vasopressin is Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly, with the cysteine residues forming a sulfur bridge. The structure of oxytocin another posterior pituitary hormone is very similar to that of the vasopressins: it is also a nonapeptide with a sulfur bridge and its amino acid sequence differs at only two positions. On the other hand, Octreotide is the acetate salt of a cyclic octapeptide. It is a long acting octapeptide with pharmacologic properties mimicking those of the natural hormone somatostatin [3].Octreotide is known chemically as L-Cysteinamide, D-phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-N- [2-hydroxy-1-(hydroxylmethyl) propyl], cyclic(2→7)disulfide;[R-(R*,R*)].It is commercially available as Sandostatin LAR® Depot (octreotide acetate for injectable suspension) and is available in a vial containing the sterile drug product, which when mixed with diluent, becomes a suspension that is given as a monthly intragluteal injection.

The octreotide is uniformly distributed within the microspheres which are made of a biodegradable glucose star polymer, D,L-lactic and glycolic acids copolymer. Sterile mannitol is added to the microspheres to improve suspendibility. Somatostatin is a 14 amino acid hormone. Octreotide is a derivative of somatostatin with a much longer half life. Both have the same therapeutic properties. The molecular weight of octreotide is 1019 daltons. It is used for the treatment of oesophageal varices. Most of the HTS procedures are still presently based on radioligand binding competition assays. Fluorescence approaches, based on the development of fluorescent ligands, offer a

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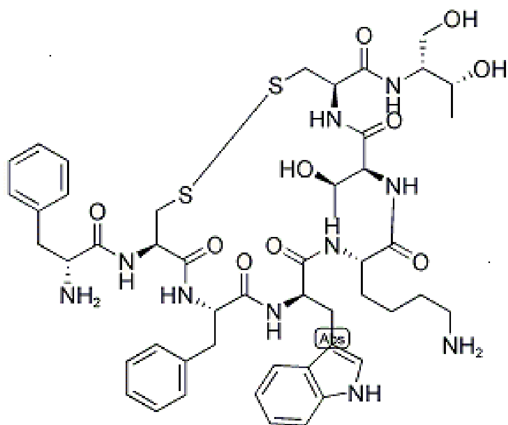
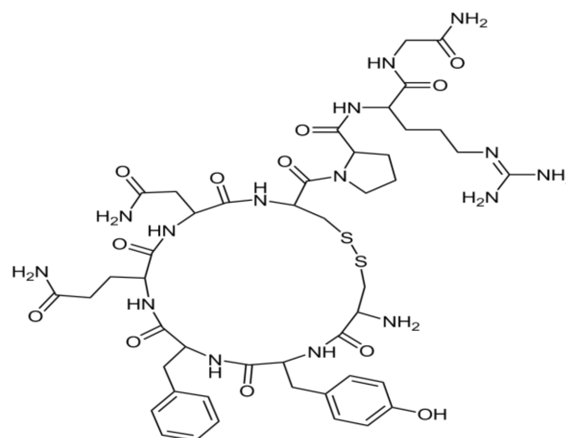
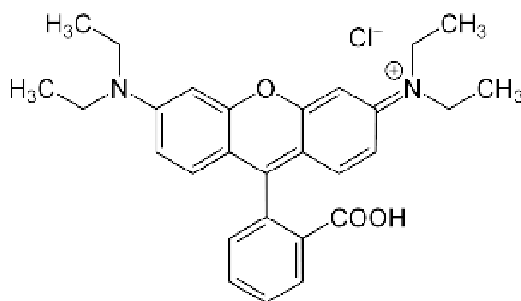
nonradioactive alternative to radioligand binding assays. In this study, we utilized the fluorescent dye rhodamine B and tagged it to vasopressin and octreotide for thin layer chromatography evaluation and determination of retention factor (Rf) values[4].



Octreotide



Vasopressin



Rhodamine B

Materials & Methods:

Chemicals and drugs used: Octreotide (United biotech Ltd, India), Arginine vasopressin (SG Pharma Private Ltd, India), Rhodamine B (Sigma Ltd ,India,), Butanol (Qualigens, India), Acetic acid (Qualigens, India), Silica gel (ACME Chemicals, India).

TLC equipment: All the equipments used for TLC used were from ACME synthetics, Mumbai, India. Acme applicator was used for thin layer chromatography, beakers, capillary pipettes for spotting solutions, rulers, hot plates (used in the hood), and gloves were used for all chemical handling procedures.

Protocol of drug and chemical usage:

Peptides: Vasopressin (4.8µg, 9.6µg), Octreotide (25µg, 50µg).

Rhodamine dye was used in a concentration 2.5mg/ml of water.

Solvent for TLC (4:1:1 of Butanol, acetic acid & water).

TLC plates (approximately 2” x 3”).

Procedure used for thin layer chromatography:

Chromatography is used to separate mixtures of substances into their components. All forms of chromatography work on the same principle. They all have a stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas)[5]. The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rate. The movement of the analyte is expressed by its retention factor (Rf) such that

$$Rf = \frac{\text{Distance moved by solute from origin}}{\text{Distance travelled by solvent from origin}}$$

Thin layer preparation:

A slurry of silica gel (stationary phase) generally in water is applied to a glass, generally 20 cm square, as a uniform thin layer by means of a plate spreader starting at one end of the plate & moving progressively to the other. The thickness of the slurry layer is of the order of 0.25mm thick. Once the slurry layer has been prepared, the plates are dried out in an oven at 100-120°C to leave the coating of stationary phase.

Sample application:

The sample is applied to the plate 2.0cm from the edge by means of capillary pipette. The sample used was octreotide & vasopressin mixed with rhodamine in two different concentrations i.e.

(i) (A) 0.5 ml octreotide (concentration= 25µg) + 0.2 ml rhodamine (concentration=2.5mg/ml)

(B) 1.0 ml octreotide (concentration=50µg) + 0.2ml rhodamine (concentration=2.5mg/ml)

(ii) (A) 0.2ml vasopressin (concentration=4.8µg) + 0.2ml rhodamine (concentration=2.5mg/ml)

(B) 0.4ml vasopressin (concentration=9.6µg) + 0.2ml rhodamine (concentration=2.5mg/ml)

A small spot of the sample was applied on the plate.

Plate development:

Separation most commonly takes place in a glass tank that contains the developing solvent (mobile phase) to a depth of about 1.5cm. The solvent system used was butanol, acetic acid and water in the ratio of 4:1:1. This was allowed o stand for at least 1hr with a lid over the top of the tank to ensure that the atmosphere within the tank becomes saturated with solvent vapour (equilibration). Unless this is done, irregular running of the solvent will occur as it ascends the plate by capillary action, resulting in poor separations being achieved. After equilibration, the lid is removed & the thin layer plate with sample is then placed vertically in the tank so that it stands in the solvent. The lid is replaced & separation of the compounds occurs as the solvent travels up the plate. One of the biggest advantages of TLC is the speed at which separation is achieved. This is commonly about 30mins & hardly ever greater than 90mins. The plate is then removed from the tank & allowed to dry.

Analyte detection:

There are several detection methods. The plate is taken after 30mins and kept in the solvent system and examined under UV detector i.e. under UV light which shows the position of UV absorbing or fluorescent compounds. Then again the plate is examined after 60mins under UV detector and Rf values were calculated.

Results

Table 1 depicts the concentration, time duration and Rf value calculations for rhodamine tagged vasopressin evaluated by TLC.

S. no.	Concentration (µg)	Time Duration (mins)	Distance travelled b solute(cms)	Distance travelle by solvent(cms)	Rf value
1.	4.8	30	6.0	6.5	0.923
2.	9.6	30	5.5	6.0	0.916
3.	4.8	60	8.0	9.0	0.888
4.	9.6	60	8.4	9.5	0.884

Table 2 depicts the concentration, time duration and Rf value calculations for rhodamine B tagged octreotide evaluated by TLC.

S.no	Concentration (µg)	Time Duration (mins)	Distance travelled b solute(cms)	Distance travelle by solvent(cms)	Rf value
1.	25	30	5.9	6.3	0.936
2.	50	30	6.0	6.7	0.895
3.	25	60	8.6	10.4	0.826
4.	50	60	8.6	9.8	0.877

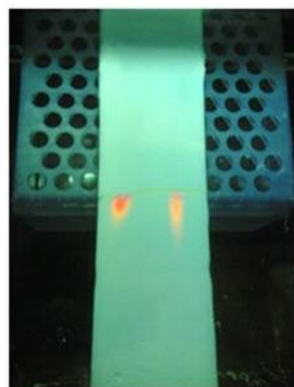


Figure: 1

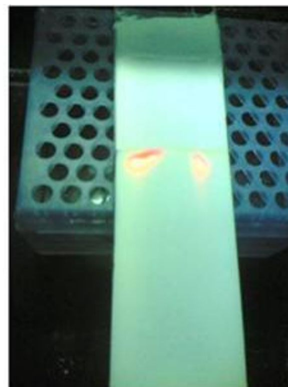


Figure: 2

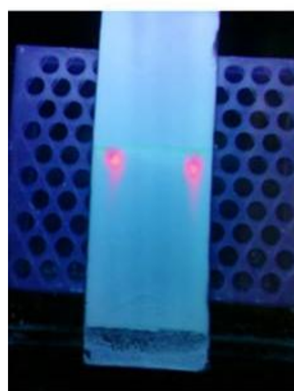


Figure: 3

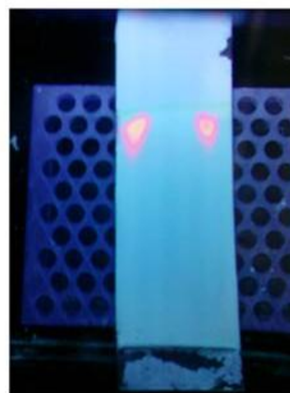


Figure: 4

Figure 1 shows the silica gel coated plates spotted with rhodamine B tagged vasopressin (4.8 µg and 9.6 µg) for chromatographic evaluation for a duration of 30 minutes. Figure 2 shows the silica gel plates spotted with rhodamine B tagged vasopressin (4.8µg and 9.6 µg) used for chromatographic evaluation for duration of 60 minutes. Figure 3 shows the silica gel coated plates spotted with rhodamine B tagged octreotide (25 µg and 50 µg) used for chromatographic evaluation for a duration of 30 minutes. Figure 4 shows the silica gel coated plates spotted with rhodamine B tagged octreotide (25 µg and 50 µg) used for chromatographic evaluation for a duration of 60 minutes.

Discussion

Thin layer chromatography has extensive application in protein chemistry. This includes recovery of peptides in microgram and nanogram quantities for further primary structural identification of peptides, peptides mapping and determination of molecular weights of peptides & proteins [6]. The various peptides and proteins have been located on thin layer chromatography by using ninhydrin, fluorescamin, iodine vapors or UV light. In our study we used rhodamine B as fluorescent material and evaluated the retention factor (Rf) of vasopressin and octreotide as shown in Table 1 & 2 and also depicted in Figures 1 to 4. Our results clearly show a very good binding and because of the increased sensitivity of the fluorescence readers and of the brightness of the fluorophores, most of these studies can be performed using fluorescence-based technologies. Fluorescent ligands selective for a given receptor family or a given receptor subtype are consequently very useful labeling and pharmacological tools. These ligands i.e vasopressin were defined as useful for the study of renal AVP receptors by fluorescence microscopy, particularly the fluorescein and rhodamine-labeled agonists. Whereas fluorescein exhibits low photostability, which restricts its use for imaging events occurring over long time periods, and therefore rhodamine fluorophore is ideal in that it possesses a high molar extinction coefficient and does not photobleach readily. The synthesis and characterization of first generations of fluorescent agonists and antagonists for AVP/OT receptors led to the discovery of very promising pharmacological tools but their use was limited to fluorescence microscopy techniques applied to the investigation of the cellular expression and localization of receptors. In this study we used optimum concentration of vasopressin and we got good elution after 30 and 60 minutes period. In earlier studies it was shown that the sensitivity of the fluorophores attached to these ligands is far from being

equivalent to that of usual radioisotopes (^{3}H, ^{125}I), and ligand binding assays could not generally be developed[7]. Thus it is not surprising that a posterior derivatization of AVP/OT analogues with bulky fluorophores like fluorescein or rhodamine at this particular position (amino acid residue 8) led to the successful development of numerous fluorescent ligands retaining high affinity, selectivity, and functional activity for these receptors. This residue is finally not crucial for binding, although it has been demonstrated to be involved in receptor subtype binding selectivity[8]. The results obtained with octreotide also suggest that its chemical configuration assimilates well with rhodamine B as a flurophore. As indicated in the introduction, GPCRs represent the single largest molecular target of therapeutic drugs currently on the market, but are also the most common target in high-throughput screening (HTS) assays for identifying potential new drug candidates. Most of these HTS procedures are still presently based on radioligand binding competition assays. Fluorescence approaches, based on the development of fluorescent ligands, offer a nonradioactive alternative to radioligand binding assays [9,10]. Fluorescence polarization and FRET (such as homogenous-time resolved fluorescence (HTRF)) techniques are even more useful since they can be achieved without the separation of the bound and free ligand fractions, leading to the development of homogenous microplate format assays with cell membrane preparation or whole cell samples. The development of these techniques has stemmed first from the elaboration of numerous sensitive microplate readers, and second from the synthesis of new fluorescent probes. Among the new fluorophores, Alexa series exhibit greater photostability and enhanced fluorescence emission than those of the classical fluoroprobes such as fluorescein and rhodamine. These properties increase the sensitivity of the assays, facilitating the development of fluorescence-based techniques. With such powerful tools, coupled with the rapidly advancing complementary imaging technologies such as fluorescence correlation spectroscopy, one is therefore becoming able to investigate therapeutically important GPCRs and their molecular relationships with bioactive ligands at the single cell level and even at the single molecule level [11,12]. This will open the way to study compartmentalization and structural organization of GPCRs in specialized microdomains of the cell plasma membrane. Thus this study provides simple and cheaper alternative for chromatographic evaluation of peptide hormones like vasopressin and octreotide using rhodamine B as a fluoroscent material.

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