SHORT THESIS FOR THE DEGREE DOCTOR OF PHILOSOPHY (PhD)

Development and practical application of radioimmunoassay (RIA) methods for measurement of the glucagon-type peptide-1 (GLP-1) and the melanin concentrating hormone (MCH)

Beáta Leleszi

UNIVERSITY OF DEBRECEN

Doctoral School of Pharmaceutical Sciences

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DEVELOPMENT AND PRACTICAL APPLICATION OF RADIOIMMUNOASSAY (RIA) METHODS FOR MEASUREMENT OF THE GLUCAGON-TYPE PEPTIDE-1 (GLP-1) AND THE MELANIN COCENTRATING HORMONE (MCH)

By Beáta Lelesz, Molecular biology MSc degree

Supervisor: Zoltán Szilvássy MD, PhD, DSc

Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the **Examination Committee**:	Árpád Tósaki PharmD, PhD, DSc
Members of the Examination Committee:	István Wittmann MD, PhD, DSc
	József Balla MD, PhD, DSc

The Examination takes place at the library of the Department of Pharmacodynamics and Pharmacokinetics Faculty of Pharmacy, University of Debrecen
11:00 am, 24th October 2017.

Head of the **Defense Committee**:	Árpád Tósaki PharmD, PhD, DSc
Reviewers:	János Gardi PhD
	Harjit Pal Bhattoa MD, PhD
Members of the Defense Committee:	István Wittmann MD, PhD, DSc
	József Balla M.D, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
1:00 pm, 24th October 2017.

LITERATURE REVIEW

Literature review of the glukagon-type peptide-1 (GLP-1)

Coding gene of proglucagon, a molecule precursor to the glucagon and other glucagon-type peptides (GLP-1, GLP-2, oxyto-modulin), was cloned in 1983. It was found that the same gene was found both in the α-cells of the pancreas and in the L-cells of the small intestine. The proglucagon produced in the two types of cells have the same structure (160 amino acids), but the post-translational process and the biologically active peptide hormones formed are completely different. Glucagon is produced in α-cells of the pancreas, GLP-1 and GLP-2 in the L-cells of the small intestine.

GLP-1 is not a particular peptide but an entire molecule group, namely it includes the following peptides: GPL-1 (1-37), GLP-1 (7-37), GLP-1 (9-37) and the amidated forms like GLP-1 (1-36) -NH 2, GLP-1 (7-36) -NH 2, GLP-1 (9-36) -NH_2. Biologically active forms are GLP-1 (7-37) and GLP-1 (7-36) -NH_2.

GLP-1 peptide hormone has many biological effects in the living organism. Improves glucose homeostasis by stimulating insulin release by nutrient intake and reducing glucagon secretion. It increases secretion of insulin from the pancreas in proportion to the blood sugar level, while reducing glucagon production and release. It enhances the growth of the β-cells of the pancreas and the expression of the gene responsible for the production of insulin. It greatly enhances insulin sensitivity. It inhibits gastric acid production and reduces gastric emptying speed, thus slowing down the hunger sensation and decreasing dietary intake. It can be seen that GPL-1 directly and indirectly affects the organization's sugar households, making it an important and studyable factor in studying the mechanism of obesity and diabetes.

Molecular diversity is a major challenge in the development of GLP-1 RIA. It is impossible to develop a RIA method that equally measures all GLP-1 peptides, but no assay can be developed which only measures one form of the six variants.

Literature review of the melanin concentrating hormone (MCH)

The melanin concentrating hormone was first isolated in 1983 from the pituitary glands of salmon and was identified its chemical structure. It has been found that MCH

consists of 17 amino acids and has been isolated from fish with a cyclic structure. In terms of its biological effect it plays a major role in the regulation of pigmentation of bony fish skin. A few years later, MCH was also isolated from rat hypothalamus. Rat and mammalian MCH are also cyclic, but not identical to those previously isolated from salmon. The mammal MCH consists of 19 amino acids (longer amino acids on the N-terminal site and amino acid substitution at 4 sites in the peptide chain), in which disulfide bridge links the Cys residues at positions 7 and 16. The peptide also contains Tyr (position 13), a radioactive marker (125I) which is an important factor for RIA development.

Experiments on chemical structure and biological activity have shown that the loop structure is essential for biological activity and that Arg (Arginine) and Tyr (tyrosine) residues in the ring play an important role in the biological activity of MCH. Mammalian MCH is formed from a precursor polypeptide molecule containing 165 amino acids in the post-translational process. MCH originally encounters the C-terminal portion of the preprohormone, and becomes free and turn out to a biologically active peptide hormone because of the proteolytic cleavage between the amino acids 145 and 146.

MCH in mammals is predominantly expressed in the lateral hypothalamus and in the zone incertal but its appearance at low levels is observed in some peripheral organs and tissues, such as pancreas, colon epithelial cells, and adipocytes.

Related to the physiological role of the peptide in mammals it has been shown to play a key role in regulating nutrition behavior. It controls energy balance, food absorption, body weight and metabolism. MCH can increase food intake, which in the long run leads to weight gain and then obesity. In addition, it is proven or supposed to be involved in the development of diabetes, intestinal inflammation, and sleep-wake cycle control.

Literature review of radioimmunoassay (RIA)

Development of ligand assays using radioisotope-labeled reagent began with radioimmunoassay or shortly RIA in the sixties of the XX. century. Steve Berson and Rosalyn Yalow used the method for measuring insulin for the first time in 1960. In ligand assays the determination of the antigen (substance to be measured) is mostly done by immunoglobulins (immunoassay) but if it is necessary receptor proteins (receptor assays) or physiological transport proteins (competitive protein binding assay (CPBA)) can be use as specific binders. RIA is based on an immunochemical reaction between the antigen and the antibodies

produced by it, in which the antigen-labeled and unlabeled form of the antigens compete for the antibody binding sites. The competition is only achieved if the number of binding sites are much less than the amount of cold and labeled antigen which are capable of binding. The detection of this method is ensured by the tracer providing radioactive material with a measurable signal.

In a good RIA the measurement limit is 0.1 fmol/ml antigen concentration. Exceptional sensitivity is due to the fact that the method combines the specificity of antigen-antibody reactions and the high sensitivity of isotope detection technology. The unrestrained popularity of the process since its development is possible because of the obtain precise concentration determination from a very small quantity of materials in series measurements. The 1977 Nobel Prize was awarded for the method (which was used to determine the hormone content of stomachs and steroids in tissues and body fluids, contributing to the revolutionary renewal of endocrinology).

OBJECTIVE

We have designed two new radioimmunoassays, the practical application of which can provide useful information to the basic research of vitality, namely nutrition, obesity, insulin resistance and type 2 diabetes at the University of Debrecen, Pharmacology and Pharmaco-therapeutics. Two peptide hormones selected for RIA development: glucagon type peptide-1 (GLP-1) and melanin concentrating hormone (MCH). These peptides based on their biological effects (regulation of insulin production, control of dietary intake) play an important role in controlling the body's energy balance and metabolism.

For the development of RIA methods, the following sub-tasks had to be solved:

- synthesis of GLP-1 and MCH peptides,
- making immunogens (linking the peptides to the carrier protein),
- immunization (production of counterbalances),
- characterization of antisera (titer, affinity, specificity),
- labeling the peptides with radioactive isotopes,
- cleaning the RIA tracer,

- Practical implementation of RIA methods (optimum measurement conditions, separation procedure selection, evaluation and control of assays),
- preparation of plasma and tissue samples for measurement.

In the newly developed GLP-1 RIA method, it was planned to use atypical antipsychotic-induced obesity and insulin resistance rat model. In the experiment, rats were treated with olanzapine and at the end of the study we measured the plasma GLP-1 levels under the fasting and glucose tolerance test and the glucose tolerance was determined.
We used our MCH RIA method for tissue MCH content definitions in our laboratory. Tissue peptide concentrations were determined from the individual stages of the gastric intestinal tract of Wistar rats, their central nervous system and other peripheral organs.

DEVELOPMENT OF RIA METHODS

During the development of the GLP-1 and MCH RIA methods, the following sub-tasks were solved:
The synthesis of the peptide derivatives required for the development of RIA methods (GLP-1 (19-37), Cys (0) -GLP-1 (19-37), MCH) was conducted by Professor Gábor Tóth at the Institute of Medical Chemistry, University of Szeged.
To link the GLP-1 and MCH peptides to the carrier protein, a Michael addition and glutaraldehyde technique was used.
Antibodies to GLP-1 and MCH were produced by immunization of rabbits. The characterization of the antisera was determined by determining their titer and affinity constant, and in the specificity assays it was shown that the antiserum "GLP-1 3/7" practically measures 100% of GLP-1 (19-37) GLP-1 (1-37), GLP-1 (7-37) and GLP-1 (9-37), but does not detect other peptides of the same hormone family.
The "MCH 1/5" antiserum used for the MCH RIA method measures 100% of mammalian MCH, modified MCH (Tyr (2), Phe (13) -MCH), but does not bind to the peptide molecule ring structure of the amino acid sequence and other peptides of the same hormone family.
The iodogenic process was used to mark GLP-1 and MCH peptides with ^{125}I isotopes. Reverse phase HPLC was used to purify mono-iodinated radioactive peptides (RIA tracer). During the marking reaction the efficiency of iodine uptake was 90% for both peptides.

After the production of the essential components for the development of the RIA methods we determined the optimum operating conditions of the methods, selected the appropriate separation procedure, the evaluation methods and the control system.
Plasma GLP-1 concentration was determined after alcohol extraction. The rat tissue MCH content was measured from a neutral extract.

PRACTICAL APPLICATION OF RIA METHODS

Our newly developed GLP-1 RIA method was used first in atypical antipsychotic-induced obesity and insulin resistance rat models. During the experiment rats were treated with olanzapine and at the end of the study we measured the plasma GLP-1 levels under the fasting and glucose tolerance test and the glucose tolerance was determined. Plasma GLP-1 concentrations in the olanzapine-treated group showed lower values compared to the control group, while blood glucose levels were higher in the antipsychotic-treated group.
Our first application of MCH RIA was the determination of tissue peptide concentrations from certain stages of the gastrointestinal tract of rats, their central nervous system and other peripheral organs. Our results on the distribution of gastrointestinal tract and peripheral organs in the MCH content provided completely new literature data. Very high MCH concentrations were measured in the small intestine, mainly in the duodenum, followed by jejunum and ileum. Also high MCH concentrations were found in the pancreas and kidneys. Lower MCH content was detected in trachea and liver, very low values were measured in the other examined tissue samples (fundus, antrum, colon, rectum, lung, skeletal muscle, heart). Using our high sensitivity RIA method our research group found low but well-measured MCH levels in human plasma (unpublished data).
Based on our experimental results and scientific knowledge we assume that MCH has a dual role in the body. On the one hand, neurotransmitter in the central nervous system and on the other hand a gastrointestinal circulating hormone released by eating, which can play an important role not only in controlling food intake but also in digestion. It is presumed to have an effect on the development of diseases such as abnormal obesity or abnormal tenderness. The clinical application of our high sensitivity and specific MCH RIA method can open up new perspectives for the diagnosis of unidentified disorders (eg. MCH producing adenoma).

SUMMARY

The thesis summarizes the development of two biologically active peptide hormones, namely glutamine-type peptide-1 (GLP-1) and melanin concentrating hormone (MCH), as well as the results of their primary practical application. Both peptides play an important role in controlling the energy balance of the body and after being an important research field in our institute for studying the development of nutrition, obesity, insulin resistance and type 2 diabetes so we have found it important to develop these new RIA processes to ensure a high level of laboratory background to make their daily routine use available.

Synthesis of the GLP-1 and MCH peptides required for the development of the methods and the production of immunogens for the immunization of rabbits (linkage of the peptides to the BSA carrier protein) was carried out at the Institute of Medical Chemistry at the University of Szeged by Professor Gábor Tóth. The other steps of the development of RIA methods include the production of antibodies by immunization of rabbits, characterization of antiserum (titer, affinity, specificity), ^{125}I isotope-labeled RIA tracer (radioactive labeling, HPLC purification), optimization of RIA processes and the practical application of the methods (plasma GLP-1 and measurement of tissue MCH levels) was performed in our institute's isotope laboratory.

For our GLP-1 RIA method the calibration curve D_{50} value after 10 determinations: 15.18 ± 2.02 fmol/ml. The detection limit of the assay for the GLP-1 peptide was 0.4 fmol/ml.

For MCH RIA the calibration curve D_{50} value is 11.93 ± 1.78 fmol/ml, the detection limit concentration for MCH is 0.2 fmol/ml.

Our RIA methods for measuring GLP-1 and MCH are highly susceptible and specific for the hormones to be measured. Both can be used to determine plasma and tissue peptide concentrations.

KEYWORDS

RIA, GLP-1, MCH, ^{125}I, HPLC purification, radioactive labeling

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Subject: PhD Publikációs Lista

Candidate: Beáta Lelesz
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List of publications related to the dissertation

1. **Lelesz, B.**, Szilvássy, Z., Tóth, G. K., Tóth, A., Enyedi, A., Felszeghy, E. N., Varga, A., Juhász, B., Németh, J.: Radioanalytical methods for the measurement of melanin concentrating hormone (MCH) and detection its receptor in rat tissues.
J. Radioanal. Nucl. Chem. 310 (3), 1325-1333, 2016.
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2. **Lelesz, B.**, Tóth, G. K., Peitl, B., Hegedűs, C., Drimba, L., Sári, R., Szilvássy, Z., Németh, J.: Description and application of a novel glucagon-like peptide-1 (GLP-1) radioimmunoassay.
J. Radioanal. Nucl. Chem. 299, 157-164, 2013.
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IF: 1.415

List of other publications

3. Fürjes, G., **Lelesz, B.**, Tóth, G. K., Arday, A., Szilvássy, Z., Varga, A., Berényi, E., Németh, J.: Comparative distribution of somatostatin and thrittene bioactive peptides in the central nervous system of rat measured by radioimmunoassay.
   J. Radioanal. Nucl. Chem. 311, 1741-1749, 2017.
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4. Tamás, A., Jávorhazy, A., Reglődi, D., Sarlós, D. P., Bányai, D., Semjén, D., Németh, J., **Lelesz, B.**, Fülöp, D. B., Szántó, Z.: Examination of PACAP-Like Immunoreactivity in Urogenital Tumor Samples.
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   J. Radioanal. Nucl. Chem. 292 (1), 113-118, 2012.
   DOI: http://dx.doi.org/10.1007/s10967-011-1516-x
   IF: 1.467

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CONFERENCE APPEARANCES

Horvath G., Nemeth J., **Lelesz B.**, Tamas A., Reglodi D., Kiss P.:
Environmental enrichment changes the levels of PACAP in the central nevous system.
The 11th International Symposium on VIP, PACAP and Realted Peptides
Pécs, 2013. augusztus 27-31.

Tamas A., Javorhazy A., Csanaky K., Ragnhildstveit E., Vikjord S.A., Sarlos P.D., Sarszegi Zs., Zapf I., Szanto Z., Faludi B., Molnar T., Nemeth J., Banki E., **Lelesz B.**, Reman Gy., Reglodi D.:
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Changes in PACAP levels following environmental enrichment in the central nervous system of rats.
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Lelesz B., Tóth A., Juhász B., Szilvássy Z., Németh J.:
Radio-analitikai módszerek alkalmazása a melanin-koncentráló hormon (MCH) mérésére és receptorának detektálására.
Magyar Framakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok Közös Tudományos Konferenciája (FAMÉ)
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Lelesz B., Szilvássy Z., Tóth A., Juhász B., Kiss R., Németh J.:
Radioanalytical methods for the measurement of melanin-concentrating hormone (MCH) and detection its receptor in rat tissues.
8th National Congess with Internation Participation of the Romanian Society for Cell Biology
Nagyvárad, 2016. június 8-12.

Lelesz B., Szilvássy Z., Tóth A., Juhász B., Kiss R., Németh J.:
Radioanalitkai metodikák kifejlesztése a melanin koncentráló hormon (MCH) szöveti peptid tartalmának meghatározására és receptorának kimutatására patkányban.
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Debrecen, 2016. december 8-10.

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Radio-analitikai módszerek alkalmazása a melanin-koncentráló hormon (MCH) mérésére és receptorának detektálására.
Magyar Klinikai Farmakológusok XVIII. Továbbképző Kongresszusa
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