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CYCLIC CHOLECYSTOKININ ANALOGUES EXHIBIT HIGH BLOOD STABILITY AND BINDING AFFINITY WITH CHOLECYSTOKININ RECEPTOR

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ABSTRACT

Recently, incidence of Cholecystokinin (CCK) receptor is recognized as a factor that determines the aggressive phenotype of pancreatic cancer. In this study, a novel Cholecystokinin (CCK) analogues; DOTA-Nle-cyclo (Glu-Trp-Met-Asp-Phe-Lys-NH2) (DOTA-cCCK) and DOTA-Nle-cyclo (Glu-Trp-Nle-Asp-Phe-Lys-NH2) (DOTA-[Nle]-cCCK) were synthesized and radiolabeled and the targeting abilities on the CCK receptor were evaluated for new CCK receptor targeting agents searching. Peptides were prepared through a solid phase synthesis method and their purity was over 98%. DOTA is the chelating agent for 68Ga-labelling, which the peptides were radiolabeled with 68Ga by a high radiolabeling yield (>98%). Peptides were stable over 98% by incubation in mouse blood at 37°C for 2 h. A competitive displacement of 125I-CCK8 on the AR42J human pancreatic carcinoma cells revealed that 50% inhibitory concentration value (IC50) were 12.31 nM of DOTA-cCCK and 1.69 nM of DOTA-[Nle]-cCCK. Stable in the blood of both DOTA-cCCK and DOTA-[Nle]-cCCK, but the binding rate with the CCK receptor on AR42J cells, DOTA-[Nle]-cCCK confirmed better than DOTA-cCCK. Therefore, it is concluded that 68Ga-DOTA-[Nle]-cCCK can be potential candidate as a targeting modality for the CCK receptor over-expressing tumors and further studies to evaluate their biological characteristics are needed.

Keywords: Gallium-68 (^{68}Ga), DOTA, Cholecystokinin, Tumor Targeting, Cyclic Peptide

1. INTRODUCTION

A CCK receptor is known as a receptor that is over expressed in tumor cells (Chakravarty *et al*., 2013; Oikonomou *et al*., 2008). Its incidence is higher than other cancer in lung cancer and pancreatic cancer (Korner *et al*., 2010). The CCK receptor subtype can be distinguished pharmacologically by the affinity of gastrin (Sosabowski *et al*., 2009). The CCK2 receptor has a high affinity of gastrin, but the CCK1 receptor has a lower affinity to gastrin (Liu *et al*., 2011). Tyr residues of the receptor binding peptide have a critical role in receptor specificity (Stone *et al*., 2007). If this tyrosine residue is sulfated, the peptide shows a high affinity for both receptors. This is displayed in the cell types of small cell lung cancer rich in CCK2 receptor affinity of medullary thyroid cancer and some stromal ovarian cancers (Roosenburg *et al*., 2011).

It has been shown that the targeting moiety of CCK peptide is the C-terminal amino acid sequences Trp-Met-Asp-Phe-NH_2 for retaining the receptor binding affinity and preserving the biological activity of CCK-like peptides (Martin-Martinez, 2005). Hence, the N-terminal region of the peptide can be used for radiolabeling and a number of potent CCK analogues have been labeled with a lot of radionuclides such as ^{99m}Tc, ^{111}In, ^{90}Y, ^{64}Cu, ^{177}Lu, ^{68}Ga, or ^{18}F for targeting CCK receptor-expressing cancer cells (D'Andrea *et al*., 2008; Heppeler *et al*., 2000).

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Among the radionuclides available, ^{68}Ga decays by a half-life of 1.13 h with the emission of beta-rays (1899 keV) and gamma-rays (511 keV) and it came into the spotlight for the imaging of tumors. Thus, ^{68}Ga can monitor the *in vivo* localization of injected therapeutic radiopharmaceuticals during treatment performing a dosimetric evaluation (Velikyan *et al*., 2005). The well-known chelating agent of radiolabeling peptides with ^{68}Ga is 1,4,7,10-tetraazacyclododecane-1,4,7,10-Tetraacetic Acid (DOTA) (Beylergil *et al*., 2013). ^{68}Ga exists primarily in an oxidation state of 3+ and is stabilized by hard donor atoms such as nitrogen or oxygen.

Several methods have been studied to improve the stability of peptide in the blood (Ocak *et al*., 2011). A method for improving the reliability has been demonstrated in studies of the target peptide melanoma through cyclization of an internal peptide (Lim *et al*., 2012). Therefore, the stability and functionality of the cyclic structure of the CCK analogues are confirmed.

In this study, we employed cyclic CCK as a linker of DOTA to prepare radiolabeled candidates for CCK receptor targeting. A novel DOTA-cCCK and DOTA-[Nle]-cCCK were synthesized and radiolabeled and IC_{50} of the peptides by ^{125}I-CCK8 on human pancreatic tumor cells were evaluated.

2. MATARIALS AND METHODS

2.1. Cell Culture

The AR42J pancreatic carcinoma cells were obtained from the American Type Culture Collection (ATCC) and grown in 100 mm culture dishes (Corning, Corning, NY, USA). The cells were cultured in RPMI-1640 (LONZA, Walkersville, MD, USA), supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin and 100 g mL^{-1} streptomycin (Sigma, Milan, Italy) in an atmosphere of 5% CO_2 in air at 37°C for up to approximately a 90% confluence.

2.2. Peptides

The peptides were prepared through the use of an automated Multiple Biomolecular Synthesizer (Peptron, Daejeon, Republic of Korea). The DOTA-Nle-Cyclo(Glu-Trp-Met-Asp-Phe-Lys-NH_2) (DOTA-cCCK) and DOTA-Nle-Cyclo(Glu-Trp-Nle-Asp-Phe-Lys-NH_2) (DOTA-[Nle]-cCCK) were synthesized by applying a standard Fmoc (fluorenylmethyloxycarbonyl) strategy as detailed in **Fig. 1**. Briefly, Fmoc-amino acid conjugated 4-Methylbenzhydrylamine (MBHA) resin was used as an anchor polymeric support for a solid phase synthesis. After removing the Fmoc protecting group from resin-bounded Fmoc-Met-OH under a standard cleavage condition (20% Piperidine in N,N-Dimethylformamide), the linear sequence peptide was prepared through the sequential coupling of Fmoc-Nle-OH, Fmoc-Glu-OH, Fmoc-Trp(tBoc)-OH, [Fmoc-Met-OH (DOTA-cCCK), Fmoc-Nle-OH (DOTA-[Nle]-cCCK)], Fmoc-Asp-OH, Fmoc-Phe-OH, Fmoc-Lys-OH, Fmoc-4 aminobenzoic acid and Fmoc-Ala(SO_3H). The resulting peptides were cleaved from the polymeric support by treatment with a mixture solvent of 90% TFA containing 2.5% Triisopropylsilane (TIS), 2.5% Ethanedithiol (EDT), 2.5% thioanisole and 2.5% deionized water (TFA: TIS: EDT: Thioanisole: H_2O = 90:2.5:2.5:2.5:2.5). The crude products were purified using a Shimadzu HPLC equipped with a Capcell pak C18 column on a binary gradient system at a flow rate of 1.0 mL min^{-1} using an elution solvent of 0.1% Trifluroacetic Acid (TFA) in water (A) and 0.1 % TFA in acetonitrile (B) with a gradient elution profile of (B): 0-10% in 2 min; 10-40% in 10 min; and 40-70% in 2 min. The molecular mass was analyzed on an LC-MS.

2.3. Radiolabelling

Peptides were dissolved in a 50 mM sodium acetate buffer (pH = 5.5) to give a concentration of 10^{-6} mole mL^{-1}. 37 MBq of a $^{68}GaCl_3$ solution diluted in a 5.5M HCl was injected into 1×10^{-8} mole of a peptide solution vial to give a final volume of 1 mL and heated at 110°C for 7 min. The radiolabeling yield and radiochemical purity/stability of the radiolabeled compound were analyzed by a Waters Chromatograph equipped with an X-Terra C-18 column. The column was eluted with a binary gradient system with a flow rate of 1.0 mL min^{-1} using an elution solvent of 0.1% TFA in 5% acetonitrile and 0.1% TFA in 95% acetonitrile. The gradient elution profile based on the solution of 0.1% TFA in 95% acetonitrile is as follows: 0%, 5 min; 0-100%, 9 min; 100%, 6 min; 100% and 2 min with 100% of 0.1% TFA in 5% acetonitrile.

2.4. Blood Stability Assay

^{68}Ga-labeled peptides were added to 200 µL of mouse blood and incubated at 37°C for 1 or 2 h. About 100 µL aliquots of the incubations were mixed with 500 µL of MeOH and acetonitrile mixed solution (1:1) and incubated at RT for at least 5 min to remove the blood proteins. The aliquots were centrifuged at 13,000 rpm for 10 min and analyzed through an HPLC analysis.

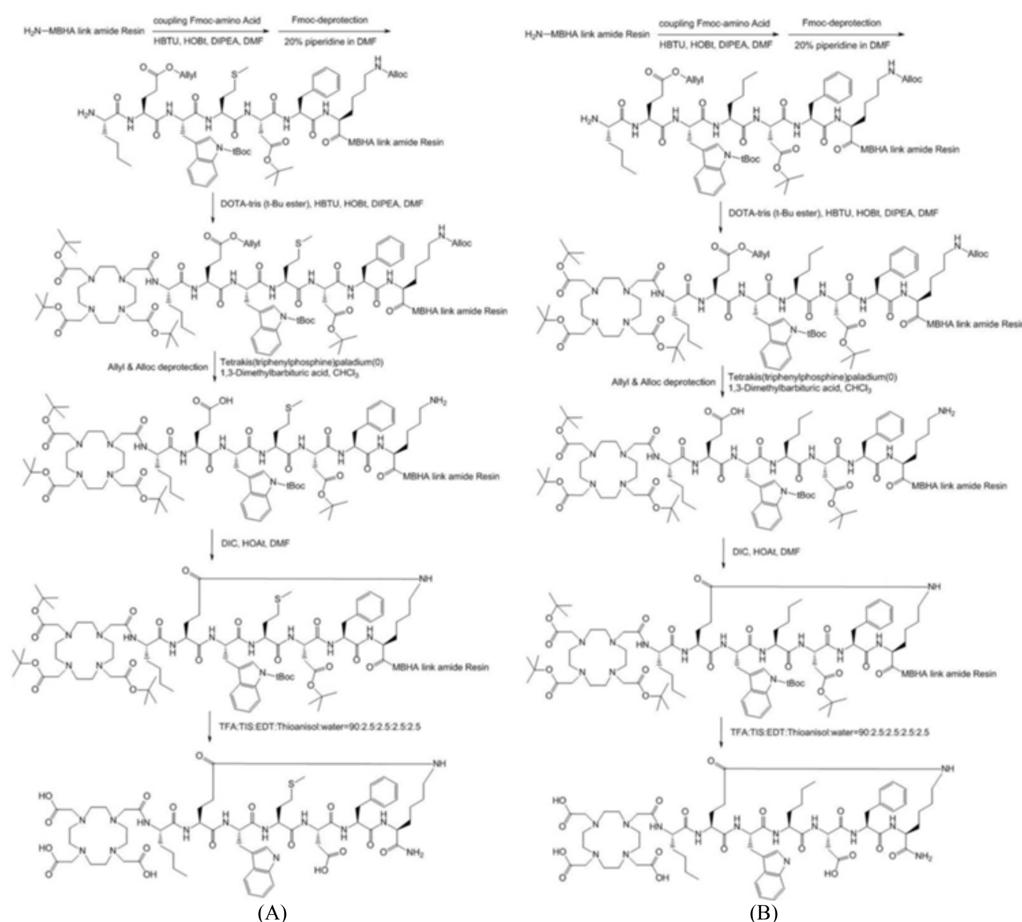


Fig. 1. Solid phase synthesis route and their formula of DOTA-cCCK (A) and DOTA-[Nle]-cCCK (B). The sequence of peptides was DOTA-cCCK: DOTA-Nle-Cyclo(Glu-Trp-Met-Asp-Phe-Lys-NH_2) and DOTA-[Nle]-cCCK: DOTA-Nle-Cyclo(Glu-Trp-Nle-Asp-Phe-Lys-NH_2)

2.5. Receptor Binding Study

The *in vitro* receptor binding of the ^{68}Ga labelled peptides was studied on AR42J cells. Cells were cultured to confluency in 12-well plates (Corning Life Sciences, Corning, NY, USA). Cells were incubated with 800 to 10,000 cpm ^{125}I-CCK8 in a binding buffer (DMEM with 0.1% w/v bovine serum albumin). After incubation at RT for 1 h, the cells were washed the times with ice-cold PBS and counted using a gamma-counter (Perkin-Elmer, Boston, MA, USA) to determine the cell associated radioactivity.

2.6. IC_{50} Determination

The 50% Inhibitory Concentration (IC_{50}) for binding the CCKR of peptides was determined on the AR42J cell line in a competitive binding assay. AR42J cells were grown to confluency in 12-well plates. Cells were washed three times with a binding buffer. After 10 min incubation at RT with a binding buffer, unlabelled peptides were added in a range of 0.001 to 1000 nM together with a trace amount of ^{125}I-CCK8 (10,000 cpm mL^{-1}, Perkin-Elmer, USA). After incubation at RT for 1 h, the medium was collected and cells were washed three times with ice-cold PBS. The cells were solubilized with 1 N NaOH for 5 min and the cell-associated radioactivity was determined using a gamma-counter. IC_{50} was defined as the peptide concentration at which 50% of binding without a competitor was reached. IC_{50} values were calculated using GraphPad Prism software (Version 5.00 for Windows, GraphPad Software, San Diego CA, USA).

2.7. Statistical Analysis

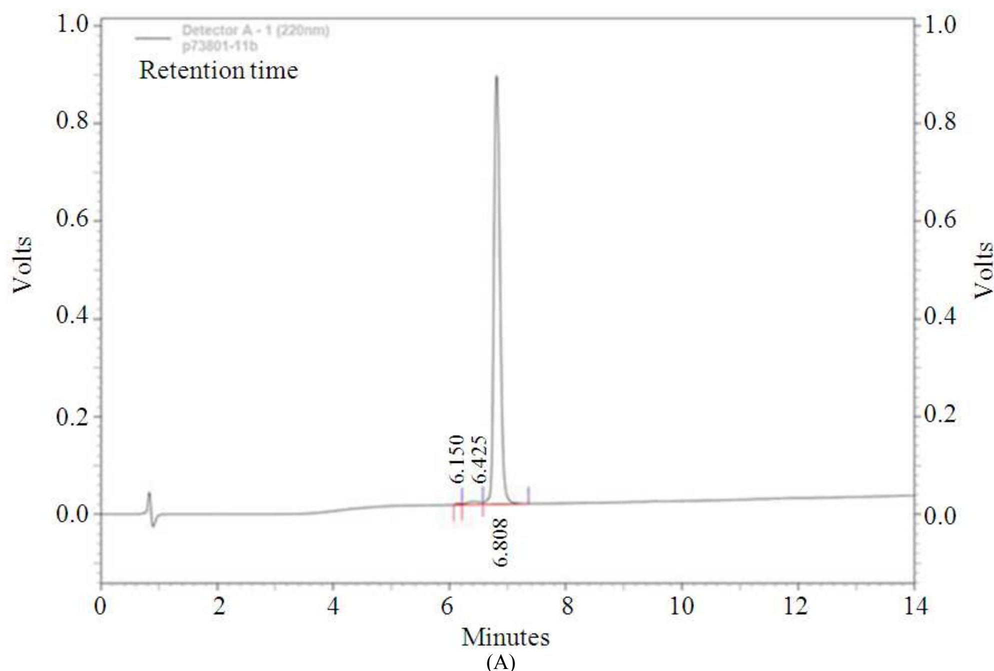
All mean values are given as mean±Standard Deviation (SD). A statistical analysis was performed using a Welch's corrected unpaired Student's t test or one-way analysis of variance using GraphPad Prism computer fitting program. The level of significance was set at $p<0.05$.

3. RESULTS

Nonradioactive peptides were synthesized by solid phase peptide synthesis following the standard Fmoc strategies, as shown in **Fig. 1**. The retention time of the analytical HPLC for DOTA-cCCK was found to be 6.80 and 7.19 min for DOTA-[Nle]-cCCK, resepectively and the chemical purities were over 98% (**Fig. 2A-D**). The measured ion peaks [M+1 (m/z)] were consistent with the calculated values of the proposed formula. MS data of DOTA-cCCK and DOTA-[Nle]-cCCK were 1334 and 1316. The final peptide sequence of DOTA-cCCK was DOTA-Nle-Cyclo(Glu-Trp-Met-Asp-Phe-Lys-NH_2) and DOTA-gluBBN was DOTA-Nle-Cyclo(Glu-Trp-Nle-Asp-Phe-Lys-NH_2).

The new conjugates, ^{68}Ga-DOTA-cCCK and ^{68}Ga-DOTA-[Nle]-cCCK were routinely prepared in a high yield (>98%) by adding $^{68}GaCl_3$ to an aqueous solution (pH 5.5 ammonium acetate) of the peptides at 110°C for 7 min. The HPLC chromatogram of ^{68}Ga-DOTA-cCCK (**Fig. 3A**) and ^{68}Ga-DOTA-[Nle]-cCCK (**Fig. 3B**) showed a retention time of 12.12 min and 12.29 min, respectively. As shown in **Fig. 4**, ^{68}Ga-DOTA-cCCK and ^{68}Ga-DOTA-[Nle]-cCCK were stable in mouse blood for 2 h. In contrast, 41% of noncyclic CCK peptide, ^{68}Ga-DOTA-HHHHHH-Gly-Trp-Nle-Asp-Phe-NH_2 was degraded for 2 h (data not shown). Because the difference between cyclic and noncyclic peptide is cyclization, it seems that the structure using Lys as a cyclic agent is more stable than noncyclic ^{68}Ga-DOTA-HHHHHH-Gly-Trp-Nle-Asp-Phe-NH_2. This might be caused by the cyclization.

The *in vitro* CCK receptor binding affinities and specificities of DOTA-cCCK and DOTA-[Nle]-cCCK were assessed through a competitive displacement assay using ^{125}I-CCK8 as the radioligand. It was found that DOTA-cCCK and DOTA-[Nle]-cCCK were able to compete with ^{125}I-CCK8 bound to AR42J pancreatic carcinoma cells (**Fig. 5**). In other peptide of CCK, IC_{50} value of DOTA-sCCK8, sulfated cholecystokinin fragment 26-33, is 11.6 nM (Roosenburg *et al*., 2011). However, IC_{50} values of new cyclic peptides were 12.31 and 1.69 nM for DOTA-cCCK and DOTA-[Nle]-cCCK, respectively. It seems that the structure using Nle instead of Met in a CCK analogue is a higher binding affinity than the original CCK analogue in cyclic structures.



(A)

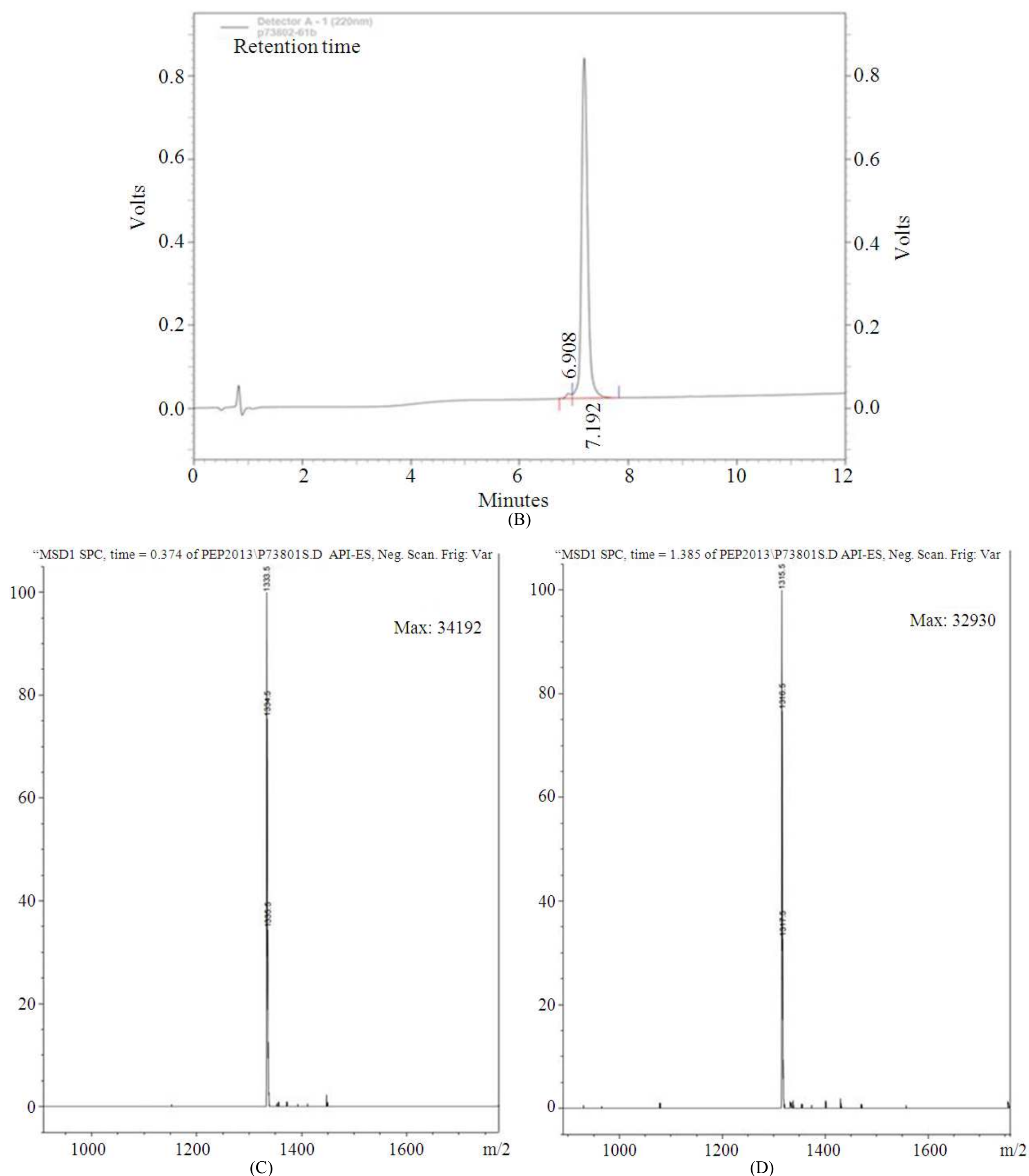


Fig. 2. HPLC analysis of DOTA-cCCK (A) and DOTA-[Nle]-cCCK (B) and LC/MS profiles of DOTA-cCCK (C) and DOTA-[Nle]-cCCK (D). The crude products were purified by Shimadzu HPLC equipped with a Capcell pak C-18 column and the molecular mass was analyzed on LC-MS. The purity of the peptides was over 98% and the final MS data of the peptides were equal to the calculated value of the proposed formula

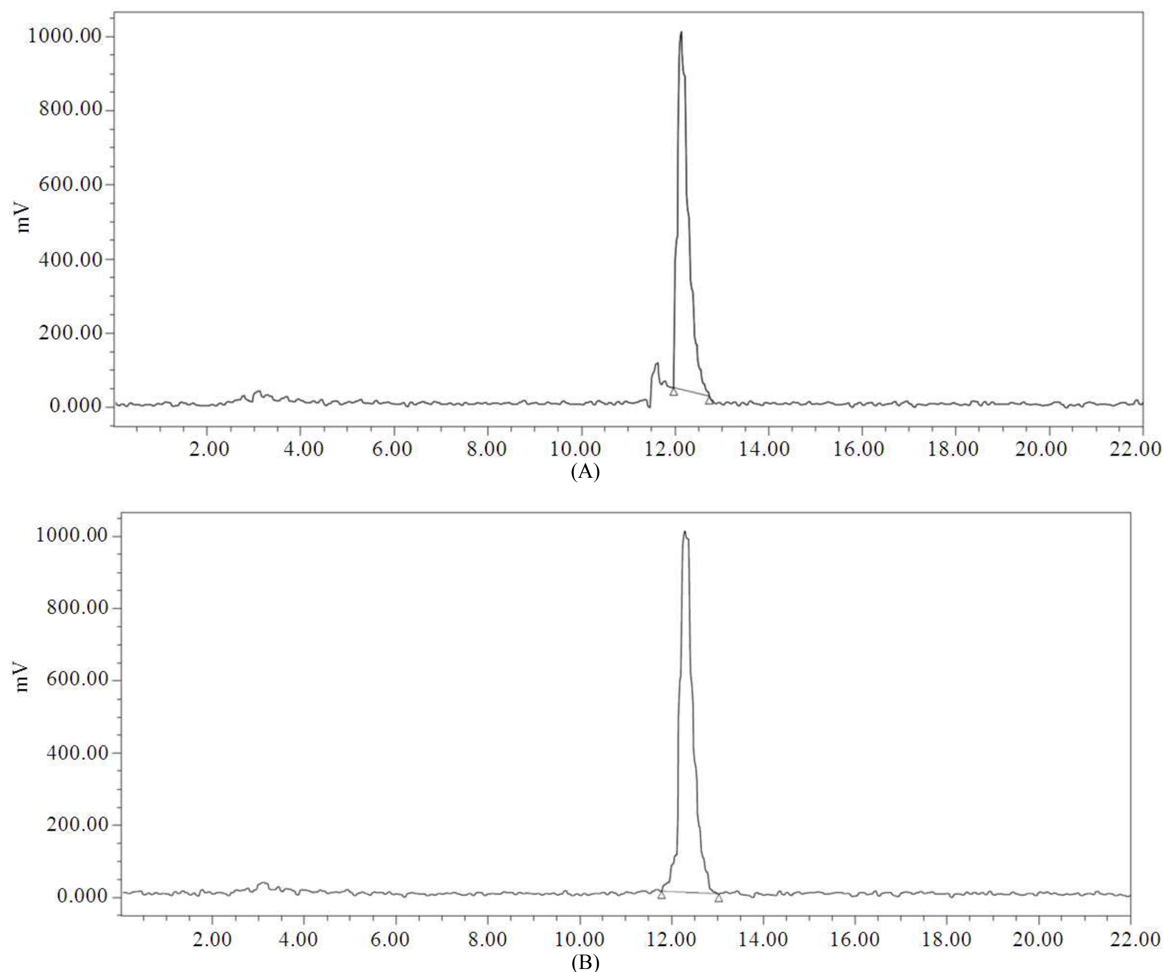
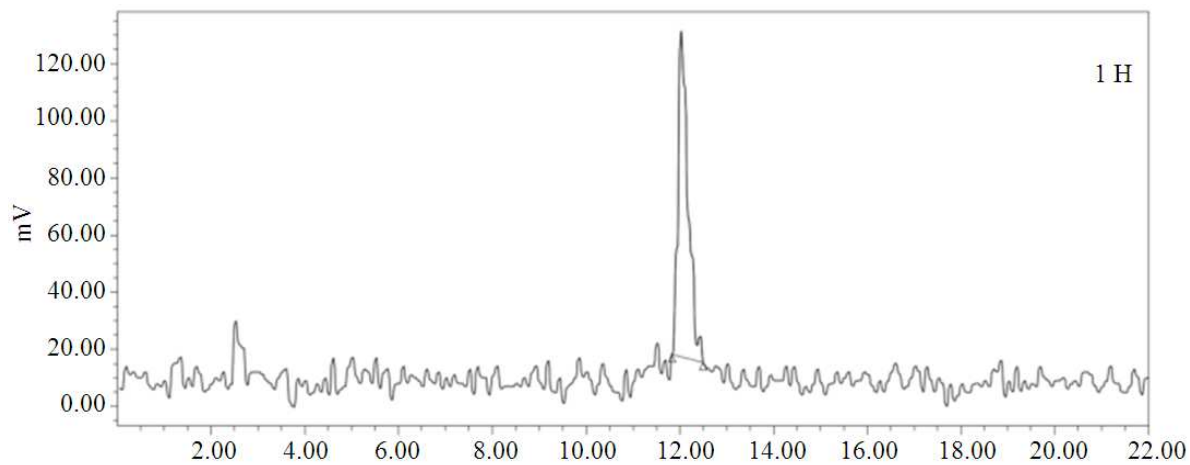


Fig. 3. Typical profiles of ^{68}Ga-DOTA-cCCK (A) and ^{68}Ga-DOTA-[Nle]-cCCK (B) determined by HPLC analysis using a C-18 column. The radiochemical purity of the peptides was over 98 % and further purification was not needed. Retention time: ^{68}Ga-DOTA-cCCK at 12.124 min and ^{68}Ga-DOTA-[Nle]-cCCK at 12.290 min



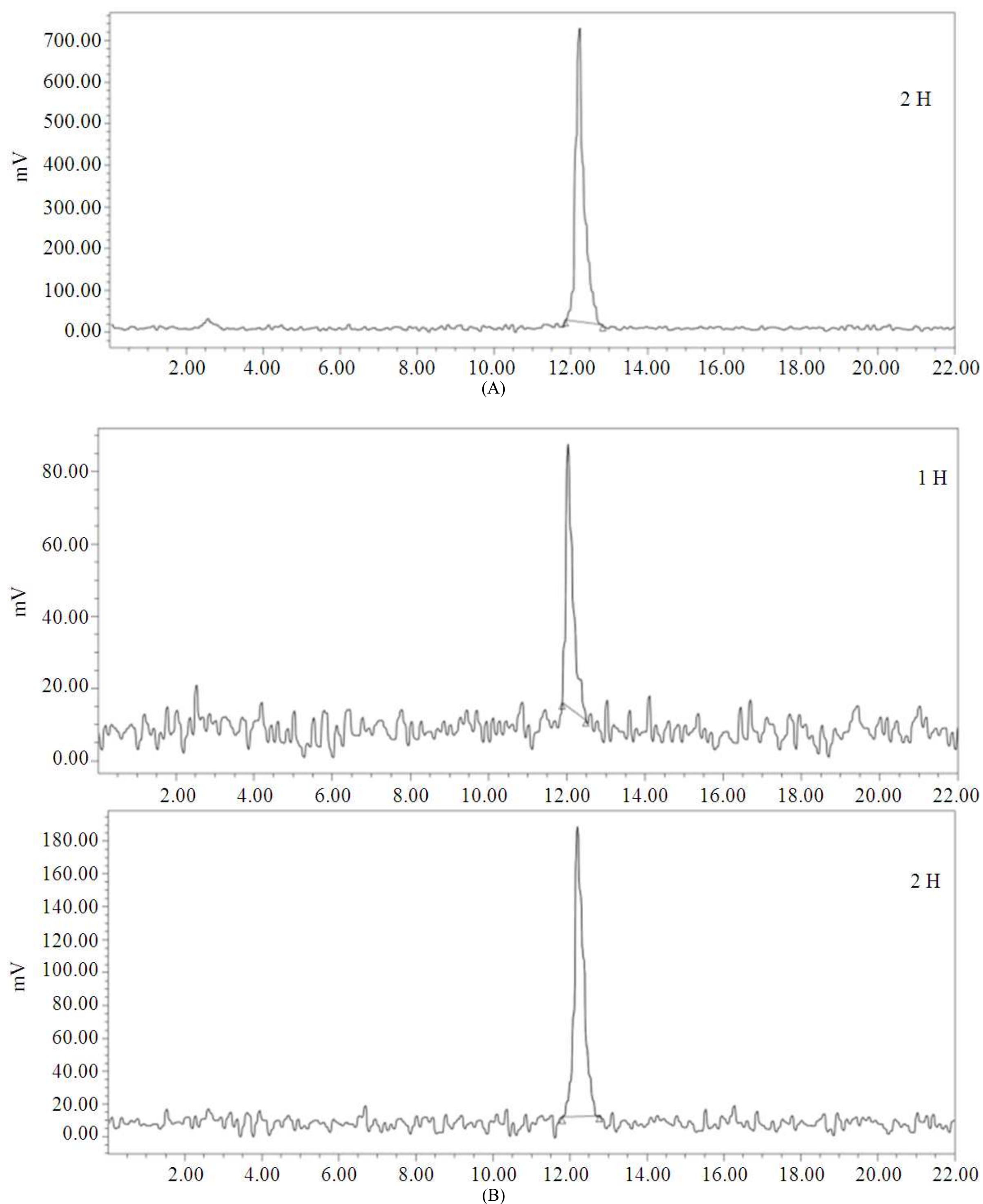


Fig. 4. Mouse blood stability of 68Ga-DOTA-cCCK (A) and 68Ga-DOTA-[Nle]-cCCK (B). 68Ga-DOTA-cCCK and 68Ga-DOTA-[Nle]-cCCK remained over 98% at 1hr and 2 h in mouse blood

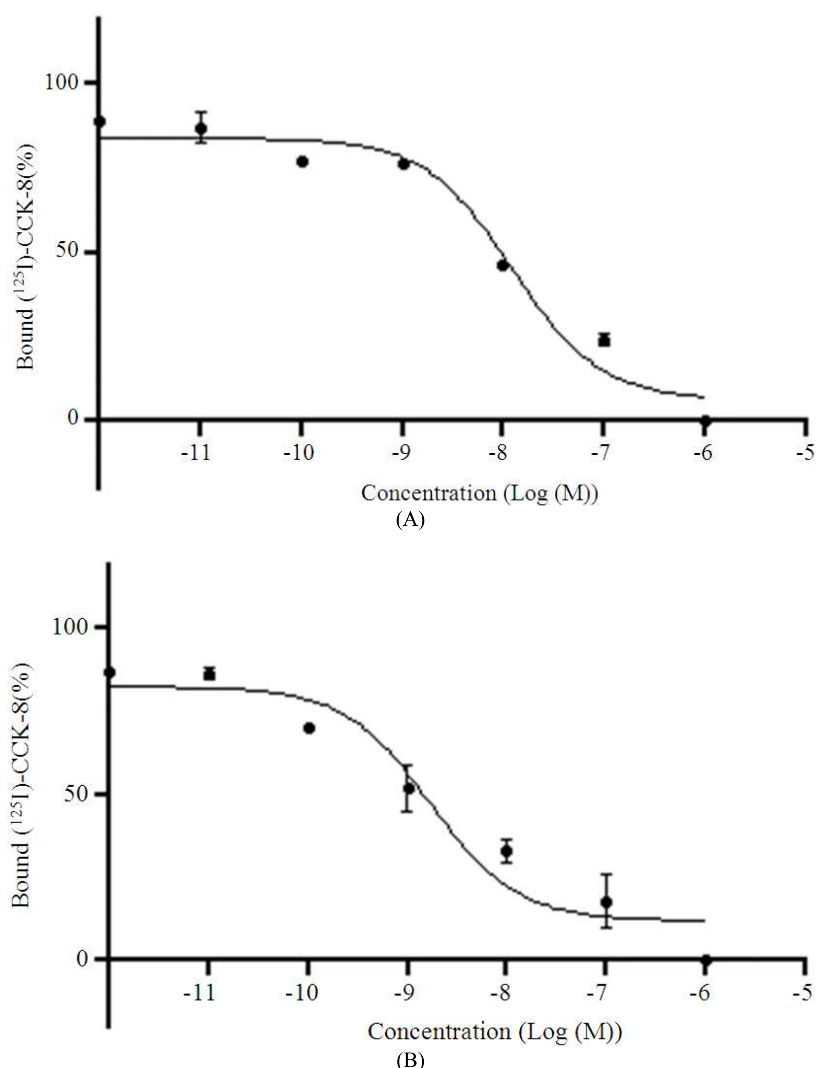


Fig. 5. Competitive binding of ^{125}I-CCK8 (Perkin-Elmer, USA) on AR42J cells by treatment of DOTA-cCCK (A) and DOTA-[Nle]-cCCK (B). Results expressed as a percentage of binding are mean ± SD in triplicate. 1×10^5 AR42J cells were incubated at RT for 1 hr with 10,000 cpm of ^{125}I-CCK8 in the presence of increasing concentrations of the peptides (0.001~1000 nM) in a 1 ml binding buffer. The IC_{50} of DOTA-cCCK was 12.31 nM and of DOTA-[Nle]-cCCK was 1.69 nM indicating highly nanomolar binding affinity on the CCK receptor

4. DISCUSSION

The incidence of the CCK receptor is higher in lung cancer and pancreatic cancer than other cancers (Brabez *et al*., 2013; Korner *et al*., 2010). Studies of tumor-associated via CCK peptides are actively performed, has been recognized possible. However, it has emerged as a point through the research conducted, improved stability in blood and excretion rate on kidney (Breeman *et al*., 2008).

Several methods have been studied to improve the stability of the peptide in blood. There is a way to improve the stability through the cyclization of the peptide therein, which has been demonstrated in studies of the targeting peptide of melanoma. Thus, by the cyclic structure of the peptide CCK, it was confirmed targeting functionality and stability of this material (Lim *et al*., 2012).

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improve the reliability through the cyclization of the peptide therein, which has been demonstrated in studies on the target peptide of melanoma. Thus, based on the cyclic structure of the peptide CCK, the target functionality and stability of this material were confirmed.

Bifunctional Chelating Agents (BFCA) are used for the preparation of many radiolabeled compounds. In particular, DOTA is able to strongly chelate many radionuclides such as ^{177}Lu, ^{111}In, ^{149}Pm, ^{212}Pb, ^{90}Y and ^{68}Ga. Additionally, ^{68}Ga emits strong energy β-rays (1899 keV) and γ-rays (511 keV) and its half life is short (1.13 h). Thus, ^{68}Ga is considered a suitable radionuclide for performing dosimetry and the imaging of tumors or metastatic deposits. DOTA-cCCK and DOTA-[Nle]-cCCK were also easily labeled with ^{68}Ga and an imaging study might be possible in the next investigations.

Two peptides showed a high stability. However, DOTA-[Nle]-cCCK showed a higher binding affinity than DOTA-cCCK on the CCK receptor. Therefore, the pharmacokinetic characteristics and therapeutic efficacy of ^{68}Ga-DOTA-[Nle]-cCCK should be evaluated in the next investigation. If ^{68}Ga-DOTA-[Nle]-cCCK shows fast excretion in the kidney it can be expected to be a good pharmaceutical agent in further *in vivo* studies.

5. CONCLUSION

The newly synthesized cyclic CCK peptides are stable in the blood. In particular, DOTA-[Nle]-cCCK showed high binding affinity with CCK receptor on AR42J cells. In further studies, *in vivo* metabolism and bio-distribution researches of ^{68}Ga-DOTA-[Nle]-cCCK are necessary and if studies with longer half-life therapeutic radioisotopes than ^{68}Ga have been performed, DOTA-[Nle]-cCCK can be expected as therapeutic agents.

6. ACKNOWLEDGMENT

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