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Research Article

The preparation of $^{123/125}$I-clioquinol for the study of Aβ protein in Alzheimer's disease

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Summary

Clioquinol or 5-chloro-7-iodo-8-hydroxyquinoline (CQ) is a lipophilic hydroxyquinoline currently under investigation for the treatment of Alzheimer's disease by targeted copper and zinc chelation. CQ was radiolabelled with no-carrier-added iodine-123/125 to give high specific activity analogs for SPECT imaging and biochemical studies. ^{123}I-clioquinol (^{123}I-CQ) was prepared by direct iodination of the activated hydroxyquinoline using a variety of oxidants. Highest yields (> 85%) and purity were obtained when chloramine-T or iodogen at pH 4–5 was used. Large-scale radiolabelled ^{123}I-CQ was purified by reverse-phase HPLC using a C-18 small 'Rocket®' column. The radiochemical purity of ^{123}I-CQ as assessed by analytical HPLC was 99.1 $\pm$ 0.6% ($n = 9$) with specific activity higher than 3 GBq/nmol for ^{123}I-CQ and 80 MBq/nmol for ^{125}I-CQ. The total preparation time was 40 min and starting from 3.7 GBq of iodine-123, more than 2.5 GBq of formulated ^{123}I-CQ as radiopharmaceutical was available for clinical investigations. Using the same method of preparation, ^{125}I-CQ was produced in radiochemical and chemical purity higher than 97% with a specific activity of 80 MBq/nmol.

Key Words: ^{123}I-clioquinol; Alzheimer's disease; radioiodination

Introduction

Alzheimer's disease (AD) has been characterized by the accumulation of insoluble amyloid plaques formed by the aggregation of a 40–43-residue

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peptide called β-amyloid (Aβ) to form insoluble fibres. This aggregation is enhanced by the presence of high concentrations of metal ions, particularly Cu^{2+} and Zn^{2+}, whereby Aβ acts as a metalloprotein with high affinity for these ions to form the insoluble Aβ plaques.[1] Although high concentrations of copper and zinc have been found in the neo-cortex of AD patients,[2] it is the presence of copper and its strong binding to Aβ which has been implicated in oxidative damage and toxicity through the generation of hydrogen peroxide and other destructive reactive oxygen species.[3] On the other hand, the accumulation of zinc in amyloid plaques appears to be the brain's response at reducing this oxidative damage caused by Aβ.[4] Consequently, potential treatments for AD have been proposed through the selective chelation of copper.[5,6] This hypothesis was recently supported by the solubilization of Aβ plaques from post mortem AD brain tissue by Cu/Zn chelators.[7] Clioquinol or 5-chloro-7-iodo-8-hydroxyquinoline (CQ) is a lipophilic, halogenated hydroxyquinoline that is permeable to the intact blood brain barrier with suitable properties for the chelation of Zn and Cu.[8] CQ has been used extensively for 20 years as an anti-amebic, until it was withdrawn due to the development of subacute myelo-optic neuropathy, a condition later found to be caused by vitamin B_{12} deficiency.[9] However, more recently, oral treatment of transgenic mice that overproduce Aβ with CQ has also led to a significant reduction of these amyloid deposits.[10] Furthermore, a phase two study with CQ as a potential treatment for AD was completed with encouraging results.[11]

CQ has an iodine atom ortho to the hydroxyl group of the quinoline molecule, which is amenable to radiolabelling with iodine-123/125. Although CQ has been radiolabelled with iodine-125 and carbon-14 previously,[12,13] its method of preparation, large-scale radiosynthesis, purification and stability to our knowledge has not been published.

In this work, the preparation and purification of no-carrier-added (nca) ^{125}I- and ^{123}I-CQ for performing pharmacokinetic studies in mice as well as clinical SPECT studies in humans is presented.

Results and discussion

Radiolabelling

^{123}I-CQ was prepared by direct electrophilic iodination of the 5-chloro-8-hydroxyquinoline (cloxyquin) precursor with nca $Na^{123}I$ in the presence of an oxidant. As with tyrosine, the hydroxyl group in the 5 position of cloxyquin is activating for direct regiospecific ortho-iodination.[14] Chloramine-T (CAT), iodogen, peracetic acid and hydrogen peroxide were employed as oxidants under various concentrations and pH conditions to provide the reactive electrophilic iodine species *in situ*.[15–19]

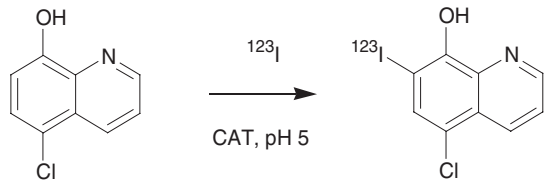

Figure 1. Radiolabelling of 5-chloro-7-[^{123}I]iodo-8-hydroquinoline

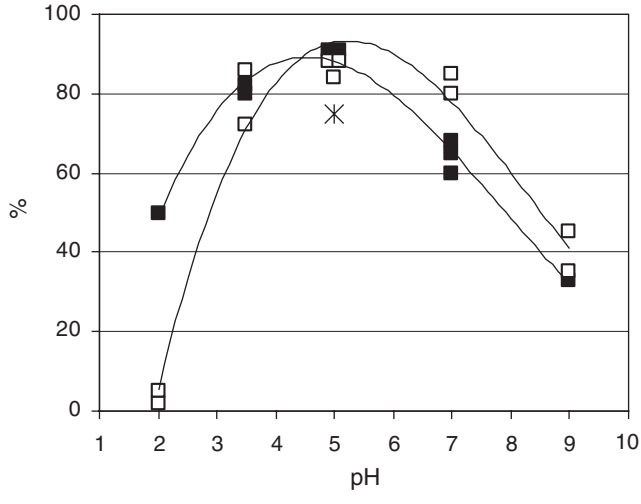

Figure 2. Radiolabelling yield (in %) of ^{123}I-CQ using CAT (■ = 100 µg, * = 50 µg) and iodogen in solution (□) as oxidants versus pH

Both chloramine-T and iodogen gave radioiodinated products in yields well in excess of 85%. The use of peracetic acid and dilute hydrogen peroxide gave radiochemical yields of less than 10% (Figure 1).

The radiolabelling yields obtained with CAT and iodogen are displayed in Figure 2. With CAT (100–200 µg) the optimum radiolabelling conditions resulted from reactions conducted at pH 5, yielding radiolabelling yields of 90% and independent of the amount of cloxyquin precursor used (50–200 µg). Reducing the amount of CAT (50 µg), decreased the radiochemical yield to 75%. The average radiochemical yields in acidic (pH 2–3), neutral (pH 7–7.5) or basic (pH 9–9.5) conditions were also significantly lower (35–80%).

Iodogen in ethanol solution and at pH (4–7) gave radioiodinated CQ in radiochemical yields of 85–95%. However, as above, reactions in acidic (pH 2–3) or basic (pH 9–10) conditions, gave radiochemical yields of 5 and 40%, respectively. Coating the iodogen on the walls of the reaction vessel also led to radiochemical yields of less than 5%. The use of the non-chlorinated

oxidants hydrogen peroxide, and particularly peracetic acid, gave low yields of ^{123}I-CQ (5–10%).

After quenching with $Na_2S_2O_5$, the reaction mixture was purified by reverse-phase HPLC (Systems 1 or 2 below) to give ^{123}I-CQ in radiochemical purity exceeding 98%.

Although iodogen gave slightly higher radiochemical yields than CAT, reactions with iodogen indicated the presence of an unidentified UV product in close proximity to the ^{123}I-CQ product peak. CAT gave less unlabelled by-products than iodogen, allowing easier HPLC purification and was thus selected for high-scale radiopharmaceutical preparation.

Stability studies

One of the requirements for the ^{123}I-CQ radiopharmaceutical is to maintain adequate chemical and radiochemical stability over 24 h to allow its overnight transport to the clinical centres. The stability of the ^{123}I-CQ was hence analysed in various formulations, including different pH and reducing conditions.

In solutions of 0.1 M HCl (acidic, pH 1–2), 0.25% ascorbic acid in saline (pH 2–7), and 0.1 M NaOH (basic, pH 10–12), the rate of ^{123}I-CQ decomposition was more than 10% in 1 h. Decomposition was significantly reduced by the addition of 1% benzyl alcohol in saline at pH 6.5, giving a decomposition rate of 10% in 24 h. When dissolved in the same solution and frozen at -80°C in dry ice, the decomposition rate was further reduced to 5%

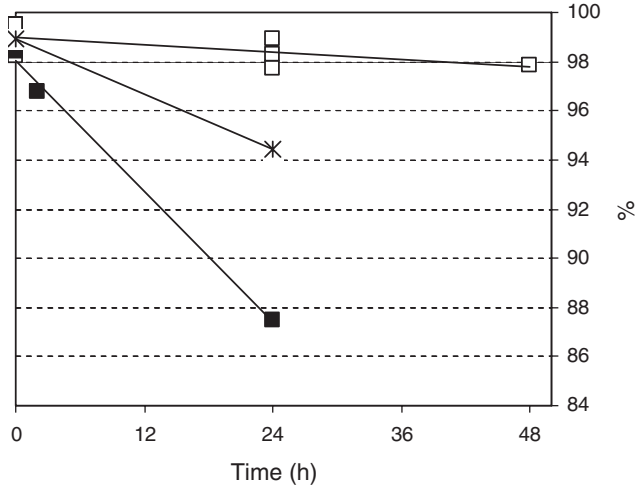

Figure 3. Time course of unchanged ^{123}I-CQ (in %) as determined by analytical HPLC. ^{123}I-CQ kept at room temperature in acidic (□) and neutral (■) pH conditions and in dry ice at neutral pH (*)

in 24 h. In 1% benzyl alcohol in saline containing 10% of the mobile phase of the HPLC 2 system (pH 2.5), a 1% degradation of ^{123}I-CQ was observed in 48 h when the solution was kept at room temperature (Figure 3).

Radiopharmaceutical preparation

The large-scale preparation of ^{123}I-CQ was achieved using the optimized conditions described above. Using 100 µg of cloxyquin, 100 µg of CAT at pH 4–5, radiolabelling yields of $87 \pm 6\%$ ($n = 13$) were routinely obtained in 2 min at room temperature. The low mass of reagents in the reaction mixtures makes it possible to use small reverse-phase HPLC 'Rocket®' columns for purification. Dilute phosphoric acid and ethanol in the HPLC solvent system were chosen because they allow easy subsequent neutralization and they are non-toxic at low concentration in injectable solutions. In these conditions and with a flow rate of 1.6 ml/min, the radiotracer eluted at the retention time of 7.3 min, well separated from the precursor ($R_t = 2.8$ min) and an unlabelled by-product ($R_t = 4.8$ min) (Figure 4).

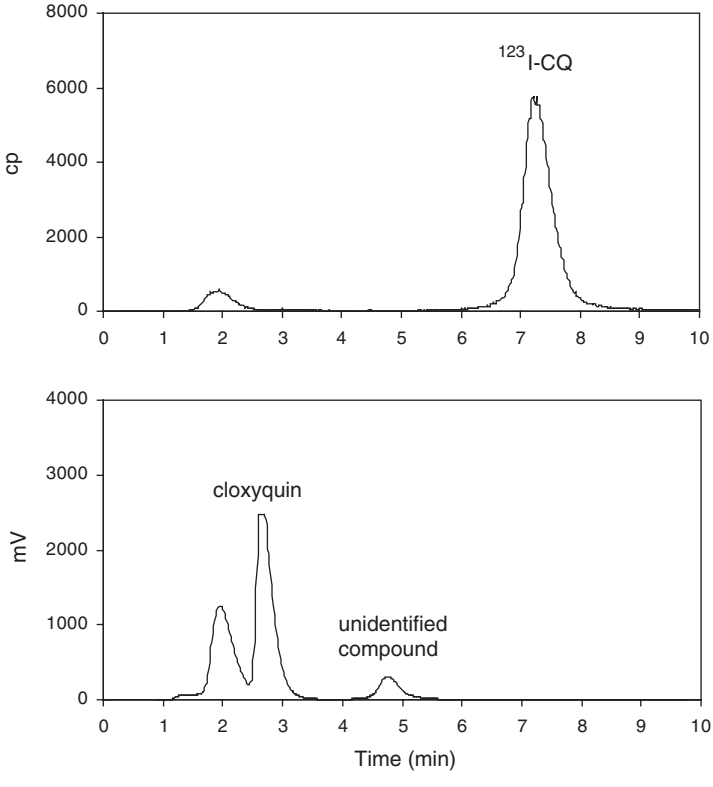

Figure 4. Radio and UV chromatograms of large-scale ^{123}I-CQ preparation, using small column (Rocket®)

For pharmaceutical preparation, 15 ml saline containing 1% benzyl alcohol was added to the 1.6 ml collected HPLC fraction. The solution was sterilized

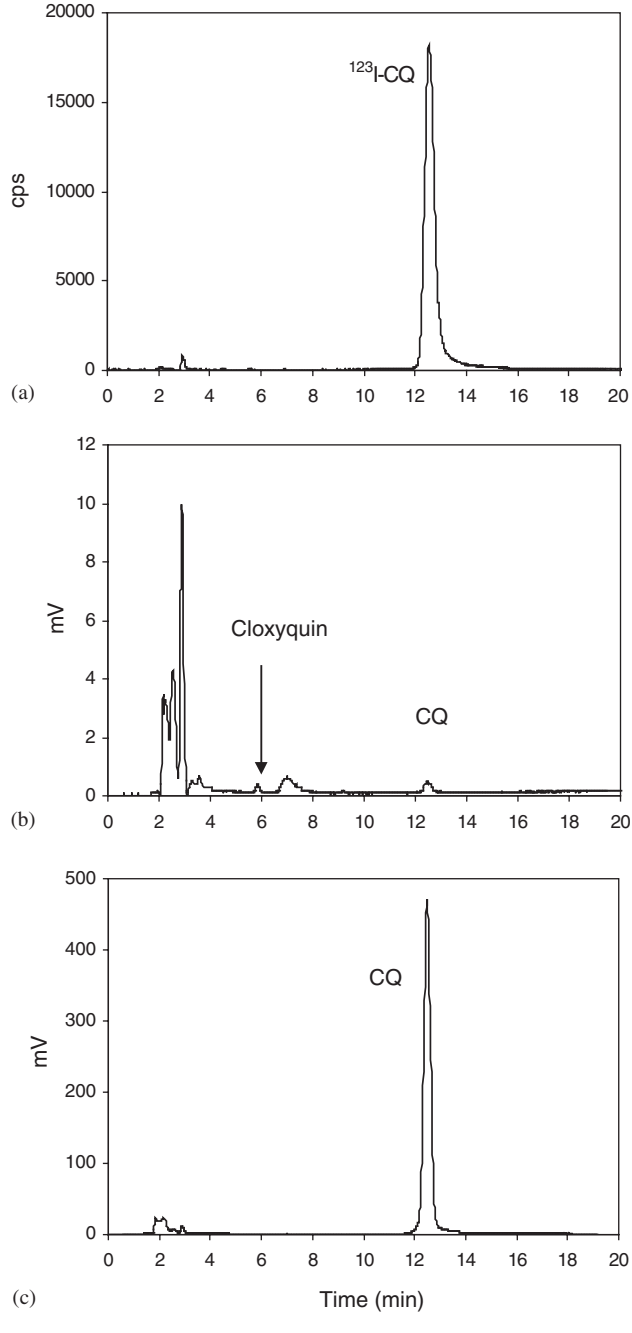

Figure 5. Quality control chromatograms of a 50 µl sample of ^{123}I-CQ after formulation. Radioactivity detection: panel (A). UV detection at 254 nm: panels (B) and (C). In panel (C), the sample was spiked with 1 µg CQ

through a 0.22 μm membrane and 85–90% of the activity was recovered in the sterile vial. An aliquot of this solution was then used for quality control. Quality control testing included chemical and radiochemical purity, sterility and pyrogenicity.

For clinical applications, the sterilized saline solution was neutralized with 200 μl 1 M $NaHCO_3$ sterile solution to pH 6–7 prior to patient administration. Due to the instability of ^{123}I-CQ in neutral conditions the preparation was used within 1 h after neutralization. In these operating conditions ^{123}I-CQ pharmaceutical preparation was proven to be pyrogen free in accordance with the LAL test and the sterility was confirmed retrospectively. ^{123}I-CQ was characterized by co-injecting the corresponding unlabelled analog in an analytical HPLC (system 3) and followed by simultaneous UV and radioactivity detection (Figure 5).

The radiochemical purity as assessed by analytical HPLC was $99.1 \pm 0.6\%$ ($n = 9$). On the UV chromatogram, three peaks were observed at retention times of 5.85, 7.00 and 12.45 min corresponding to cloxyquin ($k' = 1.72$), an unidentified compound ($k' = 2.26$) and CQ ($k' = 4.79$).

The maximum amount of the precursor cloxyquin and CQ detected in the 50 μl samples used for QC were 2.5 ng (14 pmol) cloxyquin and 1 ng (3.3 pmol) CQ. Assuming the unidentified compound is the chloro analogue of CQ, its amount was estimated to 5 ng (25 pmol).

Consequently, we found that the specific activity of ^{123}I-CQ was 3 GBq/nmol, a value half that of the theoretical specific activity of iodine-123 at carrier-free level.

The total preparation time was 40 min and starting from 3.7 GBq of iodine-123, more than 2.5 GBq of ^{123}I-CQ as formulated radiopharmaceutical was available for clinical investigations.

Preparation of ^{125}I-CQ

The radiochemical yield of ^{125}I-CQ preparation was 80–85%. The radiotracer was kept in acidic conditions in the mobile phase, at a concentration of 50 MBq/ml without any degradation for 2 days. Aliquots of this formulation were neutralized with 1 M $NaHCO_3$ to pH 6–7 just before use. The radiochemical and chemical purity of ^{125}I-CQ were 99 and 97%, respectively, and the specific activity was found to be the same as carrier-free iodine-125: 80 MBq/nmol.

Lipophilicity

The lipophilicity of ^{123}I-CQ was ascertained in order to explain its *in vivo* properties. The lipophilicity values of CQ were 2.5, 3.3 and 3.64, for octanol/buffer partition, HPLC and fragmental approach, respectively. The differences

in these values could be explained by the different contribution of the ionic character of CQ in the lipophilicity determination.[20] Nevertheless, the lipophilicity of CQ is in the range for optimal extraction by the brain.[21]

Experimental

General

CQ (5-chloro-7-iodo-8-hydroxquinoline), cloxyquin (5-chloro-8-hydroxyquinoline) CAT and iodogen were purchased from Sigma-Aldrich. Other chemicals were used as purchased without further purification. Solvents for chromatography were of HPLC grade. Iodine-123 was produced by the National Medical Cyclotron, Sydney, Australia, using the ^{124}Xe(p, 2n) reaction and delivered as Na^{123}I in 0.1 M NaOH solution at a concentration of 37 GBq/ml. The radioactivity was measured with a radioactive dose calibrator (Capintec).

HPLC

System 1, small-scale preparation. The optimization of the ^{123}I-CQ labelling conditions were developed using semi-preparative HPLC. After radiolabelling, the crude ^{123}I-CQ reaction mixtures were injected onto a reverse-phase C18 column (C-18 Alphabond 300 × 10 mm, Alltech) and eluted with a mobile phase consisting of TFA:CH_3CN:H_2O, 0.1:50:50, v/v/v, at a flow rate of 2.5 ml/min. The eluent was monitored with an UV detector at 254 nm (Linear, model 200) and a NaI radioactivity detector (Berthold). The iodinated radiotracer was collected (R_t = 18 min) and the solvent evaporated. The radiochemical yield was obtained by comparing the activity of the collected fraction with the starting activity.

System 2, large preparative scale ^{123}I-CQ purification. After radioiodination, the crude reaction mixture was purified by HPLC on a Waters, 510 pump, and a small semi-preparative reverse-phase column (C-18 Alltima Rocket®, 3 μm, 7 × 53 mm, Alltech). The mobile phase consisted of an ethanol:0.1 M H_3PO_4, 60:40 v/v with a flow rate of 1.6 ml/min. A UV detector (Linear, model 200) set at 254 nm and a NaI radioactivity detector monitored the eluent. The radioactive peak eluting at the retention time of the authentic CQ (7.3 min) was collected and the activity measured. Using this small column the ^{123}I-CQ was collected in a small volume (approximately 1.6 ml) ready for dilution and formulation with saline containing 1% benzyl alcohol as stabiliser.

System 3, quality control and determination of specific activity. Chemical, radiochemical purity and specific activity were assessed on an analytical HPLC system consisting of a Varian (model 9002) solvent delivery system, a UV detector, λ = 254 nm, (Linear, model 200) and a γ radioactivity detector.

Analytical samples of ^{123}I-CQ were injected onto a reverse-phase column (Alltima-C18, 5 μm, 4.6 × 150 mm Alltech), eluted at 0.6 ml/min with THF:0.1 M H_3PO_4, 50:50 v/v.

Radiolabelling

Optimization studies for the preparation of ^{123}I-CQ by direct radioiodination was achieved by classical radioiodination methods including CAT, iodogen, peracetic acid and hydrogen peroxide as oxidants.

Chloramine-T. To a solution of cloxyquin (100–200 μg, 0.5–1 μmol in 50–100 μl of ethanol) was added nca $Na^{123}I$ (1 GBq, 25 μl 0.1 M NaOH). Varying solutions from pH 1 to 9 were then prepared by adding 50 μl of 0.1 M HCl, 1 M NaH_2PO_4, 1 M Na_2HPO_4, or water. A desired amount of CAT (25–200 μg, 0.1–1 μmol) in a volume of 25–100 μl water was added and the reaction was allowed to proceed at room temperature for 2–5 min. The reaction was stopped by the addition of $Na_2S_2O_5$ (0.1 ml, 10 mg/ml) and the reaction profile analysed by radio-HPLC (system 1). The radiolabelling yield and chemical purity of each reaction mixture was then measured.

Iodogen solid phase. Iodogen (400 μg, 1 μmol) was coated onto a glass vial by evaporation of a chloroform solution under nitrogen. Cloxyquin (200 μg, 1.1 μmol in 200 μl of 1 M CH_3CO_2H) and nca $Na^{123}I$ (1 GBq, 25 μl 0.1 M NaOH) was added to the vial and the reaction mixture allowed to stand at room temperature for 30 min with occasional shaking. The labelling process was stopped by directly injecting the reaction mixture onto the HPLC (system 1).

Iodogen in solution. To a solution of cloxyquin (200 μg, 1 μmol in 100 μl of ethanol) was added nca $Na^{123}I$ (1 GBq, 25 μl 0.1 M NaOH). Varying solutions from pH 1 to 9 were then prepared as for the CAT reactions above by adding 100 μl of 0.1 M H_3PO_4, 1 M NaH_2PO_4, 1 M Na_2HPO_4, or water. Iodogen (100–200 μg, 0.23–0.45 μmol in 50 μl ethanol) was then added and the reaction allowed to proceed at room temperature for 5 min. The reaction mixture was halted by the addition of $Na_2S_2O_5$ (0.1 ml, 10 mg/ml) and analysed by HPLC (system 1) as above.

Peracetic acid. Peracetic acid (3.2% in 100 μl acetic acid) was added to a glass vial containing cloxyquin (200 μg, 1.1 μmol in 50 μl ethanol) and nca $Na^{123}I$ (1 GBq, 25 μl 0.1 M NaOH). After 30 min, the labelling process was stopped by the addition of $Na_2S_2O_5$ (0.1 ml, 10 mg/ml) and the reaction mixture analysed by HPLC (system 1).

Hydrogen peroxide. Hydrogen peroxide (50 μl of 10% in water) was added to a glass vial containing cloxyquin (200 μg, 1.1 μmol in 50 μl ethanol), nca $Na^{123}I$

(1 GBq, 25 µl 0.1 M NaOH) and 20 µl 1 M HCl. After 15 min at room temperature, $Na_2S_2O_5$ (0.1 ml, 10 mg/ml) was added to stop the reaction and the mixture analysed by HPLC (system 1).

Radiopharmaceutical preparation of ^{123}I-CQ

Nca $Na^{123}I$ (3.7 GBq, 100 µl in 0.1 M NaOH) was added to a glass vial containing cloxyquin (0.1 mg, 0.6 µmol) dissolved in ethanol (100 µl). The solution was buffered at pH 5 by adding 100 µl 0.1 M NaH_2PO_4. A solution of CAT (100 µg, 0.35 µmol in 40 µl water) was then added and the reaction mixture allowed to stand at room temperature for 2 min. After quenching of the reaction with $Na_2S_2O_5$ (0.5 mg in 100 µl water) the mixture was purified by semi-preparative HPLC (system 2). Using a flow rate of 1.6 ml/min, the radioactivity peak corresponding to ^{123}I-CQ was collected between 6.8 and 7.8 min in a volume of 1.6 ml. The ^{123}I-CQ solution was then diluted with 15 ml saline containing 1% benzyl alcohol to reach a radioactivity concentration of 160 MBq/ml. The solution was then sterilized through a 0.22 µm filter membrane (Millex GV, Millipore) in a sterile environment and stored in these acidic conditions (pH 2–3) until its use (up to 24 h) the following day. Just before clinical investigations, the filtered ^{123}I-CQ solution is neutralized with 200 µl sterile 1 M $NaHCO_3$ to reach a pH of 6–7.

Quality control

For quality control and specific activity measurements, 50 µl of the final radioactive solution was injected onto the reverse-phase analytical HPLC (system 3). The radiochemical purity was determined by analysing the radioactive peaks observed in the radiochromatogram. The amounts of CQ and cloxyquin in the sample were determined from the area under the UV peak and compared to their respective standard solutions. The specific activity of the solution was then calculated from the activity of iodine-123 in the sample.

Stability

The stability of the ^{123}I-CQ in solution was studied under different formulation conditions including varying pH, reducing conditions and temperature. Aliquots of ^{123}I-CQ, 50 MBq in 100 µl (ethanol: 0.1 M H_3PO_4, 60:40 mobile phase) from a bulk radiochemically pure solution were diluted with 1 ml of – (a) 0.1 M HCl (pH 1), (b) 0.1 M NaOH (pH > 12), (c) 0.25% ascorbic acid in saline (pH 2), (d) 1% benzyl alcohol in saline (pH of mobile phase = 2.5) and (e) 1% benzyl alcohol in saline (pH 6.5, buffered with 10 µl 1 M $NaHCO_3$) and kept for 48 h at room temperature. ^{123}I-CQ stability of the final solution was also checked after storing at −80°C for 24 h. Aliquots (50 µl)

of these solutions were withdrawn and analysed over a 24 h period using HPLC (system 3).

Preparation of ^{125}I-CQ

For the preparation of ^{125}I-CQ, the same labelling procedure as described above was used, starting with 100 MBq $Na^{125}I$, (30 µl in 0.1 M NaOH) (Amersham). The mixture was purified by semi-preparative HPLC (system 2) with a γ-detector dedicated for iodine-125 (Flow-count, Bioscan). ^{125}I-CQ was collected between 6.8 and 7.8 min and kept in the mobile phase (ethanol, 0.1 M H_3PO_4, 60:40) until its $NaHCO_3$ neutralization for radiopharmacological purposes.

Lipophilicity

Lipophilicity of ^{123}I-CQ was assessed using octanol/buffer partitioning, HPLC and fragmental calculation methods.[20] In octanol/buffer partitioning, the distribution of 1 MBq ^{123}I-CQ between 1 ml 1-octanol and 1 ml 0.15 M NaH_2PO_4/Na_2HPO_4 (pH = 7.4) was performed by the flask shake method using successively, vortex agitation and centrifugation, 7 times. The radioactivity of aliquot of organic and aqueous phases was measured in a γ-counter and the ratio ($P_{7.4}$) was calculated. Lipophilicity ($\log P_{7.4}$) was determined as the mean of the last 5 partitions.[22] Via the HPLC method, using a methanol, phosphate buffer (pH = 7.4) mobile phase, the lipophilicity of CQ was estimated by comparing its retention time to standards of known $\log P$ value.[23] The fragmental calculation method was performed using Chemdraw® software.

Conclusion

$^{123/125}$I-CQ, was prepared by direct electrophilic radioiodination with nca iodine-123/125. CAT and iodogen appear to be the best competitive oxidizing agents for the preparation of ^{123}I-CQ providing excellent radiochemical yields of 85–90%. The radiolabelling reaction carried out in the presence of low amounts of precursor and CAT or iodogen at room temperature and in sodium phosphate buffer (pH 5) is very efficient. The rapid HPLC purification using the small semi-preparative 'Rocket®' columns allows excellent recovery of pure radiotracer possessing a very high specific radioactivity. CAT has been selected for large-scale preparation of ^{123}I-CQ due to lower levels of UV impurities close to the radioactive peak under the HPLC conditions described above. ^{123}I-CQ is stable under acid conditions for overnight transport. Finally, these methods of radiolabelling and purification allow the large-scale preparation of ^{123}I-CQ and delivery for the clinical investigation of Alzheimer's disease.

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