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Development of a high-performance liquid chromatographic method for the determination of a new potent radioiodinated melanoma imaging and therapeutic agent

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ABSTRACT

N-(2-diethylaminoethyl)-6-iodoquinoxaline-2-carbamide (ICF 01012) is a new melanoma imaging agent showing promising properties for application in internal radionuclide therapy. We developed an analytical protocol for detection of ICF 01012 in biological samples using HPLC. The proposed method was first validated using standard of ICF 01012 and four potent metabolites of this compound and then applied to follow the metabolic fate of [^{125}I]ICF 01012 after injection in melanoma-bearing mice. The results demonstrate that this method exhibits a good linearity (r^2 = 0.9947), specificity and acceptable accuracy. This simple method appears convenient and sufficient for pharmacokinetic studies on [^{125}I]ICF 01012.

1. Introduction

Cutaneous melanoma is a highly invasive cancer characterized by a fast and often fatal progression [1]. Early diagnosis of lesions is therefore essential. However, the limited efficacy of conventional treatments for disseminated melanoma makes the development of new therapeutic approaches targeting melanoma metastases indispensable [2,3].

For several years, a series of radioiodinated benzamide derivatives described to selectively concentrate into melanin rich cells, in particular melanoma cells have been developed as radiopharmaceuticals [4–10]. One of them, *N*-(2-diethylaminoethyl)-4-iodobenzamide ([^{123}I]BZA, Fig. 1A) [11] was the first to be selected for testing in a Phase II clinical trial for the detection of melanoma and their metastases [12,13]. A pharmacokinetic study of BZA showed that the main metabolization pathway in human subjects was the cleavage of the amide bond and a progressive dealkylation of the compound [12]. The affinity of this compound for melanoma is due to a cellular internalization and binding to melanin pigment within melanosomes [14–17].

Besides the development of BZA, radioiodinated molecules have been selected with the aim of finding potent derivatives useful for targeted internal radionuclide therapy. One compound *N*-(2-diethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (ICF 01012, Fig. 1B), exhibited an original pharmacokinetic profile: high, sustained and specific tumor concentration with a rapid clearance from non-target organs (Fig. 1C) [18,19]. Such a profile makes this compound promising for application in targeted radionuclide therapy. Indeed, our preliminary characterization of [^{131}I]ICF 01012 in melanoma-bearing mice were encouraging (unpublished results). In this context, a more complete study of *in vivo* ICF 01012 metabolism was warranted.

To trace the metabolic fate of ICF 01012 in B16 F0-melanoma-bearing mice and perform a detailed and quantitative analysis, we chose high-performance liquid chromatography (HPLC). This analytical method is widely employed for the detection and quantification of various drugs due to its sensitivity, repeatability and specificity. Here, we report a simple and specific RP-HPLC method to identify ICF 01012 and its expected metabolites after intravenous injection in melanoma-bearing mice. The expected metabolites have been chosen based on our previous pharmacokinetic study of BZA [12]. All the standard molecules (*i.e.*, ICF 01012 and four potential metabolites) have a quinoxaline ring that possesses a

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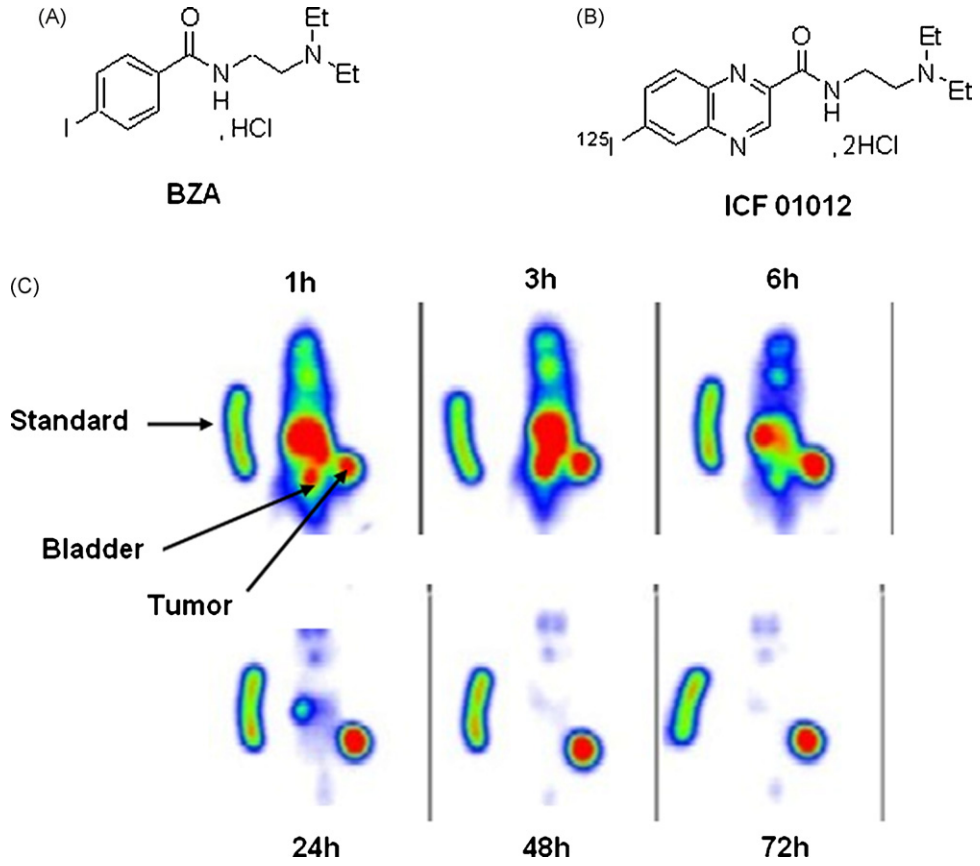

Fig. 1. Chemical structures of BZA (A) and [^{125}I]ICF 01012 (B). Scintigraphic imaging of a B16 F0-melanoma-bearing C57Bl6 mouse at different times after i.v. injection of [^{125}I]ICF 01012 (Gamma IMAGER BIOSPACE dedicated for small animal imaging) (C).

characteristic UV absorption peak at 254 nm (Figs. 1B and 2A). This property enabled us to validate the HPLC analysis method with non-radioactive standard molecules. For the biological studies, [^{125}I]ICF 01012 was detected with a flow scintillation analyzer as the amount of ICF 01012 was too low to be seen with UV detector. The different compounds found were identified by analogy of retention time. The validation study indicates that the protocol is appropriate for evaluating the biodistribution and metabolism of [^{125}I] ICF 01012 in biological samples.

2. Experimental

2.1. Instrumentation

The analyses were carried out using a series HP 1100 HPLC system (Hewlett Packard) equipped with an UV detector and a Radiomatic™ 500TR series Flow Scintillation Analyzer (Packard). Data acquisition and processing were performed using HP ChemStation software (Hewlett Packard) for UV absorption or FLO-One software (Packard) for radioactive detection. Electrospray ionization mass spectra (ESI-MS) were obtained on a TSQ 7000 ThermoQuest Finnigam (Les Ulis, France). The samples were analyzed in CH_3OH/H_2O (1/1, containing 1% HCOOH) in positive mode or in CH_3OH/H_2O (1/1, containing 1% NH_4OH) in negative mode, at a final concentration of 8–12 pmol/μL. Each ESI-MS spectrum was recorded by averaging 10 spectra. Column chromatography was performed with Merck neutral aluminum oxide 90 standardized (63–200 μm). Melting points were determined on a Reichert-Jung-Koffler apparatus without correction. NMR spectra (400 or 200 MHz for ^{1}H and 100 or 50 MHz for ^{13}C) were recorded on a Bruker Avance 400 or Bruker AM 200 instruments using $CDCl_3$ or DMSO-d_6 as solvent. Microanalyses were performed by the Analytical Laboratory of the CNRS (Vernaison, France) for the elements indicated.

2.2. Synthesis of standard compounds

2.2.1. 6-Iodoquinoxaline-2-carboxylic acid

A solution of ethyl 6-iodoquinoxaline-2-carboxylate (1.30 g, 3.96 mmol) in 2N sodium hydroxide/ethanol (1/1, v/v, 6 mL) was refluxed for 30 min. After return back to room temperature, the reaction mixture was acidified (pH 4–5) with acetic acid and cooled to 0 °C. The resulting white precipitate was removed by filtration and dried in a vacuum oven (yield 91%; m.p. 242–244 °C). NMR ^{1}H (200 MHz, DMSO-d_6) (7.97 (d, 1H, J = 8 Hz), 8.15 (d, 1H, J = 8 Hz), 8.52 (s, 1H), 9.36 (s, 1H). NMR ^{13}C (50 MHz, DMSO-d_6) (98.0, 131.4, 137.2, 138.9, 140.1, 142.8, 146.6, 148.8, 165.7. ESI-MS m/z 298.6 $[M–H]^-$ ($C_9H_5IN_2O_2$ = V169 = M1). Found: C, 34.06%; H, 1.79%; N, 8.36%; Calculated for CHNO; C, 36.03%; H, 1.68%; N, 9.34%.

2.2.2. N-(6-Iodoquinoxalin-2-yl)glycine

To a solution of 6-iodoquinoxaline-2-carboxylic acid described above (221 mg, 0.74 mmol) in dry dichloromethane (10 mL), under argon and at 0 °C, dry *N*,*N*-dimethylformamide (110 μL) and thionyl chloride (220 μL, 3.03 mmol) was added dropwise. The reaction mixture was stirred at reflux for 3 h. After cooling to room temperature, the solvent was removed under reduced pressure to afford crude 6-iodoquinoxaline-2-carbonyl chloride. This was resuspended in dry dichloromethane (10 mL), and was added, under argon, to a solution of ethyl glycinate (76 mg, 0.74 mmol)

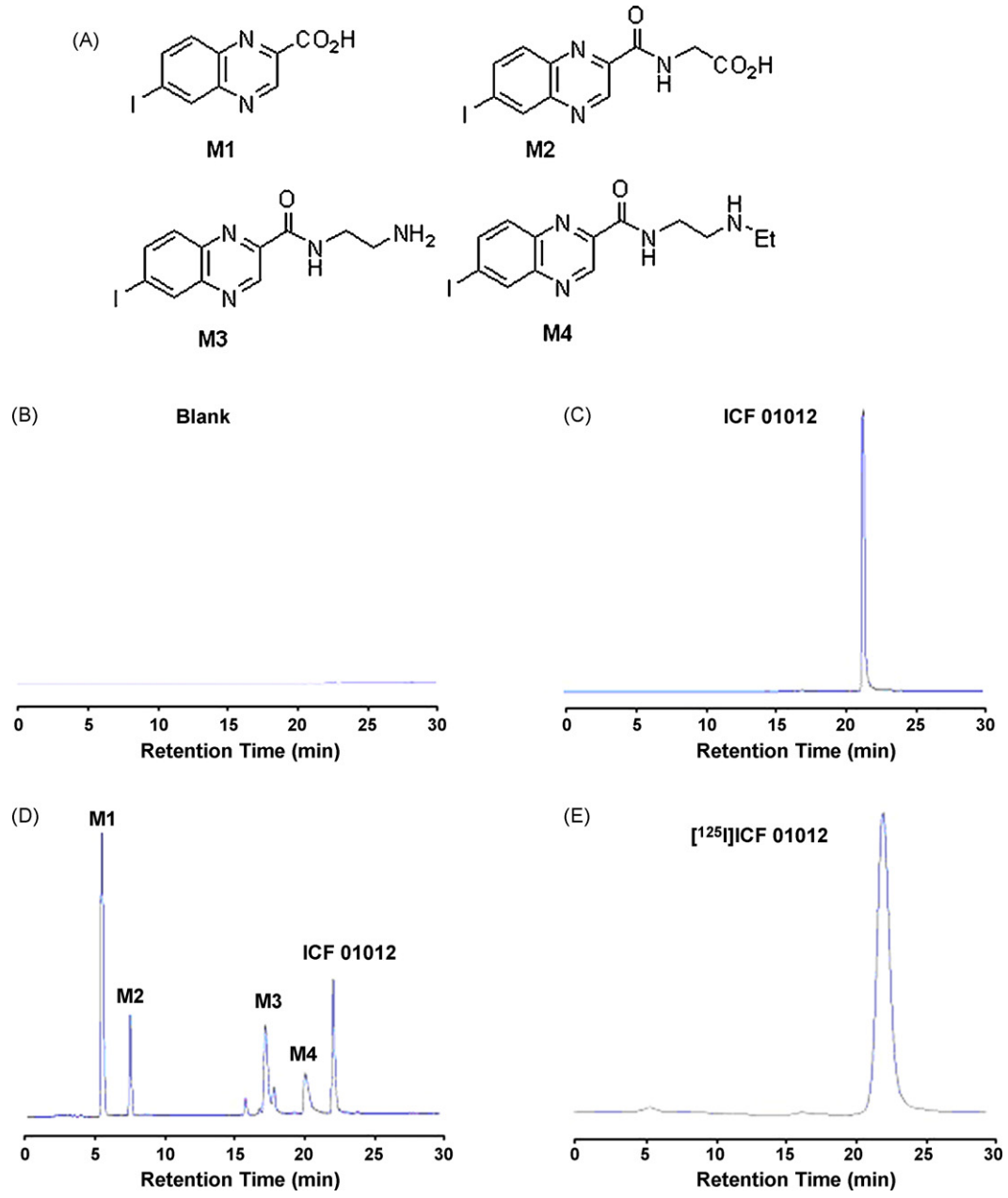

Fig. 2. Chemical structures of expected metabolites of ICF 01012 (A). HPLC chromatograms of a blank (B), a solution of ICF 01012 in water (400 μg/mL) (C) and a mixture of ICF 01012 and its expected metabolites in water (D) with UV detector. Retention time was 22.00 min for ICF 01012, 5.60 min for 6-iodoquinoxaline-2-carboxylic acid (**M1**), 7.60 min for *N*-(6-iodoquinoxalin-2-yl)glycine (**M2**), 17.20 min for *N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide (**M3**) and 20.20 min for *N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (**M4**). Radiochromatogram of [^{125}I]ICF 01012 (4.44×10^{-2} MBq) (E). Retention time was 22.5 min.

and triethylamine (104 μL, 0.74 mmol) in dry dichloromethane (1 mL). The mixture was stirred at room temperature for 24 h and then evaporated to dryness under reduced pressure. The residue was diluted with water (10 mL) and extracted with dichloromethane (3×20 mL). The organic layers were collected, dried ($MgSO_4$), filtered and evaporated under reduced pressure. The crude product was chromatographed (Al_2O_3, CH_2Cl_2/EtOH, 99/1, v/v) to afford ethyl *N*-(6-iodoquinoxalin-2-yl)glycinate (yield 34%, m.p. 142–144 °C). NMR ^{1}H (200 MHz, $CDCl_3$) δ 7.97 (d, 1H, J = 8 Hz), 8.15 (d, 1H, J = 8 Hz), 8.52 (s, 1H), 9.36 (s, 1H); NMR ^{13}C (50 MHz, $CDCl_3$) δ 98.0, 131.4, 137.2, 138.9, 140.1, 142.8, 146.6, 148.8, 165.7. To a solution of ethyl *N*-(6-iodoquinoxalin-2-yl)glycinate (50 mg, 0.13 mmol) in tetrahydrofuran (2 mL) water (2 mL) and lithium hydroxide monohydrate (16 mg, 0.38 mmol) was successively added. The mixture was stirred at room temperature for 24 h, diluted with water (2 mL), acidified (pH 4–5) with acetic acid and extracted with ethyl acetate (3×10 mL). The organic layers were collected, dried ($MgSO_4$), filtered and evaporated under reduced pressure to afford desired *N*-(6-Iodoquinoxalin-2-yl)glycine (yield 69%, m.p. 185–187 °C). NMR ^{1}H (400 MHz, DMSO-d_6) δ 4, 04 (d, 1H, J = 6 Hz), 7.95 (d, 1H, J = 9 Hz), 8.25 (d, 1H, J = 9 Hz), 8.65 (s, 1H), 9.27 (t, 1H, J = 6 Hz), 9.46 (s, 1H), 12.80 (m, 1H); NMR ^{13}C (100 MHz, DMSO-d_6) δ 41.0, 99.6, 130.8, 137.6, 139.0, 139.9, 143.7, 144.0, 144.2, 163.1, 170.7. ESI-MS m/z 355.9 [M–H]$^-$. Found: C, 37.15%; H, 1.99%; N, 11.52%; Calculated for $C_{11}H_8IN_3O_3$; C, 36.40%; H, 2.26%; N, 11.77%.

2.2.3. N-(2-Aminoethyl)-6-iodoquinoxaline-2-carboxamide dihydrochloride salt

To a solution of 6-iodoquinoxaline-2-carbonyl chloride in dry dichloromethane (10 mL) obtained from 6-iodoquinoxaline-2-carboxylic acid (200 mg, 0.67 mmol) according to the procedure described above, under argon, a solution of *tert*-butyl *N*-(2-aminoethyl)carbamate (105 μL, 0.67 mmol) and triethylamine (93 μL, 0.67 mmol) in dry dichloromethane (1 mL) was added dropwise. The mixture was stirred at room temperature overnight and a 1N aqueous sodium bicarbonate solution was added (15 mL). The mixture was decanted and the aqueous layer was extracted with dichloromethane (3 × 20 mL). The organic layers were combined, dried ($MgSO_4$) and evaporated under reduced pressure to afford *N*-[2-(*tert*-butylcarbamoyl)ethyl]-6-iodoquinoxaline-2-carboxamide (yield 79%, m.p. 181–183 °C). NMR ^{1}H (400 MHz, $CDCl_3$) δ 1.42 (s, 9H), 3.46 (t, 2H, J = 6 Hz), 3.66 (q, 2H, J = 6 Hz), 5.05 (m, 1H), 7.81 (d, 1H, J = 9 Hz), 8.08 (dd, 1H, J = 9, 2 Hz), 8.32 (m, 1H), 8.62 (d, 1H, J = 2 Hz), 9.62 (s, 1H). NMR ^{13}C (100 MHz, $CDCl_3$) δ 28.4 (3C), 40.5 (2C), 79.9, 98.5, 130.7, 138.4, 139.6, 140.0, 143.7, 144.1, 144.2, 156.6, 163.6. This compound (183 mg, 0.41 mmol) was resuspended, under argon, in a 2N hydrochloric acid solution in anhydrous ether (10 mL). The mixture was stirred at room temperature for 20 h and the solvent was evaporated under vacuum. The residue was then suspended in anhydrous ether (10 mL) and the mixture was stirred, under argon, overnight at room temperature. The precipitate was collected by filtration to give *N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide dihydrochloride salt (yield 74%, m.p. 286–288 °C). NMR ^{1}H (400 MHz, DMSO-d_6) δ 3.05 (m, 2H), 3.65 (q, 2H, J = 6 Hz), 7.92 (d, 1H, J = 9 Hz), 8.18 (m, 3H), 8.24 (dd, 1H, J = 9, 2 Hz), 8.61 (d, 1H, J = 2 Hz), 9.28 (t, 1H, J = 6 Hz), 9.45 (s, 1H); NMR ^{13}C (100 MHz, DMSO-d_6) δ 36.8, 38.4, 99.6, 130.7, 137.5, 138.9, 139.9, 143.5, 144.4 (2C), 163.6. ESI-MS m/z 343.1 $[M+H]^+$. Found: C, 32.12%; H, 3.09%; N, 13.58%; Calculated for $C_{11}H_{11}IN_4O$, 2HCl; C, 31.83%; H, 3.16%; N, 13.50%.

2.2.4. N-(2-Ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide dihydrochloride salt

To a solution of 6-iodoquinoxaline-2-carbonyl chloride in dry tetrahydrofuran (10 mL) obtained from 6-iodoquinoxaline-2-carboxylic acid (200 mg, 0.67 mmol) according to the procedure described above, under argon, *p*-nitrophenol (93 mg, 0.67 mmol) and a solution of triethylamine (95 μL, 0.68 mmol) in dry tetrahydrofuran (5 mL) were added dropwise. The solution was stirred at 50 °C for 18 h. After cooling to room temperature, dichloromethane (30 mL) was added and the resulting solution was washed with a 5% aqueous sodium carbonate solution (20 mL). The aqueous layer was extracted with dichloromethane (5 × 15 mL) and the organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The resulting precipitate was triturated with ether (5 mL) and collected by filtration to afford *p*-nitrophenyl 6-iodoquinoxaline-2-carboxylate (yield 80%; m.p. 228–230 °C). NMR ^{1}H (400 MHz, DMSO-d_6) δ 7.71 (d, 2H, J = 7 Hz), 8.09 (d, 1H, J = 9 Hz), 8.30 (dd, 1H, J = 9, 2 Hz), 8.41 (d, 2H, J = 7 Hz), 8.72 (d, 1H, J = 2 Hz), 9.60 (s, 1H). To a solution of this compound (75 mg, 0.18 mmol) in dry tetrahydrofuran (10 mL), under argon, the *N*-ethylethylenediamine (19 μL, 0.18 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 18 h and evaporated under reduced pressure. The residue was chromatographed (Al_2O_3, CH_2Cl_2/EtOH, 97/3, v/v). To the resulting amine was added successively, under argon, dry dichloromethane (5 mL) and a 2N hydrochloric acid solution in anhydrous ether (10 mL). The mixture was stirred at room temperature for 10 min and the solvent was evaporated under vacuum. The residue was then resuspended in anhydrous ether (10 mL) and the mixture was stirred, under argon, at room temperature for one night. The precipitate was collected by filtration to give *N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide dihydrochloride salt (yield 54%; m.p. 262–264 °C). NMR ^{1}H (200 MHz, DMSO-d_6) δ 1.21 (t, 3H, J = 7 Hz), 2.96 (m, 2H), 3.13 (m, 2H), 3.70 (q, 2H, J = 7 Hz), 7.92 (d, 1H, J = 9 Hz), 8.24 (dd, J = 9, 2 Hz), 8.62 d, 1H, J = 2 Hz), 9.05 (m, 2H), 9.33 (m, 1H), 9.34 (t, 1H, J = 6 Hz), 9.45 (s, 1H); NMR ^{13}C (100 MHz, DMSO-d_6) δ 10.9, 35.5, 41.9, 45.6, 99.6, 130.7, 137.5, 138.9, 139.9, 143.5, 144.4 (2C), 163. ESI-MS m/z 371.1 $[M+H]^+$. Found: C, 36.45%; H, 3.78%; N, 12.64%; Calculated for $C_{13}H_{15}IN_4O$, 2HCl; C, 35.24%; H, 3.87%; N, 12.95%.

2.3. Solvents

Methanol and aqueous ammonia were obtained from SDS (France) and from Riedel-De Haën Ag Seelze-Hannover (Germany), respectively. All solvents used were of HPLC grade.

2.4. Chromatographic conditions

A reverse phase Zorbax Extend-C18 column (150 mm × 4.6 mm), with a 5 μm particle size from Agilent Technologies (Interchim, Montluçon, France) was used as stationary phase. The mobile phase was composed of 0.2% (v/v) aqueous ammonia in ultra-pure water (Solvent A) and 0.2% (v/v) aqueous ammonia in methanol (Solvent B). The column was eluted with a 20-min linear gradient from 70:30 (A/B) to 0:100 (A/B) at a 0.5 mL/min flow rate and the total run time was 30 min. The column was maintained at 20 °C. Absorption was measured at 254 nm or radioactivity was detected with the radioactive liquid scintillation detector as described above.

2.5. Preparation of standards

For chromatographic analyses, standards (ICF 01012, *N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide dihydrochloride salt and *N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide dihydrochloride salt) were diluted with ultra-pure water at 400 μg/mL and further diluted with water at the desired concentration (6.25–200 μg/mL). 6-Iodoquinoxalin-2-carboxylic acid was diluted with 0.2% (v/v) ammoniac in methanol and *N*-(6-iodoquinoxalin-2-yl)glycine with methanol:water (1:1, v/v).

2.6. Validation method

The linearity, intra- and inter-day precision and specificity were validated to show the reliability of the analytical method. The linearity of the system was determined using standard calibration curves constructed as follow: ICF 01012 peak area versus the nominal concentrations of ICF 01012. Linear regression analysis was performed to assess the linearity and to generate the standard calibration equation.

The precision of the chromatographic system was evaluated using repeatability which was carried out using intra-day precision. Intra-day precision was defined as Relative Standard Deviation (R.S.D.) calculated from the values measured from three samples at five concentrations in one day (n = 3).

The inter-day precision was also studied by comparing three samples at five different concentrations on three different days (n = 3) and the results documented as R.S.D.

The specificity of the method was determined by comparing the chromatograms of ICF 01012 standard with the chromatograms of a mixture of ICF 01012 and expected metabolites of this molecule and with the chromatograms of blanks (ultra-pure water analytes-free).

Table 1
Intra- and inter-day precision of ICF 01012

ICF 01012 concentration (μg/mL)	R.S.D. (%)
Intra-day ($n = 3$)	
6.25	0.72
12.5	0.40
50	0.20
200	0.16
400	0.26
Inter-day ($n = 3$)	
6.25	1.59
12.5	1.09
50	0.55
200	0.60
400	2.10

The limit of detection (LOD) is defined as the lowest concentration of a chemical compound that an analytical process can reliably differentiate from background levels. The limit of quantification (LOQ) is defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability [20]. The LOD and LOQ were calculated as follows: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation of the lowest standard concentration and S is the slope of the standard curve.

2.7. Animal experiments

2.7.1. Tumor model

Three-hundred thousand of B16 F0-melanoma cells in 0.1 mL of PBS were subcutaneously injected in male C57Bl6 mice (Iffa-Credo, France). After 15 days, [^{125}I]ICF 01012 diluted in 0.9% NaCl (0.1 μmol/0.2 mL, 1.85–3.3 MBq/animal), was injected through the tail vein. At intervals (15 min, 1 h, 6 h, 24 h, 48 h and 8 days), the [^{125}I]ICF 01012-treated mice were sacrificed and blood and B16 F0-melanoma were removed. Radioactivity contained in blood and tumors was then extracted using the method described below.

2.7.2. Experimental protocol

To determine the concentration of [^{125}I]ICF 01012 present in B16 F0 primary tumors after i.v. administration of the compound, total radioactivity was released by extraction with methanol: 0.1N NaOH and then analyzed by HPLC. Tumors (150–300 mg) were homogenized in 9 mL of methanol and 1 mL of 0.1N NaOH. For blood, 0.5 mL was mixed with 8.5 mL of methanol and 1 mL of 0.1N NaOH. The total radioactivity in the homogenate was determined with a gamma counter. The mixture was then shaken for 30 min at room temperature and centrifuged at 5000 rpm for 10 min. The released radioactivity in the supernatant was determined and the supernatant was evaporated to dryness with a rotary evaporator. Extraction recovery was calculated as activity in the supernatant: total activity of the homogenate ratio multiplied by 100.

The residue was reconstituted with 1 mL of methanol and vortex mixed. One hundred micro-liters of this solution were subsequently injected for HPLC analysis. The relative abundance of the metabolites was determined based on the percentage of total radioactivity in each peak relative to the entire radiochromatogram. Then, the quantification of the different radiolabeled chemical entities expressed as nmol/g was obtained as follows: % of a given metabolite × [^{125}I]ICF 01012 biodistribution values of blood or tumor (nmol equivalent/g).

3. Results and discussion

3.1. Separation and specificity

Under the chosen chromatographic conditions, specificity was indicated by the absence of any interference at retention times of peaks of interest as evaluated by chromatograms of a blank, a solution of ICF 01012 (400 μg/mL) and a mixture of ICF 01012 and its expected metabolites (Fig. 2B–D). All the compounds were well separated with a UV detector and displayed the following retention times: 22.00 min for ICF 01012, 5.60 min for 6-iodoquinoxaline-2-carboxylic acid (**M1**), 7.60 min for *N*-(6-iodoquinoxalin-2-yl) glycine (**M2**), 17.20 min for *N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide (**M3**) and 20.20 min for *N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (**M4**) (Fig. 2C and D). In parallel, the retention time of [^{125}I]ICF 01012 (22.50 min) was determined with a flow scintillation analyzer (Fig. 2E). The delay of 0.50 min observed between UV and radioactive detection is explained by the fact that the flow scintillation analyzer was placed after the UV detector in the analysis chain.

3.2. Linearity of the calibration curve

Over the ICF 01012 concentration range (6.25–400 μg/mL) regression analysis showed an excellent linearity between UV absorption and ICF 01012 concentrations. The calibration curve could be represented by the following linear regression equation: $y_{\text{ICF 01012}} = 54.313x + 308.83$ ($r^2 = 0.9947$), where y is area, x is concentration in μg/mL and r is correlation coefficient. Three replicates of each concentration of ICF 01012 (6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL) were performed and the relative standard deviations obtained were less than 3.5% for all concentrations.

The measured LOD and LOQ values for ICF 01012 using this method with an UV detector were 0.14 ± 0.05 and 0.43 ± 0.15 μg/mL, respectively.

3.3. Precision

Precision data for intra- and inter-day solution of ICF 01012 are presented in Table 1. The intra-day precision (% R.S.D.) was 0.72, 0.40, 0.20, 0.16 and 0.26 for 6.25, 12.5, 50, 200 and 400 μg/mL, respectively. The inter-day precision (% R.S.D.) was <2.10% for all concentrations used.

3.4. Stability

A solution of ICF 01012 stored at room temperature and injected after 15 days did not show any appreciable change in assay value. The percentage of the Area Under the Curve (% AUC) was 99.07 ± 0.34% when freshly prepared ICF 01012 was injected for HPLC analysis and 98.87 ± 0.33% after 15 days. Similarly, the standard solution of [^{125}I]ICF 01012 did not show

Table 2
Biodistribution of [^{125}I]ICF 01012 in blood and tumor following i.v. injection of 0.1 μmol of [^{125}I]ICF 01012 in B16 F0-melanoma

Tissues	15 min	1 h	6 h	24 h	48 h	8 days
Blood	1.78 ± 0.06	1.77 ± 0.34	0.97 ± 0.25	0.16 ± 0.02	0.06 ± 0.03	0.01 ± 0
B16	2.67 ± 0.52	26.43 ± 5.97	20.42 ± 2.45	22.22 ± 3.46	9.65 ± 2.24	2.39 ± 0.79

The data are expressed as the nmol equivalent per gram of tissue (nmol equivalent/g) (mean ± S.D., $n = 5$).

any appreciable change in the retention time or % AUC when injected after 1 month of storage at 4 °C (22.5 ± 0.08 min and 99.67 ± 0.19% versus 22.53 ± 0.05 min and 99.45 ± 0.30%, respectively).

Samples were stored at −80 °C before radioactivity was extracted. Results obtained with B16 F0-melanoma or blood samples stored during 1 week showed the same number of peaks at the same retention times as samples stored during 1 month. All the identified peaks represented the same percentage of total radioactivity in both cases. As an example, HPLC analysis of 1 week-stored 15 min B16-F0 sample showed 3 peaks at 2.87 ± 0.07, 21.05 ± 0.07 and 22.48 ± 0.03 min with 6.4 ± 0.4, 4.45 ± 0.45 and 88.75 ± 0.25% AUC, respectively. Similar results were obtained when the tumor sample was stored for 1 month: 3 peaks at 2.9 ± 0.14, 20.95 ± 0.08

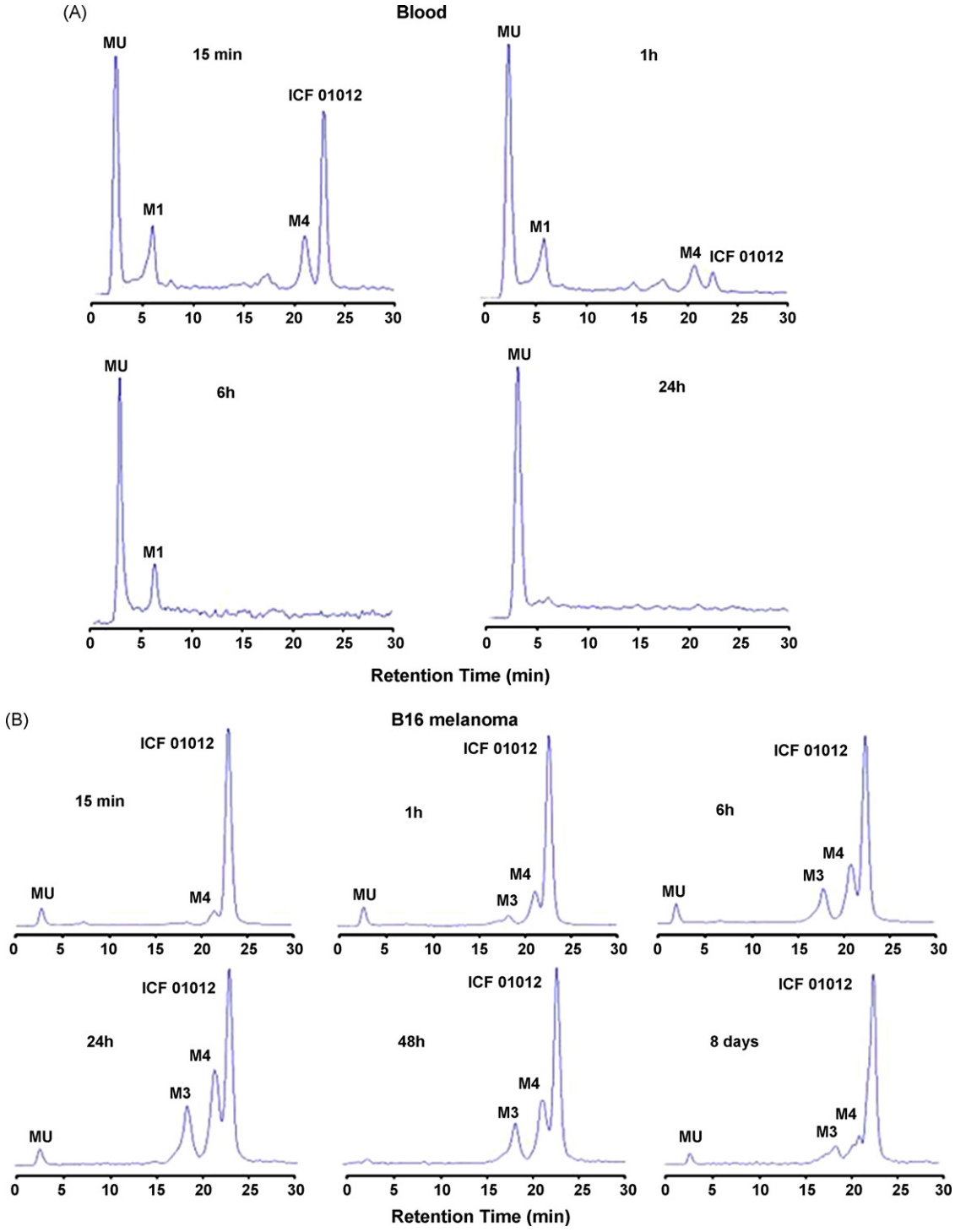

Fig. 3. HPLC radiochromatograms of: blood 15 min, 1 h, 6 h and 24 h after intravenous administration of 0.1 μmol of [^{125}I]ICF 01012 in C57Bl6 mice bearing B16 F0-melanoma (A) and B16 F0-melanoma 15 min, 1 h, 6 h, 24 h, 48 h and 8 days after administration of [^{125}I]ICF 01012 (B). Retention time was 22.50 min for [^{125}I]ICF01012, 2.90 min for the unknown peak (**MU**), 6.10 min for [^{125}I]6-iodoquinoxaline-2-carboxylic acid (**M1**), 18.10 min for [^{125}I]*N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide (**M3**) and 20.70 min for [^{125}I]*N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (**M4**).

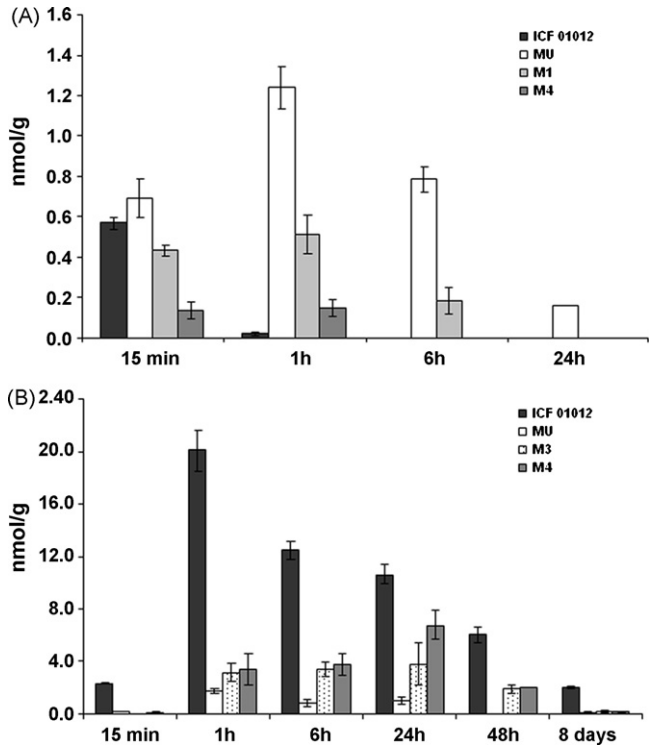

Fig. 4. Concentration of [^{125}I]ICF 01012, unknown peak at 2.90 min retention time (**MU**), [^{125}I]6-iodoquinoxaline-2-carboxylic acid (**M1**), [^{125}I]*N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide (**M3**) and [^{125}I]*N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (**M4**) in blood (A) and tumor (B) after intravenous administration of 0.1 μmol of [^{125}I]ICF 01012 in C57Bl6 mice bearing B16 F0-melanoma. Results are expressed as nmol of compound per gram of tissue, mean±S.D., $n = 5$.

and 22.55 ± 0.05 with 6.75 ± 0.15, 4.39 ± 0.41 and 90.00 ± 1.05% AUC, respectively.

3.5. Application of the method

The aim of this study was to develop a reliable, reproducible and simple method to assess the chemical entities ICF 01012 present in tumor samples.

As described above, the mice bearing B16 F0-melanoma were sacrificed 15 min, 1 h, 6 h, 24 h and 48 h and 8 days after [^{125}I]ICF 01012 intravenous injection, the blood and the tumors were removed and radioactivity was determined with a gamma counter and then extracted before samples were analyzed by HPLC. Taking together all results, extraction yield of radioactivity was 84.2% ± 10.1 for tumors and 87.7% ± 7.8 for blood. Table 2 shows the distribution of [^{125}I]ICF 01012 in blood and B16 F0-melanoma. [^{125}I]ICF 01012 is rapidly distributed to the organs since only 1.78 nmol equivalent of ICF 01012 per gram of tissue (nmol equivalent/g) was detected in blood 15 min after injection. The amount of radioactivity increased rapidly in the tumor, reaching 26.43 nmol equivalent/g of tissue 1 h post-injection. Radioactivity in B16 F0-melanoma remained stable for up to 24 h and then decreased. However, there was still 2.39 nmol equivalent/g of radioactivity 8 days after injection.

The profile of the radioactivity found in the blood and the tumors at different times after [^{125}I]ICF 01012 injection, is displayed in Figs. 3 and 4. The blood extracts were analyzed by HPLC between 15 min and 24 h post-injection as for later times the total amount of radioactivity was undetectable. By analogy of time retention, we found three peaks which corresponded to the expected compounds, *i.e.*, ICF 01012, 6-iodoquinoxaline-2-carboxylic acid (**M1**), *N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (**M4**) and an unknown peak at a retention time of 2.90 min (**MU**). This last peak was the major one and became the only form of radioactivity in the blood 24 h post-injection (Fig. 3). Fig. 4A shows the nmoles of [^{125}I]ICF 01012 per gram of tissues compared to its metabolites. The amount of unchanged compound is very low at 15 min after [^{125}I]ICF 01012 injection and undetectable from 6 h.

We identified four different peaks in B16 F0-melanoma extracts, the major peak corresponding to [^{125}I]ICF 01012. Even eight days post-injection, 83.6% of the remaining radioactivity was derived from unmodified [^{125}I]ICF 01012 rather than its metabolites. We also found radioactivity in *N*-(2-aminoethyl)-6-iodoquinoxaline-2-carboxamide (**M3**) and *N*-(2-ethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (**M4**) forms (the values ranged from 0 to 19.65% and from 4.4 to 30.57%, respectively). The last peak was unknown (**MU**) (retention time was about 2.90 min) and represented only a small proportion of the remaining radioactivity (less than 7% for the highest value) (Fig. 3). Even if total radioactivity in tumor remained stable between 1 and 24 h (Table 2), the amount of the unchanged compound [^{125}I]ICF 01012 decreased while the amount of its metabolites increased (Fig. 4B). Metabolites decreased after 48 h and nearly disappeared after 8 days. Thus, the radioactivity in B16 F0-melanoma corresponded to [^{125}I]ICF 01012. Taken together, these results suggest that the organs rapidly take up [^{125}I]ICF 01012 and that once trapped into the tumor, [^{125}I]ICF 01012 is slowly converted into different metabolites that can be expelled out of the tumor.

Using the described conditions, the different peaks detected in blood and tumors were well resolved. The exact metabolic fate of [^{125}I]ICF 01012 in B16 F0-melanoma and blood and the percentage of [^{125}I]ICF 01012 evaluated from remaining radioactivity present in tissues could be determined with the HPLC method described. This study demonstrates that the radioactivity imaged within the tumors is primarily derived from unchanged [^{125}I]ICF 01012. Taken together, the analytical data presented herein indicate that [^{125}I]ICF 01012 possesses many of the desired attributes of radiotracer and warrant further investigation for its use as radionuclide therapy agent.

4. Conclusions

The proposed method is simple and precise for the detection of [^{125}I]ICF 01012 metabolism *in vivo* with an easy characterization of the radioactivity in blood and tumors. Therefore, this method appears convenient and is likely to be suitable for carrying out extensive studies of [^{125}I]ICF 01012 metabolism in other organs, leading to the determination of the accurate metabolic fate of this promising agent for radionuclide therapy.

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