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THE RADIOSYNTHESIS OF A NEW POTENTIAL GLUCOSE TRANSPORT PROTEIN TRACER : 3-[^{125}I]IODOPHLORETIN.

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SUMMARY.

Phloretin (2',4',6'-trihydroxy-3-(4-hydroxyphenyl)-propiophenone) is a glucose transport (GLUT) blocker acting at the extracellular part of the GLUT protein. Labelling this compound with radioiodine can provide a tracer for elucidating in vitro and in vivo the role of GLUT's in cells showing an increased glucose metabolism. As the inhibition of GLUT by phloretin is supposed to occur at the 2',4',6'-trihydroxyphenyl site, the radioiodine is substituted on the 3-position of the 4-hydroxyphenyl group. Direct electrophilic substitution on this position is not possible due to highly activated sites in the trihydroxyphenyl group. 3-Bromophloretin, the precursor for radioiodination, is prepared using a new method, a saturation substitution followed by the elimination of the unstable bromine atoms. Radioiodinated phloretin is obtained with a labelling yield of 75% by applying Cu^{1+}-assisted radioiodo for bromo exchange at 140°C. Pure non carrier added tracer results from HPLC separation coupled to a mini-column preconcentration and recovery (± 70% overall yield).

Keywords: 3-[^{125}I]iodophloretin, glucose transport blocker, saturation substitution technique, Cu^{1+}-assisted labelling.

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INTRODUCTION

Up to now the research performed to develop a ^{123}I-glucose analogue for SPECT (Single Photon Emission Computerised Tomography), which in vivo would have the properties of ^{18}F-FDG (^{18}F-2-fluoro-2-deoxy-D-glucose), remained without success. 2-Iodo-2-deoxy-D-glucose or iodovinyl substituted glucose is not stable in vivo and I-benzyl glucose analogues lack carrier transport into the cell and/or are not a substrate for hexokinase (1,2,3,4).

In vivo PET (Positron Emission Tomography) has shown an increased ^{18}F-FDG uptake by tumor cells. The increased ^{18}F-FDG uptake in tumors seems to be linked to the glucose "import" constant k_1 (5). For rate limited glucose transport this can point to increased density of transport proteins. Measurement of the glucose transport proteins with an appropriate tracer can elucidate this problem and can provide an alternative for the diagnosis with SPECT of tumors characterized by an increased glucose uptake.

From the literature it is well known that phloretin is a potent competitive inhibitor of glucose binding to transport proteins. Phloretin inhibits red cell glucose transport competitively with an apparent Ki of 0.5 - 2.5 μM (6).

Krupka (7) and Krupka and Devés (8) state that phloretin is bound exclusively to the outward-facing carrier and that external glucose competes with phloretin.

All these studies on phloretin were performed by direct measurements with conventional spectrophotometric techniques or indirectly, using phloretin to inhibit the ^{14}C-labelled glucose or ^{14}C-deoxyglucose uptake.

To our knowledge, radioactive labelled phloretin and more specifically radioiodinated phloretin, has up to now never been described or used. In view of the potentialities of SPE(C)T, it was decided to develop and evaluate a radioiodinated phloretin analogue.

MATERIALS and METHODS.

Reagents: Phloridzin.2H_2O (4',6'-dihydroxy-2'-(β-D-glucosido)-3-(4-hydroxyphenyl)propiophenone.dihydrate) 1 (99%) and phloretin (2',4',6'-trihydroxy-3-(4-hydroxyphenyl)-propiophenone) 2 (98%) were purchased from Aldrich. The

other reagents used were p.a. grade (Merck) or HPLC grade (Lichrosolv quality Merck). Non carrier added sodium [125I]iodide (10^{-2}N NaOH ; 74 ± 5,5 TBq/mmol) was purchased from Nordion Europe (Fleurus, Belgium).

HPLC Equipment :

Analytical 1 : The equipment consisted of a Rheodyne injector (50 µl loop), a Hitachi 655A pump and L-6000 II controller, a 655A variable wavelength UV monitor at 288 nm, a Na(Tl) detector (Harshaw QS) and appropiate electronics (Canberra), D2000 Chromato integrator (Hitachi) and an Ankersmidt R40 one channel recorder. Quality control was achieved on a Lichrospher 125x4 mm 100 RP 8 (5µ) Merck column and a MeOH/acetate buffer 10^{-3}M : X/Y (v/v) mixture of pH 4.8 , specified for each synthesis, was used as eluent at a flow rate of 1 ml/minute.

Analytical 2 : The equipment consisted of a Rheodyne injector (50 µl loop), a Waters 510 pump, a Waters 486 tunable UV absorbance detector at 288 nm, a Radiomatic A500 (Canberra Packard) with a CaF_2-detector, a Flo-one\ data for Windows software package for instrument control and data analysis, and a HP deskjet 500 recorder. Quality control was achieved as described above.

Semi-preparative : Rheodyne injector (2.5 ml loop), a Waters M6000A pump provided with a semi-preparative pumphead, a Waters Lambdamax UV-480 monitor at 288 nm, a NaI(Tl) detector (Harshaw QS) connected to Ortec electronics, a HP 3580 and an Intersmat ICR-18 integrator.

A MeOH/acetate buffer 10^{-3} M : X/Y (v/v) mixture of pH 4.8 , specified for each synthesis, was used as eluent on respectively a Lichrocart 250x10 mm Lichrosorb RP Select B (10µ) Merck column (flow rate of 6 ml/minute) or Hibar 250x25 mm Lichrosorb RP Select B (7µ) Merck column (flow rate of 15 ml/minute).

Synthesis of phloretin 2.

A mixture of 2.12 mmol phloridzin.$2H_2O$ **1** (1g) and 30 ml 1 N hydrochloric acid was refluxed under N_2 for 90 min. After cooling to room temperature the suspension was stored during 2 hours at 4°C. After filtration on a Whatmann nr. 5 filter the precipitate was rinsed three times with 15 ml ice-cold water and

resuspended in 20 ml ice-cold water. After a second filtration the precipitate was dried under vacuum at 30°C. A final product **2** (0.558 g, very light brown powder) was obtained (Yield 96%).

Analytical HPLC (eluent : MeOH/acetate buffer 10^{-3}M : 45/55, t_R**1**= 3.9 minutes ; t_R**2**= 12 minutes) revealed a purity ⩾ 99%. As reference for identification the purchased phloretin was used.

Synthesis of 3-bromophloretin 3.

0.15 mmol phloretin **2** (41 mg) was dissolved in 15 ml glacial acetic acid. While stirring, 0.46 mmol Br_2 was added dropwise (12 μl Br_2 / 1 ml glacial acetic acid). The bromination reaction was followed up by HPLC (MeOH / acetate buffer 10^{-3}M : 55/45). When complete conversion to 3,3',5'-tribromophloretin **4** was observed, the reaction was stopped by addition of 5 ml 1M Na_2SO_3 and 20 ml water. This mixture was extracted twice with 40 ml diisopropylether. The organic phase was evaporated and the residue was dissolved in 20ml 10^{-3}N NaOH solution.

After addition of 5 ml 1M Na_2SO_3 the mixture was stirred for 30 minutes. The solution was acidified with 95% H_2SO_4 to pH 3 and the obtained suspension was filtered on a Whatmann nr. 5 filter. The precipitate was rinsed 3 times with 10 ml ice-cold H_2O, resuspended in 20 ml 10^{-3}N H_2SO_4 solution and sonicated during 5 minutes. The suspension was stored overnight at 4°C. After filtration the precipitate was respectively rinsed with 3 portions of 10 ml ice-cold 10^{-3}N H_2SO_4 solution and 5 ml ice-cold water and dried under vacuum at 30°C. A final product **3** (49 mg, light brown powder) was obtained (Yield : 95%).

Analytical HPLC (MeOH/acetate buffer 10^{-3}M : 55/45, t_R**2**= 3.54 minutes; t_R**3**= 5.96 minutes) and semi-preparative HPLC (MeOH/acetate buffer 10^{-3}M : 55/45, 6 ml/minute; t_R**2** =36 minutes, t_R**3** = 78,2 minutes) revealed a purity ⩾ 99%.

^{1}H-NMR (270 MHz,DMSO / d_6) : δ 7,28 (s,1H,Ar-H_2), δ 7 (d,1H,Ar-H_5), δ 6,8 (d,1H,Ar-H_6), δ 5,8 (s,2H,Ar-$H_{3',5'}$), δ 3.18 (t,2H,Phe-CH_2-C-), δ 2.7 (t,2H,-C-CH_2-CO-).

MS (FAB) (m/e,%RA) : 353(M^+,90), 274(5), 200(9), 187(40), 169(45), 153(100).

Synthesis of 3',5'-dibromophloretin 5.

0.455 mmol phloretin **2** (125 mg) was dissolved in 37.5 ml glacial acetic acid. While stirring, 0,91 mmol Br_2 was added dropwise (46 μl Br_2 / 2,25 ml glacial acetic acid). The bromination reaction was followed up by HPLC (MeOH/acetate buffer 10^{-3}M : 55/45). When complete conversion to 3',5'-dibromophloretin was observed, the reaction was stopped by addition of 5 ml 1M Na_2SO_3 and 75 ml H_2O. This solution was extracted twice with 80 ml diisopropylether. After evaporation of the organic phase the residue was resuspended in 30 ml 10^{-3}N H_2SO_4 solution and was stored overnight at 4°C.

After filtration on a Whatmann nr. 5 filter the precipitate was rinsed 3 times with 10 ml ice-cold 10^{-3}N H_2SO_4 and dried under vacuum at 30°C. A final product **5** (191 mg, light brown powder) was obtained (Yield : 97%). Analytical HPLC (MeOH / acetate buffer 10^{-3}M : 55/45, t_R**2**= 3.54 minutes; t_R**5**= 6,6 minutes) revealed a purity ≥ 97%.

^{1}H-NMR (270MHz,DMSO/d_6) : δ 7 (d,2H,Ar-$H_{3,5}$), δ 6.6 (d,2H,Ar-$H_{2,6}$), δ 3.4 (t,2H,Phe-CH_2-C-), δ 2.9 (t,2H,-C-CH_2-CO-).

MS (FAB) (m/e) : 431 (M^+), 433, 435.

Synthesis of 3-iodophloretin 6.

0.912 mmol 3',5'-dibromophloretin (394 mg) was dissolved in 77ml MeOH/H_2O : 7/3 (v/v). 0.912 mmol ICl was added by means of a peristaltic pump (55,1 μl ICl / 2 ml MeOH; flow rate 0.1 ml/minute) and the solution was stirred for 30 minutes. After addition of 25 ml 1M Na_2SO_3 and 75 ml 10^{-3}M NaOH the solution was stirred for another 30 minutes and acidified with 95% H_2SO_4 to pH 3. After filtration, as mentioned above, the precipitate was dissolved in HPLC eluent. After semi-preparative HPLC separation (MeOH / acetate buffer 10^{-2} M : 40/60 ; flow rate 15 ml/minute ; t_R**6** = 32 minutes), the MeOH of the eluent fraction containing the 3-iodophloretin was removed by evaporation under vacuum at 30°C. The obtained suspension was stored overnight at 4°C. After filtration the precipitate was rinsed with respectively three portions of 10 ml ice-cold 10^{-3}N H_2SO_4 solution and 5 ml of ice-cold water and then dried under vacuum at 30°C. A final product **6** (183 mg, light

brown powder) was obtained (Yield : 50%). Analytical HPLC (MeOH/acetate buffer 10^{-3}M : 55/45, t_R **6** = 8.8 minutes) revealed a purity ≫ 99%.
^{1}H-NMR (270 MHz, DMSO / d_6) : δ 7,5 (s,1H,Ar-H_2), δ 7 (d,1H,Ar-H_6), δ 6,73 (d,1H,Ar-H_5), δ 5,8 (s,2H,Ar-$H_{3',5'}$), δ 3.18 (t,2H,Phe-CH_2-C-), δ 2.7 (t,2H,-C-CH_2-CO-).
MS (FAB) (m/e,%RA) : 401 (M^+,100), 274 (5), 233 (55), 132 (8), 120 (23).

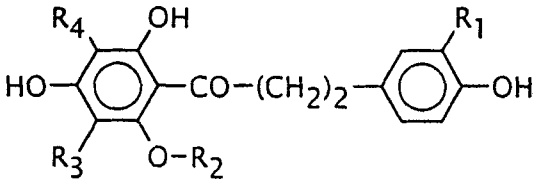
Radiosynthesis of non carrier added 3- [^{125}I]iodophloretin 7.

Stock solution : 2.5 mg $SnSO_4$, 25 mg 2,5-dihydroxybenzoic acid, 35 mg citric acid.H_2O were dissolved in 25 μl glacial acetic acid and 2250 μl H_2O (pro injection quality) .

Copper solution : 32.5 mg $CuSO_4.5H_2O$ was dissolved in 10 ml H_2O.

Reaction: A reaction mixture of 1.2 mg 3-bromophloretin **3** dissolved in 5 μl glacial acetic acid, 450 μl stock solution and 60 μl of the copper solution contained in a septum closed vial of 1 ml volume was flushed with N_2 for 5 minutes and the radioiodide solution was added through the septum. The vial, contained in an aluminum safety container, was heated in a thermoblock at 140°C for 60 minutes. The contents of the reaction vial followed by 0.5 ml rinsing solution (i.e. 0.2 ml MeOH and 0.3 ml MeOH/acetate buffer 10^{-3}M : 50/50) were sucked through a 0.45 μ filter and injected for semi-preparative HPLC separation. To the eluent fraction containing the radioactive tracer an amount of H_2O, corresponding to two times its volume, was added. The resulting solution was passed through a Baker Bond Octadecyl 100 mg column by N_2 pressure at a flow rate of 4 ml/minute. The column was rinsed with 20 ml H_2O and blown to apparent dryness with N_2. The radioactive compound was recovered in 250 μl of EtOH and stored at -18°C prior to further use. An average labelling yield of 75% was observed. Besides free radioiodine no other radiolabelled compound was present. The overall radiochemical yield amounted to 70%. HPLC in analytical and semi-preparative conditions (radioactivity detection and mass detection by sensitive UV spectrometry) revealed a radiochemical purity of at least 99% and the amount of starting bromo com pound, if present, was lower than the detection limit (10^{-13} mol) for the compound (λ_{max} **3** ~ λ_{max} **6** = 288 nm).

RESULTS and DISCUSSION.



R1	R2	R3	R4	Compound
H	β-D-glucose	H	H	**1**
H	H	H	H	**2**
Br	H	H	H	**3**
Br	H	Br	Br	**4**
H	H	Br	Br	**5**
I	H	H	H	**6**
*I	H	H	H	**7**
H	H	H	Br	**8**

Synthesis and Radiosynthesis.

As the inhibition of the glucose transport by phloretin is assumed to occur by the interaction of the trihydroxyphenyl site with the protein and in view of the lower increase of lipophilicity it was decided to substitute the iodine atom in ortho-position to the hydroxyl function, i.e. the 3-position in the 4-hydroxyphenylgroup.

In literature two synthetic pathways are proposed for phloretin (9,10) :

i. the coupling of 4-methoxyphenylpropionic acid chloride to 1,3,5-trimethoxybenzene using BF_3 in $CHCl_3$ and CCl_4 followed by deprotection,

i.i. the coupling of 4-hydroxyphenylpropionitrile to 1,3,5-trihydroxybenzene,

the first step being the reaction of acrylonitrile with phenol, both in Friedel-Crafts conditions ($AlCl_3$, dry HCl_g).

When applying the first method on 2-bromo-4-methoxyphenylpropionic acid chloride, the coupling reaction failed due to the formation of a cyclic ketone (5-methoxy-indanone, as shown by NMR analysis). In the second method, it is known from thermodynamic considerations that in the first step, quantitative para-positioning of the propionitrile group occurs by an intramolecular displacement from initial ortho-position to para-position during reflux (9). When we used 2-bromophenol, the first step failed and it would seem that the bromine atom on the 2-position disturbed the coupling reaction.

In view of the failure of the conventional methods, a new synthetic pathway had to be developed. Direct electrophilic substitution on the 3-position of the hydroxyphenyl group of phloretin was not possible due to the highly activated sites, 3 and 5 position, of the trihydroxyphenyl ketone group. Phloretin shows a pH dependent keto-enol tautomery, represented in Fig.1, with a $pK_a=7.5$ (11). Down from pH 5, 100% of the keto-form is present while, up from pH 9 the enol (dissociated) -form is approximatively quantitative.

The bonding of the bromine atoms substituted on the 3 and 5-position of the trihydroxyphenyl group (3' and 5' position in phloretin) is destabilised by the enol-form.

It is this property which is used in the synthesis we propose, based on a so-called "saturation substitution technique " : in a first step bromine atoms are substituted on the 3', 5' and 3-position of phloretin in acidic medium; in a second step the labile bromine atoms on the trihydroxyphenyl-part of the molecule are removed under alkaline conditions (100% enol-form).

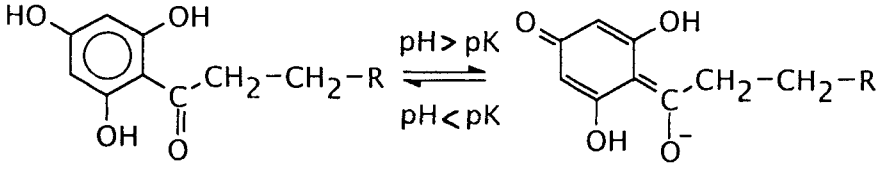


Fig.1. pH dependent keto-enol tautomery of phloretin.

In the case of iodination this strategy could not be applied as the bond-strength of the iodine atoms on the trihydroxyphenyl group is too weak, even in acidic conditions, resulting in an "on - off" equilibrium (as proved using radioactive iodine), thus not allowing substitution on the 4-hydroxyphenyl group. Therefore 3',5'-dibromophloretin was used as the substrate for electrophilic iodination on the 3-position, ortho to the hydroxyfunction.

Addition of I_2 to 3',5'-dibromophloretin dissolved in glacial acetic acid yielded an iodinated product which after treatment with an alkaline sulfite solution resulted in the starting product phloretin. It was assumed that in acidic medium a second type of keto-enol tautomery (Fig.2.) could occur, resulting in the substitution on the aliphatic double bond of an iodine atom, which is then also removed.

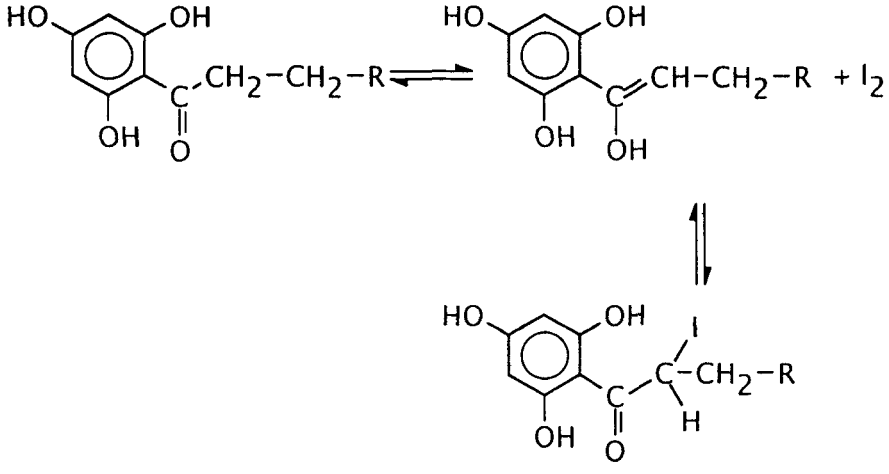


Fig. 2. Substitution of an iodine atom on the aliphatic double bond of phloretin in acidic medium; second type of keto-enol tautomery.

The use of NaI in peracetic acid (glacial acetic acid / 30% H_2O_2 : 3/2 v/v) did not yield the compound of interest and only side products were generated. Applying ICl in a methanol / H_2O mixture was successful. The labile bound bromine atoms were removed under alkaline conditions without disturbing the iodine-bond in the 3-position. With the reaction conditions described, a yield of 50% was obtained. Higher amounts of ICl, in view of increasing the yield,

had to be avoided because otherwise 3,5-diiodinated phloretin was generated. ICl could not be used for radioiodination since it must be performed under non carrier added conditions to obtain high specific activity.

When performing direct electrophilic radioiodination on 3',5'-dibromophloretin in glacial acetic acid ($^{125}I^+$ was generated by addition of 100 μl H_2O_2 to 500 μl glacial acetic acid) a labelling yield not higher than 50% was obtained and the formation of a radioactive side product (25%) was observed.

Moreover the total procedure to obtain the pure non carrier added tracer was cumbersome because of different column purifications and the debromination step before semi-preparative HPLC separation.

Therefore it was preferred to apply the Cu^{1+}-assisted nucleophilic non-isotopic exchange (12,13) using 3-bromophloretin as substrate. The labelling yield then amounted to at least 75% . The reaction mixture could readily be injected for HPLC separation. After mini-column recovery the pure non carrier added 3-[^{125}I]iodophloretin was obtained with an overall yield of about 70%. Analytical HPLC showed a purity $\geq$ 99%.

The preliminary results, obtained in vitro with ^{125}I-3-iodophloretin for binding to red blood cells and cancer cells and which will be reported elsewhere, showed that this new tracer is promising for the study of pathologies involving glucose transport proteins .

Conclusion.

3-[^{125}I]iodophloretin could not be obtained by direct electrophilic substitution, but the Cu^{1+}-assisted radioiodo for bromo exchange has been applied with success (70% overall yield) on 3-bromophloretin. This substrate was obtained by applying a new synthetic pathway: the saturation substitution technique followed by elimination of the unstable bromine atoms. This was shown to be an efficient alternative for previously described synthetic methods which failed in an initial step due to the presence of an halogen on the 4-hydroxyphenyl entity .

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