PREPARATION OF ^{18}F-LABELLED 5-FLUOROURACIL OF VERY HIGH PURITY.

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SUMMARY

5-[^{18}F]Fluorouracil was prepared by direct fluorination of uracil in acetic acid using [^{18}F]F_2 diluted in neon. The labelled product was obtained with a purity of ≥99% following preparative HPLC. Quality control consisted of two different and improved HPLC procedures combined with high resolution NMR spectroscopy for analyzing directly the HPLC elution peaks. A final formulation contained typically $4.8 \cdot 10^8$ Bq 5-[^{18}F]fluorouracil with a specific activity of $1.14 \cdot 10^{-5}$ Ci/μM.

Key Words: ^{18}F-Labelling, 5-[^{18}F]Fluorouracil, preparative HPLC, Quality Control, PET.

INTRODUCTION

5-Fluorouracil is conventionally prepared by condensation of ethyl pseudothiourea hydrobromide with α-fluoroformylacetic ester as described in an improved modification of the original Wheeler synthesis [1] by Duschinsky et al. [2]. Preparations by direct fluorination methods of uracil are repeatedly described in the literature, but have never gained considerable importance in preparative organic chemistry, except presumably the process published through a patent by Giller et al. [3].

A critical examination of former reports concerning direct fluorination of pyrimidines and derivatives thereof [4-8] suggested that the initially formed 5-fluoro-,6-OR- disubstituted primary products required further chemistry for isolating 5-fluorouracil, which was then obtained in a rather moderate yield following an expended separation and purification. Essentially the same was observed in typical low scale reactions when ^{18}F-labelled

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5-fluorouracil was prepared, although degradation and over-fluorination was not a problem with these preparations. The main contamination of 5-[^{18}F]fluorouracil consisted of considerable amounts of unreacted uracil, which initially was present in a large excess, and which was difficult to remove quantitatively from the usual 5-[^{18}F]fluorouracil preparation.

Fowler et al. [9] first communicated a rapid and practical procedure for labelling 5-fluorouracil with ^{18}F. This synthesis, which accurately is the most convenient one, has been successfully applied by Vine et al. [10]. Minor modifications of this procedure, using [^{18}F]acetylhypofluorite, were reported for the synthesis of the ^{18}F-labelled uracil nucleosides [11,12], which were also applicable to the 5-[^{18}F]fluorouracil preparation, and which recently were the subject of some mechanistic and stereochemical considerations [7,8] already known in general by the work of Robins et al. [5].

In our hands, none of the methods mentioned above proved to deliver 5-[^{18}F]fluorouracil of a purity suitable for the difficult PET-supported study of in-vivo pharmakokinetics of this clinically important chemotherapeutic agent. We focused therefore our work on the final isolation of a very pure 5-[^{18}F]fluorouracil and developed a procedure which allowed the separation of an unexpected pure labelled drug from other labelled and non-labelled contaminants [13] for the first time since the initial introduction of this tracer. Quality control was performed by two different HPLC procedures with the direct analysis of HPLC elution peaks through high resolution ^{1}H- and ^{19}F-NMR spectroscopy. This was definitely shown as the only way of ensuring a reproducible and invariable high quality formulation of the labelled drug.

EXPERIMENTAL

Reagents. Uracil and 5-fluorouracil were commercial analytical grade reagents (Janssen). Solvents generally were of p.a. quality and were used without further purification. 5-Fluorouracil for i.v. application was a FARMITALIA product (Fluoroblastin 500).

The Ne/F_2 gas mixture (2% F_2 in Ne, Matheson or BOC) was used as delivered without scrubbing a possible HF contamination. [^{18}F]F_2 was obtained by the ^{20}Ne(d,α)^{18}F reaction from a gas target system (pure nickel) containing 1% F_2 in Ne [14].

Thin layer chromatography was done on glass backed silica plates (silica 60F_{254}, Merck) using CH_3CN/H_2O mixtures, by volume: 99.7/0.3 for micro scale reactions ($\leq 10^{-4}$ M); 70/30 for the larger scale reactions ($\geq 10^{-3}$ M); R_f of 5-fluorouracil was $\approx$0.47. Labelled

contaminants appeared at $0.5 < R_f < 0.9$. Radioactivity was recorded qualitatively using a Berthold thin layer scanner. It was applied for roughly monitoring the preparation and purification steps.

Analytical HPLC was performed using a Merck-Hitachi 655A-12 pump equipped with a photodiode array detector (L-3000, Merck-Hitachi). UV-detection occured at two wavelengths simultaneously, at 250 nm and at the UV maximum, 270 nm, of 5-fluorouracil. Radioactivity was monitored by a sodium iodide-photomultiplier tube. An ionisation chamber (Robotron), calibrated for ^{18}F, was used for quantitative measurements of eluate fractions. Columns were either the organic base analysis column Aminex HPX-72S (Bio-Rad), or LiChrospher 100 CH-18/2 (Merck). The HPX-72S column (300x7.8 mm) consisted of 11μ particles of 8% cross-linked divinylbenzene styrene co-polymer, loaded with sulfate anions. It was operated at a constant 70 °C (Eldex column heater) with a flow rate of 1 ml/min of 0.125 M $(NH_4)_2SO_4$ solution. A micro-guard anion exchange cartridge (Aminex SA, 40x4.6 mm) was mounted in front of the column. The LiChrospher column (250x4 mm) consisted of 5μ reversed phase material and was operated at room temperature with 0.5 ml/min of $2 \cdot 10^{-3}$ M NaH_2PO_4 buffer solution at pH = 3.6.

Preparative HPLC was carried out using a Waters 600 multi solvent delivery system equipped with a U6K injection valve having a sample loop of 5 ml volume. UV-trace was recorded at 270 nm; radioactivity was monitored as above. Radioactive fractions were collected and counted separately in the previously calibrated ionisation chamber. The column (250x21 mm) was prepared from 5μ reversed phase material HD-Sil-18-5s-80 obtained from Orpegen GmbH, Heidelberg [15]. It was operated at 15 ml/min with $2 \cdot 10^{-2}$ M NaH_2PO_4 buffer solution at pH = 7.8.

NMR analysis of analytical and preparative HPLC elution peaks was made following fraction collection, confirming the final purity of our preparations, and for checking the efficiency of the respective HPLC procedures. Radioactive fractions contained sufficent fluorine carrier and were additionally analyzed by ^{19}F-NMR spectroscopy. Samples were evaporated to dryness, dissolved again in D_2O, and measured in 5 mm sample tubes. External ^{19}F shift referencing was performed by inserting a capillary with C_6F_6 in C_6D_6. The C_6F_6 signal (-87.28 ppm) was calibrated against 10^{-3} M trifluoroacetic acid. ^{1}H shift values (ppm) were referenced using 2,2,3,3-tetradeutero-3-trimethylsilyl-propionic acid (= 0 ppm). Measurements were made on a AM-500 FT NMR spectrometer (Bruker). Typical resolution was 0.26 Hz/point for ^{1}H, and 1.09 Hz/point for ^{19}F spectra. Integrated peaks of uracil at δ = 5.788 and δ = 7.517

with $^3J_{5,6}$ = 7.66 Hz, and 5-fluorouracil at δ = 7.656 and δ_F = -93.8 with $^3J_{HF}$ = 5.25 Hz, were used for analysis.

Preparation of 5-[^{18}F]fluorouracil. About $4.5 \cdot 10^{-4}$ M of F_2 diluted to 1% in Ne were labelled with ^{18}F by the ^{20}Ne(d,α)^{18}F nuclear reaction. A typical irradiation (15 μAh, 10-12 μA) delivered an average of $3.4 \cdot 10^9$ Bq (≈92 mCi) [^{18}F]F_2 which was then bubbled slowly (50 ml/min) through 40 ml of a suspension of uracil in acetic acid containing at least $2.5 \cdot 10^{-5}$ M uracil per ml. The radioactive solution was transferred into an evaporation flask, which also fitted to a sublimation apparatus. The acetic acid was distilled off until dryness at a bath temperature of 90 °C and under a mild regulated vacuum, so that the distillation rate was about 2ml/min. During this procedure 45-50% of the initially trapped radioactivity was lost into the distillate and, to a smaller amount, into the waste. The remaining white, sometimes slightly yellow, residue contained ≈ 40% of 5-[^{18}F]fluorouracil together with some further labelled material (see figure 1). Subsequent vacuum sublimation (205-210 °C bath temperature; $2 \cdot 10^{-3}$ bar; 25 min) resulted in the recovery of 70% of the target compound.

This rather crude product - also containing considerable amounts of unreacted uracil - was further purified by preparative HPLC (see figure 1). The sublimate ($9.9 \cdot 10^7$ Bq) was dissolved in 4 ml of $2 \cdot 10^{-2}$ M NaH_2PO_4 buffer solution of pH = 7.8, filtered (Millipore HV, 0.45 μm) and then was purged on the HD-Sil-18-5s-80 column through a 5 ml sample loop. The 5-[^{18}F]fluorouracil radioactivity eluted with a k' = 1.85, calculated from the radioactive trace. The void volume was determined separately in a previous run by injecting a small amount of urea (instead of thiourea, which is usually used) dissolved in the elution buffer. Labelled contaminants were separated with k_1' = 0.35, k_2' = 0.57, and k_3' = 0.96. The resolution, R, between 5-fluorouracil and uracil was determined as R = 1.36 [16]. The actual recovered radioactivity represented $7.4 \cdot 10^8$ Bq, which was 75% of the injected amount (the remainder was discarded into the waste). Among these 8.2% eluted with the first two fractions (k_1' and k_2'), 14.3 % with the third peak (k_3'), and 76.5% ($5.6 \cdot 10^8$ Bq, 15 mCi) together with 5-fluorouracil (percentages corrected for decay with respect to the time of loading the sample loop).

Consequently the final yield of isolated 5-[^{18}F]fluorouracil was 16% ($5.4 \cdot 10^8$ Bq) related to the initially trapped radioactivity. It had a guaranteed purity of ≥99%. Preparation and purification consumed 95 min. Typically 17.5 mg of ^{18}F-labelled 5-

fluorouracil were obtained (≈30% chemical yield, determined by osmometric measurements).

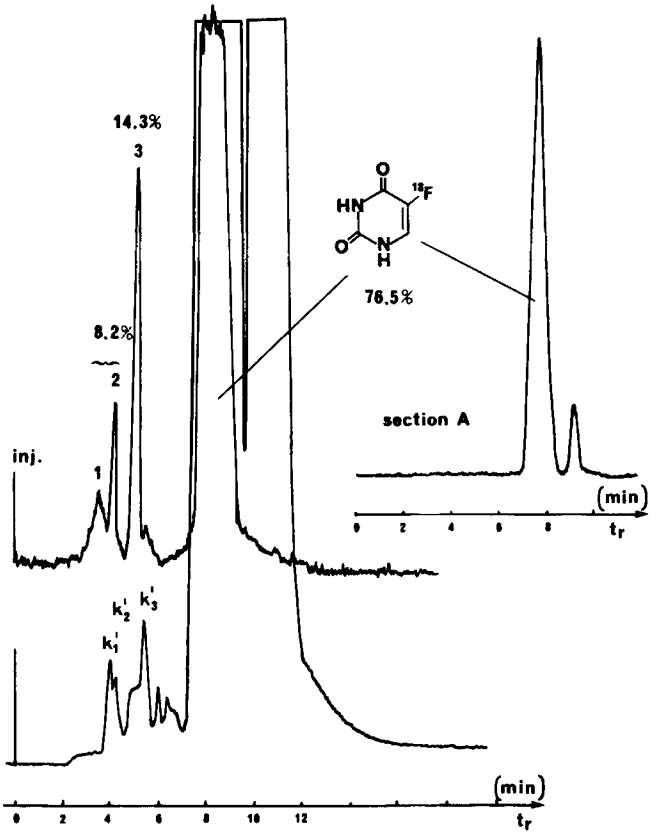

Figure 1. Preparative HPLC of 5-[^{18}F]fluorouracil. Section A indicates the uracil content of the product fraction, when sampling occured over the whole baseline peak width of the radioactive peak. 1, 2, and 3 were labelled byproducts, present before and remaining following sublimation.

The 5-[^{18}F]fluorouracil was obtained in a volume of 30 ml of buffer solution and was then evaporated to dryness. The product was redissolved in 3 ml of Fluoroblastin 500 for i.v. injection. This solution was then made isotonic by adding the required amount of sterile water, as determined by an osmometer. Filtration through a 0.22 μm sterile filter delivered 8-10 ml 5-[^{18}F]fluorouracil with a radioactivity of $4.8 \cdot 10^8$ Bq (13 mCi) ready for injection. The volume depended on the chemical yield of 5-fluorouracil and the sampled amount of buffer, but was always within the

indicated range. The specific activity A^* of the preparation was at this time $1.14 \cdot 10^{-5}$ Ci/μM ($\epsilon = \ln(A_i/A^*) = 18.83$, with A_i as the specific activity of the perfect carrier free state).

Analysis of 5-[^{18}F]fluorouracil preparations. A small sample ($2.7 \cdot 10^5$ Bq) of 5-[^{18}F]fluorouracil, separated through preparative HPLC, was loaded on the LiChrospher 100 CH-18/2 analytical column. Elution (0.5 ml/min) with a $2 \cdot 10^{-3}$ M NaH_2PO_4 buffer at pH = 3.6 confirmed that 5-[^{18}F]fluorouracil was >99% pure. The compound had a retention time of t_R = 11.6 min (k'_{FU} = 2.2) with this column. Resolution between 5-fluorouracil and uracil was R = 1.3. The selectivity was $\alpha = k'_{FU}/k'_U = 1.2$ as determined separately using 10^{-6} M standard solutions of 5-fluorouracil and uracil respectively.

An identical sample was analyzed by a second HPLC procedure, using the Aminex HPX-72S column. 5-[^{18}F]fluorouracil eluted from this column at t_R = 21.4 min (1 ml/min of 0.125 M $(NH_4)_2SO_4$). Resolution was R = 2.7, and α = 2.2 (k'_{FU} = 2.34). Although this column had a much better resolution and selectivity, one would expect it to separate semi-preparative samples easily. This was not the case. Considerable tailing of the uracil elution peak through a large sample loading contaminated the 5-fluorouracil fraction and repeated runs were necessary for obtaining a pure preparation. On the contrary the preparative reversed phase column inverted the order of elution ($k'_{FU} < k'_U$), which circumvented such difficulties.

DISCUSSION

5-[^{18}F]Fluorouracil, which was absolutely free from contaminants, could be obtained exclusively through preparative HPLC on a reversed phase material, which was especially prepared in collaboration with Orpegen GmbH, Heidelberg [15]. The most important characteristic of the column was the fact that uracil and 5-fluorouracil changed the order of elution when going from analytical (pH = 3) to preparative application (pH = 7.8) solely by changing the ionic strength and pH value. This was strictly required in order to avoid contamination of 5-fluorouracil by uracil. Additionally, 5-[^{18}F]fluorouracil was obtained with the correct pH in the final solution to be injected to patients [17]. Figure 2 and figure 3 instructively demonstrate the separation profile of the Orpegen column (**A**) compared to the LiChrospher material (**B**). No separation at all could be obtained at 5 < pH < 7 with the Orpegen column. The LiChrospher column separated Uracil and 5-fluorouracil well at 2.5 < pH < 7, but no separation at all

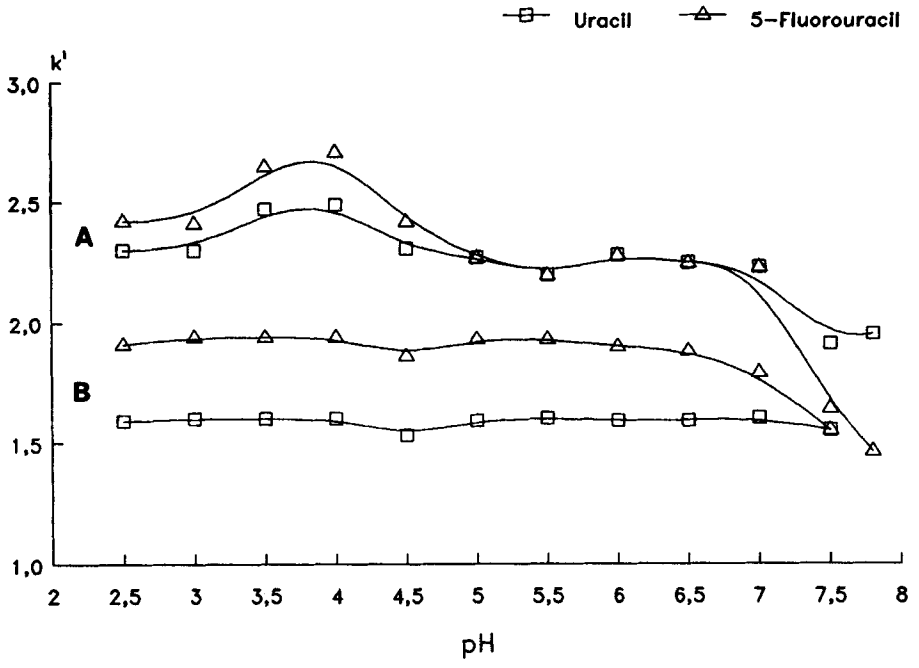

Figure 2. k' of 5-fluorouracil and uracil as function of pH; **A** for the HD-Sil-18-5s-80 column, **B** for the LiChrospher column.

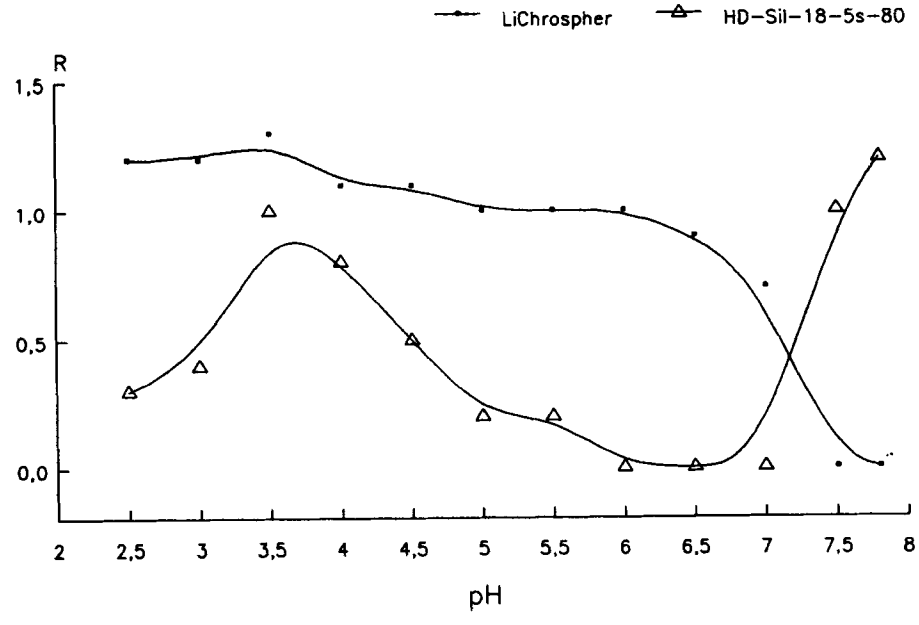

Figure 3. Dependence of resolution R between 5-fluorouracil and uracil upon pH of the eluent.

was obtained at pH > 7. The LiChrospher column was used to control the separation efficiency of the preparative set-up.

As indicated above, 76.5% of collected radioactivity eluted with 5-fluorouracil from the preparative column (see fig.1). Reinjection of this fraction onto the same column resulted in the chromatogramm as shown in section A of figure 1. Only the UV trace was recorded in this case. Peak integration and integrated ^{1}H-NMR spectra confirmed that the uracil contamination was as low as 4.4% (0.8 mg) of the amount eluted in total, i. e. along the whole baseline peak width of the radioactive peak. Uracil contamination was reduced to an undetectable trace when sampling time was reduced accordingly. Radioactivity was lost during this procedure, e.g. the isolated yield of 5-[^{18}F]fluorouracil dropped by 20% when sampling occured 1.5 min only, but purity reached 100%.

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[13] All compounds produced through direct fluorination of uracil could be separated and analyzed quantitatively, but are not included in this report. Very much work was invested into the separation of the already known, but previously not isolated, intermediates 5-fluoro-6-hydroxy-, 5-fluoro-6- acetoxy-, 5,5-difluoro-6-hydroxy-, and 5,5-difluoro-6- acetoxy- compounds. Analytical support for a 5-fluoro-5H- pyrimidine-2,4-dione as an intermediate was also established. A discussion of all these data went beyond this work and will be published in a comprehensive further report, as they formed part of the PhD of E. H.

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[15] Prepacked columns are now commercially available through ORPEGEN GmbH, Czernyring 22, D-6900 Heidelberg, F. R. G.

[16] $R = [t_R(2) - t_R(1)]\{0.5[w(1) + w(2)]\}^{-1}$ with $t_R(2) > t_R(1)$ and w = baseline peak width.

[17] The pH did not change after evaporation of solvent, re-dissolution with Fluoroblastin, and dilution by water. The final solution was usually at pH = 7.8 - 8.