

Reduced dimethylaminoethanol in [^{18}F]fluoromethylcholine: an important step towards enhanced tumour visualization

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Received: 3 February 2010 / Accepted: 19 May 2010 / Published online: 18 June 2010
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Abstract

Purpose [^{18}F]Fluoromethylcholine ([^{18}F]FCho) is a radiotracer generally used for tumour visualization in patients. Due to high levels of dimethylaminoethanol (DMAE) remaining in [^{18}F]FCho solutions synthesized by currently available methods, tumour visualization might be compromised.

Methods An improved purification method involving an optimized purification step for reducing the levels of DMAE was conceived. The physiological explanation for the interference of residual DMAE in [^{18}F]FCho pharmacokinetics was further elaborated in a xenograft mouse model. **Results** The use of a series of polymer solid-phase extraction cartridges (Oasis HLB/WCX), instead of the commonly used combination of tC18 and Accell CM cartridges, reduced DMAE levels from 402.2 ± 49.6 ppm to 3.0 ± 0.5 ppm. Subsequent in vitro tests proved that (1) [^{18}F]FCho uptake was reduced in the presence of DMAE at concentrations above $0.5 \mu\text{M}$ and (2) DMAE is a competitive inhibitor of [^{18}F]FCho transport. In vivo experiments in xenograft mouse models corroborated reduced tumour uptake at DMAE plasma levels of about $2.5 \mu\text{M}$ as found in patients injected with contaminated [^{18}F]FCho.

Conclusion Residual DMAE, even at levels below choline plasma concentrations found during fasting, compromises

[^{18}F]FCho uptake in vivo and care should be taken to avoid its interference in molecular imaging with [^{18}F]FCho.

Keywords Dimethylaminoethanol · ^{18}F -Choline · Choline transporters · Positron emission tomography · Tumour visualization

Introduction

Although tumour cells are typically characterized by amplified proliferation rates, they also show increased levels of choline transporters [1] and increased expression of the enzyme choline kinase [2]. The latter accounts for choline phosphorylation and, consequently, influences incorporation of the resulting phosphorylcholine into the tumour cells. Since phosphorylcholine is metabolically trapped in the cell, the use of radiolabelled choline and choline analogues for tumour visualization with PET is a rational approach [3]. Several studies have shown the potential of [^{11}C]- and [^{18}F]-labelled choline as radiotracers for the detection of prostate, brain and breast cancers, and hepatocarcinoma [4–9].

[^{18}F]Fluoromethylcholine ([^{18}F]FCho) is commonly produced by nucleophilic addition of dimethylaminoethanol (DMAE) to gaseous [^{18}F]fluorobromomethane ([^{18}F]FCH₂Br). The crude mixture is usually purified on a silica-based cation exchange cartridge (Accell CM, Waters), thus giving [^{18}F]FCho. However, using this purification step, Kryza et al. [10] have reported high levels of residual DMAE in the final [^{18}F]FCho solution. Remarkably little attention has been paid to this observation, particularly when it is taken into account that DMAE uptake in tumour cells is two to seven times higher than that of choline [11]. Moreover, DMAE acts as an inhibitor of choline transport across the blood–brain barrier [12], in alveolar type II

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epithelial cells ($K_{i \text{ DMAE}}=7 \mu\text{M}$, $K_{m \text{ Cho}}=18 \mu\text{M}$) [13], and in fetal rat cerebral hemispheres ($K_{i \text{ DMAE}}=6 \mu\text{M}$) [14]. Preliminary in vitro experiments by Kwee et al. [15] have shown reduced [^{18}F]FCho and [^{18}F]fluoroethylcholine uptake in the presence of DMAE (up to 1 mM) in prostate cancer cells. According to these findings, DMAE can be considered to reduce tumour uptake of [^{18}F]FCho and thereby compromise tumour visualization. Therefore, an alternative purification method with improved chromatographic steps was designed with the aim of producing [^{18}F]FCho with higher purity (i.e. lower DMAE levels). In an attempt to demonstrate the importance of DMAE contamination on [^{18}F]FCho uptake, in vitro (using F98 glioma cell lines) and in vivo (using a xenograft mouse model) experiments were carried out in which the standard Accell CM purification method was compared with our newly developed purification method.

Materials and methods

Materials

Potassium carbonate, potassium bicarbonate, dibromomethane, ethylenediaminetetraacetic acid (EDTA), nitric acid, *N,N*-dimethylaminoethanol and absolute ethanol were purchased from Sigma-Aldrich (St. Louis, MO). Kryptofix (K_{222}) and fluoromethylcholine chloride standard were obtained from ABX (Radeberg, Germany). Dry acetonitrile (ACN) and ammonium hydroxide (28–30% aqueous solution) were purchased from Acros Organics (Geel, Belgium). Physiological saline and water for injection were obtained from B. Braun Medical (Sheffield, UK). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), penicillin/streptomycin mixture, L-glutamine, trypan blue stain, and cell dissociation medium were obtained from Lonza (Basel, Switzerland). Athymic Nu/Nu mice were obtained from Charles River Laboratories International (Janvier, France). The F98 glioma-derived tumour cell line was obtained from

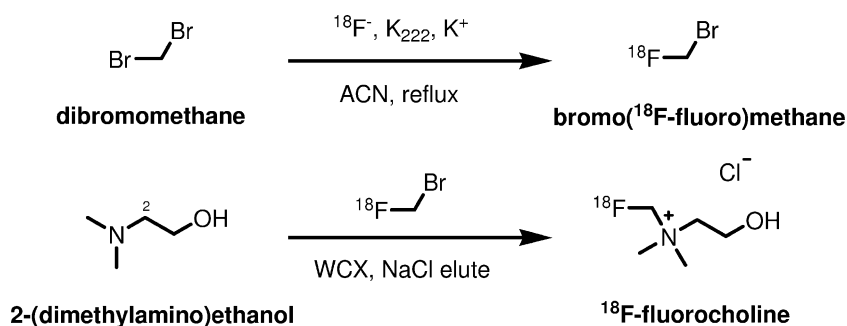
ATCC (Manassas, VA). All solid-phase extraction (SPE) cartridges (Sep-Pak plus tC18, Sep-Pak Accell plus CM (Light), Oasis WCX plus, Oasis HLB plus, Sep-Pak Light Accell plus QMA, and Sep-Pak plus silica) were obtained from Waters (Milford, MA).

Methods

[^{18}F]Fluoromethylcholine synthesis

The synthesis was performed on a fully automated Scintomics module (Fürstfeldbruck, Germany). [^{18}F]Fluoride was produced via the ^{18}O (p,n) ^{18}F nuclear reaction in a Cyclone 18/18 cyclotron (IBA Group, Louvain-la-Neuve, Belgium) by irradiation of enriched water (IBA Group). After transfer to the hot cell, [^{18}F]fluoride was isolated from enriched water using a silica-based anion exchange cartridge (QMA) previously conditioned with potassium bicarbonate solution (0.25 g/5 ml) and water (10 ml). [^{18}F]FCho was synthesized according to a previously reported method [10, 16] principally involving nucleophilic addition of DMAE to gaseous [^{18}F]FCH₂Br (Scheme 1). Formation of the latter was achieved by eluting [^{18}F]fluoride from the QMA cartridge using a mixture of 3.5 mg potassium carbonate, 18.8 mg Kryptofix 222, 1.92 ml ACN and 80 μl water. Subsequent azeotropic evaporations (using 2 \times 1 ml dry ACN) stimulated the nucleophilic activity of [^{18}F]fluoride. Addition of CH₂Br₂ (300 μl in 2 ml dry ACN) combined with heating until reflux yielded [^{18}F]FCH₂Br. Gas chromatographic separation of the crude mixture, including [^{18}F]FCH₂Br (boiling point 8°C, retention time 8–16 min) and CH₂Br₂ (boiling point 80°C, retention time 19 min) was carried out as described by Iwata et al. [16] on four serial silica cartridges with a nitrogen stream at 30 ml/min as the mobile phase. [^{18}F]FCho was eventually formed by passing the fraction containing gaseous [^{18}F]FCH₂Br over a SPE cartridge loaded with 400 μl DMAE. The chemical purity of the resulting [^{18}F]FCho solution with various SPE cartridges was evaluated as described below.

Scheme 1 Reaction scheme for [^{18}F]fluoromethylcholine synthesis



Purification with tC18 and Accell CM SPE cartridges (tC18/ACM)

This method, which is referred to as tC18/ACM, is the benchmark in [^{18}F]FCho purification and is used as a reference method to evaluate new purification methods. The plus tC18 silica-based cartridge was loaded with DMAE (400 μl) and the resulting [^{18}F]FCho was purified on a serially switched Accell CM cartridge preconditioned with HCl and H₂O or EtOH (Table 1) [3, 10]. Purification of the product was completed by successive washing steps of the cartridges with EtOH and H₂O. The product was eluted with 0.9% NaCl. Both Accell CM light and regular cartridges (which vary only in the amount of packing material) were evaluated. Experiments were performed in triplicate.

Purification with Oasis HLB and WCX SPE cartridges (HLB/WCX)

Alternatively, 400 μl DMAE was loaded on a polymer Oasis HLB, which was placed in line with a polymer-based weak cation exchange cartridge (Oasis WCX). Purified [^{18}F]FCho was obtained after successive washing steps of both cartridges with 6% NH₄OH, 6% NH₄OH/EtOH (50/50, v/v), EtOH, and H₂O. [^{18}F]FCho was eluted with a 0.9% NaCl solution (Table 1). Experiments were performed in triplicate. This method, using Oasis HLB and a WCX cartridge, is referred to as HLB/WCX and was used in all subsequent in vivo and in vitro experiments.

Quality control

Residual DMAE levels were determined by high-pressure liquid chromatography using an IC PAK Cation M/D (3.9 \times 150 mm, 5 μm ; Waters, Milford, MA) as the solid phase. The mobile phase consisted of an aqueous solution

of 0.1 mM EDTA and 4 mM HNO₃ pumped isocratically at 1 ml/min. The eluate was detected by refractive index (RI 2414, Waters). The identities of DMAE and [^{18}F]FCho were confirmed by comparison of the observed retention times (7.65 min and 11.01 min, respectively) with the retention times obtained after injection of the standard reference compounds. Residual amounts of DMAE with each purification method were obtained from a calibration curve and are expressed as parts per million (ppm, w/v).

In vitro experiments with tumour-derived cell lines

Cultivation of F98 glioma cells Cells were cultured in DMEM supplemented with 1% penicillin/streptomycin mixture, 1% L-glutamine, and 10% FBS under an atmosphere containing 5% CO₂ in a humidified incubator at 37°C until confluence. On the day of the experiment, the culture medium was discarded, the cells were washed twice with HBSS and harvested with a cell dissociation medium. After centrifugation of the cells (4 min, 1,100 rpm, 4°C), the supernatant was removed and the cells were resuspended in PBS (0.01 M, pH 7.4) until the desired concentration was reached. The cells were counted using trypan blue staining and a Neubauer counting chamber.

Saturation experiment F98 glioma cells (10⁶ suspended in 150 μl PBS) were incubated with increasing amounts of [^{18}F]FCho (0.0185–5.18 MBq) in 100 μl 0.9% NaCl ($n=6$), and therefore the residual levels of DMAE increased proportionally. At the same time, another incubation experiment was performed: F98 glioma cells (10⁶ suspended in 100 μl PBS) were incubated with increasing amounts of [^{18}F]FCho (0.0185–5.18 MBq) in 100 μl 0.9% NaCl and spiked additionally with 3.6 μM DMAE (dissolved in 50 μl PBS, $n=6$). The specific activity was 35.7 GBq/ μmol [^{18}F]FCho. Cells were placed in an incubator on a rotor

Table 1 Purification methods. The traditional tC18/Accell CM method (light and regular cartridges) and our newly developed Oasis HLB/WCX method

	tC18/Accell CM light	tC18/Accell CM regular	Oasis HLB/WCX
Conditioning	Accell: 10 ml 0.5 M HCl, 10 ml H ₂ O or 10 ml EtOH	Accell: 10 ml 0.5 M HCl, 10 ml H ₂ O or 10 ml EtOH	WCX: no conditioning
DMAE loading	400 μl DMAE on tC18	400 μl DMAE on tC18	400 μl DMAE on Oasis HLB
Washing (successive steps)	10 ml H ₂ O, 10 ml EtOH	10 ml H ₂ O, 10 ml EtOH	10 ml 6% NH ₄ OH, 10 ml 50/50 6% NH ₄ OH/EtOH, 10 ml EtOH, 10 ml H ₂ O
Elution	10 ml 0.9% NaCl	10 ml 0.9% NaCl	10 ml 0.9% NaCl
Residual DMAE (ppm w/v, \pm SD, $n=3$)			
No conditioning	–	–	3.04 \pm 0.5
Accell conditioned with HCl/H ₂ O	144.1 \pm 39.9	432.6 \pm 54.6	–
Accell conditioned with HCl/EtOH	143.0 \pm 31.4	402.2 \pm 49.6	–

(Labincoco, Breda, The Netherlands) at 8 rpm, and after 30 min cell uptake was quenched at 0°C. The tubes were centrifuged for 20 s at 13,000 rpm (Heraeus Biofuge Pico, Labcare, UK), the supernatant was removed, and the cell pellet was washed twice with ice-cold PBS (200 µl). Finally, the pellet radioactivity was counted in a Packard Cobra γ -counter equipped with five 1"×1" NaI(Tl) crystals (Canberra, Meriden, CT).

Incubation with increasing levels of DMAE F98 glioma cells (10^6 suspended in 100 µl PBS) were incubated with 0.0925, 2.22, and 5.18 MBq [^{18}F]FCho in 0.9% NaCl (100 µl) and at each radioactivity level the cultures were spiked additionally with 0.1, 0.5, 1.0, 2.5 and 3.6 µM DMAE (dissolved in 50 µl PBS). The specific activity of [^{18}F]FCho was steady within an incubation experiment with different DMAE levels, but varied in the range of 35.8 to 36.4 GBq/µmol during the incubation experiments with different [^{18}F]FCho levels. F98 glioma cells were placed in the incubator on a rotor at 8 rpm and after 30 min the uptake was quenched at 0°C. The cells were centrifuged (20 s, 13,000 rpm), the supernatant was removed, and the cell pellet was washed twice with ice-cold PBS (200 µl). Finally, the pellet radioactivity was counted in a Packard Cobra γ -counter. Tracer uptake in the presence of additional DMAE was normalized to the incubation of [^{18}F]FCho with the highest obtainable purity, i.e. produced by HLB/WCX purification and containing 3.04 ± 0.53 ppm DMAE. These incubation experiments were performed in triplicate.

Biodistribution in a xenograft athymic Nu/Nu mouse model

Athymic Nu/Nu mice were injected subcutaneously with 5×10^5 F98 glioma cells suspended in serum-free cell medium (200 µl). Mice bearing tumours of about 0.5 cm³ were used in the biodistribution study. Food was withheld from the mice 4 h prior to tracer injection. At the start of the experiment, mice (weighing about 22 g, female) were intravenously injected in a tail vein with 0.444 MBq [^{18}F]FCho (in 100 µl 0.9% NaCl) that was corrected to 0.01, 0.446 and 17.86 µg DMAE, eventually corresponding to final plasma levels of 0.05, 2.5 and 100.19 µM DMAE (assuming a blood volume of 7% of body weight).

Animals were killed at 5, 20 and 60 min after injection by cervical dislocation under isoflurane sedation. Blood was obtained immediately by cardiac puncture. Then all organs were isolated, rinsed with PBS, blotted to dryness, weighed and counted for radioactivity in a Packard Cobra γ -counter. Aliquots (10 µl) of the injection medium were counted and weighed. Subsequently, syringes were weighed before and after injection in order to allow accurate determination of the injected dose. Biodistribution was

averaged in four mice per time point and per DMAE concentration. Results are expressed as percentage of injected dose per gram tissue (%ID/g) calculated according to the expression: counts per minute in tissue sample/counts per minute injected $\times 100$ /tissue weight in grams. All animal experiments were conducted in accordance with Belgian legislation and the experimental designs were approved by the local ethics committee of the University Hospital in Ghent (UZGent; ECD 08/48).

Statistics

All statistical tests were performed using GraphPad Prism 5.00 for Windows (San Diego, CA; www.graphpad.com). Due to the expected inhibitory effect of residual DMAE on [^{18}F]FCho uptake by tumour cells, a one-tailed paired *t*-test was used for all the in vitro test results. In the biodistribution studies, brain, lung and tumour tissues were evaluated using the one-tailed Mann-Whitney test, since the inhibitory effect of DMAE on choline uptake has been proved in these organs. Other organs were analysed using the two-tailed Mann-Whitney test. Differences were regarded as statistically significant for $p<0.05$.

Results

[^{18}F]Fluoromethylcholine synthesis

[^{18}F]FCho was synthesized in less than 50 min with a reasonable radiochemical yield of $29.4\pm 3.1\%$ (non-decay-corrected) and a radiochemical purity of more than 95%, regardless of the type of purification method used (Fig. 1a). The specific activity of the [^{18}F]FCho solution was 36.0 ± 2.4 GBq/µmol. Extensive quality controls [17] showed that [^{18}F]FCho solutions were within the following specifications: pH 7–10; $\text{K}_{222} < 50$ ppm; ACN < 410 ppm; EtOH $< 5,000$ ppm; solutions were sterile and the level of pyrogenicity was < 2.5 endotoxin units/ml. Residual DMAE levels varied with the type of SPE cartridge used and are part of the scope of this work.

Purification methods

The use of tC18/ACM for the separation of radiolabelled choline and DMAE, as described in previously [3, 10, 16, 18–20], was compared with an optimized HLB/WCX method. The commonly used tC18/ACM method resulted in high residual levels of DMAE (> 100 ppm) regardless of whether water or ethanol was used for Accell CM conditioning (Table 1), but the Accell CM light cartridge (160 mg packing material) performed better than the corresponding regular cartridge (360 mg packing material)

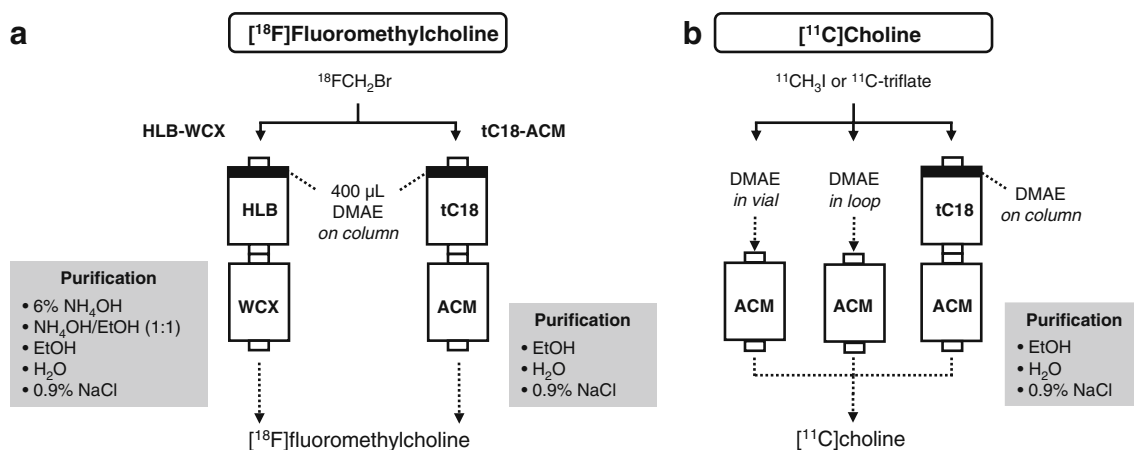


Fig. 1 Various purification methods used in (a) [¹⁸F]FCho synthesis and (b) [¹¹C]choline synthesis (HLB polymer-based reversed-phase sorbent, WCX polymer-based weak cation exchange sorbent, ACM silica-based weak cation exchange sorbent)

in reducing DMAE levels (143 vs. 402 ppm). However, the combination of Oasis HLB and WCX (each 225 mg of packing material) reduced the level of residual DMAE in the final [¹⁸F]FCho solution to 3 ppm. The effects of the higher purity of [¹⁸F]FCho on its incorporation into tumour cells was investigated in the following in vitro and in vivo experiments.

In vitro experiments with tumour-derived cell lines

Saturation experiment F98 glioma cells were incubated with increasing amounts of [¹⁸F]FCho. The corresponding saturation curve was plotted, with a good fit ($R^2=0.9748$) according to the Michaelis-Menten equation using nonlinear regression (Fig. 2). F98 glioma cells were also incubated with increasing amounts of [¹⁸F]FCho in the presence of additional DMAE (3.6 μM). The corresponding

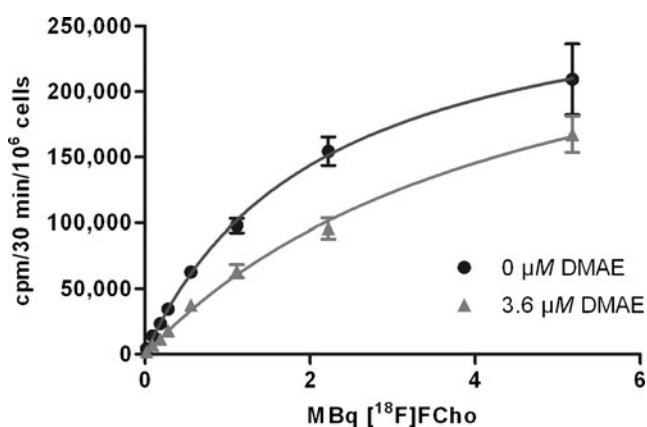


Fig. 2 Saturation curves plotted according to the Michaelis-Menten equation. Counts per minute in 10⁶ F98 glioma cells after 30 min incubation with [¹⁸F]FCho are expressed in relation to the activity (MBq) of [¹⁸F]FCho added. The experimental data points ±SD are shown. The circles and triangles represent 0 and 3.6 μM additional DMAE added

saturation curve was also plotted with a good fit ($R^2=0.9871$) according to the Michaelis-Menten equation.

Because functional characterization of the choline transporter was not required and the specific activity of the [¹⁸F]FCho solution was steady, the results of both experiments are expressed as counts per minute in 10⁶ F98 glioma cells after 30 min incubation in relation to the activity (MBq) of [¹⁸F]FCho added. Thus from the graphs, V_{max} and the Michaelis-Menten constant (K_m) were calculated as 295,982±11,972 cpm/30 min/10⁶ cells and 2.16±0.19 MBq (means±SD), respectively, for the saturation experiment without additional DMAE. Determination of DMAE allowed the evaluation of the possible inhibitory effect of DMAE on the [¹⁸F]FCho uptake by tumour cells. The experiment with additional DMAE (3.6 μM) showed a V_{max} of 315,432±18,627 cpm/30 min/10⁶ cells and a K_m of 4.67±0.47 MBq (means±SD).

Incubation with increasing levels of DMAE The incubation experiments with added DMAE and [¹⁸F]FCho ≤ K_m showed a significantly lower uptake with respect to the reference starting from 0.5 μM DMAE ($p<0.05$; Fig. 3a, b). With [¹⁸F]FCho > K_m a corresponding decreasing trend in [¹⁸F]FCho uptake in relation to the amount DMAE added was observed even though a significant difference was seen only at 3.6 μM DMAE (Fig. 2c). Since it can be concluded that residual DMAE compromises [¹⁸F]FCho uptake in tumour cells, experiments with clinically relevant DMAE levels were designed in which the effects on [¹⁸F]FCho incorporation in various organs of a xenograft tumour model were evaluated.

Biodistribution in a xenograft athymic Nu/Nu mouse model

Increasing DMAE levels significantly reduced [¹⁸F]FCho incorporation into brain at all time points and lungs at

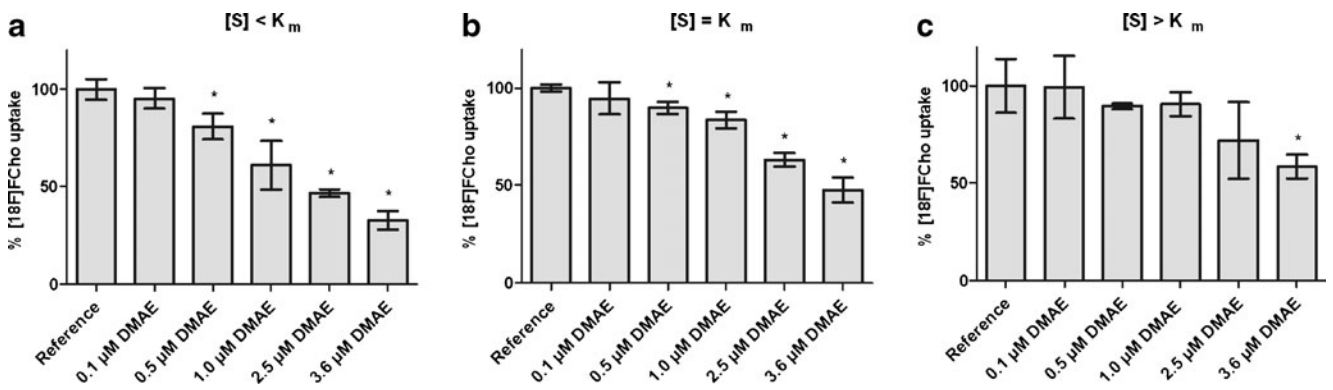


Fig. 3 Incubation of [¹⁸F]FCho with increasing levels of DMAE (0, 0.1, 0.5, 1.0, 2.5 and 3.6 μM). Data are presented as percent [¹⁸F]FCho uptake in relation to the reference, which was obtained by incubation of HLB/WCX-purified [¹⁸F]FCho containing 3 ppm

DMAE. **a** 0.09 MBq [¹⁸F]FCho; **b** 2.22 MBq [¹⁸F]FCho; **c** 5.18 MBq [¹⁸F]FCho. Values are means±SD. **p*<0.05 vs. the reference, unilateral paired *t*-test

5 min after injection (*p*<0.05, Mann-Whitney test; Fig. 4a and Table 2). Analogously, a significant reduced tumour uptake from 2.575 to 0.998%ID/g was seen at 20 min after injection, the time point of highest tracer uptake (Fig. 4b). The kidneys and heart demonstrated reduced [¹⁸F]FCho uptake in the presence of 0.05 μM DMAE compared with 100 μM DMAE. Heart uptake was significantly different at all time points, whereas the kidneys showed a significant difference only at 60 min after injection. The other organs appeared to be insensitive to the level of residual DMAE, since no significant differences were observed (Table 2).

Discussion

Accell CM cartridges are commonly used for solid-phase purification of [¹⁸F]- and [¹¹C]- labelled choline used for

tumour visualization in patients. Kryza et al. were the first to report high remaining levels of DMAE (up to 375 ppm for a 3-ml solution) using the standard tC18/ACM purification method [10]. Contamination with DMAE typically arises from conditioning the silica-based cation exchange cartridge with acid (HCl) and water, resulting in the protonation of DMAE and interaction with the cation exchange cartridge. Even subsequent washing with ethanol failed to reduce DMAE levels significantly (Table 1). The use of a polymer-based Oasis HLB/WCX cartridge and subsequent washing with 6% NH₄OH (pH 12), as evaluated in the current work, was found to be more effective in reducing residual DMAE levels down to 3 ppm. Besides obtaining higher purity, the optimized synthesis allowed the effect of residual DMAE on [¹⁸F]FCho incorporation to be explored (by DMAE spiking) in various organs (in vivo) as well as tumour uptake (in vitro and in vivo).

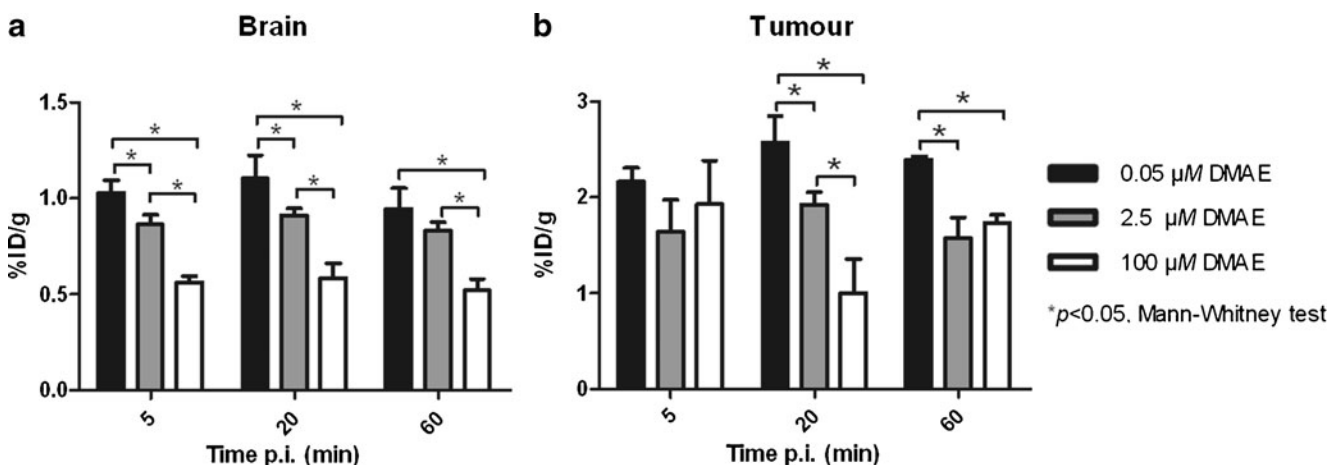


Fig. 4 Uptake of [¹⁸F]FCho in (a) the brain and (b) tumour of F98 glioma tumour-bearing nude Nu/Nu mice injected with [¹⁸F]FCho spiked with additional DMAE that resulted in DMAE plasma levels of

0.05 μM (black bars), 2.5 μM (grey bars) and 100.19 μM (white bars). Data are presented as means±SD. **p*<0.05, Mann-Whitney test

Table 2 Uptake of [^{18}F]FCho in various tissues of F98 glioma tumour-bearing athymic Nu/Nu mice injected with [^{18}F]FCho spiked with additional DMAE that resulted in final DMAE plasma levels of 0.05, 2.5 and 100.19 μM . Mice were killed 5, 20, and 60 min after injection. Values are mean %ID/g tissue \pm SD ($n=4$)

Tissue	Minutes after injection	DMAE plasma concentration (μM)		
		0.05	2.5	100.19
Blood	5	0.68 \pm 0.22	0.56 \pm 0.11	0.64 \pm 0.11
	20	0.79 \pm 0.19	0.67 \pm 0.04	0.62 \pm 0.04
	60	0.58 \pm 0.11	0.57 \pm 0.11	0.42 \pm 0.04*
Heart	5	16.5 \pm 1.52	15.0 \pm 0.59	10.8 \pm 0.65*
	20	15.3 \pm 2.14	11.6 \pm 1.55	9.93 \pm 1.67*
	60	14.3 \pm 1.79	11.6 \pm 2.63	8.78 \pm 1.67*
Stomach	5	5.97 \pm 0.74	5.77 \pm 0.77	5.02 \pm 0.44
	20	6.59 \pm 1.34	5.99 \pm 0.34	5.30 \pm 0.69
	60	6.85 \pm 0.83	6.51 \pm 0.90	5.16 \pm 0.69
Spleen	5	5.99 \pm 1.08	6.18 \pm 0.68	5.37 \pm 1.22
	20	4.51 \pm 0.43	4.04 \pm 0.82	4.58 \pm 0.67
	60	5.04 \pm 2.14	5.75 \pm 1.10	5.86 \pm 0.67
Large intestine	5	3.14 \pm 0.38	3.44 \pm 0.72	1.82 \pm 1.07
	20	3.21 \pm 0.75	1.60 \pm 1.10	1.66 \pm 0.40*
	60	2.34 \pm 1.92	3.08 \pm 0.21	2.80 \pm 0.40
Small intestine	5	8.54 \pm 0.76	4.32 \pm 4.13	5.96 \pm 0.70*
	20	6.22 \pm 2.10	4.35 \pm 1.67	6.37 \pm 0.27
	60	6.20 \pm 2.24	6.28 \pm 1.30	6.66 \pm 0.27
Pancreas	5	9.10 \pm 2.02	8.26 \pm 2.46	7.14 \pm 0.78
	20	8.48 \pm 1.51	6.45 \pm 1.42	7.19 \pm 2.02
	60	8.75 \pm 2.39	8.16 \pm 2.10	6.86 \pm 2.02
Liver	5	8.58 \pm 1.49	8.54 \pm 1.22	9.93 \pm 1.82
	20	10.4 \pm 3.39	7.94 \pm 1.50	10.5 \pm 0.56
	60	8.80 \pm 2.59	9.35 \pm 2.57	10.1 \pm 0.56
Kidney	5	33.9 \pm 4.35	20.5 \pm 23.6	35.7 \pm 11.6
	20	39.9 \pm 7.19	31.2 \pm 7.90	34.0 \pm 1.31
	60	51.2 \pm 3.39	40.7 \pm 7.53	39.6 \pm 1.31*
Lung	5	23.3 \pm 1.40	18.6 \pm 3.00*	11.8 \pm 1.43*†
	20	19.4 \pm 6.64	18.1 \pm 5.07	14.9 \pm 1.20
	60	21.8 \pm 7.00	13.5 \pm 10.3	14.2 \pm 1.20
Brain	5	1.02 \pm 0.07	0.86 \pm 0.05*	0.56 \pm 0.03*†
	20	1.10 \pm 0.12	0.91 \pm 0.03*	0.39 \pm 0.06*†
	60	0.94 \pm 0.11	0.83 \pm 0.05	0.52 \pm 0.06*†
Tumour	5	2.16 \pm 0.26	1.64 \pm 0.67	1.50 \pm 1.07
	20	2.58 \pm 0.54	1.92 \pm 0.23*	1.00 \pm 0.17*†
	60	2.39 \pm 0.07	1.57 \pm 0.38*	1.73 \pm 0.17*

* $p < 0.05$ from 0.05 μM DMAE,
† $p < 0.05$ from 2.5 μM DMAE,
Mann-Whitney test.

With respect to the use of [^{18}F]FCho in patients, feasible plasma levels of DMAE were estimated from the respective contamination remaining after the various purification methods (Table 3). The lower bound of the ranges was ascertained by the amount of radioactivity needed for a [^{18}F]FCho scan (i.e. 259 MBq/70 kg) [21]. The upper bound was defined by the maximum volume that can be administered, according to the quality control of residual solvents [17]. These specified limits led, for our HLB/WCX purification, to the range 0.005–0.068 μM DMAE in patients. Analogously, tC18/ACM purification results in

0.225–3.208 μM DMAE for the light cartridge and 0.632–9.024 μM DMAE for the regular cartridge. These ranges were applied in in vitro and in vivo experiments to evaluate the effect of clinically relevant DMAE levels on [^{18}F]FCho incorporation.

In vitro experiments in F98 glioma cells showed that [^{18}F]FCho uptake, according to the Michaelis-Menten kinetics, is saturable and therefore must be carrier-mediated. The most important specific binding sites of this choline transporter, as determined by structure–activity relationship studies, are (1) an anionic binding site for the

Table 3 DMAE plasma levels obtained by different purification methods. Data were determined for patients weighing 70 kg (with 5 l of blood) and a synthesis yield of 3.7 GBq/10 ml physiological solution. The lower bound was ascertained by the amount needed for a[¹⁸F]FCho scan (259 MBq). The upper bound was obtained by the maximum volume that can be administered to patients according to European Pharmacopoeia guidelines for fluorodeoxyglucose [¹⁸F] injection [17]

Purification method	DMAE (ppm)	DMAE injected (μg)		DMAE plasma concentration (μM)	
		Lower bound	Upper bound	Lower bound	Upper bound
HLB/WCX	3.04	2.13	30.40	0.005	0.068
tC18/ACM light	143.0	100.1	1430	0.225	3.208
tC18/ACM regular	402.2	281.5	4022	0.632	9.024

quaternary ammonium, (2) a hydroxyl binding site, and (3) a binding site with a limited distance between the quaternary nitrogen and hydroxyl group of approximately 3.3 Å [22]. Considering its structure, DMAE, which is protonated at physiological pH, is a possible substrate for the choline transporter [11]. Moreover, the saturation experiment (Fig. 2) showed an altered Michaelis-Menten constant after DMAE addition, while the V_{\max} remained constant. It can thus be concluded that DMAE acts as a competitive inhibitor for [¹⁸F]FCho transport across the cell membrane in the tumour cells. To illustrate this inhibitory effect, generally a concentration of [¹⁸F]FCho equal to the Michaelis-Menten constant (K_m) is used. But given the fact that radiopharmaceuticals in vivo are assumed to be within the linear uptake region of the tumour and the fact that the determined K_m value is excluded from the linear region of the saturation curve, the in vitro inhibition experiments were performed at a concentration below, equal to and higher than the Michaelis-Menten constant (2.22 MBq). Under each of these conditions, the inhibitory effect of DMAE on [¹⁸F]FCho uptake by tumour cells was demonstrated. At [¹⁸F]FCho levels $\leq K_m$, the most relevant conditions for the in vivo situation, tracer uptake was reduced starting from 0.5 μM DMAE added ($p < 0.05$). This concentration is, according to the ranges given above (Table 3), easily generated in patients injected with the radiotracer prepared by the traditional tC18/ACM purification method. In contrast, DMAE concentrations found in [¹⁸F]FCho prepared with our newly developed WCX purification method (with resulting patient plasma DMAE levels ≤ 0.07 μM) failed in vitro to induce a significant reduction in [¹⁸F]FCho uptake by F98 glioma cells. We can therefore conclude that DMAE exhibits a dose-dependent inhibitory effect on [¹⁸F]FCho transport across the cell membrane due to competition. Since it influences the subsequent incorporation of [¹⁸F]FCho in tumour cells by choline kinase tumour visualization might be compromised.

Concurrently, a biodistribution study was performed to evaluate the inhibitory effect of residual DMAE on [¹⁸F]

FCho uptake by various organs. It was found that administration of increasing amounts of DMAE (up to 100 μM) induced a significant reduction in [¹⁸F]FCho uptake in brain, lungs, heart, and tumour, but no significant differences were observed in other tissues. These results can be explained by the expression of ubiquitously distributed choline transporters, of which three different types have been characterized: (1) high-affinity choline transporters (CHT), (2) intermediate-affinity choline transporter-like proteins (CLT), and (3) low-affinity but polyspecific organic cation transporters (OCT) [23]. Molecular characterization of these transporters is usually carried out using RT-PCR and functional characterization by evaluating the inhibitory effects of other cationic drugs including hemicholinium-3 (HC-3), tetraethylammonium chloride (TEA), acetylcholine, guanidine, and others, on the choline transporter [22]. Our biodistribution data showed that residual DMAE had an inhibitory effect similar to that of HC-3 on the various choline transporters. DMAE levels (2.5 μM) led to a reduced [¹⁸F]FCho uptake, with respect to 0.05 μM DMAE, in particular lung, brain, and tumour tissues [12–14]. These tissues mainly express CHT and CLT which are particularly sensitive to HC-3 inhibition ($K_{i \text{ HC-3}} = 0.0001\text{--}0.1$ μM) and show an easily saturated choline uptake ($K_{m \text{ choline}} < 10$ μM) [22, 24, 25]. Only relatively high DMAE levels (100 μM) led to reduced [¹⁸F]FCho uptake, with respect to 0.05 μM DMAE, in the heart and kidneys, whereas no reduction could be induced in the liver. OCTs, which are less sensitive to HC-3 inhibition ($K_{i \text{ HC-3}} = 100$ μM) and show a poorly saturated choline transport ($K_{m \text{ choline}} = 30\text{--}100$ μM), mainly prevail in these tissues [22, 23].

OCTs include six different subtypes, with different substrate and inhibitor specificities [26]. This provides an explanation for the reduced [¹⁸F]FCho uptake in specific organs (heart and kidneys) and the absence of reduced [¹⁸F]FCho uptake in other tissues. DMAE plasma levels of 100 μM are only generated in laboratory animals, so these observations are not relevant for patients. Clinically relevant residual DMAE levels (2.5 μM) surely give proof

of an inhibitory effect on [^{18}F]FCho uptake in tissues expressing CHT and CLT, among which are brain, lungs and tumour. Even DMAE levels ($2.5\ \mu\text{M}$) lower than the plasma levels of choline during fasting ($7\text{--}10\ \mu\text{M}$) [27] compete with [^{18}F]FCho tumour uptake. This emphasizes the importance of DMAE reduction and monitoring, secondary to fasting, during [^{18}F]FCho experiments in patients and particularly in laboratory animals. Due to the small blood volume and higher amounts of injected radioactivity, experimental animals easily generate higher DMAE plasma levels and are as a consequence more sensitive to inhibition of [^{18}F]FCho uptake. This fact is crucial when PET scans are used as a diagnostic tool for tumour visualization or for therapeutic response monitoring in small laboratory animals and patients. Indeed, reduced [^{18}F]FCho uptake due to residual DMAE, which acts as a competitive inhibitor of [^{18}F]FCho, can lead to poor tumour visualization and misjudgement in therapeutic monitoring. It is thus important to reduce and determine DMAE levels in the final product by implementing a good purification method and adequate quality control.

In addition to current findings on perturbed [^{18}F]FCho uptake in the presence of residual DMAE, it is worth noting that such contamination possibly occurs in [^{11}C]choline solutions as well. Indeed, in both synthetic procedures DMAE is the common precursor while ACM cartridges are used for purification (Fig. 1). Since residual DMAE is a competitive inhibitor of choline transporters, [^{11}C]choline uptake could thus be altered accordingly. In this respect, Rosen et al. have already demonstrated that uptake of [^{11}C]choline in rat brain is reduced because of the presence of residual DMAE [28].

Conclusion

Our improved purification method, which is easy to implement, resulted in a favourable DMAE reduction (from 145 to 3 ppm) without loss of [^{18}F]FCho yield during radiosynthesis. The optimized synthetic method (HLB/WCX) led to superior [^{18}F]FCho uptake in tumour cells and tumour tissues than the tC18/ACM method. In the future DMAE cut-off levels that guarantee a maximum [^{18}F]FCho uptake and optimal molecular imaging performance need to be determined.

Conflicts of interest None.

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