Video Article

Investigations on the Ga(III) Complex of EOB-DTPA and Its ⁶⁸Ga Radiolabeled Analogue

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Abstract

We demonstrate a method for the isolation of EOB-DTPA (3,6,9-triaza-3,6,9-tris(carboxymethyl)-4-(ethoxybenzyl)-undecanedioic acid) from its Gd(III) complex and protocols for the preparation of its novel non-radioactive, i.e., natural Ga(III) as well as radioactive ⁶⁸Ga complex. The ligand as well as the Ga(III) complex were characterized by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry and elemental analysis. ⁶⁸Ga was obtained by a standard elution method from a ⁶⁸Ge/⁶⁸Ga generator. Experiments to evaluate the ⁶⁸Ga-labeling efficiency of EOB-DTPA at pH 3.8–4.0 were performed. Established analysis techniques radio TLC (thin layer chromatography) and radio HPLC (high performance liquid chromatography) were used to determine the radiochemical purity of the tracer. As a first investigation of the ⁶⁸Ga tracers' lipophilicity the *n*-octanol/water distribution coefficient of ⁶⁸Ga species present in a pH 7.4 solution was determined by an extraction method. *In vitro* stability measurements of the tracer in various media at physiological pH were performed, revealing different rates of decomposition.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54334/>

Introduction

Gadoxetic acid, a common name for the Gd(III) complex of the ligand EOB-DTPA¹, is a frequently used contrast agent in hepatobiliary magnetic resonance imaging (MRI).^{2,3} Due to its specific uptake by liver hepatocytes and high percentage of hepatobiliary excretion it enables the localization of focal lesions and hepatic tumors.^{2,5} However, certain limitations of the MRI technique (e.g., toxicity of the contrast agents, limited applicability in patients with claustrophobia or metal implants) call for an alternative diagnostic tool.

Positron emission tomography (PET) is a molecular imaging method, wherein a small amount of a radioactive substance (tracer) is administered, upon which its distribution in the body is recorded by a PET scanner.⁶ PET is a dynamic method that allows for high spatial and temporal resolution of images as well as quantification of the results, without having to deal with the side-effects of MRI contrast agents. The informative value of the obtained metabolic information can be further increased by combination with anatomical data received from additional imaging methods, as most commonly achieved by hybrid imaging with computed tomography (CT) in PET/CT scanners.

The chemical structure of a tracer suitable for PET must include a radioactive isotope serving as positron emitter. Positrons have a short life-span since they almost immediately annihilate with electrons of the atom shells of surrounding tissue. By annihilation two 511 keV gamma photons with opposite direction of movement are emitted, which are recorded by the PET scanner.^{7,8} To form a tracer, PET nuclides may be bound covalently to a molecule, as is the case in 2-deoxy-2-[¹⁸F]fluoroglucose (FDG), the most extensively used PET tracer.⁷ However, a nuclide may also form coordinative bonds to one or several ligands (e.g., [⁶⁸Ga]-DOTATOC^{9,10}) or be applied as dissolved inorganic salts (e.g., [¹⁸F] sodium fluoride¹¹). Altogether, the structure of the tracer is crucial as it determines its biodistribution, metabolism and excretion behavior.

A suitable PET nuclide should combine favorable characteristics like convenient positron energy and availability as well as a half-life adequate for the intended investigation. The ⁶⁸Ga nuclide has become an essential force in the field of PET over the last two decades.^{12,13} This is mainly due to its availability through a generator system, which allows on-site labeling independently from the vicinity of a cyclotron. In a generator, the mother nuclide ⁶⁸Ge is absorbed on a column from which the daughter nuclide ⁶⁸Ga is eluted and subsequently labeled to a suitable chelator.^{6,14} Since the ⁶⁸Ga nuclide exists as a trivalent cation just like Gd(III)^{10,13}, chelating EOB-DTPA with ⁶⁸Ga instead would yield a complex with the same overall negative charge as gadoxetic acid. Accordingly, that ⁶⁸Ga tracer might combine a similar characteristic liver specificity with the suitability for PET imaging. Although gadoxetic acid is purchased and administered as disodium salt, in the following context we will refer to it as Gd[EOB-DTPA] and to the non-radioactive Ga(III) complex as Ga[EOB-DTPA], or ⁶⁸Ga[EOB-DTPA] in case of the radiolabeled component for the sake of convenience.

To evaluate their applicability as tracers for PET, radioactive metal complexes need to be examined extensively in *in vitro*, *in vivo* or *ex vivo* experiments first. To determine the suitability for a respective medical problem, various tracer characteristics like biodistribution behavior and clearance profile, stability, organ specificity and cell or tissue uptake need to be investigated. Due to their non-invasive character, *in vitro* determinations are often performed prior to *in vivo* experiments. It is generally acknowledged that DTPA and its derivatives are of limited suitability as chelators for ^{68}Ga due to these complexes lacking kinetic inertness, resulting in comparably fast decomposition when administered *in vivo*.¹⁴⁻²⁰ This is primarily caused by *apo*-transferrin acting as a competitor for ^{68}Ga in plasma. Nevertheless, we investigated this new tracer concerning its possible application in hepatobiliary imaging, wherein diagnostic information may be provided within minutes post-injection^{3,4,21-23}, thereby not necessarily requiring long-term tracer stability. For this purpose we isolated EOB-DTPA from gadoxetic acid and initially performed the complexation with natural Ga(III), which exists as mixture of two stable isotopes, ^{69}Ga and ^{71}Ga . The complex thus obtained served as non-radioactive standard for the following chelation of ^{68}Ga . We used established methods and simultaneously evaluated their suitability for determining the ^{68}Ga -labeling efficiency of EOB-DTPA and to investigate the lipophilicity of the new ^{68}Ga tracer and its stability in different media.

Protocol

1. Preparation of EOB-DTPA and Ga[EOB-DTPA]

Caution: Please consult all relevant material safety data sheets (MSDS) of the used organic solvents, acids and alkalines before use. Perform all steps in a fume hood and use personal protective equipment (safety glasses, gloves, lab coat).

1. Isolation of EOB-DTPA from gadoxetic acid

- Put 3 ml of 0.25 M gadoxetic acid injectable solution into a flask. Add 500 mg (5.6 mmol) of oxalic acid to the stirred solution.
- After stirring for 1 hr, filter the suspension through a frit using reduced pressure. Wash the residue three times with 3 ml of water, respectively.
- Combine the aqueous filtrates and equip the solution with a pH electrode. Add 12 M hydrochloric acid to the filtrate until the pH is about -0.1.
- Remove the solvent *in vacuo* to yield a colorless residue. Store under inert gas.
- Wash the residue thoroughly (at least three times) with ethyl acetate to remove the excess of oxalic acid. Dry the residue *in vacuo*.
- Redissolve the residue in 2 ml of water at room temperature and then cool the solution in an ice bath. Without removing the ice bath, add 0.5 M aqueous sodium hydroxide solution dropwise until the formation of a colorless gluey solid is observed.
- Remove the water by decantation. Wash the solid two more times with 1 ml of cold water. Dry the solid *in vacuo* to yield the first product fraction.
- Isolate a second product fraction from the combined fractions of decanted water *via* column chromatography (silica, methanol/water 4/1).²⁴ Remove the solvent *in vacuo*.
- If the thus obtained solid is not pure white, redissolve it in 1 ml of water, add 10 ml of ethanol and subsequently 10 ml of diethyl ether to precipitate the product. Filter through a frit using reduced pressure and dry *in vacuo*.
- Combine both solid fractions of EOB-DTPA and perform NMR spectroscopic,²⁵ mass spectrometric²⁶ and elemental²⁷ analyses.

2. Synthesis of Ga[EOB-DTPA]

CAUTION: Store solid Ga(III) chloride under a dry inert atmosphere, since upon contact with air, moisture or grease decomposition takes place, resulting in corrosive fumes and formation of yellow, brown or black impurities.

- Prepare a 0.11 M stock solution by dissolving 1.94 g (11.0 mmol) of Ga(III) chloride in 100 ml of water. Dilute 1 ml of 25% aqueous ammonia solution with 4 ml of water.
- Dissolve 80 mg (0.15 mmol) of EOB-DTPA in a flask in 10 ml of water. If necessary, heat the solvent to achieve complete dissolution.
- Add 1.4 ml (0.15 mmol) of the Ga(III) chloride stock solution. Equip the flask with a stirrer and pH-electrode. Add diluted aqueous ammonia solution dropwise until the pH of the solution is approximately 4.1. Stir at room temperature for 30 min.
- Remove the solvent *in vacuo*. Place the residue in a flask, equipped with a stillhead with a central and parallel side neck. Equip the central neck with a cooling finger and the side neck with a vacuum pump outlet.
- Heat the residue under reduced pressure (125 °C, 0.6 mbar). Periodically remove sublimated ammonium chloride (visible as white coating of the glass surface) from the cooling finger and still head, as well as from the upper parts of the flask with a slightly wet cloth. Continue the process until there is no visible formation of new sublimate.
- To remove final traces of ammonium chloride wash the residue three times with 0.5 ml of hot methanol, respectively. Dry the colorless residue *in vacuo*. Perform NMR spectroscopic,²⁵ mass spectrometric²⁶ and elemental²⁷ analyses.

2. General Labeling Procedure

CAUTION: All experiments including direct or indirect contact with radioactive substances must be undertaken by trained personnel only. Please use appropriate shielding equipment. Collect any radioactive waste separately and store and dispose in accordance with valid regulations.

1. Elution of the generator

Note: A 40 mCi $^{68}\text{Ge}/^{68}\text{Ga}$ generator with the mother nuclide bound as oxide on dodecyl-3,4,5-trihydroxybenzoate silica was used. Elution and purification may be performed manually or, as was the case in this procedure, as a combined automated process using a peristaltic pump and dispenser unit.

- Prepare solutions of 5.5 M, 1.0 M and 0.05 M hydrochloric acid. Prepare a solution of 5.0 M sodium chloride containing 25 μl of 5.5 M hydrochloric acid per ml. Prepare a buffer solution of pH 4.6 by combining 4.1 g sodium acetate, 1 ml HCl (30%) and 2.5 ml glacial acetic acid and diluting the mixture with water to 50 ml.
- Precondition the PS-H⁺ cartridge by flushing it slowly with 1 ml of 1.0 M hydrochloric acid and subsequently 5 ml of water.
- Elute the silica column of the generator with 4 ml 0.05 M HCl.¹² Load the ^{68}Ga eluate onto the PS-H⁺ cartridge.

4. Flush the cartridge with 5 ml of water and subsequently dry it with 5 ml of air. Elute the ^{68}Ga from the cartridge with 1 ml 5.0 M acidified sodium chloride solution.²⁸
2. **Labeling of EOB-DTPA with ^{68}Ga**
 1. Dissolve 1 mg (1.9 μmol) of EOB-DTPA in 1 ml of water. From this solution take 100 μl (0.19 μmol) and dilute them with 9.9 ml of water to prepare a 19 μM (10 $\mu\text{g/ml}$) stock solution of EOB-DTPA.
 2. Remove 50 μl (equaling 22-29 MBq) of the solution containing ^{68}Ga and put into a vial. Add 50 μl (0.5 μg) of a 19 mM stock solution of EOB-DTPA and 300 μl of buffer to raise the pH to 4.0. Shake briefly and incubate the solution at room temperature for 5 min. Remove an aliquot of 1-5 μl and put to HPLC or TLC analysis.
 3. Perform radio HPLC analysis on a reversed phase (RP) C18 column.²⁹ Use the following mobile phase: A - water/trifluoroacetic acid (99.9%/0.1%), B - acetonitrile/trifluoroacetic acid (99.9%/0.1%), gradient: 06 min 80% A $\rightarrow$ 0% A (0.5 ml/min), 610 min 0% A (0.5 ml/min).
 4. Determine the peak intensities of the radio HPLC signals as area under curve. Calculate the labeling yield as radiochemical purity (RCP) of the tracer as follows:

$$\text{RCP} = A_{\text{Ga-EOB-DTPA}} / (A_{\text{Ga}} + A_{\text{Ga-EOB-DTPA}}) \cdot 100\%$$

$$A_{\text{Ga-EOB-DTPA}}: \text{area under curve of } ^{68}\text{Ga}[\text{EOB-DTPA}]$$

$$A_{\text{Ga}}: \text{area under curve of free } ^{68}\text{Ga}$$

3. Labeling Efficiency

1. Perform labeling procedures as described in section 2. Use a consistent range of starting activity of ^{68}Ga eluate, e.g., 22-29 MBq (40-140 μl , depending on the freshness of the eluate).
2. Add the required amount of buffer solution to adjust the pH to 3.8-4.0 (40-190 μl , depending on the volume of ^{68}Ga eluate). Add the required amount of ligand stock solution (10-70 μl of a 19 mM solution).
3. Add the required amounts of water to adjust the overall volume of each labeling probe to 1.75 ml. Mix thoroughly and let the sample stand for 5 min at room temperature. Perform HPLC analysis as described in section 2 to determine the labeling yield.
4. Perform labeling procedures with amounts of ligand between 0.1 μg and 0.7 μg in steps of 0.1 μg . Perform experiments in triplicates for each ligand concentration. Calculate the mean yield and standard deviation.

4. In Vitro Stability

1. **General procedure and preparations**
 1. Dissolve a tablet of phosphate buffered saline (PBS) in 200 ml of deionized water to prepare a PBS stock solution with a phosphate concentration of 10 mM.
 2. Perform labeling of 22-29 MBq ^{68}Ga with 0.5 μl of EOB-DTPA stock solution, as described in section 2. Depending on the volume of the ^{68}Ga eluate, adjust the amount of buffer, as described in section 3. Withdraw samples of labeling solution containing 6-12 MBq of tracer to perform stability measurements.
 3. Perform radio TLC analysis on 80 mm silica gel coated aluminum plates using 0.1 M aqueous sodium citrate as eluent and analyze the plates with a TLC radioactivity scanner.³⁰ Determine the intensities of the TLC signals as area under curve. Calculate the RCP of the tracer as follows:

$$\text{RCP} = A_{\text{Ga-EOB-DTPA}} / (A_{\text{Ga-free}} + A_{\text{Ga-EOB-DTPA}} + A_{\text{Ga-colloidal}}) \cdot 100\%$$

$$A_{\text{Ga-EOB-DTPA}}: \text{area under curve of } ^{68}\text{Ga}[\text{EOB-DTPA}]$$

$$A_{\text{Ga-free}}: \text{area under curve of free } ^{68}\text{Ga}$$

$$A_{\text{Ga-colloidal}}: \text{area under curve of colloidal } ^{68}\text{Ga}$$
 4. Calculate $\text{RCP}_t / \text{RCP}_0$ for every time point. Plot the thus standardized RCP vs. time difference since the starting point $t = 0$ min.

$$\text{RCP}_t = \text{RCP of } ^{68}\text{Ga}[\text{EOB-DTPA}] \text{ at time point } t$$

$$\text{RCP}_0 = \text{RCP of } ^{68}\text{Ga}[\text{EOB-DTPA}] \text{ at } t = 0 \text{ min.}$$
2. **Stability in phosphate buffered saline (A)**
 1. To 65 μl of labeling solution add 150 μl of PBS stock solution and 60 μl of sodium hydroxide solution (0.1 M) to raise the pH to 7.4. Mix thoroughly.
 2. Remove an aliquot of 1-5 μl to perform TLC analysis ('starting point'). Immediately store the solution in an incubator at 37 $^{\circ}\text{C}$ and remove aliquots to perform TLC analysis at representative time points over 3 hr.
3. **Stability towards excess of apo-transferrin in PBS (B)**
 1. To 120 μl of labeling solution add 50 μl of PBS stock solution and 430 μl of sodium hydroxide solution (0.1 M) to raise the pH to 7.4. Add 40 μl of a solution of apo-transferrin (25 mg/ml). Mix thoroughly.
 2. Remove an aliquot of 1-5 μl to perform TLC analysis ('starting point'). Immediately store the solution in an incubator at 37 $^{\circ}\text{C}$ and remove aliquots to perform TLC analysis at representative time points over 3 hr.
4. **Stability in human serum (C)**
 1. To 500 μl of human serum add 25 μl of labeling solution and 45 μl of sodium hydroxide solution (0.1 M) to raise the pH to 7.4. Mix thoroughly.
 2. Remove an aliquot of 1-5 μl to perform TLC analysis ('starting point'). Immediately store the solution in an incubator at 37 $^{\circ}\text{C}$ and remove aliquots to perform TLC analysis at representative time points over 3 hr.

5. Determination of Distribution Coefficient LogD

1. Perform labeling procedures as described in section 2. To 50 μ l of labeling solution add 20 μ l of PBS stock solution and 170 μ l of sodium hydroxide solution (0.1 M) to raise the pH to 7.4.
2. Withdraw 200 μ l from that solution and put it into a plastic V-vial. Add 200 μ l of *n*-octanol. Close the vial and vortex for 2 min. Then centrifuge the sample at 1,600 x g for 5 min.
3. Remove triplicates of 40 μ l from the *n*-octanol phase and the aqueous phase each and put them in separate V-vials. Be careful not to mix up the layers.
4. Measure the activity of each sample in a gamma well counter for 30 sec. For each sample immediately repeat the measurement twice and thereof calculate the mean activity $\bar{A}_t$ in counts per minute (cpm). List the thus gained $\bar{A}_{t,W1}$, $\bar{A}_{t,W2}$ and $\bar{A}_{t,W3}$ (activities in aqueous samples) and $\bar{A}_{t,O1}$, $\bar{A}_{t,O2}$, $\bar{A}_{t,O3}$ (activities in *n*-octanol) along with the respective time point *t* of their determination.
5. Define the time point of the measurement of the last sample as t_0 . Determine and list Δt in min by calculating $\Delta t = t - t_0$. Perform decay correction of $\bar{A}_t$, using the following formula:

$$\bar{A}_0 = \bar{A}_t \cdot 2^{(\Delta t/68 \text{ min})}$$
6. Calculate $\bar{A}_{0,W}$ as the mean of $\bar{A}_{0,W1}$, $\bar{A}_{0,W2}$ and $\bar{A}_{0,W3}$ as well as $\bar{A}_{0,O}$ as the mean of $\bar{A}_{0,O1}$, $\bar{A}_{0,O2}$ and $\bar{A}_{0,O3}$. Calculate logD using the following formula:

$$\log D = \log[(\bar{A}_{0,O} \cdot 40 \mu\text{g})/(\bar{A}_{0,W} \cdot 33 \mu\text{g})]$$
7. Perform the entire experiment in triplicates and calculate the mean logD along with its standard deviation.

Representative Results

The ligand EOB-DTPA and the non-radioactive Ga(III) complex were analyzed via ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectroscopy, mass spectrometry and elemental analysis. The results listed in **Table 1** and depicted in **Figures 1-6** verify the purity of the substances.

Elution of the $^{68}\text{Ge}/^{68}\text{Ga}$ generator yielded solutions of 400-600 MBq ^{68}Ga . The described labeling procedure results in the formation of the desired tracer $^{68}\text{Ga}[\text{EOB-DTPA}]$, indicated as radio HPLC peak exhibiting a retention time of 2.8 min (**Figure 7**). Comparison with the retention time of the $\text{Ga}[\text{EOB-DTPA}]$ standard in the UV-vis detector at 220 nm (2.7 min, **Figure 8**) confirms successful labeling. Uncoordinated ^{68}Ga is detected as radio peak at 2.1 min (**Figure 7**). The ^{68}Ga -labeling efficiency of EO-BDTPA was investigated by determining the labeling yield as a function of the ligand concentration via HPLC (**Figure 9**). The yields were determined in triplicate and standard deviations were calculated.

Depending on the pH and the concentration of anions present in solution, uncoordinated or non-labeled ^{68}Ga may exist in various species, e.g., gallates or insoluble hydroxide.³¹ The generalized term "free ^{68}Ga "³² is used for all non-labeled species in solution except the hydroxide, which is generally referred to as "colloidal ^{68}Ga ". Under the described analysis conditions, free ^{68}Ga moves with the solvent front ($R_f = 1.0$) on a TLC plate. Colloidal ^{68}Ga cannot be detected via HPLC, while on a TLC plate it appears as activity at the origin ($R_f = 0$). A representative chromatogram of a TLC plate analyzed with a TLC radioactivity scanner is shown in **Figure 10**. The tracer exhibits different retention behavior, depending on whether a sample of labeling solution (pH 3.8-4.0, $R_f = 0.3$) or a sample of physiological pH ($R_f = 0.5$) was analyzed.

To investigate the stability of the tracer, freshly labeled $^{68}\text{Ga}[\text{EOB-DTPA}]$ was added to samples of physiological pH, containing diluted PBS (phosphate concentration 5.5 mM, **A**), excess of apo-transferrin (1.6 mg/ml in diluted PBS with a phosphate concentration of 0.8 mM, **B**) and human serum (**C**), respectively. Over time, the radiochemical purity (RCP_t) of tracer in the samples was determined via TLC. The percentage of intact tracer was calculated as the ratio of RCP_t at the respective time points and RCP₀ at the starting point (**Table 2**). This was necessary due to the labeling solutions containing tracer of differing RCP₀ (93-96%). The thus standardized percentage of intact tracer is depicted as a function of time in **Figure 11**.

For the determination of logD aqueous samples of tracer in a diluted PBS solution were prepared. The samples were mixed with *n*-octanol, centrifuged and subsequently aliquots were removed to determine the activity concentration in both phases. Activity values and subsequent calculation of logD thereof are depicted in **Table 3**. The mean logD value is 3.54 ± 0.08 .

Figure 1. ^1H -NMR spectrum of EOB-DTPA. The spectrum was recorded in D_2O at 400.1 MHz. [Please click here to view a larger version of this figure.](#)

Figure 2. $^{13}\text{C}\{^1\text{H}\}$ -NMR spectrum of EOB-DTPA. The spectrum was recorded in D_2O at 100.6 MHz. [Please click here to view a larger version of this figure.](#)

Figure 3. MS of EOB-DTPA (electrospray ionization (ESI), methanol, negative mode). [Please click here to view a larger version of this figure.](#)

Figure 4. ¹H-NMR spectrum of Ga[EOB-DTPA]. The spectrum was recorded in D₂O at 400.1 MHz. [Please click here to view a larger version of this figure.](#)

Figure 5. $^{13}\text{C}\{^1\text{H}\}$ -NMR spectrum of Ga[EOB-DTPA]. The spectrum was recorded in D_2O at 100.6 MHz. [Please click here to view a larger version of this figure.](#)

Figure 6. MS of Ga[EOB-DTPA] (ESI, methanol, negative mode), along with a detailed depiction of the isotope pattern of the molecular peak. [Please click here to view a larger version of this figure.](#)

Figure 7. Representative HPLC chromatogram of a sample of ^{68}Ga [EOB-DTPA] containing in parts uncoordinated ^{68}Ga , as recorded by the radioactivity detector. Uncoordinated ^{68}Ga exhibits a retention time of 2.1 min, while the tracer is detected at 2.8 min. [Please click here to view a larger version of this figure.](#)

Figure 8. Representative HPLC chromatogram of the standard substance Ga[EOB-DTPA], as detected in the UV-vis channel at 220 nm. The retention time of the cold standard is 2.7 min. [Please click here to view a larger version of this figure.](#)

Figure 9. Depiction of ^{68}Ga -labeling efficiency of EOB-DTPA. The labeling yield as determined *via* HPLC is plotted as a function of the concentration of EOB-DTPA (22-29 MBq starting activity, pH 3.8-4.0, 5 min, RT). The standard deviation is depicted by error bars. [Please click here to view a larger version of this figure.](#)

Figure 10. Representative TLC chromatogram revealing different ^{68}Ga species. A sample of ^{68}Ga [EOB-DTPA] in diluted PBS (phosphate concentration 5.5 mM, pH = 7.4) was analyzed after 110 minutes of incubation. Exemplary distribution of colloidal ^{68}Ga ($R_f = 0$), ^{68}Ga [EOB-DTPA] ($R_f = 0.5$) and free ^{68}Ga ($R_f = 1.0$) on a 70 mm TLC plate as detected by a TLC radioactivity scanner is presented. Counts are decay corrected. [Please click here to view a larger version of this figure.](#)

Figure 11. Stability determinations of ^{68}Ga [EOB-DTPA] in different media. The decay corrected, standardized percentage of intact tracer as determined via TLC, is depicted as a function of time. [Please click here to view a larger version of this figure.](#)

analysis	unity	result of analysis					
		EOB-DTPA			Ga[EOB-DTPA]		
¹ H NMR (D ₂ O, 400.1 MHz)	δ	7.26 (d, ³ J _{H,H} = 8.1 Hz, 2H)			7.27 (d, ³ J _{H,H} = 8.6 Hz, 2H)		
		7.00 (d, ³ J _{H,H} = 8.1 Hz, 2H)			7.02 (d, ³ J _{H,H} = 8.6 Hz, 2H)		
		4.13 (q, ³ J _{H,H} = 6.9 Hz, 2H)			4.20-2.91 (m, 21H)		
		3.91-2.78 (m, 19H)			1.38 (t, ³ J _{H,H} = 7.0 Hz, 3H)		
		1.38 (t, ³ J _{H,H} = 6.9 Hz, 3H)					
¹³ C{ ¹ H} NMR (D ₂ O, 100.6 MHz)	δ	173.8, 170.5, 170.1, 157.3, 130.5, 127.8, 115.3, 64.4, 62.5, 57.3, 53.6, 53.3, 52.8, 48.6, 31.3, 13.9			174.8, 174.3, 174.0, 169.8, 157.5, 130.6, 127.9, 115.4, 64.6, 64.4, 62.0, 57.8, 57.6, 57.3, 53.4, 52.0, 50.1, 32.1, 13.9		
MS (ESI, negative mode)	m/z	calcd.			calcd.		
		527.2 [M]			595.1 (67%) [M(⁷¹ Ga)]		
					594.1 (26%) [M(⁶⁹ Ga)+1]		
					593.1 (100%) [M(⁶⁹ Ga)]		
		found			found		
		526.2 [M-H]			595.1 (19%) [M(⁶⁹ Ga)-H+1]		
					594.1 (70%) [M(⁷¹ Ga)-H]		
					593.1 (27%) [M(⁶⁹ Ga)-H+1]		
					592.1 (100%) [M(⁶⁹ Ga)-H]		
EA	%	C	H	N	C	H	N
		calcd.	calcd.	calcd.	calcd.	calcd.	calcd.
		50.64	6.47	7.70	42.92	5.68	8.17
		found	found	found	found	found	found
		50.66	6.16	7.71	42.72	5.42	7.98

Table 1. Results of NMR spectroscopic, MS and elemental analyses performed for EOB-DTPA and Ga[EOB-DTPA]. Relative MS peak intensities are given in %, assignment to peaks are given in square brackets. Elemental CHN values were calculated for C₂₃H₃₃N₃O₁₁·H₂O (EOB-DTPA) and (NH₄)_{0.75}H_{1.25}[C₂₃H₂₈GaN₃O₁₁]·2H₂O (Ga[EOB-DTPA]).

A			B			C		
t [min]	composition	RCP _t /RCP ₀	t [min]	composition	RCP _t /RCP ₀	t [min]	composition	RCP _t /RCP ₀
0	93 / 2 / 5	1.00	0	93 / 3 / 4	1.00	0	96 / 1 / 3	1.00
15	89 / 6 / 5	0.96	10	91 / 4 / 5	0.98	20	93 / 4 / 3	0.97
25	91 / 5 / 4	0.98	25	89 / 5 / 6	0.96	40	93 / 5 / 2	0.97
45	90 / 6 / 4	0.97	40	89 / 6 / 5	0.96	65	90 / 8 / 2	0.94
65	88 / 7 / 5	0.95	70	85 / 8 / 7	0.91	95	84 / 13 / 3	0.88
95	86 / 8 / 6	0.92	100	82 / 12 / 6	0.88	125	78 / 19 / 3	0.81
125	84 / 10 / 6	0.90	130	82 / 13 / 5	0.88	155	73 / 22 / 5	0.76
155	83 / 13 / 4	0.89	160	75 / 18 / 7	0.81	185	69 / 28 / 3	0.72
185	78 / 13 / 9	0.84	180	72 / 20 / 8	0.77			

Table 2. Stability determination of ⁶⁸Ga[EOB-DTPA] in different media. The RCP of ⁶⁸Ga[EOB-DTPA] in media A, B and C was determined via TLC at given time points. The composition of the samples is given as percentages in % of tracer / free ⁶⁸Ga / colloidal ⁶⁸Ga. The percentage of intact tracer is standardized as ratio of RCP_t/RCP₀. RCP₀ is the respective RCP of the tracer at t = 0 min.

	$\bar{A}_{0,x}$				
phase	1	2	3	$\bar{A}_0$	logD
W	561479	574321	574847	570216	-3.47
O	152	160	166	159	
W	683769	689959	702529	692086	-3.63
O	131	132	139	134	
W	704161	718133	715094	712463	-3.51
O	177	187	186	183	
					logD $\pm$ SD
					-3.54 $\pm$ 0.08

Table 3. Determination of logD. Decay corrected values $\bar{A}_{0,x}$ of three aliquots (x = 1, 2, 3) removed from each phase (W: aqueous, O: n-octanol) of a sample. All activities are given in cpm. LogD is calculated as described in section 5 of the protocol. The experiment was repeated twice.

Discussion

EOB-DTPA is accessible through a multi-step synthesis³³ but may just as well be isolated from available contrast agents containing gadoxetic acid. For this purpose, the central Gd(III) ion can be precipitated with an excess of oxalic acid. After removing Gd(III) oxalate and oxalic acid the ligand can be isolated by precipitation in cold water at pH 1.5. However, in order to enhance yields column chromatography of the filtrate can be performed instead or as a follow-up procedure. Either method yields the analytically pure ligand in total yields of 70% (Figures 1-3, Table 1).

We found that in order to isolate Ga[EOB-DTPA] adjusting the pH with ammonia solution is advantageous compared to the use of sodium hydroxide, since the by-product ammonium chloride may be removed from the very hydrophilic residue *via* sublimation. Under the aforementioned conditions this process takes place slowly. Since non-negligible amounts of chloride were still detectable after five days, the remaining salt was washed out with methanol. Although this work-up procedure results in partial loss of Ga[EOB-DTPA], the product was obtained in analytical purity with an overall yield of 46% (Figures 4-6, Table 1). For the isolation of both EOB-DTPA and its Ga(III) complex, the use of reversed phase chromatography should be considered as an alternative method of purification, especially since decomposition of silica gel is likely when using highly polar solvents.

The labeling process of EOB-DTPA required the use of highly pure solvents, chemicals and metal-free equipment to avoid the presence of competing metal ions, due to ⁶⁸Ga being present in nanomolar amounts (2 MBq of ⁶⁸Ga in a 1.75 ml sample equal a nuclide concentration of 0.14 nM). Labeling of EOB-DTPA to ⁶⁸Ga occurs at pH 3.8-4.0 within five minutes at room temperature. Investigations on the ⁶⁸Ga-labeling efficiency require determination of the labeling yield while keeping the reaction conditions pH, temperature and reaction time as well as starting activity of ⁶⁸Ga constant or in a justifiable range. For each data point (*i.e.*, ligand concentration) the experiment should be performed at least three times to provide a reasonable confidence level, since the concentrations of both ligand and ⁶⁸Ga are very low and the labeling yield therefore sensitive to even slight deviations of the reaction conditions. For example, as the ⁶⁸Ga eluate ages, aliquots of increasing volume need to be withdrawn to provide a constant starting activity, thereby requiring increasing volumes of buffer. Furthermore, aging of the eluate results in increasing concentrations of the decay product ⁶⁸Zn, which itself might act as a competitor for ⁶⁸Ga, thus negatively affecting labeling efficiency.^{13,34,35} Practically quantitative labeling of 22-29 MBq ⁶⁸Ga is achieved under the aforementioned conditions with amounts of EOB-DTPA $\geq 0.7 \mu\text{g}$ (Figure 9), with contents of free ⁶⁸Ga $\leq 2\%$ and about 5% of colloidal ⁶⁸Ga present in samples.

While HPLC provided superior baseline separation of free ⁶⁸Ga and ⁶⁸Ga[EOB-DTPA], it is not suited to detect colloidal ⁶⁸Ga. We therefore chose TLC to determine the RCP during stability measurements, wherein the quantification of transferrin or protein-bound ⁶⁸Ga was required. We found baseline separation acceptable for this purpose (Figure 10); however, the use of size exclusion chromatography or filtration methods^{15,36} to remove colloidal fractions, followed by HPLC analysis, might be considered as alternatives. The ⁶⁸Ga complex exhibits a stronger retention on TLC plates ($R_f = 0.3$) if the sample is withdrawn directly from labeling solution as opposed to samples at physiological pH ($R_f = 0.5$). We suggest this observation might be explained by different protonation states of the complex.

In vitro stability determinations of ⁶⁸Ga tracers are usually performed in PBS^{15,17} or alternative buffer systems mimicking the physiological pH³⁷, as well as in solutions containing apo-transferrin³⁷, which is the main competitor for ⁶⁸Ga in blood, or in human serum^{15,17}. In our experiments the addition of 0.1 M sodium hydroxide solution to PBS was required to adjust the pH of the samples to 7.4. We could not assert that the phosphate concentration influences the rate of degradation, since stability experiments in solutions of varying phosphate concentration (0.8 mM and 5.5 mM (A)) yielded non-reproducible results. However, we found that a solution B, containing apo-transferrin (1.6 mg/ml, which is within range of the normal plasma content³⁸) and 0.8 mM phosphate (human blood usually exhibits a phosphate level of 0.8–1.5 mM^{39,40}), causes decomposition at a rate comparable to that observable in human serum (C). In solutions AC, after 185 min the content of colloidal ⁶⁸Ga had increased by about 24%, while the content of free ⁶⁸Ga had increased by 11% in solution A, 17% in solution B and 27% in solution C (Table 2). The fact that ⁶⁸Ga formed by tracer decomposition is predominantly present as free ⁶⁸Ga as opposed to colloidal or protein-bound ⁶⁸Ga in B and C might be due to transferrin saturation or comparably slow transferrin binding rates.⁴¹ The observed overall low stability (Figure 11) of ⁶⁸Ga[EOB-DTPA] is comparable to tracers featuring similar DTPA derived chelators.^{15,16,18} Usually, information on the early arterial and venous perfusion phase of the liver are gained by performing MRI scans within the first 3 minutes^{4,21} after administering Gd[EOB-DTPA], while the hepatocyte presence is detected in the delayed phase 20 minutes^{3,4,23} up to several hours^{21,22} after injection. After 20 minutes in human serum 93% of ⁶⁸Ga[EOB-DTPA] remain intact. Expectedly, the signal-to-noise ratio by that time would have deteriorated due to increasing amounts of ⁶⁸Ga-transferrin, which is present in plasma and tissue expressing transferrin receptors, as well as free ⁶⁸Ga gallate.^{41,42}

For predicting a tracers tissue distribution *n*-octanol/water partition coefficients logP or distribution coefficients logD can be determined as ratio of activity concentrations in the two phases. By definition, the logD parameter does not differentiate between multiple species present in a medium, which makes it suitable for our experiments due to the possibility of different protonation states of the tracer as well as its decomposition in the aqueous phase. To determine logD by extraction the aqueous medium is usually buffered with PBS to mimic blood conditions.^{17,43-45} For aforementioned reasons we used diluted PBS, exhibiting a phosphate concentration of 0.8 mM and physiological pH. Following extraction with *n*-octanol and centrifugation, the removal of several aliquots from the same phase allows for inaccuracies caused by pipetting to be reduced. Due to the very low activity concentrations in *n*-octanol one should be careful to avoid cross-contamination with the aqueous phase and to ensure quantitative transfer into a separate vial. Distribution coefficients determined by this procedure were reproducible, and while they allow for a rough estimation of lipophilicity, a direct comparison to a logP of Gd[EOB-DTPA] is not possible. Due to the specificity of Gd[EOB-DTPA] resulting not primarily from lipophilicity but rather its hepatobiliary uptake additional experiments in living subjects or cells would be necessary to provide more extensive information on the biodistribution as well as stability *in vivo* of ⁶⁸Ga[EOB-DTPA]. Altogether, an application as imaging agent for the perfusion and early hepatobiliary phase is imaginable.

Disclosures

The authors have nothing to disclose.

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