True Tracer Radiolabeling of Gadolinium Complex of 10-(2-Hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7 triacetic Acid (HP-DO3A)

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SUMMARY

True tracer radiolabeling, purification, and analysis of a gadolinium complex of HP-DO3A (10- (2-hydroxy-propyl)- 1,4,7,10-tetraazacyclo dodecane-1,4,7-triacetic acid) was achieved by the reaction of carrier-added $^{153}GdCl_3$ and excess free ligand. Two analytical methods (ITLC and HPLC) were developed to determine the purity of radiolabeled samples.

Key Words: Gadolinium, Magnetic Resonance in Imaging, MRI, Chelates, ITLC, HPLC, Biodistribution.

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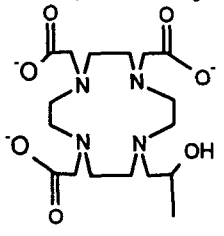
1. INTRODUCTION

Gadolinium (Gd) chelates are used clinically as contrast agents in Magnetic Resonance Imaging (MRI) [1,2]. Gd^{3+} ion hydrolyzes under physiological conditions and is poorly tolerated in animals. Consequently, it must be complexed with powerful chelating agents. The class of ligand in current use is the multidentate polyaminocarboxylates, of which the macrocyclic ligand, HP-DO3A, [10-(2-hydroxy-propyl)-1,4,7,10- tetraazacyclododecane-1,4,7 -triacetic acid, structure given below) is an example [3,4].



In vivo evaluation of the Gd chelate [5] requires radiolabeling with ^{153}Gd, a nuclide with a half-life of 241.6 days emitting low energy gamma rays (97.4, 103.2 keV) . For biological studies it is critically important that the uncomplexed Gd (free Gd) be very low, and that ^{153}Gd be a true tracer of the Gd in the injectate:

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$$\frac{[^{153}Gd]_{free}}{[^{153}Gd(HP-DO3A)]} = \frac{[Gd]_{free}}{[Gd(HP-DO3A)]}$$

If this equality does not hold, inaccurate biodistributions may result [6].

In a typical procedure the radiolabeled samples of chelates for biological studies are prepared by an exchange method. In this procedure the cold Gd(chelate) is mixed with $^{153}GdCl_3$ (in 0.1 M HCl). The reaction mixture is heated at 80°C and the pH is raised to 7. For relatively labile polyaminocarboxylates this method seems to work well. However, there are obvious concerns over the complete exchange of ^{153}Gd with the Gd in the Gd(chelate) due to the relatively sluggish exchange kinetics (half-life of hours) of Gd(chelate) complexes of the more powerful chelating aminocarboxylates[7].

In the present work we report synthesis, purification, and analysis of ^{153}Gd(HP-DO3A) by a method which yields extremely low levels of free $^{153}Gd/Gd$, and wherein the true tracer definition is fulfilled.

2. MATERIALS AND METHODS

2.1. Materials

A sample of $^{153}GdCl_3$ with a known radioconcentration and specific activity was obtained from the Oak Ridge National Laboratories, Oak Ridge, TN. The chemical purity of the sample was > 95 wt.% with several trace metal impurities, e.g. [Fe], 0.3% and [Ti], 0.2%. The radionuclidic purity (the ratio of ^{153}Gd to other radionuclides) was 99.999%. A solution of $GdCl_3$ (Research Chemicals, Phoenix, AZ) was prepared, and the sample concentration was determined by EDTA titration using Xylenol Orange as an indicator [8]. The chelating agent, HP-DO3A, was synthesized by a literature procedure [3]. The sample solutions of the ligand were prepared from the analytically pure solid sample. The concentration of the ligand sample solutions was determined by an acid-base or a back titration method.

Mobile phases for analytical and preparative HPLC were prepared from acetonitrile (Baxter), TRIZMA base (Fisher), acetic acid (EM Sciences), EDTA (Fisher), and deionized distilled water. Dilute solutions of hydrochloric acid and sodium hydroxide were used for slow adjustment of the pH. All other chemicals were reagent grade. Silica gel impregnated glass fiber sheets (ITLC-SG) were purchased from Gelman Sciences, Inc. (Ann Arbor, MI). The sheets were cut into 1 x 10 cm strips prior to use.

2.2 Instruments

A Radiometer PHM-82 pH meter, with a combination glass electrode was used for all pH measurements. An LKB-Wallace gamma counter was used for the determination of radioactivity in the sample. The window used to count ^{153}Gd radioactivity was machine channels 20-140 inclusive. This window measures the gamma radiation at 97.4, and 103.2 k eV.

The HPLC system was a two pump, either Rainin Instrument or Beckman model 110, with a Rheodyne injection valve containing a 20 or 500 μL sample loop. The HPLC system was interfaced with a Macintosh SE computer and three detectors: a Hitachi F-1050 for fluorescence, an Applied Biosystem for UV/Vis, and a Beckman 170 for radionuclides. The radionuclide detector is capable of detecting high energy beta and mid to low energy gamma radiation. Fluorescence measurements were made using an excitation wavelength of 274 nm and an emission wavelength of 312 nm. Integration of peak areas was performed either by a Shimadzu RC-3A chromatopac/plotter or by a Dynamax software. At least triplicate injections were made for all HPLC analyses.

2.3 Methods

Two methods were used to determine free Gd in gadolinium chelates [9,10]. In the first method the free Gd was precipitated as $Gd(PO_4)$ by addition of KH_2PO_4 ($K_{sp}^{Gd(PO_4)} = 10^{-22}$). Then the sample was applied to a TLC plate and developed, leaving precipitated Gd at the origin. Free Gd was also measured in the form of $Gd(EDTA)^-$, which was separated from the Gd(HP-DO3A) sample using HPLC in the second method. The mole percentage of free Gd in the chelate was determined from a calibration curve.

2.3.1. Instant Thin Layer Chromatography (ITLC). For an ITLC analysis a 10 cm TLC plate was prepared as follows: a line was drawn at 1.5 cm to mark the origin, followed by another line at 4.0 cm to be included for the counts at origin. A line at 9.0 cm was drawn as the solvent front line. To measure the recovery, precision, and accuracy, samples were prepared as follows: the pH of the sample of $GdCl_3/^{153}GdCl_3$ was first adjusted to 7, either with NaOH or some buffer such as tris acetate or acetate/acetic acid. A known concentration of KH_2PO_4 (usually 67 mM) was added to precipitate free Gd. The sample was vortexed and applied to the ITLC plate. The plates were air dried for 10-20 minutes and developed in 50% aq. methanol containing 10% ammonium acetate by wt. Some control experiments with no addition of phosphate were also carried out. After developing,

the plates were dried, cut, placed in a counting tube, and counted in a LKB gamma counter. Each tube was counted for one minute and the average was used for the calculations. Similarly, for the analysis of the ^{153}Gd(HP-DO3A) samples, phosphate was added and the sample was transferred to the plate. No reaction between the chelate and phosphate was detected.

2.3.2 HPLC Analysis. A Nucleosil C_{18} reversed phase (4.6 X 250 mm) column from Jones Chromatography was used. The column was equilibrated with degassed mobile phase containing 10 mM EDTA and 50 mM Tris acetate buffer at pH 7.4 and 2% or 4% acetonitrile. EDTA was used to scavenge free Gd in the sample. Solutions of variable amounts of Gd/^{153}Gd or preformed Gd(EDTA)$^-$/^{153}Gd(EDTA)$^-$ were used for development and validation of the method. These sample solutions were made either by diluting a radioactive $^{153}GdCl_3$ sample solution with carrier $GdCl_3$ or by reacting $GdCl_3$/$^{153}GdCl_3$ with the buffer containing Tris Acetate and EDTA. No reaction between EDTA in the mobile phase and the chelates was observed. A Partisil ODS-3, RAC (12.5 X 100 mm) column from Whatman was used for preparative HPLC.

2.4 Synthesis of Gd(HP-DO3A)

The solid sample of Gd(HP-DO3A) used as a marker was synthesized by a literature procedure [3] and was characterized by elemental and mass spectral analysis.

2.5.1 Synthesis of ^{153}Gd(HP-DO3A): Method 1. A sample of $^{153}GdCl_3$ (1.32 mL, radioactivity concentration =7.60 mCi/mL, Specific Activity = 15.78 mCi/mg Gd_2O_3) in 1.0 M HCl was mixed with 0.25 mL $GdCl_3$ (0.0885 mmoles) in a 3 dram vial. The mixture was heated for 15 min at 80°C in a heating block. An excess of solid HP-DO3A (110 mg, 0.275 moles) was added to the reaction mixture. After mixing and heating at 80°C for 45 min, the pH of the reaction mixture was adjusted to 2 with 5 N NaOH. The sample was heated for an additional 8 h, and the pH of the mixture was adjusted to 3, 4, and 6 at two h intervals. The final pH of the reaction mixture was adjusted to 6.5 and the mixture was concentrated to 1.0 mL, and then diluted with 1.0 mL of 1.0 M cold Gd(HP-DO3A). The cold Gd(HP-DO3A) was analytically pure with free Gd and free ligand, <0.002% mole % (by the HPLC detection method). A portion of the sample was purified by a semi-prep reversed phase HPLC (Column: Partisil ODS-3 RAC, mobile phase: 1% acetonitrile and 99% water, flow rate: 0.5 mL/min, Detector: Beckman 170 radionuclide

detector). The recovery of the purification was quantitative. An HPLC analysis of the product showed the absence of free Gd or the free ligand.

Method 2. The sample was prepared by a minor modification of method 1. At the end of the reaction, ^{153}Gd(HP-DO3A) was added to cold Gd(HP-DO3A). The cold Gd(HP-DO3A) was analytically pure with free Gd and free ligand <0.002 mol% by HPLC.

3. RESULTS AND DISCUSSION

3.1 Synthesis of ^{153}Gd(HP-DO3A). Radiolabeling of gadolinium complexes by ^{153}Gd tracer may be achieved by mixing the radionuclide with preformed Gd(HP-DO3A), allowing acid-assisted isotope exchange to occur. In this procedure the pH of the mixture of $^{153}GdCl_3$/Gd(HP-DO3A) is lowered to 2 by adding HCl. The mixture is heated at 80°C and the pH of the reaction mixture is raised to 7.0 very slowly. The method works for some relatively labile aminocarboxylate complexes, such as DTPA. However, due to the kinetic inertness of the macrocyclic aminocarboxylate complexes of Gd [7] complete exchange is extremely difficult to achieve. The carrier-added method of radiolabeling of new MRI contrast agents requires the free ligand. When the equilibrated mixture of labile $^{153}Gd/GdCl_3$ is reacted with the free acid form of the ligand, the ligand cannot discriminate between ^{153}Gd and Gd. The crude and HPLC purified material both maintained constant $^{153}Gd/^{153}$Gd(HP-DO3A) and Gd/Gd(HP-DO3A) ratios. The radiolabeled chelate produced by this procedure would give a true representation of the biodistribution of free and chelated gadolinium.

3.2 ITLC Method of Analysis of ^{153}Gd(HP-DO3A). To validate the procedure, attempts were made to demonstrate recovery, precision, and accuracy of the ITLC method. Several experiments with no-carrier-added and carrier-added samples were performed. For carrier added samples, most of the radioactivity was recovered at the origin. The percent free Gd was also the same whether the precipitation was carried out in the test tube or on the TLC plate, indicating good mass transfer of the precipitate. For no-carrier-added cases all of the radioactivity did not stay at the origin and that the recovery of the experiment was not quantitative. However, on addition of carrier the percentage of total counts at the origin and the recovery were both increased to at least 90%.

A major problem in the experiment with no-carrier-added samples of ^{153}Gd(HP-DO3A)/Gd(HP-DO3A) was that the mass transfer was not efficient for samples at pH 7.

This could be due to the formation of insoluble $Gd_x(OH)_y$ in the test tube, perhaps in colloidal forms, prior to phosphate precipitation. Mass transfer was particularly poor when the time between the sample preparation and ITLC analysis was not controlled. Although each sample was vortexed before aliquots were taken, particles still might have been unevenly distributed in the solution or adhering to the walls of the container. The pH adjustment was a necessary step during preparation of the ^{153}Gd radiolabeled samples. The carrier-added studies alleviated the problem of the low recovery from poor mass transfer, i.e. most experiments showed greater than 90% recovery.

Another problem for no-carrier-added samples was that as much as 15% of the total CPM (counts per min) was found in the middle section of each ITLC plate. Similar results (33-54%) were observed in the control experiments without the phosphate precipitating agent. These two observations suggest that not all of the free Gd species move with the solvent front as might be expected in the absence of phosphate at low pH, nor does the $GdPO_4$ or $Gd_x(OH)_y$ always stay at the origin. Phosphate precipitation may be incomplete, or small particles may be moving with the solvent front.

Table I. Results of ITLC Analysis of No-Carrier-Added and Carrier Added Experiments with $GdCl_3$[a].

$[^{153}Gd]$ μCi/mL	$[Gd]_t$ mM	Final pH	% counts origin	% counts center	Total Recovery %
1. pH adjusted to 7, 10 min before adding phosphate:					
0.32	0.007	7.3	81±3[b]	14±2[b]	95
0.33	0.209	7.3	90±2	6±1	118
0.32	2.090	7.3	99±1	1±1	100
2. pH adjusted to 7, 2 days before adding phosphate:					
0.32	0.007	7.2	84±3[b]	15±1[b]	72
0.33	0.209	7.2	92±4	7±1	109
0.32	2.090	7.2	99±1	1±1	101

[a]10μL sample applied to ITLC plate. [b]"No-carrier-added" experiment.

We conclude that the ITLC method is valid to determine free [Gd] at the 0.1% level in a carrier-added radiolabeled Gd chelate solution with a relative standard deviation of $\leq 20\%$. The same method is not suitable for no-carrier-added samples.

3.3 HPLC Method To Determine Free Gd. To improve the limit of detection (LOD) and the precision of the determination of the free Gd in a radiolabeled sample, an HPLC/radionuclide detection method was developed. This method was a modification of

the HPLC/fluorescence detection method used for free [Gd] in cold Gd(HP-DO3A) samples [9]. In the HPLC/fluorescence detection method, a calibration curve of the fluorescence intensity vs. the concentration of Gd as $Gd(EDTA)^-$ was established. The fluorescence intensity of the $Gd(EDTA)^-$ peak in the sample of interest was compared with the calibration curve to determine free [Gd]. Although the same calibration method could be used with radionuclide detection, a simpler method without an external calibration curve was developed. Normalized peak areas of $Gd(EDTA)^-$ for free Gd in Gd(HP-DO3A) were compared. In a typical analytical experiment, a 20 mL sample of GdL is injected onto an HPLC column. The free ^{153}Gd is determined as $^{153}Gd(EDTA)^-$ at a low KCPM scale and the chelate at a higher KCPM scale on the Beckman radionuclide HPLC detector.

3.3.1 Limit of Detection (LOD) and Linearity of HPLC Detection Method. When a known amount of $^{153}Gd(EDTA)^-$ or $^{153}GdCl_3$ was injected onto the HPLC system under the above mentioned conditions, the $^{153}Gd(EDTA)^-$ peak appeared with a retention time of 2.7-2.8 min. For quantitative determinations, samples were diluted successively with the appropriate EDTA buffered solution, and analyzed immediately. As shown in Figure 1, normalized peak areas decreased proportionally with the radioconcentration.

The radioconcentration was varied from 0.035 to 147 μCi/mL. A plot of the normalized peak areas (normalized to 1000 KCPM) vs. radioconcentration is shown in Figure 1. Linear-least-squares analysis of the data gave a slope of $(2.21\pm0.03) \times 10^5$ mV-s/mmol of Gd ($r^2 = 0.995$). A plot of the radioconcentrations <1.5 μCi/mL is shown in the insert. When the radioconcentration of the sample was less than 0.035 μCi/mL, the results were varied and relative errors in peak area determination were as high as 50%. Below a radioconcentration of 0.01 μCi/mL, the results were not accurate. At this low concentration, only two out of three injections gave identical results.

Also, peaks at this concentration were split. Thus, it is estimated that the LOD of the Beckman 170 detector is about 0.01 μCi/mL for a 20 μL injection at a flow rate of 1 mL/min. Based on the calculation of the radioactive events in the detector and the background events, the LOD of the method is normal [11]. We conclude that the HPLC/radionuclide detection method for the determination of free [^{153}Gd] has a linear radioconcentration range of 0.01 to 147 μCi/mL. Below the concentration of 0.035 μCi/mL, the determinations are of poor accuracy and precision.

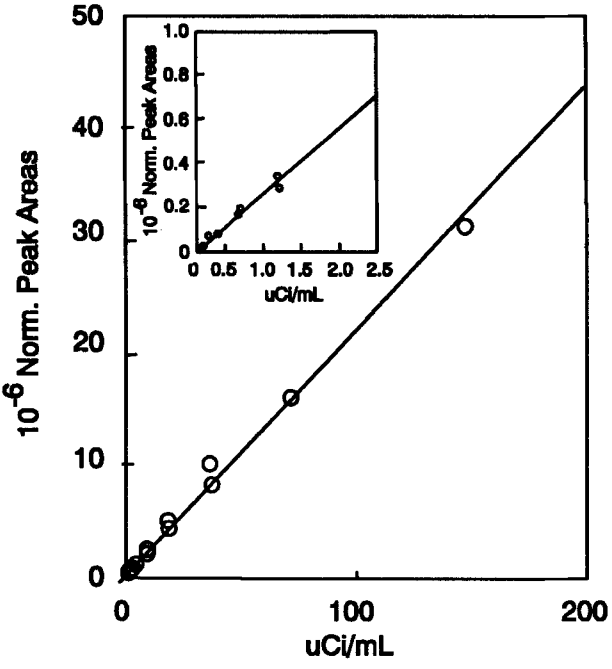

Figure 1. Dependence of normalized peak areas on the radioactivity concentration. The insert in the figure shows linearity at very low radioactivity concentration.

3.3.2 Recoverability of Radioisotope Detection Method. The recoverability of the HPLC column (Nucleosil, C_{18} reversed phase) was determined by comparing the activity of a radiolabeled Gd(EDTA)$^-$ sample before and after loading it onto the column. In a typical experiment, the column was first detached from the HPLC system, and 20 μL of sample was passed through the loop and collected. This sample was counted on a LKB gamma counter. The experiment was then performed with the column connected to the HPLC system. The eluant was collected in the usual way and counted on the counter. Triplicate injections were made for each concentration. The percent recovery value of each run was calculated from the relationship given below:

% Recovery = (Activity onto the column/Activity off the Column) x100

For both carrier-added and no-carrier-added samples the recovery was 100% (Table II). An example of the method to determine free Gd in a true tracer radiolabeled sample of Gd(HP-DO3A) is shown in Figure 2. A sample which had a trace amount of free Gd was analyzed by this method. First, the KCPM scale was adjusted low to detect the free Gd peak as Gd(EDTA)$^-$. Then the scale was changed to integrate the major peak due to Gd(HP-DO3A). The percentage of the free Gd was calculated as 0.005% from

Table II. Results On the Recoverability of the RadionuclideHPLC Detection Method.

[153Gd] μCi/mL	$[Gd]_t$, mM	Activity onto the Column (CPM)	Activity off the Column (CPM)	% Recovery
1. No carrier added experiment:				
1.78	0.0385	87281	91081	104.4
		86955	90190	103.7
		88190	89297	101.3
		-------	------	------
		87475±640	90189±892	103.0±2.0
2. Carrier added experiment:				
1.78	0.155	87467	92426	105.7
		87210	88544	101.5
		88213	87074	98.7
		------	------	------
		87630±521	89348±2765	102.0±3.5
1.78	1.20	89380	90558	101.3
		88588	88092	99.4
		86506	87806	101.5
		-------	-------	--------
		88158±1484	88819±1513	100.7±1.2
1.78	11.68	84698	91205	107.7
		86806	90796	104.6
		88400	89754	101.5
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		86635±1857	90585±748	104.6±3.1

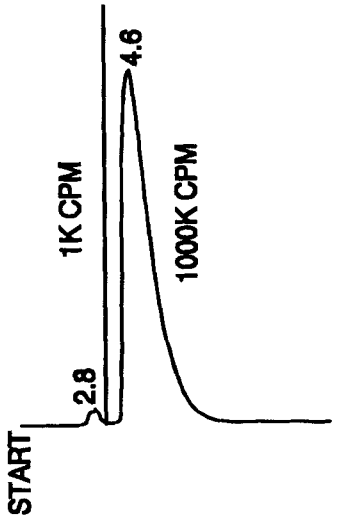

Figure 2. HPLC chromatogram of true tracer radiolabeled Gd(HP-DO3A). The conditions used were: Column: Nucleosil C_{18} reversed phase (250 X 4.6 mm), Mobile Phase: 4% acetonitrile + 96 % buffer (10 mM EDTA + 50 mM tris acetate, pH 7.4), Flow Rate: 1 mL/min, and Detector: Beckman 170 radionuclide detector.

the normalized peak areas of $Gd(EDTA)^-$ and Gd(HP-DO3A).

To validiate the true tracer radiolabeling method, we prepared a sample of $^{153}Gd/Gd$(HP-DO3A) with relatively high free $^{153}Gd/Gd$ concentration. The sample was analyzed for free ^{153}Gd and ^{153}Gd(HP-DO3A) by the HPLC/radionuclide detection method developed in this work and for free Gd and Gd(HP-DO3A) by the HPLC/fluorescence method [9]. The calculated ratios were: [$^{153}Gd/^{153}Gd$(HP-DO3A) = 0.036±0.001 and [Gd]/[Gd(HP-DO3A)] = 0.035±0.001.

4. CONCLUSIONS

In the present work we have presented a method by which true tracer radiolabeled gadolinium chelates with very low free Gd may be prepared. For analysis of radiolabeled chelate, we also developed an HPLC method with high precision and low LOD.

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[11] For a 20 μL sample with 0.01 μCi/mL activity the residence time in the detector would be ~1s, which translates into 0.2 nano Ci. This activity corresponds to ~480 CPM, which is well above the background counting rate (~100 CPM) in the ^{153}Gd channel.