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Purification of *p*-nitrobenzyl C-functionalized diethylenetriamine pentaacetic acids for clinical applications using anion-exchange chromatography

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Abstract

A general process for the purification of large quantities (5–10 g) of *p*-nitrobenzyl C-functionalized diethylenetriamine pentaacetic acids is reported. The method of choice to achieve purification for clinical applications is anion-exchange chromatography.

Keywords: Radioligands; Preparative chromatography; Diethylenetriamine pentaacetic acids; Monoclonal antibodies

1. Introduction

The use of radiolabeled monoclonal antibodies (mAbs) continues to receive attention [1]. A variety of metallic radionuclides have been employed to expand the choices of emission characteristics and half-lives for diagnostic (γ and β^+ emitters) and therapeutic (β^- and α emitters) applications [2,3].

Use of a C-functionalized diethylenetriamine pentaacetic acid (DTPA) (Fig. 1) for the modification of mAbs and subsequent radiolabeling with either ^{111}In, ^{90}Y, ^{212}Bi and other radionuclides has been previously reported [4–6]. The C-functionalized ligand 1B4M-DTPA has since been extensively investigated in pre-clinical trials for radiolabeling mAbs and continues to be employed in clinical trials for radioimmunotherapy (RIT) with ^{90}Y [7]. Additionally, the CHX-A and CHX-B DTPA ligands continue to be investigated for future clinical trials with the α-emitters ^{212,213}Bi [8].

These ligands also serve as sequestering agents for gadolinium and are useful for the preparation of MRI contrast enhancement reagents. In particular, the use of complexed Gd dendrimer macromolecular constructs have been shown to produce significantly greater molar relaxivity than commercial products currently in the clinic [8].

In response to increasing demands for a supply of this ligand for all of the above applications, a reproducible and reliable process for the synthesis and purification of the 1B4M-DTPA has been developed. While synthesis of the ligand was amenable to being increased in scale, purification of the ligands has proven to be challenging. To meet this challenge, we turned our attention to developing an ion-exchange chromatography method.

Herein we report our results on the preparation and use of the chloroacetate form of AG1 anion-exchange resin for the routine, reproducible purifica-

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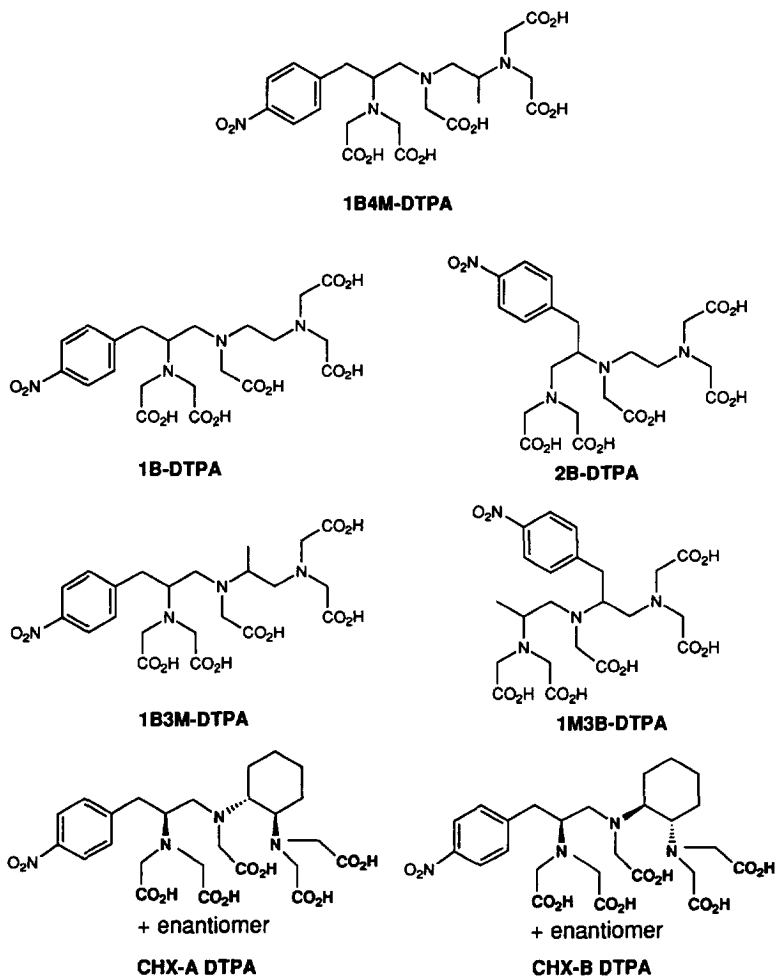

Fig. 1. C-Functionalized *p*-nitrobenzyl-DTPA chelating agents.

tion of C-functionalized *p*-nitrobenzyl-DTPA chelating agents. The processes described in this report allow for the preparation of substantial amounts of these compounds in analytical purity, suitable for use in clinical applications. The preparation and purification of 1B4M-DTPA is presented here as an example of this methodology.

2. Experimental

2.1. Reagents

All chemicals and solvents were purchased from either Fluka (Ronkonkoma, NY, USA) or Aldrich (Milwaukee, WI, USA). The parent diethylenetriamine trihydrochloride was prepared as described in the literature [10]. Ultrapure water was secured from a Hydro purification system (Research Triangle Park, NC, USA) and used for preparation of all aqueous solutions. Ion-exchange resins (AG1-X8, particle size 38–75 μm, acetate form) and (AG50W-X8, particle size 38–75 μm, H^+ form) were obtained from Bio-Rad Labs. (Hercules, CA, USA). Chemical analyses were performed by Atlantic Microlabs. (Norcross, GA, USA).

^1H and ^{13}C NMR were obtained using a Varian Gemini 300 instrument and chemical shifts are reported in ppm on the δ scale relative to TMS, TSP or solvent. Proton chemical shifts are annotated as

follows: ppm [multiplicity, integral, coupling constant (Hz)]. Fast atom bombardment mass spectra (FAB-MS) were taken on a Extrel 400.

2.2. Apparatus

The components of the ion-exchange chromatography system (Gilson, Middleton, WI, USA) were the following: a MiniPuls 2 peristaltic pump, a Holochrome UV variable-wavelength detector, a Model 202 fraction collector and an N2 strip chart recorder. Detection of the chelating agents was performed at 345 nm. The columns used for the resin beds were purchased from Pharmacia (Uppsala, Sweden).

The HPLC system components (Beckman Instruments, Fullerton, CA, USA) were as follows: a pair of 114M pumps, a 165 dual- wavelength variable-UV detector monitoring at 254 and 280 nm, controlled through a 406 analogue interface module, running under System Gold software on a 486 personal computer. A 210A Altex injector with a 50 μl loop and a Beckman 25×4.6 cm Ultrasphere ODS 5 μm column were also used. A 25 min gradient from 100% 0.05 *M* Et_3N/HOAc to 100% MeOH at 1 ml/min was employed for all HPLC chromatography.

2.3. Resin preparation

Anion-exchange resin (AG1-X8, particle size 38–75 μm, HOAc form, ca. 430 ml) was suspended in water and poured into a Pharmacia column (80×2.6 cm) and washed with water (600 ml). A 2.66 *M* NaOH, 0.05 *M* $Ba(OH)_2$ solution was freshly prepared from boiled water (2 l), filtered, and protected from the atmosphere (Ascarite). The solution was immediately pumped at 2 ml/min through the column until conversion to the hydroxide form was complete. The resin was then washed with water (0.7 l) at 3 ml/min to insure that the eluant was neutral before proceeding.

The freshly prepared hydroxide form resin was then washed with a 1 *M* $ClCH_2CO_2H$ solution (2 l) at 3 ml/min to convert the resin to its final form. After the conversion was complete, the residual acid was washed from the resin with water until the pH of the eluant was >5.0 (ca. 3.5 l). The top 1–2 cm of resin typically appeared discolored and was siphoned off and discarded. The $ClCH_2CO_2H$ resin was then washed from the column, bottled and stored in the refrigerator until needed.

2.4. Purification of 1B4M-DTPA

Typically, crude penta-*tert.*-butyl ester was prepared from the parent diethylenetriamine trihydrochloride (10 g, 27.7 m*M*) by alkylation with *tert.*-butyl bromoacetate (32.4 g, 166 m*M*) with Na_2CO_3 (26.2 g, 247 m*M*) in dimethylformamide (DMF) (200 ml). After an extractive work-up, this material was treated with trifluoroacetic acid (ca. 100 ml) for 18 h. The acid was removed by rotary evaporation with residual amounts minimized by holding the crude penta acid at 0.01 mm at room temperature for 18 h.

This material was taken up in minimal water and loaded onto a cation-exchange resin (AG50W-X8, particle size 38–75 μm, H^+ form) column (30×1.6 cm). The column was washed with water until the eluant was ca. pH 5.0 and then the crude product was eluted from the resin with 2 *M* NH_4OH (1 l). The ammonia solution was concentrated to leave a dark yellow-brown solid which was dried under vacuum.

The solid (5–6 g) was taken up in minimal water, loaded onto a column of the chloroacetate form anion-exchange resin prepared above (20×1.2 cm), and washed with water (ca. 200 ml) to remove any materials not bound to the resin. The product was then eluted from the column with a linear gradient, 0.0 *M* to 1.0 *M* aqueous $ClCH_2CO_2H$ (2 l total volume) and collected in 88 test tubes (150×18 mm). Typically, the pure product was found in the second half of the gradient as monitored at 345 nm. The resin was then washed with water back to initial conditions, and the process repeated to purify the remainder of the crude material. The relevant fractions were combined and the aqueous solution concentrated. The vast majority of the $ClCH_2CO_2H$ was removed by extraction with Et_2O leaving the product in a ca. 150 ml of water. Residual $ClCH_2CO_2H$ was eliminated by continuous extraction with Et_2O for 72 h. The aqueous solution from the extractor was lyophilized to leave the analytically pure product as a white powder. After washing the column back to initial conditions, this resin was routinely useful for up to ca. 20 more gradient

elutions before being discarded due to extreme discoloration of the resin bed. However, no changes in chromatographic characteristics were noted at this point of use of the resin.

^{1}H NMR (^2H_2O) δ 8.27 (d, J=8.0), 8.26 (d, J=8.0), 7.59 (d, J=8.0), {integral over this range=4 H}, 4.0–2.9 (m, 20 H), 1.35 (d, J=6.5), 1.27 (d, J=6.5), 1.19 (br.d, J=5.0), 1.05 (m), {integral over this range=3 H}; ^{13}C NMR (^2H_2O) δ 181.29, 180.81, 179.17, 178.44, 178.26, 151.00, 150.75, 149.42, 133.45, 127.14, 127.01, 62.62, 62.54, 61.00, 60.53, 59.38, 58.89, 58.04, 57.80, 57.70, 57.56, 57.07, 56.16, 54.95, 36.25, 35.40, 14.15, 13.66 {diastereomers plus contributions from regioisomers and diastereomers thereof}; HPLC t_R=10.1 min; FAB-MS (glycerol) 543 (M^++1); anal. calc. for $C_{22}H_{30}N_4O_{12}$;C, 48.70; H, 5.58; N, 10.33. Found: C, 48.85; H, 5.69; N, 10.16.

3. Results and discussion

Iodine isotopes have been a traditional technique for radiolabeling proteins, but their use has inherent limitations. Use of metallic radionuclides has expanded the choices of emission characteristics and half-lives to better match the biological applications and half-lives, be it diagnostic or therapeutic, while obviating the difficulties associated with radio-iodination of immunoproteins, their fragments and their engineered variants [2,3].

This approach requires use of chelating agents that must meet the minimum requirements of kinetic and thermodynamic stability in vivo. Failure to meet this pre-requisite leads to in vivo dissociation of the isotope and its subsequent deposition into organs which contributes to toxicity [4–6].

Initial studies with a C-functionalized DTPA confirmed an octadentate ligand to be a necessary attribute for optimal in vivo stability of the ^{90}Y complex [4]. However, biodistribution experiments employing this ligand indicated need for greater stability. Addition of an alkyl substituent had been recognized to increase metal complex stability constants [11]. Thus, a methyl group was added to the structure of the base compound to produce the 1B4M-DTPA [10]. This second generation C-functionalized ligand, after being extensively investigated in pre-clinical trials for radiolabeling mAbs, continues to be employed in clinical trials for RIT with ^{90}Y [6,7]. Finally, third generation C-functionalized DTPA ligands have been synthesized [5], and of these, the CHX-A DTPA continues to be investigated for clinical trials [7] with ^{111}In, ^{90}Y and the α-emitters ^{212,213}Bi [8].

Additionally, use of chelated metals for medical applications has included the use of gadolinium as an MRI T_1 relaxation agent for contrast enhancement. Expansion of initial experiments employing starburst PAMAM dendrimeric constructs [9] to pre-clinical experiments to determine optimum molecular size, extent of ligand conjugation, clearance and imaging quality and comcommitant toxicity studies was quickly found to require far greater amounts of ligands than were easily prepared previously. Concurrently, our laboratory had interests in determining the solution equilibrium, acid dissociation and serum stability of the yttrium complexes of all of the C-functionalized DTPA derivatives to attempt correlation of physical properties with observed in vivo results [12]. These requirements, coupled with additional clinical RIT demands, provided the impetus for development of a dependable source of pure 1B4M-DTPA that was also applicable to all of the *p*-nitrobenzyl C-functionalized DTPA ligands.

A detailed report for both the triamine precursor and 1B4M-DTPA itself has been reported elsewhere [10] and the modified experimental presented above was provided for completeness of the purification process. The prior synthesis did not employ *tert.*-butyl bromoacetate in DMF (Fig. 2) and the product was isolated in small quantities after semi-preparative HPLC as an ammonium salt. Modification of the alkylation was found to be more efficient with less incomplete alkylation products being found while greatly reducing the possibility of contamination with extraneous metals. The penta ester thus formed could be cleanly and easily cleaved by treatment

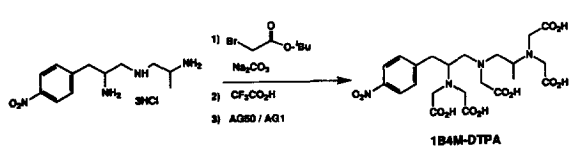

Fig. 2. 1B4M-DTPA synthesis.

with trifluoroacetic acid. After elution from the cation-exchange resin, the crude ammonium salt could be stored until purification by anion-exchange chromatography was convenient.

A gradient elution of AG1 anion-exchange resin had previously been employed to obtain analytically pure C-functionalized ethylenediamine tetraacetic acid (EDTA) [13]. Direct application of this method with the commercially available AG1 resin failed to produce any purified DTPA material, and in fact, formic acid elution failed to liberate DTPA from the resin. In our hands, anion-displacement chromatography, reported by Sawyer et al. [14] for purification of a structurally related diethylenetriamine pentaacid, consistently resulted in recovery of the crude material.

Alternately, purification was thought possible at the *tert.*-butyl ester stage by simple column chromatography using basic alumina. This method had in fact been employed using a fairly large column (50×4 cm) which allowed for the production of 2–4 g of the penta ester. Unfortunately, mass specta of this material routinely possessed a significant ion which corresponded to 44 u greater than the molecular mass. Careful isolation of this constant contaminant in the trailing portion of the "pure" product eluting from the basic alumina. Subsequent NMR analysis indicated this material was the result of a CO_2 insertion occurring during the alkylation step (Fig. 3) [15]. As this method failed to easily and reproducibly eliminate this impurity, was somewhat slow and tedious and not suitable for automation, and as the anion-exchange chromatography described herein met these criteria, purification of the penta ester at the scale required for clinical was discarded.

Re-examination of the anion-exchange chromatography option led us to hypothesize that elution with a stronger acid than formic acid (pK_a=3.75) would be

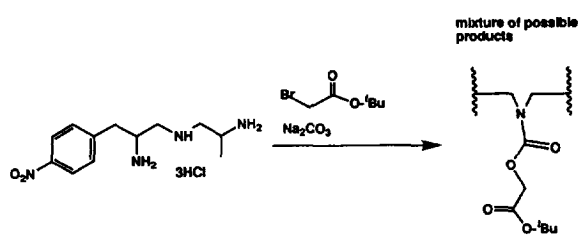

Fig. 3. Products of CO_2 insertion during alkylation.

necessary for removing an analogous DTPA from AG1 resin [16] after comparison of the pK_{a1} values of EDTA (2.21) and DTPA (2.14) [17]. Examination of the chlorinated acetic acid series revealed the pK_a values of the mono- to tri- substituted acids to be 2.85, 1.48 and 0.70, respectively, and that perhaps chloroacetic acid would be useful. To test this hypothesis, preparation of the chloroacetate form of AG1 anion-exchange resin was required. Attempts to use acetate or formate form of AG1 resin for chromatography with chloroacetic acid as an eluant gave erratic results. The conditions reported here for the resin preparation are both reproducible and highly dependable. A key observation revealed that protection of the resin from CO_2 during the conversion process minimized formation of bubbles within the resin bed which would contribute to irregular flow, and thus, incomplete conversion, and later, poor chromatography results. Of paramount importance was the removal or minimization of the concentration of trifluoroacetic acid used in the ester cleavage via AG50 cation-exchange resin. Bypassing this step and attempting to directly apply acidic crude material onto the chloroacetate form of AG1 anion-exchange resin negated isolation of purified product.

Anion-exchange chromatography was very straightforward using the chloroacetate resin at a 5–7 g scale of the crude material isolated from the cation-exchange resin. Use of greater loading resulted in inconsistent results on this column. This step essentially eliminated all of the impurities including the products from CO_2 insertion, incomplete alkylation and alkylation of trace aniline formation frequently observed from excess reduction in the preparation of the triamine. The scale of this operation could be roughly doubled but required increased resin bed (30×2.6 cm) and a 3 l gradient elution (ca. 18 h for completion). Simple extraction by organic solvent of the combined concentrated fractions of pure product, as confirmed by analytical HPLC, left the product in an aqueous solution. Lyophilization at this point produced a white solid, which by ^1H NMR, contained a residual amount of $ClCH_2CO_2H$ (0.5–1.5 equiv.). While further manual extraction was possible, a lighter than water continuous extraction apparatus was far more convenient for removing the last traces of $ClCH_2CO_2H$. Concen-

tration and lyophilization, after the continuous extraction process, during which two anion-exchange chromatography elutions could be performed, provided a white powder identical (^{1}H, ^{13}C-NMR, FAB-MS, HPLC) in all respects to the originally reported 1B4M-DTPA [10], in good yield from the triamine (4.5–5.5 g, 30–36%), ready for use in clinical applications.

The NMR spectra data for 1B4M-DTPA were somewhat complicated by the fact that this compound was prepared without any stereocontrol. This led to production of all four possible stereoisomers, existing as two pairs of diastereomers. Additionally, a small percentage of the 1B3M-DTPA regioisomer also generated in all four possible configurations was observed as evidenced by the two sets of signals at 1.15 ppm and 1.05 ppm.

Analytical HPLC chromatograms of the crude product prior to (Fig. 4) and after elution from the AG1 chloroacetate resin (Fig. 5) provide additional validation for the purification process. While HPLC chromatography was monitored at both 254 and 280 nm, impurities due to over reduction (anilines) were best observed at 254 nm as reported here. All aryl nitro group compounds were easily monitored at 254 nm and other impurities were obviated by the synthetic route. In general, purity prior to ion-exchange chromatography varied from 70–80%. While the purified material could range from 90–98%, routinely acceptable elemental analyses have been obtained for the product. The peak at ca. 8.75 min was the result of alkylation of over reduction material in the triamine precursor. The other major contaminant at ca. 14.5 min has been tentatively attributed to the product from CO_2 insertion and alkylation, while the remaining trailing peaks were from the various incomplete alkylation reaction products. None of these impurities has any significant impact on further applications as hydrogenation of the aryl nitro group and subsequent treatment with thiophosgene leads to the product used for protein modification. These impurities are then either inert to this chemistry, or converted to thioureas, and after at this point, all are inert to interaction with protein and would be easily eliminated via size-exclusion HPLC.

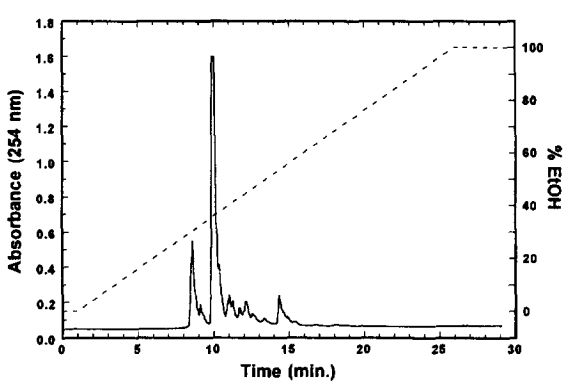

Fig. 4. HPLC chromatogram showing impurities in 1B4M-DTPA after elution from AG50 cation-exchange resin.

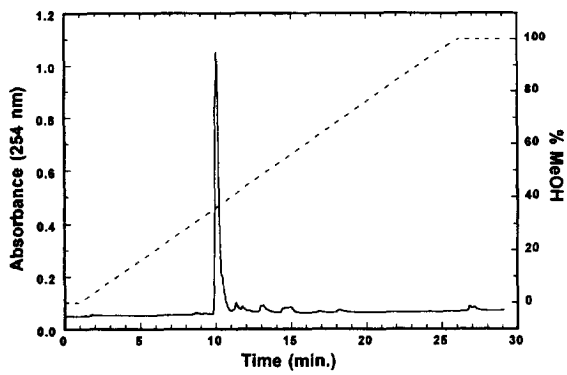

Fig. 5. HPLC chromatogram showing impurities in 1B4M-DTPA after elution from AG1 anion-exchange resin.

Finally, as a proof of generality, all of the remaining DTPA derivatives (Fig. 1) have been successfully purified by this methodology for further studies of their solution chemistry [12]. Elution characteristics for all of these compounds were consistent throughout the series with the product being isolated in precisely the same portion of the gradient elution profile.

4. Conclusions

A simple, dependable and reproducible process for the preparation and, most importantly, the purification of C-functionalized *p*-nitrobenzyl DTPA derivatives has been developed. The use of anion-exchange chromatography with gradient elution of the chloroacetate form of AG1 resin successfully separated the impurities due to incomplete alkylation and reaction byproducts. In spite of the absence of an absolute

correlation between pK_a of eluant solution and the acid being eluted from AG1 resin, requirement of a stronger acid, chloroacetic versus formic acid, for elution of DTPA versus EDTA was demonstrated. After removal of the chloroacetic acid, the C-functionalized DTPA was useful for clinical applications.

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