

Research Paper

Pre-clinical evaluation and efficacy studies of a melanin-binding IgM antibody labeled with ^{188}Re against experimental human metastatic melanoma in nude mice

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Abbreviations: RIT, radioimmunotherapy; TCEP, tris(2-carboxyethyl) phosphine hydrochloride; DTT, dithiothreitol

Key words: clinical trial, melanoma, melanin-binding antibody, radioimmunotherapy, ^{188}Re -Rhenium

Purpose: Currently there is no satisfactory treatment for metastatic melanoma. Radioimmunotherapy (RIT) uses the antigen-antibody interaction to deliver lethal radiation to target cells. Recently we established the feasibility of targeting melanin in tumors with ^{188}Re -labeled 6D2 mAb to melanin. Here we carried out pre-clinical development of ^{188}Re -6D2 to accrue information necessary for a Phase I trial in patients with metastatic melanoma.

Results: TCEP proved to be effective in generating a sufficient number of -SH groups on 6D2 to ensure high radiolabeling yields with ^{188}Re and preserved its structural integrity. ^{188}Re -6D2 was quickly cleared from the blood with the half-life of approximately 5 hrs and from the body—with the half-life of 10 hr. The doses of 0.5, 1.0 and 1.5 mCi significantly ($p < 0.05$) slowed down A2058 tumor growth in nude mice, also causing release of melanin into the extracellular space which could provide additional target for repeated treatments. Transient effects of RIT on WBC and platelet counts resolved by day 14 post-treatment

Experimental design: Tris(2-Carboxyethyl) Phosphine Hydrochloride (TCEP) was evaluated as potential agent for generation of -SH groups on 6D2 mAb. TCEP-treated 6D2 mAb was radiolabeled with ^{188}Re and its radiochemical purity and stability was measured by ITLC and HPLC and its immunoreactivity—by melanin-binding ELISA. The pharmacokinetics, therapeutic efficacy and acute hematologic toxicity studies were performed in nude mice bearing lightly pigmented A2058 human metastatic melanoma tumors.

Conclusions: We have developed radiolabeling and quality control procedures for melanin-binding ^{188}Re -6D2 mAb which

made possible currently an on-going Phase I clinical trial in patients with metastatic melanoma.

Introduction

Melanoma poses an increasing health problem that affects about 40,000 patients each year in the United States and an estimated 100,000 world-wide. While primary melanomas that are localized to the skin can be successfully treated by surgical removal, there is no satisfactory treatment for metastatic melanoma, a condition that currently has an estimated five year survival of 6%. Targeted radionuclide therapy has evolved into an efficient modality for cancer patients in whom standard anti-neoplastic therapies have failed.¹ One type of targeted radionuclide therapy—radioimmunotherapy (RIT) takes advantage of the specificity of the antigen-antibody interaction to deliver localized lethal doses of radiation to target cells using radiolabeled antibodies.^{2,3} The clinical success of FDA-approved drugs such as Zevalin[®] and Bexxar[®] (anti-CD20 monoclonal antibodies labeled with ^{90}Y -Yttrium (^{90}Y) and ^{131}I -Iodine (^{131}I), respectively) for the treatment of relapsed or refractory B-cell non-Hodgkin lymphoma (NHL) demonstrates the potential of RIT as an anti-neoplastic strategy. Encouraging reports on the use of RIT as an initial treatment for follicular lymphoma⁴ support the use of RIT as first-line therapy for this malignancy. Hence, the increasing acceptance of RIT for certain lymphomas combined with the development of a technical infrastructure to support this type of therapy have created a favorable environment for the development of radionuclide therapy for metastatic melanoma provided that suitable targets can be identified.

Melanoma owes its name to the presence of the pigment melanin. Given that even amelanotic melanomas contain some melanin, this pigment presents a potential target for development of radionuclide therapy of metastatic melanoma. Historically, melanin was not considered a target for RIT because it is an intracellular pigment outside the reach of a specific antibody. Because melanomas are rapidly growing, cell turnover releases melanin pigment into the extracellular space that can be targeted for delivery of cytotoxic

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radiation by radiolabeled melanin-binding antibodies. Experimental results have established the feasibility of targeting melanin released from dead melanoma cells in tumors with radiolabeled antibodies⁵ and peptides.⁶ Furthermore, this strategy is attractive because melanin in normal tissues is not accessible to the antibody by virtue of its intracellular location. To test this hypothesis we employed a murine IgM mAb known as 6D2 generated from mice immunized with melanin produced by the fungus, *Cryptococcus neoformans*.⁷ This antibody also binds human melanin since both fungal and human melanins have structural similarities⁸ and are negatively charged. Nude mice bearing MNT1 pigmented human melanoma tumors were treated with mAb 6D2 labeled with 1.5 mCi of the β -emitter 188-Rhenium (¹⁸⁸Re). Mice treated with radiolabeled mAb 6D2 manifested inhibition of tumor growth and prolonged survival. MAb 6D2 bound tumor melanin but did not bind to normal melanized tissues in C57BL6 black mice. The mechanism of melanoma targeting with mAb 6D2 involved antibody binding to extracellular melanin released during tumor cell turnover or to dying tumor cells with damaged or permeable membranes. These results provided the basis for the pre-clinical development of radiolabeling of ¹⁸⁸Re-6D2 mAb. Consequently we carried out additional pre-clinical development of ¹⁸⁸Re-6D2, including pharmacokinetics, efficacy and acute hematologic toxicity studies in a metastatic human melanoma model in mice to accrue additional information necessary to support a Phase I trial in patients with metastatic melanoma.

Results

Influence of TCEP concentration and incubation time on mAb 6D2 SH group generation. The efficiency of TCEP in generating -SH groups was estimated from the radiolabeling yields with ¹⁸⁸Re, while the structural integrity of TCEP-treated mAb 6D2 was assessed with non-reducing SDS-PAGE and size exclusion HPLC. Lower concentrations of TCEP (2:1 and 10:1 TCEP to 6D2 molar ratios) were inefficient at generating -SH groups resulting in low radiolabeling yields with ¹⁸⁸Re of 25% (Table 1). In contrast, TCEP to 6D2 molar ratios of 50:1 and 100:1 led to 70–72% radiolabeling yields (Table 1). However, at the 100:1 molar ratio there was some fragmentation of 6D2 mAb relative to that observed for lower concentrations (Fig. 1). Consequently, a 50 molar excess of TCEP over 6D2 mAb was selected for subsequent experiments.

In experiments assessing incubation time, we noted that 5- and 15-min incubations resulted in 41–42% radiolabeling yields with ¹⁸⁸Re, 30- and 60-min incubations increased the yield to 71–72%, while longer incubation times up to 240 min produced no further yield (Table 2). SDS-PAGE analysis of mAb 6D2 samples treated with TCEP for different times and then labeled with “cold” Re demonstrated that after short incubation times with TCEP (up to 30 min) most of 6D2 remained as an intact IgM molecule while 120 min incubation caused significantly more fragmentation of the IgM (Fig. 2A). We therefore selected an incubation time of 30 min and a 50:1 molar ratio of TCEP to mAb 6D2 as the optimal conditions for

Table 1 Influence of TCEP molar excess over 6D2 mAb on radiolabeling yields with ¹⁸⁸Re

TCEP molar excess over 6D2	Radiolabeling yield, %
0	10
2	25
10	25
50	72
100	70

Incubation was carried out at room temperature for one hour.

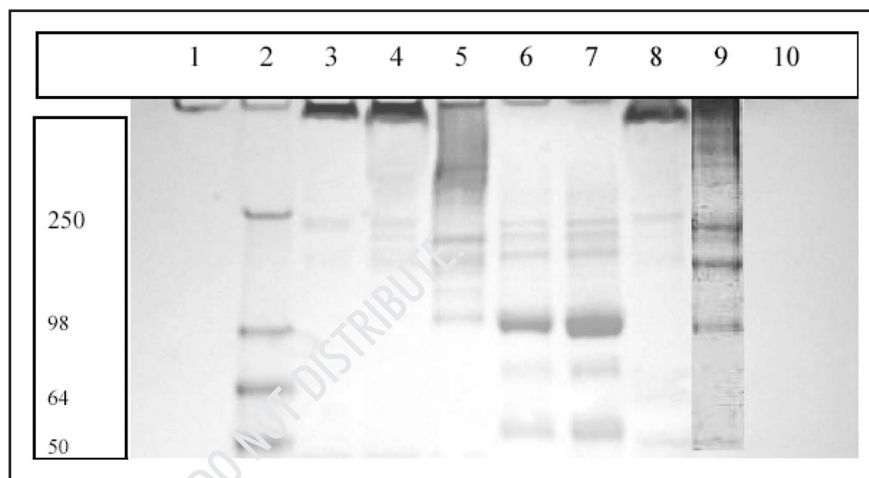


Figure 1. Structural integrity of 6D2 mAb after treatment with TCEP. For comparative purposes samples of 6D2 treated with DTT as in⁵ are also shown. Non-reducing SDS-PAGE (4–20% Tris-Glycine gel) was used. Lane # 1: empty; Lane # 2: Pre-stained MW markers: 250 kDa myosin, 98 kDa BSA, 64 kDa glutamic dehydrogenase, 50 kDa alcohol dehydrogenase; Lane # 3: affinity purified 6D2 standard; Lane # 4: 6D2:TCEP, 1:10 molar ratio; Lane # 5: 6D2:TCEP, 1:100 molar ratio; Lane # 6: 6D2 treated with DTT; Lane # 7: the same; Lane # 8: mouse myeloma IgM standard; Lane # 9: 6D2:TCEP, 1:50 molar ratio; Lane # 10: empty.

Table 2 Influence of incubation time of 6D2 mAb with 50 molar excess of TCEP on radiolabeling yields with ¹⁸⁸Re

Time of reduction with TCEP, min	Radiolabeling yield, %
5	42
15	41
30	72
60	71
120	66
240	33

Incubation at room temperature.

generating -SH groups on 6D2 mAb. Interestingly, however, although non-reducing SDS-PAGE showed fragments in antibody preparations labeled with “cold” Re (Fig. 2A), the antibody eluted from the size exclusion HPLC column as a single peak at 6.7 min similar to that of the intact native 6D2 (Fig. 2B) for all preparations (Fig. 2C–F). Blaunstein et al.¹⁰ observed the same inconsistency between SDS-PAGE and size exclusion HPLC of TCEP-treated murine IgG

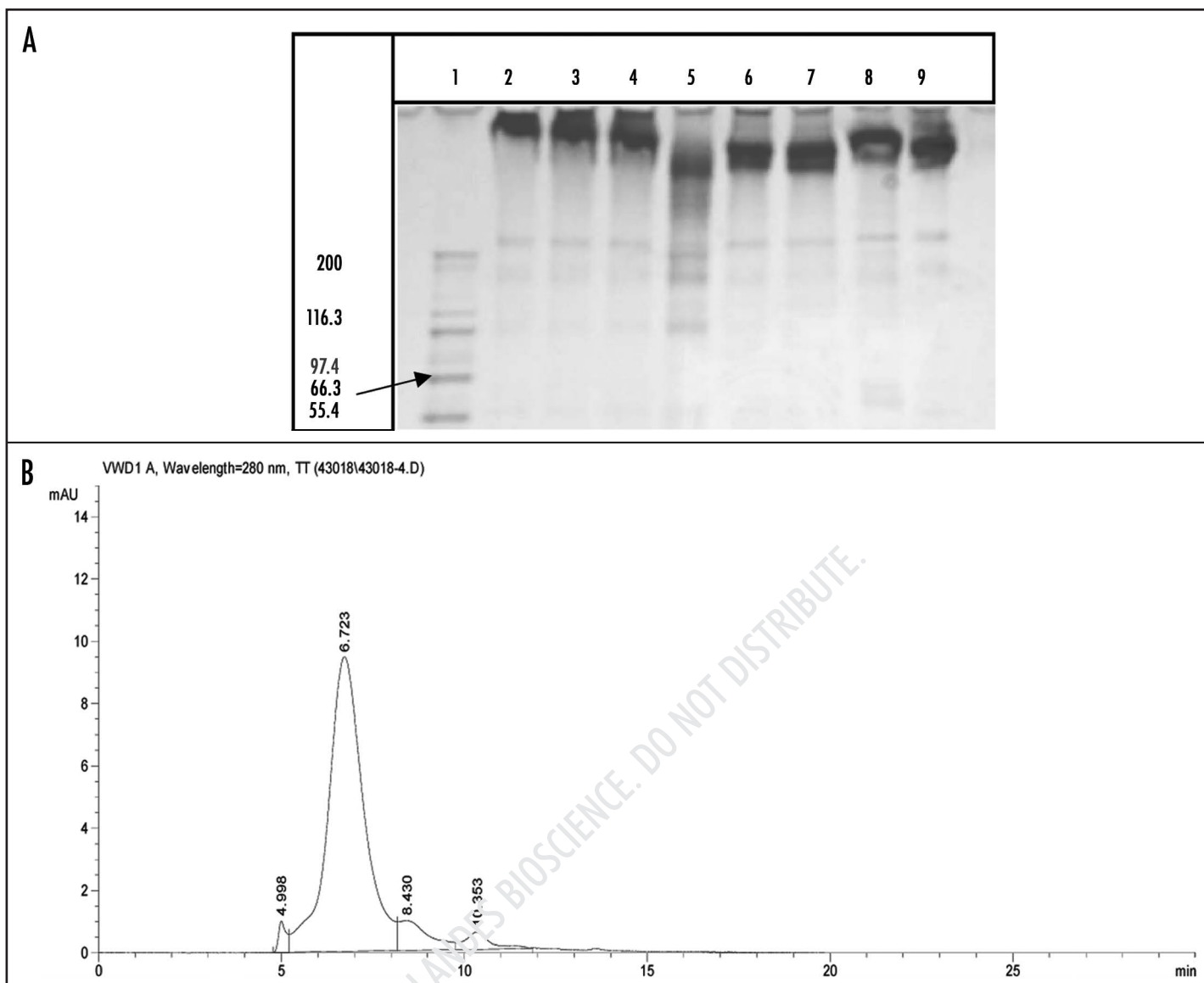


Figure 2. Structural integrity of 6D2 mAb after treatment with TCEP at a TCEP:mAb ratio of 50:1 for various periods of time. The samples were subsequently labeled with “cold” rhenium. Non-reducing SDS-PAGE (4–20% Tris-Glycine gel) was used. (A) SDS-PAGE: Lane # 1: Pre-stained MW markers Myosin 200 kDa, B galactosidase 116.3 kDa, Phosphorylase b 97.4, Bovine Albumin 66.3 kDa, Glutamic Dehydrogenase, 55.4 kDa; Lane # 2: 6D2, 5 min treatment; Lane # 3: 6D2, 15 min treatment; Lane # 4: 6D2, 30 min treatment; Lane # 5: 6D2, 120 min treatment; Lane # 6: 6D2, 30 min treatment; Lane # 7: 6D2, 60 min treatment; Lane # 8: Sigma Std. IgM; Lane # 9: 6D2 reference standard. (B–E) size exclusion HPLC of 6D2: (B) 6D2 reference standard; (C) 6D2, 5 min treatment; (D) 6D2, 30 min treatment; (E) 6D2, 120 min treatment.

radiolabeled with ^{99m}Tc, and Michaelsen et al.¹¹ reported similar findings for a human IgG1. In both reports, the discordance between SDS-PAGE and HPLC did not affect the immunoreactivity of mAbs towards their respective antigens. The fragmentation apparent by SDS-PAGE might reflect electrophoretic forces pulling apart an antibody held together by fewer disulfide bridges as a result of TCEP treatment. The size exclusion HPLC, on the other hand, allows detection of the antibody in its form in solution.

Radiochemical purity, stabilization of ¹⁸⁸Re-6D2 with L-ascorbic acid and “cold” 6D2 and melanin-binding ELISA. Because radiolabeling yields with ¹⁸⁸Re of TCEP-treated 6D2 were 71–72%, post-radiolabeling purification was necessary. After passing ¹⁸⁸Re-6D2 through a size exclusion HiPrep column, the radiochemical purity of ¹⁸⁸Re-6D2 was 92–93% with <1% radiocolloids, as

determined by SG-ITLC. Approximately 70% of the total amount of the antibody was recovered from the HiPrep column. The purified antibody was immediately stabilized with 0.2 mg/mL L-ascorbic acid, which is widely used as a radioprotector for radiopharmaceuticals. In addition to its radioprotective properties, L-ascorbic acid also decreased the pH of ¹⁸⁸Re-6D2 in saline from 5.3 to 4.8; the lower pH may have further stabilized the ¹⁸⁸Re radiolabel on the antibody.¹² The radiochromatographic profile of the purified ¹⁸⁸Re-6D2 stabilized with L-ascorbic acid is shown in Figure 3A. We also evaluated the stability of ¹⁸⁸Re radiolabel on the antibody after freezing the radiolabeled mAb at -80°C immediately after preparation and thawing 24 hr later. Both SG-ITLC (91% radiochemical purity) and radiochromatography (Fig. 3B) showed the ¹⁸⁸Re radiolabel remained attached to the antibody. The addition of “cold”

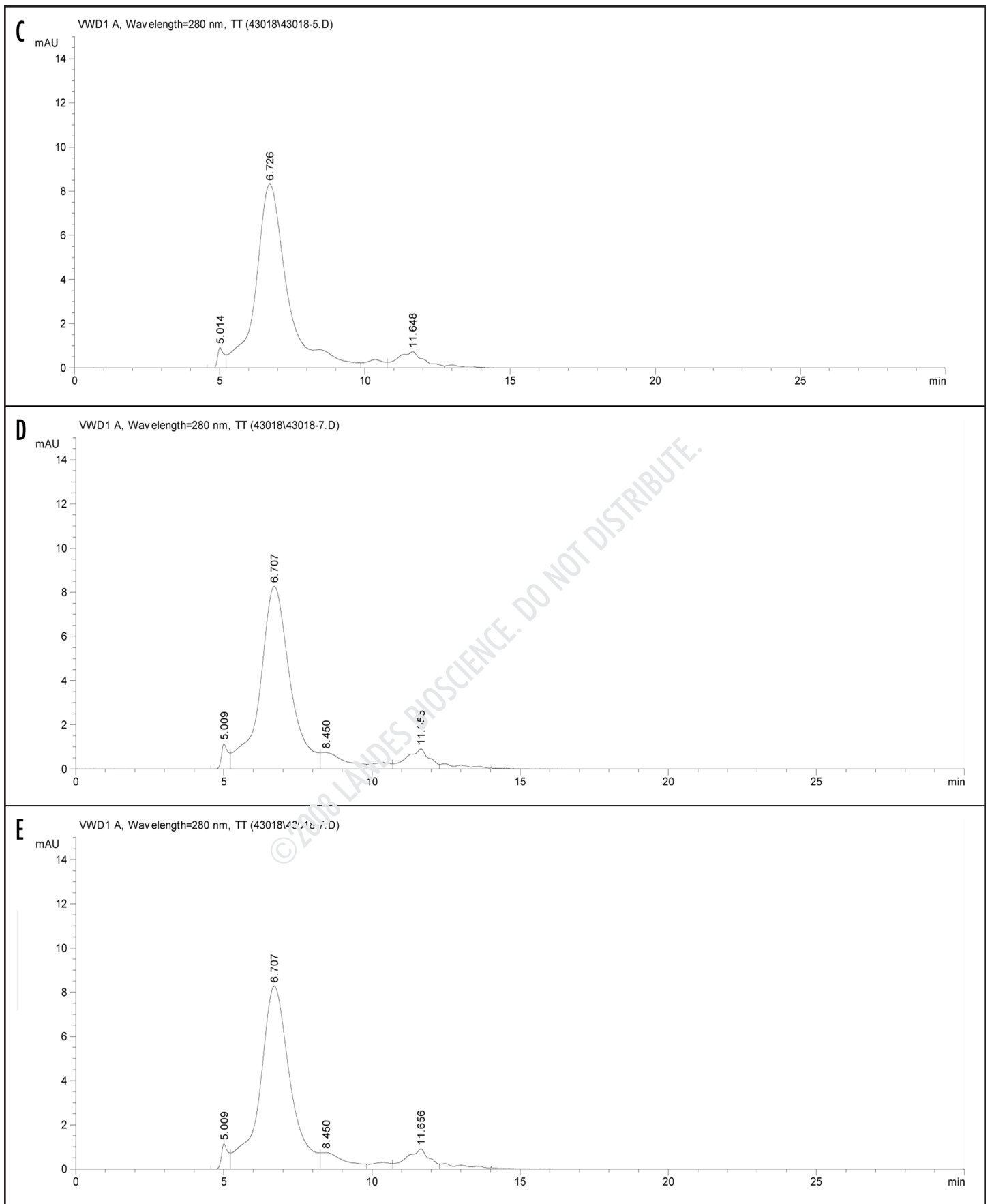


Figure 2 C-E. See legend, page 1118.

