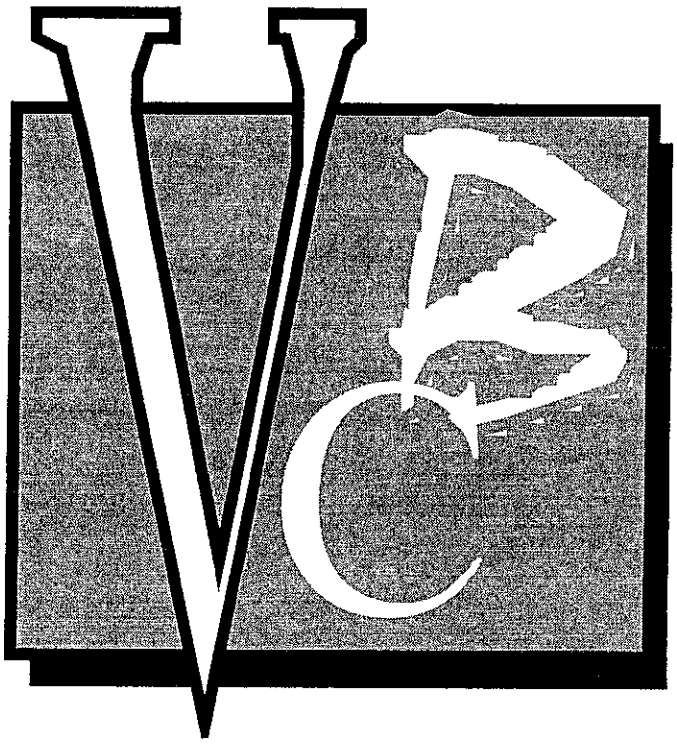
EQUIDYNE-VBC PROJECT D:

RETENTION OF STRUCTURAL/POTENCY CHARACTERISTICS OF INJECTABLE DRUGS AND MODEL COMPOUNDS

INVESTIGATIONAL PROTOCOL # EQUA-001



REPORT

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INTRODUCTION

Needle-free injectors were initially developed for mass inoculations and have been extensively utilized by military and civilian travelers. In recent years, needle-free delivery technology has gained momentum in other clinical areas, such as insulin administration in type 1 diabetes, heparin administration, and administration of pain medications. Needle-free delivery products in development and on the market employ various different technologies. Some are based purely on mechanical pressure (provided by a spring mechanism), some generate pressure using a CO_2 cartridge, whereas others employ more esoteric technologies such as ultrasound.

The Equidyne Systems, Inc. Injex device is an FDA-approved needle-free delivery device which utilizes a mechanical pressure technology to propel 0.05-0.5 ml of solution through a 0.006" orifice under a pressure of ~3000 psi. This pressure is sufficient to sub-cutaneously inject pharmaceutical compounds. However, this pressure may also be sufficient to damage such compounds to the extent that they may lose pharmacologic activity or physical characteristics.

In the case of the former, efficacy studies are required. In the case of the latter, analytical studies may be employed to determine whether physical characteristics such as molecular structure, three dimensional conformation, molecular weight, and ionic charge are retained. The objective of this study is to determine whether model compounds retain their physical characteristics post-injection with the Injex™ needle-free delivery device. Comparisons to delivery via a control as well as a standard syringe will be performed.

METHODS

The compounds investigated encompassed a range of molecules from small (<1000 Daltons) to large (close to 1 million Daltons). Model compounds comprise the majority of materials tested; however, an intra-muscularly delivered drug is also analyzed. Although the Injex is not cleared for IM delivery at this time, this compound is included for future information.

Drug

Thyroxine (T4, molecular weight 776.9 Daltons) was purchased from Sigma Chemical (St. Louis, MO). This compound is equivalent in molecular weight, structure, and purity to Synthroid®, which is commercially marketed by Boots Pharmaceutical, Inc.

Model Compounds

To investigate molecular damage across a wide range of compound molecular weights, molecular weight standards (Product #151-1901) were purchased from Bio-Rad Laboratories (Hercules, CA). This mixture of compounds includes the following compounds:

1) Cyanocobalamin (Vitamin B-12) – 1350 Daltons
2) Myoglobin – 17000 Daltons
3) Ovalbumin – 44000 Daltons
4) Immunoglobulin G (bovine) – 158,000 Daltons as a monomer
5) Thyroglobulin – 670,000 Daltons as a dimer

Preparation of compounds

Solid thyroxine was weighed out using a microbalance. Due to solubility issues (thyroxine is insoluble in water) a 1 mg/ml solution of T4 was made in weakly alkaline sodium carbonate buffer, pH 9.0. The thyroxine solution was stored overnight at 2-8 °C prior to analysis. The Molecular Weight Standards are supplied as a lyophilized substance. They were reconstituted in 0.5 ml of distilled water and microfuged for 3 minutes at 3000 rpm to remove particulate matter. The reconstituted material was stored overnight at 2-8 °C prior to analysis.

Delivery of compounds

For each compound and each delivery method, 6 deliveries were performed, i.e. 0.3 ml was delivered twice into each of three receptacles by a Becton-Dickinson 0.5 ml Syringe and another 6 aliquots of 0.3 ml were delivered into a second set of 3 receptacles by the Injex. This resulted in 0.6 ml delivered to each receptacle. Each receptacle was thereafter treated as a separate sample. A pre-delivery retention of each compound was kept as a matched study Control.

The total amount of thyroxine delivered was clinically relevant. For instance, most hypothyroidemic subjects require less than 200 μg/day of Synthroid®. Therefore, delivery of the 1 mg/ml thyroxine solution in 300 μl 'doses' resulted in 300 μg of drug delivered per injection.

High Performance Liquid Chromatography (HPLC)

Size Exclusion HPLC (SEC-HPLC) was performed on the Molecular Weight Standards in the Control sample or post-injection with the Injex or Syringe only. A Rainin (Emeryville, CA) Dynamax HPLC system was fitted with a 300 x 7.8 mm Bio-Sil SEC 250-5 column (Bio-Rad, Hercules, CA). The pore size of this silica-based column is 500 Angstroms and the molecular weight separation range for proteins is 10,000-300,000 Daltons. The system was equilibrated with 50 mM sodium phosphate, 150 mM sodium chloride, 0.01% sodium azide buffer adjusted to pH 6.8 (all buffer reagents were from Sigma Chemical, St. Louis, MO). A Hamilton syringe was used to inject 50 μl of each sample. The syringe was cleaned scrupulously between injections. The flow rate was 1.0 ml/min and detection of eluting compounds was performed with a spectrophotometer set at 280 nm wavelength (A_{280}).

Polyacrylamide gel electrophoresis (PAGE)

Molecular Weight Standards in the Control and post-injection with the Injex or Syringe were run on pre-cast 4-20% Tris-Glycine gradient gels (Bio-Rad, Hercules, CA). Broad-spectrum PAGE standards (Bio-Rad) were run to calibrate the gels . Silver staining of protein bands was performed using the Silver Stain Plus kit (Bio-Rad), which is based on a derivation of the method of Gottleib and Chavko.

Thyroxine and insulin immunoassays

Thyroxine samples in the Control and post-injection with the Injex or Syringe were analyzed by immunoassay on the Abbott Laboratories (Abbott Park, IL) AxSym analyzer. The dynamic range of this assay is 1.05 – 24 μg/dL. Thyroxine assays were performed by Biomedical Testing Services, Inc. (San Diego, CA) per the manufacturer's directional insert.

Data analyses

For SEC-HPLC, elution time for each peak (minutes) and peak area (μV-seconds) were determined by the Dynamax software. Means and SDs were computed for the three separate injections of the Control, Injex, and Syringe samples. The Dunnett's t-test was used to compare the average elution time and peak area for each of the peaks from the Injex and Syringe samples to the Control. Student's t-tests were then employed to perform all pairwise combinations of the average elution time and peak area for the Control, Injex, and Syringe samples.

Immunoassay results were analyzed using Dunnett's t-test and Students t for all pairwise combinations in an identical manner to the HPLC analysis. The JMP software (SAS Institute, Cary, NC) was used for all statistical investigations.

RESULTS

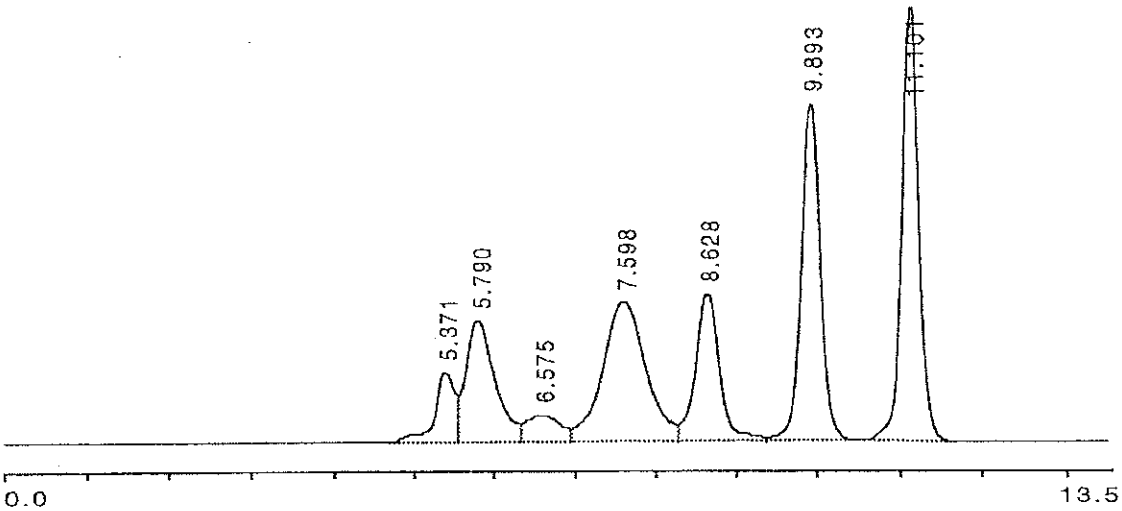
Size Exclusion High Performance Liquid Chromatography

SEC-HPLC separates molecules based on molecular weight and three-dimensional conformation (tertiary structure). Any loss in molecular weight or change in structure would be detected by a shift in peak retention time or peak area vs. the Control. For instance, a molecule that is sheared into smaller subunits would generate small additional peaks, each of which would be retained longer on the column than the Control.

As mentioned in the Methods section, only the Molecular Weight Standards were analyzed by this method. Thyroxine was inappropriate since it is too small to separate from possible degradation products on this column.

A typical chromatogram of the Molecular Weight Standards in the Control sample is displayed in Figure 1 on the next page. Note that 7 peaks are detected and integrated by the Dynamax software. The additional two small peaks are a trimer of thyroglobulin (Peak 1) and a dimer of IgG (Peak 3). Since these peaks separate acceptably from their major species counterparts (thyroglobulin dimer and IgG monomer), they were used for subsequent data analysis. A full description of each peak is provided in Table 1.

Figure 1: Example SEC-HPLC Chromatogram of Control Sample



Analysis: Channel A

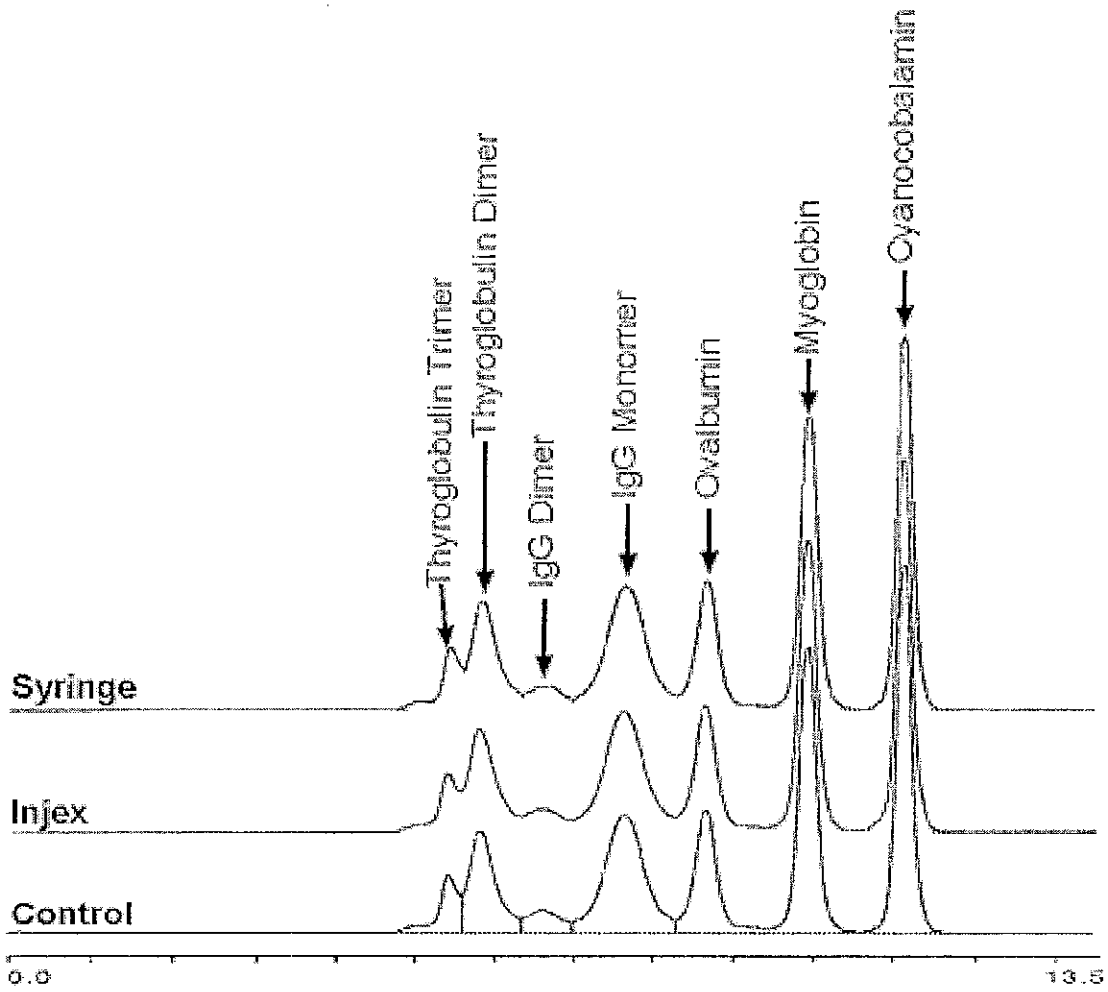
Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	5.371	N1	30004	520401	5.236
2	5.790	N2	52284	1265726	12.735
3	6.575	N3	11866	348530	3.506
4	7.598	N4	59861	2131650	21.448
5	8.628	N5	62884	1223567	12.311
6	9.893	N6	143960	2136610	21.498
7	11.101	N	184466	2311761	23.261
Total Area				9938245	99.995

Table 1: Peak Identification

Peak # from example in Figure 1	Identification	Molecular Weight (Daltons)	Pertinent physical characteristics
1	Thyroglobulin Trimer	~ 1 Million	Complex protein, precursor of Thyroxine
2	Thyroglobulin Dimer	670,000	As w/trimer
3	Bovine IgG Dimer	316,000	Complex protein w/ carbohydrate moieties
4	Bovine IgG Monomer	158,000	As w/dimer
5	Ovalbumin	44,000	Complex single chain protein; contains mannose and glucosamine oligosaccharide residues
6	Myoglobin	17,000	Complex single chain protein with iron-porphyrin group
7	Cyanocobalamin (Vitamin B-12)	1350	Corrin ring and ribonucleotide structure

As a visual comparison, Figure 2 is an overlay of representative chromatograms from the Control, Injex, and Syringe samples. There are 7 clear peaks in each chromatogram, peak retention times are nearly identical, and peak areas are visually similar. (Note: Baseline drops for integration purposes are shown in the Control trace as an example. They were removed in the Injex and Syringe traces for viewing ease.)

Figure 2: SEC-HPLC chromatogram overlay



To determine whether statistical differences exist, average peak retention time (minutes) and peak area (μV-seconds) from the Control, Injex, and Syringe runs were analyzed by Dunnett's t-test and Students-t (raw data and analyses can be found in Appendix A of this report). The results of the peak retention time analysis are shown in Table 2. Average retention times and standard deviations are nearly identical. There are no statistical differences vs. the Control by Dunnett's method and pairwise differences were insignificant by Students-t.

Table 2: Average retention times in minutes (mean of 3 injections) and standard deviation (SD)

Compound	Control Average Retention Time (SD)	Injex Average Retention Time (SD)	Syringe Average Retention Time (SD)
Thyroglobulin Trimer	5.384 (0.028)	5.382 (0.034)	5.392 (0.033)
Thyroglobulin Dimer	5.800 (0.028)	5.800 (0.032)	5.806 (0.034)
Bovine IgG Dimer	6.582 (0.029)	6.583 (0.029)	6.590 (0.033)
Bovine IgG Monomer	7.610 (0.027)	7.606 (0.03)	7.610 (0.033)
Ovalbumin	8.633 (0.028)	8.632 (0.029)	8.633 (0.034)
Myoglobin	9.893 (0.028)	9.891 (0.028)	9.888 (0.033)
Cyanocobalamin	11.100 (0.026)	11.098 (0.025)	11.091 (0.030)

Dunnett's t: all p>0.05 vs. the Control
Students-t: all pairwise combinations p>0.05

Table 3 displays the results of the analysis of peak area. By Dunnett's method, there are no significant differences between the Injex and the Control. However, in all cases except Cyanocobalamin, peak area from the Syringe samples is greater than both the Control and the Injex. Similar results are seen with the Students-t analysis. The Syringe samples display higher peak area than the Control in all cases except cyanocobalamin. The Syringe is also higher than the Injex in all comparisons.

Table 3: Average peak area (mean of 3 injections) and standard deviation (SD)

Compound	Control Average Peak Area (SD)	Injex Average Peak Area (SD)	Syringe Average Peak Area (SD)
Thyroglobulin Trimer	515566 (9566)	527176 (12698)	551894 (5179)*†
Thyroglobulin Dimer	1282737 (21134)	1281709 (20479)	1377025 (12443)*†
Bovine IgG Dimer	357469 (1686)	352177 (4234)	372989 (7004)*†
Bovine IgG Monomer	2194942 (35476)	2175910 (50791)	2320701 (26701)*†
Ovalbumin	1278375 (11314)	1254526 (28330)	1332448 (18312)*†
Myoglobin	2225994 (21930)	2192205 (52002)	2324128 (28909)*†
Cyanocobalamin	2421607 (34579)	2381640 (67225)	2521790 (49986)*§

** Dunnett's t, p<0.05*
†Students-t, p<0.05 vs. Control and Injex
§Students-t, p<0.05 vs. Injex

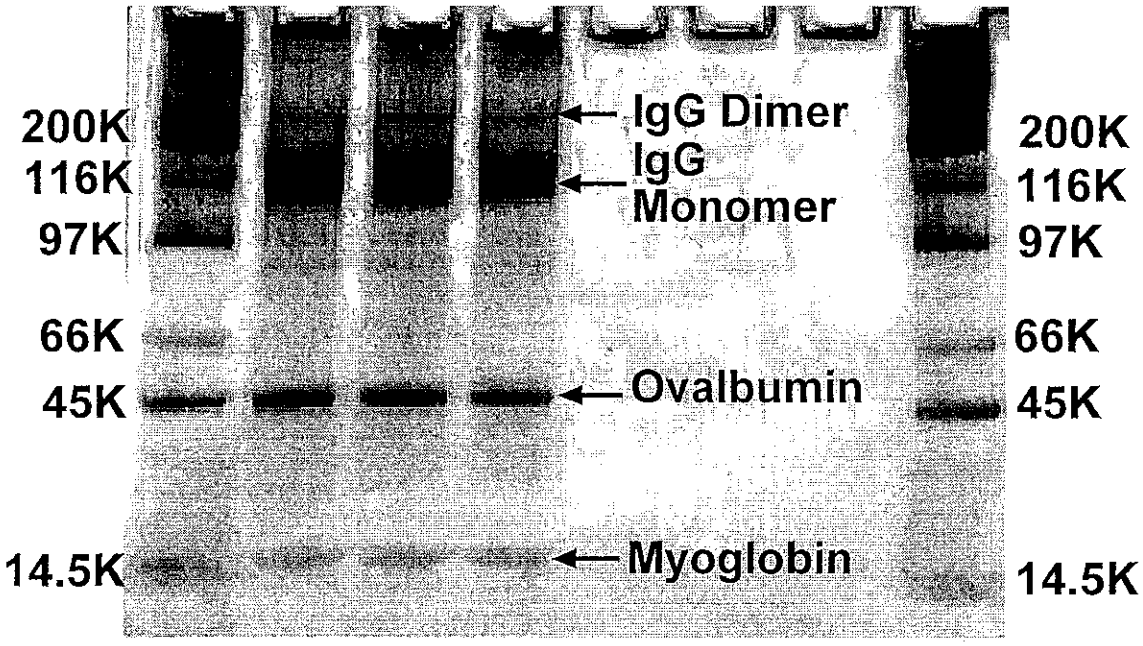
Clearly, peak retention time from Injex samples is equivalent to the Control and Syringe samples. Peak area from the Injex samples is equivalent to the Control. However, the Syringe samples are statistically different from both the Control and the Injex. At this time, there is no good explanation for this observation. According to Dalton's law, matter can neither be created nor destroyed. The higher values for the Syringe system would suggest that matter is being created.

Polyacrylamide gel electrophoresis (PAGE)

Aliquots of the Molecular Weight Standards analyzed by HPLC were diluted appropriately and loaded with precision pipettors in 20 μg amounts on a 4-20% Tris-Glycine gradient gel. Broad spectrum gel electrophoresis standards were run in Lanes 1 and 8. Figure 3 is a scan of the gel following silver staining. Molecular weights of the PAGE standards are shown (1K = 1000 Daltons) and the Control, Injex, and Syringe samples are shown in Lanes 2, 3, and 4, respectively. Due to the significant mass of Thyroglobulin, it was prevented from entering the resolving portion of the gel and is therefore not visible. Additionally, Cyanocobalamin does not contain moieties capable of staining with the silver reagent and is therefore not visible.

Clearly, the Injex sample is equivalent to both the Control and Syringe samples. The density of the bands is visually equivalent, the major bands appear to have their assigned molecular weights (as determined from the gel standards), and no degradative products of any of the compounds are visible. Additionally, molecular charge characteristics have been retained. Any effect would have caused a shift in mobility of the compounds within the electrical field applied to the gel.

Figure 3: 4-20% Tris-Glycine Gel of Molecular Weight Standards in the Control, Injex, and Syringe samples vs. the Gel Standards



Interestingly, the Syringe samples don't appear to yield bands with greater grayscale density than the Control or Injex, yet these are the same samples that yielded higher Peak Area in the HPLC analysis. If anything, there is lower density in the Syringe sample (note the lighter ovalbumin band as an example).

Thyroxine immunoassay analysis

Two dilutions of the Control, Injex, and Syringe samples were made into alkaline buffer and characterized by immunoassay on the Abbott AxSym analyzer. The results (Mean µg/dL ± SD) are shown in Table 4.

Table 4: Thyroxine immunoassay results

Dilution	Control Average Thyroxine	Injex Average Thyroxine	Syringe Average Thyroxine
1	4.6 ± 0.2	3.8 ± 0.2	5.8 ± 0.6*†
2	5.7 ± 0.2	4.9 ± 0.3	9.7 ± 1.1*†

** Dunnett's t, p<0.05 vs. the Control*
†Students-t, p<0.05 vs. Control and Injex

As with the HPLC study, the Injex is statistically equivalent to the Control. However, the Syringe sample displays higher values than both the Control and Injex. Again, there is no good explanation.

CONCLUSIONS

In all analyses, samples delivered by the Injex are equivalent to Control solutions. These Control solutions were pristine stock solutions that had encountered neither the Injex, nor a syringe. Based on the HPLC and PAGE data, the use of the Injex does not cause loss of molecular structure, molecular weight, or molecular charge characteristics. The Thyroxine immunoassay shows that use of the Injex does not cause any change in immunoreactivity which, after all, is a function of molecular structure, conformation, and other physical characteristics.

This study is limited since it does not examine all pharmaceuticals that might potentially be injected with the Injex. However, this study was not intended to answer the question of molecular damage for all potential pharmaceuticals. Rather, the intent was to demonstrate a lack of molecular damage in a set of model compounds. This has been achieved. Further molecular damage and efficacy studies with specific pharmaceuticals are recommended as part of the overall marketing plan for the Injex.

APPENDIX

SUMMARIZED RAW DATA AND JMP STATISTICAL ANALYSES