THE REMOVAL OF CREMOPHOR® EL FROM PACLITAXEL FOR QUANTITATIVE ANALYSIS BY HPLC-UV

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A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
Of the Requirements for the Degree of Master of Science

Department of Chemistry and Biochemistry

University of North Carolina Wilmington

2005

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ABSTRACT

The most common technique for analysis of drug substances is high performance liquid chromatography (HPLC) using a variable wavelength detector (UV). Cremophor® EL (ethoxylated castor oil, CrEL) is a non-ionic emulsifier that is used to assist in the solubilization of hydrophobic drugs such as the antineoplastic agent, paclitaxel. However, the molecular weight distribution of CrEL creates difficulties in the analysis of potency and related substances of hydrophobic drugs via UV detection.

Using a precipitation method, CrEL was selectively removed from the paclitaxel by pre-treating the injectable dosage form with mercuric chloride and analyzing the filtered extract by HPLC-UV. The removal of CrEL from the injectable dosage form of paclitaxel by precipitation with mercuric chloride resulted in a solution that was suitable to quantitate active and related substances by HPLC-UV. The precipitate was then characterized by nuclear magnetic resonance, infrared spectrophotometry and x-ray diffraction to elucidate its mechanism of action. The combination of these analyses provided sufficient evidence that the complex formed between CrEL and mercuric chloride is from the coordination of the mercury atom to the ether oxygens present in the CrEL.

ACKNOWLEDGEMENTS

Without the financial contributions and use of facilities at aaiPharma, this thesis would have never come to fruition. I am most grateful for aaiPharma's generosity and their continued support. I would also like to express my inestimable indebtedness to Dr. Dan DeVido for making this thesis a reality. His ability to theorize and orchestrate the challenges encountered in the pharmaceutical industry is truly admirable. I would like to thank Dr. John Tyrell, who without his enthusiasm and unremitting insight, my long-gestated work may have had no end. Dr. Tyrell's sincerity, humor, and unwavering belief were more motivating than he will ever know. I am grateful for Dr. Robert Hancock who provided me with unbounded assistance. I also want to thank Dr. Bob Whittle and Linda Whittall for all the insight, suggestions, and X-ray work performed in this project. Finally, I find difficulty in expressing my gratitude to Dr. Pam Seaton who munificently provided me with the technical support and assistance needed to make this research successful.

DEDICATION

I dedicate this thesis to my newborn daughter, Malina.

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INTRODUCTION

For a drug to be marketed in the United States it must be effective, convenient, and most importantly, safe. Since parenterals are administered by injection, the quality of the finished drug product must be significantly higher than other dosage forms (Avis, et al., 1992). The standards set forth by the U.S. Food and Drug Administration (FDA) and United States Pharmacopoeia (USP), a private organization that works in collaboration with the FDA, ensure that the quality (identity, purity, quantity, and potency) of a drug product is as stated by the manufacturer (USP, 2005). Federal law requires manufactures, processors, and packagers of drugs to take a proactive approach to ensuring that their products are of the highest quality. The current Good Manufacturing Practices (cGMP), which are regulations promulgated by the FDA's Code of Federal Regulations (CFR), provide the framework for companies to minimize or eliminate contamination and erroneous data. The FDA's guidelines and standards for determining the quality of products generally include a series of methods that must be performed by an analytical laboratory to determine their chemical composition, both quantitatively and qualitatively.

In addition to cGMP, cost and convenience of administration are major concerns that affect the pharmaceutical industry. Analytical methods must be developed so that the drug products can be tested in their finished, ready-to-deliver, product form. However, some drug products must be manufactured using a two-vial system where one vial contains the active pharmaceutical ingredient and the other vial contains the solubilizer or excipient. The two vials are analyzed separately and once the products are released, the consumer mixes the vials immediately before use. This two-vial system is mandated

because there has not been a suitable technique or method developed that allows for the components to be accurately analyzed when combined or due to the instability of the drug product. Analytical laboratories must continually develop novel methods that allow for the quality of drug products to be determined in the presence of its excipients.

The primary goal of new drug formulations and reformulation of established drug products is to enhance bioavailability and effectiveness of the active pharmaceutical ingredient (API). The recent advances in novel drug formulations have substantially increased the amount of research and development required to bring a product to market. Costs are further escalated because of certain unseen challenges that are encountered when developing new analytical methods that determine the drug's quality and stability.

The analytical tool that is used most extensively in determining the quality of drug products is high performance liquid chromatography (HPLC) (Snyder, 1997). Using HPLC methodology, the potency of compounds can be rapidly established by comparing a known reference standard with the analytes of interest. However, if peaks in a chromatogram attributed to the active or its related substances or degradents elute at the same retention times of the excipients or solubilizers inaccurate results will be obtained. Therefore, HPLC methods that accurately and specifically quantitate finished drug products by selectively removing interfering excipients with a sample preparation before the analyses are needed. Some techniques, like liquid-liquid extraction, preparative HPLC purification, and solid phase extraction (SPE) have been used to remove interfering excipients prior to HPLC analysis. Techniques like liquid-liquid extraction and SPE that depend on solvent selectivity can not be used for some excipients because they are soluble in such a diverse range of solvents.

The major challenge in analyzing hydrophobic parenteral dosage forms by HPLC-UV is the interference of the many peaks produced by the large molecular weight distribution of excipients such as CrEL. The retention times of the peaks due to solubilizing agents are sometimes the same as the related impurities and degradation products present in a drug product causing inaccurate results to be obtained. The analysis of newly derivitized formulations of hydrophobic drugs such as paclitaxel, for example, interfere with its excipient making the peaks due to the active and its related compounds very difficult to quantitate by HPLC-UV.

Paclitaxel (Taxol®, Bristol-Myers Squibb Company) is one drug product that has posed a challenge from an analytical standpoint. Paclitaxel is a potent natural product that is harvested from the bark of the Pacific Yew tree, *Taxus brevifolia*. Its chemical structure is depicted in Figure 1.

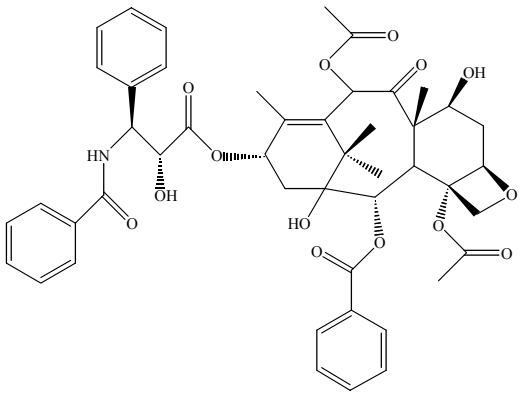


Figure 1. Molecular structure of paclitaxel.

Paclitaxel is an antineoplastic agent that is indicated for the treatment of non-small cell lung cancer, refractory breast cancer, epithelial ovarian cancer, and potentially for multiple sclerosis and Alzheimer's disease (Halford, 2005). In 1977, the National

Cancer Institute (NCI) was faced with significant obstacles pertaining to the formulation of paclitaxel. The major challenge in developing its formulation was due to the absence of ionizable functional groups and a bulky fused ring system, which makes paclitaxel poorly soluble in aqueous media (less than 0.1μg/ml)(Suffness, 1995).

There are a variety of vehicles that have proven to be effective in transporting hydrophobic drugs within the human circulatory system. Mostly, the use of nonionic solubilizers has been applied by investigators in cancer chemotherapy because of their effectiveness. One of the non-aqueous formulations proposed and accepted by NCI for paclitaxel was a formulation consisting of the emulsifier/solubilizer, polyethylene glycol triricinoleate 35 (Cremophor® EL, BASF) and ethanol (Suffness, 1995). CrEL is an FDA approved amphiphilic polymer that is used as a solubilizer for the delivery of a variety of hydrophobic parenteral drugs. It is a viscous liquid that is formed by the reaction of ethylene oxide with castor oil at a molar ratio of 35:1 (BASF, 1998). A major component (80%) of CrEL consists of a hydrophobic glycerol-polyethylene glycol ricinoleate which is shown in Figure 2 (BASF, 1998).

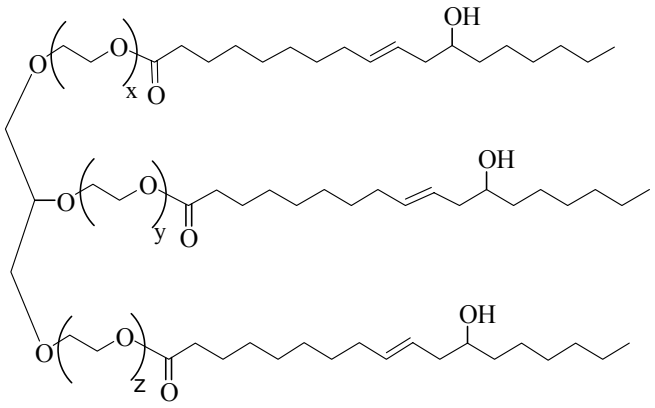


Figure 2. Molecular structure of CrEL. Σ(x,y,z) = 30-33

The remaining 20% of CrEL contains 7% polyethylene glycol, 10% glycerol polyoxyethylene, and 3% non reacted castor oil (Meyer, et al., 2002). Nonionic surfactant molecules, such as CrEL, have no formal ionic charge. Its polarity arises from the combined effects of the many ether linkages joined to the nonpolar moiety (Yalkowsky, 1999). CrEL, like most surface active agents, contain flexible nonpolar alkyl chains that are oriented toward the hydrophobic core where the drug resides. The polar region forms the hydrophilic corona that hydrogen bonds with the water molecules. A schematic of this arrangement of CrEL molecules forming a micelle around a paclitaxel molecule is illustrated in Figure 3. The threshold concentration above which micelles form, that is, the critical micelle concentration (CMC) for CrEL in an aqueous solution was previously determined by cryogenic temperature transmission electron microscopy (cryo-TEM) to be 97-102 μg/mL (Szebeni, et al., 2001).

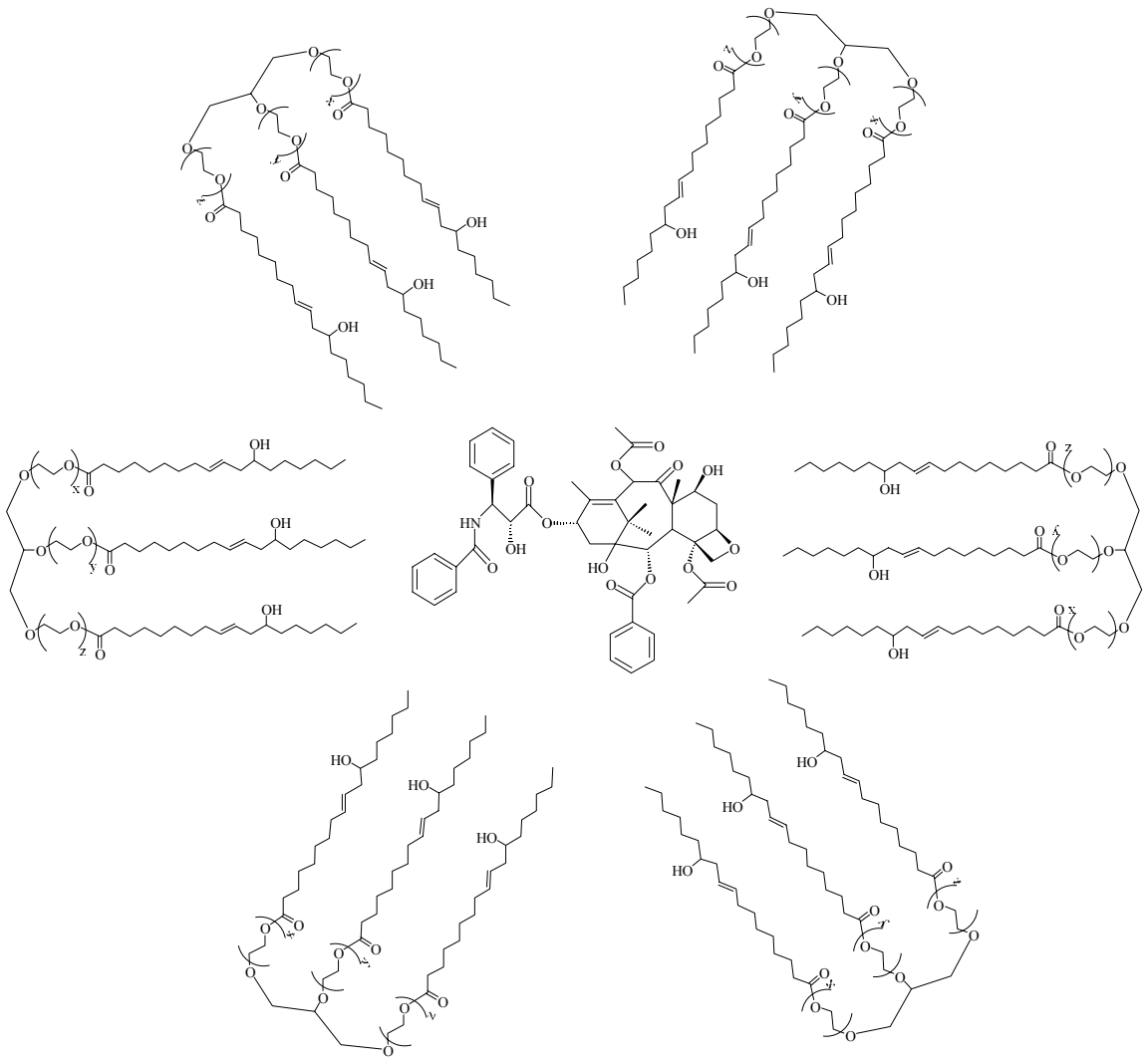


Figure 3. A schematic of the micellar arrangement of CrEL surrounding a paclitaxel molecule. (Note: Ethylene oxides in parenthesis are 10-11 units in length.)

One of the unique properties of CrEL that BASF chemists discovered is that it is soluble in the presence of many electrolytes. Most all salts and acids, provided their concentrations are not too excessive, will not cause CrEL to precipitate out of solution (BASF, 1999). However, mercuric chloride and compounds that contain phenolic hydroxyl groups, such as resorcinol, tannin, and phenol may cause precipitation at certain concentrations (BASF, 1999). Based on these unique properties, a method for CrEL-containing drug products can be developed that allows CrEL to be removed by precipitation before the analysis of parenteral drug products by HPLC-UV. The pre-treatment of an injectable hydrophobic drug product containing CrEL with mercuric chloride will allow for the analysis to proceed with negligible interference from the CrEL.

When CrEL is subjected to a saturated ethanolic solution of mercuric chloride, a dense, white precipitate is formed. When a majority of the CrEL is precipitated out of a solution in this manner, mercuric chloride may be selective enough to complex with and remove the interfering excipient from the injectable dosage form. Once CrEL is removed, the drug product can be analyzed by HPLC-UV with no interference from the CrEL.

This novel precipitimetric approach to analyzing paclitaxel in a one vial system can also be applied to other hydrophobic drugs containing CrEL. In order to apply this precipitimetric method to other CrEL-containing finished drug products, it is critical to investigate mercuric chloride's mechanism of action. If mercury specifically complexes certain functional groups present in both the solubilizer and the API, the method would be rendered less suitable. The precipitation reaction was studied using a variety of

techniques including qualitative analysis, Fourier transform infrared spectrophotometry (FTIR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and X-ray diffraction.

A rapid qualitative analysis was performed to explore the potential sites where the reaction might take place. In the qualitative analysis study, compounds that represented key functional groups of CrEL were reacted with ethanolic mercuric chloride to determine what part of the CrEL molecule is interacting with the mercuric chloride. Certain functional groups that were candidates for the mercuric chloride reaction were alcohols, ethers, and olefins.

The most valuable instrumental technique employed to elucidate the mechanism of interaction was ^{1}H-NMR and ^{13}C-NMR. The goal of the NMR studies was to support the theory of mercuric chloride complexing with the ether units present in CrEL. Special attention was given to the changes that took place in the chemical shifts when CrEL reacted with mercuric chloride.

Supporting data was collected using techniques in FTIR and X-ray diffraction. If the complex is formed between the ethers and the mercury ion, then changes in the degrees of motion for the ether bonds should be prevalent in the IR spectra. Furthermore, an X-ray diffraction pattern of an isolated crystal of the mercury-complexed CrEL would be sound evidence to support the proposed theory of this precipitation reaction.

Since the discovery of paclitaxel's use as a chemotherapy drug, there have been a variety of studies pertaining to its purification, preparation and application. Most of the recent published data addresses the application and the resulting clinical effects of the intravenously administered paclitaxel and/or CrEL (Ghassempour, et al., 2003, Gelderblom, et al., 2002, Meyer, et al., 2001, Kunkel, et al., 1999, Gelderblom, et al.,

1999, Huizing, et al., 1998, and Sparreboom, et al., 1996). One method that particularly addresses the chromatographic analysis of paclitaxel and CrEL was developed by Lillian Shao and David Locke (Shao and Locke, 1997). The method they developed utilizes micellar electrokinetic capillary chromatography to separate paclitaxel and related taxanes (Appendix B) in the bulk drug as well as its injectable dosage form.

More recently, Ciutaru, et al. developed and validated a HPLC method that evaluates paclitaxel's related taxanes in its finished injectable form (Ciutaru, et al., 2003). Ciutaru, et al. obtained chromatographic data that showed baseline resolution of the related substances in the presence of CrEL. It was noted that there was difficultly in the separation of related taxane peaks in the sample that contained CrEL. The method was optimized and validated so that the peaks of the CrEL did not interfere with the related taxanes.

The methods proposed by Shao and Locke and Ciutaru, et al. may be suitable for analyzing the pure natural product, paclitaxel, in the presence of CrEL. However, the peaks in the chromatographic analysis due to derivitized formulations of paclitaxel interfere with the peaks of CrEL, making quantitation of the active and related compounds difficult to analyze. In HPLC analysis, as illustrated here, it is paramount to analyze finished products without interference from the excipient matrix.

EXPERIMENTAL

Equipment

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance DRX400 multinuclear spectrometer. All the compounds used in the NMR analysis were dissolved and read in d_6-acetone. The spectra were acquired and processed with XWINN-NMR® software.

Infrared (IR) absorption spectra were obtained with a Mattson Genesis Series Fourier transform infrared (FTIR) spectrophotometer and then processed with Winnfirst® software.

X-ray crystallographic data was obtained on a Nonius Kappa CCD diffractometer and a Siemens D500 X-ray powder diffraction instrument. All measurements were carried out at 23 ˚C using monochromated Cu (K_α) radiation (λ=1.54178Å). The data was processed with Maxus Solution® software.

The high performance liquid chromatography (HPLC) components used were a Hewlett-Packard 1100 autosampler, Hitachi L-7100 quaternary gradient pump, and a Waters 2487 dual wavelength absorbance detector. An auxiliary HPLC system was used that consisted of the same components as listed above except a Waters 717Plus Autosampler was used. The column used throughout was a Phenomenex Curosil, 250mm x 4.6 mm, with a 5 μm particle size bonded with pentafluorophenyl (PFP) groups. The column was maintained at ambient temperature. Separations were achieved using a mixture of acetonitrile and water at 40% acetonitrile for mobile phase A and 70% acetonitrile for mobile phase B. A gradient elution, flowing at 1.0 mL/min (except where noted), was performed from 0 to 25 minutes, 90% mobile phase A, from 25 minutes to 60

minutes, 100% mobile phase B, and from 60.1 minutes to 70 minutes 90% mobile phase A. These steps in the gradient, which significantly increased the runtime were critical in eluting the excipient, CrEL and other retained impurities from the precipitation reaction. Analytes were detected at 228 nm. The injection volume was 10μL. All chromatographic data was acquired and processed with Waters Millennium® 4.0.

Chemical Information

See Appendix A for the list of chemicals and manufacturers used for each type of analysis/study performed.

Procedure

Sample Preparations

The stock solution of paclitaxel was prepared in a similar manner to the formulation of the injectable dosage form that is currently marketed (PDR, 2005). Paclitaxel was dissolved in a 50% v/v solution of CrEL in ethanol at a concentration of 25 mg/mL. Working standard solution (1) was prepared by diluting the stock solution in ethanol to obtain a final concentration of 0.5 mg/mL, which is approximately the final concentration (0.3 mg/mL to 1.2 mg/mL) of the drug when being administered by intravenous infusion. Working solution (2) was prepared at the same concentration as (1) except using an intermediate solution containing saturated mercuric chloride in ethanol, centrifuging and immediately filtering the supernatant with a Gelman Acrodisc 0.45 μm borosilicate glass hydrophilic polypropylene (GHP) filter before diluting to the same concentration (0.5 mg/mL). The pellet remaining in the centrifuge tube was transferred to

a Buchner funnel and washed with ethanol then dried over silica gel, under vacuum, for three days.

Polyethylene glycol 400 (PEG 400) was dissolved in ethanol to furnish a 50% v/v solution. To a tube containing 5 mL of this solution, 10 mL of saturated ethanolic mercuric chloride was added and mixed vigorously. To a tube containing 5 mL of triglyme, 10 mL of saturated ethanolic mercuric chloride was added and mixed vigorously. The resulting precipitates from each reaction were washed and dried like the CrEL precipitate.

Rate of Precipitation Reaction

To estimate the rate of the mercury- CrEL reaction, a preliminary time study was performed. A 50:50 mixture of saturated ethanolic mercuric chloride and CrEL was vortexed for 30 seconds and immediately centrifuged at 5000 rpm for ten minutes. The supernatant was decanted into a test tube, capped, and placed at ambient laboratory conditions for 48 hours. No further precipitation was evident. Based on these findings, the reaction of mercuric chloride in ethanol with CrEL was determined to be immediate.

Preparations for NMR Analysis

For the ^{1}H-NMR analysis, the dried CrEL precipitate was weighed into d_6-acetone to obtain a concentration of 20 mg/mL. A preparation of the unreacted CrEL was prepared similarly. The ^{13}C-NMR analyses of the same components were obtained in the same solvent but at a concentration of 50 mg/mL. The same experiments were run with PEG 400 at the same concentrations as CrEL. The d_6-acetone peak was used for calibration (2.04 ppm for ^{1}H-NMR spectra and 29.94 ppm for ^{13}C-NMR spectra). The

^{1}H-NMR integral regions were relative to the calibrated acetone peak that was arbitrarily set at 1.00.

Preparations for FTIR Analysis

Ten milligrams of the CrEL precipitant was triturated intimately with 4 drops of mineral oil (nujol) in a mortar. The mixture was suspended between two sodium chloride plates and the spectrum was obtained in a blank subtracted background. Ten milligrams of the triglyme precipitant was prepared similarly. The spectrum of the unreacted CrEL and triglyme were also obtained under the same conditions as the precipitant.

Preparations for X-ray Analysis

Using a microscope, a representative crystal was selected from the dried CrEL precipitant and mounted on a glass fiber with epoxy cement. The data was collected by ω-2θ scans.

Preparations for HPLC Analysis

A mixture of thirteen related taxanes was commercially purchased from Hauser Chemical Research Company. The list of the taxanes and their structures is located in Appendix B. The taxanes in this mixture represent the most commonly found related substances present when analyzing the bulk, natural drug product, paclitaxel. For the recovery study, working solution (3) was prepared with paclitaxel at 0.5 mg/mL and spiked with the thirteen taxane mixture at 1.0% using the intermediate ethanolic mercuric chloride solution described above. A control of paclitaxel at 0.5 mg/mL, working standard (4), was spiked with the thirteen taxane mixture in absence of CrEL. A blank preparation of 50% v/v CrEL in ethanol and saturated ethanolic mercuric chloride was made in a centrifuge tube. The resulting mixture was centrifuged at 5000 rpm for 10 min.

A portion of the supernatant was decanted into a GHP syringe filter, filtered, and diluted to the same concentration as the final working standard solutions.

RESULTS AND DISCUSSION

Over the past several years, there have been a multitude of studies published that evaluate the complexation of metal ions with ethers and etheral polymers (Maunu, et al., 1987, Varshney, et al., 1991, Doxsee, et al., 1993, Costero, et al., 1998, Shou, et al., 1999). A majority of these reports, however, have been of crown ether complexes and other non-polymer, well-characterized molecules containing ethers. Nonetheless, their results provide insight into the use of metal ions to complex organic compounds.

The etheral polymer, CrEL that is used in the preparation of the hydrophobic drug, paclitaxel has to be selectively removed so valid quantitative and qualitative chromatographic data for the finished product can be obtained. A unique property of CrEL that will allow for its removal from the parenteral formulation of paclitaxel, is by selectively precipitating with metal ions.

Precipitimetric Analysis

BASF, the manufacturer of CrEL, reports that CrEL is unstable in solutions containing mercuric chloride in addition to phenolic hydroxyl compounds (BASF, 1997). A series of experiments was performed to determine the most effective agent for removing CrEL from a solution by precipitation. A solution of CrEL was prepared at a 50% v/v dilution in ethanol to mimic the solubilizing concentration in finished dosage forms of paclitaxel. The metal ions in addition to mercury that were mixed with CrEL are listed in Table 1 (manufacturers are listed in Appendix A). When saturated solutions of

the metal ions listed in Table 1 were added to the 50% v/v ethanolic CrEL solution, most yielded no visible precipitate. If a complex did form with these metals, the metal-complex was soluble and was not further investigated due to the lack of a precipitate. BASF also reported that some organic substances, such as resorcinol and phenol, may cause precipitation. Experimentation with these phenolic hydroxyls yielded no observable result. The use of organic substances as precipitating agents was also not pursued further.

Table 1. Compounds that were mixed with 50% v/v CrEL in ethanol to investigate alternative precipitating agents.

Compound	**Insoluble precipitate with CrEL**
Mercuric chloride	Yes
Mercuric iodide	No
Mercuric bromide	Yes (slight)
Barium chloride	No
Mercuric acetate	Yes (slight)
Resorcinol	No
Phenol	No
Ferric chloride	No
Lanthanum chloride	No
Cobalt chloride	No
Copper chloride	No
Zinc chloride	No
Nickel chloride	No
Silver chloride	No
Stannous chloride	No
Magnesium chloride	No
Lead acetate	No
Lithium chloride	No

The basis of selectivity for metal ions in open chain ligands such as CrEL has previously been investigated from a thermodynamic and geometric standpoint (Hancock, 1992). In addition to favorable entropy effects, and to a much lesser degree, enthalpy effects, the stability of the CrEL-metal complex in ethanol is strongly related to the size of the metal ion being chelated. Typically, metals such as lead(II), mercury(II), and barium(II) that have an ionic radius greater than 1.0 Å, will strongly bind to CrEL where as metals that have ionic radii smaller than 1.0 Å will not. For the metal to form an insoluble complex, the metal ion must also maintain its bonds with the cation so as to produce a neutral ethanol-insoluble polymer. Based on the hard/soft acid base principle, mercury(II) is a soft acid that strongly binds chloride ions where as lead(II), a borderline hard acid and barium(II) a hard acid do not maintain their bonds to chloride ions during complexation (MacKay, et al., 2002). This theory supports the reason why the only metal salt that successfully yielded a sufficient quantity of precipitated CrEL was mercuric chloride.

The interaction of mercury and CrEL was initially thought to take place at the carbon – carbon double bond with the π electrons acting as the nucleophile, the mercury as the electrophile, and the chloride ions possibly as the leaving group thus forming a stable mercurinium compound, as illustrated in Figure 4 (Cotton, et al., 1988).

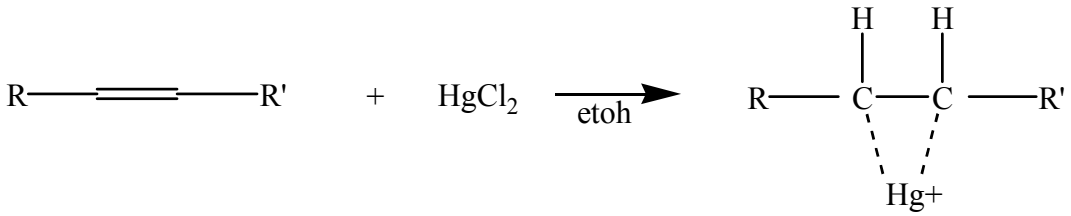
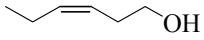


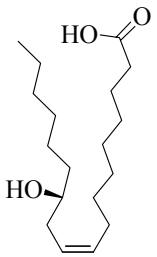
Figure 4. A schematic of an olefin complex, forming a mercurinium compound.

Another experiment was performed with short alkenes to investigate the possibility of the interaction illustrated in Figure 4. 1-Octene and 2-heptene were used as representative olefins and mixed with a saturated ethanolic solution of mercuric chloride. No visible precipitate was immediately obtained. The solutions were covered with a pin-punctured piece of foil and left for about 14 days. White crystals formed at the bottom of each of the reaction tubes. Using a microscope, a representative crystal was selected from the 1-octene reaction tube and mounted for an X-ray diffraction pattern. It was determined that the crystals that were in the tube were of the recrystallized mercuric chloride, thus proving this theory incorrect. Based on the result obtained with the 1-octene solution, the 2-heptene solution was not analyzed by X-ray diffraction. From this experiment it can be inferred that the reaction appears to be taking place with the ether linkages and not the olefin as initially thought.

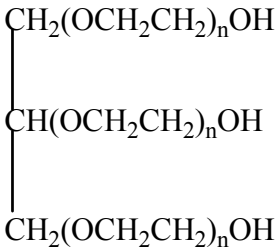
To determine which moiety of the CrEL structure was interacting with the mercury, a rapid qualitative analysis using compounds that represent the major functional groups was tested. The compounds that simulate the particular regions of CrEL, cis-3-hexen-1-ol, castor oil, glycerol ethoxylate, and polyethylene glycol (PEG 400) were used. There structures are depicted in Figure 5. They were each individually reacted with saturated ethanolic mercuric chloride solution. Of these four compounds, PEG 400 and glycerol ethoxylate were the compounds that, when mixed with mercuric chloride formed a white, insoluble salt.



Cis-3-hexen-1-ol



Castor oil (~90% cis-ricenoleic acid)



Glycerol ethoxylate, n=6-7

Polyethylene Glycol (MW 400), n≈9

Figure 5. Molecular structures of chemicals used for simulating the functional groups present in CrEL.

Other compounds that were investigated contained linear chains of ethers, like diglyme and tryglyme. Diglyme (Figure 6) proved unsuccessful in producing an insoluble precipitate where as triglyme, with one more ethylene unit, successfully complexed with mercuric chloride to form a white precipitate.

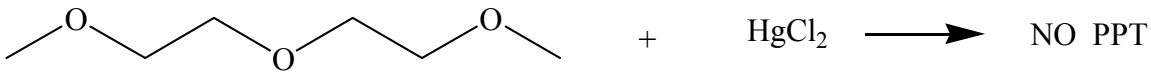


Figure 6. Reaction of diglyme and mercuric chloride.

Perhaps the reason diglyme was unsuccessful at complexing a mercury (II) ion is because this cation typically forms four-coordinate tetrahedral structures in monomers where diglyme has only three coordinating ether oxygens (Helm, et al., 2002). Furthermore, diglyme may not be large enough to geometrically allow the oxygens to chelate the mercury atom. Triglyme, with its all-ether structure, is very similar to crown ethers in that they dissolve many inorganic salts by chelating the cation (Figure 7) (Glymes, 2005). The white, solid complex that triglyme formed with mercury was insoluble in ethanol, much like that seen in CrEL and PEG 400.

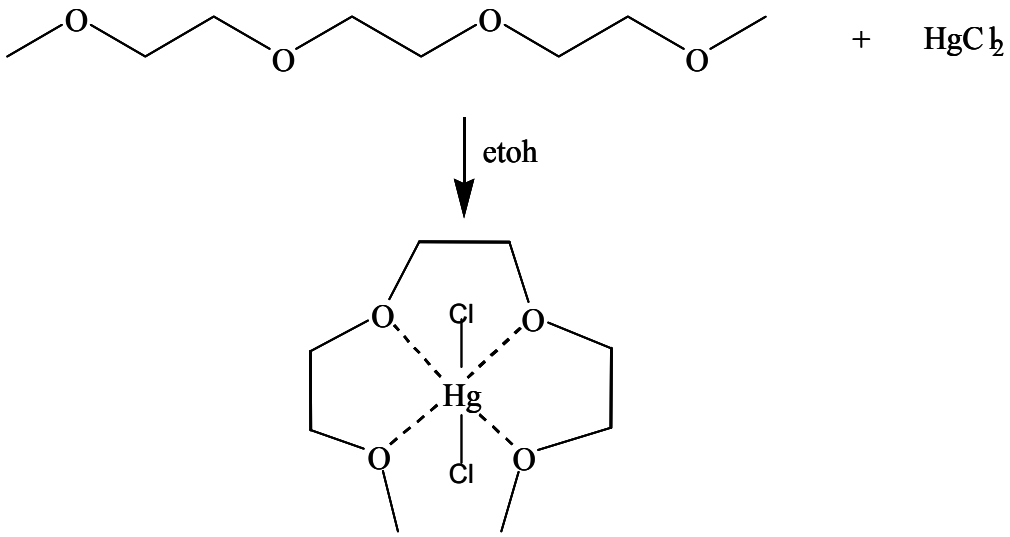


Figure 7. Reaction of triglyme and mercuric chloride to form an insoluble complex.

With mercuric chloride being the only salt that furnished a precipitate with CrEL in ethanol, the method was further optimized to separate the solid CrEL from the solution. Based on membrane selection guides, hydrophobic drug literature, and previous projects run using these solvents and compounds, a GHP syringe filter was selected for this method (Williams, et al., 2001). No filter study was performed on the method developed here. The precipitate that was formed from reacting mercuric chloride with CrEL was immediate when performed in ethanol and was easily removed from the solution by filtration.

NMR Analysis

Nuclear magnetic resonance spectroscopy was utilized to map the changes that took place in the carbon-hydrogen framework when CrEL complexed with mercury. The information derived from both the ^{1}H-NMR and ^{13}C-NMR spectra proved to be extraordinarily useful for determining the change in the chemical environment when mercury is complexed with CrEL. Even though a molecule the size and complexity of CrEL is difficult to fully interpret by NMR spectroscopy, many structural features can still be recognized.

The greatest amount of information that was gained from the NMR experiments was from the change in chemical shifts when the CrEL was complexed with mercuric chloride. When a spectrum of the pure polymer was obtained and compared to the mercury-complexed CrEL, the chemical (magnetic) environments of certain nuclei were effected. For instance, Figure 8 shows the overlay of the ^{1}H-NMR spectra of the raw polyethoxylated castor oil (CrEL) and the mercury-complexed CrEL. In regards to chemical shift changes present in these spectra, the most notable variation is the

absorption of the hydrogens adjacent to the electronegative ether oxygens. In the CrEL spectrum, the largest ether absorption is at 3.58 ppm and when mercury is complexed with CrEL, it shifts downfield to an absorption of 3.66 ppm. From this 0.1 ppm shift, it is evident that there is a removal of electron density surrounding the nuclei of the ether protons when mercury is present. Similar shifting patterns in ^{1}H-NMR were seen in previous spectroscopic studies focusing on the polyether coordination of alkali metal cations (Popović, 2000 and Isab, 1995). Another obvious difference in the two spectra is the absorption at approximately 2.84 ppm (Figure 8). When the CrEL was complexed with the mercuric chloride, filtered and dried, it caused the broad peak at 2.84 ppm to split into two distinct sharper peaks. The presence of residual water in raw, liquid CrEL could potentially be masking the –OH groups present in the CrEL. To correctly identify these absorptions as protons contributed from a functional hydroxyl group, another experiment was performed. The NMR tubes containing CrEL and the mercury-complexed CrEL that were dissolved in *d_6*-acetone, 50 μL of deuterated water (D_20) was added, mixed and the NMR spectra obtained (see Figure 9 and 10). The broad peak that was initially observed in CrEL is absent after the D_2O was added. As seen in Figure 10, the two peaks that were present at about 2.84 ppm are absent after the addition of D_2O indicating that the exchange of similar protons found in the raw CrEL was evident. The protons were replaced with deuterium as illustrated below, thus proving that these were –OH protons.

$$\text{R-O-H} + \text{D-O-D} \rightarrow \text{R-O-D} + \text{D-O-H}$$

The absorptions for CrEL and CrEL-Hg complex were also integrated relative to the solvent, *d_6*-acetone. The difference in integration between the two compounds is

negligible. See Appendix C for the spectra containing the integrated absorptions. No changes in chemical shifts of alkene protons (5.40 ppm), methylene protons of the ricenoleic fatty acids (1.20 ppm) and glycerol protons (4.85 ppm and 4.15 ppm) were observed. In conclusion, the ^{1}H-NMR data suggests that mercuric chloride is being complexed by the ether oxygens present in CrEL.

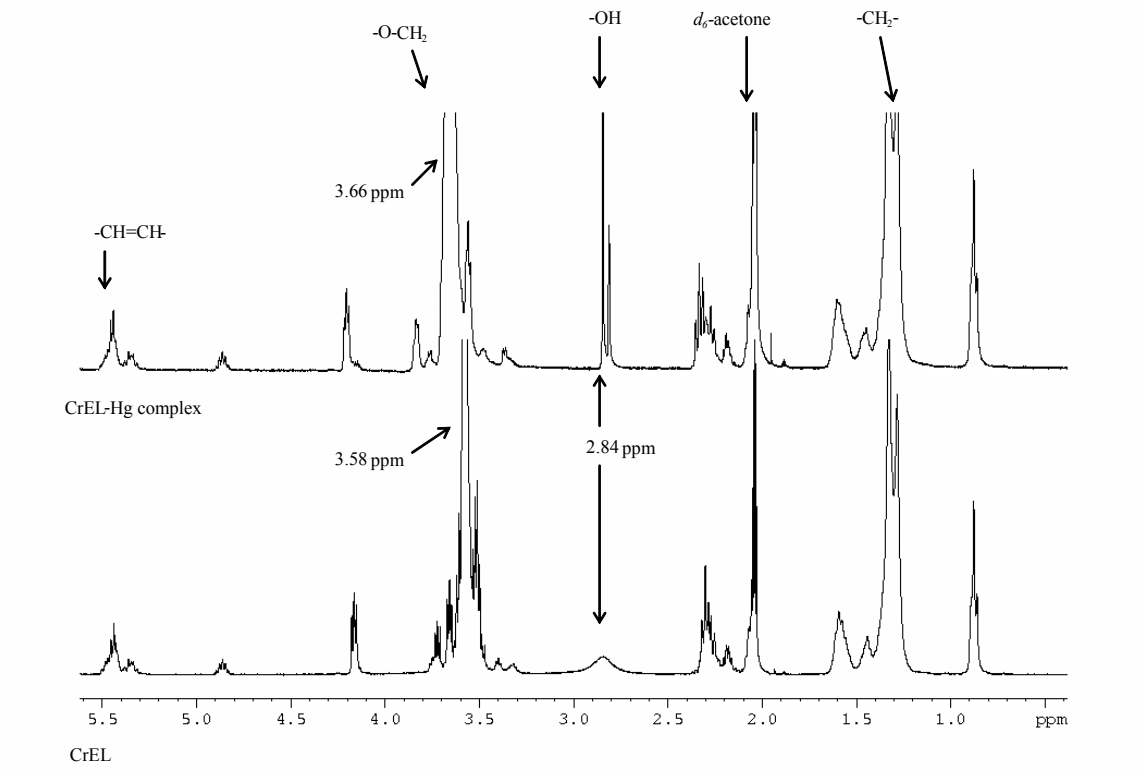


Figure 8. Overlay of CrEL (bottom) and Hg-complexed CrEL (top) ^{1}H-NMR spectra.

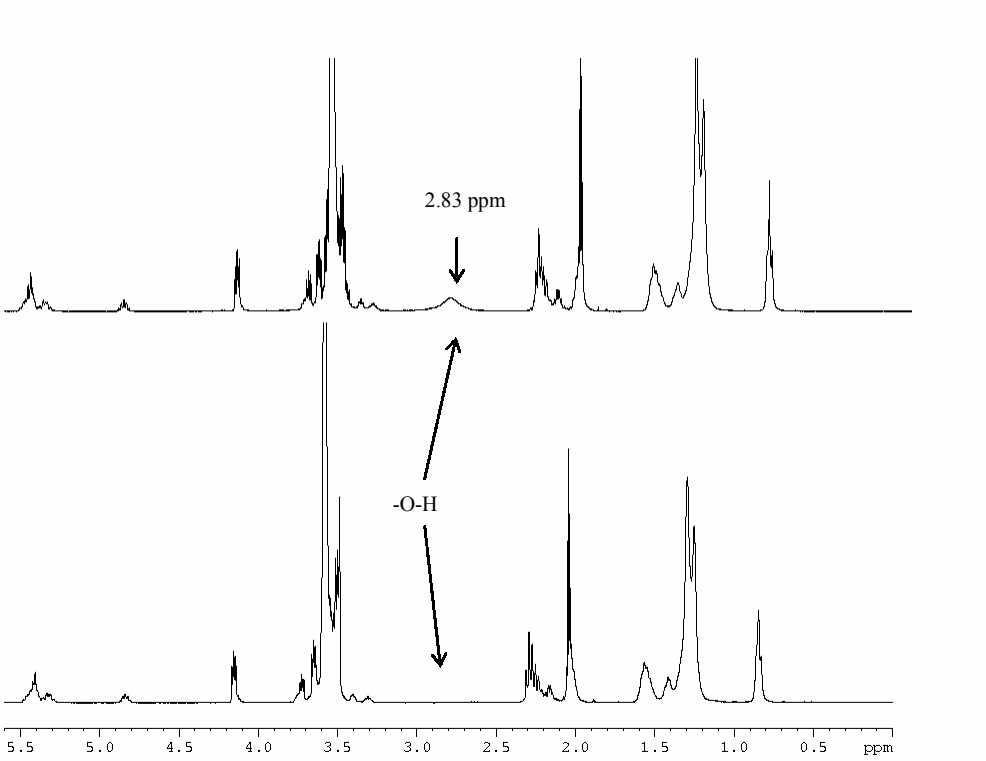


Figure 9. Overlay of CrEL(top) and CrEL-deuterated water (bottom) ^{1}H-NMR spectra.

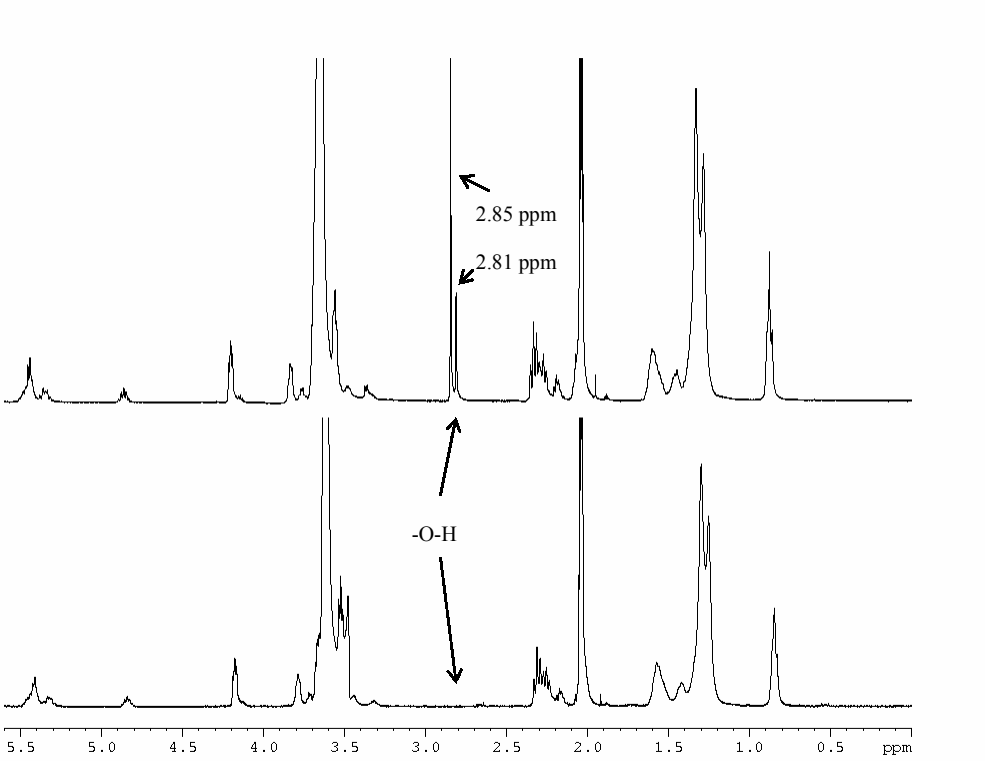


Figure 10. Overlay of Hg-complexed CrEL (top) and Hg-complexed CrEL-deuterated water ^{1}H-NMR spectra (bottom).

The ^{13}C-NMR spectra of CrEL and the Hg-CrEL complex provided further support of the results from the ^{1}H-NMR studies. The ^{13}C-NMR spectra furnished results that were unexpected at first in the region where carbons bound to oxygen absorb. The chemical shifts of carbons single-bonded to oxygen generally absorb between 80 ppm and 40 ppm. Figures 11-13 illustrate the specific absorptions of CrEL and the Hg-CrEL complex. In Figure 11, the region where the alkane carbons absorb (40 ppm to 20 ppm) is shown. As expected, there is no noticeable shifting that takes place in this region. Figure 12 illustrates the region where there is significant shifting when mercury is complexed with CrEL. In the area between 80 ppm and 60 ppm there are absorptions that arise from the carbons bearing oxygens in CrEL. The chemical shifts are upfield in the spectrum of the Hg-complexed CrEL, due to a shielding effect on the carbons adjacent to the ether oxygens and the esters present. The carbonyl carbons of CrEL, which absorb at approximately 174 ppm, do not show any effect when mercury is complexed as seen in Figure 13. The results obtained in the ^{13}C-NMR study further supports that the ether oxygens present in CrEL are complexing with the mercuric chloride.

A survey of the recent literature pertaining to ^{13}C-NMR shifts in mercury ether complexes was performed to investigate these shifts that are in contrast (opposite shifting) to those found in the ^{1}H-NMR spectra. One study by Gray and Duffey included a spectroscopic study that addressed how metallocrown ethers bind heavy metal cations such as Hg^{2+} (Gray and Duffey, 1995). The ^{13}C-NMR data was obtained for a penta-ether molybdenum compound that was complexed with mercuric chloride. When the mercury atom complexed with the ethers of the molybdenum compound, as further-evidenced by X-ray diffraction studies, the chemical shift of the carbons flanked by the ether oxygens

were strongly shielded and required a higher applied field to bring them into resonance. This upfield shift of the ether absorptions in the molybdenum complex supports the ^{13}C-NMR shifting of CrEL illustrated in Figure 12. Thus, this change in the chemical (magnetic) environment of the C-O region shows support that the CrEL is complexing with the mercury ion. The lack of changes in the alkene region between 150 ppm to 100 ppm of CrEL and Hg-complexed CrEL proves that the olefin present in CrEL is not involved in the complexation reaction.

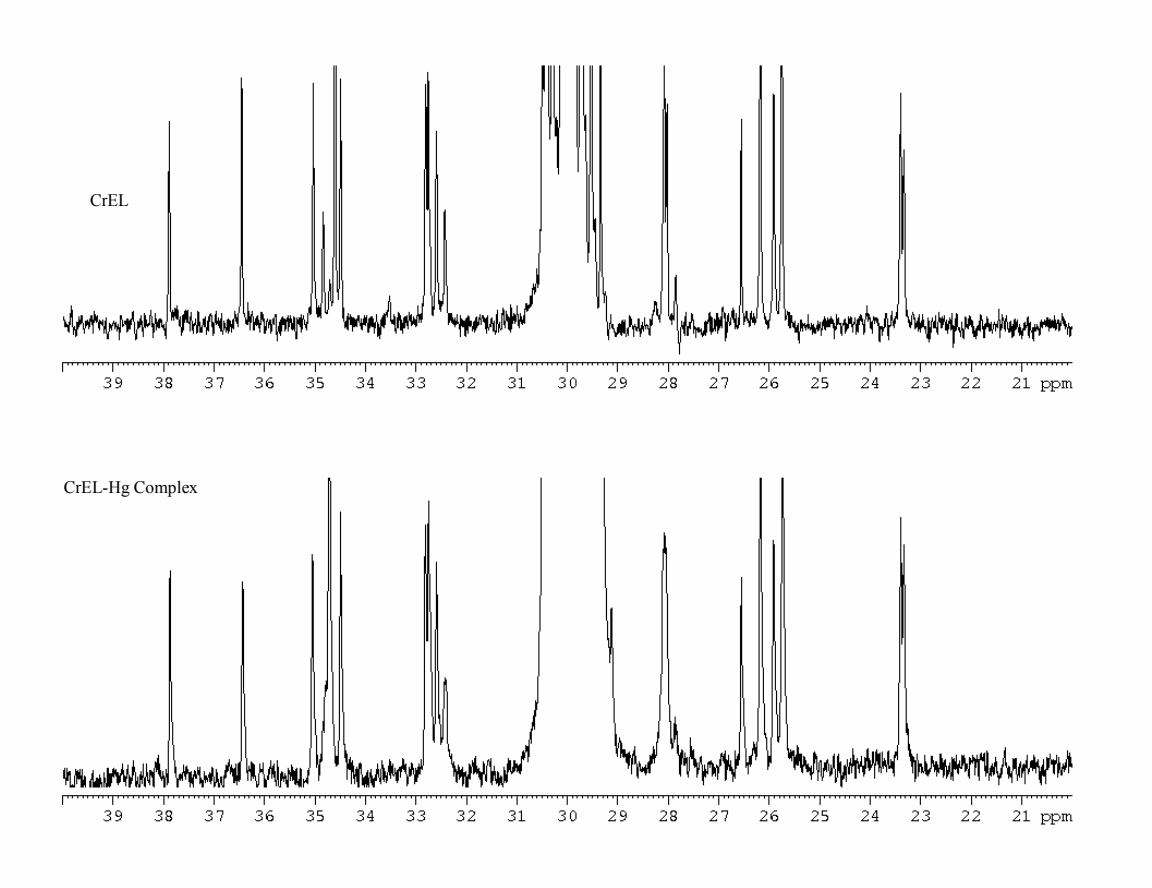


Figure 11. Overlay from 40 ppm to 20 ppm of CrEL (top) and CrEL-Hg complex (bottom) ^{13}C-NMR spectra.

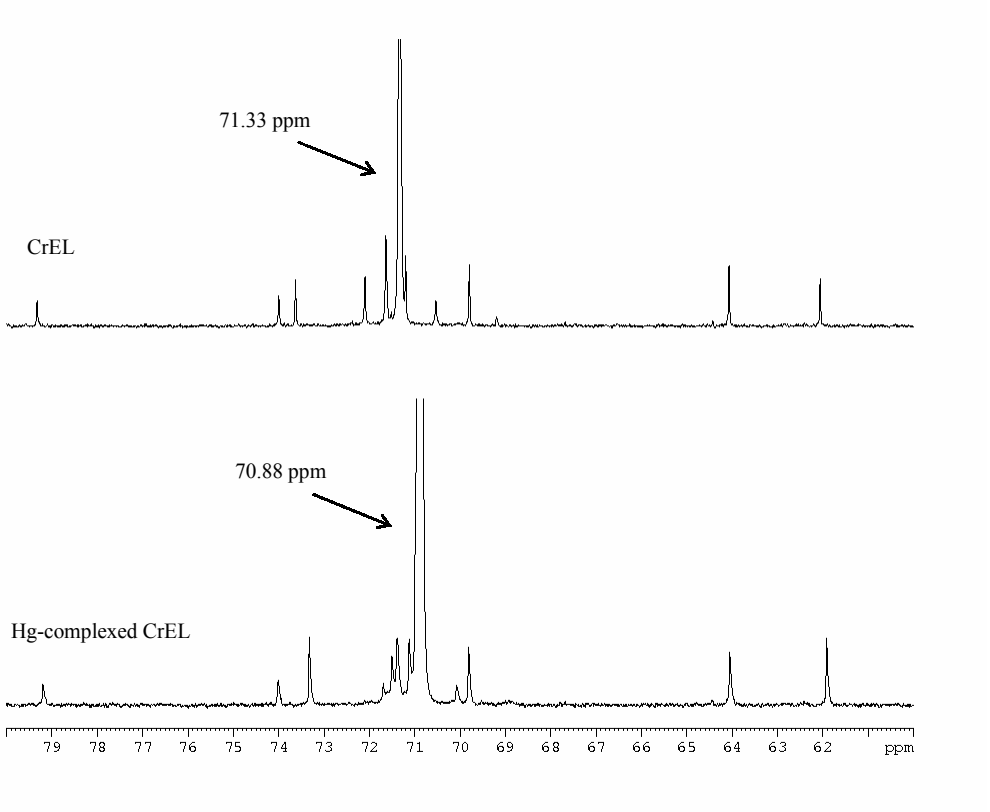


Figure 12. Overlay from 80 ppm to 60 ppm of CrEL (top) and CrEL-Hg complex (bottom) ^{13}C-NMR spectra.

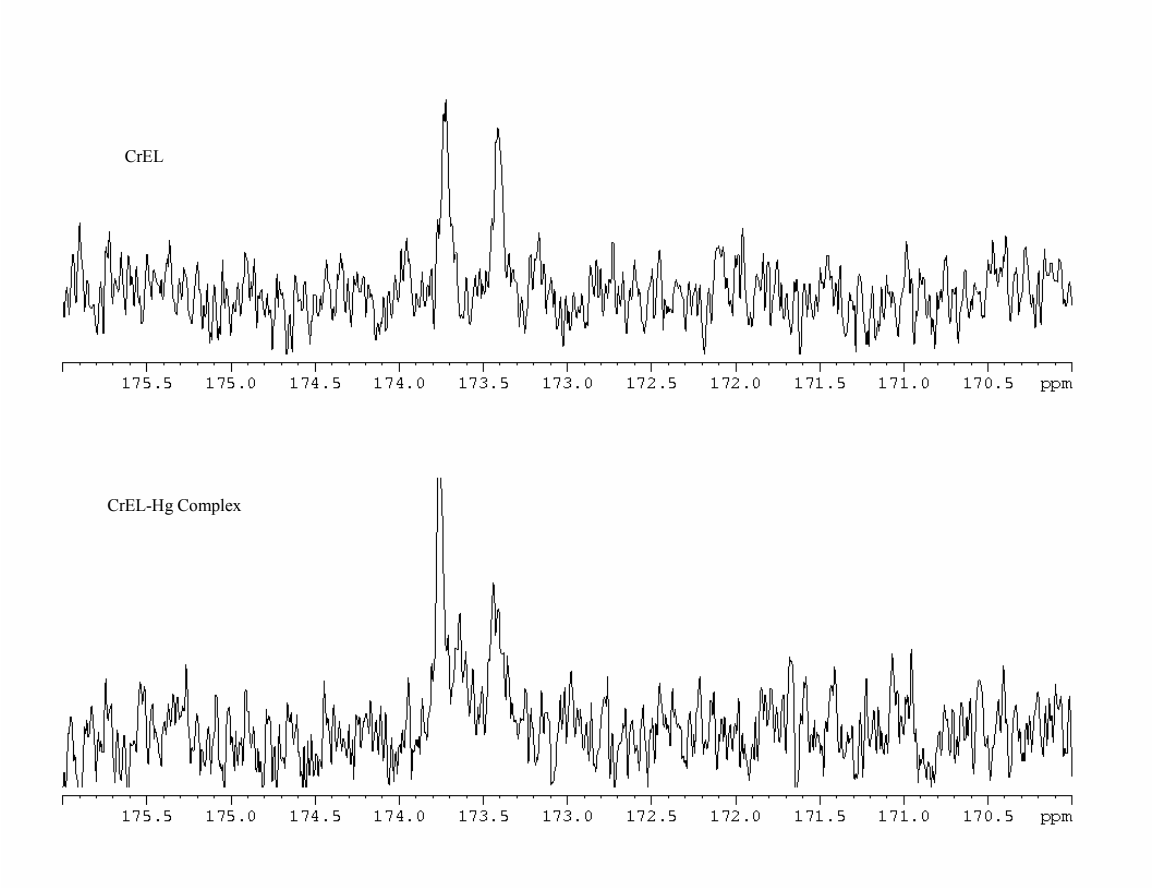


Figure 13. Overlay from 176 ppm to 170 ppm of CrEL and Hg-complexed CrEL ^{13}C-NMR spectra.

To further support the theory that the mercury is coordinating with the electronegative oxygens present in CrEL, another NMR spectroscopy experiment was performed on PEG 400, a compound that reacts with mercuric chloride in a similar fashion to CrEL. The spectra in Figure 14 are of PEG 400 and PEG 400 complexed with mercuric chloride, prepared in a similar manner as CrEL. The full scale spectra can be found in Appendix C. Figure 14 shows a significant shift of the ethylene protons when complexed with the mercury. The major absorption for PEG 400 at 3.57 ppm has shifted to 3.66 ppm when complexed with mercury. This deshielding effect corresponds to the results seen in the CrEL shifts (0.1 ppm shift). The peak also exhibits more individual peaks over a broader region as previously observed with the CrEL-Hg complex.

The data obtained from the ^{13}C-NMR spectra for PEG 400 was analogous to the data obtained for the CrEL. Figure 15 shows an overlay of the PEG 400 and the Hg-complexed PEG 400 ^{13}C-NMR spectra. There is an upfield shift in the ether absorption region from 71.31 ppm in the PEG 400 to 70.83 ppm in the Hg-complexed PEG 400. This -0.5 ppm change in the absorption is indicative of a shielding effect due to the complexation of mercuric chloride, which is analogous to the results obtained in the studies performed with CrEL.

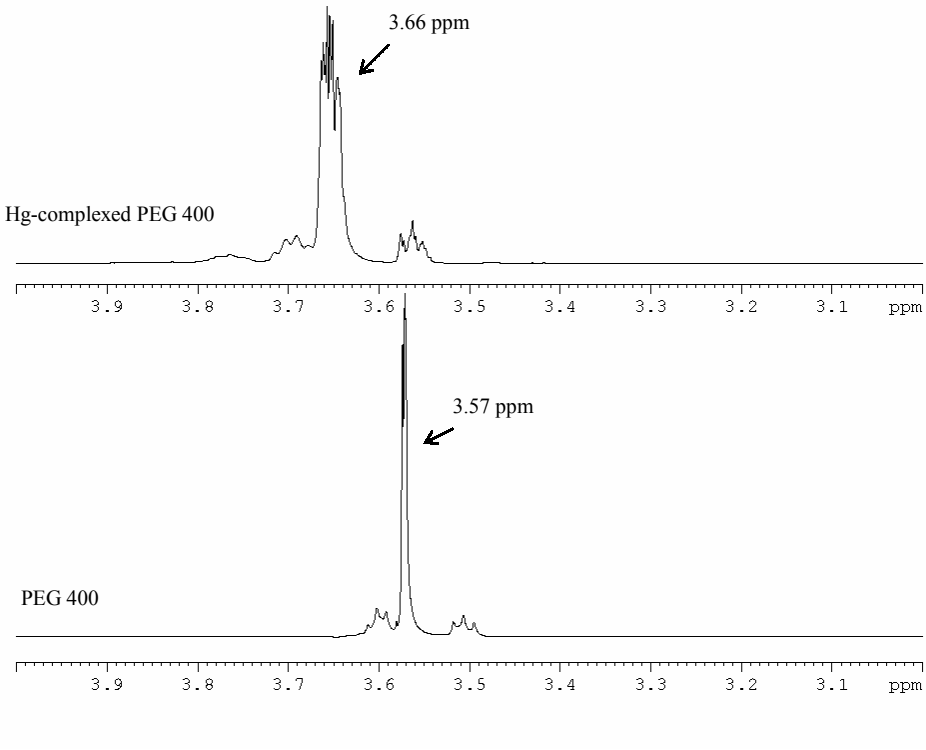


Figure 14. Overlay of PEG 400 and Hg-complexed PEG400

1H-NMR spectra.

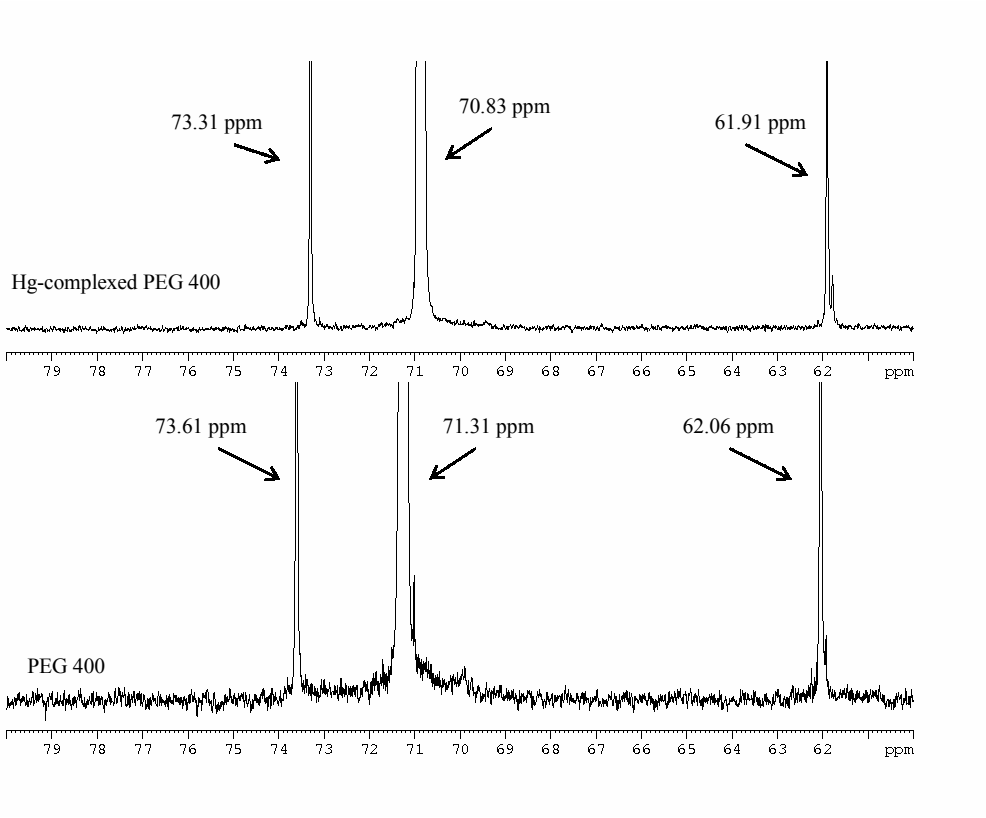


Figure 15. Overlay of PEG 400 and Hg-complexed PEG 400

^{13}C-NMR spectra.

FTIR Analysis

The interactions between metal ions, like divalent mercury, and polymers have been shown to be weak in nature (Caykara, et al., 2004). Although binding forces such as coordination bonds, hydrogen bonds, and charge-transfer interaction are generally weak, they act cooperatively to affect the molecular environment during complex formation (Caykara, et al., 2004). To aide in understanding these possible interactions and changes in the molecular environment when the polymer CrEL is complexed with mercuric chloride, techniques in Fourier transform infrared spectrophotometry (FTIR) were employed. FTIR was used to investigate the vibrational changes in certain functional groups present in CrEL and the Hg-complexed CrEL. The particular functional groups of interest were the alkene, ester, and ether absorption regions.

Figure 16 shows the FTIR spectra obtained from 4000 cm^{-1} to 500 cm^{-1} for the pure polymer CrEL and Figure 17 shows the solid Hg-compexed CrEL. The pure CrEL and the complexed solid were triturated with mineral oil (Nujol), suspended between two sodium chloride plates and read using the infrared spectrophotometer. The absorption bands at 1375 cm^{-1} and 1460 cm^{-1} in addition to the broad absorption at 2900 cm^{-1} are partly contributable to the Nujol that was used to suspend the samples. In comparison of the two CrEL-containing spectra found in Figure 16 and Figure 17, the most notable difference between the two spectra is the hydroxyl region (3600 cm^{-1}) due to residual water, the ether region (1100 cm^{-1}), and the ester region (1730cm^{-1}). The absence of the –OH stretch in Figure 17 is due to drying the CrEL-mercury complex in a vacuum over silica gel. The residual moisture present in the raw, liquid CrEL was not able to be removed by drying in a vacuum over silica gel. The C-O bending due to the ether

linkages at 1100 cm^{-1} is substantially reduced when complexed with mercuric chloride. The ester peak found at 1730 cm^{-1} also shows a significant decrease is absorption when complexed with mercuric chloride. No absorptions were observed for the alkene absorption regions (3020-3100 cm^{-1} and 1650-1670 cm^{-1}).

To support the observed changes in the IR spectra that were obtained with the CrEL preparations, the same study was performed with triglyme, as illustrated in Figure 18 and Figure 19. The same effect was observed in the ether region (1100 cm^{-1}) for triglyme when it was complexed with the mercuric chloride as illustrated in Figure 19. From the FTIR data that was obtained for the CrEL it is apparent that etheral oxygens present in the molecule have a significant effect on the complexation with the cation, mercury. This theory is supported by the similar results obtained for the ether, triglyme.

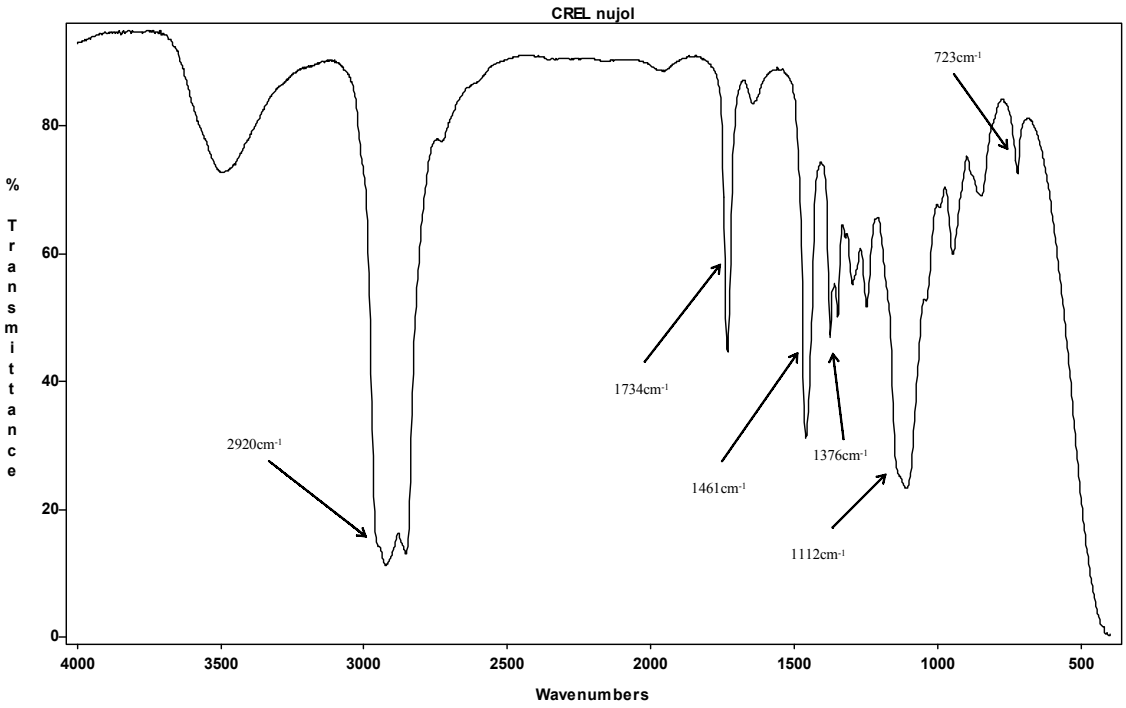


Figure 16. IR spectrum of CrEL.

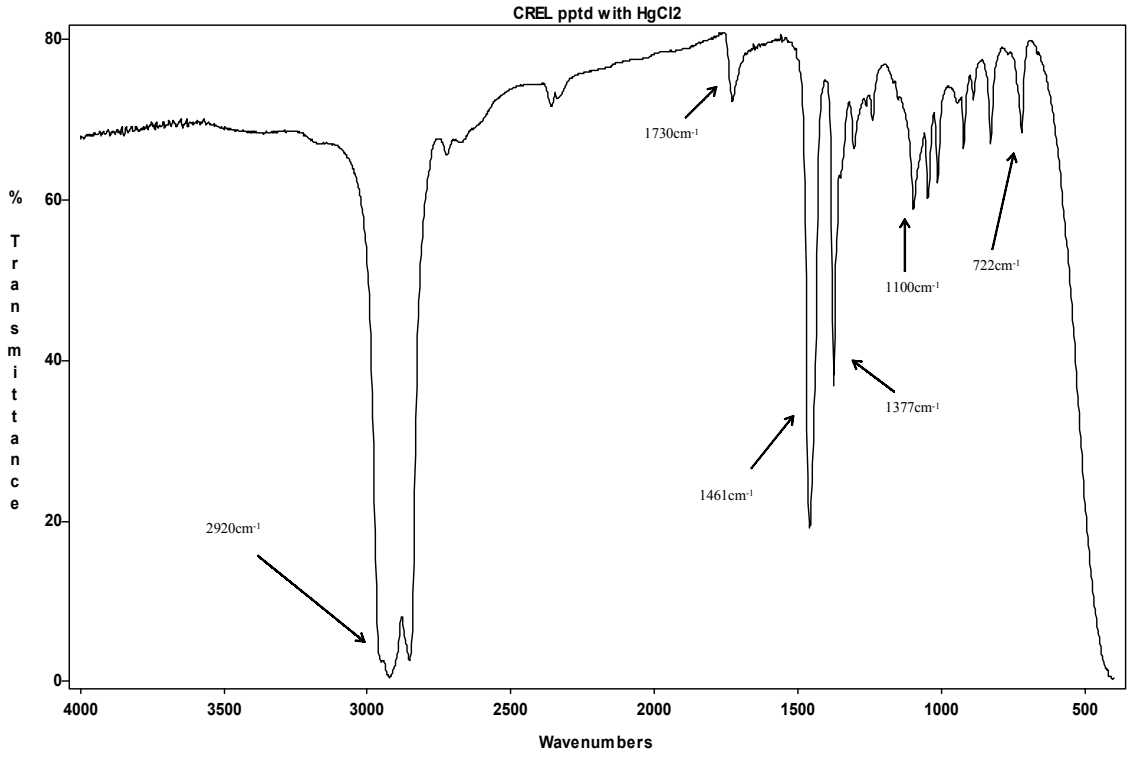


Figure 17. IR spectrum of Hg-complexed CrEL.

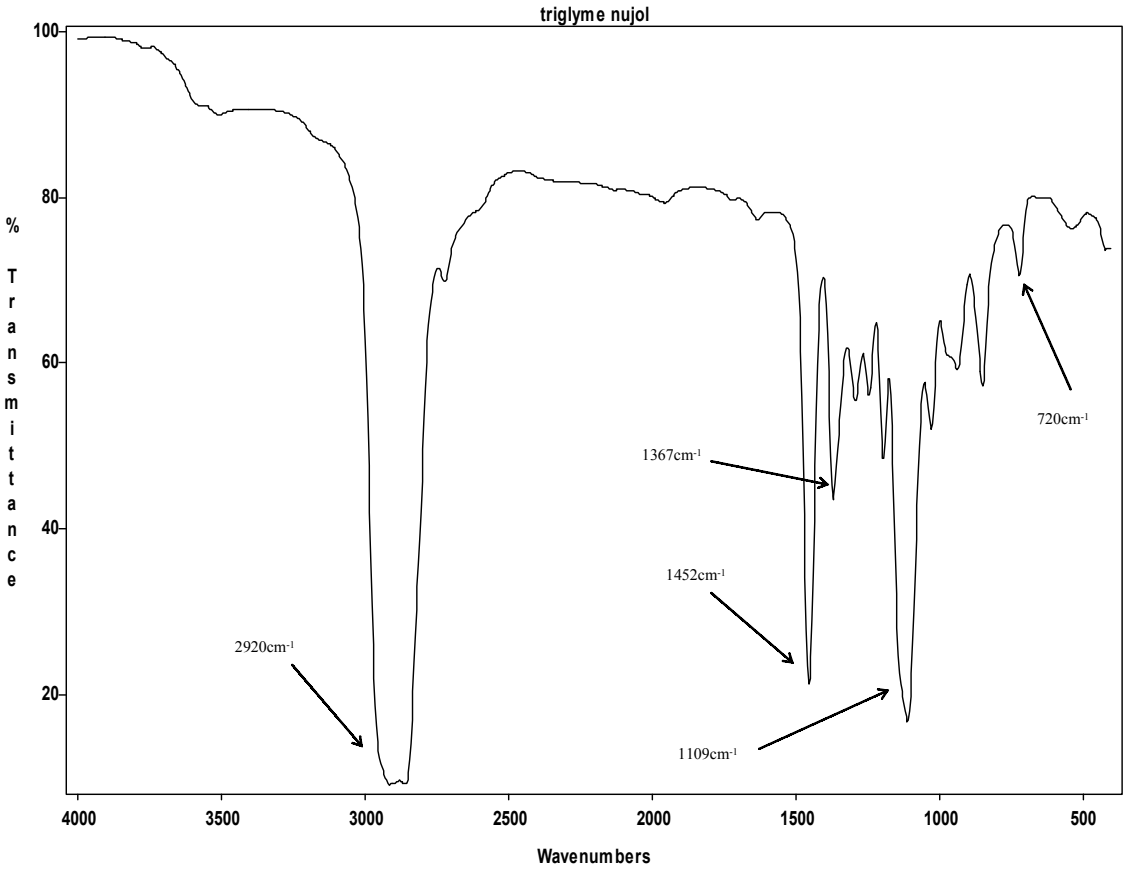


Figure 18. IR spectrum of triglyme.

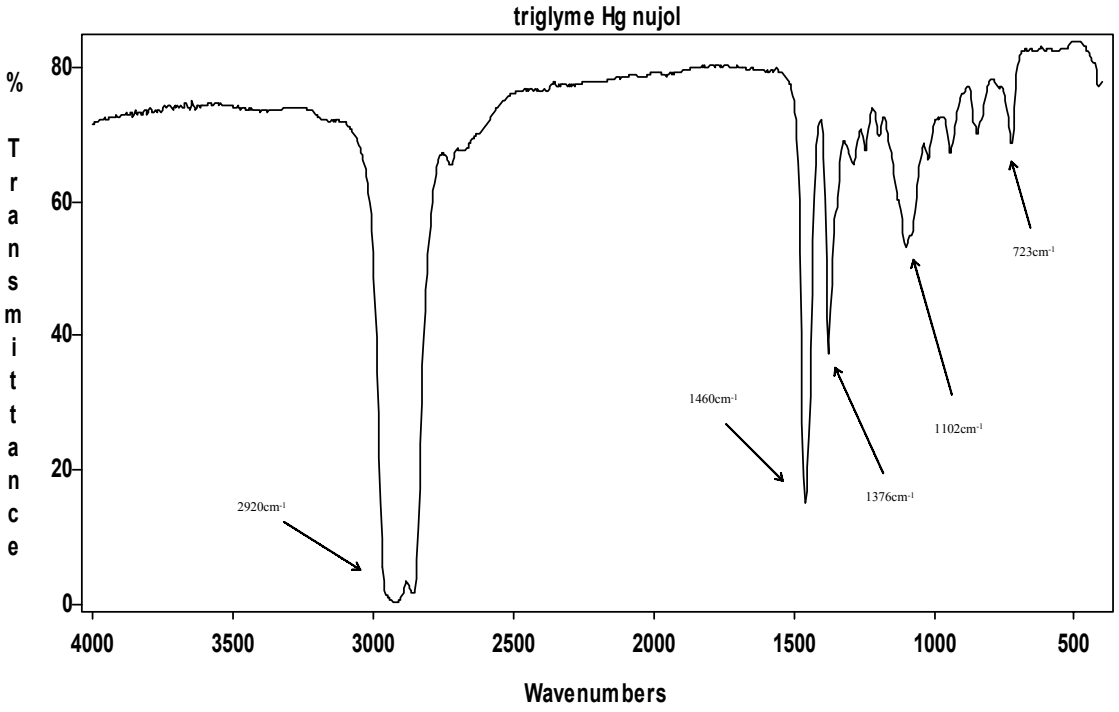


Figure 19. IR spectrum of Hg-complexed triglyme.

X-ray Analysis

An X-ray diffraction study was performed to aid in the elucidation of the mechanism of action for the precipitation of the CrEL. For single-crystal diffractometry, it is essential to obtain a crystal that is of sufficient size (0.1-0.3 mm), shape (no physical distortion) and uniform in its internal structure (Stout and Jensen, 1989). The selected crystal must be pure at the atomic, ionic, or molecular level so as to provide meaningful X-ray diffraction data. Therefore, the technique utilized to grow crystals from the liquid phase becomes paramount. One of the preferred methods, and the one selected here, is to grow crystals from a saturated solution that is allowed to slowly evaporate.

The first experiment performed attempted to show that the mercury atom was complexing with the olefinic region of CrEL and then precipitating out of solution. To simulate this reaction, 1-octene was mixed in a flat-bottom glass tube with an excess of a saturated ethanolic solution of mercuric chloride then covered with a punctured piece of aluminum foil. After the ethanol evaporated, a single white crystal was isolated using a microscope, mounted on a glass fiber and placed in the gonimeter head. The data points that were collected showed that the crystal was recrystallized mercuric chloride and that no reaction resulting in crystallization with the olefin present in 1-octene took place.

Another X-ray study was performed on the precipitate obtained from the reaction of CrEL with the ethanolic solution of mercuric chloride. Many attempts to isolate a satisfactory crystal of the CrEL complex were tried using different solvents (dimethyl sulfoxide, acetonitrile, ethanol, acetone, methanol, and dimethyl formamide) as well as different divalent metal atoms (mercury, copper, cobalt, and zinc). The only substantial crystals that were obtained were from the expected CrEL in ethanolic mercuric chloride.

Other solvent evaporations (methanol and dimethyl formamide) with mercuric chloride furnished microcrystals that were unsuitable for analysis.

The diffractogram in Figure 20 is of the large crystal-like solid obtained from the precipitation reaction of CrEL and ethanolic mercuric chloride. Since CrEL is not a pure and distinct compound that can produce sizable, uniform crystals when complexed with mercuric chloride, the quality of the diffraction pattern detailed in Figure 20 is expected. From this diffraction pattern (Figure 20) and its tabulated data (Appendix D), it is evident that the precipitate obtained is both amorphous and crystalline in nature due to the fluctuating baseline and the sharp peaks respectively. To draw conclusive evidence from this diffraction pattern, however, a significant amount of further research is needed to develop an effective recrystallization technique for an isolated CrEL molecule so as to properly identify the peaks present and thus, its true crystal structure.

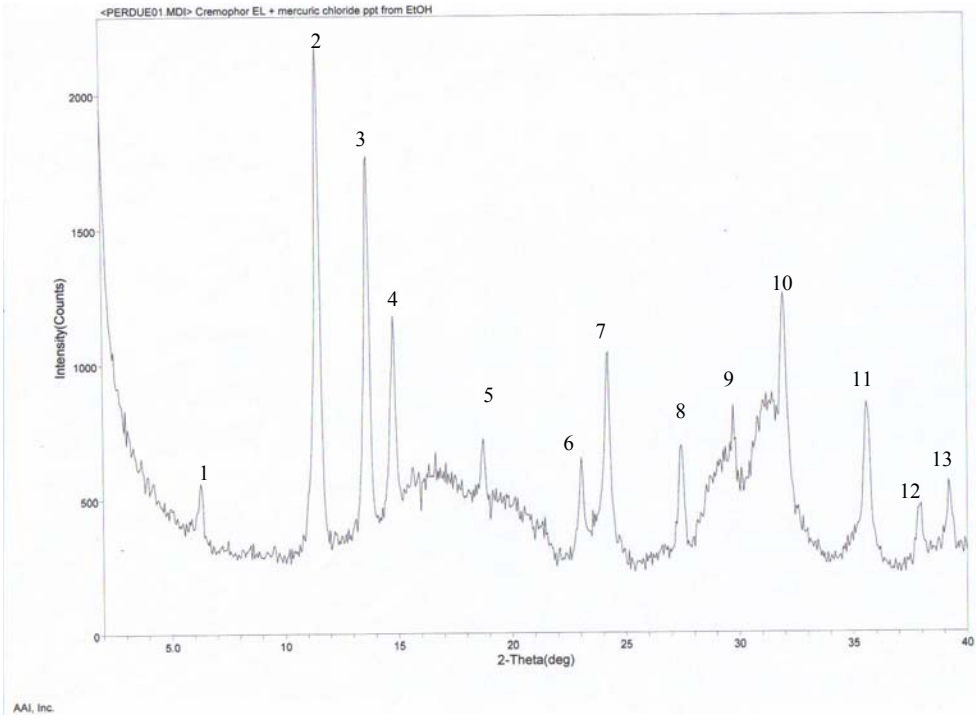


Figure 20. Diffractogram of Hg-complexed CrEL.

HPLC-UV Analysis

Reverse-phase high performance liquid chromatography with ultra-violet detection was utilized to physically separate and detect the chemical compounds present in the parenteral formulation of paclitaxel. The extracts obtained from the bark and the needles of the Pacific Yew tree contain a variety of taxanes in addition to paclitaxel, which sometimes appear at low levels in the finished drug product. Over the last two decades, a significant amount of research has been conducted on the separation of these taxanes using HPLC. Until recently, bonded phases containing cyano-, phenyl-, and octadecyl- groups where used (Shao and Locke, 1997). A new type of column with a PFP bonded phase has been designed specifically for the taxanes which is remarkably efficient. PFP phases are unique in their selectivity toward compounds containing aromatic rings and other electron-rich groups as compared to alkyl-modified silicas. Thus, the PFP bonded phase was selected for its ability to retain analytes with functional groups that possess π electron systems as well as other electron-rich functional groups. Nevertheless, a method must be developed that allows not only the separation of the taxanes but the excipient from the taxanes as well. It is unfortunate that the excipient (CrEL) that is required to make the drug soluble, has almost the identical absorption spectrum ($\lambda_{max} \approx 230$ nm) as paclitaxel (Shao and Locke, 1997). This may cause significant interference with coeluting peaks that are contributable to the drug product.

Dilutions of CrEL with ethanol were first prepared and injected onto the PFP column and adjustments to the gradient and the injection volume were made so as to optimize the chromatography. The precipitation reaction was applied to a 50% v/v ethanolic solution of CrEL and injected onto the column. The resulting chromatograms

are overlayed in Figure 21. It is evident from Figure 21 that the removal of CrEL is highly effective by the mercuric chloride precipitimetric method. The many peaks between 17 min and 50 min caused by the large molecular weight distribution of CrEL are removed. The ramp in the gradient of the mobile phase allowed for compounds retained from the mercuric chloride precipitation reaction to be removed from the column as is evidenced at 65 min in Figure 21.

Initial HPLC testing was performed on the paclitaxel raw material that was prepared at 50 mg/mL in 50% v/v ethanolic CrEL and diluted with water to have a working concentration of 1.0 µg/mL. Paclitaxel was diluted to 1.0 µg/mL to establish the sensitivity of the instrument. The dilutions were initially made in water so as to prevent the peak splitting that sometimes happens when organic liquids are injected into an aqueous/organic phase system. It was observed during the analysis that the complexation was not immediate and complete. Over a period of a week, the quantity of the precipitate continued to increase in the filtered aliquot placed in the autosampler vial. When the dilutions of stock solutions were carried out in ethanol instead of water, the reaction was immediate and complete as evidenced by the absence of further precipitation after the initial precipitate was centrifuged and filtered. As a result, all dilutions of stock solutions were exclusively prepared with ethanol and not water. No peak-splitting was observed in this method.

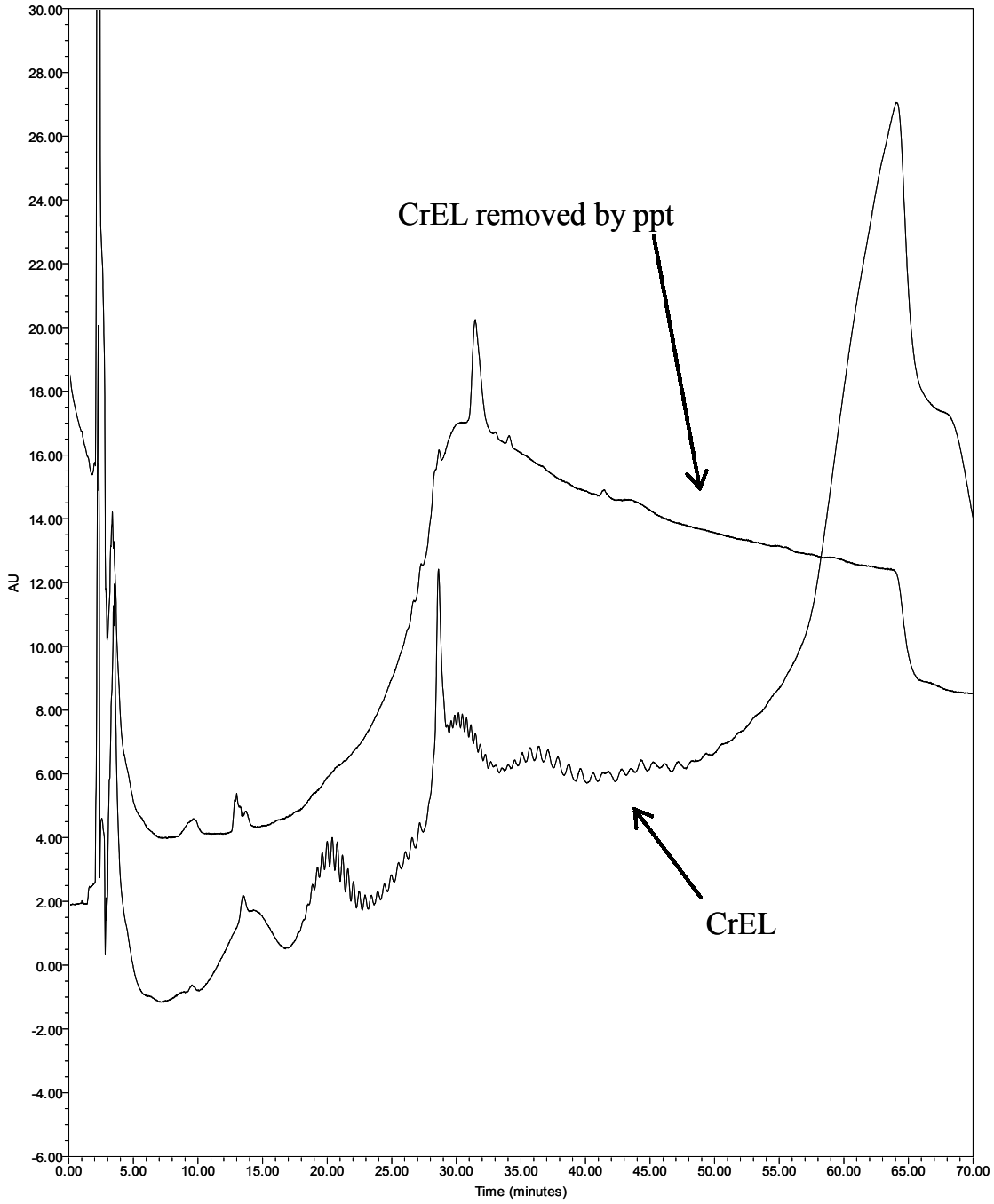


Figure 21. Chromatographic overlay of a 1.0% v/v ethanolic solution of CrEL (bottom) and the solution after precipitation and filtration (top).

To demonstrate the broad range in which the solubilizer elutes, 1.0 mL of a 0.6 mg/mL paclitaxel solution in ethanol was spiked into 2.0ml of a 50% v/v CrEL ethanolic solution. The chromatogram in Figure 22 illustrates this very large distribution of CrEL. For drug substances that exhibit a greater hydrophobicity than paclitaxel and use additional CrEL in their parental formulation when analyzing by HPLC will be an issue.

In order to determine if the precipitation reaction influences the drug product, an experiment was performed with paclitaxel (25 mg/mL in 50% v/v ethanolic CrEL) diluted to 0.5 mg/mL. In Figure 23, the top chromatogram is of the 0.5 mg/mL paclitaxel solution and the bottom chromatogram is paclitaxel treated with saturated ethanolic mercuric chloride and diluted to 0.5 mg/mL. The chromatograms illustrated in Figure 24 were acquired on the auxiliary system described in the experimental section with the flow set at 1.5 mL/min. A majority of the CrEL was removed with the mercuric chloride precipitation reaction and paclitaxel was not affected, as evidenced by the minor change in the area counts of its peak (percent recovered: 95.4%).

To illustrate the selectivity of mercuric chloride for CrEL, Figure 24 shows a chromatographic overlay of two preparations of the related substances. The bottom chromatogram is a preparation of paclitaxel prepared at 0.5 mg/mL in ethanol and spiked with a commercially purchased mixture of 13 taxanes at 0.1% of the 25 mg/mL paclitaxel solution. The top chromatogram is of a preparation made at the same concentrations as the control with CrEL present (10 μL/mL). A substitution of saturated ethanolic mercuric chloride solution was used during an intermediate dilution step and filtered as stated in the procedure. System suitability was established with five replicate injections of the

control solution with a percent-relative standard deviation (%RSD) at less than 3.0% for all 14 components.

The elution order of the taxanes is broadly related to the molecular size and increasing hydrophobicity. The retention time of the taxanes increase as the number of acetylated hydroxyl groups and xylosyl groups are added to the taxane ring, (Shao and Locke, 1997). The selectivity for the retention of taxanes is contributed by the π-π interactions of the fluorines on the stationary phase with the carbonyl oxygens, the principal electron-rich groups in the taxanes. In Table 2, the retention times and area counts are listed for each of the related substances (taxanes) present in the control solution illustrated in the bottom chromatogram in Figure 24. Table 3 lists the retention times, area counts, and percent recovery of the related substances after precipitating CrEL from the solution prepared at the same concentration as the control (top chromatogram in Figure 24). Since the same concentrations were used for both preps, the percent-recovery values were obtained by taking the ratio of the area counts of sample to the area counts of control and multiplying by 100%. From the percent-recovery results illustrated in Table 3, it is evident that the complexation of mercuric chloride does not interfere with paclitaxel or its related substances. This allows for the peaks of interest in the drug product to be accurately determined and quantitated.

The preparations illustrated in Figure 24 contained some impurities that were not identified. There is one peak at 6.0 minutes in the top chromatogram that is an artifact of a compound retained on the system and is diminishingly present in replicate injections of the solution. Fortunately, this peak does not interfere with the related taxanes. There are smaller extraneous peaks present in both chromatograms that may be due to either the

degradation of paclitaxel, additional taxanes present in the commercially purchased mixture or impurities in the CrEL. The literature suggests that certain grades of CrEL contain a sufficient amount of water to cause degradation of the paclitaxel (Shao and Locke, 1997). The additional peaks seen in the chromatographic analysis of the paclitaxel used in this method may be attributed to the CrEL used in this analysis. The CrEL is of pharmaceutical grade. However, the container has been exposed to atmospheric moisture during the last year and was not further purified. The moisture value for CrEL determined by Karl Fisher titrimetric methods was 2.5%.

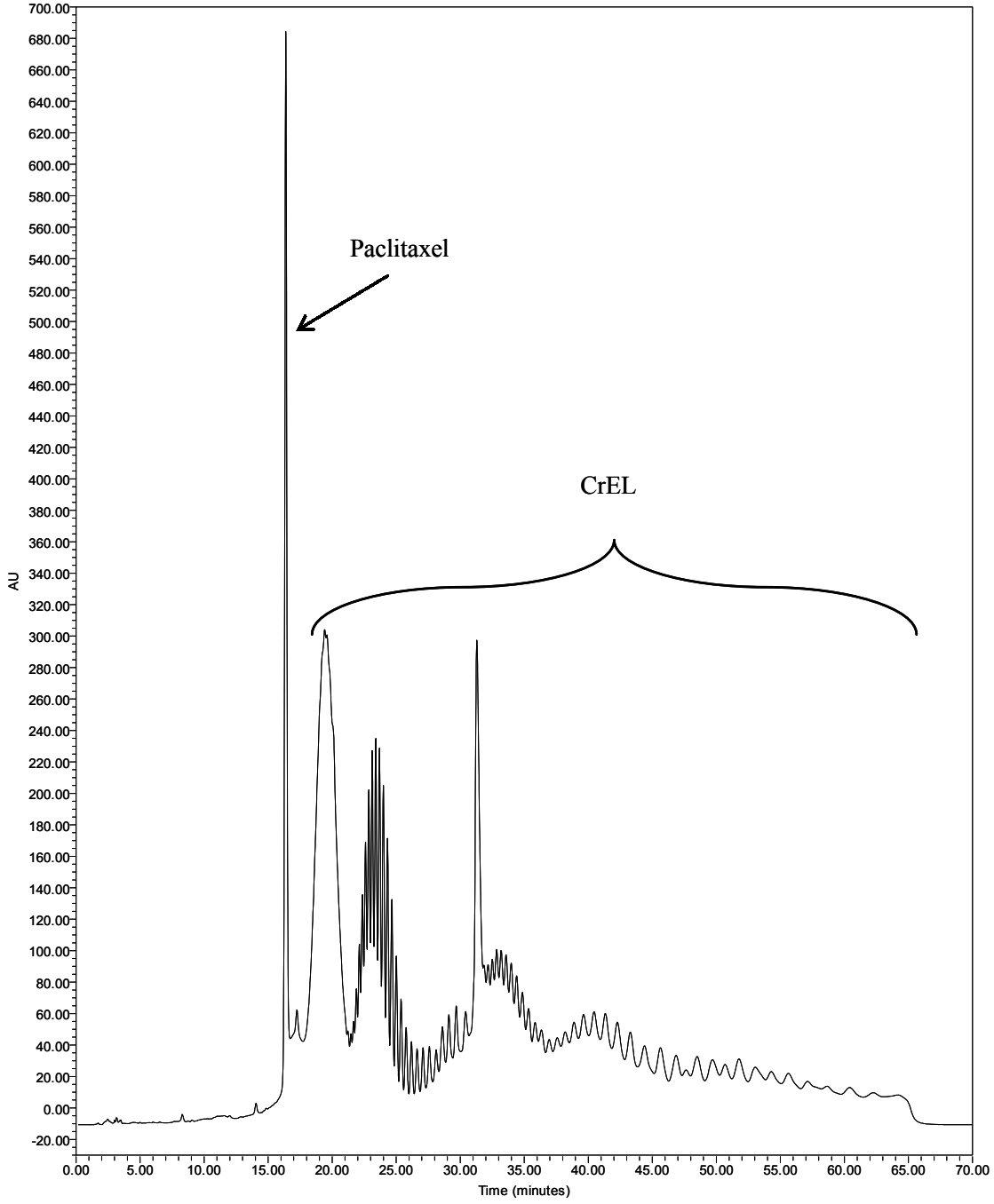


Figure 22. Chromatogram of paclitaxel (0.3mg/mL) dissolved in 50%CrEL in ethanol.

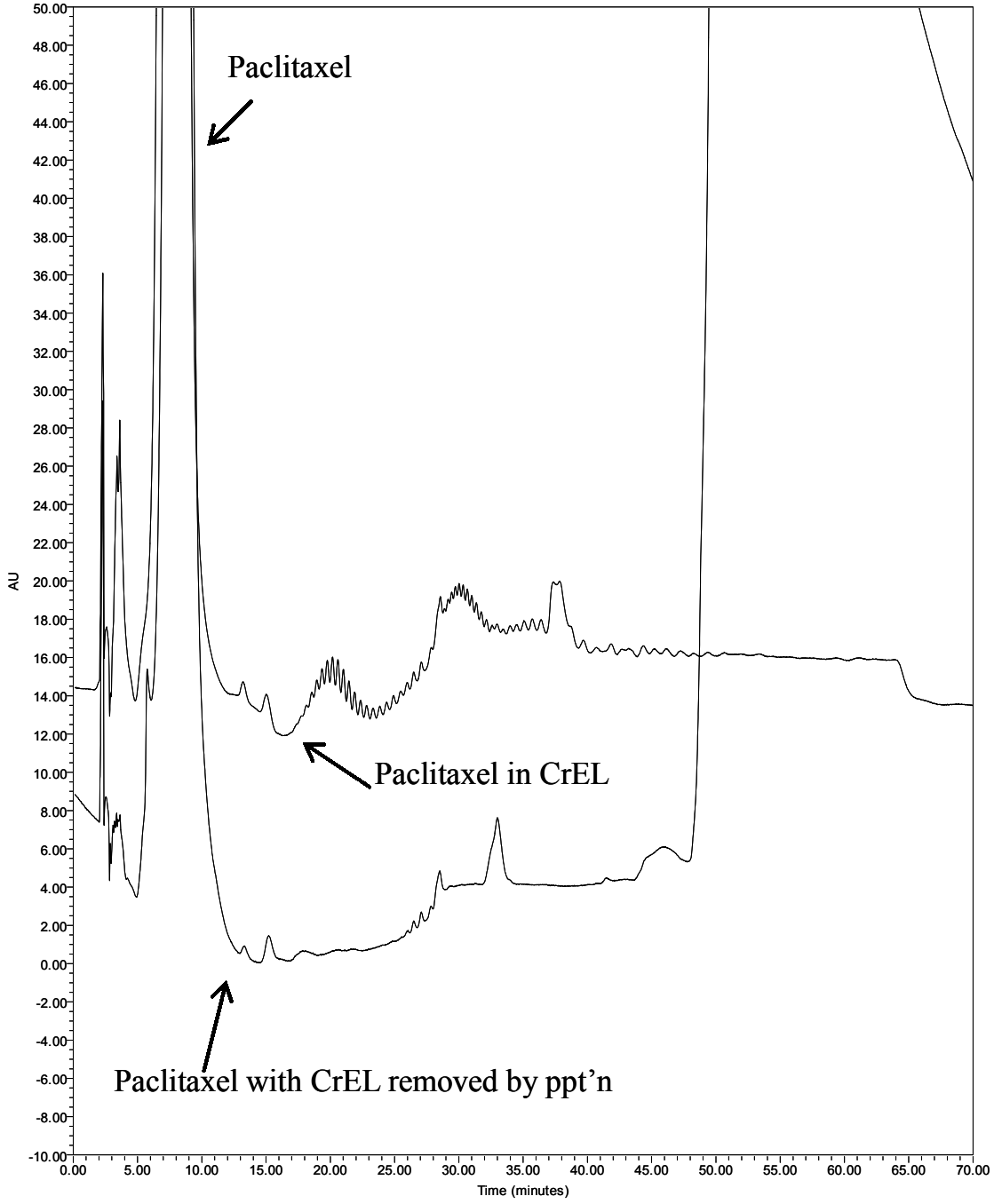


Figure 23. Chromatographic overlay of paclitaxel (0.5 mg/mL) in CrEL and paclitaxel with CrEL removed by mercuric chloride precipitation.

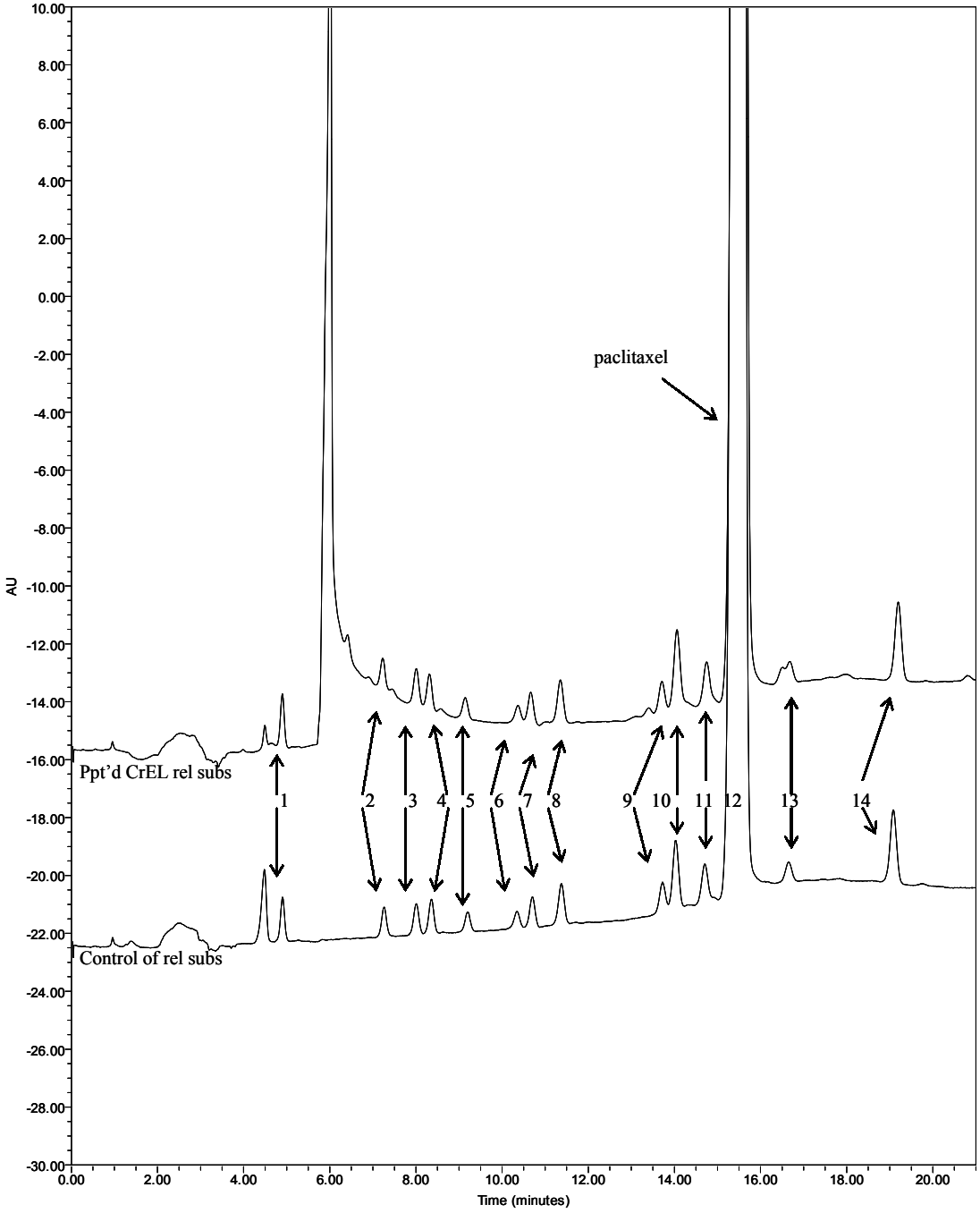


Figure 24. Chromatogram of precipitated and filtered paclitaxel (0.5 mg/mL) in ethanolic CrEL solution spiked with related substances mixture (0.1%) overlayed with a control solution prepared at the same concentration with no CrEL.

Table 2. Retention times, and area counts for control preparation of paclitaxel and its related substances.

Number/Name	RT Control (min)	Area Control
1. 10-Deacetylbaccatin III	4.89	10340
2. Baccatin III	7.24	8382
3. 7-Xylosyl-10-deacetyl-taxol B	7.99	8710
4. Taxinine M	8.33	9905
5. 7-Xylosyl-10-deacetyl-taxol	9.17	5547
6. Degradation peak unknown	10.31	4861
7. 7-Xylosyl-10-deacetyl-taxol C	10.66	9180
8. 7-Xylosyl-taxol	11.34	12244
9. 10-deacetyl-taxol	13.68	7814
10. Cephalomannine (taxol B)	13.99	20912
11. 7-Epi-10-deacetyl-taxol	14.66	14422
12. Taxol	15.44	9755687
13. Taxol C	16.61	6860
14. 7-Epi-taxol	19.05	26675

Table 3. Retention times, area counts, and percent-recovery after the precipitation of CrEL from paclitaxel and its related substances.

Number/Name	RT Sample (min)	Area Sample	%Recovery
1. 10-Deacetylbaccatin III	4.90	11520	111
2. Baccatin III	7.23	7679	92
3. 7-Xylosyl-10-deacetyl-taxol B	8.01	10092	116
4. Taxinine M	8.31	9359	94
5. 7-Xylosyl-10-deacetyl-taxol	9.14	6136	111
6. Degradation peak unknown	10.37	4352	90
7. 7-Xylosyl-10-deacetyl-taxol C	10.67	9198	100
8. 7-Xylosyl-taxol	11.35	14292	117
9. 10-Deacetyl-taxol	13.71	8820	113
10. Cephalomannine (taxol B)	14.05	26271	126
11. 7-Epi-10-deacetyl-taxol	14.75	16160	112
12. Taxol	15.52	10753090	110
13. Taxol C	16.68	6806	99
14. 7-Epi-taxol	19.19	28873	108

CONCLUSION

For drugs that are poorly soluble in water and are most efficacious by parenteral administration, solubilizers such as CrEL are a necessary excipient. Furthermore, the need for quantitatively and qualitatively determining these finished products will always be mandated by the FDA. The sample treatment demonstrated here can be applied to hydrophobic drugs in their finished product form that would otherwise suffer from specificity issues due to CrEL.

The additional analysis performed on CrEL and mercury-complexed CrEL encompassed the use of NMR, FTIR and X-ray to aide in determining the mechanism of action. The data obtained from these experiments support the theory that CrEL is selectively removed from a solution by divalent mercury binding to the polyether moiety of the CrEL. Furthermore, the evidence collected from the instrumental analyses presented here is supported by the literature referenced herein.

The goal of this work was to establish a sensitive and selective reversed-phase HPLC method for the determination of a hydrophobic drug with CrEL removed by a precipitimetric method. As an application of this method, Taxol® *for injection concentrate* was prepared as the drug product because of its well known structure and many related substances. The use of mercuric chloride to remove the solubilizer, CrEL, from Taxol® *for injection concentrate* is adequate for the proposed objective. The recovery results for the HPLC analysis of the 13 related taxanes demonstrate the theory that mercury is selective enough to remove CrEL without removing the active, paclitaxel and its related substances.

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APPENDIX

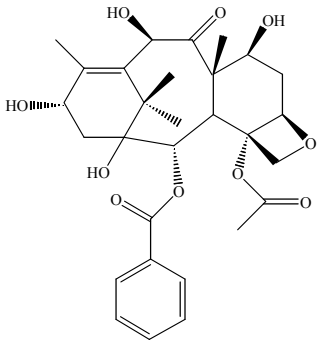
Appendix A. Reagents used for each study.

Type of analysis/ study	**reagent**	**Manufacturer/ supplier**
Precipitation	Mercuric chloride	Aldrich
	Mercuric iodide	Aldrich
	Mercuric bromide	Aldrich
	Barium chloride	Aldrich
	Mercuric acetate	Aldrich
	Polyethylene glycol (mw 400)	Mallinckrodt
	Diglyme (diethylene glycol dimethyl ether)	Aldrich
	Triglyme (triethylene glycol dimethyl ether)	Aldrich
	Glycerol ethoxylate	Aldrich
	Cis-3-hexene-1-ol	Aldrich
	Resorcinol	Aldrich
	Cremophor® EL	BASF
	Ethanol	Aaper
	Water	Milli-Q Ultra Pure
	Cobalt chloride	Aldrich
	Zinc chloride	Aldrich
	Nickel chloride	Aldrich
	Silver chloride	Aldrich
	Stannous chloride	Aldrich
	Magnesium chloride	Aldrich
	Lead acetate	Fisher
	Lithium chloride	Fisher
	Ferric chloride	Fisher
	Lanthanum chloride	Fisher
	Castor oil	Fisher
	Phenol	Aldrich
Solubility	Dimethyl sulfoxide	Burdick and Jackson
	Carbon tetrachloride	Aldrich
	Chloroform	Mallinckrodt
	Dichloromethane	Aldrich
	Acetonitrile	Burdick and Jackson
	Ethanol	Aaper
	Acetone	Mallinckrodt
	Mercuric chloride	Aldrich
	Cremophor® EL	BASF
	Methanol	Fisher

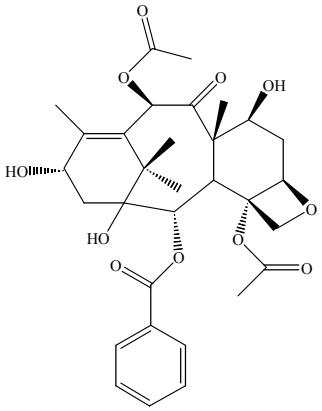
NMR	Cremophor® EL	BASF
	Mercuric chloride	Aldrich
	Ethanol	Aaper
	Deuterated acetone	Aldrich
	Deuterated water	Aldrich
IR	Mineral oil (Nujol)	Sigma
	Mercuric chloride	Aldrich
	Cremophor® EL	BASF
	Ethanol	Aaper
X-ray	Cremophor® EL	BASF
	Mercuric chloride	Aldrich
	Ethanol	Aaper
	Methanol	Fisher
	Isopropanol	Fisher
	Dimethyl formamide	Burdick and Jackson
	Cobalt chloride	Aldrich
	Zinc chloride	Aldrich
	Nickel chloride	Aldrich
	Silver chloride	Aldrich
	Lead acetate	Fisher
HPLC	Paclitaxel	AAI Pharma
	Acetonitrile	Burdick and Jackson
	Cremophor® EL	BASF
	Ethanol	Aaper
	Water	Milli-Q Ultra Pure
	Mercuric chloride	Aldrich
	13-Taxanes mixture	Hauser Chemical Research

Appendix B. Molecular structures of paclitaxel and related substances (taxanes).

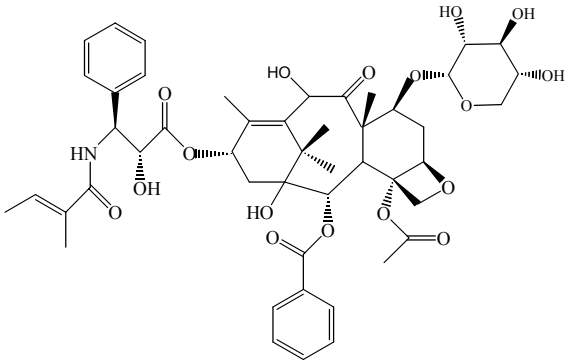
1. 10-Deacetylbaccatin II



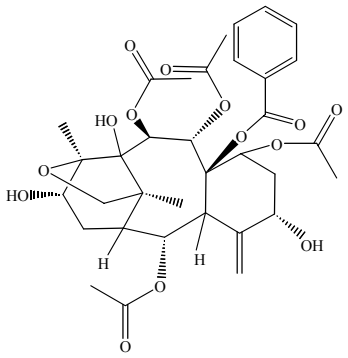
2. Baccatin III



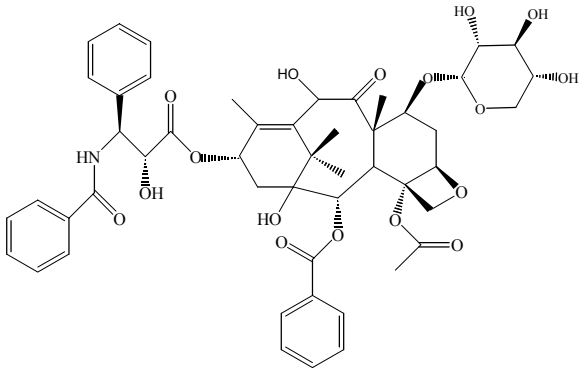
3. 7-Xylosyl-10-deacetyl-taxol B



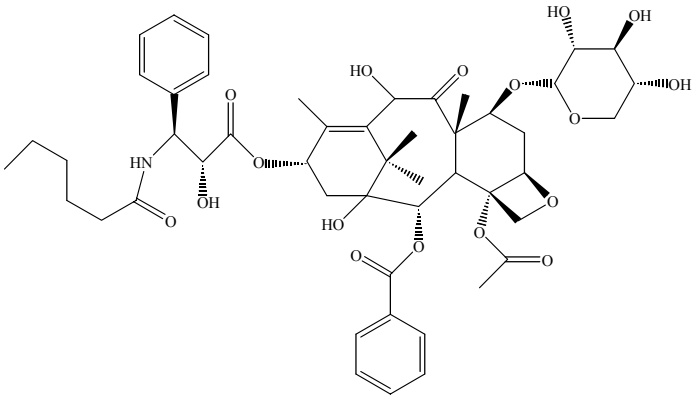
4. Taxinine M



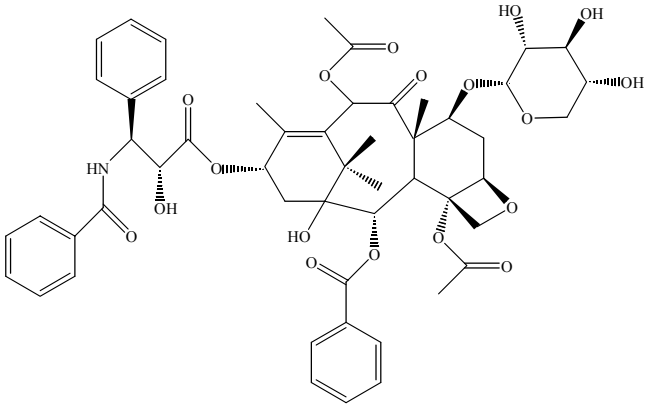
5. 7-Xylosyl-10-deacetyl-taxol



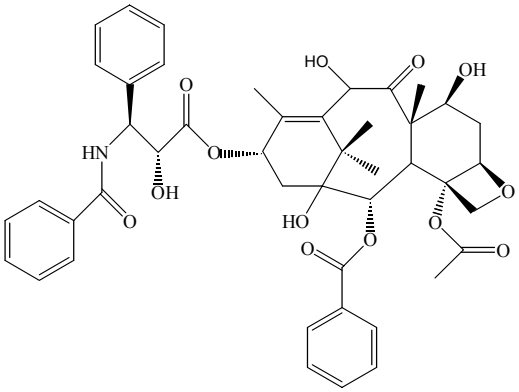
6. 7-Xylosyl-10-deacetyl-taxol C



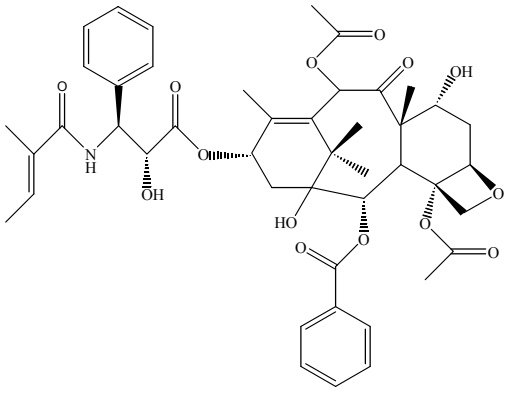
7. 7-Xylosyl-taxol



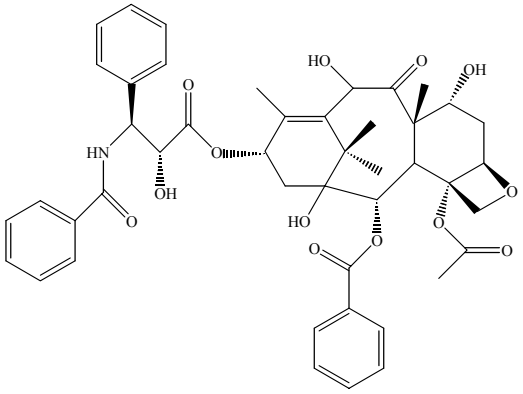
8. 10-Deacetyl-taxol



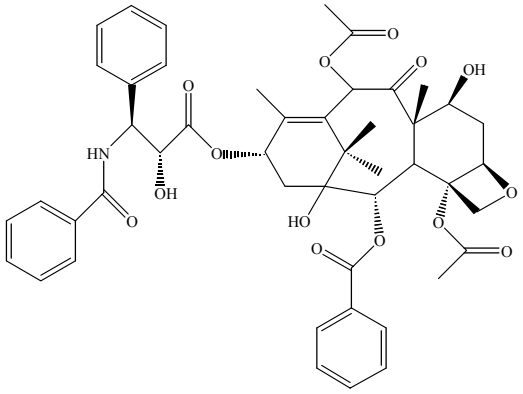
9. Cephalomannine



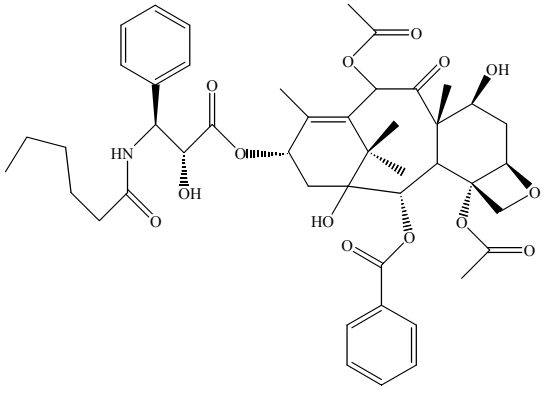
10. 7-Epi-10-deacetyl-taxol



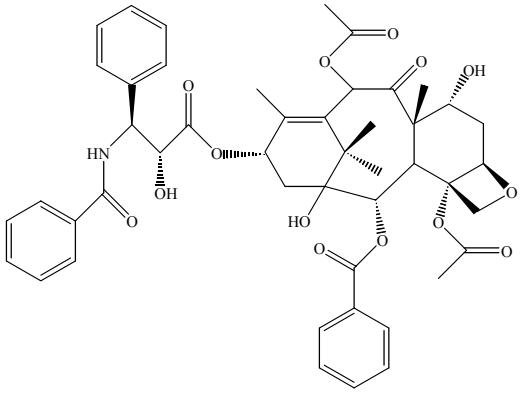
11. Paclitaxel



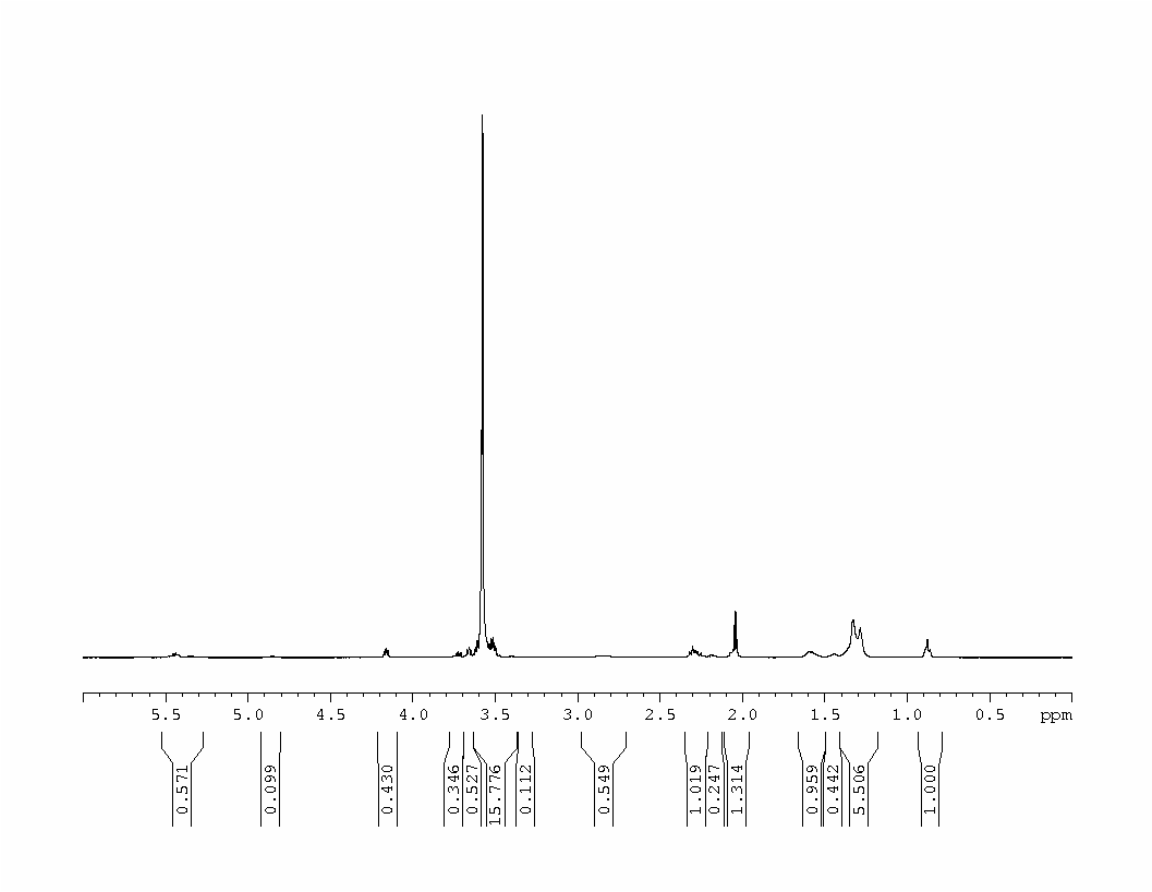
12. Taxol C



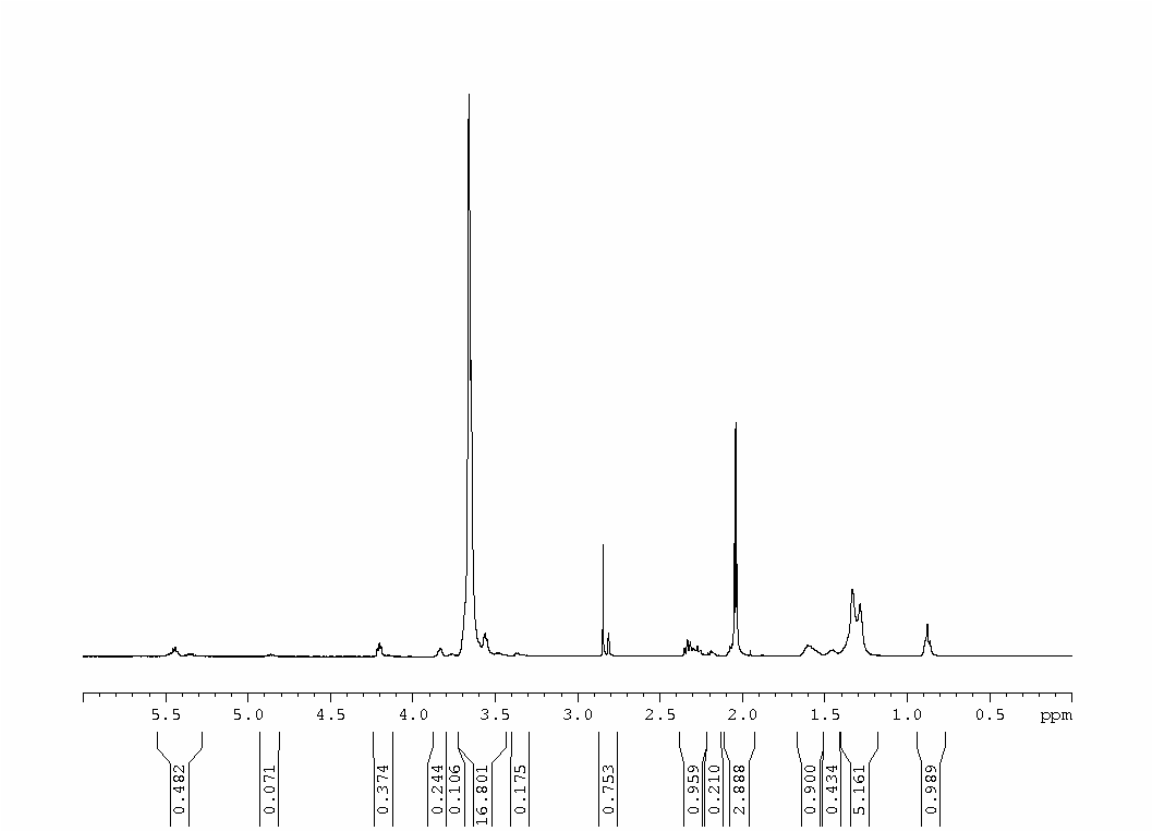
13. 7-Epi-taxol



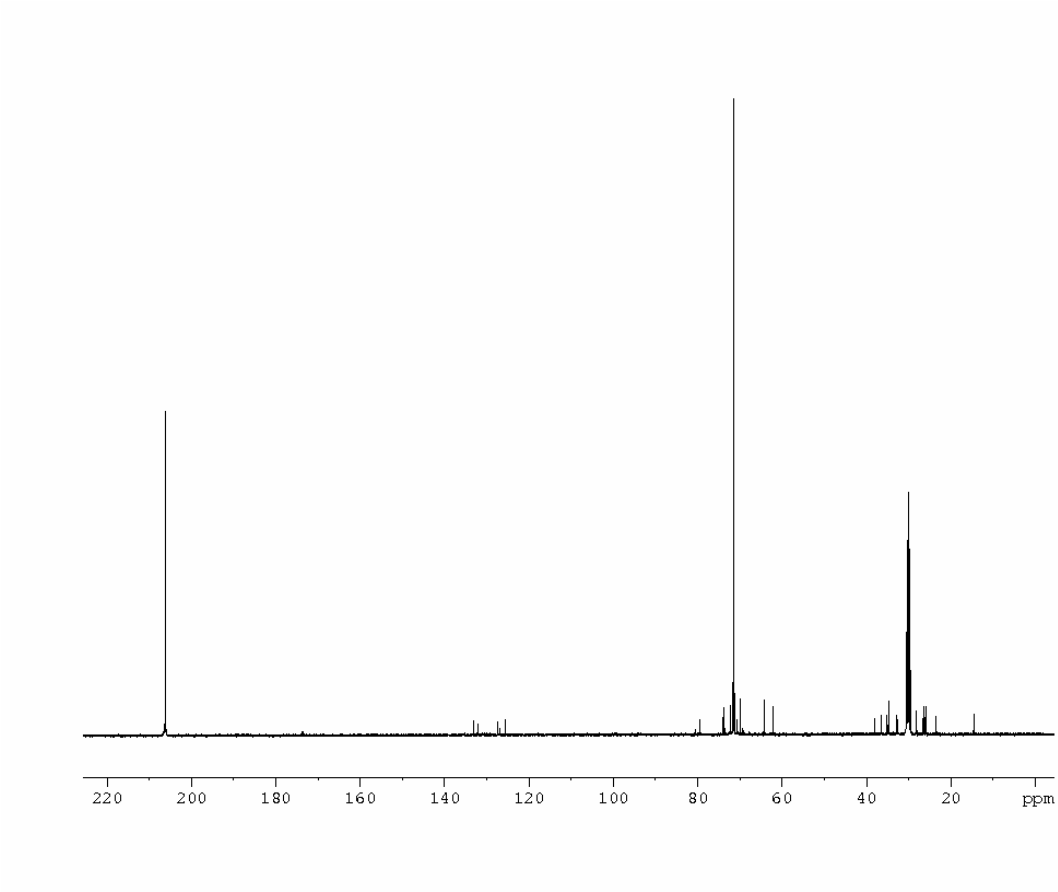
Appendix C. Additional 1H-NMR and ^{13}C-NMR spectra.



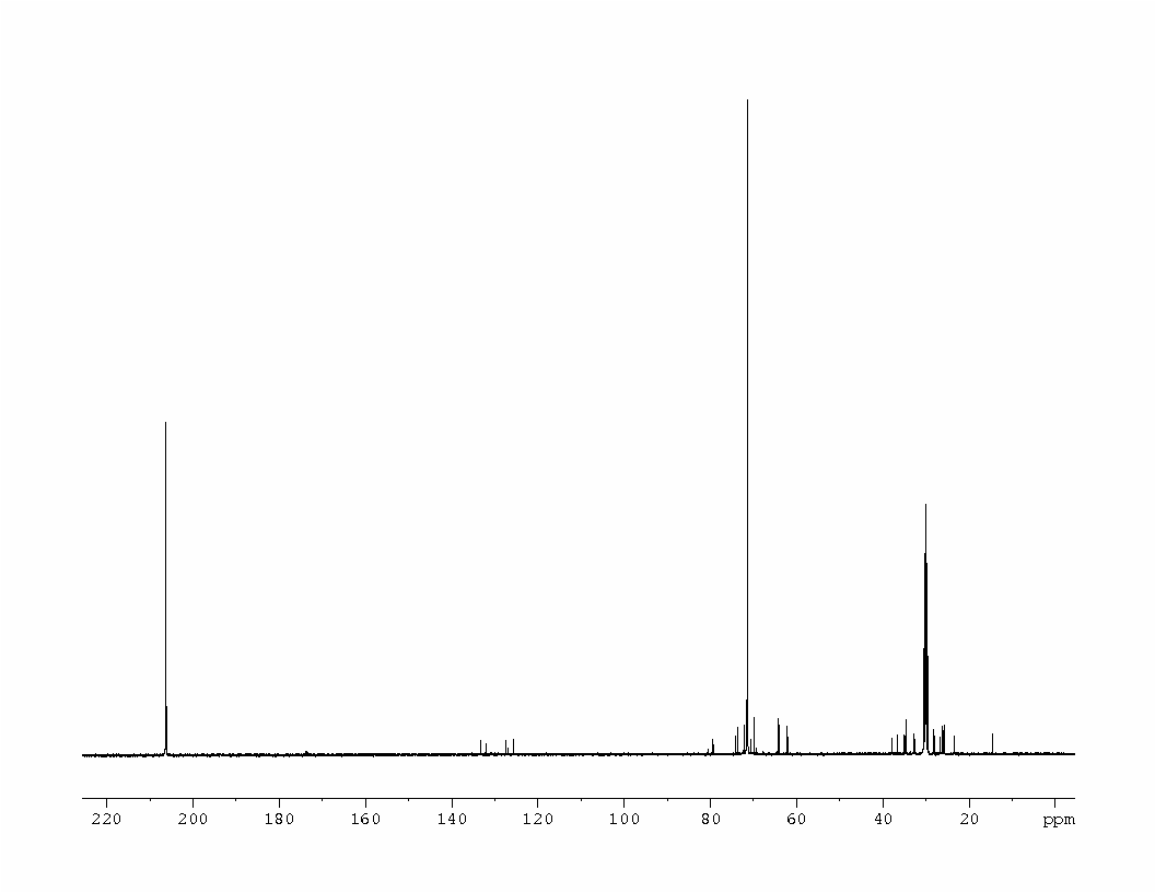
Fullscale, integrated ^{1}H-NMR spectrum of CrEL.



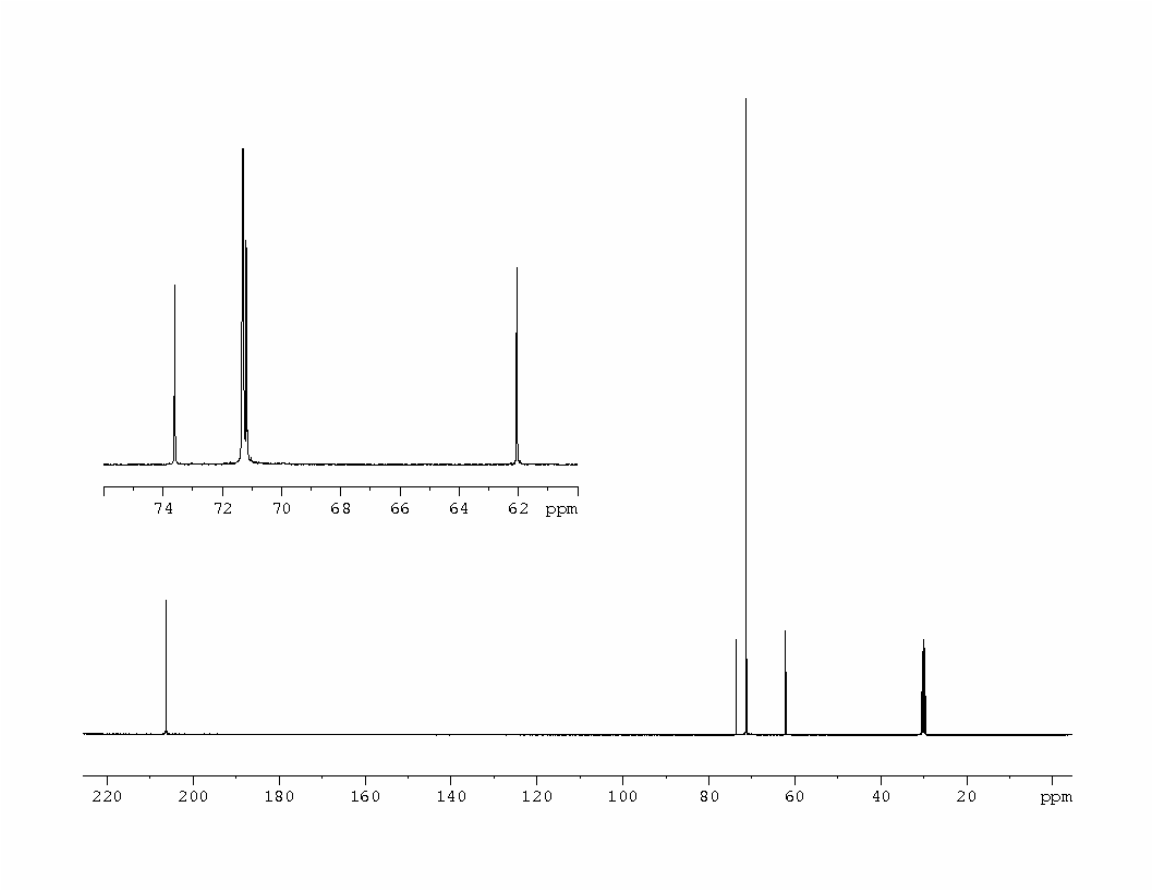
Fullscale, integrated ^{1}H-NMR spectrum of CrEL-Hg complex.



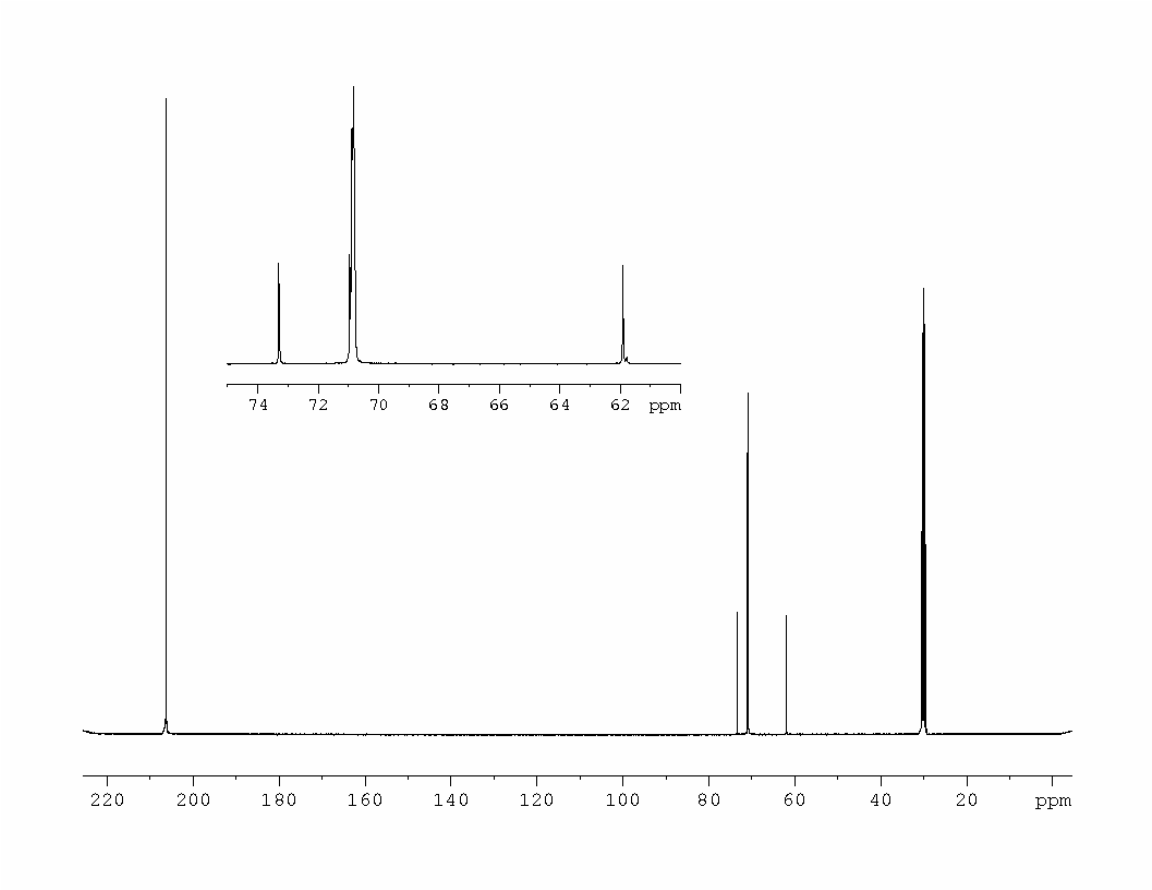
^{13}C-NMR spectrum of CrEL.



^{13}C-NMR spectrum of CrEL-Hg complex.



^{13}C-NMR spectrum of PEG 400.



^{13}C-NMR spectrum of PEG 400-Hg complex.

Appendix D. X-ray tabulated data for Figure 20.

ID	2-Theta	D(A)	Peak	%P	Area	A%
1	6.30	14.02	201	10.9	50	8.2
2	11.50	7.69	1847	100.1	598	100.0
3	13.69	6.47	1364	73.8	392	64.1
4	14.80	5.98	683	37.0	182	30.4
5	18.74	4.73	216	11.7	45	7.4
6	23.05	3.86	291	15.8	66	10.9
7	24.21	3.67	714	38.7	242	40.4
8	27.45	3.25	367	19.9	107	17.8
9	29.75	3.00	283	15.3	56	9.3
10	31.95	2.80	893	48.3	470	78.7
11	35.60	2.51	580	31.4	265	44.3
12	37.98	2.37	177	9.6	43	7.3
13	39.21	2.30	243	13.2	66	11.0