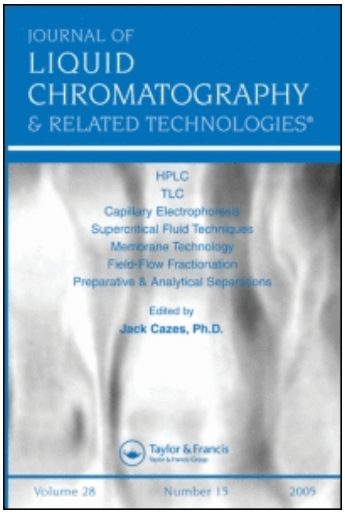

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DETERMINATION OF LOW MOLECULAR WEIGHT MOLECULES IN CREAMS AND OINTMENTS BY GEL PERMEATION CHROMATOGRAPHY

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ABSTRACT

The analysis of low molecular weight compounds can frequently be achieved by small molecule gel permeation chromatography (SMGPC). In the SMGPC mode, large molecules are excluded, while separation of the analytes is based on the effective size of the compounds of interest in solution. It is possible to analyze two compounds whose molecular weight differ by 10% or more by the judicious selection of the mobile phase. For instance, the separation of tolnaftate (the active ingredient of an anti-fungal preparation) and BHT (which was present as an antioxidant), was accomplished using methylene chloride, a non-hydrogen bonding solvent. For this assay, sample preparation is very simple and the overall analysis takes only 12 minutes.

INTRODUCTION

Separation of mixtures by Gel Permeation Chromatography (GPC) is based on the effective size in solution of the various

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components of the sample. It is most commonly used in the analysis of polymeric materials, although the ability of the technique to separate low molecular weight compounds (mw<2000) has been known for over twenty years (1). Until recently, however, small molecule gel permeation chromatography (SMGPC) has not been commonly used to separate low molecular weight compounds because very long analysis times were required to adequately separate these compounds.

Today, the commercial availability of high efficiency GPC columns has significantly shortened the analysis time required for the separation of low molecular weight compounds. These columns, which contain packings with a nominal particle size of 5μ, lead to retention times that are similar to those observed from other modes of liquid chromatography. High efficiency columns, with up to 15,000 theoretical plates/foot, provide a significant improvement in separation capability relative to earlier generations of GPC columns (2). The use of SMGPC leads to a number of significant benefits, relative to reverse phase HPLC. These include extremely simple sample preparation and a minimal effort required for methods development. In addition, the chromatographer knows with certainty when the separation is over, since all compounds will be eluted within the total volume of the system. In SMGPC, there is no concern about highly retained samples, which may create difficulties when chromatographic modes based on a retention mechanism are used.

In SMGPC, the separation of a mixture is based on the size of the various components in the mixture relative to the size of the pores of the GPC packing. Small molecules can enter many of the pores in the stationary phase, while larger molecules are excluded from many or all of these pores. The selective permeation of a molecule into the pores of a column packing is based on molecular size and, therefore, it is possible to construct a calibration curve for a column in which the log molecular weight is plotted vs the elution volume. A typical calibration curve is shown in Figure 1 for the elution of a number of pharmaceuticals on a series of 100 Å Ultrastyragel

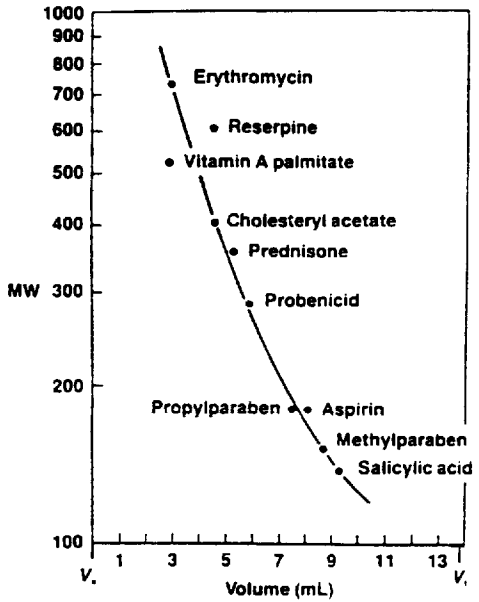


Figure 1. Calibration Curve for some low-molecular weight pharmaceutical compounds on 100 A Ultrastyragel columns in series. Mobile Phase THF, flow rate 2 mL/min. (Reprinted from reference 3 with permission).

columns (3). The reader will observe that reserpine and vitamin A palmitate do not fall directly on the curve. The explanation for these phenomena lies in the concept of the "effective size of the molecule." Reserpine has a very compact structure, while vitamin A palmitate is a long rod-like molecule and appears larger than what might be expected from a simple consideration of the molecular weight. As a rough rule of thumb, SMGPC is frequently able to separate compounds which have a molecular weight difference of 10%. Note, for example, that SMGPC can separate n-C_{20} H_{42} from n-C_{22} H_{46}, similarly methylparaben (MW=152) can be readily separated from salicylic acid (MW=138) (2). Both of these pairs represent approximately molecular weight difference of approximately 10%.

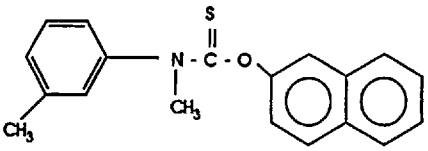
In GPC, there is no attraction between the analyte and the stationary phase due to the use of a mobile phase which is a good solvent for the sample. However, the solvent itself may "interact" with the compounds of interest via hydrogen bonding. This will depend on the nature of the solvent and the functional groups present in the sample. It is possible that steric considerations also may affect the number of solvent molecules associated with the compounds under discussion. In favorable cases it is possible to effect the GPC separation of two or more compounds with similar molecular weight by a judicious choice of mobile phase (3). For instance, two similarly sized molecules that are not separable in one solvent (e.g. a non-hydrogen bonding solvent) may be separable in another solvent (e.g. where one of the analytes is capable of hydrogen bonding). In this situation, the effective size of one of the molecules is larger than the other due to the associated solvent molecule(s).

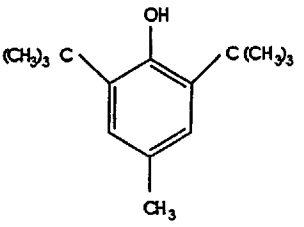
In this paper, we will describe the application of SMGPC to the analysis of the active ingredient and the stabilizer in an antifungal cream.

EXPERIMENTAL

Gel permeation chromatograms were obtained using an HPLC system which consisted of a Model 590 solvent delivery system, a Model 481 absorbance detector and a Model R-401 differential refractometer (all manufactured by the Waters Chromatography Division of Millipore Corporation, Milford MA 01757). The mobile phases were tetrahydrofuran and methylene chloride (HPLC grade), obtained from Burdick and Jackson, Inc.,(Muskegon, MI). The columns were Ultrastyragel (either 100 Å or 500 Å pore size). As discussed in reference 4, the indicated "Å Value" refers not to the actual median pore diameter but to the exclusion limit for the column (e.g the 500 Å pore size column will exclude all polystyrene molecules having an excluded chain length greater than 500 Å).

A commercial antifungal cream was purchased at a local pharmacy and used as received. The active ingredient was tolnaftate





TOLNAFTATE **BHT**

I **II**

Figure 2. Structure of Tolnaftate (I) and BHT (II)

(I), with BHT (II) present as a stabilizer; the structures of these compounds are shown in Fig. 2. Other components of the cream were propylene glycol, monoamylamine, carbomere 934P, polyethylene glycol and titanium dioxide. The sample was dissolved in THF as described below and filtered thru a 0.45μm Millex SR cartridge (Millipore Corporation, Bedford MA) to remove the titanium dioxide. In all experiments described below, the clear filtrate was injected onto the gel permeation column.

RESULTS AND DISCUSSION

In a preliminary experiment, the filtered cream sample (0.7g/5mL) was injected onto a 500 Å Ultrastyragel column, eluted with THF and monitored by a differential refractive index detector. The observed chromatogram is shown in Figure 3a. The early featureless peak is due to all the high molecular components. Tolnaftate and BHT co-elute at approximately 9.5 minutes. The high molecular weight peak and the tolnaftate/BHT peak are separated.

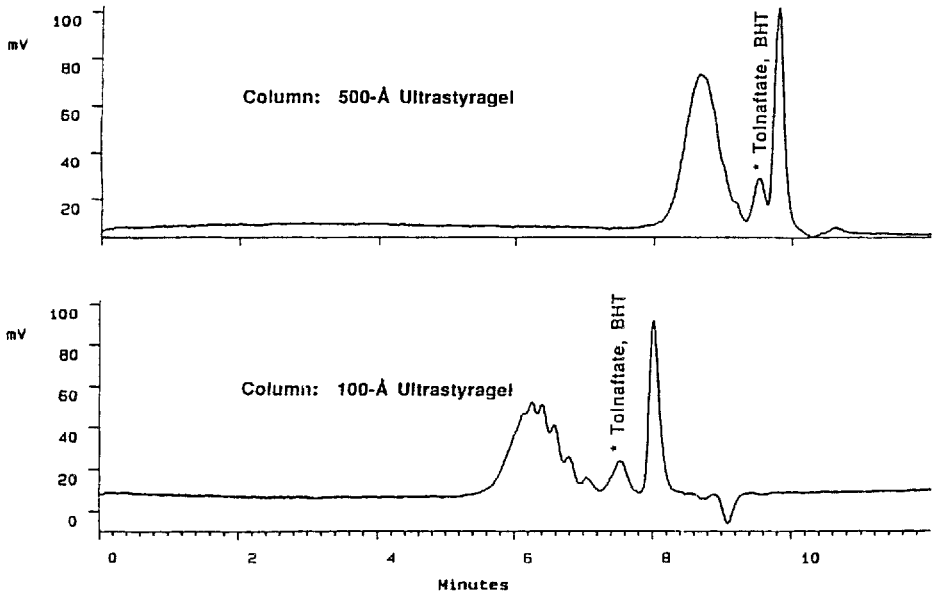


Figure 3. Separation of antifungal cream. Solvent THF, 1 mL/min, Refractive Index Detector, a) 500 A Ultrastyragel, b) 100 A Ultrastyragel

The fact that tolnaftate and BHT co-elute is somewhat of a surprise, since the size, shape, and molecular weight of the two compounds are quite different (e.g. the molecular weight of tolnaftate is 307, while the molecular weight of BHT is 220). Since the ultimate goal of this work was a sensitive assay for tolnaftate and BHT in the cream, a series of experiments to optimize the resolution and sensitivity was undertaken.

Selection of the pore size to optimize resolution. When the sample was injected onto an Ultrastyragel column with 100 Å pores, the resultant chromatogram (Figure 3b) provided a small improvement in the separation of the early eluting high molecular weight compounds. More importantly, it provided improved resolution between this peak and the peak for the compounds of interest. In

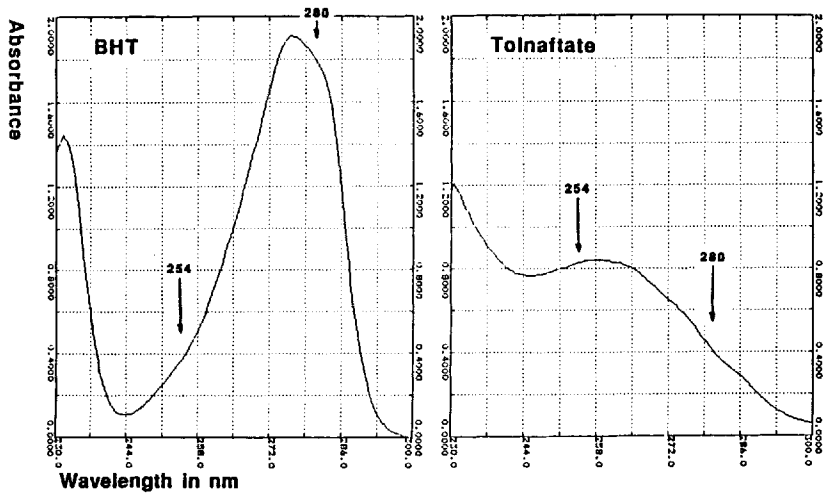


Figure 4. Absorbance Spectra of BHT and Tolnaftate

addition, the peak for tolnaftate and BHT is well resolved from the last peak in the chromatogram.

Selecting the detector to optimize sensitivity. Both tolnaftate and BHT absorb strongly in the UV, thus an absorbance detector can provide better sensitivity than a refractive index detector. The absorbance spectra of these two compounds are shown in Figure 4. At 254 nm (and at longer wavelengths), the absorbance of the other materials in the cream is negligible. The absorbance maximum of tolnaftate is at 258 nm, while the absorbance maximum of BHT is at 275 nm. Replacing the refractive index detector with an absorbance detector provides the chromatogram shown in Figure 5.

In the selection of the optimum wavelength for detection, we considered the relative concentration of the two compounds of interest in the sample. Since BHT is present at a considerably lower concentration, a wavelength of 280 nm was used, since that is very close to its wavelength of maximum absorbance. Although this reduces the intensity of the tolnaftate by approximately 50%, it should

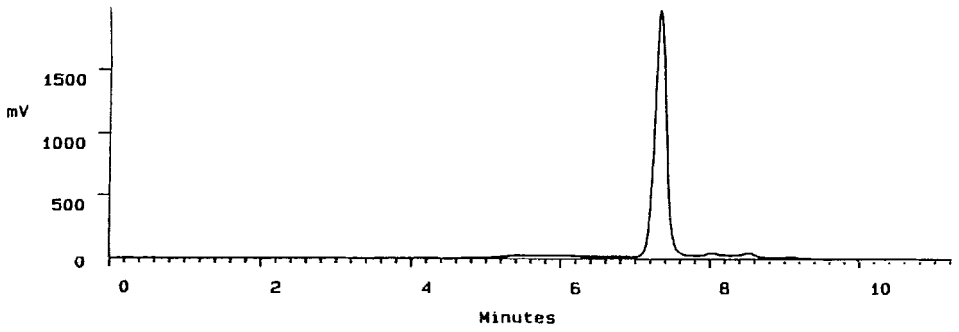


Figure 5. Separation of antifungal cream. Solvent:THF, 1 mL/min, 100 A Ultrastyragel column. Absorbance detector set at 280 nm.

not pose any difficulty since the concentration of the active ingredient is high.

Selecting the appropriate solvent to maximize resolution. In GPC, the separation is based on the effective size in solution of the compounds of interest. Since some solvents can interact with analyte molecules by phenomena such as hydrogen bonding, the choice of solvent may have a significant effect on the effective size of an analyte in solution. Tetrahydrofuran is capable of forming hydrogen bonds with BHT, thus effectively making the molecule larger. The fact that BHT co-elutes with the tolnaftate indicates that a significant amount of hydrogen bonding is probably occurring. In order to effect a separation of the two compounds, a solvent which is less capable of forming hydrogen bonds was used.

Using methylene chloride as the mobile phase, the chromatogram shown in Figure 6 was obtained. Tolnaftate and BHT are baseline separated and can be readily quantitated. The improved separation of the compounds of interest may be ascribed to the lack of hydrogen bonding of methylene chloride to BHT so that BHT and tolnaftate exhibit different sizes in solution. Comparing the behavior of these molecules in THF to that in methylene chloride, it appears that the absence of hydrogen bonding enhances the size difference

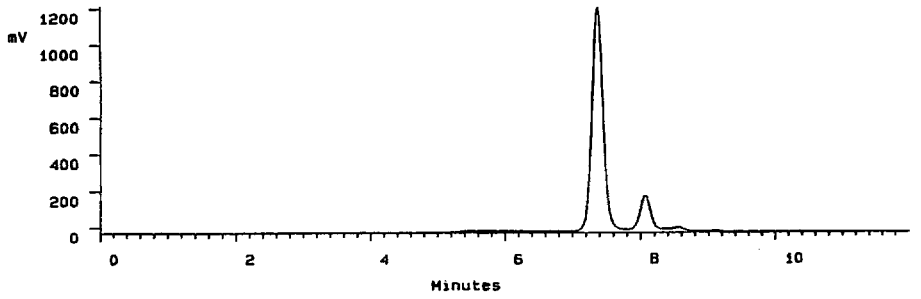


Figure 6: Separation of antifungal cream. Solvent:Methylene Chloride, 1 mL/min. 100 A Ultrastyragel column. Absorbance detector set at 280 nm.

between BHT and tolnaftate so that the smaller effective molecular size for BHT results in a longer retention time.

Analysis of Tolnaftate and BHT in Creams. The optimum conditions for the analysis of Tolnaftate and BHT by SMGPC is with a column with small pores (100 Å), a non-hydrogen bonding solvent and absorbance detection at 280 nm. Sample preparation consists simply of dissolution, filtration and injection. The cream was dissolved in THF rather than CH_2Cl_2 because THF appeared to solubilize the cream more effectively. The presence of THF in the 5µl injection did not affect the chromatographic performance of the system. Commercial antifungal treatments contain approximately 1% of the active ingredient, thus a typical injection represents a sample concentration of 8.5µg/5µl. This is contrasted to the USP procedure for the cream, in which the sample is dissolved in chloroform, washed twice with base, washed twice with acid, washed with water, filtered, and diluted for measurement of the absorbance (5). The USP procedure does not involve an HPLC separation, and could be subject to error due to unexpected interferences which have similar solubility and absorption properties. The two procedures are compared directly in Table I.

TABLE I

Comparison of USP Standard Procedure to SMGPC Procedure

USP	SMGPC
Dissolve in Chloroform	Dissolve in Tetrahydrofuran
Wash with 0.1n NaOH (2x)	
Wash with 0.1n HCl (2x)	
Wash with water	
Filter the organic layer	Filter through Millex-SR Cartridge
Adjust volume, mix	
Dilute 1:9, mix	
Measure UV-absorption	Inject

CONCLUSION

The analysis of tolnaftate demonstrates the ease of using SMGPC. In this assay, the analyst simply dissolves the sample in methylene chloride, filters the solution and injects it onto the column. The assay is rapid; it takes only 12 minutes from dissolution to completion of the separation. It should be noted that SMGPC allows for the analysis of more concentrated samples than other modes of HPLC. Since it is more difficult to overload the SMGPC column than a standard reverse phase column, a broader dynamic range of detection can be obtained (6).

Small Molecule Gel Permeation Chromatography is a very powerful analytical technique for the separation of complex mixtures. The technique is complementary to reverse phase HPLC, as it provides a mechanism of separation by molecular size. The choice of solvent can effect significant changes in the separation; molecules of similar molecular weight which differ in their ability to form hydrogen bonds can frequently be separated by the choice of an appropriate solvent.

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