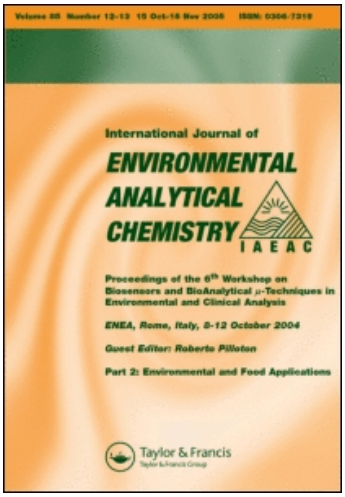

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Gel Permeation for the On Line Removal of Proteins from Plasma Samples Prior to HPLC†

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Gel permeation chromatography (GPC) has been investigated for the on line removal of proteins from plasma samples prior to their analysis by HPLC. The results show that GPC is a mild and effective way to remove proteins from plasma samples. It can very well be coupled on line to HPLC, providing the solutes are suitable for preconcentration on the analytical column itself or on a small pre-column, after the GPC. Under these conditions excellent reproducibility and accuracy can be obtained.

KEY WORDS: Gel permeation chromatography (GPC), plasma samples, HPLC, on line removal of proteins.

INTRODUCTION

Reversed phase liquid chromatography has shown to be eminently suitable for the analysis of compounds in biological samples. However, the direct analysis of compounds in these samples is often not possible because of the presence of proteins. These proteins tend to denaturate in the water/organic solvent mixtures used as mobile phase and precipitate on the top of the column or show a strong tendency to adsorb irreversibly on the hydrophobic column packings. In both cases the column deteriorates in a short time. Therefore proteins must be absent in the final injection solution.

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There are several methods available to remove proteins from biological samples such as precipitation, dialysis, ultrafiltration. Alternatively the compounds can be separated from proteins by selective liquid–liquid extraction.[1] Although these methods are quite effective, they are laborious and time consuming and moreover are difficult to automate. Other separation techniques that are capable to separate proteins from low molecular weight compounds and have better features for automation are solid phase extraction[2,3] and gel permeation chromatography. Solid phase extraction has the disadvantage that apart from the solutes also proteins adsorb on the solid phase. When desorbing the solutes also the proteins are released from the solid phase and thus injected into the analytical column.

In this study we present the results of an investigation to apply gel permeation chromatography as an on line clean up step to remove proteins from plasma samples prior to the analysis by HPLC. Gel permeation lends itself for on line coupling with reversed phase and ion-exchange chromatography (i.e. multi dimensional liquid chromatography) as has been previously demonstrated.[4–6]

EXPERIMENTAL

Apparatus

The schematical representation of the whole set up is given in Figure 3 and Figure 5. The set up was built with two pumps: a constant flow pump (type 4140 Kipp en Zonen, Delft, The Netherlands) and a reciprocating pump (Orlita, Giessen, F.R.G.); two high pressure injection valves (Rheodyne, 7010, Berkeley, U.S.A.) and a spectrophotometer (Spectroflow 757, Kipp en Zonen). The dimensions of the analytical, gel permeation and pre-concentration columns were 250×4.6; 80×6.2; 160×6.2; 250×9.4; and 30×2.0 mm ID respectively. The analytical and gel permeation columns were commercially available (Dupont), the pre-concentration column was packed by means of a slurry technique.

Materials

All solvents and chemicals were of analytical grade and purchased from Merck (Darmstadt, F.R.G.). The column packings used were

Hypersil ODS (Shandon, U.K.) particle size 5 μm and Zorbax ODS 7 μm (pre-packed columns, Dupont). The gel permeation column was a Zorbax GF 250 column, commercially available from Dupont (Wilmington, U.S.A.).

RESULTS AND DISCUSSION

In order to determine optimum conditions for the removal of proteins from plasma samples by GPC, on line coupled to HPLC, a number of experiments were carried out: the elution behaviour of proteins and some test solutes were measured on the GPC column; the possibilities of on-column and pre-column concentration and the reproducibility and quantitative aspects were investigated.

Selection of the conditions for gel permeation chromatography

Plasma contains about 6.5 g of proteins/100 g plasma. These proteins consist of about 60% of albumins (Mw 67,000–134,000), 35% of globulins (Mw 160,000), 4% of fibrinogen (Mw 330,000) and 1% of lipoproteins (Mw $\approx$ 200,000). The ideal packing for GPC in this study should show non-adsorptive properties, a complete exclusion of proteins from the pores, but allowing a complete permeation of the low molecular weight compounds. In that case the resolution between proteins and low molecular weight compounds is the largest and constant for all solutes. However, such an ideal packing is not yet available. We therefore decided to use the Zorbax GF 250 column of Dupont. The packing in this column consists of a zirconium-stabilized silica of which the surface is chemically modified with hydrophylic groups. The packing has small adsorptive properties towards proteins, is stable up to pH = 8 and shows an excellent column efficiency.

The permeation range of the GPC columns (size 250 × 9 mm) was investigated with a number of proteins and dextranes with different molecular weights, using 0.15 M phosphate buffer pH = 7 as mobile phase. The graphs of log molecular weight versus retention volume are given in Figure 1. The curve of proteins and dextranes do not match. This can be attributed to the different shape of both types of macromolecules[7] (e.g. sphere and rod shape). However, from both

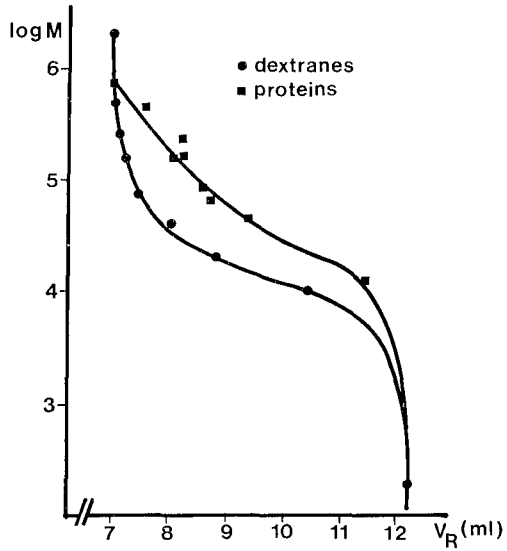

FIGURE 1 Relationship between log molecular weight (Mw) and retention volume (V_R) of dextranes and proteins on the Zorbax GF-250 gel permeation column.
Mobile phase: 0.05 M Na_2HPO_4 pH = 7.
Detection: for dextranes refractive index (Waters, Framingham, U.S.A.) and for proteins UV detection ($\lambda = 254$ nm).

curves it can be seen that the permeation limits range from 400,000 to 4,000 Mw. Figure 1 shows that there is a 3 ml difference in elution volume between albumine, the major protein in plasma, and compounds with a molecular weight $<4{,}000$. This difference is large enough to separate proteins from low molecular weight compounds in plasma as is demonstrated in Figure 2. However, on this column the solute peak elutes in a relatively large volume (≈ 2 ml), which might give rise to problems with the pre-concentration step. In order to diminish the dilution in the GPC, two smaller sized home-packed GPC columns were investigated on their efficiency to separate plasma proteins from small molecules (see Figure 3). As can be seen in this figure, a considerable overlap between proteins and test solute occurs on the 80×6 mm ID column. However, on the 160×6 mm ID column the separation is quite good and seems to be suitable to remove effectively the proteins from small plasma samples. The 250×9 mm and 160×6 mm ID columns were used in all further studies.

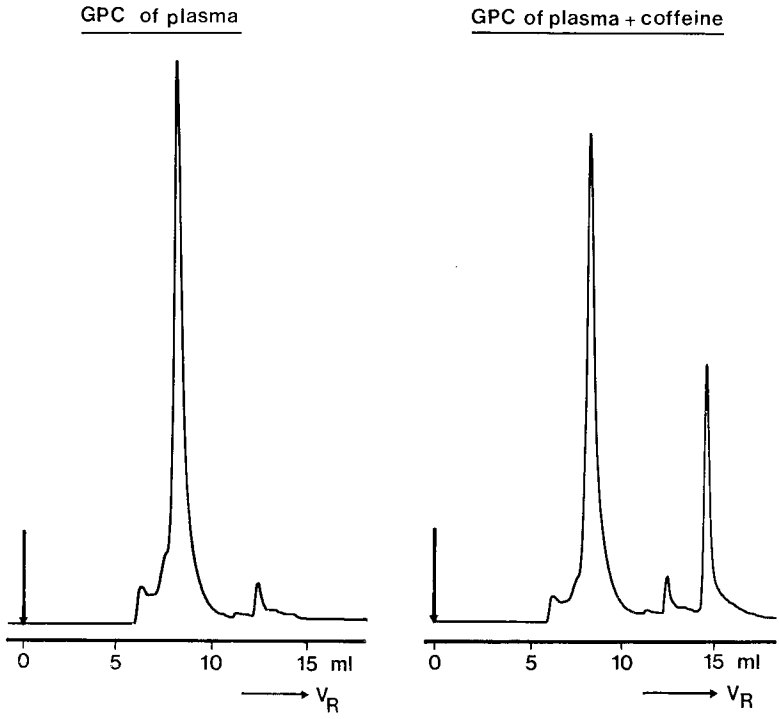

FIGURE 2 Chromatogram of a plasma sample spiked with coffeine on the Zorbax GF-250 gel permeation column (250 × 9.4 mm ID).
Mobile phase: see Figure 1.

In order to determine whether all plasma proteins are eluted from the GPC column, the protein content was measured in the original injection solution and in the collected GPC eluate. The protein content was determined according to the method described by Lowry.[8] The measurements show that the recovery of proteins is between 80–90%. In order to check whether a part of the proteins is adsorbed on the column packing or the observed loss can be ascribed to the applied analysis method, the GPC column was washed with methanol after ten times injection of plasma samples. No proteins were found in this methanol fraction. It can therefore be concluded that the plasma proteins elute completely from the GPC column under the selected conditions and that the loss is due to the imprecision of the analysis method.

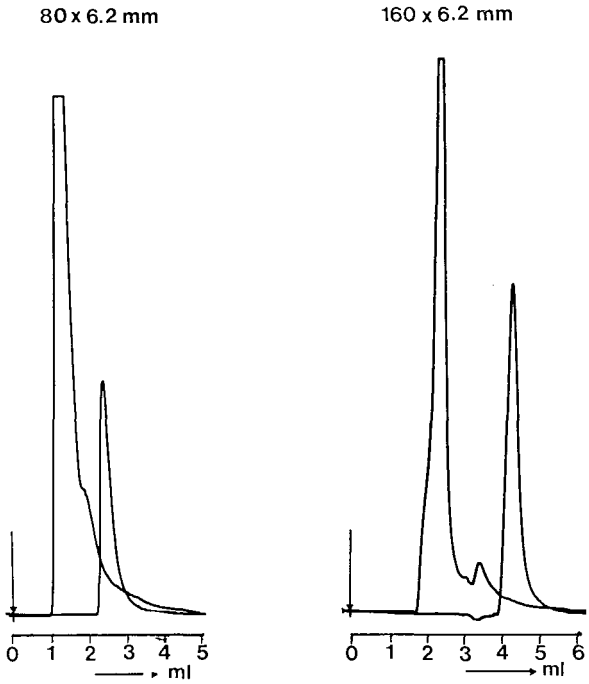

FIGURE 3 Chromatogram of a plasma sample spiked with phenobarbital on two GPC columns with different length.
Mobile phase: see Figure 1.

To collect correctly that fraction of eluate which contains the solutes of interest, it is necessary to know their elution behaviour on the GPC column. We therefore measured the retention volumes of a variety of frequently used drugs on the GPC column. In the ideal case (i.e. no adsorption onto the support) all low molecular weight compounds will have the same elution volume. However, it appears that some of the selected solutes show a significant larger elution volume than expected on basis of the permeation data as is shown in Figure 1. This indicates that a mixed retention mechanism (GPC and adsorption) occurs for these solutes.

Although the occurrence of adsorption gives rise to a better resolution between solutes and proteins, it excludes the collection of all low molecular weight compounds in a small defined eluate fraction. In other words, for each solute this fraction has to be

determined by measuring its retention volume on the GPC column. Despite this drawback, the extra retention due to adsorption can simplify the separation on the analytical column because of the more selective cut in the gel permeation chromatogram.

It appears that the nature of the adsorption is of the reversed phase type and can be decreased by adding a small amount of methanol to the mobile phase. A methanol percentage up to 10% does not harm the proteins. However, the addition of methanol has only sense when the solutes still can be pre-concentrated before injection into the analytical column (mainly rather hydrophobic solutes).

On line coupling of GPC and HPLC

When coupling the GPC with the analytical column one is confronted with the fact that the solutes of interest are eluted from the GPC column in a relatively large volume ($\approx$1–2 ml). For quantitative work it is necessary to inject this whole volume onto the analytical column. However, such large injection volumes deteriorate tremendously the column performance of common sized analytical columns. It is therefore necessary to decrease significantly the volume of the collected fraction without loss of solutes. A very elegant way to realize this is by preconcentrating the solutes on a solid support.[2] This requires that the solutes show a large retention on the solid support in the presence of the sample solvent. Fortunately, the mobile phase applied for the GPC separations in our study appears to be a very weak solvent in reversed phase chromatography. This offers the possibility to pre-concentrate many solutes from the collected GPC fractions on reversed phase packings. There are two approaches to realize this in an on line way:

i) The collected fraction is directly injected into the analytical column. In this way pre-concentration occurs on the top of the column (on-column concentration).[9]

ii) The collected fraction passes first a small pre-concentration column, placed in the sample loop of an injection valve, then the solutes are injected onto the analytical column by desorbing them from the small pre-column (on pre-column concentration).[2]

Both approaches were investigated.

On-column concentration

The set up for the on line GPC-HPLC combination, using pre-concentration on the analytical column is schematically shown in Figure 4. The desired fraction of the eluate from the GPC column is trapped in a large sample loop of an injection valve and then injected onto the analytical column. The plug of sample solvent displaces the original mobile phase at the top of the column, by which the solutes strongly adsorb on the column packing. When the whole sample has been injected, the original mobile phase enters the column again and elutes the adsorbed solutes from the top of the column. It will be obvious that the injection of a large plug of solvent, deviating from the mobile phase composition, gives rise to a significant disturbance of the phase system and consequently in the chromatogram as can be seen in Figure 5. Despite this disturbance, it can be seen from this figure that the test solute appears as a sharp, well resolved peak. This indicates that with this on-column concentration technique, large sample volumes can be injected without losing much in column performance.

The main disadvantage of the technique is the occurrence of the disturbance of the phase system. This causes that the retention is not well defined anymore and moreover depends on the injection volume. However, under standardized conditions reproducible and accurate data can be obtained.

On pre-column concentration

The set up for the on pre-column concentration is schematically shown in Figure 6. The selected eluate fraction of the GPC is sent

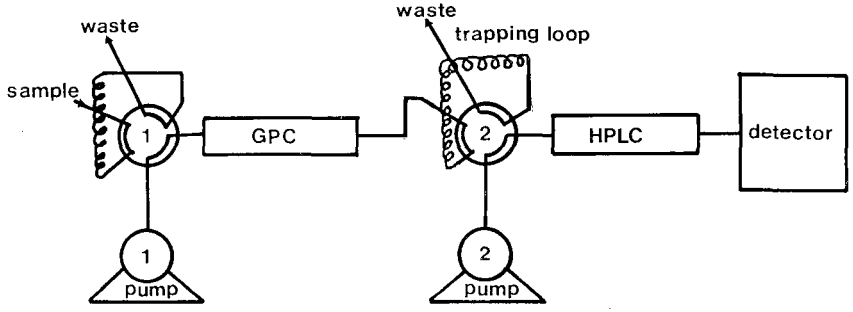

FIGURE 4 Schematic representation of the set up for on column concentration.

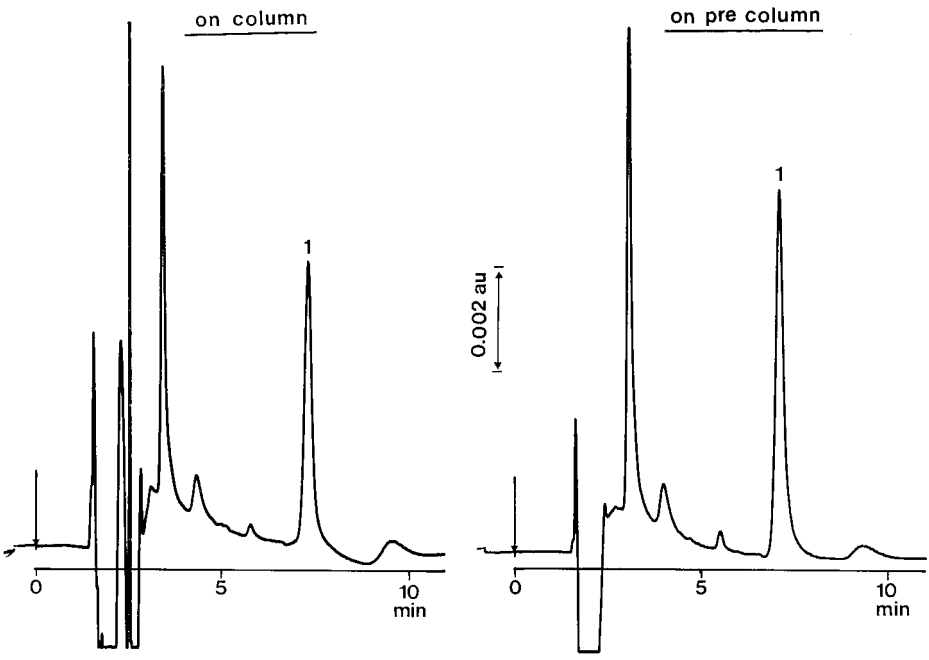

FIGURE 5 Chromatogram of a spiked plasma sample (primidon) obtained with the on-column and on pre-column concentration technique.

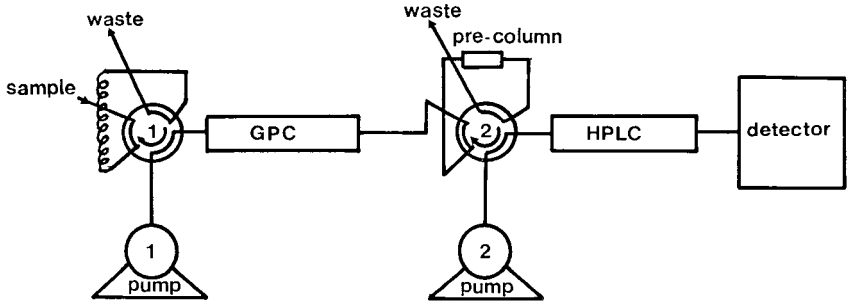

FIGURE 6 Schematic representation of the set up for on pre-column concentration.

through a small pre-column. This pre-column is filled with a packing that adsorbs strongly the solutes when in contact with the sample solvent (e.g. the GPC mobile phase). By switching of the valves the pre-column is placed in the mobile phase stream to the analytical column. When the elution strength of the mobile phase is much larger than that of the sample solvent, the solutes desorb instantaneously from the pre-column and are injected into the analytical column. With this set up a significant smaller sample volume is injected ino the analytical column compared to the on-column concentration technique. This small volume hardly disturbs the chromatogram as can be seen in Figure 5. Moreover, the pre-column protects the analytical column from contamination, which may lengthen the column life time. The performance of the analytical column was not seriously influenced by injecting the sample via the pre-column. The theoretical plate height drops with about 5–10% for solutes with a k' of 4 to 8.

Effect of the injection volume

The influence of the injected volume of plasma on the resolution between proteins and small molecules on the GPC column was investigated by injecting different volumes of plasma spiked with a test solute on the 250 × 9 mm ID column. Figure 7 shows the

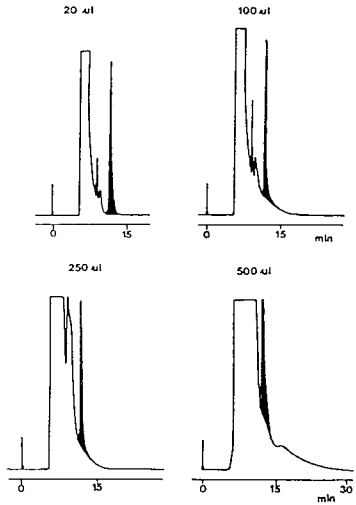

FIGURE 7 GPC chromatograms obtained by injection of different volumes of spiked plasma (primidon).

chromatogram obtained on the GPC column with four different injection volumes of plasma. It shows that the resolution between proteins and solute on the GPC column is still acceptable up to about 200 μl. However, at larger volumes the tail of the protein peak elutes at the position of the small molecule due to volume overload of the column and thus starts to interfere. However, this overlap does not mean that the collected fraction of the test solute is not suitable for further analysis by HPLC. In contrary the concentration of the proteins in the collected fraction is very small compared to that in the original plasma sample and it was found that this does not harm seriously the HPLC analysis. From these results it can be concluded that about 500 μl of plasma can be "treated" on the GPC column to remove effectively the proteins from the low molecular weight compounds.

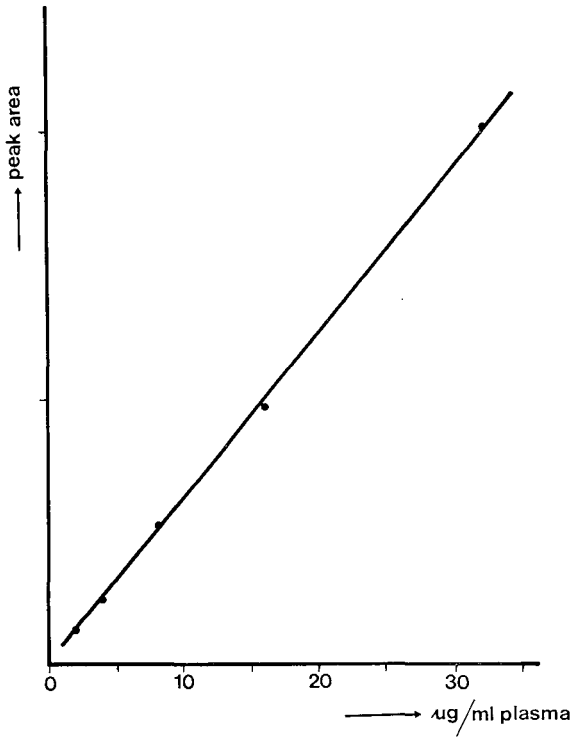

FIGURE 8 Calibration curve for phenobarbital with GPC-HPLC and on column pre-concentration.

Quantitative aspects

The reproducibility and linearity of the GPC-HPLC combination was investigated with the on pre-column concentration configuration by injection of 100 μl of spiked plasma samples and measurement of the peak areas. The reproducibility was found to be $\pm 1.1\%$ ($n=6$). The calibration curve is shown in Figure 8. As can be seen a high degree of linearity was found with the selected test solute phenobarbital. Similar results were obtained with primidon and salicyclic acid, drugs which show another degree of protein binding than phenobarbital. This indicates that most probably the drugs are completely released from the proteins during their transport through the GPC column. However, if this also occurs with drugs with very strong protein binding properties has not yet been investigated. Work in this direction is now in progress.

Conclusions

The main conclusions of this study can be summarized as follows:

—Gel permeation chromatography is an effective and mild way to remove proteins from plasma samples.
—This technique lends itself to on line coupling with HPLC, providing the solutes of interest can be pre-concentrated (on column or on pre-column) after the GPC.

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