The porphyrias are a group of metabolic diseases characterized by the abnormal production and excretion of porphyrins. The clinical diagnosis of porphyria is based on the qualitative and quantitative determination of the pattern of excreted porphyrins which has been shown to be characteristic of the type of porphyria (1).

The analytical methods currently used for porphyrin analysis are difficult, laborious and expensive. We recently (2) developed a high pressure liquid chromatographic technique for the quantitative analysis of the urinary porphyrins. We have now studied the application of a high pressure liquid chromatographic method to the analysis of fecal porphyrins, which may include, besides the 2 to 8 carboxylated compounds, a heterogeneous group of sub-uroporphyrin compounds (3,4).

Experimental

Fecal Sample Extraction and Column Chromatography

20 ml of methanol and 2 ml of BF₃-Et₂O were added to the fecal sample (.5 g wet or .2 g dry), and the solution was stirred overnight. The esterified material was extracted with two 100 ml portions of methylene dichloride after the addition of 80 ml of water. The methylene dichloride solution was dried over sodium sulfate, filtered and the solvent removed on a rotary evaporator.

The residue was dissolved in a minimum of methylene dichloride and chromatographed on 30 g of silica gel Woelm activity IV in a 1.6 cm x 45 cm glass column. The silica was packed in the column using methylene dichloride/methanol
(99:5:0.5 v/v), and was eluted with the same solvent to remove a yellow-brown material. Elution by methylene dichloride/ethyl acetate/methanol (45:15:10) gave the 2 to 8 carbonyl porphyrins followed by a band of sub-uro material. The eluent between the major porphyrin band and the sub-uro material was fractionally collected and compared to uroporphyrin on silica gel TLC (methylene dichloride/ethyl acetate/methanol, 75:30:5 v/v) to prevent any carbonyl porphyrin loss. The appropriate fractions were pooled and the solvent was removed on a rotary evaporator. The 2 to 8 carbonyl porphyrin residue was dissolved in methylene dichloride (1.0 ml/0.10 g feces) and analyzed by HPLC.

Column Chromatography of the Sub-Uro Material

The sub-uro residue was dissolved in a minimum of methylene dichloride/methanol (98:2 v/v) and chromatographed on 10 g of silica gel Woelm activity IV which had been packed using the same solvent. After collection of the eluted band the solvent was removed on a rotary evaporator. The residue was dissolved in methylene dichloride (1.0 ml/0.10 g feces) and analyzed by HPLC.

High Pressure Liquid Chromatography

Samples were analyzed on a Waters Associates (WA) ALC 202 using % in x 2 ft column with Polyamide, Corasil C8, Basic Alumina, Neutral Alumina, Porasil C or Corasil II packings (6). A Cary 17 spectrometer with a detection cell as described previously (2) was used for detection at 300 and 403.5 nm with a chart speed of 0.4 in/min and for visible scans (350-650 nm) taken during chromatographic analysis. The 254 nm chromatogram was recorded using a Waters Associates UV detector.

The gradient for sample elution was generated using a 12.5 ml loop in a Waters Associates valve and loop injector. The loop was made from 3.8 meters of 2 mm I.D. stainless steel tubing. The pump was constantly supplied with the higher polarity solvent and uses displacement of the lower polarity solvent from the loop for proper sample elution. The time from loop "injection" to sample injection is about 2.5 minutes.
Solvent Preparation

*n*-Propanol and triethylamine (TEA) were reagent grade. The light petroleum (30-60°) and methylene dichloride used for HPLC were glass distilled and contained 10 μg TEA/100 ml. The glass distilled methylene dichloride-TEA solution was used within 5 days of distillation. Older solutions exhibited altered chromatographic properties.

Analysis of the 2 to 8 Carboxyl Porphyrin Sample

The sample was eluted with light petroleum (30-60°) (TEA)/methylene dichloride (TEA) (40:100) followed by light petroleum (30-60°)-TEA/methylene dichloride-TEA/n-propanol (15:100:50). The 40:100 solution was placed in the loop in preparation for each injection. Solvent flow was 1.0 ml/min (< 50 psi). The eluted porphyrins were characterized by comparison with authentic samples as described previously (2).

Analysis of the Sub-Uro Material

The sample was eluted with methylene dichloride-TEA/n-propanol (100:2) followed by methylene dichloride-TEA/n-propanol (90:10). The 100:2 solution was placed in the loop in preparation for each injection. Solvent flow was 1.0 ml/min (< 50 psi). The visible spectra from the chromatogram were obtained by stopping the solvent flow and scanning the desired wavelength range.

RESULTS AND DISCUSSION

Evaluation of Available Column Packings

Chromatography of Porphyrin Free Acids

The development of a chromatographic system using Polyamide was unsuccessful because a solvent combination which would elute Proto-porphyrin IX could not be found. A reverse phase (C18) column was tested with combina ions of:

- Methanol
- 2% Ammonium Hydroxide
- Acetonitrile
- H2O

The samples eluted with tailing or as very broad peaks.
Chromatography of Porphyrin Methyl Esters

The adsorption packings were tested with combinations of:

<table>
<thead>
<tr>
<th>Methylene Dichloride</th>
<th>Propanol</th>
<th>Ethyl Propionate</th>
<th>Triethylamine (TEA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Ethyl Acetate</td>
<td>Petroleum</td>
<td>(30-60°C)</td>
<td></td>
</tr>
<tr>
<td>Propyl Acetate</td>
<td>Propyl Propionate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Basic alumina gave poor resolution with broad peaks, while Porasil C gave good resolution but extremely broad peaks. Samples chromatographed on neutral alumina tailed too much to give acceptable resolution.

Corasil II gave good resolution and peak width using a methylene dichloride/light petroleum (30-60°C)/n-Propanol/Triethylamine (TEA) system. This packing was applicable to a simple gradient system. If an electronic programmer were available methylene dichloride/n-propanol (0.3-1.0% n-propanol) would probably give a good chromatogram. However, for routine clinical analysis the operationally simpler system was best.

A comparison of standard and urine samples on Corasil II and Porasil T (Fig. 1) demonstrates that Porasil T gives the better resolution in the 4 to 8 carboxyl region and is well suited to the analysis of urine samples in which the 4 to 8 carboxyl porphyrins is important. For the analysis of fecal samples in which the 2 to 4 carboxyl porphyrins dominate Corasil II is preferred but for routine clinical needs either column is equally suitable.

Generation of a Gradient

The use of a valve and loop injector provided a convenient and reproducible method for the generation of a gradient. The baseline at 254 nm (Fig. 2) illustrates the change between the two solvent systems. This is supported by the tight uroporphyrin peak in the reference chromatogram because this compound elutes as the propanol/amine is flushed from the column.

Fecal Analysis

Boron trifluoride/methanol has been shown (5) to be an efficient method for the esterification of fecal porphy-
Fig. 1. Comparison of a urine sample chromatogram on Corasil II (upper) and Porasil T (lower). Note the difference in elution time for the 4 to 8 carboxylated porphyrins. Absorption not to scale.

We combined this procedure with column chromatography on silica with methylene dichloride/ethyl acetate/methanol to give porphyrin samples which were suitable for HPLC analysis; this gave a convenient separation of the 2 to 8 carboxyl porphyrins from the sub-uro compounds. The latter was rechromatographed on a methylene dichloride/methanol column. With each fraction, fluorescence remained on the top of the column.
The spike at -2 minutes is caused by an air bubble and marks the change to lower polarity solvent. Methylene dichloride is injected at 0 minutes; the solvent front appears at 1.5 minutes, and the higher polarity solvent begins to elute at 11 minutes. The peak at 20 minutes is discussed in the text.
Fig. 3. Chromatogram of Fecal samples VGH and SA; absorption not to scale.
Fig. 4. Chromatogram and visible spectra of the sub-uro fraction.
The adsorption of the 300 and 403.5 nm chromatograms is on scale.
The visible spectra A-D were taken at the points indicated.
The 400 nm region of A-C and D is reduced by a factor of 2.5.
The results of the analysis of two fecal samples for the 2 to 8 carboxyl porphyrins are given in Table I and Figure 3.

The preliminary results of the chromatographic analysis of the sub-uro group illustrate that HPLC will provide information on these compounds. The highly complex sub-uro fraction (VGH) has been investigated at 403.5 and 300 nm (Figure 4).

The tracing at 300 nm suggests the presence of compounds which do not absorb strongly in the Soret region. Figure 4 also shows the advantage of visible spectra measured during a chromatographic scan. All four spectra (A-D) demonstrate the presence of porphyrins and spectrum C demonstrates the presence of a major non-Soret component.

Acknowledgement
This work was supported by the Canadian National Research Council and the United States National Institutes of Health (AM 17999).

REFERENCES

7. The polyamide packing was a nylon type absorbent bonded to an impermeable bead available from Reeve Angel under the name Pellamidon. The Corasil C\textsubscript{18} was a linear 18-carbon alkane bonded to a silica coating on an impermeable bead. It is sized at 37-50 μ and is available from Waters Associates. The basic and neutral Woelm aluminas were 18-30 μ, from Waters Associates. The Porasil C was a spherical totally porous silica of 37-75 μ size, from Waters Associates. The Corasil II was a silica coating on an impermeable bead. It was 37-50 μ from Waters Associates.