The Chromatographic Separation of Uroporphyrin I and III Octamethyl Esters

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Uroporphyrins I and III have been separated by high performance liquid chromatography on a microporasil column using n-heptane:glacial acetic acid:acetone:water (90:60:30:0.05 by volume). By using uro [3H]porphyrin I and uro [14C]porphyrin III it was shown that less than 2% cross contamination occurred during the chromatographic separation. The same system may be used to separate uro through protoporphyrins (as their esters) by simply increasing the acetone concentration.

Of the four possible isomers of uroporphyrin (designated by the Roman numerals I–IV) two are of biochemical interest. These are the Type I and Type III isomers. Uroporphyrinogen III, the fully reduced form of uroporphyrin III, is the biosynthetic precursor of all known biologically functional tetrapyrroles including the hemes, chlorophylls, and corrinoids. In nature under normal conditions the Type I isomer is only encountered in trace amounts. However, in certain disease states, considerable quantities of the Type I isomer are produced and excreted. In erythropoietic porphyria (1,2), for instance, there is a relative deficiency of one of the two enzymes required for the formation of uroporphyrinogen III. As a consequence, uroporphyrin I is produced and excreted in detectable quantities. The disease is inherited in an autosomal recessive fashion. Thus only individuals that are homozygous actually manifest the symptoms and suffer the effects of the disease. Heterozygotes show no overt symptoms. They do, nevertheless, have a reduced level of uroporphyrinogen III cosynthase. Levin (2) has clearly demonstrated that it is possible to detect the lowered level of uroporphyrinogen III cosynthase in heterozygote individuals.

Diagnosis of the heterozygote condition requires that one be able to differentiate quantitatively uroporphyrin isomers of Types I and III. Until the advances reported in this paper, this differentiation and quantitation could only be achieved...
with confidence by the tedious process of decarboxylation and chromatography of the resulting coproporphyrins. An example which demonstrates the importance of detecting the heterozygote can be found in the bovine artificial insemination industry. A single bull can sire as many as $3 \times 10^8$ offspring. The records show that several bulls, heterozygote for the erythropoetic trait, have been "in service" long enough to spread this gene rather widely in the dairy cattle industry.

The chromatographic separation of uroporphyrin isomers I and III was first reported in 1953 (3). At the same time other methods, including melting point depression and X-ray diffraction pattern analysis, were introduced to allow analysis of the uroporphyrins (4,5). The latter two methods have proven to be tedious and unreliable. The problem with most chromatographic methods is exemplified by the experiments of Bogorad and Marks (6) in which they studied the Falk and Benson thin-layer chromatographic (tlc) method using radioactively labeled uroporphyrin esters. Although in these experiments the mixtures of porphyrin esters separate into two spots, the labeling experiments showed a high percentage of each isomer in both spots. It has been postulated that the two isomers form molecular complexes (7) which prevent the independent interaction of the molecules with adsorbant and solvent. The method of Falk and Benson, however, particularly as modified and improved by Cornford and Benson (8), continues to be of some use in limited situations, with obvious caveats.

The column chromatography system of Chu and Chu permits the isolation of pure uroporphyrin III and uroporphyrin I esters (9,10), as verified by decarboxylation of the uroporphyrins followed by chromatography of the resulting coproporphyrins (12, 13). However, this method is not practical as a quantitative analytical technique. Another reported separation of the uroporphyrin isomers is that of Mundschenk (14). This system involves the separation of the isomers in the free acid form. The apparent disadvantages of this system are among other things the $20-30$ h required for the separation.

We have successfully achieved analytical resolution of the octamethyl esters of uroporphyrins I and III using a high performance liquid chromatographic (hplc) system. The separation is effected in 90 min using an isocratic recycling solvent system. Experiments with mixtures of $^3$H- and $^{14}$C-labeled isomers have shown that cross contamination amounts to not more than 2%.

**EXPERIMENTAL**

**Materials.** Uroporphyrin I was prepared enzymatically from δ-aminolevulinic acid (15). Uroporphyrin III was isolated from the livers of Sprague–Dawley rats which had been maintained for many months on a diet supplemented with hexachlorobenzene. Both compounds were purified as the octamethyl esters by thin-layer chromatography (16). Isomeric purity was verified by chemical decarboxylation (11) to the corresponding coproporphyrins which were then chromatographed by the 2,6-lutidine method (12). Uroporphyrin I octamethyl ester labeled with $^3$H was prepared by ester exchange with tritiated methanol in methylene chloride using sulfuric acid as catalyst. The uroporphyrin III octamethyl ester labeled with $^{14}$C was likewise prepared by ester exchange with $[^{14}$C]methanol in methylene chloride. Solvents were reagent grade and the sample of 8–2 carboxyporphyrins was the standard kit available from Porphyrin Products.

**Instrumentation.** The high performance liquid chromatography system employed was a Waters Associates Model ALC 202 liquid chromatograph with a Cary Model 17 spectrophotometer detection system used at 403 nm (17). Scintillation counting was carried out on a Nuclear Chicago Mark II system employing a $^{13}$Ba external standard.

**Chromatography.** The final uroporphyrin
separations described were achieved using two μ-porasil columns (Waters Associates) in series and a solvent system of n-heptane:glacial acetic acid:acetone:water (90:60:30:0.05 by volume) at a flow rate of 1.5 ml/min in the recycling mode. The uro to proto-porphyrin separation used a single μ-porasil column and a solvent system of n-heptane/glacial acetic acid/acetone/water (90/60/90/05 by volume) at a flow rate of 1.5

Fig. 1. Separation of approximately equal quantities (1.0 μg) of uroporphyrins I and III. (Conditions described under Experimental.)

Fig. 2. Separation of approximately 1:9 quantities (1.0 μg total) of uroporphyrins I and III. (Conditions described under Experimental.) (A) Uro III > Uro I. (B) Uro I > Uro III.
Although this system would, on some occasions, give baseline resolution both the retention times and degree of resolution were varied and unpredictable.

The system which we have finally developed, using n-heptane, acetic acid, acetone, and water gives totally reproducible results and when the system is recycled acceptable resolution is achieved. Figures 1 and 2 illustrate the separation of the isomers and indicate that it is independent of their relative concentrations. The sensitivity of the system is indicated by the fact that these analyses were carried out using approximately 10 μg porphyrin/injection.

Bogorad and Marks, in an analysis of the separation procedure of Falk and Benson (3), demonstrated isotopically that cross contamination occurred, see above (6). We therefore carried out the following experiments with labeled uroporphyrin isomers.

A solution containing uro [3H]porphyrin I and uro [14C]porphyrin III was separated by the hplc system described above. The eluent was monitored optically and fractions were counted for both their 14C and 3H

![Figure 3](image1.png)

**Fig. 3.** Separation of uro [3H]porphyrin I and uro [14C]porphyrin III. (Conditions described under Experimental.)

Typical uroporphyrin injections (1.0 μg) gave 90 ± 5% off-column recovery.

**RESULTS**

Initially, separation of the two isomers was achieved using an isocratic solvent system of glacial acetic acid and n-heptane.

![Figure 4](image2.png)

**Fig. 4.** Separation of the uro-(8-carboxyl) to proto(2-carboxyl)porphyrins. (Conditions described under Experimental.)
content. The results of a typical separation are given in Fig. 3, where we have found that essentially no (less than 2%) cross contamination, as opposed to peak overlap, has occurred.

Although baseline separation has not been achieved with the recycling regime used here, deconvolution of the optical output and radioactive assay of collected fractions show that the isomers are greater than 92% resolved. In addition the low degree of trailing observed allows for direct integration of the curves.

This same chromatographic system can be used for the separation of porphyrins containing eight through two carboxylic acid groups. The resolution and time of analysis (Fig. 4) are comparable to those reported previously (18). Consequently this chromatographic system can be used for both the analysis of the isomeric uroporphyrins as well as for mixtures of uro- through protoporphyrins without the lengthy delays necessitated by column equilibration with drastically different solvent mixtures.

DISCUSSION

Despite the large number of solvent systems that we and others have employed in attempts to separate uroporphyrins I and III only the present system employing an organic acid has proven to be successful. The difficulty in chromatographically separating porphyrins in general, and the uroporphyrins in particular, has been speculated to be due to their high degree of association. We suggest that in the chromatographic system employed here N-protonation of the porphyrins might effectively reduce this association and may account for the observed separations. In the solvent system employed in this study (n-heptane, acetic acid, acetone, water) protonation of the uroporphyrins was not observed spectroscopically. However, as the dielectric constant of the medium is changed, acetic acid can protonate the uroporphyrins. Thus in a 1/1 (v/v) mixture of glacial acetic acid/methanol uroporphyrin III partially exists (1/3) as the mono-N-protonated cation (19). If a similar protonation occurs in the microenvironment of the silica column association should be lowered and separation would be enhanced.

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REFERENCES