A simple method for the separation and quantification of urinary porphyrins

DT LEAHY, TG BRIEN

From the Department of Pathology, University College, Earlsfort Terrace, Dublin 2, Ireland

SUMMARY A simple routine method for the separation and quantification of urinary coproporphyrin and uroporphyrin using anion-exchange resin columns is described.

The coproporphyrin is first removed from the urine by ether extraction. The anion exchange resin column is then used to isolate the uroporphyrin from the aqueous residue.

The proposed method is compared with an existing method developed by Rimington in terms of recovery and reproducibility. Results from 15 urine specimens analysed by both methods are compared.

The proposed method yielded lower values for coproporphyrin and higher values for uroporphyrin than the established method, but there was a good correlation between the two methods. This and its relative simplicity make it suitable for routine use.

Although simple screening tests for the presence of raised porphyrin concentrations in urine are available these do not separate uroporphyrin from coproporphyrin and hence quantification of the individual porphyrins present in the sample cannot be achieved. However, since porphyrins exhibit a strong Soret band absorbance between 400 and 410 nm, spectrophotometry of extracts of urine may give an indication of the predominant porphyrin present in the sample. The absorbance peak occurs at about 401 nm for coproporphyrin and around 406 nm for uroporphyrin.

Quantification of urinary copro- and uroporphyrins can be achieved using the method of Rimington. However, this method involves multiple extraction with several solvents, is long and tedious and is unsuitable for handling more than a few specimens at a time. In this paper we describe a simple and rapid procedure for the quantitative estimation of uroporphyrin and coproporphyrin in the same urine sample employing only one solvent extraction and ion-exchange chromatography.

Material and methods

Coproporphyrin and uroporphyrin standards were purchased from Sigma Ltd, Poole, England. Porphyrin chromatography columns containing 1g of Dowex 1 × 8, 50–100 mesh resin were obtained from Bio Rad Laboratories, Richmond, California.

RIMINGTON’S METHOD

This is the classical solvent extraction method of Rimington (1971) as recommended by the Association of Clinical Pathologists (ACP). It was slightly modified by the substitution of n-butanol extraction at pH 3:2 for cyclohexanone in the final stages.

Urine (12.5 ml) is pipetted into a separating funnel and acidified with 1:25 ml of glacial acetic acid. The coproporphyrins are extracted twice with 25 ml of ether. The ether extracts are combined and the aqueous phase is retained for determination of uroporphyrin.

The combined ether extracts are washed with successive portions of dilute sodium acetate solution until the aqueous phase no longer shows red fluorescence under ultraviolet light. These washings are combined, twice shaken with an equal volume of fresh ether and then added to the original aqueous urine residue. The ether is combined with the initial ether extracts and the whole washed with dilute iodine solution and then with distilled water.

Coproporphyrin is extracted from the ether by shaking with successive 2 ml portions of 5% hydrochloric acid until the extract no longer shows red fluorescence under ultraviolet light. The acid extract is quantified spectrophotometrically.

The combined aqueous phases are brought to a pH of 3-2 and extracted twice with n-butanol. Two
volumes of ether are added to the pooled butanol extracts and uroporphyrin is extracted by shaking with successive 2 ml volumes of 5% HCl until the extracts no longer show red fluorescence. The uroporphyrin content of the pooled acid extracts is measured by spectrophotometry.

PROPOSED METHOD
The proposed method is as follows: an aliquot of the urine is acidified with glacial acetic acid to between pH 4-0 and pH 4-5. The acidified urine (10 ml) and 3 ml of acetate buffer (pH 4-8, 4·5 M) are added to a separating funnel and extracted twice with 15 ml of ether to remove the coproporphyrins. The aqueous phase is reserved for further treatment and the combined ether extracts are washed with 10 ml of distilled water.

The coproporphyrin is extracted from the ether with 2 × 2 ml of 0·1 N HCl. The absorbance of the pooled extracts is read at 380 nm, 430 nm and at the absorbance peak (about 401 nm).

Of the aqueous residue, 4 ml is applied to the ion-exchange column. The column is allowed to drain and is then washed with 10 ml distilled water. Uroporphyrin is eluted with 2 × 2 ml of 3 N HCl and the absorbance of the eluate is measured at 380 nm, 430 nm and at the peak absorbance (around 406 nm).

Calculations

\[
A_{\text{max}} = \text{peak absorbance}
\]

\[
A^k = 2A_{\text{max}} - (A^{380} + A^{430})
\]

With and Pedersen,3 from studies of purified porphyrins, derive calculation factors (f) where \( f \times A^k \) gives the concentration of the porphyrin in \( \mu g/100 \) ml. For coproporphyrin in 0·1 N HCl f = 72·9 and for uroporphyrin in 3 N HCl f = 90·2.

Urinary coproporphyrin (\( \mu g/100 \) ml):

\[
A^k \times 72·9 \times \frac{\text{volume of acid extract (4 ml)}}{\text{volume of urine taken (10 ml)}} = A^k \times 29·2
\]

Urinary uroporphyrin (\( \mu g/100 \) ml):

\[
A^k \times 90·2 \times \frac{\text{original urine volume (10 ml) + buffer (3 ml)}}{\text{original urine volume (10 ml)}} = A^k \times 117·3
\]

No allowance is made in the calculations for incomplete extraction of the porphyrins.

To convert to SI units (\( \mu mol/l \)) multiply by 0·0153 for coproporphyrin and 0·012 for uroporphyrin.

Recovery experiments were performed by adding either coproporphyrin or uroporphyrin standard to urine low in porphyrins. The concentration of the stock standard was determined in dilute HCl using a Beckman DU spectrophotometer immediately before addition to the urine, which was then analysed by the relevant method. Coproporphyrin and uroporphyrin were determined by the proposed method on random day urine samples from 30 healthy males aged 18 to 40 years. Samples from 15 patients with raised porphyrins on the initial screen or in whom individual porphyrins had been specifically requested were investigated using both methods. Not all of these patients were subsequently confirmed as suffering from porphyria.

Results

RIMINGTON’S METHOD
Mean recovery of coproporphyrin added to normal urine in amounts from 0·038 \( \mu mol/l \) to 0·305 \( \mu mol/l \) was 106·2% while mean recovery of uroporphyrin added in amounts varying from 0·119 \( \mu mol/l \) to 0·894 \( \mu mol/l \) was 60·9%. (See Tables 1 and 2.)

PROPOSED METHOD
Within-batch reproducibility of the proposed method was assessed on an urine with an endogenous coproporphyrin level of 0·24 \( \mu mol/l \) and an endogenous uroporphyrin level of 1·00 \( \mu mol/l \). The CV for coproporphyrin was 1·57% and for uroporphyrin 2·18% (n = 10). Between-batch reproducibility on another urine with mean coproporphyrin and uroporphyrin levels by this method of 0·18 \( \mu mol/l \) and 1·02 \( \mu mol/l \) respectively was 4·2% for coproporphyrin and 7·2% for uroporphyrin (n = 10).

Mean recovery of coproporphyrin added to normal urine at concentrations ranging from 0·038 \( \mu mol/l \) to 0·305 \( \mu mol/l \) was 67% while the mean recovery of uroporphyrin added in concentrations from 0·104 \( \mu mol/l \) to 0·835 \( \mu mol/l \) was 83·6%. (See Tables 1 and 2.)

LINEARITY
Using pure standards in acid solution porphyrin concentration was linear with absorbance up to 1·53 \( \mu mol/l \) for coproporphyrin and 1·94 \( \mu mol/l \) for uroporphyrin. This is in agreement with Rimington4 who found that porphyrins in acid solution obey Beer’s Law up to an absorbance of about 1·0.

REFERENCE RANGE
For random urine specimens mean coproporphyrin level was 0·053 ± 0·03 \( \mu mol/l \) with an upper 95% limit of 0·113 \( \mu mol/l \) and mean uroporphyrin was 0·009 ± 0·008 \( \mu mol/l \), upper reference limit 0·025 \( \mu mol/l \).

These results are in good agreement with the range quoted by Fogstrup and With5 who found an upper reference limit of 0·155 \( \mu mol/l \) for total porphyrin in random urine. Values for coproporphyrin and uroporphyrin in random urine have not, to our knowledge, been cited in the literature.
Table 1  Recovery of coproporphyrin added to urine low in porphyrins

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<th>Rimington's method</th>
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<th>Proposed method</th>
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<tbody>
<tr>
<td>Added</td>
<td>Recovered %</td>
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<td>Added</td>
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<tr>
<td>0.038</td>
<td>0.040</td>
<td>104</td>
<td>0.038</td>
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<tr>
<td>0.076</td>
<td>0.081</td>
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<td>0.153</td>
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<tr>
<td>0.305</td>
<td>0.322</td>
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Table 2  Recovery of uroporphyrin added to urine low in porphyrins

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<th>Proposed method</th>
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<tbody>
<tr>
<td>Added</td>
<td>Recovered %</td>
<td></td>
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<tr>
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<td>0.608</td>
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<tr>
<td>0.894</td>
<td>0.532</td>
<td>58</td>
<td>0.835</td>
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The reference range quoted by Rimington is that of Fernandez, Henry and Goldenberg. These authors used 24-hour urine and so their range is not strictly comparable with ours.

However, if we extrapolate our range on the basis of an arbitrary 24-hour urine volume of 1500 ml a relative comparison with Rimington’s range may be made. On this basis our range gives an upper reference limit of 111 µg/24 h for coproporphyrin and 30 µg/24 h for uroporphyrin as against Rimington’s upper limits of 161 µg/24 h and 30 µg/24 h respectively.

Results for the 15 patients’ urines analysed by both methods are compared in Figs 1 and 2.

Fig. 1  Comparison of coproporphyrin concentrations in 15 patient urines as assessed by Rimington’s ACP method and the proposed method.

Fig. 2  Comparison of uroporphyrin concentrations in 15 patient urines as assessed by Rimington’s ACP method and the proposed method.
p < 0.001. In the case of uroporphyrin the mean values by the proposed method were higher than Rimington's method but once again a good correlation was obtained r = 0.951, p < 0.001.

Of the 15 urines analysed by the proposed method nine were within the reference range, five were typical of porphyria cutanea tarda and one was indicative of variegate porphyria. Analysis of the series by Rimington's method gave the same diagnostic conclusions. The diagnosis of porphyria was supported in each case by clinical information.

Discussion

Recoveries of added porphyrins mentioned in the literature vary. With 7 reported coproporphyrin recoveries in the range 80 to 105% and uroporphyrin recoveries of 59 to 97% using a solvent extraction procedure. Fernandez, Henry and Goldenberg also using a solvent extraction procedure reported recoveries of 90 to 106% for coproporphyrin and 79 to 109% for uroporphyrin. Martinez and Mills 8 using ion-exchange resin method achieved coproporphyrin recoveries of 82 to 106% while uroporphyrin recovery varied from 87 to 101%.

The recovery figures for the proposed method are much lower than those in the literature for coproporphyrins and slightly lower for uroporphyrins but this should be judged in conjunction with the speed and simplicity of the procedure relative to the other methods quoted. In porphyrin urines while the relative proportions of the raised fractions may differ depending on the method used, the proposed method will indicate the type of porphyria present with similar efficiency to Rimington's method. Thus this simple rapid method provides information of equivalent clinical value to that of the more complex and time-consuming multiple solvent extraction method. The proposed method offers to the smaller laboratory the means to perform urinary porphyrin analysis which might not previously have been considered feasible.

References

2 Rimington C. Quantitative determination of porphobilinogen and porphyrins in urine and porphyrins in faeces and erythrocytes. ACP Broadsheet 70, 1971.

Requests for reprints to: Dr TG Brien, Dept. of Pathology, University College, Earlsfort Terrace, Dublin 2, Ireland.