Plasma Porphyrins in the Porphyrias

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Background: As an aid in the diagnosis and management of porphyria we have developed a method to fractionate and quantify plasma porphyrins and have evaluated its use in various porphyrias.

Methods: We used HPLC with fluorometric detection to measure plasma concentrations of uroporphyrin I and III, heptacarboxyl III, hexacarboxyl III, pentacarboxyl III, and coproporphyrin I and III. We studied 245 healthy subjects, 32 patients with classical porphyria cutanea tarda (PCT), 12 patients with PCT of renal failure, 13 patients with renal failure, 8 patients with pseudoporphyria of renal failure, 3 patients with acute intermittent porphyria, 5 patients with variegate porphyria, 5 patients with hereditary coproporphyria, and 4 patients with erythropoietic protoporphyria.

Results: Between-run CVs were 5.4–13%. The recoveries of porphyrins added to plasma were 71–114% except for protoporphyrin, which could not be reliably measured with this technique. Plasma porphyrin patterns clearly identified PCT, and its clinical sensitivity equaled that of urine porphyrin fractionation. The patterns also allowed differentiation of PCT of renal failure from pseudoporphyria of renal failure.

Conclusions: The assay of plasma porphyrins identifies patients with PCT and appears particularly useful for differentiating PCT of renal failure from pseudoporphyria of renal failure.

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At present, the principal biochemical tests for differentiating the porphyrias involve the measurement of porphyrins and their metabolites in urine, feces, and blood (1, 2). Except for the use of erythrocyte porphobilinogen deaminase activity to confirm the diagnosis of acute intermittent porphyria (AIP),1 enzyme assays have little to offer in the routine investigation of these diseases. Enzyme assays often are difficult to perform, and the relevant tissues are difficult to obtain. In addition, the results may not reflect the clinical state because penetration of the overt disease in subjects inheriting a porphyrin enzyme deficiency is often low (~10%). Thus, although the identification of enzyme defects appears to be a useful approach in diagnosis, their use in porphyrias is largely confined to research facilities, where they are used to trace inheritance patterns of the diseases and to identify the exact nature of a porphyria. Genetic analysis provides a more accurate diagnosis and other benefits in AIP, variegate porphyria (VP), hereditary coproporphyria (HCP), and erythropoietic protoporphyria (EPP), provided the patient has one of the known point mutations (3); however, these techniques currently are confined to a few specialized laboratories.

The use of urine and fecal assays, however, is not problem free. Patients are reluctant to provide samples of feces, and there is always uncertainty about whether a 24-h urine collection is complete. Random samples of urine can be used if the ratios of the porphyrins to the creatinine content are determined; however, specimen collection centers often have difficulty in prorating the amount of preservative to add to random urine samples, and therefore, most specimens are inadequately preserved. We have therefore developed a plasma porphyrin method and have evaluated its use, compared with traditional methods, in the differential diagnosis of some porphyrias.

Materials and Methods

PLASMA PORPHYRINS

Reagents and chemicals. Porphyrin calibrators were obtained from Porphyrin Products. The calibrators included coproporphyrin III dihydrochloride, uroporphyrin III dihydrochloride, 2-vinyl-4-hydroxymethyl deuteroporphyrin IX dihydrochloride, deuteroporphyrin IX, protoporphyrin IX, and a porphyrin marker kit containing a

1 Nonstandard abbreviations: AIP, acute intermittent porphyria; VP, variegate porphyria; HCP, hereditary coproporphyria; EPP, erythropoietic protoporphyria; PCT, porphyria cutanea tarda; and PCTRF, PCT of renal failure.
mixture of uroporphyrin, coproporphyrin, and hepta-, hexa-, and pentacarboxylic acid porphyrins of the I isomer series. All solvents were of HPLC grade. Extraction columns (Trifunctional C\textsubscript{18}, Sep-Pak Plus cartridges; volume, 3 mL, containing 500 mg of sorbent) were purchased from Waters. Syringe filters (GHP Acrodisc 13, 0.45 \(\mu\)m pore size) were obtained from Gelman Sciences.

**Equipment.** HPLC was performed on a Varian 5500 HPLC (Varian Canada) equipped with a Bio-Rad AS-100 autosampler (Bio-Rad Laboratories Canada) and a Shimadzu RF-1501 spectrofluorometer (Mandel Scientific).

Samples. Heparin-treated plasma was used. Blood samples were centrifuged immediately after collection, and the plasma was frozen at \(-20^\circ\)C. All data were derived from samples we received in our role as a porphyrin reference laboratory and were checked for proper preservation and transportation (samples frozen and transported in dry-ice). All abnormal data include the complete range of results in a particular disease classification, disease stratification having been made by an experienced clinician and porphyrin chemist (J.T.H) after the perusal of porphyrin patterns in blood, urine, and feces and erythrocyte porphobilinogen deaminase activities as well as discussions with the patients’ physicians.

“Normal” samples were chosen by visual inspection of the porphyrin results of all specimens processed. Because the porphyrias usually produce relatively large deviations from normal, we feel that visual selection of normal data is reliable. The central 95% reference intervals were determined nonparametrically (4).

Calibrators and controls. Stock calibrators were prepared in either 3 mmol/L HCl (coproporphyrin III and the porphyrin markers) or 1 mmol/L HCl (uroporphyrin III). Calibrators were made in expired plasma obtained from the hospital blood bank at concentrations of 5, 25, and 100 nmol/L for each porphyrin fraction. This matrix was found to be porphyrin free. The internal standard (2-vinyl-4-hydroxymethyl deuteroporphyrin IX) was dissolved in 6 mmol/L HCl and diluted with deionized water to give a final concentration of 5 \(\mu\)mol/L. Controls were prepared in a similar fashion to the plasma-based calibrators to give target values of \(\sim\)15 and 75 nmol/L for each fraction.

**Extraction procedure.** The method for the extraction of porphyrins from plasma was derived largely from the report of Kennedy and James (5). Three milliliters of calibrators, controls, or patient plasmas were pipetted into 15-mL conical centrifuge tubes. Internal standard solution (50 \(\mu\)L) was added to each tube. A 5-mL aliquot of acetonitrile–6 mmol/L HCl (1:1, by volume) was added, followed by thorough mixing. The mixture was sonicated for 25 s, using a probe sonicator, and then centrifuged at 1850g for 10 min. The supernatant was decanted into a 50-mL glass test tube. The pellet was reextracted with 5.0 mL of acetonitrile–6 mmol/L HCl, followed by sonication and centrifugation as above. The supernatants were combined and deionized water was added to bring the total volume to 50 mL.

Sep-Pak cartridges (Waters) were conditioned with 10 mL of acetonitrile and then 15 mL of deionized water under reduced pressure. The combined extracts were passed through the columns under reduced pressure, and the eluates were discarded. The columns were washed with 2 mL of deionized water and dried under reduced pressure for 1 min. The porphyrins were eluted from the column by the sequential addition of 0.5 mL of 1.2 mmol/L HCl in acetonitrile, 0.5 mL of 0.5 mmol/L HCl in acetonitrile, and finally 0.5 mL of acetonitrile. The eluates were then dried at 60°C under a stream of nitrogen. The porphyrins were redissolved in 125 \(\mu\)L of concentrated HCl, diluted with 375 \(\mu\)L of deionized water, and passed through a Gelman 0.45 \(\mu\)m syringe filter to remove particulates before chromatography. We used 5–20 \(\mu\)L for chromatography.

Chromatography. The porphyrins were separated on a reversed-phase C\textsubscript{18} column, using gradient elution at a flow rate of 2.0 mL/min and a total run time of 15 min. The initial gradient concentrations were 27% solvent A (methanol) and 73% solvent B (1 mol/L ammonium acetate, pH 5.16). The gradient conditions were set to give the following ratios at the given time points: 1 min, 45% A, 55% B; 4 min, 90% A, 10% B; 5 min, 90% A, 10% B; 5.5 min, 85% A, 15% B; 7 min, 85% A, 15% B; 7.5 min, 27% A, 73% B. The spectrofluorometer wavelength settings were 404 nm (excitation) and 624 nm (emission). Plasma-based calibrators subjected to the same extraction procedure as patient samples were used to construct three-point calibration curves based on peak height ratios for each porphyrin fraction. The concentrations of porphyrins in patient samples were then estimated from this curve.

**Porphyrrins from Urine and Feces**

Urine porphyrins were fractionated and quantified using the method of Johnson et al. (6). Fecal porphyrins were fractionated and quantified using a modification, to achieve direct standardization, of the method of Pudek et al. (7).

**Erythrocyte Protoporphyrin**

Erythrocyte protoporphyrin was measured using the method of Piomelli (8), and erythrocyte porphobilinogen deaminase was measured using the method of Piepkorn et al. (9).
ETHICS
Our experimental protocol was in accordance with the recommendations of the 1975 Declaration of Helsinki on Human Experimentation, as revised in 1996, and our hospital’s ethics committee.

Results
Typical chromatograms showing the separation of the calibrators and plasma from a healthy subject are shown in Fig. 1A and B, respectively.

RECOVERY OF PORPHYRINS FROM PLASMA
The recovery of individual porphyrins added to porphyrin-free blood bank plasma at 15 and 75 nmol/L was 89–114% for all fractions except for coproporphyrin III. The recovery of this compound was 71–99% at 15 nmol/L and 72–84% at 75 nmol/L. The recovery of protoporphyrin was low and variable, and attempts to quantify this fraction in plasma were abandoned.

IMPRECISION
The between-run imprecision was determined using two concentrations of each fraction (15 and 75 nmol/L) measured once a day for 30 days. At 15 nmol/L, the CVs were 5.4–13%, with CVs for most fractions <10%. At 75 nmol/L, CVs of 5.8–9.6% were observed. Coproporphyrin III showed the most imprecision.

Table 1 shows the plasma porphyrin results obtained.

![Fig. 1. Typical plasma chromatograms of external calibrators in pooled serum (A), a healthy patient (B), a patient with PCT (C), and a patient with renal failure (D).](Image)

1, uroporphyrin I; 2, uroporphyrin III; 3, heptacarboxyl; 4, hexacarboxyl; 5, pentacarboxyl; 6, coproporphyrin I; 7, coproporphyrin III; 8, internal standard.
with HPLC for a variety of porphyrias and includes our health-related reference interval. Plasma porphyrin concentrations were low in subjects without porphyria (Table 1). Reports of the fractionation of porphyrins are rare; to our knowledge, this is the first comprehensive report of their reference intervals.

PORPHYRIA CUTANEA TARDA

In porphyria cutanea tarda (PCT), the diagnosis was first established by the demonstration of its characteristic HPLC pattern in urine. Plasma porphyrins demonstrated an easily recognizable pattern of moderate-to-large increases of both uroporphyrin isomers and a pronounced increase of heptacarboxyl III (the largest increase of all the measured fractions), usually followed by a diminishing increase of hexacarboxyl III, pentacarboxyl III, and the coproporphyrin isomers (Fig. 1C). Heptacarboxyl was always increased, even when all other fractions were not (usually patients undergoing phlebotomy treatment). In mild disease, plasma and urine heptacarboxyl concentrations were equally sensitive in detecting disease.

PCT OF RENAL FAILURE (PCTRF)

Plasma porphyrin fractionation was performed on samples from 12 patients with the typical skin lesions of PCT who were undergoing regular hemodialysis for severe chronic renal failure and who had no other risk factors for PCT. The results are shown in Table 1 and indicate that these patients had the typical plasma porphyrin pattern of PCT. Fecal samples were obtained from three patients, and the HPLC patterns were normal or were similar to that of mild PCT. However, our technique does not identify isocoproporphyrin. Thus, plasma porphyrin analysis would be useful in the diagnosis and management of cases of PCTRF.

RENAI FAILURE

Plasma porphyrin patterns were also studied in 13 patients undergoing standard 4-h hemodialysis for severe chronic renal failure (predialysis serum creatinine, 509-1289 μmol/L). The results for these patients were normal or showed modest increases of uroporphyrin I and III and heptacarboxyl porphyrin (Table 1 and Fig. 1D), clearly different from the findings in PCTRF.

PSEUDOPORPHYRIA OF RENAL FAILURE

Plasma porphyrin fractionation was performed on samples from eight other patients with severe renal failure and with skin lesions typical of PCT. The results are shown in Table 1, and indicate that their patterns resemble that of chronic renal failure and are clearly different from that of PCT. These patients were therefore labeled as having “pseudoporphyria of renal failure”.

AIP

Plasma porphyrin analysis was performed on samples from three patients with this disease, and the porphyrin pattern was normal or showed small-to-modest increases of uroporphyrins I and III and coproporphyrins I and III.

VP

The plasma porphyrins in samples from patients with VP were normal or showed a slight increase of uroporphyrin III.

HCP

The plasma porphyrins in samples from patients with HCP were normal (Table 1).

EPP

The plasma porphyrins in samples from patients with EPP were normal or showed small-to-marked increases of coproporphyrins I and III.

Table 1. Reference intervals (in nmol/L) for plasma porphyrin quantification in healthy subjects and patients with various porphyrias.

<table>
<thead>
<tr>
<th>Porphyrin fraction</th>
<th>Healthy subjects (n = 245)</th>
<th>PCT (n = 32)</th>
<th>PCTRF (n = 12)</th>
<th>Renal failure (n = 13)</th>
<th>Pseudoporphyrin of renal failure (n = 8)</th>
<th>AIP (n = 3)</th>
<th>VP (n = 5)</th>
<th>HCP (n = 5)</th>
<th>EPP (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uroporphyrin I</td>
<td>0–11</td>
<td>5–549</td>
<td>84–1103</td>
<td>4–50</td>
<td>4–37</td>
<td>6.9–16</td>
<td>0.5–7.2</td>
<td>0.4–4.9</td>
<td>0</td>
</tr>
<tr>
<td>Uroporphyrin III</td>
<td>0–3</td>
<td>5–220</td>
<td>51–293</td>
<td>0–12</td>
<td>0–9.6</td>
<td>3.4–9.6</td>
<td>0.2–8.8</td>
<td>0.3–4.4</td>
<td>0</td>
</tr>
<tr>
<td>Heptacarboxyl II</td>
<td>0–5</td>
<td>10–402</td>
<td>24–1132</td>
<td>1–8.2</td>
<td>0–4.4</td>
<td>0.4–0.9</td>
<td>0–3.8</td>
<td>0–1.7</td>
<td>0–0.2</td>
</tr>
<tr>
<td>Hexacarboxyl II</td>
<td>0–2</td>
<td>4–90</td>
<td>0–62</td>
<td>0–1.6</td>
<td>0–1</td>
<td>0</td>
<td>0–2.9</td>
<td>0–0.5</td>
<td>0</td>
</tr>
<tr>
<td>Pentacarboxyl II</td>
<td>0–2</td>
<td>2–41</td>
<td>0–18</td>
<td>0–1.6</td>
<td>0–0.5</td>
<td>0.4–1.7</td>
<td>0–3.7</td>
<td>0–1.0</td>
<td>0</td>
</tr>
<tr>
<td>Coproporphyrin I</td>
<td>0–10</td>
<td>0–16</td>
<td>0–12</td>
<td>0–5.1</td>
<td>0–7.7</td>
<td>1.4–36</td>
<td>0–5.9</td>
<td>0–3.5</td>
<td>0.8–174</td>
</tr>
<tr>
<td>Coproporphyrin III</td>
<td>0–12</td>
<td>0–26</td>
<td>0–5.4</td>
<td>0–3.3</td>
<td>0–4.5</td>
<td>3–24</td>
<td>0.2–8.7</td>
<td>0–9.3</td>
<td>2.2–203</td>
</tr>
</tbody>
</table>
porphyrin fractionation is, however, technically more demanding than HPLC analysis of urine porphyrins.

The use of plasma porphyrin concentrations in the management of PCT is not new (2), but most authors have simply measured plasma total porphyrins (10, 11) or plasma total uroporphyrin and total coproporphyrin (no intermediates) or plasma peak fluorescence by fluorometric scanning techniques (12, 13).

Although plasma total porphyrins and plasma uror and coproporphyrins may be helpful in following the progress of known cases of PCT, they are not diagnostically discriminative. Plasma peak fluorescence can provide a specific diagnosis of VP because of the characteristically discriminative. Plasma peak fluorescence can provide a specific diagnosis of VP because of the characteristic peak between 621 and 627 nm (13), whereas patients with EPP may have a peak at 636 nm. However, a fluorescence emission maximum between 618 and 622 may be present in samples from healthy subjects and those with AIP, HCP, congenital erythropoietic porphyria, PCT, renal failure, and cholestasis (12, 13) and therefore cannot differentiate these conditions. Our plasma porphyrin fractionation provides a positive diagnosis of PCT, the commonest porphyria, as well as a means to assess the effects of treatment.

It should be noted that in PCT there often are marked increases of plasma coproporphyrin concentrations, although this metabolite is located beyond the metabolic enzyme block. This phenomenon is well known. Some of this coproporphyrin may be derived from nonenzymatic decarboxylation of the preceding porphyrins in the pathway. It is also likely, however, that some excess coproporphyrin is produced by overreaction of the feedback control mechanism controlling porphyrin production in the presence of a partial enzyme deficiency in the pathway. Specifically, we postulate that, whatever the controlling mechanism, when this is activated by diminished heme synthesis in PCT, it overreacts in correcting the abnormality, thereby producing excess porphyrins beyond the enzymatic block. Jacob and Doss (14) have termed this phenomenon, “counterregulatory compensatory enhancement”. The controlling mechanism in the hepatic porphyrins, including PCT, is probably induction of the enzyme aminolevulinic acid synthase by diminished heme synthesis (15).

PCTRF
This syndrome has been well described previously (16–18). Some authors (11, 16) have reported increased plasma porphyrin concentrations in PCTRF, but they have simply measured plasma total uroporphyrin or total porphyrins, which do not necessarily provide a diagnostic pattern because a similar profile for plasma total uroporphyrin and total porphyrins can be seen in other porphyrias. Our method provides a specific diagnosis of PCTRF and clearly differentiates it from pseudoporphyria of renal failure. One case of PCTRF demonstrated reduced plasma porphyrin concentrations when her hemoglobin was allowed to fall to 90 g/L (data not shown).

RENAL FAILURE
Increased plasma porphyrins in renal failure have been reported previously (16, 19, 20), but most authors simply measured plasma total porphyrins or only a few fractions. Gebril et al. (21) measured plasma total uroporphyrin, heptacarboxyl, and total coproporphyrin in renal failure patients and found a pattern similar to the one in our study. Our results support the findings of others that the plasma uroporphyrin:heptacarboxyl ratio in chronic renal failure exceeds that in patients with PCT and normal renal function, whereas patients with PCTRF have intermediate values (21, 22).

We found a greater increase of plasma uroporphyrin I compared with uroporphyrin III in renal failure, similar to the reports of Seubert et al. (17), but contrary to the findings of Day and Eales (22). The plasma pattern in renal failure is most likely largely attributable to a failure of urinary excretion, the usual elimination route of these more polar porphyrins, although some have postulated increased porphyrin production in renal failure (23).

We also studied nine patients before and after hemodialysis with a polysulfone low-flux membrane. Although the concentrations of most fractions (particularly the uroporphyrin isomers) fell after dialysis, the pattern (modest increase of the uroporphyrins and heptacarboxyl) remained much the same (data not shown). Greater porphyrin exchange could probably be achieved with a high-flux membrane (11), and studies with this are ongoing. Only one stool sample was obtained from one of our patients with chronic renal failure, and it showed a normal HPLC pattern (data not shown).

PSEUDOPORPHYRIA OF RENAL FAILURE
This disease has also been described previously (24). The dermatologic lesions of this disease are, on visual inspection, indistinguishable from those of PCTRF. One patient with severe skin lesions was followed for 18 months, and his skin lesions remained unchanged. His plasma porphyrin pattern also remained unchanged, implying that pseudoporphyria is not merely a mild early version of PCTRF, but a different clinical entity. Our results would imply different etiologies for pseudoporphyria of renal failure and PCTRF, the latter perhaps related to increased hepatic iron stores, the consequence of repeated hemodialyses and/or iron therapy in the presence of impaired iron incorporation into erythrocyte hemoglobin. Our observation that plasma porphyrins in pseudoporphyria of renal failure are similar to those in chronic renal failure without pseudoporphyria but are lower than those in PCT is contrary to the report of Gafter et al. (20), who reported that plasma porphyrins in patients with pseudoporphyria of renal failure were higher than those found in renal failure patients without skin lesions and were similar to those of patients with PCT. They postulated that pseudoporphyria of renal failure might be related to perturbation of the heme synthesis pathway by retained aluminum.

Two of our patients with pseudoporphyria of renal
failure had fecal porphyrin HPLC fractionation performed, and the results were essentially normal (data not shown).

**PLASMA PORPHYRINS IN THE ACUTE PORPHYRIAS**

Our plasma porphyrin assay was less helpful in the acute porphyrias (AIP, VP, and HCP).

**AIP.** Not surprisingly, plasma porphyrin concentrations can be normal in AIP (Table 1). It is interesting that these metabolites, which are beyond the metabolic enzyme block in AIP, can be present in the plasma in excess, albeit to a modest extent. Our stability studies on frozen plasma have shown that plasma porphyrins are stable at −20 °C, and our samples were always frozen as soon as they were centrifuged. Therefore, we do not believe that there could have been much in vitro interconversion of the porphyrins in our experimental system. This leads us to speculate that some minimal in vivo conversion of excess porphobilinogen might have occurred, perhaps from extrahepatic sites (25). However, if this occurs, it must be small in extent because patients with AIP never exhibit the dermatologic lesions associated with in vivo porphyrin accumulation. To investigate the likelihood of porphobilinogen being converted to porphyrins in vitro, we added porphobilinogen to plasma to a concentration of approximately 1 mmol/L [a concentration that could be reached in AIP (26)] and froze the samples at −20 °C. Aliquots were taken for plasma porphyrin measurement, and no difference was detected between the samples with added porphobilinogen and samples without added porphobilinogen after 1 week in storage.

**VP.** Unfortunately, in our plasma fractionation, we have been unable to quantitatively recover protoporphyrin added to plasma samples consistently; therefore, we do not attempt to quantify this fraction, but simply note the presence or absence of a protoporphyrin peak and initiate further testing as outlined below. Three of our five patients with VP had pronounced protoporphyrin peaks when our technique was used. Plasma porphyrins are partly bound to proteins (27); protoporphyrin, being water insoluble, is bound more strongly. We have attempted to break this bond physically with sonication and enzymatically with proteases without success. In VP, this exercise is particularly difficult because the bond appears to be strong [covalent, see Ref. (28)]. In addition, chemical treatment could perhaps yield some hematoporphyrin, with a loss of fluorescence, whereas enzymatic digestion could produce porphyrin-peptide fragments (X porphyrins) with a loss of stoichiometric relationships. Thus, by our technique, plasma porphyrin fractionation was not particularly helpful in the diagnosis of VP.

Plasma from patients with VP often demonstrates a peak of fluorescence at 625 nm (excitation 405) (12, 13, 29, 30), the structure of which is unknown. We have only adopted this test recently and, therefore, have data on only a few of our patients. Plasma peak fluorescence, although lacking complete sensitivity in latent VP (30), is apparently more sensitive than that for fecal porphyrins (13). We have, therefore, recently adopted it rather than our plasma fractionation as the plasma test of choice for VP and now use it together with fecal porphyrin quantification for diagnosis of this disease.

One of our patients with VP and fresh skin lesions demonstrated a slight increase of plasma uroporphyrin III.

**HCP.** The plasma porphyrins were normal in patients with HCP, although at least one patient had active skin lesions when the specimen was taken.

**EPP.** When our technique was used, the plasma porphyrins were normal in two of our four cases of EPP in whom plasmas were analyzed. The two patients with abnormal results were particularly florid cases that demonstrated marked increase of plasma coproporphyrin I and coproporphyrin III. Thus, plasma porphyrin assays using our technique, would not be helpful in the diagnosis of this disease. This is not surprising because protoporphyrin, which we would expect to be the plasma porphyrin most increased in EPP, is poorly recovered in our method. In any event, the definitive tests for EPP—erythrocyte protoporphyrin quantification by solvent fractionation (8) together with demonstration of erythrocyte fluorescence—are far easier to perform than our plasma porphyrin method.

Only one of our abnormal plasmas was scanned fluorometrically using the technique of Poh-Fitzpatrick (29), and in this plasma, an emission peak was prominent at 636 nm, which is characteristic of this disease (12). A plasma from one of our patients with mild disease failed to demonstrate this peak, showing that this test lacks sensitivity in mild cases.

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**References**