Phenol and p-Cresol Accumulated in Uremic Serum Measured by HPLC with Fluorescence Detection
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We developed a simple and sensitive high-performance liquid chromatographic (HPLC) method that uses fluorescence as a detector for quantifying serum phenol and p-cresol in uremic patients on hemodialysis. Identification of phenol and p-cresol was confirmed by liquid chromatography/mass spectrometry. Because the HPLC method requires only simple extraction by ethyl acetate and does not require further steps such as derivatization, it is simple and rapid compared with gas chromatography or gas chromatography/mass spectrometry. Concentrations of phenol and p-cresol in uremic serum were significantly \( p < 0.01 \) higher than those in normal serum. Reduction rates of phenol and p-cresol by hemodialysis were lower than those of urea and creatinine, suggesting a protein-binding property of phenol and p-cresol. This method will be useful for monitoring serum phenols in dialyzed patients as an index of hemodialysis adequacy.

Additional Keyphrases: chromatography, reversed-phase - hemodialysis - GC/MS compared

Phenol and p-cresol markedly accumulate in the serum of undialyzed and dialyzed uremic patients \((1-6)\) and play a role in the development of uremic coma \((7)\) and defective platelet aggregation \((8, 9)\). Phenol and p-cresol are synthesized in the small intestine from tyrosine through 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid, respectively, by intestinal bacteria (Figure 1) \((10-13)\). Aerobic bacteria such as Enterobacteriaceae \((Escherichia coli etc.)\) and Streptococcus tend to produce phenol from tyrosine, whereas anaerobic bacteria such as Clostridium and Bacteroides produce p-cresol \((10-13)\). These phenols are absorbed from intestine and normally excreted into urine. In uremia, renal clearance of the phenols is impaired, leading to accumulation of the phenols in the blood.

The serum concentrations of phenol and p-cresol in uremic serum have been determined by using colorimetric nitroaniline diazotization \((2)\), gas chromatography \((GC) (1)\), or gas chromatography/mass spectrometry \((GC/MS) (3, 4)\). Plasma phenol in patients treated with p-cresol in regional nerve blocks was determined by GC/MS \((4)\). The colorimetric method is not as specific for phenols as are GC and GC/MS and cannot quantify phenol and p-cresol separately. Quantification of phenols by GC or GC/MS is laborious and time consuming because it requires complex extraction and derivatization before analysis. Reversed-phase high-performance liquid chromatography (HPLC), which does not require derivatization, has been used for measuring environmental phenols with electrochemical detection \((15)\) and urinary cresols with ultraviolet detection \((16)\). However, the HPLC method with electrochemical detection has not been applied to determine serum phenols. When ultraviolet detection was used for determination of serum phenols, the HPLC chromatograms were more complex, with many interfering peaks, and the peaks of phenol and p-cresol could not be clearly recognized. We developed a reversed-phase HPLC method for measuring phenol and p-cresol in uremic serum. The method is specific and sensitive for phenol and p-cresol, uses fluorescence as a detector, and requires only simple extraction before HPLC.

Materials and Methods

Subjects

The study included 17 patients \((10\) men and 7 women) receiving hemodialysis for 4 h, three times per week. They had been undergoing periodic dialysis for 4.2 (SD 4.0) years \((range 0.7-12.6)\) years. Their mean age was 57.4 (SD 11.1) years \((range 34-69\) years). Blood samples were taken before and after hemodialysis and allowed to clot. The control subjects were a group of 10 healthy subjects \((6\) men and 4 women).

1. Nonstandard abbreviations: LC/MS, liquid chromatography/mass spectrometry; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; APCI, atmospheric-pressure chemical ionization; and SIM, selected ion monitoring.
Sample Preparation

For quantification of serum phenol and \( p \)-cresol, 1 \( \mu \)g of \( p \)-ethylphenol in 10 \( \mu \)L of distilled water was added as an internal standard to the serum sample (0.1 mL), which was then acidified to pH 1.0 with 1 mol/L HCl, saturated with 0.1 g of NaCl, and extracted by shaking for 10 min with 0.3 mL of ethyl acetate. After centrifugation at 1300 \( \times \) g for 5 min, 10 \( \mu \)L of the supernate was used for HPLC.

For quantification of phenol and \( p \)-cresol, calibration lines were obtained by analyzing standard solutions. Standard solutions containing phenol and \( p \)-cresol at concentrations of 0.1–10 mg/L in distilled water were treated similarly to urine and serum samples.

HPLC

We used a chromatographic assembly consisting of a Model 880-50 degasser, a Model 851-AS autosampler, a Model 880-PU pump, a Model 820-FP fluorescence detector, a Chromatocorder 12, and a column [Finepak Sil C18S, 4.6 mm (i.d.) \( \times \) 150 mm; all from Jasco, Tokyo, Japan]. A mobile phase of distilled water/acetonitrile (70/30 by vol) was delivered at a flow rate of 1 mL/min at ambient temperature. Figure 2 shows the fluorescence excitation and emission spectra for phenol and \( p \)-cresol.

The eluate was monitored by detection of fluorescence (excitation at 260 nm and emission at 305 nm).

Liquid Chromatography/Mass Spectrometry (LC/MS)

A Hitachi (Tokyo, Japan) M-1000S quadrupole mass spectrometer equipped with atmospheric-pressure chemical ionization (APCI) and a Hitachi-6200 LC pump was used for LC/MS. The column was Finepak Sil C18S (Jasco), 4.6 mm (i.d.) \( \times \) 150 mm. The mobile phase, distilled water/acetonitrile (70/30 by vol), was delivered at a flow rate of 1 mL/min at ambient temperature. Desolvation temperature was 399 °C, vaporization temperature was 300 °C, and drift voltage was 40 V. LC/APCI-MS was analyzed in negative-ion mode, because negative-ion mass spectra for phenol and \( p \)-cresol showed more definite and more sensitive quasi-molecular ions than did their positive-ion mass spectra.

All results are expressed as mean \( \pm \) SD. Statistical analysis was done by using the Welch test.

Results

The calibration lines relating the concentrations (y) of phenol and \( p \)-cresol to their peak areas (x) were obtained from the HPLC chromatograms. The correlation coefficients of the calibration lines for phenol (\( y = 5.07 x + 0.26 \)) and for \( p \)-cresol (\( y = 5.92 x - 0.26 \)) were 0.99993 and 0.99991, respectively. There were no interfering peaks in the HPLC chromatograms of the serum samples for quantification of phenol and \( p \)-cresol.

The intrasay coefficients of variation (CVs) for phenol and \( p \)-cresol in a uremic serum sample were 4.0% and 5.3%, respectively, and those in a standard solution of 5 mg/L were 1.9% and 2.2%, respectively. The intrasay CVs for phenol and \( p \)-cresol in the uremic serum sample were 7.8% and 8.3%, respectively. The recoveries of phenol and \( p \)-cresol added into the uremic serum at a concentration of 5 mg/L were 103.8 \( \pm \) 2.8% and 95.9 \( \pm \) 9.7%, respectively.

Figure 3 shows HPLC chromatograms of authentic phenol and \( p \)-cresol, an extract from uremic serum, and an extract from normal serum. The peaks of phenol and \( p \)-cresol in the uremic serum can be clearly recognized because there are no interfering peaks. The concentrations of phenol and \( p \)-cresol were markedly increased in the uremic serum compared with those in normal serum.

Figure 4 shows negative-ion APCI mass spectra of phenol, \( p \)-cresol, and \( p \)-ethylphenol (internal standard).
The base peaks at m/z 93, 107, and 121 are (M–H)⁻ ions of phenol, p-cresol, and p-ethylphenol, respectively. To confirm the identification of phenol and p-cresol in the HPLC chromatogram of uremic serum, we analyzed the same sample extract by LC/MS. Figure 5 shows a selected ion monitoring (SIM) chromatogram of standards (a) and the uremic serum (b) analyzed by LC/MS. The peaks at retention time of 5.8 and 8.9 min in the SIM chromatogram of uremic serum were identified as phenol and p-cresol, respectively, by monitoring m/z 93, the (M–H)⁻ of phenol, and m/z 107, the (M–H)⁻ ion of p-cresol. Thus, the peaks at m/z 5.8 and 8.9 min in the HPLC chromatogram (Figure 3b) were confirmed to be phenol and p-cresol by LC/MS.

Table 1 shows the serum concentrations of phenol, p-cresol, urea, and creatinine in normal and uremic serum. Serum concentration of phenol and p-cresol were markedly increased in the uremic patients compared with the healthy subjects. Prehemodialysis serum concentrations of phenol showed significant correlation with those of p-cresol (r = 0.48, P < 0.05). Prehemodialysis serum concentrations of phenol and p-cresol did not show any correlation with those of urea and creatinine. Serum concentrations of phenol and p-cresol in the uremic patients decreased after hemodialysis. However, reduction rates of phenol and p-cresol by hemodialysis were significantly (P < 0.01) lower than those of urea and creatinine.

Discussion

In uremic patients, phenol and p-cresol are markedly accumulated in the serum and are considered to be responsible for uremic coma and bleeding tendencies (1–9). The uremic undialyzed patients with serum phenol concentrations of ~100 μmol/L showed uremic coma and gastrointestinal bleeding. Although the dialyzed patients showed increased phenol concentrations (maximum 60 μmol/L) and p-cresol (maximum 111 μmol/L), the long-term effects of the increased concentrations of phenol on dialysis patients are not yet known. Phenol and p-cresol are also reported to be cocarcinogens for mouse skin (17). An increase in malignancies, especially malignant renal tumors, is observed in dialyzed patients. However, it is not known whether the increased concentrations of serum phenol in the dialyzed patients are responsible for the increase in malignancies, and further study is required to elucidate the long-term effects of these increases.

Phenol and p-cresol are synthesized by intestinal bacteria through 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid, respectively, from tyrosine (10–13). Urinary phenols originate from bacteria in the small intestine (18). Aerobic bacteria mainly produce phenol, whereas anaerobic bacteria produce p-cresol (10–13). Phenols accumulate in uremic serum as a result of decreased renal clearance (5, 6). Other phenols, such as catechol, hydroquinone, homocatechol, and 2-methoxyresorcinol, also accumulate in uremic serum (4), but their concentrations in uremic patients are much lower than those of phenol and p-cresol.

We have developed a simple and sensitive HPLC method for measuring phenol and p-cresol in uremic serum. Identification of phenol and p-cresol was confirmed by LC/MS. Because the method requires only simple extraction by ethyl acetate and does not require further dehydration, evaporation, and derivatization, the method is much simpler than GC (1) or GC/MS (4).
With fluorescence used for detection, the method is highly specific and sensitive for phenol and p-cresol, with no interfering peaks on the chromatogram. The specificity and sensitivity of the newly developed HPLC method are comparable with GC/MS, which we developed for quantification of phenols in uremic serum (4). The sensitivity of GC/MS was reported to be 1.06 µmol/L (14). The fluorescence HPLC assay is able to monitor these concentrations and can be used to monitor phenol concentrations in plasma of patients treated with phenol in regional nerve blocks. However, the GC/MS method requires laborious sample preparation and expensive equipment and is not suited to routine clinical examination.

Serum concentrations of phenol and p-cresol in our patients were comparable with the concentrations determined by GC/MS (4). However, Wengle and Hellström (1) reported that no unconjugated phenol and p-cresol could be detected in uremic serum by GC. The discrepancy between the results can be explained by the better specificity and sensitivity in our method. Conjugated phenol and p-cresol (mostly sulfate and partly glucuronide) are also reported to accumulate in uremic serum (4). To quantify the conjugated phenols, one should treat the serum with sulfatase and (or) β-glucuronidase before extraction. Because unconjugated phenols are more toxic than conjugated phenols, monitoring the unconjugated form in serum is clinically more important.

The reduction rates of phenol and p-cresol by hemodialysis were significantly lower than those of urea and creatinine. Because the molecular masses (Da) of phenol (94) and p-cresol (108) are similar to urea (60) and creatinine (113), the lower reduction rates of phenols are probably due to their protein-binding properties, as suggested by Wardle and Wilkinson (2).

With our method, serum concentrations of phenol and p-cresol can be easily and rapidly quantified. The newly developed HPLC method will be useful for monitoring serum phenols in dialyzed patients as an index of hemodialysis adequacy.

References