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Diagnostic meaning of blood *p*-cresol concentration in forensic autopsy cases

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ABSTRACT

In some forensic autopsy cases there are high concentrations of *p*-cresol in the blood. In vivo, *p*-cresol is the only isomer yielded by intestinal bacteria and is excreted into urine. We investigated the diagnostic meaning of *p*-cresol in the blood of forensic autopsy cases. Blood samples from 110 autopsy cases within 48 h of the postmortem interval (PMI) and 10 healthy adults were examined. Blood with *p*-cresol-d_8 as an internal standard was analyzed using a GC–MS/MS method. Using acid and heat deproteinization, free (F) and conjugated (non-protein bound: C; protein bound: PC) *p*-cresol were individually analyzed. The *p*-cresol concentrations were 1.39 ± 0.86 μg/ml [mean ± SD] and 1.18 (0.19–18.80) μg/ml [median (range)] in healthy adults and autopsy cases, respectively. The *p*-cresol showed no significant relationship to age, sex, fasting duration, survival duration, or PMI. No significant differences were found between causes of death. Significantly higher levels of C *p*-cresol were found in cases with atherosclerosis in the basilar or renal arteries, or stenosis in the coronary artery. Significantly higher levels of *p*-cresol except F were found in cases with hyalinosis of the kidney. Cases with low BMI also showed significantly higher *p*-cresol concentrations. The 22 cases of abnormally high total *p*-cresol were investigated. It was considered that high concentrations of *p*-cresol could be an indicator of certain diseases and physical conditions that effect the production, absorption, metabolism, circulation, and excretion of *p*-cresol. Measuring the levels of *p*-cresol may provide valuable information about the antemortem physical conditions.

1. Introduction

Cresols from manufactured products are a mixture of 3 isomers: *p*-, *m*-, and *o*-cresol. In the absence of external exposure, only *p*-cresol is produced in vivo. *p*-Cresol (4-methylphenol), with a molecular weight of 108.1 Da, is a volatile phenol and a precursor of uremic retention solutes. It is a product of the metabolism of tyrosine and phenylalanine by intestinal anaerobic bacteria [1–6]. During passage through the colonic mucosa and liver, *p*-cresol is conjugated to mostly *p*-cresylsulfate and less to *p*-cresylglucuronide [6–12], which are then excreted in the urine. Dysfunction of the kidneys results in the accumulation of uremic toxins in the circulating blood and tissues [13–15].

Previous studies have suggested that the presence of *p*-cresylsulfate contributes to the progression to renal failure [16], cardiovascular complications and mortality in chronic kidney disease (CKD) patients [17,18].

Pioneering research has focused on the concentration and toxicity of *p*-cresol. *p*-Cresol has been shown to principally circulate in the form of its sulfate conjugate [10,19]. It binds tightly to plasma proteins, mostly albumin (molecular weight: 66 kDa) [20]. Therefore, this toxin is incompletely removed by hemodialysis (HD) even though its molecular mass is sufficiently low enough to pass through the dialysis membrane [19,21].

In forensic autopsy cases, toxicological analysis contributes important information for diagnosing the cause of death. In the routine toxicological screening of blood, we encountered high *p*-cresol concentrations in HD cases.

In the present study, we investigated the diagnostic meaning of *p*-cresol in the blood of forensic autopsy cases.

2. Materials and methods

2.1. Cases

There were 251 cases autopsied from April 2015 to October 2017 in the Department of Forensic Medicine of Fukuoka University. It was considered that postmortem changes, especially hyperplasia of bacteria might produce *p*-cresol. Thus, only cases within 48 h of the postmortem

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interval (PMI) were selected. The blood from 110 autopsy cases and 10 healthy adults was examined. All specimens were kept at −30 °C until use.

The cause of death, survival duration, and PMI had been diagnosed based on the autopsy, pathological, toxicological, and other examination findings. Further, clinical records and police investigation records were referenced for these diagnoses.

A summary of the autopsy cases, including age, sex, cause of death, fasting duration, survival/agony duration, PMI, and *p*-cresol concentration, as well as the data on the healthy adults are shown in Tables 1 and 2.

2.1.1. Grouping of the cases

The cases were grouped according to sex, age, body mass index (BMI), cause of death, fasting duration, survival duration, and PMI (Tables 1 and 2).

In cases over 18-year-old, body weight was classified by BMI, except in cases where the BMI was not calculatable. Cases where the BMI was lower than 18.5 were classified as the "low BMI" group. Cases with a BMI between 18.5 and 25 were classified as the "standard BMI" group. Cases with a BMI of 25 or more were classified as the "high BMI" group.

2.1.2. Organ weight

The weights of the heart, kidneys, and liver were measured and grouped based on the average weight for each age [22].

Cases where the weights were lower than the mean minus one standard deviation (SD) were classified as the "low weight" group. Cases higher than the mean + SD were classified as the "high weight" group and cases in between were classified as the "standard weight" group.

2.1.3. Atherosclerosis/stenosis

Cases where stenosis was observed in the coronary artery were designated the "stenosis" group, regardless of the level. Cases without stenosis were designated the "non-stenosis" group.

Cases where atherosclerosis was observed in the basilar and renal arteries were also assigned to the "atherosclerosis" group, regardless of the level. Cases without atherosclerosis were designated the "non-atherosclerosis" group.

2.1.4. Pathological changes

A 15 × 20 mm section of tissue from heart, liver, and kidneys was observed microscopically.

In the heart, if fibrosis in the myocardial layer [23] was observed it was assigned to the "pathological change" group. In the liver, cases where steatosis [24] was observed were also placed in the "pathological change" group. Also, cases where fibrosis or inflammatory cell infiltration [23] was observed in the liver were included in the "pathological change" group. In the kidneys, cases where hyalinosis [25] was observed were also placed in the "pathological change" group.

2.1.5. ***High blood*** *p-cresol concentration cases*

Cases where the concentration of *p*-cresol in the blood was over two times the standard deviation of the mean *p*-cresol concentration in the blood of ten healthy adult volunteers were designated "abnormally high". In these abnormally high blood *p*-cresol cases, the antemortem conditions were investigated.

2.2. Materials

Standards of *o*-, *m*-, and *p*-cresol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). The deuterated internal standard (IS), *p*-cresol-d_8, was obtained from C/D/N Isotopes Inc. (Quebec, Canada). NaCl, $MgSO_4$, and ethyl acetate were also obtained from Wako. Ampules of *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + Trimethylchlorosilane (TMCS) (99:1, v/v, 100 μl) were purchased from Supelco (Bellefonte, USA). *n*-Propyl acetate was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Concentrated sulfuric acid was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Amicon® Ultra - 4 centrifugal filter units, 17.3 mm in diameter and 124 mm in length, were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Analysis of p-cresol

We analyzed *p*-cresol concentrations using methods derived from a study by H. de Loor, *et al.* [10]. The study by de Loor found that most of the *p*-cresol in serum was in its sulfated form (> 95%), except for a small proportion that was glucuronidated (< 5%). They also described a method of acid and heat deproteinization that could convert almost all the *p*-cresylsulfate into *p*-cresol.

2.3.1. Free, conjugated, and total p-cresol

The amount of *p*-cresol was determined after pretreatment of the blood sample in one of three ways. "Free *p*-cresol" (F) was defined as the non-protein-bound and unconjugated *p*-cresol. "Conjugated *p*-cresol" (C) was defined as the non-protein-bound and conjugated *p*-cresol. "Protein-bound *p*-cresol" (PC) was defined as the protein-bound and conjugated *p*-cresol. "Total *p*-cresol" was the addition of F *p*-cresol, C *p*-cresol, and PC *p*-cresol. The concentration of C and PC *p*-cresol were determined by calculation. The pretreatment of each sample based on the category of *p*-cresol to be analyzed is described in the following sections. Each blood sample (0.1 ml) was diluted with 0.9 ml of distilled water prior to pretreatment.

2.3.1.1. F p-cresol. The sample was added to a centrifugal filter unit. After centrifugation at 3500 rpm (2330 × *g*) for 60 min, the filtrate was transferred to a glass test tube.

2.3.1.2. F and C p-cresol. The sample was added to a centrifugal filter unit. After centrifugation at 3500 rpm (2330 × *g*) for 60 min, the filtrate was transferred to a glass test tube along with 0.1 ml of concentrated sulfuric acid. The tube was immediately sealed and thoroughly mixed, and then incubated at 90 °C for 30 min. After heating, the solution was allowed to cool to room temperature.

2.3.1.3. Total p-cresol. The sample and 0.1 ml of concentrated sulfuric acid were added to a glass test tube. The tube was immediately sealed and thoroughly mixed, and then incubated at 90 °C for 30 min. After heating, the solution was allowed to cool to room temperature.

2.3.2. Extraction

Two milliliters of ethyl acetate, 1 ml of *n*-propyl acetate, IS solution (*p*-cresol-d_8, 1 μg), and 0.5 g of NaCl were added to the sample, followed by thorough mixing. After centrifugation at 3000 rpm (1710 × *g*) for 5 min, the organic layer was transferred into another glass test tube and 0.2*g* $MgSO_4$ and 0.1*g* NaCl were added. After thorough mixing and centrifugation at 3000 rpm (1710 × *g*) for 1 min, the organic layer was transferred to another glass test tube and evaporated to just short of dryness under nitrogen. The sample was heated using a microwave at 500 W for 30 s, and 100 μl of BSTFA + TMCS (99:1, v/v) was immediately added to the tube. The test tube was sealed and heated using a microwave at 500 W for 90 s twice (microwave-accelerated derivatization [26,27] of *p*-cresol). The derivatized sample was transferred to an autosampler vial for GC–MS/MS analysis.

2.4. Chromatography conditions

The GC-EI-MS/MS system was GCMS-TQ8030 (Shimadzu, Kyoto, Japan). The column used was ZB SemiVolatiles (2 m × 0.18 mm i.d., film thickness of 0.5 μm, Phenomenex, USA) / BPX5 (4 m × 0.15 mm i.d., film thickness of 0.25 μm, SGE Analytical Science, Australia) [28].

Electron ionization was employed at a voltage of 70 eV. The carrier