Isolation and Identification of Canine Plasma Components Suspected as Ureemic Toxins

Mina KAWAMURA, Fumihito OHASHI1,2, Yoshiko NAGATA1,2, Nobuharu TAKAI3, Hiroyuki MOTOIE, Ryohei NISHIMURA, Nobuo SASAKI, and Akira TAKEUCHI

Department of Veterinary Surgery, Faculty of Agriculture, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113, 1Department of Veterinary Surgery, College of Agriculture, University of Osaka Prefecture, 1–1 Gakuen-cho, Sakai, Osaka 593, 2Kyoritsu College of Pharmacy, 1–5–30 Shibakoen, Minato-ku, Tokyo 105, and 3The Institute of Industrial Science, The University of Tokyo, 7–22–1 Roppongi, Minato-ku, Tokyo 106, Japan

(Received 25 September 1992/Accepted 7 December 1992)

ABSTRACT. Suspected uremic substances contained in four fractions, which had been selected as the suspected canine uremic peaks in the previous study, were isolated by two stages of preparative liquid chromatography (PLC) from plasma of uremic dogs treated with the ligation of the ureter, and then their physicochemical properties were examined. The primary separation of the suspected uremic peaks were performed with the same anion exchange resin as used in the analytical HPLC in the previous study. Analytical reverse phase HPLC showed that three of 4 suspected uremic peaks almost consisted of single substances, but the other contained several substances. Main subfractions of these peaks were successfully isolated by the secondary stage reverse phase PLC. By means of thin layer chromatography, ultraviolet absorption spectrometry and proton-nuclear magnetic resonance spectroscopy, components of 4 main peaks were confirmed to be small molecules such as a pyridine derivative, uric acid, hippuric acid and kynurenic acid, respectively.—KEY words: canine, 1H-NMR, plasma uremic substance, preparative liquid chromatography, small molecular substance.


A great deal of efforts have been made to isolate and identify uremic toxins from body fluids and dialysates of uremic patients, especially after the proposal of the middle molecule hypothesis [2]. In most of these studies, target substances are those found increasingly in body fluids of patients with advanced uremia [5, 6, 9, 10, 14, 15]. In dogs, as in other animals, scarcely any uremic substances other than creatinine, urea nitrogen and electrolytes have been related to clinical symptoms of renal failure.

In our previous study [8], four peaks in the chromatograms of sera were found to be significantly correlated to serum creatinine concentrations in uremic dogs. In order to be recognized as a uremic toxin, a compound is requested to be chemically identified. The present study was aimed to isolate major components from the four uremic peak fractions by two stages of preparative liquid chromatography (PLC) and to examine physicochemical properties of isolated compounds by thin layer chromatography (TLC), ultraviolet (UV) absorption spectrometry and proton-nuclear magnetic resonance (1H-NMR) spectroscopy.

MATERIALS AND METHODS

Collection of plasma from uremic dogs: Under general anesthesia with combined xylazine (2 mg/kg, i.m.) and pentobarbital sodium (12.5–15.0 mg/kg, i.v.), bilateral ureters of 4 adult mixed-breed dogs (2 males and 2 females, average body weight, 9.8 kg) and 2 German Shepherd dogs (a male and a female, average body weight, 29.5 kg) were ligated. Intravenous fluid therapy was performed for 5 days following the ligation to maintain water and electrolyte balances as normal as possible. On the third and the fourth postoperative days, amounts of blood equivalent to approximately 2.4% of body weight were drawn from these dogs. On the fifth day, each dog was exsanguinated after treatment with heparin calcium (500 IU/kg, i.v.) under general anesthesia with pentobarbital sodium (25 mg/kg, i.v.). Plasma was then separated by centrifugation.

Isolation of uremic peak substances: Before fractionation, analytical HPLC for the plasma was undertaken by means of the same procedures in our previous study [8] to reconfirm the aimed peaks. Major components were isolated from significant peak fractions by means of two stages of PLC; anion exchange PLC was followed by reverse phase PLC.

Pretreatment of plasma: A total of 4,550 ml of pooled plasma was deproteinized with trichloroacetic acid (TCA) at a final concentration of 3% (wt/vol) and the resultant supernatant was lyophilized. The extracts were redissolved in 150 ml of distilled water. In order to avoid deterioration of resins during the two procedures of PLC, concentrated plasma extracts were applied to Hitachi-gel #3019 packed in a stainless-steel column (250 × 20 mm I.D.); the plasma extracts were eluted with distilled water at a rate of 4 to 5 ml/min for about 100 min. The eluent was concentrated to about 30 ml by lyophilization and filtered through a 0.45–μm membrane filter.

The primary stage PLC: A HPLC system (Model 655, Hitachi) was equipped with a solvent programming system, a double beam spectrophotometer (Model 150–20) and an injector (Model 7125, Rheodyne). The same anion exchange resin CDR-10 used in the analytical HPLC [8] was packed in both a main column (250 × 28 mm I.D.) and a precolumn (50 × 8 mm I.D.). Separation of specimen was achieved by the elution program with a
linear gradient of 0.006 M to 6.0 M ammonium acetate buffer (pH 4.40) (Table 1) and operating conditions were as follows; column temperature: 60°C, detection wavelength: 260 nm, injection volume: 5 μL [16]. Eluent for every peak was collected. Following the primary stage PLC, 100 μL of each peak fraction was checked by the anion exchange analytical HPLC [8] in order to determine the peak fractions containing components of S7, S10, S18 and S23. Successively each aliquot of 100 μL from these fractions was also applied to a reverse phase analytical HPLC system to know possibility of further separation and their retention times in this system. The reverse phase analytical HPLC was carried out by a HPLC system (LC-6A, Shimadzu) composed of a controller (SCL-6A), a UV detector (SPD-6A) and a Chromatopak (C-R3A). TSK gel ODS-120A (Toyo Soda) was packed into a stainless column (250 × 4.6 mm I. D.). The elution program is shown in Table 2 and operating conditions were as follows; flow rate: 0.7 ml/min, column temperature: ambient, detection wavelength: 254 nm. The rest of each fraction containing components of S7, S10, S18 and S23 was lyophilized and then the extract was dissolved in distilled water for the secondary stage PLC.

**The secondary stage PLC:** The secondary stage PLC was carried out with a liquid chromatographic system (Model 5000, Varian) equipped with an injector (Model 7120, Rheodyne), a UV detector (SPD-6A, Shimadzu) and a Chromatopak (C-R1A, Shimadzu). The same resins as in the reverse phase analytical HPLC were packed into both a main column (300 × 21.5 mm I. D.) and a precolumn (50 × 8 mm I. D.). Separations of those subfractions were achieved by the elution program as shown in Table 3 and operating conditions were as follows; column temperature: ambient, detection wavelength: 254 nm, injection volume: 1.5 ml. Eluent for each aimed peak was collected and each fraction was lyophilized again for the following analyses.

**Physicochemical analyses:**

1. **TLC**

High performance TLC was carried out by using a plate, Silica Gel 60 F254 (Merck & Co., Inc.), as a stationary phase and mixed fluid of chloroform, methly alcohol and acetic acid (10:7:3) as a mobile phase. After being developed, the plate was inspected for the rate of flow (Rf) of each spot at both 254 and 366 nm light.

2. **UV absorption spectrometry**

After each lyophilized sample was dissolved in distilled water, its UV absorption was serially recorded at wave length between 185 and 370 nm in a double beam spectrophotometer (Model 200-20, Hitachi).

3. **1H-NMR spectroscopy**

Each fraction sample was dissolved in 0.4 ml of deuterium oxide (D2O) or sodium deuteroxide (NaOD) solution in D2O purchased from Sigma (U. S. A.), poured into a glass tube with outer diameter of 5 mm and its 1H-NMR spectra were recorded at 300 MHz in the pulse Fourier transform mode on a spectrometer (AM 300, Bruker). Chemical shifts were determined by absorbance of D2O at 4.6 ppm down-field from tetramethylsilane. Scanning was continued to be integrated 1400-10000 times until satisfactory spectra were gained.

**Reference compounds:** The following analytical grade reagents were used as reference compounds in both the analytical reverse phase HPLC and the physicochemical analyses: uracil (Wako, Tokyo, Japan), uric acid (Wako), p-aminophenuric acid (Wako), urocanic acid (Sigma), p-aminobenzoic acid (Wako), adenosine (Wako), 5-hydrox-L-tryptophan (Sigma), theobromine (Sigma), quinuclidic acid (Wako), hippuric acid (Wako) and kynurenic acid (Sigma). Solutions of reference compounds were prepared in distilled water for HPLC, TLC and UV absorption spectrometry and in D2O or NaOD for 1H-NMR spectroscopy.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
<th>Mobile phase (%)</th>
<th>Mobile phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.3</td>
<td>0.006 M buffer</td>
<td>6.0 M buffer</td>
</tr>
<tr>
<td>15</td>
<td>4.5</td>
<td>0.006 M buffer</td>
<td>0.006 M buffer</td>
</tr>
<tr>
<td>150</td>
<td>80</td>
<td>100</td>
<td>0.006 M buffer</td>
</tr>
<tr>
<td>300</td>
<td>50</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>380</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>450</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

TFA: Trifluoro acetic acid.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>60.0</td>
<td>30</td>
</tr>
<tr>
<td>70.0</td>
<td>0</td>
</tr>
<tr>
<td>90.0</td>
<td>0</td>
</tr>
<tr>
<td>90.1</td>
<td>100</td>
</tr>
</tbody>
</table>

0.05% TFA in H2O (pH 2.23) 0.05% TFA-Acetonitrile (1:1, v/v)
RESULTS

HPLC of plasma obtained from uremic dogs: The chromatographic pattern obtained by the analytical anion exchange HPLC of plasma from a dog with uremia induced by ureteral ligation is similar to those of sera obtained from gentamicin-induced uremic dogs [8].

Isolation of uremic peak fractions: Figure 1 shows a chromatogram of the primary stage PLC of the concentrated uremic plasma extracts. Approximately thirty peaks were clearly separated. The analytical anion exchange HPLC analysis of every peak fraction assigned the retention times of peaks S7, S10, S18 and S23 in the primary stage PLC as indicated in Fig. 1.

Figures 2a-d show chromatograms of peak fractions S7, S10, S18 and S23, respectively, in the analytical reverse phase HPLC. The ratios of the maximum peak areas of S7, S10, S18 and S23 to total UV absorbing areas were 84.3, 50.8, 68.0 and 93.0%, respectively. Fraction S10 was suspected to contain several UV absorbing compounds. Only major peaks were subjected to separation by the secondary stage PLC, since a considerable amount of sample was required for subsequent identification. Major subfractions of S7, S10, S18 and S23 are quoted hereafter as S7a, S10a, S18a and S23a, respectively and these subfractions were isolated by the secondary stage PLC.

Physicochemical properties of four main fractions: Retention times of S7a, S10a, S18a, S23a and reference compounds on the reverse phase analytical HPLC are shown in Table 4. Retention times of S10a, S18a and S23a are closely coincident with those of uric acid or p-aminohippuric acid, quinaldic acid or hippuric acid, and kynurenic acid, respectively.

(1) TLC

Table 5 shows Rfs in TLC of fractions S10a, S18a and S23a, together with those of five reference compounds.

![Fig. 1. A chromatogram of concentrated and pooled plasma extracts from experimentally induced uremic dogs by the first stage preparative liquid chromatography.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7a</td>
<td>19.0</td>
</tr>
<tr>
<td>S10a</td>
<td>13.9</td>
</tr>
<tr>
<td>S18a</td>
<td>30.3</td>
</tr>
<tr>
<td>S23a</td>
<td>33.6</td>
</tr>
<tr>
<td>Uric acid</td>
<td>13.1</td>
</tr>
<tr>
<td>p-Aminohippuric acid</td>
<td>14.3</td>
</tr>
<tr>
<td>Urocanic acid</td>
<td>16.0</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>17.0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>17.5</td>
</tr>
<tr>
<td>5-Hydroxy L-tryptophan</td>
<td>24.1</td>
</tr>
<tr>
<td>Theobromine</td>
<td>25.4</td>
</tr>
<tr>
<td>Quinarchid acid</td>
<td>30.0</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>30.3</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>33.6</td>
</tr>
</tbody>
</table>
Table 5. Rf value in TLC of suspected uremic fraction S7a, S10a, S18a, and S23a and reference compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7a</td>
<td>N.D.</td>
</tr>
<tr>
<td>S10a</td>
<td>0.52</td>
</tr>
<tr>
<td>S18a</td>
<td>0.81</td>
</tr>
<tr>
<td>S23a</td>
<td>0.58</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.52</td>
</tr>
<tr>
<td>p-Aminohippuric acid</td>
<td>0.61</td>
</tr>
<tr>
<td>Quinoidal acid</td>
<td>0.75</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>0.79</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>0.58</td>
</tr>
</tbody>
</table>

N.D.: Not detected.

Figs. 3a-d. Ultraviolet absorption spectra of the compound from fraction S7a (a), S10a, and uric acid (b), S18a, and hippuric acid (c) and S23a, and kynurenic acid (d).

Fig. 4. Proton-nuclear magnetic resonance spectra of S7a, S18a, S23a, hippuric acid and kynurenic acid.

Fraction S7a was not detected in TLC. Rfs of S10a, S18a, and S23a closely approximated to those of uric acid, hippuric acid and kynurenic acid, respectively. Fraction S23a, quinoidal-acid and kynurenic acid showed positive fluorescence at 366 nm.

(2) Spectrum of UV absorption

UV absorption spectra of fractions S7a, S10a, S18a, S23a, and the reference compounds, uric acid, hippuric acid and kynurenic acid, are shown in Figs. 3a-d. Fraction S7a had the maximum absorbing wave lengths (λmax) at 207, 259 and 295 nm; S10a at 219, 230, and 295 nm; S18a at 200, 226 and 275 nm; and S23a at 190, 213, 242, 330 and 347 nm. Uric acid had λmax at 219, 230, and 295 nm; hippuric acid at 202, 226 and 275 nm; and kynurenic acid at 190, 213, 242, 331 and 346 nm. These spectra closely corresponded with those of S10a, S18a and S23a, respectively.

(3) Spectrum of 1H-NMR

Figure 4 shows 1H-NMR spectra for S7a and S18a, and hippuric acid in D2O and S23a and kynurenic acid in NaOD solution. A spectrum for S10a was not obtained. Chemical shift values (δ) for S7a were 2.00, 3.42, 6.45, 6.50, 7.75, 7.83 and 8.17 ppm; 3.81, 7.31, 7.34, 7.35, 7.41, 7.53 and 7.60 ppm for S18a; and 6.29, 6.78, 6.82, 7.01, 7.03, 7.06, 7.19, 7.21, 7.50 and 7.53 ppm for S23a. Hippuric acid had δs of 8.11, 7.61, 7.59, 7.41, 7.39, 7.34, 7.32 and 3.83 and kynurenic acid had those of 6.28, 6.78, 6.82, 7.01, 7.03, 7.06, 7.19, 7.22, 7.49 and 7.52. 1H-NMR spectra of S18a and S23a were essentially identical to those of standard preparation of hippuric acid and kynurenic acid, respectively.

DISCUSSION

The chromatographic pattern of canine uremic substances in this study was found to be similar to that recorded in the previous study [8], and appropriate amounts of peaks corresponding to S7, S10, S18 and S23 were obtained.

The primary stage PLC was successfully employed in the primary separation of uremic peaks S7, S10, S18 and S23 and additional analysis using the reverse phase HPLC was necessary for further confirmation of purity or possibility of complete isolation. Thus, the uremic peaks S7, S18 and S23 were shown to contain almost single compounds, while S10 consisted of several UV absorbing compounds. These results indicated that PLC with the same resin as used in the analytical reverse phase HPLC would be suitable for the secondary stage PLC in separation of subfractions of not only S7, S18 and S23 but also S10. Although components in subfractions of S10 deserved to be identified, the amounts of fraction S10 were too low for subfractionation except S10a.

In the present study, 1H-NMR spectroscopy was used for determination of chemical structure of components under investigation in addition to TLC analysis and UV spectroscopy. TLC easily detects these substances and UV spectroscopy is suitable for broad chemical identification. Additionally, they are employed in the identification of substances by being compared with reference compounds. At present, NMR spectroscopy is one of the most effective and reliable method in determination of the chemical structure of compounds.

S7 fraction was shown to contain almost single substance by analytical reverse phase HPLC. In the spectrum of 1H-NMR for S7a, δs of 6.45–8.17 ppm and δ of 3.42 ppm suggested the presence of aromatic heterocycle and neighbor radical of nitrogen, respectively. Additionally, one of λmax in UV absorption analysis was 259 nm, which
was not contradictory to a pyridine ring. These results may
be enough to characterize S71 to be a pyridine derivative,
but identification of S71 requires further analyses. In
human medicine, several pyridine metabolites at high
concentrations were also found in uremic plasma [4, 11].
Among these pyridine derivatives, N-methyl-2-pyridone-
5-formamidoacetic acid was found to inhibit protein
synthesis in liver homogenates [4]. Accordingly, pyridine
derivatives also could be a candidate of uremic toxins in
dogs.

S10 fraction was shown to contain several compounds
by the analytical reverse phase HPLC, and the retention
time of subfraction S101 was closely coincident with those
of uric acid and p-aminohippuric acid. As for TLC, a spot
was detected at 254 nm but not at 366 nm. RF for S101 was
compatible with that of uric acid, but differed from that of
p-aminohippuric acid. These results suggested that S101
consisted of a single substance and would be uric acid. In
fact, the spectrum of UV absorption for S101 was in good
agreement with that of uric acid. The spectrum of
1H-NMR of S101, on the contrary, was not obtained as
well as uric acid. One reason might be that uric acid
showed keto-enol tautomerism and lacked in structural
stability. For further identification, S101 assumed a violet
color by uricase-peroxidase method. These results
indicated that a component of S101 was uric acid (Mr=168.11
Da). Uric acid is the end-product of purine metabolism in
humans. On the other hand, most breeds of dogs transform
uric acid to allantoin in the liver. Therefore, serum
concentrations of uric acid in uremic dogs may be
much lower than that in uremic humans.

S18 fraction was shown to be consisted of almost a
single compound by the analytical reverse phase HPLC,
and retention time of subfraction S181 was almost coinci-
dent with quininalic acid and hippuric acid. As for TLC, RF
of fraction S181 coincided closely with that of hippuric
acid. Although RF of fraction S181 was also agreed with that
of quininalic acid to some extent, quinaldic acid was the only
fluorescent compound detected at 366 nm among S181,
quininalic acid, and hippuric acid. These results suggested
that S181 would be hippuric acid. The spectrum of UV absorp-
tion and 1H-NMR for hippuric acid were completely coincident
with those of S181. These results indicated that the component of S181
was hippuric acid (Mr=179.17 Da). Recently, hippuric acid is shown to have significant
negative correlation to residual renal function in humans
[17] and to inhibit protein binding of drug [7, 13, 18, 20]
and urate transport at the cortical tubules [3]. These will
suggest that this component might also be a possible
candidate for a uremic toxin in dogs.

S23 fraction was found almost certainly to contain a
single compound by the analytical reverse phase HPLC,
and retention time of subfraction S231 was completely
coincident with that of kynurenic acid. RF in TLC and
spectra of UV absorption and 1H-NMR for S231 were
completely equivalent to those for kynurenic acid, respec-
tively. These results indicated that the component of S231
was kynurenic acid (Mr=189.16 Da). Kynurenic acid is
one of tryptophan metabolites and is excreted in urine in
several mammalian species, especially in dogs [12, 19].
Although it has not been suspected as a uremic toxin in
humans, it may be a peculiar uremic toxins to dogs.

From all of these results, S71, S101, S181 and S231 would
represent a pyridine derivative, uric acid, hippuric acid
and kynurenic acid, respectively, all of which are aromatic
compounds with small molecular weight.

It is the first study to identify small molecular substances
related to uremia in dogs with some coincidence with
those in humans. These substances are worthwhile to
investigate their pathophysiological effect on toxicity for
further studies.

REFERENCES

1. Asatoor, A. M. 1968. Retention of pseudouridine and

B. H. 1972. Hemodialyzer evaluation by examination of solute
98–105.

G. 1975. Uricosuric agents in uremic sera. Identification of
indoxyl sulfate and hippuric acid. J. Clin. Invest. 55:
1142–1152.

Inhibition of protein synthesis by N-methyl-2-pyridone-
5-formamidoacetic acid and other compounds isolated from

5. Cueille, G., Man, N. K., Sausse, A., Farges, J. P., and
Funck-Brentano, J. L. 1981. Technical aspects on middle
molecules: separation, isolation, and identification. Artif.
Organs 4 (Suppl.): 8–12.

6. Cueille, G., Man, N. K., Sausse, A., Farges, J. P., and
Funck-Brentano, J. L. 1981. Characterization of sub peak

E. A., and Depner, T. A. 1986. Isolation and chemical
identification of inhibitors of plasma ligand binding. Kidney

8. Kawamura, M., Ohashi, F., Murakami, F., Takai, N.,
of uremic peaks in dogs by anion exchange high performance
liquid chromatography. J. Vet. Med. Sci. 54:
951–955.

9. Koide, K., Toyama, J., Inoue, N., Koshikawa, S., Akiza-
wa, T., Takahashi, K., Hitaka, S., Yamane, Y., Nakao,
M., Uehara, Y., and Nishimura, Y. 1986. Detection of
uremic peaks by high performance liquid chromatography
and their behavior. Jpn. J. Nephrol. 28: 1101–1110 (in
Japanese).

10. Koide, K., Toyama, J., Inoue, N., Koshikawa, S., Akiza-
wa, T., Takahashi, K., Hitaka, S., Yamane, Y., Shinoda,
K., Nakao, M., Uehara, Y., and Nishimura, Y. 1986.
Uremic peak 2a in high performance liquid chromatography
- Acidic components and their membrane permeability.

11. Kramer, B., Seligson, H., Seligson, D., and Baltrush,
H. 1964. Isolation of N-methyl-2-pyridone-5-carboxamide
from hemodialysis fluid obtained from uremic patients.


