Study on the Analysis of Capsaicin Glucuronide in Rat Urine by Liquid Chromatography-Mass Spectrometry after Enzymatic Hydrolysis

Masaaki Noami,*[a,c] Kazuo Igarashi,[b] Fumiyo Kasuya,[c] Masayuki Ohta,[c] Mieko Kanamori-Kataoka,[d] and Yasuo Seto[d]

[a]Forensic Science Laboratory, Wakayama Pref. Police H. Q., 1 Nishi, Wakayama 640–8313, Japan, [b]Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061–0212, U.S.A, [c]Faculty of Pharmaceutical Sciences, Kobe Gakuin University, 518 Arise, Ikawadanicho, Nishi-ku, Kobe 651–2180, Japan, and [d]National Research Institute of Police Science, 6–3–1 Kashiwanoha, Kashiwa, Chiba 277–0882, Japan

(Received May 6, 2006; Accepted August 2, 2006)

We established a method for determining capsaicin glucuronide in rat urine samples using liquid chromatography-mass spectrometry combined with enzymatic hydrolysis. Capsaicin was not detected in urine samples of rats administered capsaicin intraperitoneally (i.p., 2 and 4 mg/kg), but after hydrolysis with β-glucuronidase, extraction with methanol and solid-phase extraction, capsaicin was clearly detected by liquid chromatography-mass spectrometry with electrospray ionization. The limit of detection was obtained to be 0.1 ng/ml in urine sample matrix. The conditions for the enzymatic hydrolysis of the conjugate were optimized for β-glucuronidases from *Ampullaria*, *Escherichia coli*, bovine liver, *Helix pomatia* and *Patella vulgata*. The optimal conditions among those examined [pH (3.3–9.0), temperature (37–70°C) and enzyme amount (50–2500 U)] for 1 ml of the urine sample were as follows: β-glucuronidase from *Ampullaria* (pH 4.2, 45°C, 500 U), from *Escherichia coli* (pH 6.0–7.2, 37°C, 500 U), from bovine liver (pH 5.0, 45°C, 500 U), from *Helix pomatia* (pH 5.0, 60°C, 1250 U) and from *Patella vulgata* (pH 3.8, 45–60°C, 2500 U). Among the enzymes examined, β-glucuronidase from *Ampullaria* was found to be suitable for hydrolysis of the conjugate. Under the optimal conditions of *Ampullaria* β-glucuronidase, the incubation of 1 ml of urine sample for 90 min was sufficient for hydrolysis of capsaicin glucuronide in the urine sample. The urinary recovery values of capsaicin collected for 0–48 hr after administration of 2 and 4 mg/kg capsaicin to rats were 1.4 and 1.1%, respectively.

Key words —— capsaicin, glucuronide conjugate, enzymatic hydrolysis, β-glucuronidase, liquid chromatography-mass spectrometry, urine

INTRODUCTION

Capsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-8-methyl-*trans*-6-nonenamide Fig. 1] is one of the main pungent ingredients, or capsaicinoids, in oleoresin capsicum extractable from oily hot peppers included in pepper.[1–3] These capsaicinoids are used in lacrymating pepper spray,[4,5] and recently, crimes such as robbery, pickpocketing and rape using tear gas sprays have been increasing. Forensic toxicologists must identify such causative toxic substances in their forensic investigations.[6] Although it has been reported that capsaicin incorporated into the rat is mainly metabolized into compounds and their glucuronides (Fig. 1)[7,8] and that cytochrome P-450 converted capsaicin to various oxidized compounds,[9] the details of the *in vivo* metabolism of capsaicin and related compounds has not yet been fully elucidated. To prove exposure to pepper spray in forensic analysis, it is required to detect capsaicin and related compounds from evidence samples, especially from biological samples of casualty cases.

We previously reported on a quantification method for urinary capsaicin and its hydrolyzed compound, vanillylamine.[10] The analysis of their glucuronides in urine samples is also important for forensic analysis, as they are major metabolites in urine.[7,11] It is possible to detect glucuronides directly by liquid chromatography-mass spectrometry (LC-MS), but the analysis of hydrolyzates of glucuronides is usually more convenient because its procedure for

*To whom correspondence should be addressed: Forensic Science Laboratory, Wakayama Prefectural Police Headquarters, 1 Nishi, Wakayama-shi, Wakayama 640–8313, Japan. Tel.: +81-73-473-0110 (ext. 550); Fax: +81-73-473-0110 (ext. 549); E-mail: cfavt003@jtw.zaq.ne.jp

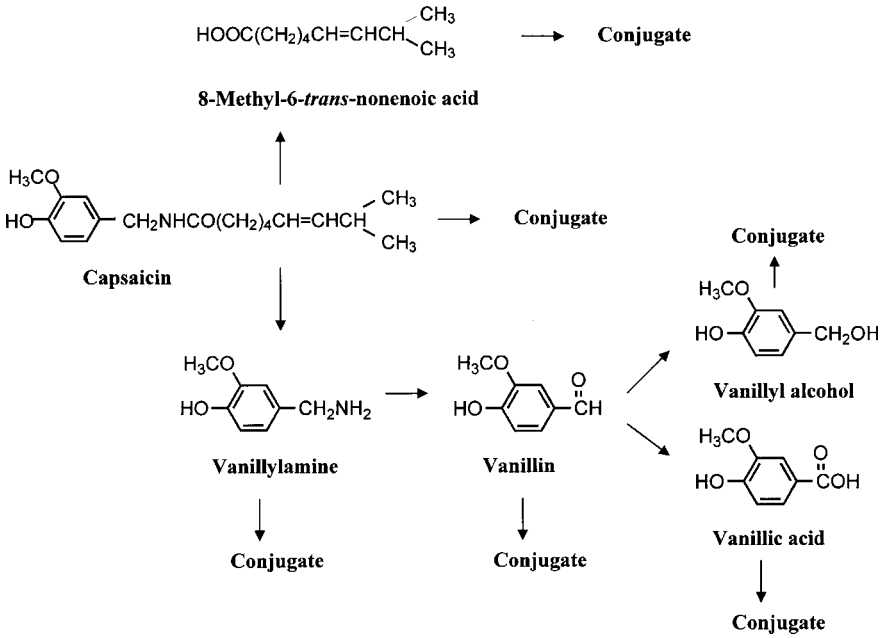

Fig. 1. Metabolic Pathways of Capsaicin in Body

analyzing the glucoronide requires an authentic conjugate and a complicated extraction procedure to exclude chromatographic interference from polar urine ingredients. The enzymatic hydrolysis condition is more moderate and generally gives cleaner extracts compared to acid hydrolysis.[12] In this report, we have adopted enzyme hydrolysis by β-glucuronidase for determination of capsaicin glucuronide in rat urine samples and have examined the procedure for determining hydrolyzed glucuronide using LC-MS. Moreover, we examined the optimal hydrolysis conditions for capsaicin glucuronide in rat urine using several commercially available β-glucuronidases.

MATERIALS AND METHODS

Materials —— Capsaicin, β-glucuronidases from *Escherichia coli* (Type IV-A), bovine liver (Type B-1) and *Patella vulgata* (Type L-II) and saccharolactone (*d*-saccharic acid 1,4-lactone) were obtained from Sigma (St. Louis, MO, U.S.A.). β-Glucuronidase/aryl sulfatase from *Helix* pomatia was obtained from Calbiochem (San Diego, CA, U.S.A.). Bond Elut Certify cartridges were obtained from Varian (Harbor City, CA, U.S.A.). Acetonitrile (HPLC-grade), methanol (HPLC-grade) and β-glucuronidase from Ampullaria were obtained from Wako Pure Chemical Industries (Osaka, Japan), and the other chemicals used were of commercial analytical grade.

The activities of β-glucuronidases used in this study were determined by the method reported by Fishman *et al.*[13] The β-glucuronidase activities found and subsequently used in this experiment were almost the same as those described on the reagent bottles.

A standard capsaicin stock solution (1 mg/ml) for urine analysis was prepared in methanol, and diluted to appropriate concentrations with methanol. The capsaicin solution (1 and 2 mg/ml) for administration (2 and 4 mg/kg) to rats was prepared in an aqueous hydoxypropyl-β-cyclodextrin mixture (4 : 1, v/v).

Animal Experiment —— Male Wistar rats weighing about 250 g were administered capsaicin [i.p., 0 (vehicle only), 2, and 4 mg/kg]. Urine samples were collected for 0–24 and 24–48 hr after administration of capsaicin, and stored at –30°C until analysis. The analyses were performed within one month after sample storage. A blank urine sample was also collected from the control rat.

Enzymatic Hydrolysis —— One milliliter of enzyme solution in an appropriate buffer (pH 3.3: 0.2 M citric acid/0.1 M disodium hydrogenphosphate buffer; pH 3.8–5.5: 0.5 M sodium acetate buffer; pH 6.0–7.8: 0.2 M sodium phosphate buffer; pH 8.5–9.0: 0.25 M sodium borate buffer) was added to 1 ml of the urine sample, and the mixture was incu-

bated for an appropriate time period at a designated temperature (37–70°C). The enzymatic reaction was stopped by adjusting the pH of the mixture to approximately 10.5 with 3 M sodium hydroxide solution under cooling in an ice bath.

Extraction —— The extraction of capsaicin from urine was performed as described previously[10] with minor modifications as follows. A Bond Elut Certify cartridge was pretreated with 4 ml water, 4 ml acetonitrile and 10 ml water. One milliliter of 10% ammonium hydroxide solution was added to the hydrolyzed urine sample (2 ml), and applied to the cartridge. Capsaicin was eluted with 4 ml acetonitrile. The eluate was evaporated to dryness, and dissolved in 500 μl of acetonitrile. A ten μl aliquot was subjected to LC-MS system.

LC-MS Conditions —— LC-MS was performed using a Micromass ZMD quadrupole mass spectrometer (Waters Co., Milford, MA, U.S.A.) equipped with electrospray ionization (ESI). The liquid chromatograph consisted of a Waters Alliance 2690 pump equipped with an autosampler and an L-column ODS column (150 × 2.1 mm i.d., 5 μm particle size, Chemicals Evaluation and Research Institute, Tokyo, Japan). The mobile phase was 2% (v/v) acetic acid solution/acetonitrile (20 : 80, v/v). The flow rate was 0.2 ml/min. The column temperature was maintained at 40°C. The following ESI operating conditions were adopted: source block temperature, 130°C; capillary voltage, +3.5 kV; cone voltages, 30 V; multiplier voltage, 650 V; and desolvation temperature, 400°C. For confirmation of the analytes, full-scan spectra were recorded in the range of *m*/*z* 80–500 at a scan duration of 1.0 sec. For quantification, analysis was conducted by the external calibration method using the protonated molecular ions of the analytes (capsaicin, *m*/*z* 306) in the selected ion-monitoring mode.

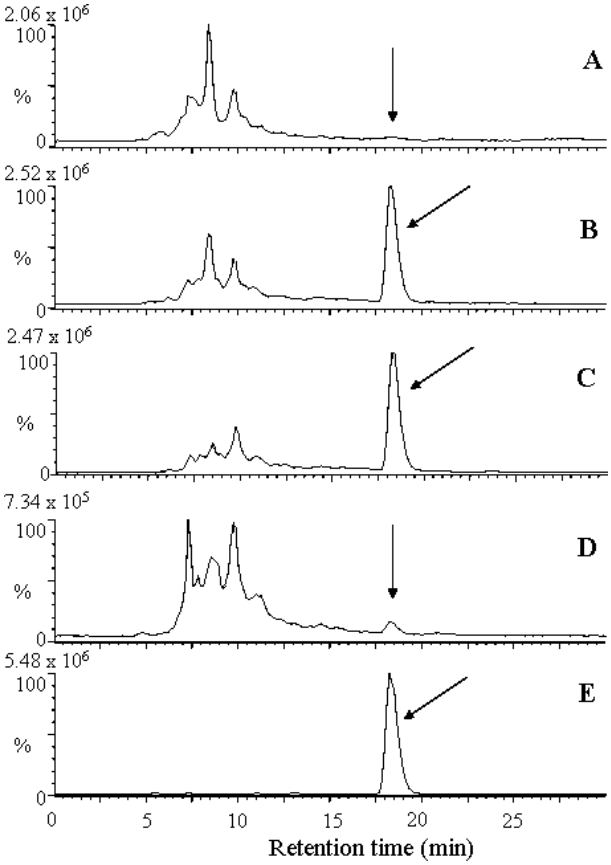

Fig. 2. Liquid Chromatography-Mass Spectrometry of Rat Urine Extracts with and without Enzymatic Hydrolysis

Hydrolysis was performed at pH 4.2 for 2 hr with β-glucuronidase from *Ampullaria* (500 U/ml urine). A: TIC of extracts obtained from urine samples of untreated rat with enzymatic hydrolysis. B: TIC of extracts obtained from urine samples of capsaicin treated rat with enzymatic hydrolysis. C: EIC (*m*/*z* 306) of extracts obtained from urine samples of capsaicin treated rat with enzymatic hydrolysis. D: EIC (*m*/*z* 306) of extracts obtained from urine samples of capsaicin treated rat without enzymatic hydrolysis. E: EIC (*m*/*z* 306) of capsaicin standard (1 μg/ml). B, C, and D, urine samples collected for 0–48 hr from rat administered capsaicin 4 mg/kg. Arrows indicated mean the ekution position of capsaicin.

RESULTS AND DISCUSSION

β-Glucuronidase Dependent Capsaicin Detection in Rat Urine

For the animal experiment, we adopted i.p. administration and a rather high dose of capsaicin (2 and 4 mg/kg) compared to the lethal dose (LD_{50} in rat, i.p.: 9.5 mg/kg[14]), in order to obtain high and reproducible concentration of capsaicin glucuronide in urine samples for establishing enzymatic hydrolysis conditions. Figure 2 illustrates typical chromatograms obtained from the solid-phase extract of the urine sample (capsaicin 4 mg/kg injection, 0–48 hr collection) with and without β-glucuronidase treatment. Capsaicin was scarcely detected on total ion chromatogram (TIC) obtained from the extract without enzyme treatment (Fig. 2A), but clearly detected on TIC from the extract with enzyme treatment (Fig. 2B). Also the peak of capsaicin detected on the extracted ion chromatogram (EIC) obtained from the extract with enzyme treatment (Fig. 2C) gave the same retention time as that from authentic standard solution of capsaicin (1 μg/ml) (Fig. 2E) and showed a well-separated peak of capsaicin from any extract matrix peaks that appeared. Capsaicin was not detected in either the control urine or the urine

sample from the vehicle-only-administered rats (data not shown). In the quantification of capsaicin supplemented to the urine extract of the control rat, the limits of detection (LODs), which was estimated from the detection limits of the target and qualifier ion peaks on each mass chromatogram (S/N ≥ 5), and satisfying the acceptable range of the ratio of qualifier to target ion responses (± 20%), were estimated from the data of a drug-free urine sample. The LODs was obtained to be 0.1 ng/ml in the selected-ion monitoring (SIM) mode. The calibration curve of the capsaicin peak areas was linear against the urinary concentration in the range 0.5–1000 ng/ml with a correlation coefficient of 0.991. Accuracy evaluated at 0.1 mg/ml was 0.074 ± 0.006 mg/ml (average ± S.D., n = 5). Within-day and between-day precisions were 6.3 and 7.1%, respectively (n = 5). Thus, the validation data guaranteed the sensitivity and quantitative efficiency of the procedure. A previous paper[10] reported that capsaicin was detected from the capsaicin-administrated rat urine at levels of 20–250 ng/ml. With β-glucuronidase (from *Ampullaria*, 500 U, pH 4.0–6.0, 45°C, 90 min) treatment, the detection level was significantly increased, to more than 10-fold. On the other hands, the detection level of capsaicin in the urine with treatment of β-glucuronidase in the presence of 100 mM saccharolactone, an inhibitor of β-glucuronidase, was almost the same (95%) as that in the absence of the inhibitor. This result indicates that the contaminated sulfatase in β-glucuronidase reagent does not give the hydrolysis of capsaicin conjugate. Capsaicin metabolites (vanillylamine and related compounds) were also detected (data not shown).

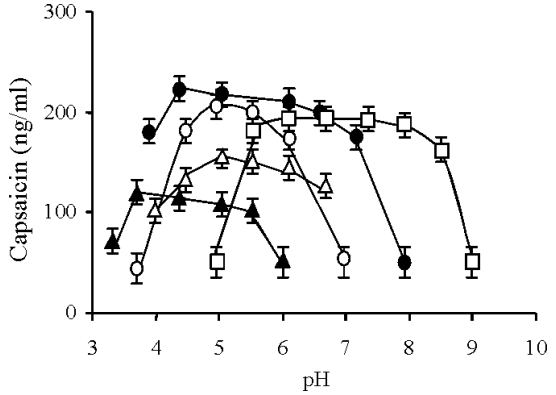

Fig. 3. Effect of pH on the Detected Capsaicin Levels in the Enzymatic Hydrolysis

Enzyme amount 500 U/ml, temperature 45°C, incubation time 120 min. β-Glucuronidases from *Escherichia coli* (□), *Helix pomatia* (△), bovine liver (○), *Patella vulgata* (▲) and *Ampullaria* (●). Each point represents the mean ± S.D. of 3 experiments.

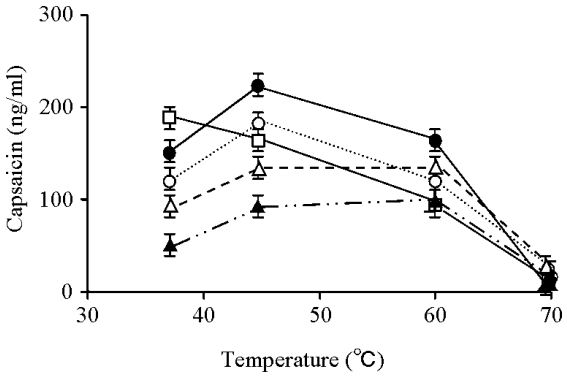

Fig. 4. Effect of Temperature on the Detected Capsaicin Levels in Enzymatic Hydrolysis

Enzyme amount 200 U/ml [except for *Ampullaria* enzyme (100 U)], pH 4.2, incubation time 120 min. β-Glucuronidases from *Escherichia coli* (□), *Helix pomatia* (△), bovine liver (○), *Patella vulgata* (▲) and *Ampullaria* (●). Each point represents the mean ± S.D. of 3 experiments.

Optimization of β-Glucuronidase Hydrolysis Conditions

The optimal hydrolysis conditions of capsaicin glucuronide with five β-glucuronidases were examined; varying pHs, incubation times, and enzyme amounts, using the urine sample collected for 0–48 hr from one rat injected 4 mg/kg capsaicin. Figure 3 shows the effect of pH on the hydrolysis levels. The hydrolysis levels of capsaicin glucuronide with enzyme were indicated as the capsaicin detection levels on the chromatogram. Bell-shape curves were obtained for all enzymes, and the levels were highest at pH 4.2 for the *Ampullaria* enzyme, pH 5.0 for the bovine liver and *Helix pomatia* enzymes, around pH 6.0–7.2 for the *Escherichia coli* enzyme, and pH 3.8 for the *Patella vulgata* enzyme. Our results concerning the optimal pH values were in agreement with the recommended pH values for β-glucuronidase reaction by the reagent company (pH 6.8 for *Escherichia coli* enzyme, pH 5.0 for bovine liver enzyme, pH3.8 for *Patella vulgata* enzyme).

Figure 4 shows the effect of incubation temperature on the hydrolysis level of β-glucuronidase treated urine. Bell-shape curves were obtained for all enzymes except for the *Escherichia coli* enzyme, and the levels were highest at 45°C for *Ampullaria* and bovine liver enzymes, and at 60°C for *Helix pomatia* and *Patella vulgata* enzymes. The level was highest at 37°C for the *Escherichia coli* enzyme; beyond this peak, the level decreased sharply, indi-

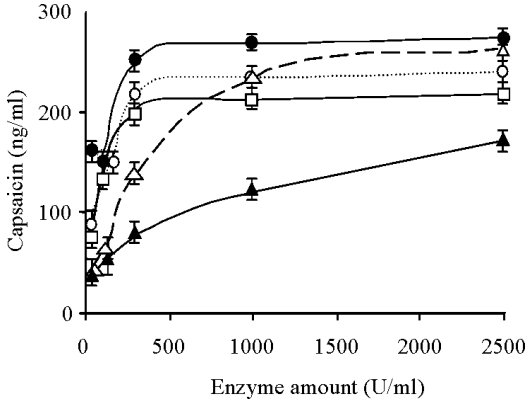

Fig. 5. Effect of Enzyme Amount on the Detected Capsaicin Levels in Enzymatic Hydrolysis

pH optimized, temperature 45°C, incubation time 90 min. β-Glucuronidases from *Escherichia coli* (□, pH 6.8), *Helix pomatia* (△, pH 5.0), bovine liver (○, pH 5.0), *Patella vulgata* (▲, pH 3.8) and *Ampullaria* (●, pH 4.2). Each point represents the mean ± S.D. of 3 experiments.

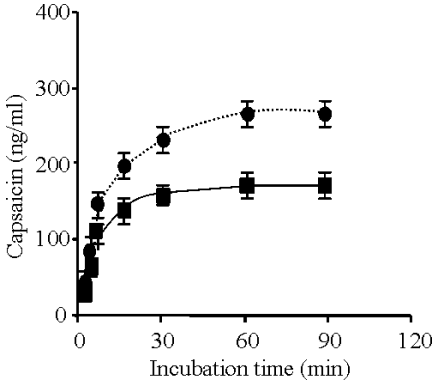

Fig. 6. Time Course of the Detected Capsaicin Levels in Enzymatic Hydrolysis

Rats were administered capsaicin [2 mg/kg (■) or 4 mg/kg (●)] intraperitoneally, and urine samples were collected for 48 hr after administration. β-Glucuronidases from *Ampullaria*, pH 4.2, 45°C, 500 U/ml. Each point represents the mean ± S.D. of 3 experiments.

Table 1. Excretion of Capsaicin and Capsaicin Glucuronide in Urine of Capsaicin-Administered Rats

Urine collection period	0–24 hr			24-48 hr		
Capsaicin administration	Volume (ml)	Concentration (μg/ml)	Excretion content (μg)	Volume (ml)	Concentration (μg/ml)	Excretion content (μg)
2 mg/kg	28	0.170	4.8	24	0.132	3.2
	24	0.152	3.6	22	0.130	2.9
	26	0.160	4.2	19	0.149	2.8
	27	0.157	4.2	25	0.126	3.2
4 mg/kg	23	0.234	5.4	24	0.199	4.8
	28	0.271	7.6	26	0.235	6.1
	27	0.227	6.1	24	0.194	4.7
	27	0.212	5.7	26	0.203	5.3

Values represent the total urine volumes, their capsaicin concentrations and excretion contents of capsaicin and its glucuronide collected both between 0 and 24 hr and between 24 and 48 hr from rats administered 2 and 4 mg/kg capsaicin intraperitoneally, respectively. Capsaicin concentrations were measured after enzymatic hydrolysis of urine samples.

cating possible enzyme inactivation at higher temperatures.

Figure 5 shows that the hydrolysis levels of capsaicin glucuronide increased with the amounts of β-glucuronidase from the five origins examined. At about 500 U/ml urine for *Ampullaria* enzyme, the hydrolysis levels reached a plateau of the highest value (about 270 ng/ml), indicating complete hydrolysis of the capsaicin glucuronide conjugate. For bovine liver and *Helix pomatia* enzymes, at 2500 U/ml the hydrolysis levels reached the highest value, which was almost compatible with the highest value in the *Ampullaria* enzyme. For the *Escherichia coli* and *Patella vulgata* enzymes, the hydrolysis levels had not peaked even at 2500 U/ml, and these levels were about 200 and 160 ng/ml. The relatively low levels in these enzymes may be compatible with the result for the effect of pH (Fig. 3) and temperature (Fig. 4). One possibility for the low level in *Patella vulgata* enzyme may be attributed to enzyme inactivation at 45°C.

As for the best commercial β-glucuronidase enzyme for capsaicin glucuronide hydrolysis, the enzyme from *Ampullaria* and its optimal hydrolysis conditions (pH 4.2, 45°C, 500 U/ml) was selected from the standpoint of pH, temperature, enzyme amount and incubation time.

Figure 6 shows the time course of the hydrolysis levels of capsaicin glucuronide under the optimized conditions. The level reached a highest-value

plateau (about 170 and 270 ng/ml) at 30 min for a 2 mg/kg dose of capsaicin and 90 min for a 4 mg/kg dose, respectively.

Our experiment did not use authentic capsaicin glucuronide, and the enzymatic hydrolysis behavior of capsaicin conjugate and stability of both capsaicin and its glucuronide were not elucidated; still, it is probable that capsaicin glucuronide was perfectly converted to free capsaicin, because the capsaicin concentrations showed a plateau (Fig. 6).

Content of Capsaicin β-Glucuronide Conjugate in the Rat Urine

Adopting the optimal enzymatic hydrolysis conditions [for 1 ml urine, 500 U of *Ampullaria* β-glucuronidase at phosphate buffer (pH 4.2) at 45°C], capsaicin levels were measured in urine samples collected for 0–24 hr and for 24–48 hr after administration of capsaicin (2 and 4 mg/kg). As shown in Table 1, the detection levels were considerably higher than the quantification limit (0.1 ng/ml). Moreover, by considering the total administration content, 0.84 ± 0.10% and 0.60 ± 0.04% of the administered dose of capsaicin were detected in urine samples collected for 0–24 hr and for 24–48 hr from the capsaicin-administered rats at 2 mg/kg doses, respectively, and 0.62 ± 0.10% and 0.52 ± 0.06% of capsaicin were detected from rats administered 4 mg/kg. Obviously, only a small portion of the capsaicin administered to the rats was recovered in the urine, and other portion may be recovered as the glucuronide conjugates of capsaicin metabolites. A further experiment is underway to establish an extraction and LC-MS quantification method for capsaicin metabolites and to investigate the effect of β-glucuronidase treatment on the detection of its metabolites.

REFERENCES

1) Govindarajan, V. S. and Sathyanarayana, M. N. (1991) Capsicum — production, technology, chemistry, and quality. part V. Impact on physiology, pharmacology, nutrition, and metabolism; structure, pungency, pain, and desensitization sequences. *Crit. Rev. Food Sci. Nutr.*, **29**, 435–474.
2) Cordell, G. A. and Araujo, O. E. (1993) Capsaicin: identification, nomenclature, and pharmacotherapy. *Ann. Pharmacother.*, **27**, 330–336.
3) Surh, Y. J. and Lee, S. S. (1995) Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. *Life Sci.*, **56**, 1845–1855.
4) Hu, H., Fine, J., Epstein, P., Kelsey, K., Reynolds, P. and Walker, B. (1989) Tear gas — harassing agent or toxic chemical weapon? *J. Amer. Med. Asoc.*, **262**, 660–663.
5) Lee, R. J., Yolton, R. L., Yolton, D. P., Schnider, C. and Janin, M. L. (1996) Personal defense sprays: effects and management of exposure. *J. Am. Opt. Assoc.*, **67**, 548–560.
6) Kataoka, M., Seto, Y., Tsuge, K. and Noami, M. (2002) Stability and detectability of lacrymators and their degradation products in evidence samples. *J. Forensic Sci.*, **47**, 44–51.
7) Kawada, T. and Iwai, K. (1985) *In vivo* and *in vitro* metabolism of dihydrocapsaicin, a pungent principle of hot pepper, in rats. *Agric. Biol. Chem.*, **49**, 441–448.
8) Surh, Y. J., Ahn, S. H., Kim, K. C., Park, J. B., Sohn, Y. W. and Lee, S. S. (1995)Metabolism of capsaicinoids: evidence for aliphatic hydroxylation and its pharmacological implications. *Life Sci.*, **56**, 305–311.
9) Christopher, A. R, William, J. E. and David, A. J. (2003) Metabolism of capsaicin by cytochrome P450 produces novel dehydrogenated metabolites and decreases cytotoxicity to lung and liver cells. *Chem. Res. Toxicol.*, **16**, 336–349.
10) Noami, M., Igarashi, K., Ueda, K., Yamamoto, S., Nogami, Y., Kasuya, F., Seto, Y. and Kataoka, M. (2004) Determination of capsaicin and its hydrolyzed metabolite vanillylamine in body fluids by liquid chromatography-mass spectrometry combined with solid-phase extraction. *Jpn. J. Forensic Toxicol.*, **22**, 33–37.
11) Oi, Y., Kawada, T., Watanabe, T. and Iwai, K. (1992) Induction of capsaicin-hydrolyzing enzyme activity in rat liver by continuous oral administration of capsaicin. *J. Agric. Food Chem.*, **40**, 467–470.
12) Hackett, L. P., Dusci, L. J., Ilett, K. F. and Chiswell, G. M. (2002) Optimizing the hydrolysis of codeine and morphine glucuronides in urine. *Ther. Drug Monit.*, **24**, 652–657.
13) Fishman, W. H., Springer, B. and Brunetti, B. (1948) Application of an improved glucuronidase assay method to the study of human blood β-glucuronidase. *J. Biol. Chem.*, **173**, 449–456.
14) Sweet, D. V. (1987) 52165. 6-Nonenamide, N-((4-hydroxy-3-methoxyphenyl)methyl)-8-methyl- (E)-. In *Registry of Toxic Effects of Chemical Substances*, U.S. Department of Health and Huamn Services, Washington, D.C.