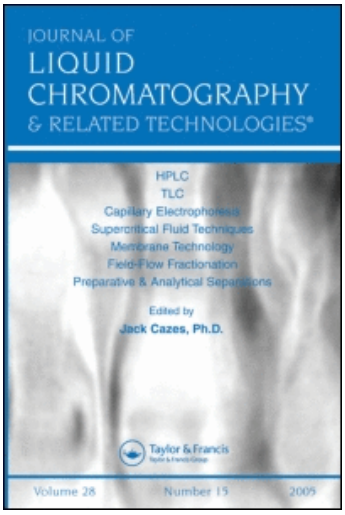

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Fluorescence Analysis for *p*-Hydroxymethamphetamine in Urine by HPLC with Post-Column Reaction

Noriyuki Kato[a]; Susumu Fujita[a]; Hiroaki Kubo[b]; Hiroshi Homma[b]
[a] Scientific Criminal Investigation Laboratory, Kanagawa Prefectural Police Headquarters, Yokohama, Japan [b] School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan

To cite this Article Kato, Noriyuki , Fujita, Susumu , Kubo, Hiroaki and Homma, Hiroshi(2005) 'Fluorescence Analysis for *p*-Hydroxymethamphetamine in Urine by HPLC with Post-Column Reaction', Journal of Liquid Chromatography & Related Technologies, 28: 19, 3099 — 3108
To link to this Article: DOI: 10.1080/10826070500295203
URL: http://dx.doi.org/10.1080/10826070500295203

Journal of Liquid Chromatography & Related Technologies®, 28: 3099–3108, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500295203

Fluorescence Analysis for *p*-Hydroxymethamphetamine in Urine by HPLC with Post-Column Reaction

Noriyuki Kato and Susumu Fujita
Scientific Criminal Investigation Laboratory, Kanagawa Prefectural Police Headquarters, Yokohama, Japan

Hiroaki Kubo and Hiroshi Homma
School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan

Abstract: A high selectivity analytical method for *p*-hydroxymethamphetamine (pOHMA) in urine is presented. After extraction, the analyte was analyzed by high performance liquid chromatography with post-column oxidation using potassium hexacyanoferrate(III) and sodium hydroxide to detect the fluorophor of pOHMA. The recovery of pOHMA is 86.5%, and the calibration curve for pOHMA is linear in the range of 5–75 μg/mL ($r^2 = 0.997$). The coefficients of variation determined for spiked samples are 2.2% for 10 μg/mL and 2.3% for 5 μg/mL pOHMA (each $n = 5$), and the detection limit for pOHMA is 1 μg/mL (20 ng; S/N = 3). Analysis for pOHMA in forensic samples is performed successfully, without interference from endogenous fluorophors, yielding concentrations in the appropriate range for methamphetamine abusers.

Keywords: Fluorescence analysis, Fluorophor, Forensic science, HPLC, *p*-Hydroxymethamphetamine, Urine

Address correspondence to Noriyuki Kato, Scientific Criminal Investigation Laboratory, Kanagawa Prefectural Police Headquarters, 155-1 Yamashita-cho, Naka-ku, Yokohama 231-0023, Japan. E-mail: n-katoh@gd5.so-net.ne.jp

INTRODUCTION

Methamphetamine (MAMP) is a stimulant of a sympathomimetic amine and is strictly controlled under Japanese law as an illegal drug. MAMP is the most widely abused drug in Japan, and is increasingly represented in criminal proceedings.[1] MAMP is metabolized in the liver after intake and is excreted mainly as amphetamine (AMP), *p*-hydroxymethamphetamine (pOHMA), and *p*-hydroxyamphetamine (pOHAP) (Fig. 1), in addition to free MAMP. In forensic laboratories, MAMP and AMP in urine are analyzed simultaneously by a routine procedure. However, testing for pOHMA should also be conducted to ensure the reliability of the results because AMP is the famous illegal drug in USA and Europe, and it is possible to criminally smuggle it into Japan. Several analytical methods for pOHMA have been described,[2–17] including methods based on high performance liquid chromatography (HPLC). Although the analysis of trifluoroacetyl or heptafluorobutyl derivatives of pOHMA by gas chromatography or other fluorescent derivatization by electrophoresis allow for the detection of pOHMA with high sensitivity, good separation, and short analytical time, these methods pose reliability problems with respect to the use of unstable derivatives and potential sensitivity to ultraviolet (UV) light. HPLC has been widely used to analyze polar substance and substances that are unsuitable for chemiluminescence or fluorescence detection due to instability upon heating or change in pH. However, HPLC usually requires large sample volumes, and involves tedious extraction and pre-derivatization procedures using reagents. Therefore, a simple yet sensitive and specific HPLC method is needed for the reliable determination of pOHMA.

Para-substituted phenolic compounds are known to yield the 2,2′-dihydroxydiphenyl derivative as a fluorophor by oxidative treatment with hexacyanoferrate(III) under alkaline conditions or with peroxidase and hydrogen peroxide.[18,19] However, there are no reported HPLC methods for the separation and detection of the fluorophor formed from pOHMA. In this study, a new forensic analytical method based on HPLC was developed for the selective and reliable detection of pOHMA. In this method, pOHMA is post-column derivatized to the fluorophor with hexacyanoferrate(III) in an alkaline medium. The proposed method is successfully used to test for pOHMA in urine.

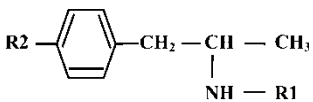

Figure 1. Chemical structures of methamphetamine and its metabolites. R1 = CH_3, R2 = H: methamphetamine; R1 = H, R2 = H: amphetamine; R1 = CH_3, R2 = OH: *p*-hydroxymethamphetamine; R1 = H, R2 = OH: *p*-hydroxyamphetamine.

EXPERIMENTAL

Materials and Reagents

pOHMA was purchased from Sigma-Aldrich (California, USA). A standard stock solution of 0.10 mg/mL pOHMA in water was prepared in advance and stored in a refrigerator at −29°C. pOHAP was synthesized by the procedure reported by Buzas et al.[20]

The Bond Elut C_{18} cartridge (100 mg/1 cc) used in the analyses was purchased from GL Science (Tokyo, Japan) and activated prior to use with 10 mL of methanol and 10 mL of water. The Oasis MCX cartridge (30 mg/1 cc) used was purchased from Nihon Waters (Tokyo, Japan) and activated prior to use by the addition of 10 mL of 2% ammonia-methanol, 10 mL of methanol, and 30 mL of water, sequentially.

Potassium hexacyanoferrate(III) was obtained from Kanto Kagaku (Tokyo, Japan). All other chemicals used were of analytical grade.

Apparatus

A schematic diagram of the system is shown in Fig. 2. The HPLC system consists of two HPLC pumps (LC-10AD$_{VP}$ and LC-10AD; Shimadzu, Kyoto, Japan), equipped with a 20 μL sample loop for the injection, a fluorescence detector (RF-10A XL; Shimadzu) with cell temperature controller (25°C) set at 320 nm for excitation and 405 nm for emission, an analytical column (Mightysil RP-18 GP; Kanto Kagaku; 150 × 3.0 mm ID, 3.0 μm particle size), an oven (RE-8010; Tosoh, Tokyo, Japan), and a data recorder (D-2000 chromato-integrator; Hitachi, Tokyo, Japan). The column temperature was left at ambient (approx. 25°C), and the oven temperature was set at 95°C to yield the fluorophor of pOHMA by reaction with the derivatization reagents in a stainless steel coil (13.3 m × 0.25 mm ID) with 10 clockwise and 10 anticlockwise turns. The mobile phase, consisting of 5% acetonitrile in

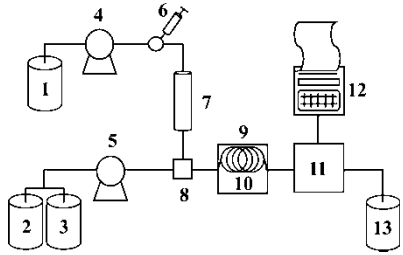

Figure 2. Schematic diagram of proposed HPLC system. 1: Reservoir (eluent), 2–3: reservoir (post-column reagent), 4–5: pumps, 6: injector, 7: separation column, 8: mixing device, 9: oven, 10: reaction coil, 11: fluorescence detector, 12: integrator, 13: waste.

25 mM phosphate buffer (pH 6.0), was supplied at a flow rate of 0.50 mL/min. The post-column reagents, 4 mM potassium hexacyanoferrate(III) and 0.2 M sodium hydroxide, were delivered separately at a flow rate of 0.125 mL/min. Thus, the total flow rate was 0.75 mL/min. Potassium hexacyanoferrate (III) and sodium hydroxide produce a yellow sediment after lengthy mixing. The pumps for the reaction reagents were separated to avoid mixing reagents during long analytical runs. The fluorescence detector and pump used to deliver the post-column reagent were washed with 6.5 M nitric acid after use.

Urine Samples

The urine samples (2 mL) analyzed in this study were samples obtained from normal subjects and forensic urine samples (residue), with MAMP and AMP removed for forensic analysis by extraction with 0.5 mL of n-hexane from samples treated with 0.2 mL of 2.5 M sodium hydroxide.

Optimization of Fluorophor Formation in Flow Injection Analysis and HPLC

The optimal reaction time for the formation of the fluorophor of pOHMA was explored by varying the length of the reaction coil (0.5–13.3 m), the reaction temperature (40–100°C), the flow rate of the mobile phase (0.1–0.5 mL/min), the pH of the alkaline medium (7.5–11.5), and the concentration of potassium hexacyanoferrate(III) (1–10 mM). The reaction temperature in flow injection analysis (FIA) was examined using the longest reaction coil (13.3 m), and the separation of pOHMA, pOHAP, and endogenous fluorophor was examined using a mobile phase consisting of 25 mM phosphate buffer and acetonitrile. Post-column reaction was employed in this experiment because pre-column methods are unable to discriminate between the S and R enantiomers or between similar structures of pOHMA and pOHAP. The post-column reagents were set at each 1/4 of the volume (total 1/2 volume) of the mobile phase.

Extraction of pOHMA

pOHMA extraction was attempted by solid-phase extraction (SPE) using the C_{18} or MCX cartridge and by liquid-liquid extraction (LLE) with acetonitrile,[21] to determine which methods provide the best removal of endogenous fluorophors in urine samples. These extractions were examined using the absolute calibration curve method for FIA. The procedures for SPE are essentially identical to those described previously (C_{18}[3] and

MCX[4]). Briefly, the urine sample (2 mL) was transferred to the activated C_{18} or MCX cartridge. The C_{18} cartridge was washed with 10 mL of water, 10 mL of 5% methanol, and 4 mL of acetonitrile, sequentially, after which the compounds retained in the cartridges were eluted with 3 mL of a mixture of acetonitrile and 1 M HCl (9 : 1 v/v). The MCX cartridge was washed with 2 mL of water and 10 mL of methanol, sequentially, and the retained compounds were subsequently eluted with 3 mL of 2% ammonia-methanol. For LLE with acetonitrile, 0.4 g of sodium carbonate was added to a 2 mL urine sample, followed by the addition of 2 mL of acetonitrile and vigorous mixing for a few seconds on a vortex-mixer. The mixture was then centrifuged at 3000 rpm for 10 min, and the supernatant (acetonitrile phase) was separated from the aqueous phase. This procedure was repeated three times and the acetonitrile solutions were combined. The final combined solution was evaporated to dryness under reduced pressure. The mobile phase of 0.50 mL was added to the residue, and 20 μL of the solution were injected to the FIA or HPLC to determine the recovery of pOHMA. LLE was carried out with acetonitrile rather than a mixture of chloroform and isopropanol, as the method used here is less toxic and considered more useful.

RESULTS AND DISCUSSION

Determination of Optimum Conditions for Fluorophor Formation

The fluorophor formation of pOHMA was investigated with 10 μg/mL of pOHMA standard solution. The effects of reaction temperature, pH of the alkaline medium, and concentration of potassium hexacyanoferrate(III) are summarized in Fig. 3. Increasing the temperature improved the fluorescence intensity to a maximum at temperatures over 90°C (Fig. 3(A)). To obtain reproducible results, the reaction temperature was, therefore, set at 95°C. Similarly, the fluorescence intensity reached an optimal level at pH greater than 10.5 (Fig. 3(B)). A potassium hexacyanoferrate(III) concentration of 4 mM yielded the highest fluorescence intensity (Fig. 3(C)), and this concentration was used in the main analyses.

Increasing the reaction time by increasing the coil length and reducing the flow rate also improved the fluorescence intensity, although the peak became slightly broader with increasing reaction time (data not shown). The highest fluorescence intensity was obtained using a 13.3 m reaction coil, and a flow rate of 0.50 mL/min for the mobile phase and 0.125 mL/min for each of the post-column reagents, corresponding to a reaction time of 77 s.

The separation of pOHAP and pOHMA was achieved using the mobile phase (5% acetonitrile in 25 mM phosphate buffer, pH 6.0) with a retention time of 8.0 and 9.0 min, respectively. Although PMAP and AMP of concentration from 1 to 500 μg/mL were analyzed for 30 minutes by this method, the fluorescence peaks were not confirmed on the chromatogram.

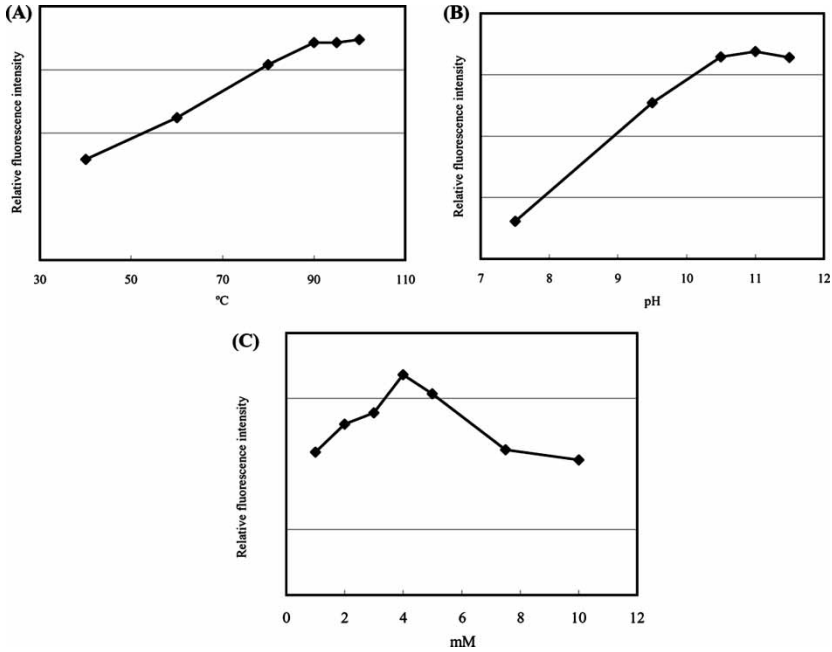

Figure 3. Effects of (A) reaction temperature, (B) pH, and (C) concentration of potassium hexacyanoferrate(III) on post-column fluorogenic derivatization of pOHMA.

Linearity, and Limits of Detection and Quantitation

The linearity of the method was evaluated by pOHMA standard solution at nine non-zero concentrations over the range of 0.1–100 μg/mL. A linear regression analysis of the calibration curves, which were obtained in the range 5–75 μg/mL yielded the equation $Y = 14237X - 86774$ ($r^2 = 0.997$) for pOHMA. The fluorescence intensity, peak height (Y), was related to the μg/mL (X) with high linearity. The limits of detection (LOD) (S/N = 3) and quantitation (LOQ) (S/N = 20) were 1 and 5 μg/mL for pOHMA, respectively.

Recovery from Sample

As shown in Table 1, SPE using the MCX cartridge and LLE with acetonitrile achieved high recovery rates for pOHMA (90.3 ± 3.5 and 93.7 ± 3.6%, respectively) by FIA, whereas SPE with the C_{18} cartridge gave very limited recovery (51.2 ± 1.3%). However, as endogenous fluorophors in the urine samples could not be eliminated by extraction with the MCX cartridge and subsequent HPLC analysis with acetonitrile, LLE extraction with acetonitrile was combined with SPE using the MCX cartridge to ensure complete removal

Table 1. Absolute recoveries of pOHMA from 10 μg/mL of spiked urine

	Extraction	Absolute recovery (mean % ± S.D.)
SPE[a]	Bond Elut C18	51.2 ± 1.3
	Oasis MCX	90.3 ± 3.5
LLE[b]	Acetonitrile	93.7 ± 3.6

Note: n = 4.
[a]SPE = Solid phase extraction; [b]LLE = liquid–liquid extraction.

of endogenous fluorophors. In this combined process, after the virgin urine samples were subjected to LLE with acetonitrile, the pH of the extract was adjusted with an equal volume of diluted HCl or phosphate buffer. The adjusted solutions were then applied to the activated MCX cartridge for extraction by the procedure outlined above. As shown in Table 2, the recovery of 10 μg/mL of pOHMA by this process was highest (86.5 ± 3.1%) for samples adjusted to pH 6.0 prior to injection onto the MCX cartridge. Under these conditions, a clear peak was produced on the chromatogram without any interference or overlap from other fluorophors (Fig. 4(B)).

Precision

The coefficient of variation (C.V.) for pOHMA in spiked urine samples was 2.2% for 10 μg/mL and 2.3% for 5 μg/mL (each $n = 5$). With these data, the same concentration of spiked samples was carried out intra-day and different concentrations carried out inter-day.

Analysis of Forensic Samples

pOHMA and pOHAP in two forensic samples of MAMP suspects determined by the proposed method were investigated. The result of one of the MAMP

Table 2. Effect of pH on recovery of pOHMA from 10 μg/mL of spiked urine

	pH (mean % ± S.D.)			
	1.5	4	6	7.8
AcCN-MCX	27.40.5	71.9 ± 9.7	86.5 ± 3.1	40.6 ± 1.1

Note: n = 3.

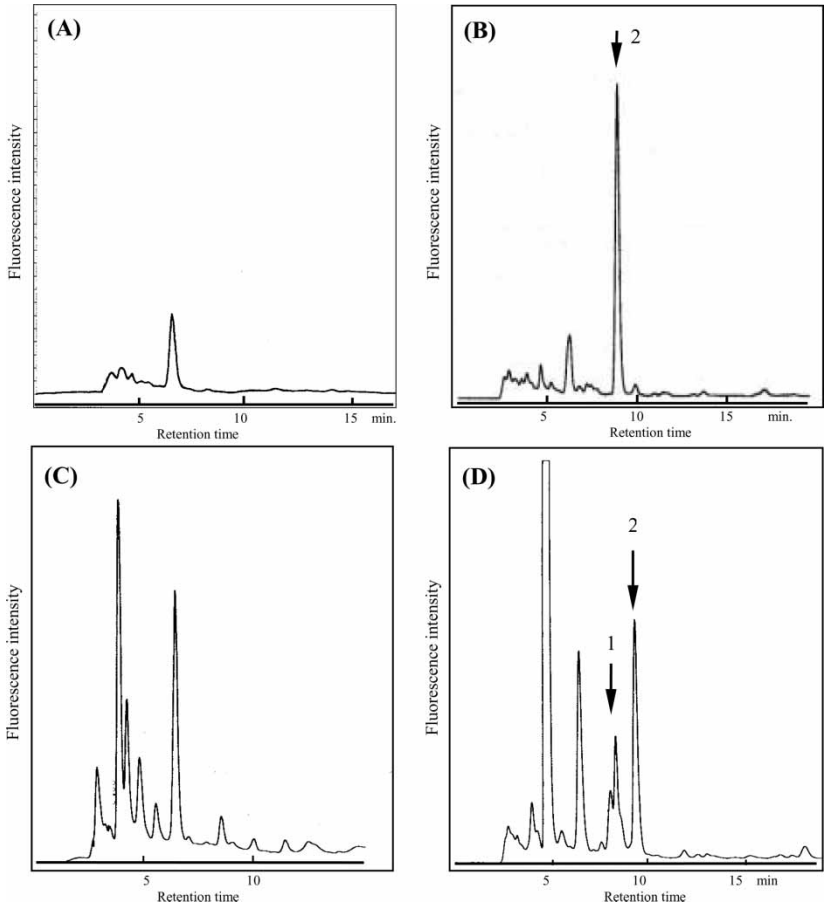

Figure 4. Chromatograms of normal and one of the forensic urine samples. A: Blank urine sample. B: Normal sample spiked with pOHMA. C: Forensic sample without post-column reaction. D: Forensic sample after post-column reaction. pOHMA concentration of this sample is 3.0 μg/mL. pOHAP and pOHMA peaks are indicated by arrows 1 and 2, respectively.

suspects is shown in Figs. 4(C) and (D). After extraction by the proposed method, the extract was dried under low pressure and, then, to the residue, was added 0.250 μL of the mobile phase due to bending of the calibration curve in the range of 5–75 μg/mL. This solution was concentrated 8X in comparison with 2 mL of forensic urine. In Fig. 4(C), the peaks of pOHMA and pOHAP did not appear on the chromatogram. pOHMA was detected as a clear symmetric peak without any interference from endogenous fluorophors, while the peak due to pOHAP was partially overlapped by endogenous interference (Fig. 4(D)). The concentrations of pOHMA in these samples,

determined by the absolute calibration curve method, were 1.2 and 3.0 μg/mL (7.39×10^{-6} and 1.82×10^{-5} M), respectively. These results are similar to those obtained for unconjugated pOHMA in the urine of MAMP abusers, as reported by Hayakawa et al.[10] Thus, the proposed HPLC technique appears to be viable as a simple forensic analysis for pOHMA.

CONCLUSION

A new analytical method for the quantitative detection of pOHMA as a fluorophor without endogenous interference, and with high selectivity and qualitative detection of pOHAP was proposed. The HPLC based technique is relatively easy and inexpensive, and was shown to be capable of detecting indicative levels of pOHMA in urine. Although this method is unable to discriminate between S and R enantiomers, it is readily applicable to the detection of para-substituted phenolic compounds in urine, irrespective of endogenous or exogenous origin.

REFERENCES

1. National Police Agency. *White paper on Police (in Japanese)*; Police Associations: Tokyo, Japan, 2003; 60.
2. Moffat, A.C.; Osselton, M.D.; Widdop, B. *Clarke's Analysis of Drugs and Poisons*, 3rd ed; Pharmaceutical Press: London, 2004; 1448.
3. Shimosato, K.; Tomita, M.; Ijiri, I. Urinary excretion of *p*-hydroxylated methamphetamine metabolites in man. Arch. Toxicol. **1986**, *59*, 135–140.
4. Katagi, M.; Nishioka, H.; Nakajima, K.; Tsuchihashi, H.; Fujima, H.; Wada, H.; Nakamura, K.; Makino, K. Direct high performance liquid chromatographic and high performance liquid chromatographic-thermospray-mass spectrometric determination of enantiomers of methamphetamine and its main metabolites, amphetamine and *p*-hydroxymethamphetamine in human urine. J. Chromatogr. B **1996**, *676*, 35–43.
5. Al-Dirbashi, O.; Kuroda, N.; Menichini, F.; Noda, S.; Minemoto, M.; Nakashima, K. Enantioselective high performance liquid chromatography with fluorescence detection of methamphetamine and its metabolites in human urine. Analyst **1998**, *123*, 2333–2337.
6. Peinhardt, G.P. High performance liquid chromatographic determination of pholedrine in human serum using ion-pair extraction and amperometric detection. J. Chromatogr. B **1999**, *726*, 309–312.
7. Nagai, T.; Kamiyama, S. Simultaneous HPLC analysis of optical isomers of methamphetamine and its metabolites, and stereoselective metabolism of racemic methamphetamine in rat urine. J. Anal. Toxicol. **1991**, *15* (6), 299–304.
8. Sukbuntherng, J.; Hutchaleelaha, A.; Chow, H.H.; Mayersohn, M. Separation and quantitation of the enantiomers of methamphetamine and its metabolites in urine by HPLC: precolumn derivatization and fluorescence detection. J. Anal. Toxicol. **1995**, *19* (3), 139–147.

9. Cheung, S.; Nolte, H.; Otton, S.V.; Tyndale, R.F.; Wu, P.H.; Sellers, E.M. Simultaneous gas chromatographic determination of methamphetamine, amphetamine, and their *p*-hydroxylated metabolites in plasma and urine. J. Chromatogr. B **1997**, *690*, 77–87.
10. Hayakawa, K.; Miyoshi, Y.; Kurimoto, H.; Matsushima, Y.; Takayama, N.; Tanaka, S.; Miyazaki, M. Simultaneous determination of methamphetamine and its metabolites in the urine samples of abusers by high performance liquid chromatography with chemiluminescence detection. Biol. Pharm. Bull. **1993**, *16* (9), 817–821.
11. Romhild, W.; Krause, D.; Bartels, H.; Ghanem, A.; Schoning, R.; Wittig, H. LC-MS/MS analysis of pholedrine in a fatal intoxication case. Forensic Sci. Int. **2003**, *133* (1–2), 101–106.
12. Iio, R.; Chinaka, S.; Tanaka, S.; Takayama, N.; Hayakawa, K. Simultaneous chiral determination of methamphetamine and its metabolites in urine by capillary electrophoresis-mass spectrometry. Analyst **2003**, *128* (6), 646.
13. Kuroda, N.; Nomura, R.; Al-Dirbashi, O.; Akiyama, S.; Nakashima, K. Determination of methamphetamine and related compounds by capillary electrophoresis with UV and laser-induced fluorescence detection. J. Chromatogr. A **1998**, *798* (1–2), 325–334.
14. Hara, K.; Kashimura, S.; Hieda, Y.; Kageura, M. Simple extractive derivatization of methamphetamine and its metabolites in biological materials with Extrelut columns for their GC-MS determination. J. Anal. Toxicol. **1997**, *21* (1), 54–58.
15. Yamamoto, T.; Takano, R.; Egashira, T.; Yamanaka, Y.; Terada, M. Simultaneous determination of methamphetamine and its metabolites in monkey urine by gas chromatography/mass spectrometry with selected ion monitoring. J. Anal. Toxicol. **1989**, *13* (2), 117–119.
16. Nakahara, Y.; Hanajiri, R. Hair analysis for drugs of abuse XXI: Effect of para-substituents on benzene ring of methamphetamine on drug incorporation into rat hair. Life Sci. **2000**, *66* (7), 563–574.
17. Peters, F.T.; Schaefer, S.; Staack, R.F.; Kraemer, T.; Maurer, H.H. Screening for and validated quantification of amphetamines and of amphetamine- and piperazine-derived designer drugs in human blood plasma by gas chromatography/mass spectrometry. J. Mass Spectrom. **2003**, *38* (6), 659–676.
18. Sato, T.L. The quantitative determination of 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) in urine. J. Lab. Clin. Med. **1965**, *66*, 517–525.
19. Zaitsu, K.; Ohkura, Y. New fluorogenic substrates for horseradish peroxidase: Rapid and sensitive assays for hydrogen peroxide and the peroxidase. Anal. Biochem. **1980**, *109*, 109–113.
20. Buzas, A.; Dufour, C. Preparation of [2-(p-hydroxyphenyl) isopropyl] methylamine. Bull. Soc. Chim. **1950**, 139–140.
21. Yoshida, M.; Akane, A.; Nishikawa, M.; Watabiki, T.; Tsuchihashi, H. Extraction of thiamylal in serum using hydrophilic acetonitrile with subzero-temperature and salting-out methods. Anal. Chem. **2004**, *76*, 4672–4675.

Received April 8, 2005
Accepted June 30, 2005
Manuscript 6618