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Determination of Methamphetamine in Human Urine by HPLC Using Post-Column Simon's Reaction

Satoshi Chinaka,*[,a] Seishi Tanaka,[a] Nariaki Takayama,[a] and Kazumasa Ueda[b]

[a]*Forensic Science Laboratory, Ishikawa Prefectural Police Headquarters, 2—1—1 Hirosaka, Kanazawa 920—8553, Japan and* [b]*Department of Chemistry and Chemical Engineering, Faculty of Engineering, Kanazawa University, 2—40 Kodatsuno, Kanazawa 920—8667, Japan*

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An HPLC method using post-column Simon's reaction (SR-HPLC) for the determination of methamphetamine (MA) has been developed. The detection limit was 1 ng, which is about 100 times more sensitive than TLC using Simon's reaction (SR-TLC). The calibration curve was linear in the range of 0.1—1000 μg/ml, and the intermediate precision of within-run and between-run assays was 1.15% and 2.12%, respectively. MA in 13 urine samples was determined by SR-HPLC and GC with flame ionization detection, and these quantitative values were strongly correlated (r^2=0.995). MA could be analyzed without interference peaks. The proposed method is highly selective and quantitative.

Key words —— methamphetamine, sodium nitroprusside, Simon's reaction, HPLC, urine

INTRODUCTION

Analysis of stimulants is an increasingly important function of the Forensic Science Laboratories (FSLs) of the Japanese Police. Stimulants that are controlled by the Stimulant Drug Control Law of Japan are methamphetamine (MA) and amphetamine (AP). MA is used by most addicts, and AP is detected in urine and hair samples as its major metabolite. Several methods have been reported for the analysis of MA and related compounds in urine and hair samples. In these methods, TLC using Simon's reaction (SR-TLC) is selective for MA,[1] GC/MS is sensitive and highly selective,[1,2] and HPLC using chemiluminescence detection is the most sensitive.[3,4] To confirm the use of MA, metropolitan and prefectural FSLs routinely analyze urine samples using a combination of SR-TLC and GC/MS methods. SR-TLC demonstrates the presence of MA, and GC/MS detects MA and AP after trifluoroacetic (TFA) derivatization.

Simon's reaction is a color reaction that produces a blue color by mixing a secondary aliphatic amine, acetaldehyde, and sodium nitroprusside (SNP) under carbonate alkaline conditions,[5] and is extremely sensitive to MA.[6] On the other hand, benzphetamine (BZP), a legal anorexic drug, was biotransformed into MA (both the illegal MA and the BZP product are S(+)-enantiomers), and SR-TLC has been reported as an easy method to detect the major metabolite of BZP, *p*-hydroxydesmethyl benzphetamine (OHnorBZP).[7,8] These results suggested that SR-TLC might distinguish MA from BZP. However, the detection limit of MA of SR-TLC is more than 100 times higher than that of GC/MS. In this report, we proposed a highly sensitive HPLC method using Simon's reaction as a post-column reaction (SR-HPLC) for the simultaneous determination of MA and OHnorBZP.

*To whom correspondence should be addressed: Forensic Science Laboratory, Ishikawa Prefectural Police Headquarters, 2—1—1 Hirosaka, Kanazawa 920—8553, Japan. Tel.: +81-76-262-1161; Fax: +81-76-265-8566

MATERIALS AND METHODS

Materials —— MA hydrochloride was obtained from Dainippon Pharmaceutical (Osaka, Japan). OHnorBZP was provided by the Forensic Science Laboratory, Osaka Prefectural Police Headquarters. Acetonitrile was of HPLC grade (Wako Pure Chemical Industries, Tokyo, Japan). SNP, ammonium acetate, and sodium carbonate were of analytical reagent grade (Wako). Acetaldehyde was of chemical grade (Wako). All other reagents used were of analytical reagent grade. Deionized, distilled water was used for all the procedures.

Urine Samples —— Urine samples were taken from MA users arrested by the Ishikawa Prefectural Police

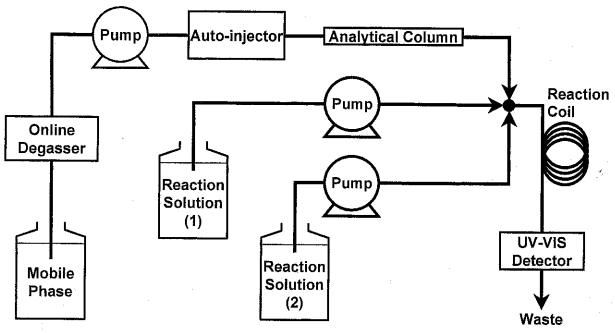

Fig. 1. SR-HPLC System

under "Voluntary Presentation". A control urine sample was collected from a healthy individual who had not taken any legal or illegal drugs. Urine samples were stored at +4℃ for 1—10 days until analysis.

SR-HPLC System and Conditions —— The SR-HPLC system was based on a Shimadzu (Kyoto, Japan) HPLC system (Fig. 1) consisting of three LC-10AD pumps, a DGU-3A on-line degasser, an SIL-10A auto-injector, and an SPD-10AV ultraviolet-visible detector, using a detection wavelength of 590 nm. The system was controlled by an FMV-5120D5 computer (Fujitsu, Tokyo, Japan), a CBM-10A communications bus module and CLASS-LC10 software (Shimadzu). An L-column ODS (150 × 4.6 mm i.d., Chemicals Inspection and Testing Institute, Tokyo, Japan) was used as an analytical column, and the eluent was 20% acetonitrile–20 mM ammonium acetate. The column temperature was ambient and the flow rate was 1.0 ml/min. Two SR-reaction solutions were mixed with the mobile phase from the analytical column using a 4-way connector. The reaction solutions were 100 mM sodium carbonate solution and 0.25% SNP–10% acetaldehyde, and the flow rates were 0.25 and 0.75 ml/min, respectively. The reaction coil was 0.5 mm i.d. × 10 m. The sample injection volume was 20 μl.

Pretreatment of Urine Samples —— To 10 ml of urine in a tube, 1 ml of 10% sodium carbonate solution and 10 ml of chloroform – iso-propanol (3 : 1, v/v) were added, and the mixture was shaken vigorously for 2 min. After centrifugation (1000 *g*, 10 min), the organic phase was transferred. This procedure was repeated two more times. All the organic phases were combined and dehydrated with about 1 g of sodium sulfate and decanted. After the addition of several drops of acetic acid, the solution was dried in a stream of nitrogen, and the residue was dissolved in 0.5 ml of methanol.[9] A 20 μl aliquot of the methanol solution was subjected to the SR-HPLC system.

GC for the Determination of MA in Urine —— The GC instrument consists of an HP5890 SERIES II gas chromatograph with hydrogen flame ionization (Yokogawa-Hewlett Packard, Tokyo, Japan), a DB-1 capillary column (0.25 mm i.d. × 30 m, J&W Scientific, Folsom, CA, U.S.A.) and an HP ChemStation analysis system (Hewlett Packard). The injection and detector temperatures were 150℃ and 250℃, respectively. The initial temperature of the column oven was 80℃ (6 min constant) and the final was 200℃ (20℃/min). The above-mentioned methanol extract was derivatized with TFA as follows: 0.2 ml each of ethyl acetate and TFA anhydride were added to 0.1 ml of the extract, and the mixture was incubated at 55℃ for 15 min. After drying under a flow of nitrogen, the residue was dissolved in ethyl acetate containing diphenylmethane as an internal standard. A 1 μl aliquot of the solution was subjected to the GC system.

RESULTS AND DISCUSSION

Effect of pH on Simon's Reaction

The reagents of Simon's reaction are sodium carbonate solution and a mixture of acetaldehyde and SNP. The effect of pH (carbonate concentration) was examined. A 0.1 ml aliquot of 2 mg/ml MA standard solution was placed in a tube, and 2 ml of sodium carbonate and/or sodium bicarbonate solution ranging from pH 8.4 (25 mM sodium bicarbonate) to pH 11.3 (100 mM sodium carbonate) and 1 ml of 0.5% SNP–10% acetaldehyde were added. After mixing, the absorbance was measured by a UV-1600PC spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 580 nm. The results are shown in Fig. 2A. As the pH increased, the absorbance increased and the reaction time decreased. At pH values lower than 10, maximum absorbance was reached within a few minutes, but at pH 11.3 the reaction was instantaneous. The higher sodium carbonate concentration (>100 mM) produced a slight increase in pH. Thus, pH 11.3 (100 mM sodium carbonate) was selected.

Effect of the Mobile Phase of HPLC on Simon's Reaction

The effects of acetonitrile and methanol, which are commonly used in the mobile phases of HPLC, were examined. In this experiment, 0.1 ml of 2 mg/ml MA standard solution, 2 ml of various concentrations of acetonitrile or methanol, 1 ml

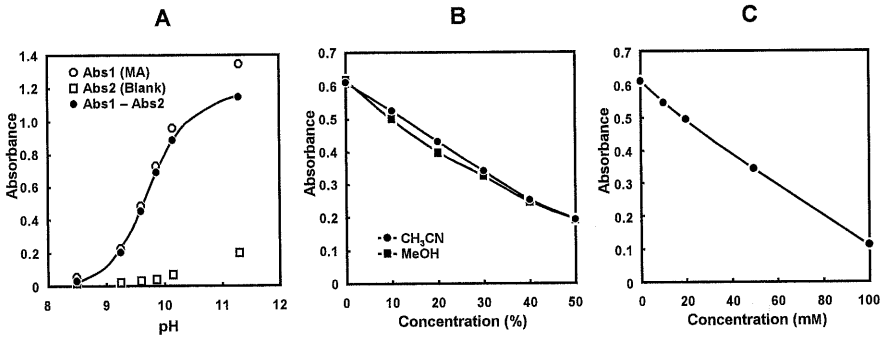

Fig. 2. Effects of (A) pH, (B) Organic Solvent Concentrations and (C) Ammonium Acetate Concentration on the Absorbance of MA by Simon's Reaction

of 100 mM sodium carbonate, and 1 ml of 0.5% SNP–10% acetaldehyde were mixed, and the absorbance of the mixture was measured immediately. The results are shown in Fig. 2B. For both acetonitrile and methanol, as the concentrations increased, the absorbance decreased in a linear fashion. The effect of the concentration of ammonium acetate as the buffer in the mobile phase was also examined, and the results are shown in Fig. 2C. As with the organic solvents, the absorbance decreased linearly with increasing ammonium acetate concentration.

HPLC Conditions for the Separation of MA and OHnorBZP

Acetonitrile or methanol and ammonium acetate concentrations were varied to find the mobile phase that would allow the simultaneous determination of MA and OHnorBZP at the lowest possible concentrations. The optimization was carried out using an ODS analytical column (150 × 4.6 mm i.d.) at a flow rate of 1.0 ml/min and ambient column temperature. The selected mobile phase was 20% acetonitrile–20 mM ammonium acetate.

Mixing Procedure of Post-Column Reagent

Two ways of mixing the mobile phase with the two post-column reagents were examined. In one method, one 3-way connector combined the mobile phase with one reagent and a second 3-way connector combined this mixture with the second reagent. In the other method (as shown in Fig. 1) a single 4-way connector was used. The results of both mixing methods were the same, therefore, the 4-way connector, which was simpler, was adopted.

Effect of Reaction Coil Length and Temperature

For these experiments, the post-column reagents were 100 mM sodium carbonate and 0.5% SNP–10% acetaldehyde, and the flow rates were 0.25 ml/min each. The inside diameter of the coil was 0.5 mm, which offered better performance for both the reactivity and peak resolution. Under these conditions, the coil lengths and temperatures which gave maximum MA peak areas were 10—20 m (Fig. 3A) and 10—20℃ (Fig. 3B),

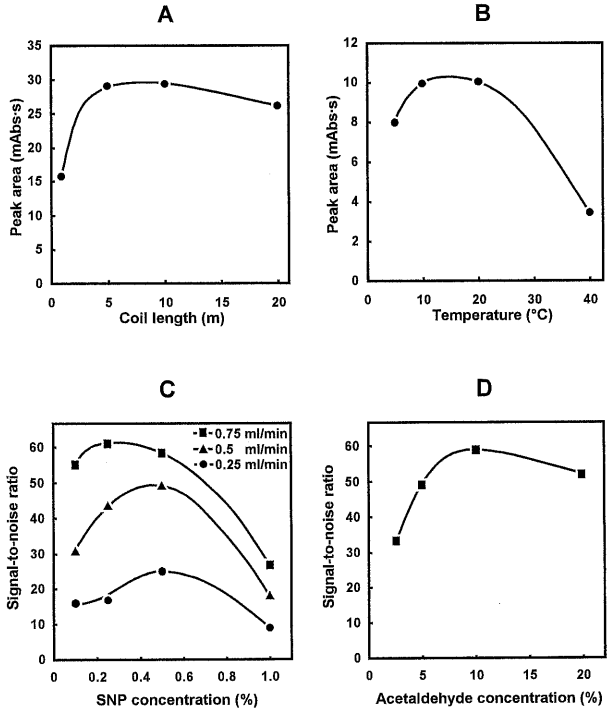

Fig. 3. Effects of (A) the Reaction Coil Length and (B) the Reaction Temperature on the Peak Area of MA by SR-HPLC

Effects of (C) the Concentration and Flow Rate of Sodium Nitroprusside and (D) Acetaldehyde Concentration on the Signal-to-Noise Ratio for the Peak of MA by SR-HPLC

respectively. Therefore, a coil length of 10 m, which provided better resolution, and room temperature (18 ± 2℃) were adopted.

Effect of Concentrations and Flow Rates of SNP and Acetaldehyde

The effects of SNP concentration and the flow rate on the signal-to-noise ratio of 1 μg/ml MA are shown in Fig. 3C. The combination of 0.25% SNP and a flow rate of 0.75 ml/min gave the highest signal-to-noise ratio. The effect of acetaldehyde concentration is shown in Fig. 3D. The maximum signal-to-noise ratio occurred at an acetaldehyde concentration of 10%. Therefore, 0.25% SNP–10% acetaldehyde (0.75 ml/min) was selected as one of the two Simon's reagents.

The Chromatogram of MA and OHnorBZP Mixed Standard Solution

The chromatogram of a standard solution containing MA and OHnorBZP (Fig. 4A) shows two prominent peaks at retention times of 9.4 and 21.3 min, respectively. A minor peak at a retention time of 13.3 min was found to be an impurity present in the synthetic OHnorBZP that was used. The spectra of the MA and OHnorBZP peaks (Fig. 5) showed maximum absorbances at 590 nm and 610 nm, respectively. Accordingly, a wavelength of 590 nm was selected to determine these compounds.

Detection Limits and Calibration Curbs

The detection limits of MA and OHnorBZP, defined as the concentration which produced a signal equal to 3 times the background noise level, were 1 ng and *ca.* 20 ng, respectively. The difference in sensitivity was caused by the difference in reactivity in Simon's reaction. The sensitivity of SR-HPLC to MA was 100 times greater than the sensitivity of SR-TLC to MA.[1] The calibration curve of MA was linear in the range 0.1—1000 μg/ml. The intermediate precision of

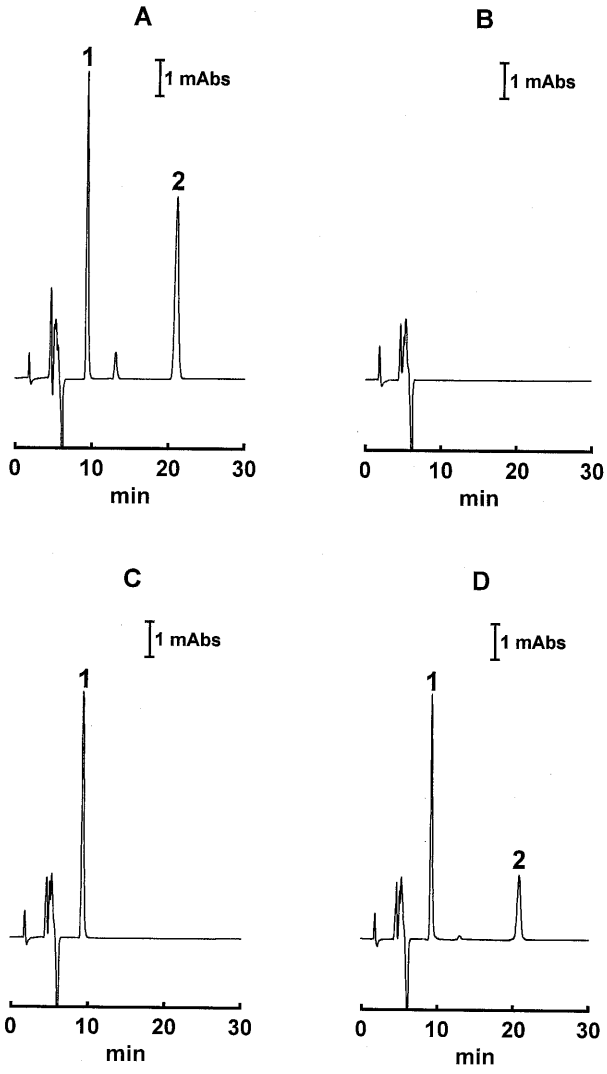

Fig. 4. Chromatograms of (A) Standard Solution, (B) Control Urine Sample and (C, D) Addict's Urine Samples

Peak 1, MA ; peak 2, OHnorBZP. (A) 20 μg/ml MA and *ca.* 200 μg/ml OHnorBZP, (B) no peaks, (C) 17 μg/ml MA, (D) 17 μg/ml MA and *ca.* 86 μg/ml OHnorBZP.

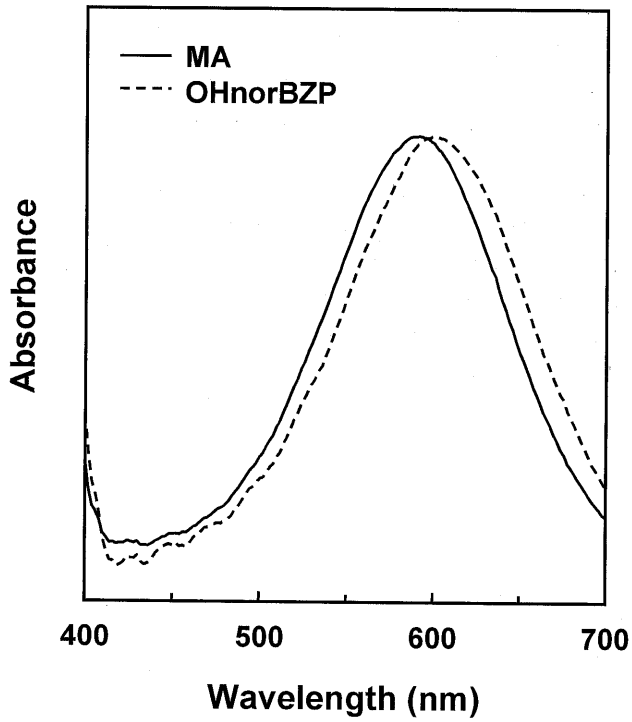

Fig. 5. Spectra of MA and OHnorBZP by SR-HPLC Using a Photodiode Array Detector

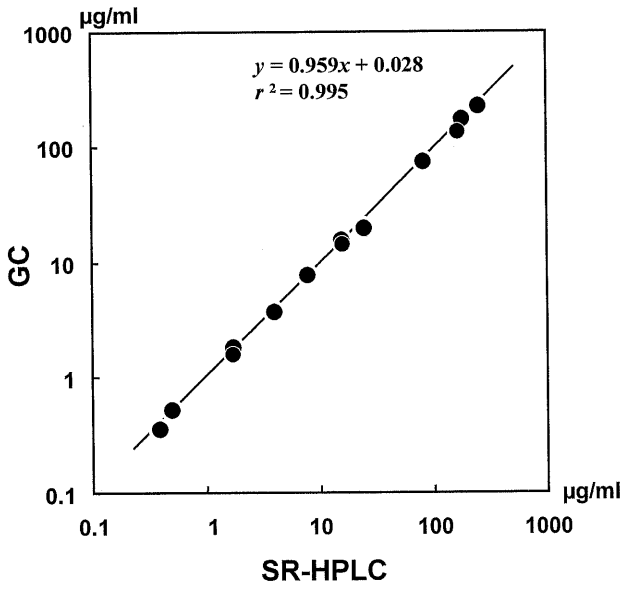

Fig. 6. Correlation between Urine MA Concentrations Measured by SR-HPLC and GC

within-run and between-run assays for 10 μg/ml MA was 1.15% ($n=10$) and 2.12% ($n=12$), respectively. This method gave good reproducibility without the use of an internal standard.

Determination of MA and OHnorBZP in Urine

An HPLC chromatogram of the control urine sample is shown in Fig. 4B. No peaks were found at the retention times of MA (9.5 min) or OHnorBZP (20.8 min). Urine samples from several dozen addicts were analyzed, and in all cases, the MA peaks were detected and were free from interference. Because none of the urine samples contained OHnorBZP, some of them were spiked with OHnorBZP. Typical chromatograms are shown in Figs. 4C and D. It can be seen that the peaks of MA and OHnorBZP are free from interference. MA concentrations in urine samples from 13 addicts were determined by SR-HPLC and GC. As shown in Fig. 6, the values obtained with the two methods were strongly correlated. Thus, SR-HPLC is a selective and quantitative method for the simultaneous determination of MA and OHnorBZP in urine without pre-column derivatization, and is superior to SR-TLC as a routine analysis method at FSLs.

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REFERENCES

1) The Pharmaceutical Society of Japan, "Yaku Dokubutsu Kagaku Shikenho Chukai (Standard Methods of Chemical Analysis in Poisoning — With Commentary)," 4th ed., Nanzando, Tokyo, pp. 287—306, 1992.
2) Suzuki S., Inoue T., Yasuda T., Niwaguchi T., Hori H., Inayama S., *Eiseikagaku*, **30**, 23—26 (1984).
3) Hayakawa K., Hasegawa K., Imaizumi N., Wong O. S., Miyazaki M., *J. Chromatogr.*, **464**, 343—345 (1989).
4) Takayama N., Tanaka S., Kizu R., Hayakaya K., *Jpn. J. Sci. Tech. Iden.*, **3**, 11—15 (1998).
5) Feigel F., Spot Tests in Organic Analysis, 7th ed., Elsevier, Amsterdam, pp. 250—251, 1966.
6) Kishi T., Kozuka H., *Kakeiken-houkoku (Reports of the National Research Institute of Police Science)*, **27**, 22—27 (1974).
7) Niwaguchi T., Inoue T., Suzuki S., *Xenobiotica*, **12**, 617—625 (1982).
8) Inoue T., Suzuki S., *Xenobiotica*, **16**, 691—698 (1986).
9) Inoue T., Niwaguchi T., *Kakeiken-houkoku (Reports of the National Research Institute of Police Science)*, **34**, 142—146 (1981).