ELSEVIER

Journal of Chromatography B, 751 (2001) 177–185

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Column-switching high-performance liquid chromatography–electrospray ionization mass spectrometry for identification of heroin metabolites in human urine

Munehiro Katagi*, Mayumi Nishikawa, Michiaki Tatsuno, Akihiro Miki, Hitoshi Tsuchihashi

Forensic Science Laboratory, Osaka Prefectural Police H.Q., 1-3-18, Hommachi, Chuo-ku, Osaka 541-0053, Japan

Received 8 March 2000; received in revised form 15 August 2000; accepted 15 August 2000

Abstract

In order to prove heroin (DAM) use, a simple, rapid and sensitive analytical method has been established by combining semi-microcolumn HPLC, a column switching technique and electrospray ionization mass spectrometry (ESI-MS). Urine samples were directly introduced to the system, and endogenous urinary constituents were removed by using on-line column switching solid-phase extraction with a strong cation-exchange (SCX) cartridge column (2.0 mm I.D.×10 mm). Heroin and its metabolites enriched on the top of the column were then successfully analyzed with excellent separation by use of a SCX semi-microcolumn (1.5 mm I.D.×150 mm), accompanied by ESI mass spectral detection. The proposed conditions are as follows: mobile phase, 10 m*M* ammonium acetate (pH 6.0)–acetonitrile (30:70, v/v) (for main separation) and 30 m*M* ammonium acetate (for trapping); flow-rates, 120 μl/min (for main separation) and 200 μl/min (for trapping); capillary voltage, +4.5 kV; cone voltage, 50 V. Linear calibration curves were obtained in the selected ion monitoring (SIM) mode using protonated molecular ions (*m*/*z* 370 for DAM, *m*/*z* 328 for MAM and *m*/*z* 286 for MOR) over the concentration ranges from 10 to 1000 ng/ml for morphine (MOR) and 1–100 ng/ml for DAM and 6-acetylmorphine (MAM). The detection limits were 0.1–3 ng/ml. Upon applying the scan mode, 2–30 ng/ml were the detection limits. The present HPLC–ESI-MS method was successfully applied to the determination of opiates in users' urine samples. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Heroin; Column-switching; HPLC–ESI-MS

1. Introduction

Drug abuse is widely considered to be one of the most serious social problems throughout the world. At present, methamphetamine (MA) is the most prevalently abused drug in Japan. Recently, abuse of other street drugs, including heroin (DAM) and amphetamine-derived designer drugs, have also been increasing [1]. DAM was not usually used along with other drugs. However, it is said that the blending of MA and DAM leads to a combination of stimulation and depression that produces a bizarre and much pleasurable effect. Quite recently, this blending is especially used by the young people in Japan.

It is well established that DAM is rapidly metabo-

*Corresponding author. Tel.: +81-6-6268-1234; fax: +81-6-6271-8066.

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(00)00469-2

lized by serum or liver esterases, or spontaneously hydrolyzed to 6-acetylmorphine (MAM) (half-life being approximately 5 min), and that MAM is then further hydrolyzed to morphine which in turn is conjugated to morphine glucuronides (Fig. 1). DAM is rarely detected in urine samples except for those collected immediately after the intake. DAM use is often evidenced by the detection of MOR from suspects' urine samples. However, MOR is also detectable in opium user's urine. Codeine (COD), which is often used as a cough medicine, can also be metabolized to MOR. Thus, in order to prove the use of DAM, it is required to detect MAM, the characteristic metabolite of DAM, in addition to MOR. However, MAM has a short detection time of 2–8 h in urine [2]. Also, a sensitive detection technique is required since the concentration of MAM is often very low compared with that of MOR.

Previously reported identification of MAM and MOR has mainly utilized gas chromatography mass spectrometry (GC–MS), owing to its high specificity and sensitivity, after derivatization of the analytes by acetylation [3–7], propionylation [8,9], trimethylsilylation [4,10], trifluoroacetylation [2–4,11,12], pentafluoropropionylation [3,4,13,14] or heptafluorobutyrylation [3,4]. Such derivatization methods are, however, tedious and time-consuming. High-performance liquid chromatography–mass spectrometry (HPLC–MS) has become increasingly utilized to confirm and quantitate analytes for forensic purposes [15–25], including DAM and its metabolites [26–28]. This is mainly due to its applicability to polar and/or thermolabile compounds without tedious derivatization. However, biological samples such as urine and serum have to be prepared prior to analysis. Column switching on-line solid-phase extraction (SPE) has been adopted to directly analyze biological and environmental samples [29–36] with an enhanced sensitivity as a highly automated methodology, though this approach has rarely been utilized in the forensic field [19,33,34].

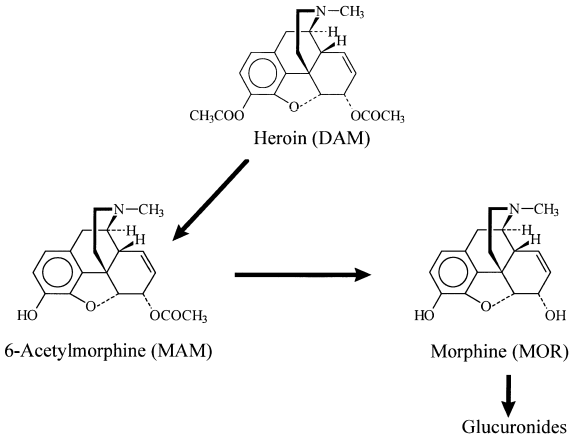

Fig. 1. Main metabolic pathway for heroin in human.

This study aims to construct an on-line analytical system to simultaneously determine DAM and its metabolites in urine by combining column switching on-line SPE and HPLC–electrospray ionization mass spectrometry (HPLC–ESI-MS).

2. Experimental

2.1. Materials

Morphine (MOR) hydrochloride was obtained from Takeda (Osaka, Japan); codeine (COD) phosphate and dihydrocodeine (DHC) phosphate from Sankyo (Tokyo, Japan). Heroin (DAM) hydrochloride was synthesized from MOR with acetic anhydride, and 6-acetylmorphine (MAM) following the method of Fehn et al. [14] in our laboratory. Stock standard solutions of DAM and MAM were prepared in methanol and those of MOR in distilled water (1 mg/ml). They were stored below 0°C and diluted to appropriate concentrations with distilled water or human urine immediately prior to the use. Acetonitrile was of HPLC grade from Wako pure Chemical Industries (Osaka, Japan), and other chemicals used were of analytical grade.

2.2. Column-switching system

A schematic diagram of the dual-column system constructed for this study is displayed in Fig. 2. The system was based on the NANOSPACE series (Shiseido, Tokyo, Japan). Two 2001 inert pumps, a 2003 auto-sampler, an H-P valve, a CS-300B column oven (Chromato Science, Osaka, Japan) set at 30°C for the analytical column, and a mass spectrometer were arranged as shown in Fig. 2.

The analytical process was as follows: a 50 μl

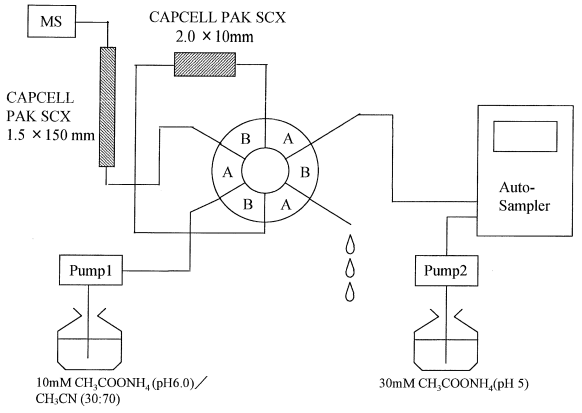

Fig. 2. Schematic diagram of the column-switching system used.

aliquot of urine sample was injected by the auto-sampler into the sample loop, and was transferred to the trapping column at a flow-rate of 200 μl/min (status A). Six minutes after the injection, the switching valve was turned to the alternate position, so that the trapping column and the analytical column were in-line (status B).

By using the backflush mode, the mobile phase used for the main separation carried the enriched analytes from the trapping column on to the analytical column at a flow-rate of 120 μl/min. After a duration of 4.0 min, the switching valve was turned back to the initial position to disconnect both columns, so as to re-equilibrate the trapping column with the mobile phase (status A). The analytes were eluted from the analytical column with the mobile phase used for the main separation. The entire eluent flow was introduced without splitting into the electrospray interface. The time required for one analytical run was approximately 30 min.

2.3. HPLC conditions

The analytical column used for the main separation was a CAPCELL PAK SCX (column dimension and particle size being 1.5 mm I.D.×150 mm and 5 μm, respectively) (Shiseido, Tokyo, Japan). The column for trapping (pretreatment of samples) was a CAPCELL PAK MF SCX cartridge column (column dimension and particle size being 2.0 mm I.D.×10 mm and 5 μm, respectively) (Shiseido, Tokyo, Japan).

The mobile phases used in the main separation and trapping were 10 m*M* ammonium acetate (pH 6.0)-acetonitrile (30:70, v/v) and 30 m*M* ammonium acetate, respectively. The flow-rates were 120 and 200 μl/min, respectively. Both main separation and trapping were carried out at 30°C.

2.4. ESI mass spectrometry

Electrospray ionization–mass spectrometry (ESI-MS) was performed by using a Platform quadrupole mass spectrometer (Micromass, Manchester, UK) in the positive-ion mode. The ESI operating parameters were as follows: ion source temperature, 70°C; capillary voltage, +4.5 kV; cone voltage, 50 V; and multiplier voltage, 650 V. Under these conditions, full scan data acquisition was performed from *m/z* 100 to 500 in centroid mode and using a cycle time of 1.0 s and an interscan time of 0.1 s.

3. Results and discussion

3.1. Sample clean-up and enrichment of analytes

In order to carry out a sensitive and accurate determination, sample preparation is one of the most essential processes in HPLC–MS analysis, particularly when samples are biological fluids such as urine and blood. Most of interfering substances in urine are empirically known to be either neutral or anionic, and these endogenous components of urine are expected to be successfully removed by sample clean-up with a strong cation-exchanger (SCX) cartridge. In fact, we have successfully applied SCX cartridges to the HPLC–MS analysis of methamphetamine and its metabolites in urine [17,19]. Therefore, the SCX cartridge described above was employed as a trapping column (pretreatment of samples) in this study.

The short trapping column removed the interfering substances in urine matrix and enriched DAM and its metabolites on the top of the column during the first 6 min of analysis. With the mobile phase described earlier, most of the regular components of urine were eluted within about 5.5 min, and DAM and its metabolites are eluted later than 25 min. Switching the valve at 6 min resulted in the transfer of the

enriched analytes on to the analytical column in the backflush mode with the mobile phase used for the main separation.

3.2. Optimization of chromatographic conditions for main separation

For the simultaneous analysis of DAM and its metabolites by HPLC, the gradient elution on an octadecylsilyl (ODS)-type column with water-rich eluent has often been utilized. This method involves a longer analysis cycle compared with isocratic solution. On the other hand, an SCX column allows the use of a mobile phase with a higher organic solvent concentration than an ODS column, which provides higher sensitivity in ESI-MS, especially when analyzing amine compounds. Therefore, an SCX-type column was employed in combination with an isocratic acetonitrile–aqueous ammonium acetate (NH_4OAc) mobile phase. In addition to DAM, MAM and MOR, DHC as well as COD, which often provide opiate-positive screening results by enzyme immunoassay, were also included as analytes when optimizing the chromatographic conditions in order to ensure sufficient peak separation.

Preliminary experiments suggested that chromatographic separation on SCX depended more largely on the buffer concentration than on the acetonitrile concentration. The concentration of NH_4OAc was first optimized on the basis of peak resolution and retention time. The higher NH_4OAc concentration led to a shorter retention time and worse resolution. At the NH_4OAc concentration of 10 m*M*, all the analytes eluted within 30 min with satisfactory resolutions.

Although lower acetonitrile concentrations resulted in larger capacity factors (k' values), acetonitrile concentrations between 60 and 70% produced sufficient peak separation. Additionally, such a higher acetonitrile concentration usually provides higher sensitivity in ESI-MS.

For further improvement, the effect of pH of mobile phase on chromatographic efficiency was examined by varying the pH of NH_4OAc solution used for preparing the mobile phase between 4 and 7. At the pH below 5, sufficient separations of COD, MOR and DHC could not be achieved. Also, acidic conditions would be preferable for keeping the lifetime of SCX column. Based on the above mentioned comparisons, acetonitrile–10 m*M* NH_4OAc, adjusted to pH 6.0, (70:30, v/v) was chosen as the mobile phase.

3.3. Instrumental parameters for ESI-MS

Spectral profiles and sensitivities are known to be dependent on the instrumental conditions. The capillary and cone voltages were optimized using the flow-injection method and employing an aqueous solution containing DAM, MAM and MOR at 1 μg/ml each. The capillary voltage was varied between 1.5 and 4.5 kV: the abundance of the base peaks, or protonated molecular ions of DAM (m/z 370), MAM (m/z 328) and MOR (m/z 286), increased proportionally with the voltage. The cone voltage was further varied between 10 and 70 V. The higher voltage produced the more extensive fragmentation, which can help the identification. However, the sensitive detection of protonated molecular ions in full scan mode is essential in forensic drug analysis. The highest abundance, as well as the best level reproducitivity of each protonated molecular ion was obtained at 50 V. Thus, the capillary and cone voltages were set at 4.5 kV and 50 V, respectively.

3.4. HPLC–ESI-MS identification

Confirmation was performed by mass spectral identification in the scan mode. Fig. 3 presents extracted mass chromatograms of the five analytes and the mass spectra of DAM, MAM and MOR, all obtained from a urine sample spiked at 100 ng/ml of each compound. For all the five analytes, one can see typical $[M+H+CH_3CN]^+$ ions in addition to predominant $[M+H]^+$ ions. In the mass spectrum of MAM, a fragment ion $[M-CH_3COO+2H]^+$ was also observed at m/z 286 with relatively high intensity.

3.5. Extraction recoveries

In order to evaluate the extraction recoveries of the established method, spiked urine samples and standard aqueous solutions were each analyzed in the SIM mode. Peak areas of $[M+H]^+$ ions were

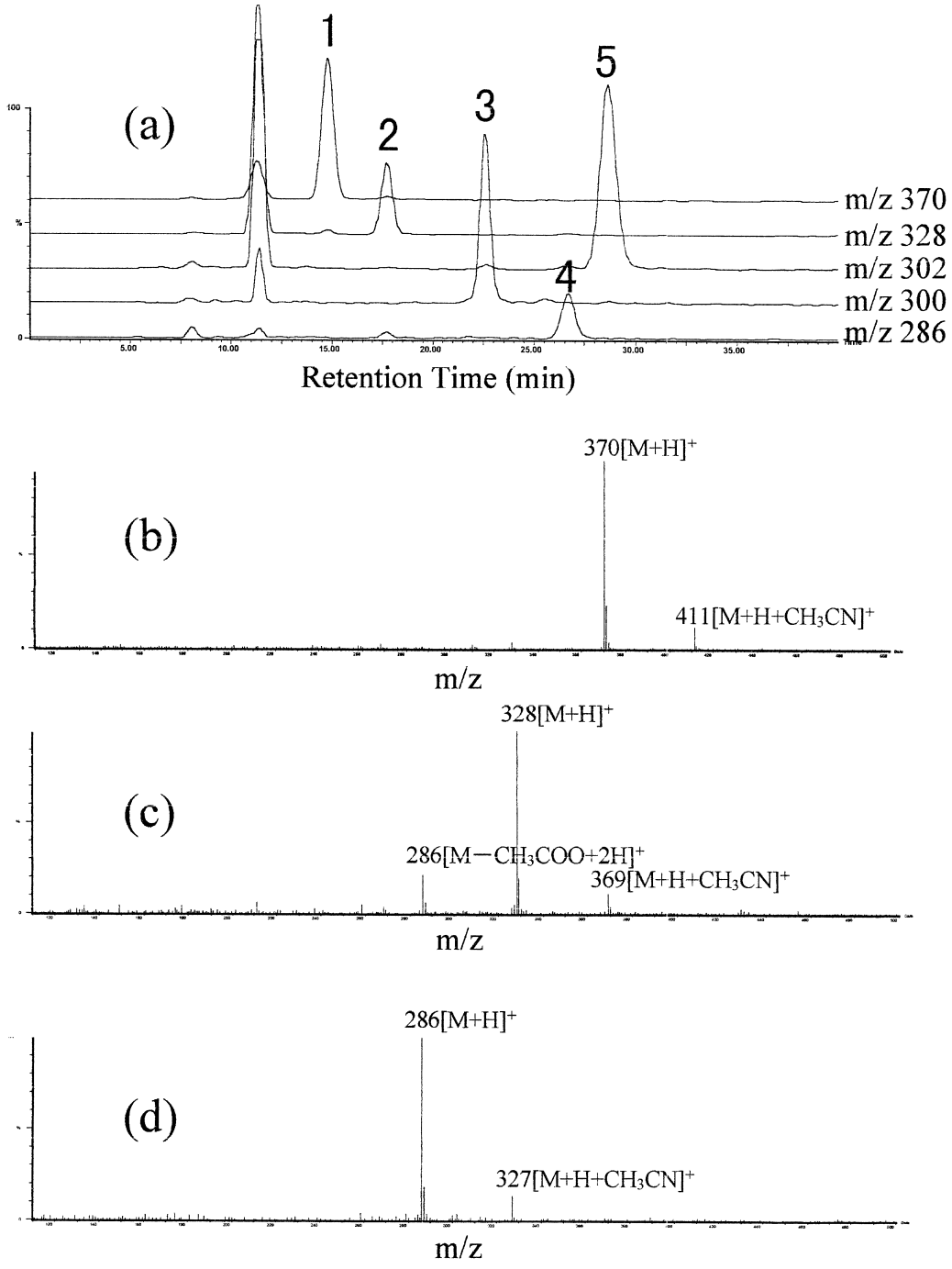

Fig. 3. Mass chromatograms obtained for a spiked urine sample (a), and mass spectra produced from peaks of DAM (b), MAM (c) and MOR (d). Eluent for main separation: 10 m*M* ammonium acetate (pH 6.0)–acetonitrile (30:70, v/v). The concentration of all the analytes was 100 ng/ml each, and 50 μl of the urine sample was injected. Other chromatographic conditions appear in the Experimental section. Peaks in (a): 1, DAM; 2, MAM; 3, COD; 4, MOR; 5, DHC.

compared for DAM, MAM and MOR. The recovery ratios for urine against those for standard solution were 99.1, 100.7 and 103.6% for DAM, MAM and MOR at the concentration of 100 ng/ml, and 94.3, 97.1 and 94.8% at 10 ng/ml, respectively ($n=5$). Urine samples basically gave similar results to those of the standard solutions, and chromatographic and spectral deterioration in peak profiles was hardly observed in this experiment.

3.6. Quantitative analysis and detection limits

Calibration curves were constructed without using an internal standard by the SIM technique, in which the $[M+H]^+$ ions (m/z 370 for DAM, m/z 328 for MAM and m/z 286 for MOR) were chosen. The analysis showed good linearity over the concentration range from 1 to 100 ng/ml for DAM ($y=3.55\times10^3x-1.35\times10^3$, $r^2=0.999$) and MAM ($y=7.80\times10^2x-5.01\times10$, $r^2=1.0$) and from 10 to 1000 ng/ml for MOR ($y=3.99\times10^2x-7.30\times10$, $r^2=0.999$).

Precision and accuracy were evaluated by analyzing drug-free urine samples spiked with known concentrations of DAM, MAM and MOR (quality control samples) in the SIM mode. The within-day relative standard deviations (RSDs) ($n=5$) were 3.7% for DAM, 4.1% for MAM and 3.4% for MOR at the concentration of 100 ng/ml, and 4.9% for DAM, 5.8% for MAM and 6.7% for MOR at 10 ng/ml. Accuracy were found to be 99.3% for DAM, 97.5% for MAM and 103.1% for MOR at the concentration of 100 ng/ml, and 93.1% for DAM, 104.8% for MAM and 106.3% for MOR at 10 ng/ml. The between-day RSDs determined from three batches over 3 days ($n=3$ for each batch) at the concentration of 100 ng/ml were calculated to be 4.3% for DAM, 3.9% for MAM and 4.5% for MOR.

Also, the sensitivities were evaluated. The detection limits in the scan mode were 2 ng/ml for DAM, 10 ng/ml for MAM and 30 ng/ml for MOR. The SIM technique by monitoring each protonated molecular ion lowered the detection limits further down to 0.1 ng/ml for DAM, 0.5 ng/ml for MAM and 3 ng/ml for MOR with a signal-to-noise ratio of 3:1. These results demonstrate high sensitivity and good reproducibility of the present method.

Subnanogram-per-milliliter detection limits for urinary opiates by GC–MS [9,11] or HPLC–MS [26,27] has been recently reported in previous papers. Although the sensitivity of the present method was somewhat inferior to those results, this method was successfully applied to the urine samples from several drug users, as detailed in the subsequent subchapter.

3.7. Application to urine samples of opiate users

This established analytical system was applied to the urine samples collected from one suspected MOR user (user A) and two suspected DAM addicts (users B and C). These samples had already been prescreened to be opiate-positive by enzyme multiplied immunoassay technique (EMIT) at our laboratory.

User A intravenously injected an ample of MOR hydrochloride solution that had been stolen from a hospital. As depicted in Fig. 4, a high concentration of MOR (concentration being 12.7 μg/ml) was exclusively detected in his urine. This led to the endorsement of MOR use.

User B intravenously injected an illicit DAM solution immediately prior to his arrest. His urine was sampled immediately after his arrest. As shown in Fig. 5, not only MAM and MOR, but also DAM and COD were detected. Also, a fairly small peak appeared in the mass chromatogram, whose mass spectrum was characterized by a predominant ion at m/z 342 and a less intense ion at m/z 383. Careful examination led to the assignment of these ions to the $[M+H]^+$ and $[M+H+CH_3CN]^+$ ions of acetylcodeine (ACOD) which usually exist in illicit DAM as a major impurity, respectively. Further examination of the HPLC–MS data revealed that this urine sample also contained methamphetamine (MA) and its metabolite amphetamine (AP) (identification of AP and MA was performed by comparing the retention time and spectral data with those of the authentic samples by using the present method).

User C intravenously injected a DAM solution at night and was found dead at home the next morning. His urine and serum were sampled during postmortem examination. As depicted in Fig. 6, MA and AP as well as MAM, ACOD, COD and MOR were detected, though no traces of DAM were detectable in urine. The present analytical method was also applied to the serum sample. Only MOR and COD

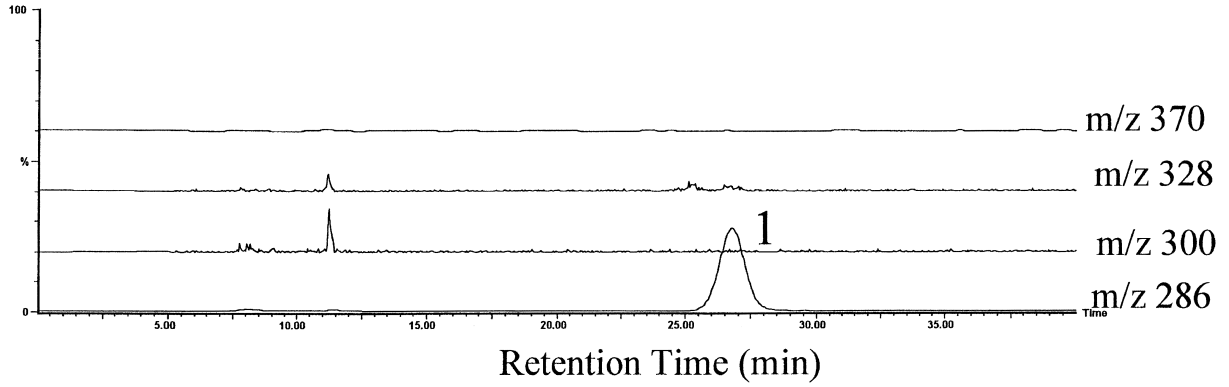

Fig. 4. Mass chromatograms obtained from a urine sample taken from a morphine user (User A). Peak: 1, MOR. The estimated concentration of MOR is 12.7 μg/ml.

were detected in the scan mode, whereas MAM was detected in the SIM mode.

The frequent detection of MA and AP along with DAM and its metabolites indicates that the blending of MA and DAM has become popular among drug users.

4. Conclusion

A rapid, accurate and sensitive analytical procedure for DAM and its metabolites in urine was established by coupling semi-microcolumn HPLC with column switching on-line SPE and ESI-MS. This method eliminated tedious manual sample processing such as extraction and derivatization, and minimized run time (i.e. 30 min per sample from sample preparation through MS data acquisition). The proposed method was not applicable at present to the analysis of MOR glucuronides that has been menthioned in some previous papers [16,26,27]. However, this may be solved by improvement of the trapping column. The usefulness of this methodology was confirmed through its applications to real foren-

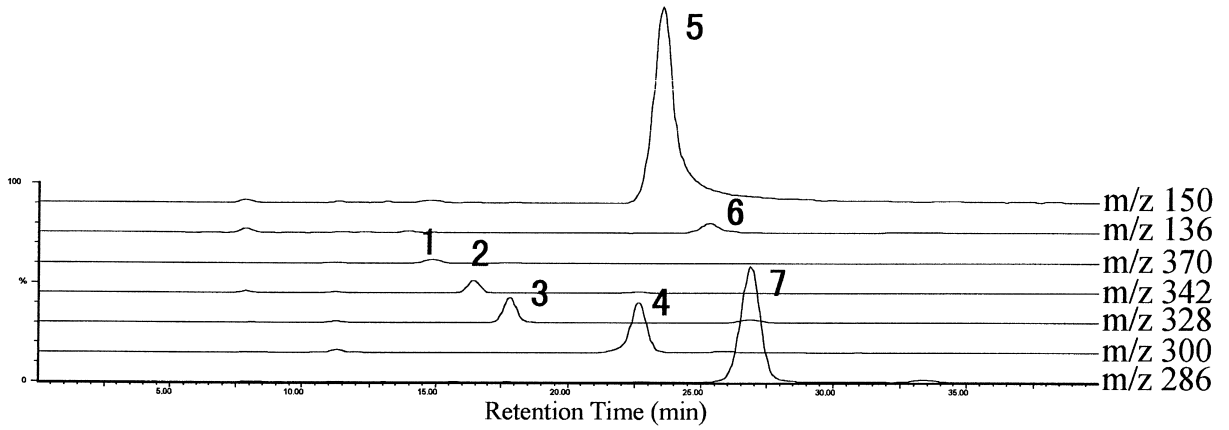

Fig. 5. Mass chromatograms obtained from a urine sample taken from a heroin user who also took MA (User B). Peaks: 1, DAM; 2, ACOD; 3, MAM; 4, COD; 5 MA; 6, AP; 7, MOR. The estimated concentrations are 0.159 μg/ml for DAM, 2.36 μg/ml for MAM, 21.6 μg/ml for MOR.

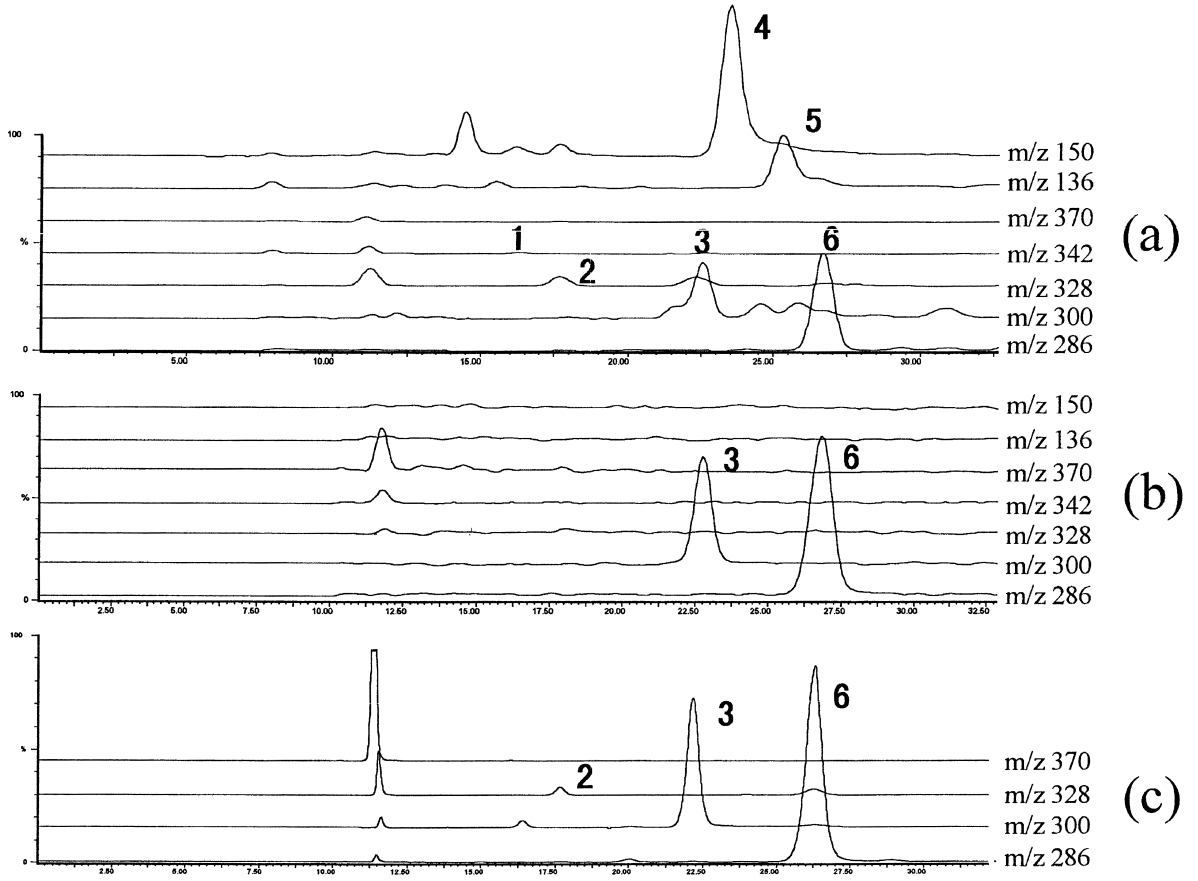

Fig. 6. Mass chromatograms obtained from the urine(a) and serum (in the full scan (b) and SIM (c) modes) samples taken from a heroin user who also took MA (User C). Peaks: 1, ACOD; 2, MAM; 3, COD; 4, MA; 5 AP; 6, MOR. The estimated concentrations are 0.279 μg/ml for MAM and 2.56 μg/ml for MOR in the urine sample.

sic samples: it especially provided high sensitivity for both MAM and ACOD that are the best indicators for DAM use [37,38]. Therefore, this method could become a powerful tool in drug enforcement that needs rapid and uncontroversial proof of DAM use.

References

[1] National Police Agency, Government of Japan, 'White Paper on Police 1994–1998 (Excerpt)', Japan Times, Tokyo, 1995–1999.
[2] B.A. Goldberger, E.J. Cone, T.M. Grant, Y.H. Caplan, B.S. Levine, J.E. Smialek, J. Anal. Toxicol. 18 (1994) 22.
[3] B.D. Paul, L.D. Mell, J.M. Mitchell, J. Irving, A.J. Novak, J. Anal. Toxicol. 9 (1985) 222.
[4] B.H. Chen, E.H. Taylor, A.A. Pappas, J. Anal. Toxicol. 14 (1990) 12.
[5] G.F. Grinstead, J. Anal. Toxicol. 15 (1991) 293.
[6] A.M. Bermejo, I. Ramos, P. Fernández, M. López-Rivadulla, A. Cruz, M. Chiarotti, N. Fucci, R. Marsilli, J. Anal. Toxicol. 16 (1992) 372.
[7] R.W. Romberg, L. Lee, J. Anal. Toxicol. 19 (1995) 157.
[8] B.D. Paul, J.M. Mitchell, L.D. Mell, J. Irving, J. Anal. Toxicol. 13 (1989) 2.
[9] J.G. Guillot, M. Lefebvre, J.P. Weber, J. Anal. Toxicol. 21 (1997) 127.
[10] S.L. Mulé, G.A. Casella, Clin. Chem. 20 (1988) 1427.
[11] E.J. Cone, P. Welch, J.M. Mitchell, B.D. Paul, J. Anal. Toxicol. 15 (1991) 1.
[12] B.A. Goldberger, W.D. Darwin, T.M. Grant, A.C. Allen, Y.H. Caplan, E.J. Cone, Clin. Chem. 39 (1993) 670.

[13] J. Schuberth, J. Schuberth, J. Chromatogr. 490 (1989) 444.
[14] J. Fehn, G. Megges, J. Anal. Toxicol. 9 (1985) 134.
[15] M. Katagi, M. Tatsuno, H. Tsuchihashi, Jpn. J. Toxicol. Environ. Health 40 (1994) 357.
[16] M. Tatsuno, M. Nishikawa, M. Katagi, H. Tsuchihashi, J. Anal. Toxicol. 20 (1996) 281.
[17] M. Katagi, H. Nishioka, K. Nakajima, H. Tsuchihashi, H. Fujima, H. Wada, K. Nakamura, K. Makino, J. Chromatogr. B 676 (1996) 35.
[18] H. Hoja, P. Marquet, B. Verneuil, H. Lotfi, B. Pénicaut, G. Lachâtre, J. Anal. Toxicol. 21 (1997) 116.
[19] M. Katagi, M. Nishikawa, M. Tatsuno, T. Miyazawa, H. Tsuchihashi, A. Suzuki, O. Shirota, Jpn. J. Toxicol. Environ. Health 44 (1998) 107.
[20] M. Nishikawa, H. Tsuchihashi, J. Toxicol.-Toxin Rev. 17 (1998) 13.
[21] H.H. Maurer, J. Chromatogr. B 713 (1998) 3.
[22] M. Nishikawa, H. Tsuchihashi, A. Miki, M. Katagi, G. Schmitt, H. Zimmer, Th. Keller, R. Aderjan, J. Chromatogr. B 726 (1999) 105.
[23] P. Marquet, G. Lachâtre, J. Chromatogr. B 733 (1999) 93.
[24] M.J. Bogusz, J. Chromatogr. B 733 (1999) 65.
[25] M. Katagi, M. Tatsuno, A. Miki, M. Nishikawa, H. Tsuchihashi, J. Anal. Toxicol. 24 (2000) 354.
[26] M.J. Bogusz, R.-D. Maier, M. Erkens, S. Driessen, J. Chromatogr. B 703 (1997) 115.
[27] P. Zuccaro, R. Ricciarello, S. Pichini, R. Pacifici, I. Altieri, M. Pellegrini, G. D'Ascenzo, J. Anal. Toxicol. 21 (1997) 268.
[28] S. Pichini, Mass Spectrom. Rev. 18 (1999) 119.
[29] K.A. Ramsteiner, J. Chromatogr. 456 (1988) 3.
[30] P.O. Edlund, L. Bowers, J. Henion, J. Chromatogr. 487 (1989) 341.
[31] J. Cai, J. Henion, Anal. Chem 68 (1996) 72.
[32] M. Zell, C. Husser, G. Hopfgartner, J. Mass Spectrom. 32 (1997) 23.
[33] H.S. Lee, K. Kim, J.H. Kim, K.S. Do, S.K. Lee, J. Chromatogr. B 716 (1998) 371.
[34] M. Katagi, M. Nishikawa, M. Tatsuno, H. Tsuchihashi, J. Chromatogr. A 833 (1999) 169.
[35] J. Slobodnik, B.L.M. van Baar, U.A.Th. Brinkman, J. Chromatogr. A 703 (1995) 81.
[36] A. Motoyama, A. Suzuki, O. Shirota, R. Namba, Rapid Commun. Mass Spectrom. 13 (1999) 2204.
[37] C.L. O'Neal, A. Poklis, J. Anal. Toxicol. 21 (1997) 427.
[38] P. Kintz, C. Jamey, V. Cirimele, R. Brenneisen, B. Ludes, J. Anal. Toxicol. 22 (1998) 425.