ELSEVIER

Journal of Chromatography B, 700 (1997) 83–91

JOURNAL OF CHROMATOGRAPHY B

Hair analysis for drugs of abuse XIX. Determination of ephedrine and its homologs in rat hair and human hair

Yuji Nakahara*, Ruri Kikura

National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan

Received 15 April 1997; received in revised form 12 June 1997; accepted 12 June 1997

Abstract

A sensitive GC–MS method was developed for the quantitative analysis of ephedrine (EP), phenylpropanolamine (PPA) and methylephedrine (ME) in animal and human hair. After washing with 0.1% sodium dodecyl sulfate, hair samples (10 mg) were added with deuterated internal standards, extracted by 1-h sonication and over night soaking in 2 ml of 5 *M* HCl–methanol (1:20) at room temperature. Following evaporation of the liquid phase, the residue was dissolved in phosphate buffer solution (pH 6.0) and purified using a solid-phase extraction procedure with Bond Elut Certify columns. Two types of derivatization were compared – using trifluoroacetic anhydride (TFAA) and pentafluoropropionic anhydride (PFPA) – for discrimination of EP and methamphetamine (MA). Derivatized extracts were analyzed by GC–MS in the EI mode using a capillary column (OV-1 equivalent). From the results comparing three GC–MS conditions, PFP-derivatives separated with a temperature gradient of 20°C/min from 60°C to 280°C gave the best resolution between EP and MA. ME was analyzed as a trimethylsilyl derivative using *N,O*-bis-trimethylsilyl acetamide at the above GC condition. The assay was linear from 0.5 to 50 ng/mg (r=0.998) and capable of detecting less than 50 pg of derivatized EP, PPA and ME on-column. Intra-assay precision was characterized by C.V. values from 5 to 16% in the concentration range of 1–10 ng/mg hair. The method was used for the quantitative determination of EP, PPA and ME in the hair obtained from three rats with dark brown hair after ten intraperitoneal injections (5 mg/kg/day) of the three drugs and from three male and one female volunteers with black hair after an oral dose of 50 mg/day of EP–HCl for three days. Hair samples were collected by shaving from the back of rats and cutting from the scalp of humans 28 days after the first dose. The incorporation rates of EP, PPA and ME into hair (the ratios of [hair concentration] to [AUC]) obtained from the animal experiment were 0.10, 0.07 and 0.03, respectively, which are a little lower than those (0.14, 0.10 and 0.04) of their desoxy-compounds, MA, amphetamine and dimethylamphetamine. EP was detected at an average of 2.25 ng/mg (n=4) in human scalp hair and at a range of 1–29 ng/mg (n=3) in human beard hair until day 14, but its metabolite (PPA) was at a trace level in the hair of the four subjects. The method was successfully used for detection of ME and EP in the hair of a neonate and its mother who was abusing Bron syrup containing ME during the pregnancy. © 1997 Elsevier Science B.V.

Keywords: Hair; Ephedrine; Phenylpropanolamine; Methylephedrine

1. Introduction

Ephedrine (EP) is a sympathomimetic amine

*Corresponding author. Fax: +81 3 3700 8764; e-mail: nakahara@nihs.go.jp

PII S0378-4347(97)00332-0

which has a central nervous system stimulating property and is therefore classified among the banned substances in sports. Some EP poisonings have also been reported [1–3]. In addition, EP has long been employed as a precursor for the illicit synthesis of methamphetamine (MA).

As it is well known [4–7], the gas chromatographic behavior of EP is similar to that of MA when analyzed as trifluoroacetyl (TFA) derivatives and in mass spectrometric detection, their major ions are similar to each other. Therefore, it has been pointed out that EP may be confused with MA in specimens, like hair, containing a very low amount of the drug.

However, very little information has been reported about the presence of EP and its homologs in hair. Moreover, the study of the disposition of these compounds in animal and human hair and of the factors that control the concentration of drugs in hair is necessary for the reliable evaluation of analytical findings. Such studies require highly sensitive analytical methods for the determination of drugs and their metabolites in the amounts of hair which can be collected from experimental animals and men.

The objective of this project was to develop a sensitive, selective and reliable analytical method for the determination of EP and its homologs in animal and human hair. Furthermore, this paper describes experiments on the incorporation into hair of EP homologs and some cases of EP intake by humans, studied by means of hair analysis.

2. Experimental

2.1. Chemicals and reagents

L-EP·HCl, L-methylephedrine (ME)·HCl and L-phenylpropanolamine (PPA)·HCl were obtained from Dainippon Pharmaceutical (Osaka, Japan). BRON syrup was obtained from SS Pharmaceutical (Tokyo, Japan). Bond Elut Certify (mixed mode cation-exchange/reversed-phase sorbent) columns were purchased from Varian (Harbor City, CA, USA); trifluoroacetic anhydride (TFAA) and pentafluoropropionic anhydride (PFPA) were purchased from Pierce (Rockford, IL, USA). N,O-bis-trimethylsilyl acetamide (BSA), lithium aluminium deuteride and all other reagent-grade chemicals were obtained from Aldrich (Milwaukee, WIS, USA).

DL-EP-d3 was synthesized by the reduction with lithium aluminium deuteride of *N*-carboethoxy-norephedrine obtained from the reaction of norephedrine and ethyl chloroformate. The PFP-derivative of this product showed a single peak on a GC–MS chromatogram and its principal peaks were *m/z* 207 (100%), 163 (32%) and 119 (23%). DL-ME-d6 was synthesized by the *N*-methylation with formic acid-d2 (Merck, Darmstadt, Germany) and paraformaldehyde-d2 (MSD isotope, Montreal, Canada). The trimethylsilyl (TMS)-derivative of this product showed a single peak on a GC–MS chromatogram and its principal peaks were *m/z* 78 (100%), 108 (1.2%) and 242 (0.8%).

2.2. Animal experiments

Three male Dark Agouti (DA) pigmented rats, aged 5 weeks (100–120 g) were used in these experiments. Before drugs were administered, the back hair of the rats was shaved with an animal electric shaver (Daitoh Electric Machine, Tokyo, Japan). The rats were then intraperitoneally administered once a day with EP·HCl, PPA·HCl, and ME·HCl (5 mg/kg) for 10 consecutive days ($n=3$), respectively.

2.2.1. Hair samples

The newly growing back hair was collected by the electric shaver 28 days after the first administration.

2.2.2. Plasma samples

Blood (400–500 μl) was collected at 5, 15, 30, 60, 120 and 360 min after injection from orbital vein plexus on the first day of administration. The blood was collected into plastic tubes containing heparin and sodium fluoride cooled in ice. The plasma samples were obtained by centrifugation at 10 000 rpm for 3 min and stored at −20°C until analysis. Areas under the curve (AUCs) were calculated by the trapezoidal rule according to our previous report [8].

2.3. Standards and solutions

Three stock solutions containing each EP·HCl, PPA·HCl or ME·HCl (1 mg/ml) in methanol for

standard curves were prepared and stored at +4°C. From these stock solutions, two working solutions were prepared. For preparation of the calibration curves, the first working solution contained EP and PPA at 5 and 0.5 μg/ml, respectively, and the second working solution contained ME and EP at 10 and 1 μg/ml, respectively. The calibration curves were prepared by adding plasma (200 μl) and urine (50 μl) with EP, PPA and ME to achieve final concentrations of 5–1000 ng/ml. Hair samples (10 mg) were added with standards of the compounds to achieve final concentrations of 0.5–50 ng/mg of hair. Only in the case of Bron use during pregnancy, was the calibration curve made in the range of 10–200 ng/mg of hair using 3 mg of hair samples.

2.4. Extraction and derivatization

2.4.1. Plasma and urine

To 200 μl of plasma sample or 50 μl of urine were added 100 μl of internal standard (I.S.) aqueous solution containing EP-d3 and ME-d6 at 1 μg/ml each and 500 μl of 0.1 *M* potassium hydrogen phosphate buffer (pH 6.0). The Bond Elut Certify column pre-activated with MeOH and 0.1 *M* potassium hydrogen phosphate buffer (pH 6.0) was loaded with the mixture and the cartridge was washed with 1 ml of water, 1 ml of 0.1 *M* acetic acid and 1 ml of water, in sequence. The column was then dried under vacuum for 5 min, rinsed with 1 ml of methanol and dried again under vacuum for 2 min. Elution was accomplished with 3 ml of MeOH–5 *M* HCl (20:1). After evaporation of the solvent under a nitrogen stream, the residue was dissolved in 200 μl of PFPA–ethyl acetate (1:1) or TFAA–ethyl acetate (1:1) and heated at 55°C for 20 min. After evaporation of solvent, the residue was redissolved in 50 μl of ethyl acetate. In the case of samples containing ME, TMS-derivatization of ME with 50 μl of BSA was carried out at 90°C for 60 min. Two μl of the ethyl acetate solution or BSA solution was automatically injected into the GC–MS system.

2.4.2. Hair samples

Rat hair and human scalp hair samples were washed three times with 0.1% sodium dodecyl sulfate (SDS) and three times with water under ultrasonication. After the samples were dried under a nitrogen stream and precisely weighed (3–10 mg), they were extracted for 1 h under ultrasonication with 2 ml of MeOH–5 *M* HCl (20:1) containing, as the I.S., 50 ng of EP-d3 and ME-d6. Following incubation at room temperature overnight, the hair was filtered off, the filtrate was evaporated under nitrogen stream and the residue was dissolved in 1 ml of 0.1 *M* phosphate buffer (pH 6.0). The solution was applied to a Bond Elut Certify column, eluted, derivatized and analyzed as above.

2.5. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis was performed using 5890 SERIES-II gas chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equipped with 7673A autosampler (Hewlett–Packard) and MSD 5971 (Hewlett–Packard). The gas chromatography (GC) was carried out with a 15 m×0.25 mm I.D., 0.25-μm cross-linked methylsilicone fused-silica TC-1 (GL Sciences, Tokyo, Japan). The injection port temperature was 250°C (splitless mode) and helium was the carrier gas (4 psi head pressure). The oven temperature was programmed from 60°C or 100°C to 280°C at a rate of 20°C/min or 10°C/min, respectively, after 0.5 min at the initial temperatures following the injection. Drugs in biological specimens were investigated by selected ion monitoring as follows; *m/z* 154, 118, 110 for TFA–EP, *m/z* 204, 118, 160 for PFP–EP, *m/z* 140, 110 for TFA–PPA, *m/z* 190, 160 for PFP–PPA, *m/z* 72 for TMS–ME, *m/z* 157 for TFA–EPd3, *m/z* 207 for PFP–EPd3 and *m/z* 78 for TMS–MEd6, respectively.

2.5.1. Quantitative analysis

The drug concentrations in the biological specimens were calculated using their peak-area ratios of the ions monitored for PFP–EP (*m/z* 204) and PFP–PPA (*m/z* 190) with PFP–EPd3 (*m/z* 207) and TMS–ME (*m/z* 72) with TMS–MEd6 (*m/z* 78). The calibration curves for determination of EP, PPA and ME were constructed by analyzing extracted and derivatized control specimens spiked with the standard solutions of these drugs and the internal standards. The calibration curves for the drugs were linear over the concentration ranges of 50–2000 ng/ml in rat plasma, 500–60 000 ng/ml in human

urine and 0.1–50 ng/mg in rat and human hair with good correlation coefficients (r=0.999). The intra-assay relative standard deviations were less than 5% in regard to plasma and urine, and from 5 to 16% in the concentration range of 1–10 ng/mg hair.

2.6. Human hair collection protocol

Control hair was obtained from healthy volunteers with black hair in our Institute and stored at room temperature in sealed glass vials until use. Three male and one female volunteers with black hair daily received 50 mg EP–HCl for 3 days. Hair samples were cut near the scalp at the vertex posterior 28 days after the first administration. As hair specimens, the 1-cm proximal samples from the cut were chosen and were finely cut with scissors. Human subjects gave informed consent prior to drug administration.

All human experiments were approved by the Human Subjects Protection Committee of the National Institute of Health Sciences, and all subjects gave written informed consent.

2.7. Human beard hair and urine collection protocol

Three male volunteers daily received 50 mg EP·HCl for 3 days. Beard samples were shaved with an electric shaver at 7 o'clock in the morning for 14 days before dosage. After every collection, the parts of the shaver were taken off and washed in ethanol under ultrasonication for 20 min.

Urine samples were collected 3.5, 6, 9, 12, 16, 23 and 24 h after the last dose in plastic tubes and stored at −20°C until use.

2.8. Human scalp hair collection protocol from a mother and a neonate in hospital

Hair samples were cut near the scalp at the vertex posterior of a neonate and its mother at the second day after the birth. The mother's hair (about 16 cm) was cut every 1-cm from the root side into 10 sections and the distal rest was section 11. The neonate's hair (3–4 cm) was cut into 3 sections 1 cm long. Each section was finely cut with scissors. We obtained an informed consent from the mother.

3. Results and discussion

3.1. Optimization of extraction time

From our previous studies [9] on the extraction of hair samples containing phenylethylamines, it has been demonstrated that methanol–5 *M* HCl (20:1, v/v) is the best solvent. Therefore, this solvent was selected for the present study. The amount of EP homologs solubilized from hair reached almost a plateau after soaking for 24-h, following 1-h ultrasonication in methanol–5 *M* HCl (20:1, v/v) (Table 1).

3.2. Discrimination between EP and MA

The gas chromatographic behavior and the main fragmentation pattern of TFA-derivatized EP is very similar to that of TFA-derivatized MA. For this reason, in order to avoid misinterpretation problems, the optimum GC conditions for the separation of the compounds were carefully studied. A test sample was made by mixing equivalent concentrations of EP and MA at 1 μg/ml. Two derivatization methods using TFAA and PFPA were compared, looking at the resolution of the peaks. Also, separation was optimized by testing the two GC temperature gradients.

As shown in Fig. 1A, the TFA-derivatives of MA and EP were not separated under the usual gradient conditions (60°C–20°C/min–280°C). It was difficult to distinguish between them even by the selected ion monitoring using m/z 118 which is a characteristic

Table 1
Extraction kinetics of ephedrine analogs extracted from hair

	EP	PPA	ME
	ng/mg		
1-h sonication	12.2 (75%)[a]	9.7 (80%)	3.6 (68%)
after sonication[b]			
2 h	13.7 (86%)	10.6 (87%)	3.9 (74%)
4 h	14.0 (88%)	10.8 (89%)	4.2 (79%)
6 h	14.5 (91%)	11.1 (91%)	4.5 (85%)
8 h	15.1 (94%)	11.5 (94%)	4.6 (87%)
24 h	16.0 (100%)	12.2 (100%)	5.3 (100%)
36 h	16.1 (101%)	12.1 (99%)	5.2 (98%)

[a] Relative percentages to the concentrations at 24 h.
[b] The cumulative time after 1-h sonication.

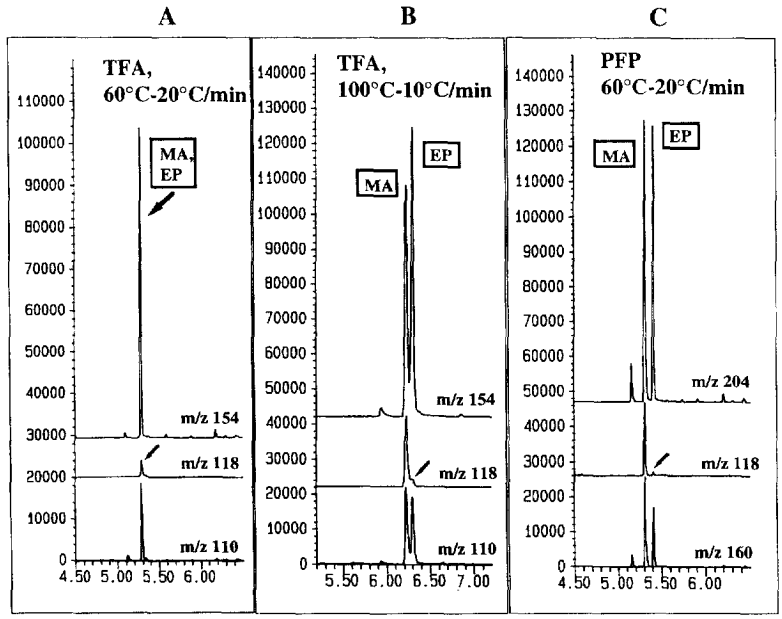

Fig. 1. GC–SIM-MS chromatograms of derivatized ephedrine and methamphetamine at three methods; (A) the TFA-derivatives obtained with oven temperature of 20°C/min from 60°C, (B) the TFA-derivatives obtained with that of 10°C/min from 100°C, and (C) PFP-derivatives obtained with that of 20°C/min from 60°C.

ion of TFA–MA and is not a fragment ion of TFA–EP. When a different gradient temperature (100°C–10°C/min–280°C) was used, the two peaks were partially separated as shown in Fig. 1B, but the resolution of ions at *m/z* 118 between TFA–EP and TFA–MA was still poor. Only the PFP–derivatives clearly showed a base-line separation on the chromatogram even in the usual analytical conditions (60°C–20°C/min–280°C) (Fig. 1C). In this case, PFP–EP and PFP–MA ions at *m/z* 118 could be clearly differentiated. For discrimination of MA and EP in hair, the chromatographic resolution is very important in addition to specific ions like *m/z* 118 ion. Therefore, our result clearly showed that PFP-derivatization is better for identification and discrimination of the two compounds than TFA-derivatization.

3.3. Animal experiments

Fig. 2 shows the pharmacokinetics of EP and PPA in plasma over 360 min after intraperitoneal administration. EP in plasma showed a peak concentration at 15 min and then decreased to less than the limit of detection at 360 min. The metabolite (PPA) showed a peak concentration at 60 min and zero at 360 min.

Fig. 3 shows the typical GC–MS chromatogram obtained from the hair extracts of rats administered with EP·HCl. EP and PPA were found at 16.4±1.67 and 0.72±0.07 ng/mg as an average, respectively. Table 2 shows the plasma AUC and hair concen-

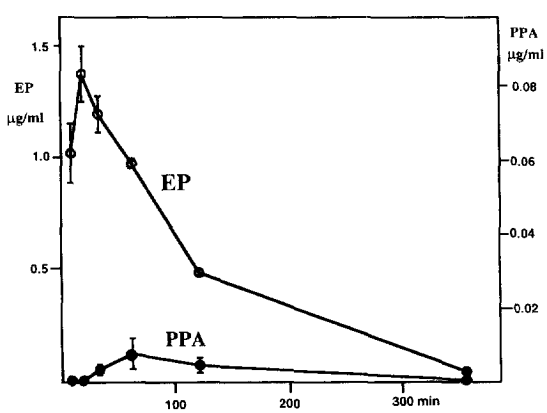

Fig. 2. Pharmacokinetics of EP and PPA in plasma over 360 min. The left axis shows the concentration of EP and the right axis that of PPA.

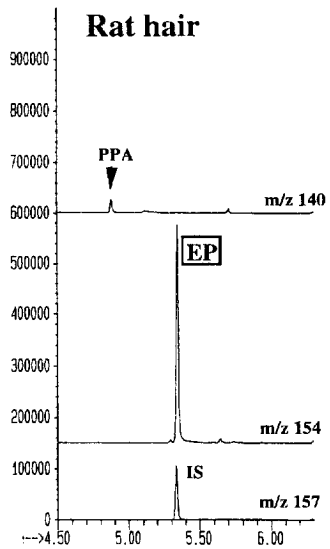

Fig. 3. Typical GC–MS chromatogram obtained from the hair extracts of rats administered with EP·HCl.

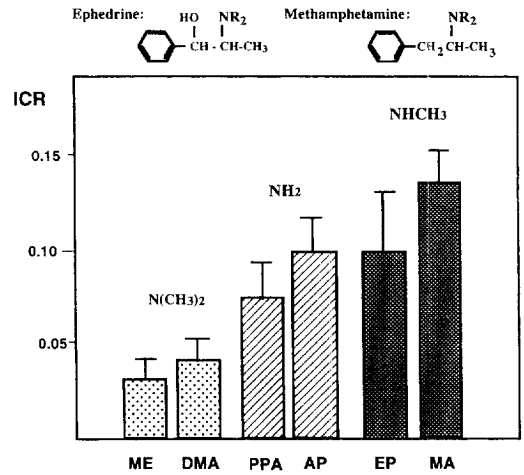

Fig. 4. Comparison of drug incorporation rates into rat hair (ICRs) between three ephedrine homologs and three amphetamine homologs. ICR means the ratios of hair concentration to plasma AUC.

trations of EP, PPA and ME when each drug was administered to the rats as a parent compound. Although their plasma AUC were almost same, the ratio of the hair concentrations of EP, PPA and ME was 1:0.75:0.32. Their incorporation rates which are represented as the ratios of hair concentrations to plasma AUCs were 0.095, 0.073 and 0.031, respectively. It means that the order of drug incorporation tendency into hair is EP>PPA>ME.

3.4. Drug incorporation tendency into hair

The drug incorporation rates into hair (ICRs) of EP, PPA and ME were 1.2–1.5 times lower than the corresponding ICRs [10] of MA, AP and dimethylamphetamine (DMA) (0.12, 0.10 and 0.045, respectively) shown in Fig. 4. It is thought that the incorporation tendencies of EP-homologs into hair might be around 80% of the corresponding MA-homologs due to the difference in hydroxy group. As shown in Fig. 4, the incorporation tendency decreased according to the order of secondary-, primary- and tertiary amines. It is not clear about the reason of this phenomenon, but it is presumed that it could depend on respective pK_a (9.6, 9.55 and 9.3) and/or melanin affinity.

3.5. Analysis of EP in scalp hair, beard and urine from four subjects

The described method was used to quantitate EP and PPA in human scalp hair, beard and urine obtained from three male and one female volunteers with black hair receiving three 50-mg oral doses of

Table 2
Rat plasma AUCs, rat hair concentrations and hair incorporation rates after EP, PPA and ME (5 mg/kg, i.p.) administration

	EP	PPA	ME
AUC (∞) in plasma (μg/ml/min)	172.8±2.5	167.7±15.6	171.7±38.9
Drug concentrations in hair	16.4±1.7	12.3±0.9	5.3±0.3
Ratio in plasma ([EP]=1.0)	1.00	0.97	0.99
Ratio in hair ([EP]=1.0)	1.00	0.75	0.32
ICR ([Hair]/[AUC])[a]	0.095	0.073	0.031

[a] ICR means the ratio of drug concentration in hair to plasma AUC.

EP·HCl for three days. All subjects were permitted to wash daily their own hair with a mild shampoo at their baths. The hair samples were collected by cutting near the scalp 28 days after the first administration. The 1-cm long proximal hair samples were chosen as the specimens. The GC–SIM-MS chromatograms obtained by the described method are presented in Fig. 5. EP in the hair samples was confirmed by the confirmatory ions (*m/z* 154 and 110) and definite relationship between retention times of EP and EP-d3. The concentrations of EP in scalp hair were 1.1, 1.5, 2.8 and 3.6 ng/mg. The metabolite, PPA, was at trace levels.

Fig. 6 shows the pharmacokinetics of the presence of EP and PPA in urine and beard. EP concentrations in urine were 59 μg/ml at the first collection (3.5 h) and gradually decreased to be less than 1 μg/ml after 24 h, although the EP concentration in urine increased once again at 9 h. PPA was also detected, but its concentrations were always less than one tenth of the parent compound (Fig. 6, left). EP in beard was found from the second day and increased up to the third day (next day of last dose) when the maximum concentration (29 ng/mg) was found. Then the concentrations began to decrease and were below the limit of detection (0.2 ng/mg) at day 14. The concentrations of PPA in beard were less than 1/15 of those of the parent compound in beard.

3.6. Analysis of the hair from a neonate and its mother who used to abuse Bron syrup during pregnancy

Bron syrup, which is a trade name of cough medicine made by SS Pharmaceutical Company (Tokyo, Japan), has been well known as an abused drug in Japan. This syrup contains mainly ME, dihydrocodeine, chlorpheniramine, and caffeine. Our analytical method was applied to detect and quantitate ME and its metabolite (EP) in hair samples obtained from a mother who used to abuse Bron syrup during pregnancy and from her newborn baby. The female neonate was born prematurely and from the day following birth she repeatedly vomited, her limbs were trembling and she was continuously crying with high-temperature. Fig. 7 shows the ME concentrations in 11 sections of mother's hair and three sections of neonate's hair. The concentrations of ME in mother's hair increased as the sections approach the proximal end up to 157 ng/mg. Similarly, the ME concentrations in neonate's hair increased up to 5.7 ng/mg from the distal to the

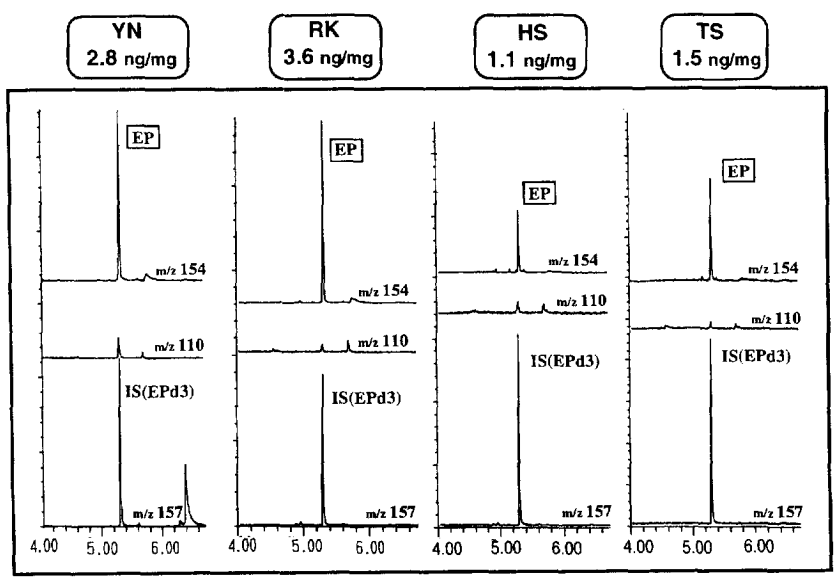

Fig. 5. GC–SIM-MS chromatograms of PFP-derivatized extracts obtained from scalp hair samples of four subjects taking orally 50 mg ephedrine·HCl daily for three days.

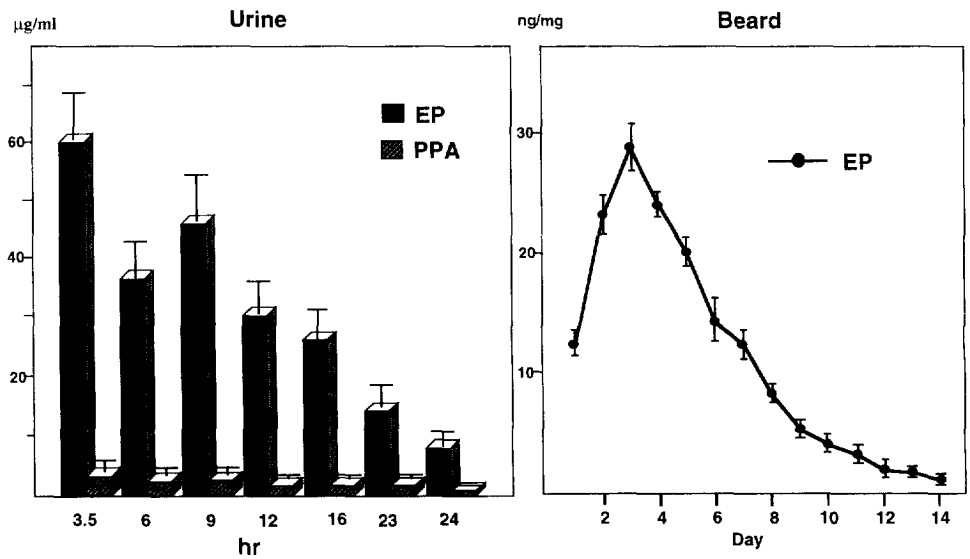

Fig. 6. Pharmacokinetics of EP and PPA in urine and beard of subjects taken orally 50 mg EP·HCl daily for three days. The urine samples were collected from the last dose and the beard samples were collected from the first dose.

proximal section. Since also dihydrocodeine, chlorpheniramine and caffeine were detected at high levels in both mother's and neonate's hair, these four drugs may have strongly affected the fetus during pregnancy. These analytical data could explain the symptomatology of the neonate just after birth.

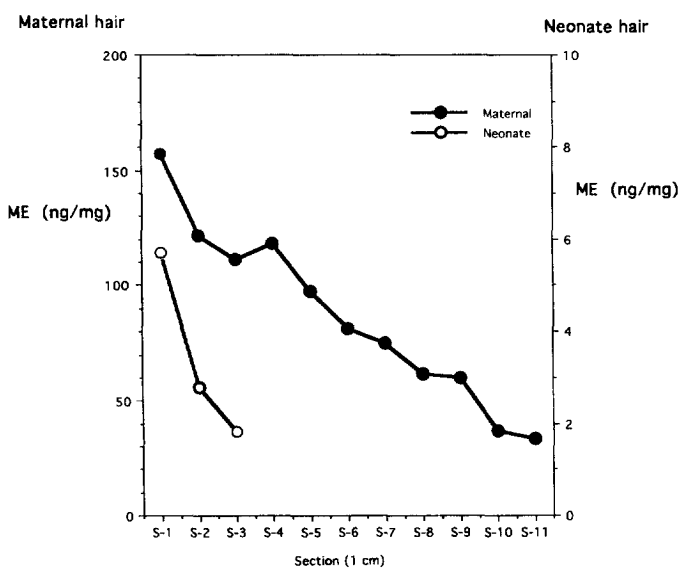

Fig. 7. ME concentrations in 11 sections of mother's hair and three sections of neonate's hair. Each 1-cm section was taken by cutting from the proximal side.

4. Conclusion

This paper described a GC–SIM-MS analysis for quantitative determination of EP, PPA and ME in human hair and beard, and rat hair. The results of a study on the chromatographic resolution between EP and MA showed that PFP-derivatization was the best choice for identification of EP homologs. The method was sensitive, selective and accurate for EP homologs in hair. The ratios of hair concentrations to plasma AUCs using the rat model experiment suggested the order of drug incorporation tendency into hair like EP>PPA>ME, which was around 80% of the corresponding MA-homologs. Data from animal and human experiments demonstrated that this method can be used to follow the disposition of EP homologs into hair. Especially, exposure of the fetus to drug abuse during pregnancy could be successfully demonstrated by hair analysis using our method.

Acknowledgements

The authors thank Dr. M. Watanabe of Fukui Red Cross Hospital for providing us with hair samples of a neonate and its mother.

References

[1] R. Backer, D. Tautman, S. Lowry, C.M. Harvey, A. Poklis, J. Forensic Sci. 42 (1997) 157.
[2] B. Levine, R. Jones, K. Klette, M.L. Smith, E. Kilbane, J. Anal. Toxicol. 17 (1993) 381.
[3] P. Pentel, J. Am. Med. Assoc. 252 (1984) 1898.
[4] W.M. Asselin, J. Anal. Toxicol. 11 (1987) 87.
[5] H.B. Wu, A.S.S. Wong, K.G. Johnson, A. Ballatore, W.E. Seifert Jr., Biol. Mass Spectrom. 21 (1992) 278.
[6] M.A. ElSohly, D.F. Stanford, D. Sherman, H. Shah, D. Bernot, J. Anal. Toxicol. 16 (1992) 109.
[7] C.L. Hornbeck, J.E. Carrig, R.J. Czarny, J. Anal. Toxicol. 17 (1993) 257.
[8] Y. Nakahara, K. Takahashi, M. Shimamine, A. Saitoh, Arch. Toxicol. 66 (1992) 669.
[9] Y. Nakahara, K. Takahashi, M. Shimamine, Y. Takeda, J. Forensic Sci. 36 (1991) 70.
[10] Y. Nakahara, R. Kikura, Arch. Toxicol. 70 (1996) 841.