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Determination of hypnotic benzodiazepines (alprazolam, estazolam, and midazolam) and their metabolites in rat hair and plasma by reversed-phase liquid-chromatography with electrospray ionization mass spectrometry

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

Sensitive determination of benzodiazepines i.e., alprazolam (ALP), estazolam (EST), and midazolam (MDZ), and their metabolites, was carried out by reversed-phase liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS). The chromatography separations were achieved using a semi-micro HPLC column (3 μm particle size; 100 × 2.0 mm, i.d.) with acetonitrile–water containing 1% acetic acid as eluent. The mass spectrometer was operated in selected-ion monitoring mode at protonated-molecular ions $[M+H]^+$ of parent drugs and the metabolites. The proposed procedure was applied to the determination in hair shaft of Dark Agouti rats after intraperitoneal (i.p.) administration of benzodiazepines twice a day for 5 days. Various metabolites together with parent drugs were identified in the hair shaft, 1-hydroxyalprazolam (1-HA) and 4-hydroxyalprazolam (4-HA) from ALP administration; 8-chloro-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine-4-one (K-EST) from EST administration; 1-hydroxymidazolam (1-HM) and 4-hydroxymidazolam (4-HM) from MDZ administration. A few unknown metabolites were also detected in the hair samples. These structures were elucidated with acetylation using acetic anhydride and pyridine. The time course studies of parent drugs and the metabolites in both hair root and plasma were also carried out after single i.p. administration of benzodiazepines. The results suggested that the concentrations of parent drugs and the metabolites in the hair samples were mainly dependent upon those in the plasma.

Keywords: Benzodiazepines; Hair analysis; Metabolic study; HPLC; Electrospray ionization mass spectrometry

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1. Introduction

Benzodiazepines such as alprazolam (ALP) and triazolam (TZ) are members of new class of 1,4-benzodiazepines characterized by high potency, oral activity and low toxicity (Fig. 1). The benzodiazepines are used as anxiolytic agents, sedative-hypnotic agents, anticonvulsants and muscle relaxants. In general, the action is adequately derived from an oral dose less than 1 mg. Although the benzodiazepines are good hypnotic drugs for management of insomnia, overdose could be fatal [1,2]. The benzodiazepines such as TZ are known to be the abused pharmaceutical drugs [3,4]. It is obvious that a reliable method for qualitative and quantitative analyses of the benzodiazepines is necessary. Thus, the highly sensitive detection is one of the important subjects in forensic and clinical sciences.

Various methods, such as immunoassay [5–8], gas chromatography (GC) coupled with various detectors [9–20] and high-performance liquid chromatography (HPLC) [21–27], are adopted for monitoring of benzodiazepines. Among them, HPLC has been proposed for the analysis of heat labile drugs because of the milder working conditions. However, the sensitivity of HPLC method using UV detection is often insufficient for the determination of drugs in real samples. Recently, HPLC coupled to mass spectrometry (MS) has been a major focus for the determination of benzodiazepines in biological specimens such as blood and urine because of its high sensitivity and selectivity [28].

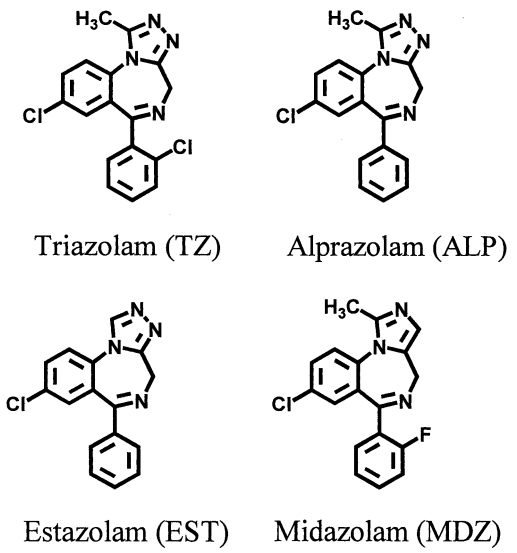

Fig. 1. Chemical structures of benzodiazepines tested.

Drug monitoring is usually based upon blood and/or urine measurements. However, the concentrations in such biological samples may only reflect dosage at sampling time. On the other hand, hair analysis provides more permanent marker of drug intake than blood and urine analyses. However, the analysis is possible to apply only limited drugs because of low concentration in hairs. Due to the progress of separation technique and detection sensitivity and selectivity, however, pmol/mg level detection of drugs in hair is possible. Thus, hair is currently an excellent specimen for resolution over relatively long periods. The analysis of hair provides a means of determining individual past history of long-term drug use, because the drug and its metabolites are deposited into the hair [29–31]. In the last decade, hair analysis for drugs of abuse such as heroin, cocaine and amphetamines gained widespread attention in forensic science. The largest number of papers on hair analysis have dealt with drugs of abuse such as cocaine and amphetamine [32]. Recent studies on hair analysis have gradually been changing to the other drug species, e.g. doping agents and therapeutic drugs [33–38]. Great interest of hair analysis in the field of drug testing seems to be due to its wide detection window. Inspite of a lot of papers regarding hair analysis, the studies of drug disposition in hair root are very rare [39–43], because it is believed that the permeation of drugs into hair from circulation of the blood is very slow. However, recent research has shown that administered drugs are relatively promptly incorporated into hair shaft *via* hair roots. The results suggest a possibility of drug monitoring in hair roots shortly after drug administration, similar to drug monitoring using blood. Thus, the investigations of the fate of drug in hair roots and the comparison with that in plasma seem to be important in order to elucidate drug disposition in hair and interpret the retrospective drug exposure.

We have previously reported the determination of TZ and the hydroxy metabolites in rat hair by HPLC coupled with ESI-MS [44]. The present study deals with the determination of the other benzodiazepines (ALP, EST, and MDZ) and their metabolites in rat hair shafts and hair roots. These concentrations in hair root at each sampling time are also compared with those in plasma after single intraperitoneal (i.p.) administration of the drugs.

2. Experimental

2.1. Materials and reagents

Estazolam (EST) (Radian International, Austin, TX), 4-hydroxyestazolam (4-HE), 4-(8-chloro-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine-6-yl)phenol (P-EST), 8-chloro-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine-4-one (K-EST), ALP (Radian International), 1-hydroxyalprazolam (1-HA) (Radian International), 4-hydroxyalprazolam (4-HA) (Biomol Research Laboratories, Plymouth Meeting, PA), midazolam (MDZ) (Radian International), 1-hydroxymidazolam (1-HM) (Lipomed, Cambridge, MA) and 4-hydroxymidazolam (4-HM) (Lipomed) were used as received. Estazolam-d_5 (EST-d_5), and 1-hydroxyalprazolam-d_5 (1-HA-d_5) made by Radian International was used as the internal standard (IS) for MS. Pyridine and acetic anhydride were of special reagent grade (Wako Pure Chemicals, Osaka, Japan). DL-Isocitric acid lactone (Wako), nicotinamide-adenine dinucleotide (NAD^+) (Wako), nicotinamide-adenine dinucleotide phosphate ($NADP^+$) (Wako) and ethylenediamine tetraacetic acid (EDTA) (Kanto Chemicals, Tokyo, Japan) were used as received. Synthesized melanin was obtained from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA), glacial acetic acid (AcOH), ethylacetate, methanol (MeOH) and acetonitrile (CH_3CN) were of HPLC grade (Kanto). Deionized and distilled water was used throughout. All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. HPLC-ESI-MS

A Hewlett-Packard HPLC 1100 series (Wilmington, DE) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) fitted with an ESI source was used. The separations of benzodiazepines and the metabolites were carried out on a Mightysil RP-18 column (100 × 2.0 mm i.d., 3 μm; Kanto) with water–acetonitrile containing 1% AcOH as the mobile phase. The flow rate of the mobile phase was 0.15 ml/min. MDZ and the metabolites were separated by an isocratic elution using water–acetonitrile–AcOH (84:16:1). The gradient elution profiles were as follows: linear gradient from water–acetonitrile–AcOH (75:25:1) to water–acetonitrile–AcOH (74:26:1) for 7 min, isocratic elution with water–acetonitrile–AcOH (74:26:1) for 10 min, linear gradient from water–acetonitrile–AcOH (74:26:1) to water–acetonitrile–AcOH (65:35:1) for 7 min, isocratic elution with water–acetonitrile–AcOH (65:35:1) for 9 min, and then linear gradient from water–acetonitrile–AcOH (60:40:1) to water–acetonitrile–AcOH (10:90:1) for 7 min. The ESI capillary temperature and capillary voltage were 275 °C and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in the positive ion mode, over a mass range of m/z 150–500, at a scan speed of 0.5 scan/s. The spectra were collected in the form of continuum data.

2.3. Animal experiments

Male Dark Agouti (DA) rats (age, 5 weeks; weight, ≈110 g) (Nippon Bio-supp. Center, Tokyo, Japan) were used. The animal experiments were conducted according to the guidelines of the Ethical Committee on Animal Experimentation of the National Institute of Health Sciences (NIHS) (Tokyo, Japan). The rats were housed at constant room temperature with an alternating 12-h light/dark cycle with free access to food and water. Each rat was housed individually in hanging wire cages to prevent contamination from bedding, urine or the saliva of other rats. Back hair of DA rats had been shaved 10 days before i.p. administration of benzodiazepines.

2.3.1. Determination in rat hair shaft

Benzodiazepines dissolved in ethanol–saline (4:6) were injected intraperitoneally twice a day with the doses (10 mg/kg: $n = 3$ at each dose) for 5 days. After 10 days from final injection, the newly grown hairs were shaved with an electric shaver and stored in a refrigerator until analysis.

The hair shaft, collected before and after administration of benzodiazepines, was washed with 1 ml of 0.1% sodium dodecylsulfate for 1 min under ultra sonication. The hair shaft was then rinsed with distilled water 3 times in the same manner and dried in a desiccator under reduced pressure.

The hairs were cut into small pieces less than 1 mm in length, and about 10 mg of hair was weighed into a glass vial. A 0.2 ml volume of methanol solution containing 100 ng of an IS (EST-d_5, or 1-HA-d_5) was added into the vial containing the hair samples. To extract the benzodiazepines and the metabolites, 2 ml of CH_2Cl_2–MeOH–28% NH_4OH (20:80:2) was added to the vials, sonicated for 1 h, and allowed to stand for 24 h at room temperature. After centrifugation at 3000 rpm ($600 \times g$) for 5 min, the upper layer was collected and evaporated to dryness in vacuo. The resulting residue was re-dissolved in 0.2 ml of mobile phase and an aliquot (50 μl) was submitted to HPLC-MS analysis.

2.3.2. Determination in rat hair root at each sampling time

A dose of benzodiazepines dissolved in ethanol–saline (4:6), 10 mg/kg of ALP, EST and MDZ, was injected intraperitoneally into the DA rats. The hair roots were plucked with a hair nipper at fixed sampling times after the injection, 0.25, 0.5, 1, 2, 4, 6, 9, 24 and 48 h. About 10 mg of the hair roots was collected and stored in a refrigerator until analysis. About 2 mg of hair root collected were weighed into a glass vial. The procedures of extraction and HPLC-MS analysis were the same as those described in Section 2.3.1. However, washing treatment was not performed to the hair root samples.

2.3.3. Determination in rat plasma at each sampling time

A dose of 5 mg/kg of benzodiazepines, dissolved in ethanol–saline (4:6), was injected intraperitoneally into the DA rats. The blood was collected at fixed times after the injection, 15 min, 30 min, 1, 2 and 4 h. A 0.2 ml of IS solution and 1 ml of *n*-hexane–CH_2Cl_2 (1:1) were added to the plasma separated by centrifugation at 1 °C and 13 000 rpm ($9000 \times g$) for 10 min. After thoroughly mixing, the separated organic layer was collected and evaporated to dryness under stream of N_2 gas. The residue was re-dissolved in 0.2 ml of mobile phase, i.e. water–acetonitrile–AcOH (75:25:1), and an aliquot (50 μl) was subjected to HPLC-ESI-MS.

2.4. Calibration curves

Each 100 ng/ml of IS solution was prepared with methanol. Suitable volumes of benzodiazepines or the metabolites solutions were mixed with 200 μl of IS solution (20 ng) in the vials and the mixtures were evaporated to dryness. Then, a 200 μl of mobile phase of HPLC was added to the residue and dissolved well. Each 50 μl of the solution was subjected to HPLC-MS. EST-d_5 was used as the IS for the determination of EST and the metabolites. Since there were no deuterated compounds corresponding to MDZ and the metabolites, EST-d_5 was used for the determination of MDZ and the metabolites.

The peak area ratios of the analytes toward IS were plotted against the concentrations of authentic benzodiazepines and the metabolites. The concentration ranges of EST, K-EST, ALP, 1-HA, 4-HA, MDZ, 1-HM, and 4-HM were 1.25–40.0, 1.25–500, 0.63–80.0, 0.31–10.0, 0.63–20.0, 0.63–20.0, 0.21–10.0, and 0.47–15.0 ng, respectively (Table 1).

2.5. Metabolic study of benzodiazepines in vitro

A liver procured from sacrificed male DA rat was perfused with 40 ml of 1.15% potassium chloride (KCl) solution containing 1 mM EDTA. After perfusion, the liver was immediately homogenized in 10 ml of phosphate buffer containing

Table 1
Concentration ranges and linear equations of calibration curves

Analytes	Concentration range (ng/injection)	Linear regression
EST	1.25–40.0	$y = 0.327x - 0.2085$ ($R^2 = 0.9916$)
K-EST	1.25–500	$y = 0.0674x - 0.1875$ ($R^2 = 0.9999$)
ALP	0.63–80.0	$y = 0.060x - 0.0883$ ($R^2 = 0.9998$)
1-HA	0.31–10.0	$y = 0.0512x - 0.0055$ ($R^2 = 0.9998$)
4-HA	0.63–20.0	$y = 0.0304x - 0.0913$ ($R^2 = 0.9942$)
MDZ	0.63–20.0	$y = 0.0365x - 0.0076$ ($R^2 = 0.9994$)
1-HM	0.21–10.0	$y = 0.0288x - 0.0087$ ($R^2 = 0.9917$)
4-HM	0.47–15.0	$y = 0.0062x - 0.0185$ ($R^2 = 0.9921$)

1.15% KCl and 1 mM EDTA. The homogenate was centrifuged at 13 000 rpm ($9000 \times g$) for 20 min at 1 °C. The supernatant was further centrifuged at 44 000 rpm ($105\,000 \times g$) for 60 min at 1 °C. The microsomal pellets were washed twice with 1 ml of the above mentioned phosphate buffer solution.

Benzodiazepine (600 nmol each), dissolved in 3 ml of phosphate buffer solution containing 1.15% KCl, 1 mM EDTA, 0.5 mM β-$NADP^+$, 3.75 mM DL-isocitric acid lactone, 1 units/ml NAD^+ and 5 mM magnesium sulfate ($MgSO_4$), were added to the resulting pellets, which were homogenized thoroughly. The homogenate was incubated at 37 °C for 20 min and left at 3 °C overnight. Then, the metabolites produced and the parent drugs unreacted were extracted with 3 ml of ethylacetate and separated by centrifugation at 3000 rpm for 10 min. The same extraction procedure was repeated 3 times. The combined ethylacetate extracts were evaporated to dryness under reduced pressure. The resulting residue was dissolved in 0.2 ml of mobile phase for chromatography and the aliquots were injected onto HPLC-MS.

2.6. Acetylation of unknown metabolites

Unknown metabolites possessing hydroxy group in the structures were acetylated with acetic anhydride and pyridine. A 200-μl solution of acetic anhydride–pyridine (1:2) was added to the dried hair extract and reacted at 65 °C for 12 h. Then, the solution was dried with a stream N_2 gas and analysed by HPLC-ESI-MS. The metabolites obtained from liver microsome were also acetylated under the conditions in hair extract.

3. Results and discussion

3.1. Optimization of chromatography and MS detection

The benzodiazepines tested are basic compounds possessing tertiary amines in their structures (Fig. 1). Therefore, positive-ion mode by ESI-MS seems to be suitable for the determination of the parent drugs and the metabolites. The production of positive ions predominantly proceeded in acidic solutions such as formic, acetic and TFAs. Among the acids tested, AcOH provided high production efficiency of protonated-molecular ions $[M+H]^+$ and low baseline noise. Therefore, acetonitrile–water containing 1% AcOH was used as the eluent for HPLC-MS.

The separation of the benzodiazepines and their possible metabolites was carried out by reversed-phase liquid chromatography using a 3 μm particle size and 2 mm diameter semi-micro column. Although ALP and one of the hydroxy metabolites (1-HA or 4-HA) were completely separated with an isocratic elution, the separation between 1-HA and 4-HA was poor. Thus, linear gradient elutions were adopted for the separation of ALP and the metabolites. As shown in Fig. 2A, ALP and the metabolites were satisfactorily separated with gradient elution, and the elution order was 4-HA > 1-HA > ALP. In the case of EST and the metabolites, the elution positions under the gradient profiles were variety found between them, i.e. P-EST > 4-HE > EST > K-EST (Fig. 3A). The result suggests that 4-HE having alcoholic hydrox-

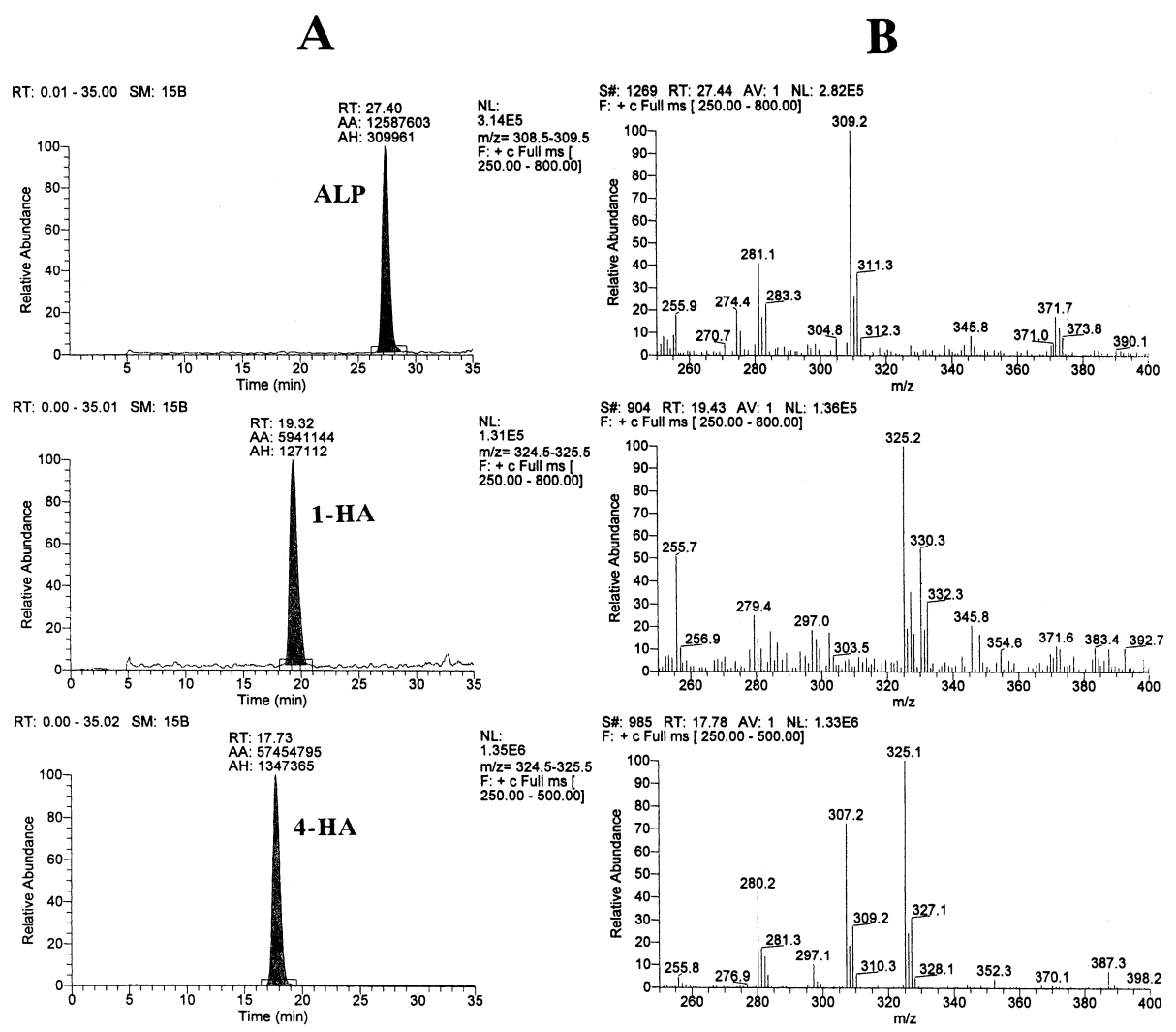

Fig. 2. Mass chromatograms and mass spectra of authentic mixture of ALP and the metabolites (A) (left panels), mass chromatograms; (B) (right panels), mass spectra.

yl group in the structure was retained more than the metabolite possessing a phenolic hydroxyl (P-EST). MDZ and the hydroxy metabolites were eluted within 10 min under the gradient elutions. Therefore, an isocratic elution, water–acetonitrile–AcOH (84:16:1), was adopted for the separation (Fig. 4A). From these chromatographic behaviors in Fig. 2A–4A, the determination of MDZ and the metabolites was performed with an isocratic elution; whereas linear gradient elutions were adopted for the other benzodiazepines (ALP and EST) and their metabolites.

The mass spectra, obtained from benzodiazepines and the possible metabolites, are shown in Figs. 2B–4B. The data indicate that the protonated-molecular ions $[M+H]^+$ of the parent drugs and almost the metabolites are strongest in all signals and seem to be suitable for the determination with SIM. The distinctive patterns of isotopic ions based upon Cl atom(s) were

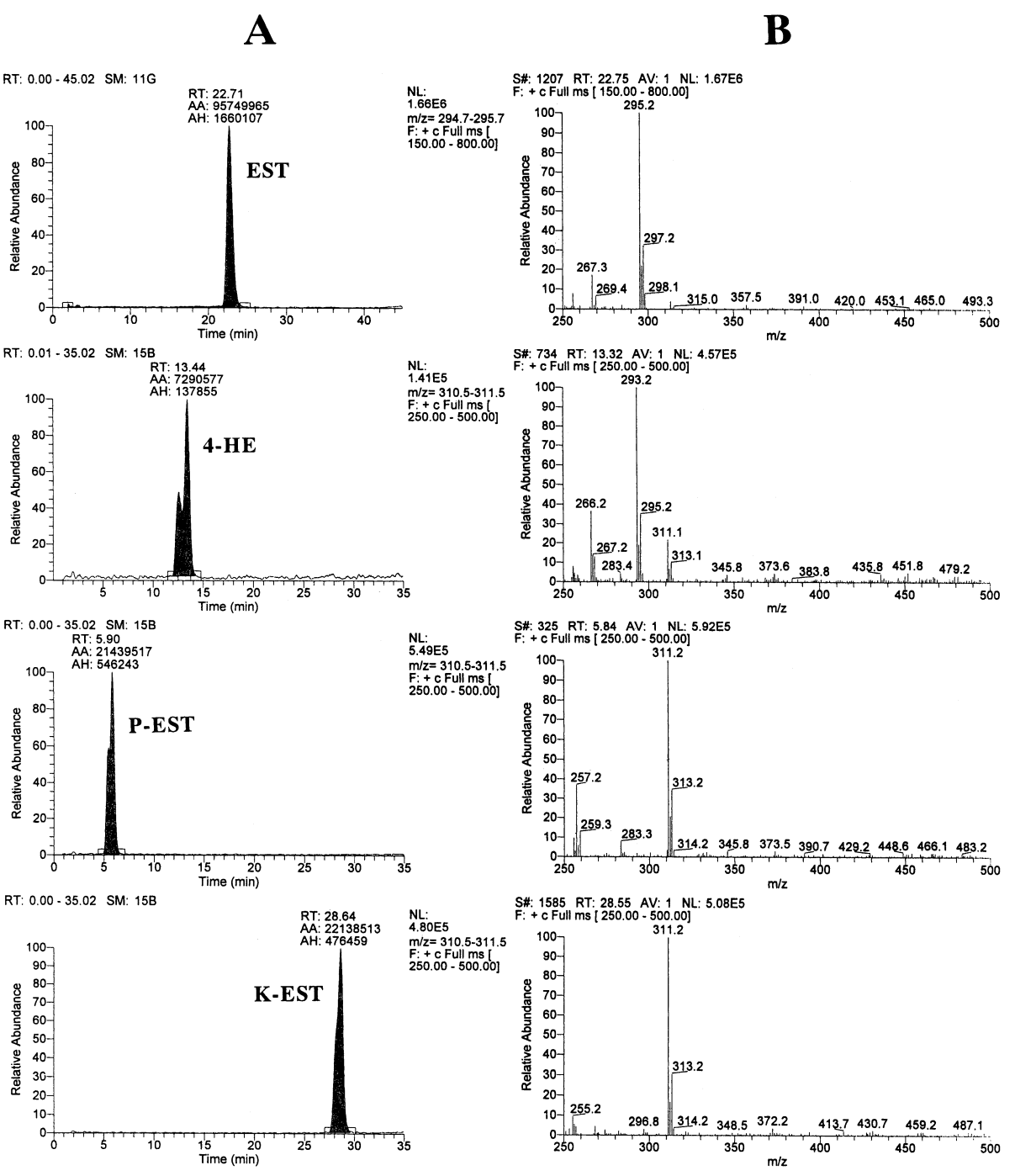

Fig. 3. Mass chromatograms and mass spectra of authentic mixture of EST and the metabolites (A) (left panels), mass chromatograms; (B) (right panels), mass spectra.

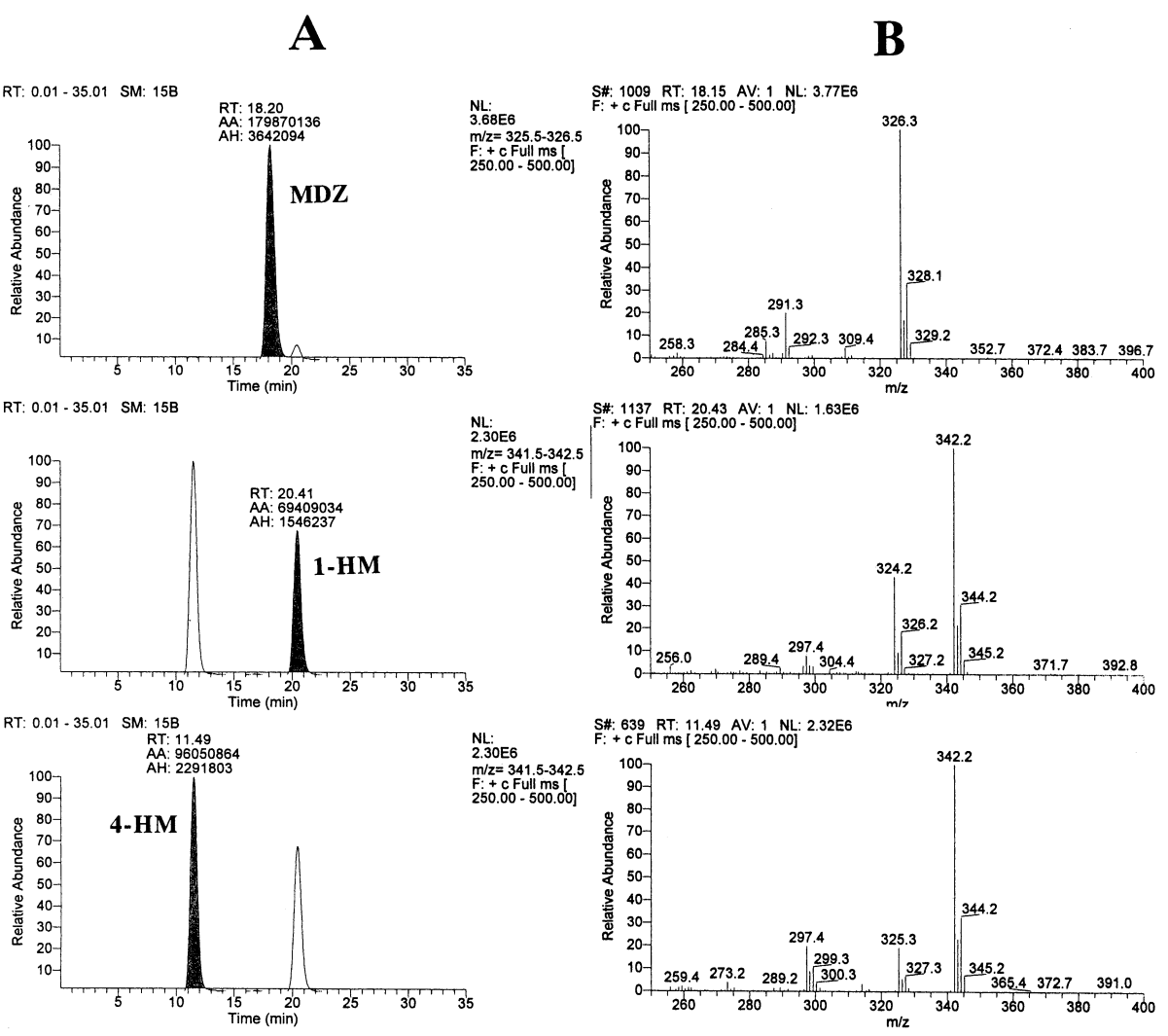

Fig. 4. Mass chromatograms and mass spectra of authentic mixture of MDZ and the metabolites (A) (left panels), mass chromatograms; (B) (right panels), mass spectra.

predominant to identify the administered drugs and their metabolites.

The calibration curves were conducted by plotted peak area (or height) ratios of IS against injected amounts of benzodiazepines and the metabolites. Good linear relations were observed with both peak area ratio and peak height ratio. In the present research, the determinations in rat hairs (shaft and root) and plasma were carried out with peak area ratio against IS. The concentration ranges and the equations of linear regression are listed in Table 1. The vertical line of the calibration curves was based upon the peak area ratios with IS; 1-HA-d_5 for the determination of ALP and the metabolites, and EST-d_5 for the determination of EST and the metabolites. For the determination of MDZ and the metabolites, EST-d_5 was used as the IS because MDZ and the metabolites labeled with deuterium were hard to obtain. Therefore, the vertical line of the calibration curve was the ratio against EST-d_5. When each 2 ng of the benzodiazepines and their metabolites was added to

approximate 10 mg of native hairs, the average recoveries with the proposed procedure were more than 88%. The precision (representative as CV value) was less than 3.2% ($n = 5$).

3.2. Determination of benzodiazepines and the metabolites in rat hair shaft

Figs. 5–7 show the mass chromatograms obtained from the extract of rat hair shafts after i.p. administration of benzodiazepines (ALP, EST and MDZ). From the comparison with authentic compounds, administered ALP and two hydroxy metabolites (1-HA and 4-HA) were identified in the hair shaft (Fig. 5). The concentrations of ALP, 1-HA and 4-HA in 1 mg hair shaft were 0.62, 0.31 and 0.76 ng, respectively. Although 1,4-dihydroxy metabolite was detected in hair shaft after administration of TZ [43,44], the peak corresponding to 1,4-dihydroxy metabolite (1,4-diHA) was not identified on the chromatogram, in spite of the similar structure to TZ. The other metabolite having the same molecular weight to 1,4-diHA, $[M+H]^+$, $m/z = 341$, was obtained from the incubation of ALP with liver microsome; however, the metabolite was not detected in rat hairs. The structure might be a N-oxide derivative (denoted as OH-O-ALP) of monohydroxy ALP (1-HA or 4-HA) because the acetylation gave the monoacetylated derivative $[M+H]_+$ $m/z = 383$ (data not shown). It was too difficult to identify the position of the N-oxide in the structure.

In the administration of EST, a more oxidized metabolite K-EST was obtained from hair shaft together with EST (Fig. 6). The hydroxy metabolites, i.e. 4-HE and P-EST, could not identify on the chromatogram, but another metabolite eluted at approximately 16 min. As the mass spectrum showed typical isotope pattern, the compound was considered to be one of the metabolites. After the acetylation of the unknown compound, the resulting derivative produced $m/z = 369$ ion corresponding to $[M+H]^+$ of monoacetyl P-K-EST (Fig. 8B). The unknown metabolite eluted near 4-HE. The N-oxide is usually retained more than original compound by reversed-phase liquid chromatography. Judging from the elution position of the unknown metabolite and the results of the acet-

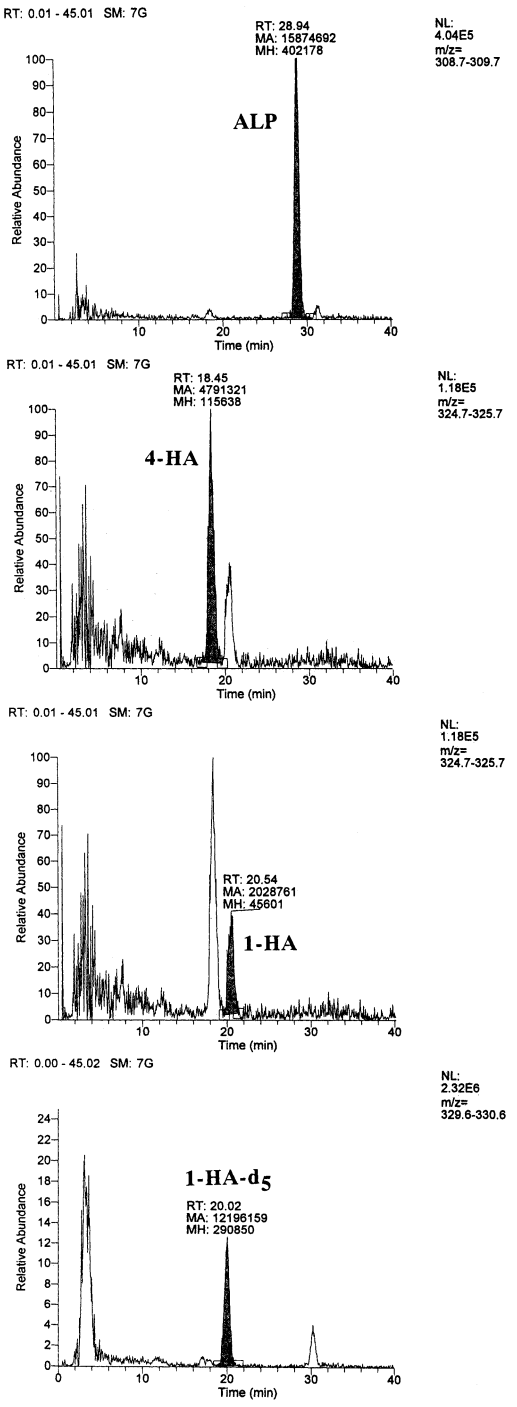

Fig. 5. Typical mass chromatograms obtained from rat hair shaft after ALP administration.

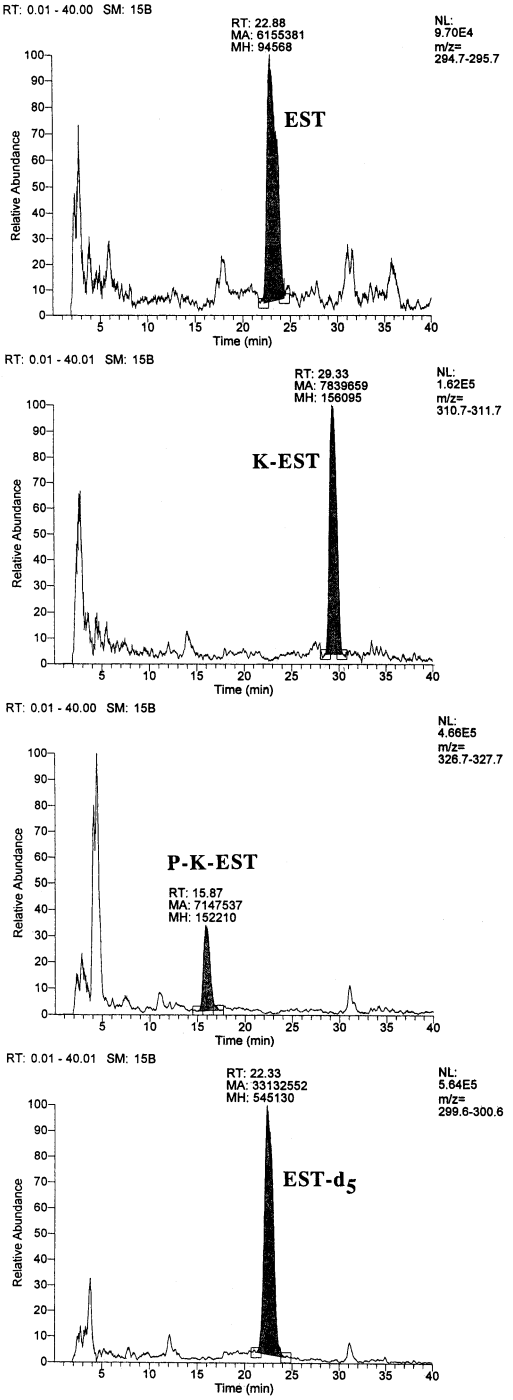

Fig. 6. Typical mass chromatograms obtained from rat hair shaft after EST administration.

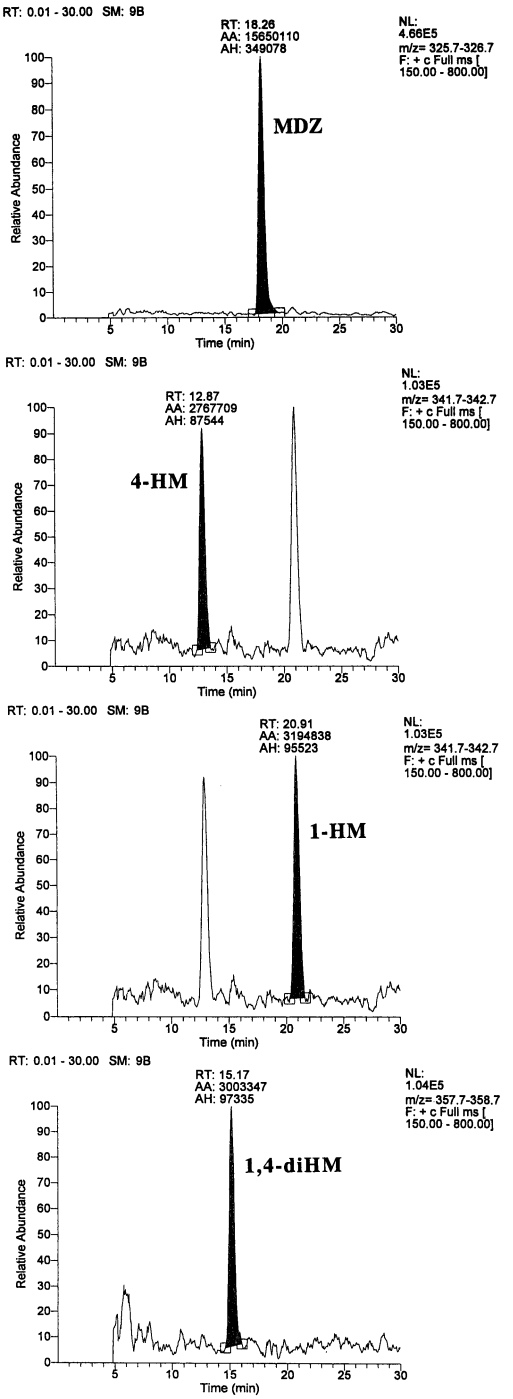

Fig. 7. Typical mass chromatograms obtained from rat hair shaft after MDZ administration.

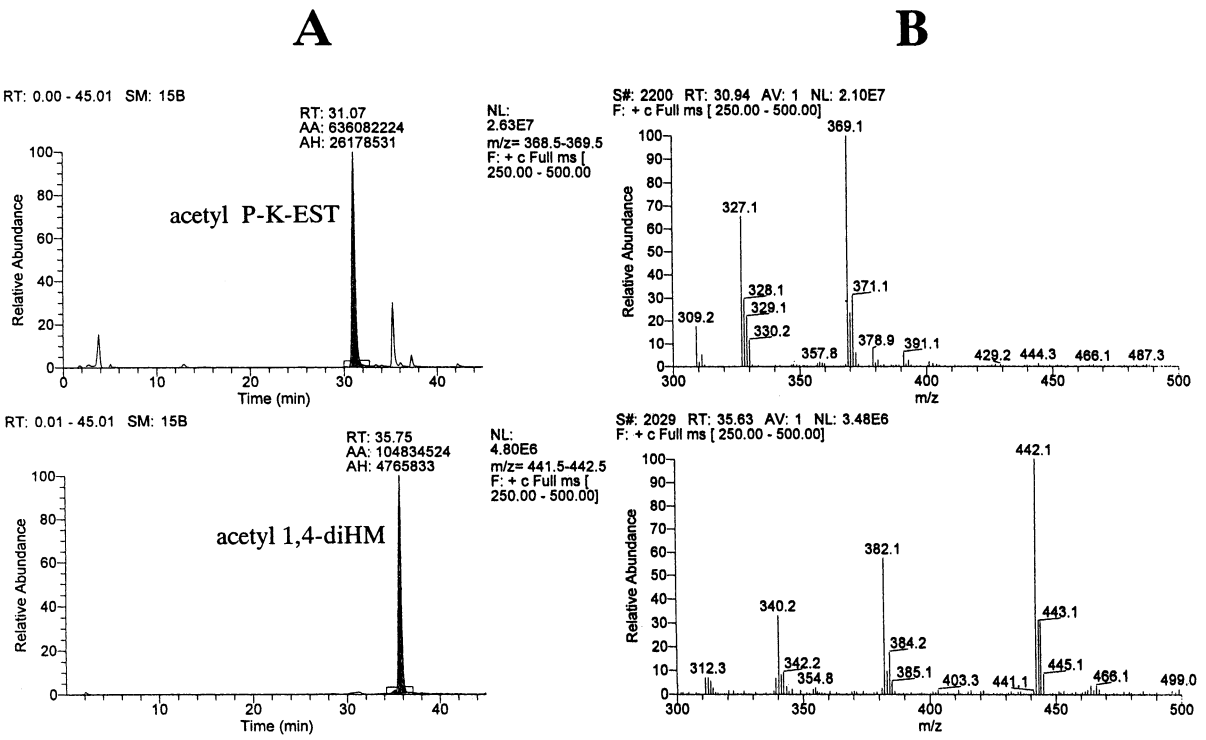

Fig. 8. Mass chromatograms and mass spectra of unknown metabolites after acetylation (A) (left panels), mass chromatograms; (B) (right panels), mass spectra.

ylation, the metabolite seems to be P-K-EST having 4-keto and phenolic OH. The concentrations of EST and K-EST were 0.30 and 0.40 ng in 1 mg hair shaft, respectively. The approximate amount of P-K-EST, calculated from the calibration curve of K-EST, was 0.39 ng/mg hair.

Since we could not obtain the deuterates of MDZ and the metabolites, EST-d_5 was used as the IS for the determination of MDZ and the metabolites in hairs. From the comparison with authentic compounds, MDZ, 1-HM and 4-HM were identified in the hair sample (Fig. 7). However, the concentrations of the three compounds were extremely low, compared with ALP and EST. A metabolite suspected as 1,4-diHM ($m/z = 358$) also appeared in the hairs. The structural elucidation was performed with acetylation. In the separation of acetylated derivative, the gradient profiles used for the analysis of the other benzodiazepines and the metabolites were adopted, because a relatively long time was necessary for the elution. The result of acetylation of the unknown metabolite supported the structure of 1,4-diHM (Fig. 8). The determination of MDZ and the metabolites was not performed due to fairly low concentrations.

3.3. Comparison of the concentration of benzodiazepines and their metabolites between hair root and plasma

The concentration difference of parent drug and the metabolites seems to be due to the incorporation ratio into hair. It is known that melanin affinity and lipophilicity of compounds are important factors for the incorporation into hairs [45–47]. As the binding ratios of TZ and the metabolites are almost comparable [43], the melanin affinity might be less effective on the incorporation into hair shafts. The lipophilicity of parent drugs and the metabolites was measured with the comparison of retention factor (k′) (data

not shown). The results suggested that high lipophilic compounds tend to be incorporated into hair shafts at higher concentrations. However, the high incorporation ratio could not be explained only the melanin affinity and the lipophilicity. The concentrations of the parent drugs and the metabolites in blood circulated seem to be also important for the incorporation into hair because hair balbs are always contacted with extracellular fluid at early time after administration. Hence, plasma concentrations at each sampling period were compared with those in hair root after single i.p. administration of benzodiazepines.

Fig. 9 shows the time courses of MDZ and the hydroxy metabolites in rat hair root and plasma after single i.p. administration of MDZ. The maximal concentrations of MDZ in the hair root and the plasma were obtained at 15 min after the administration, and then gradually decreased with time. The concentrations of the metabolites, i.e. 1-HM and 4-HM, were fairly low as comparing with MDZ. MDZ and the metabolites in the plasma disappeared at 4 h after administration. The results show MDZ was rapidly metabolized and excreted into urine. As depicted in Fig. 9A and B, the shape of curves in hair root was similar to those in plasma. The results suggest that the concentrations of MDZ and the metabolites in hair samples reflect the plasma concentrations.

The time courses of ALP and the metabolites in hair root and plasma were depicted in Fig. 10. The concentration of ALP was highest at the first sampling points in both hair root and plasma, as same results as MDZ. However, the highest concentrations of the hydroxy metabolites, 1-HA and 4-HA, were observed at 1 h after the administration in both samples. In the plasma samples, trace amounts of the metabolites which are considered to be 1,4-diHA and N-oxide of hydroxy ALP (OH-O-ALP) also appeared at every sampling times tested. However, the metabolite could be detected in hair samples. The concentrations of 4-HA in hair root were relatively higher than the plasma concentrations. It might be due to higher melanin affinity of 4-HA than 1-HA, judging from the results of TZ [43].

In the case of EST administration, K-EST was detected in the plasma at high concentrations in all sampling periods (Fig. 11B). The maximum concentration was approximately 1050 ng/200 μl plasma at 30 min. In contrast, the concentrations of parent drug and a metabolite P-K-EST were extremely low at every sampling points. The high concentrations of K-EST were observed in hair

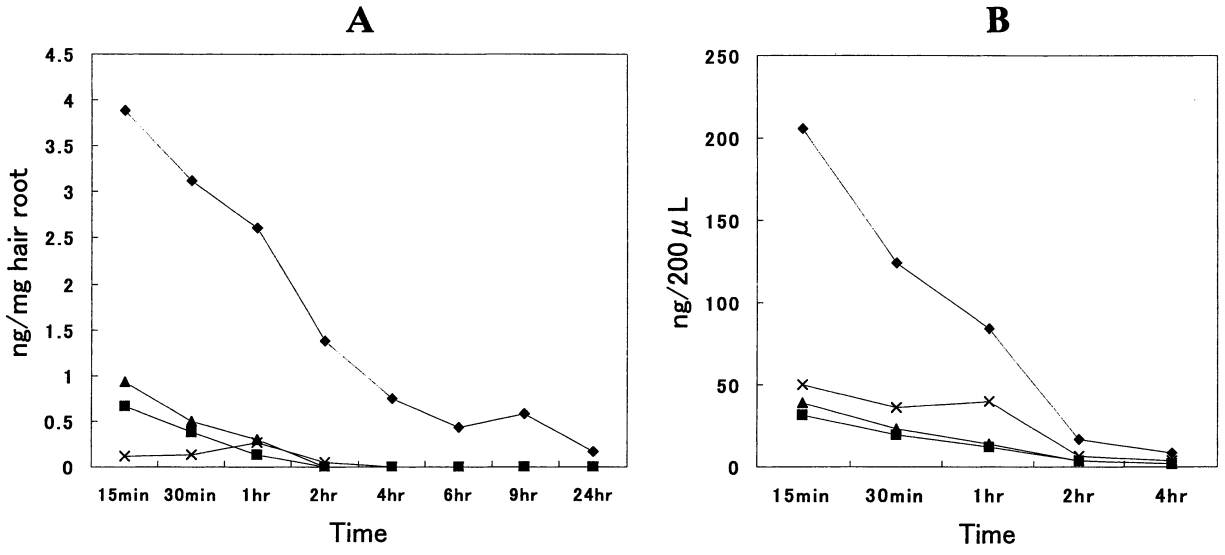

Fig. 9. Time courses of MDZ and the metabolites in rat hair root and plasma after single i.p. administration of MDZ (mean, $n = 3$). (A) Hair root; (B) plasma. ◆, MDZ; ■, 1-HM; ▲, 4-HM; ×, 1,4-diHM.

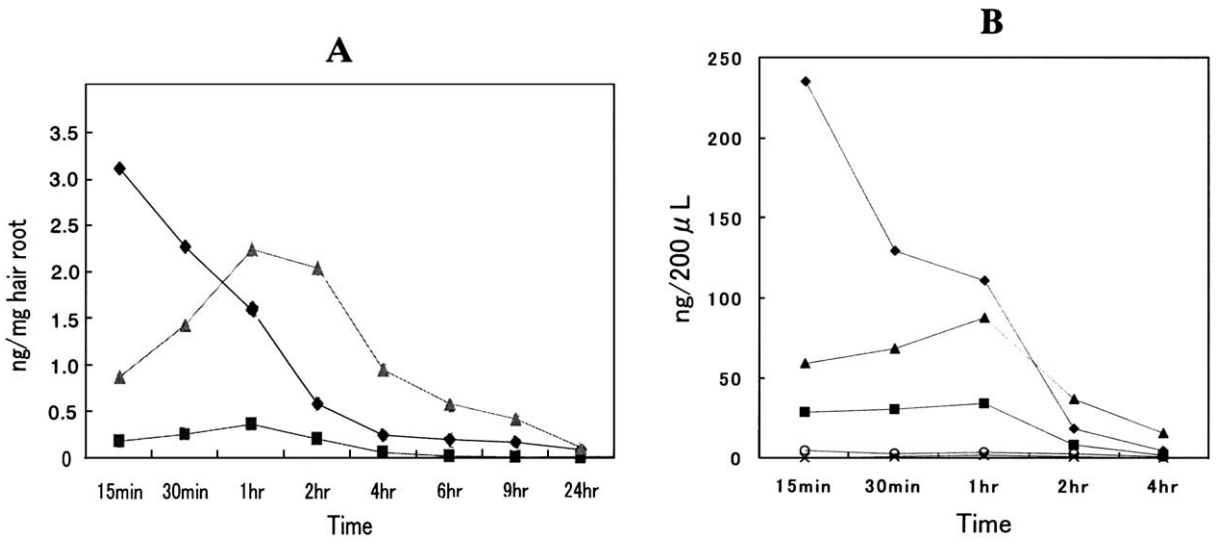

Fig. 10. Time courses of ALP and the metabolites in rat hair root and plasma after single i.p. administration of ALP (mean, $n = 3$). (A) Hair root; (B) plasma. ◆, ALP; ■, 1-HA; ▲, 4-HA; ×, 1,4-diHA; ○, OH-O-ALP.

root (Fig. 11A). The other metabolite P-K-EST was gradually increasing with time and reached maximum at 6 h after the administration. The concentration of the parent drug EST was rapidly decreased with time after administration. Inspite of limited concentration of P-K-EST in plasma, relatively high concentration was identified in hair root. The results suggest that the further oxidation of K-EST toward P-K-EST might occur in hair bulb. Judging from the results in Fig. 11, the hair concentrations of EST and the metabolites, except for K-EST, were independent of the plasma concentrations. Hence, the other factor(s) seem to be important for the incorporation into hairs.

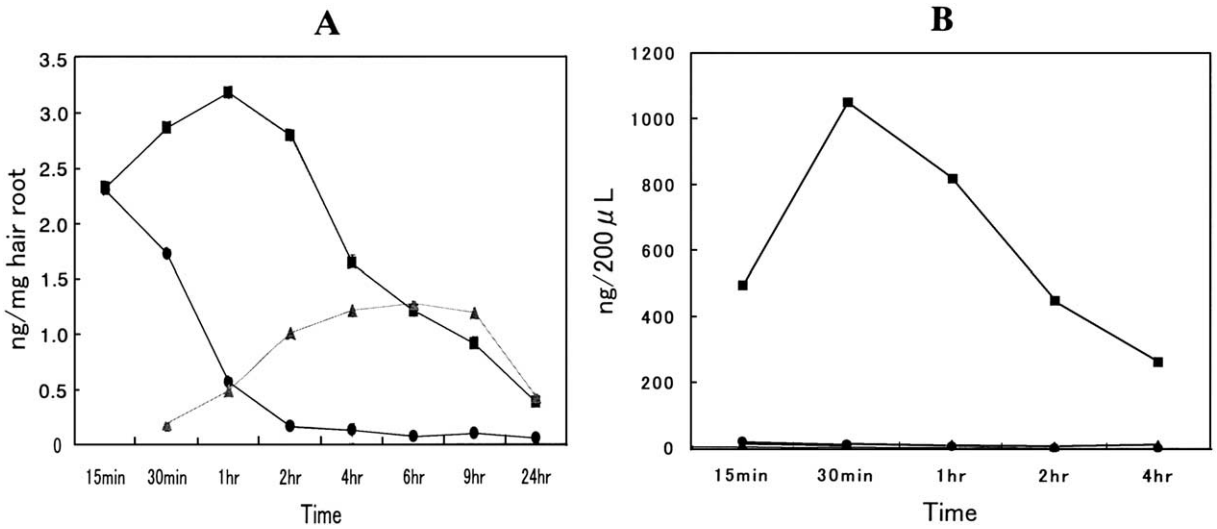

Fig. 11. Time courses of EST and the metabolites in rat hair root and plasma after single i.p. administration of EST (mean, $n = 3$). (A) Hair root; (B) plasma. ●, EST; ■, K-EST; ▲, P-K-EST.

The results of the time course study suggest that the concentrations of the benzodiazepines (ALP and MDZ) and the metabolites in hair samples mainly reflect the plasma concentrations. Consequently, the hair root could possibly be a novel specimen for metabolic study of various benzodiazepines.

4. Conclusion

Three benzodiazepines and the metabolites in rat hair samples were determined by on-line HPLC-ESI-MS. The proposed method provides trace detection of parent drugs and various kinds of metabolites without complicate pre-treatment. From the comparison between hair root and plasma, the hair concentrations of parent drugs and the metabolites are mainly dependent upon the plasma concentrations. As the method proposed is simple and sensitive, it might be useful for human hair analysis of benzodiazepine addicts. The analytical technique can also be applied to the determination of benzodiazepines and the metabolites in biological specimens such as urine and plasma. Consequently, it should allow the evaluation of pharmacokinetics of the parent drug and the active metabolites for clinicotoxicological investigations. The method utilizing hair root may be applicable for metabolic study of drugs, whereas the analysis of hair shafts seems to be useful for relative long range detection ($\sim$ 1 year) of drug and the metabolites.

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