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Journal of Chromatography A, 797 (1998) 227–236

JOURNAL OF CHROMATOGRAPHY A

Determination of sedatives and anesthetics in plasma by liquid chromatography–mass spectrometry with a desalting system

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Abstract

Though liquid chromatography–mass spectrometry (LC–MS) is a powerful tool for analysis of drugs and their metabolites, it does not allow the use of a non-volatile buffer such as phosphate buffer. We used a column-switching desalting system in combination with atmospheric pressure chemical ionization LC–MS for analysis of sedatives and anaesthetics. The drugs examined were flumazenil, butorphanol, midazolam, lorazepam, phenobarbital and flunitrazepam. The separation was carried out on a reversed-phase column using acetonitrile–0.1 *M* phosphate buffer as a mobile phase. The mass spectra are almost the same as those obtained by direct analysis and the molecular ions were clearly observed. In the analysis, phosphate buffer was completely removed with the trapping column and did not interfere with ionization of the drugs in MS. The chiral separation of lorazepam was achieved on a chiral column with UV, optical rotatory detection and MS. This method is sufficiently sensitive and accurate for the pharmacokinetic studies of these drugs in biological samples.

Keywords: Enantiomer separation; Desalting methods; Sedatives; Anaesthetics

1. Introduction

The combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) (LC–MS) is a powerful tool for analysis of drugs and their metabolites in biological samples. Many of the LC–MS interfaces have been developed for connecting a LC system with an MS instrument requiring a high vacuum condition. However, these interfaces do not allow the use of a non-volatile buffer such as phosphate buffer, which is most generally used in conventional LC, because non-volatile buffer components interfere the ionization in LC–MS. Thus, the optimal mobile phases for separation in LC have not been used in LC–MS.

We have investigated analysis of sedatives and anesthetics in biological fluids by using atmospheric pressure chemical ionization LC–MS (LC–APCI-MS) [1,2]. The use of a non-volatile mobile phase is sometimes necessary for the study of the metabolites of these drugs. In this paper, we described desalting system based on a column-switching method in combination with LC–APCI-MS. This system used two columns, an analytical column for separation of target compounds and a trapping column for desalting. In this system, a non-volatile buffer such as phosphate buffer can be used as a mobile phase to obtain the optimal separation on the analytical column. After desalting procedure, we can use most

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0021-9673/98/$19.00
PII S0021-9673(97)00928-X

appropriate solvent to eluate the analytes and to achieve the high ionization efficiency in the MS section. Additionally, this method can be used for the concentration of target compounds in biological fluids using a trapping column with a concentrating function.

The drugs examined were flumazenil, butorphanol, midazolam, lorazepam, phenobarbital and flunitrazepam. These drugs have been used in combination or used for premedication for balanced anesthesia and sedation. The chiral separation of lorazepam was achieved with a chiral column in the LC–MS system.

This method provides an important tool for the pharmacokinetic studies of these drugs.

2. Experimental

2.1. Material and reagents

The drugs used were obtained from commercial sources as follows: flumazenil and flunitrazepam (Rohypnol) (Hoffman-La Roche, Nutley, NJ, USA); butorphanol tartrate (Stadol; Bristol-Myers Squibb, Tokyo, Japan); midazolam (Dormicum; Yamanouchi Pharmaceutical, Tokyo, Japan); lorazepam (Wypax) and phenobarbital (Sankyo, Tokyo, Japan). The structures of these drugs are shown in Fig. 1. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of analytical-reagent grade.

2.2. Apparatus

Fig. 2 shows a schematic diagram of the LC–APCI-MS (Hitachi M-1200H) system and the desalting system consisting of two HPLC pumps (Hitachi L-7100) which were in operation under a controller (Hitachi M-1211).

The analytical column was Capcell Pak-ODS.UG120 (150×4.6 mm I.D., Shiseido, Tokyo, Japan) and the effluent was monitored with a UV detector (Hitachi L-7400). The trapping column was

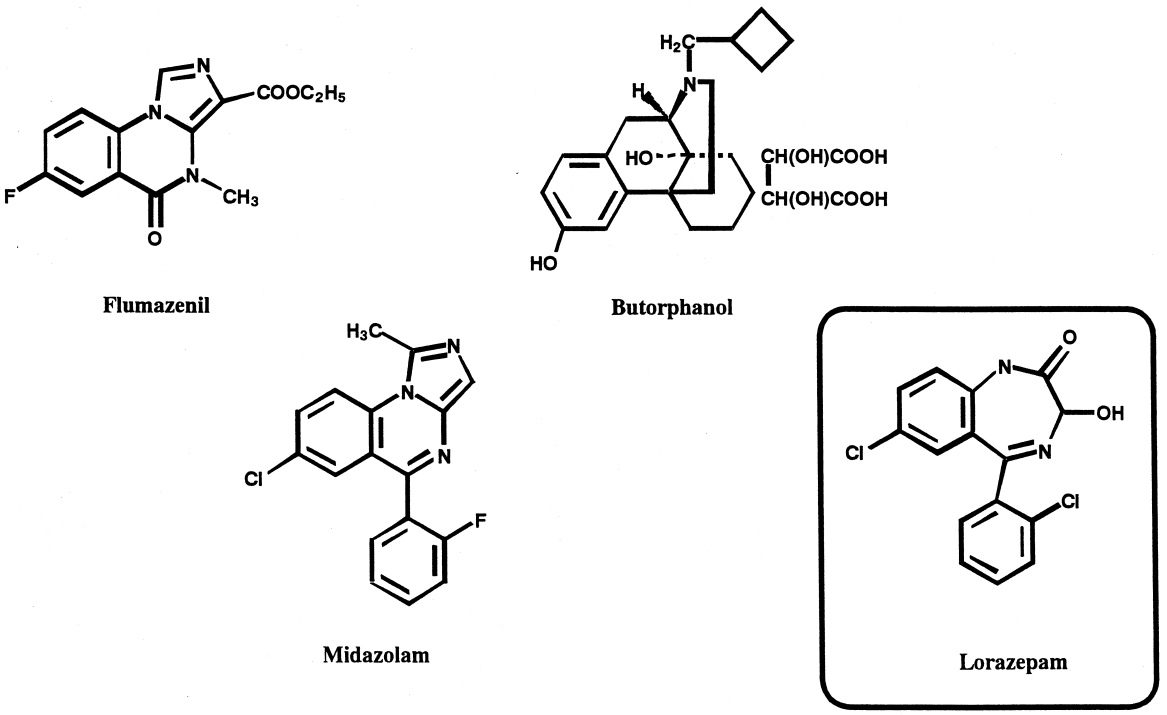

Fig. 1. The structures of flumazenil, butorphanol, midazolam and lorazepam.

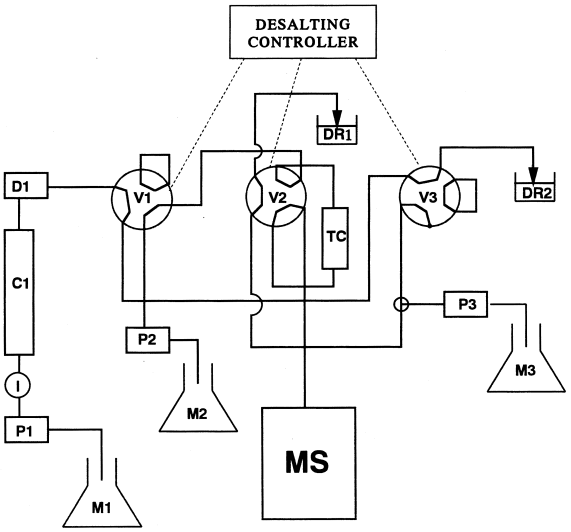

Fig. 2. A schematic diagram of the LC–APCI-MS (Hitachi M-1200H) and the desalting system. P=pump; M=mobile phase; I=injector; C=column; D=UV detector; V=six-port switching valve; DR=drain; MS=mass spectrometer.

a Capcell Pak-ODS.UG120 (30×4.6 mm I.D., Shiseido).

The chiral column used for separation of lorazepam was a Chiralpak AS and OD (150×4.6 mm I.D., Daicel, Tokyo, Japan) and the effluent was monitored with a optical rotation detector (OR-990, Jasco, Tokyo, Japan).

2.3. Sample preparation

Sample solutions were prepared by dissolving known amounts of the drugs in the mobile phase or human plasma. To remove proteins prior to injection, a plasma sample was pretreated with solid-phase extraction as described previously [1,2].

2.4. HPLC conditions

The mobile phase for the analytical column was acetonitrile–0.1 *M* potassium phosphate buffer (3:7) and the eluate was monitored at 220 nm. The flow-rate was 1 ml/min and the column temperature was 40°C.

In the desalting system, the washing (desalting) solvent of the trapping column was water and the backflush solvent introduced into the MS system was

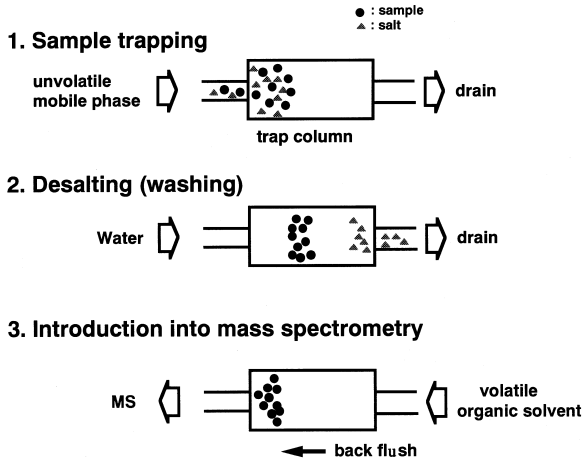

Fig. 3. The schematic illustration of desalting procedures.

methanol. A schematic illustration of desalting procedures is shown in Fig. 3.

The mobile phase for chiral separation was *n*-hexane–2-propanol–ethanol (5:5:1) and the eluate was monitored at 254 nm. The flow-rate was 1 ml/min and the column temperature was 25°C.

2.5. MS conditions

The drift voltage was 40 V and multiplier voltage was 1800 V. The desolvation temperature was 390°C and nebulizer temperature was 170°C. The backflush solvent was introduced into the MS system at the flow-rate of 1 ml/min.

3. Results and discussion

There have been several reports for the pharmacokinetic studies of sedatives and anaesthetics [3–10]. Many of these reports used a reversed-phase column and a non-volatile mobile phase such as phosphate buffer for the HPLC determination of the drugs. Non-volatile mobile phase cannot be used directly in the LC–MS system, a powerful tool for the pharmacokinetic studies of these drugs. We then examined the desalting system with the column-switching method.

The desalting procedure (Fig. 3) was follows; the sample was injected onto the analytical column (C1),

and the eluate was monitored with UV detector (D1) at 220 nm. The fraction containing the target drug was introduced into the trapping column (TC) by switching and washed with water. Then drug was backflushed by methanol from the trapping column into the MS system. The trapping time and the washing time were 17 s each and backflush time was 30 s. These trapping procedures were repeated at times of the number of the drugs in the sample.

3.1. Mass chromatograms and mass spectra

Fig. 4 shows mass and UV chromatograms of a mixture of flumazenil, butorphanol and midazolam obtained using the desalting system in combination with LC–APCI-MS. The well resolved chromatograms were obtained with acetonitrile–0.1 *M* potassium phosphate buffer as the eluent at a flow-rate of 1 ml/min, and the mass spectra are shown in Fig. 5. The spectra are almost the same as those obtained by direct analysis. The quasi-molecular ions $[M+H]^+$ of flumazenil, butorphanol and midazolam were clearly observed at m/z 304, 328 and 326, respectively, as base peaks.

Fig. 6 shows mass and UV chromatograms and the mass spectrum of phenobarbital in the negative-ion mode. The molecular ion $[M-H]^-$ of phenobarbital was clearly observed at m/z 231 as a base peak.

Flunitrazepam is prescribed for insomnia and as a pre-operative anesthetic. It was reported that the US Drug Enforcement Administration (DEA) will put the ''date rape'' drug, flunitrazepam, in the same category as heroin and LSD as abuse of the drug is becoming more serious. The mass and UV chromatograms and the mass spectrum of flunitrazepam are shown in Fig. 7. The molecular ion $[M+H]^+$ of flunitrazepam was observed at m/z 314 as a base peak.

As phosphate buffer was completely removed from the trapping column in the above experiments, there was no adverse effect of phosphate on the mass chromatograms and mass spectra.

3.2. Calibration curves and precision

The linearities between the amount of the drugs and the peak area in the mass chromatogram in the selected-ion monitoring (SIM) mode were obtained up to 1 μg. The relationship calculated between the peak area (y) and the amounts of the drug (x) and the correlation coefficients (r) were as follows: flumazenil: $y=2.00x+0.16$ ($r^2=0.970$); butor-

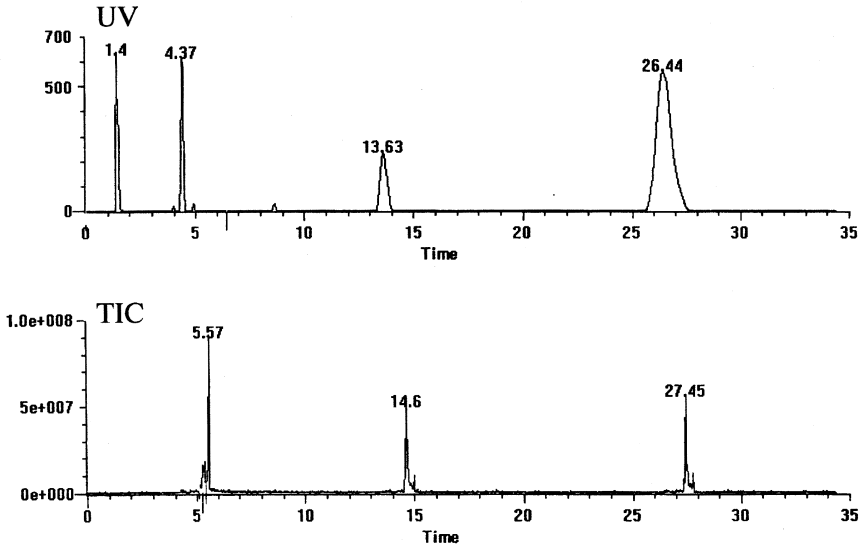

Fig. 4. Mass and UV chromatograms of a mixture of flumazenil, butorphanol and midazolam obtained using desalting system in combination with LC–APCI-MS. Chromatographic conditions are described in Section 2.4.

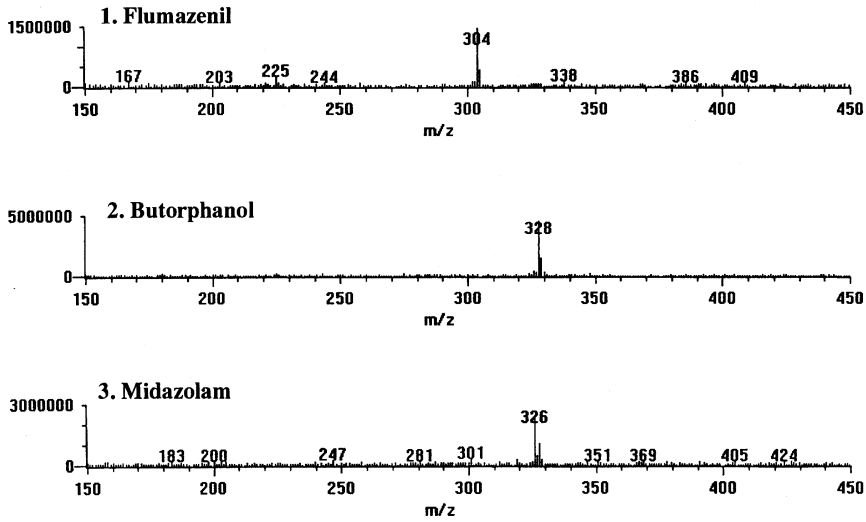

Fig. 5. Mass spectra of a mixture of flumazenil (1), butorphanol (2) and midazolam (3).

phanol: $y=1.00x+0.23$ ($r^2=0.983$); midazolam: $y=0.95x+0.100$ ($r^2=0.997$).

The lower limits of quantification for fulmazenil, midazolam and butorphanol were 1.4, 1.3 and 10.7 ng, respectively, at a signal-to-noise ratio of 3.

Vries et al. [10] reported on a comparison of HPLC, GC and GC–MS for the determination of total and unbound midazolam in human plasma. It was concluded that GC was preferred for routine plasma assays with a sensitivity of 0.02 μg/ml, HPLC analysis was less sensitive (0.1 μg/ml) and GC–MS was used for analysis validation.

The present method is sufficiently sensitive and accurate to measure pharmacokinetic parameters.

A column-switching method can also be used for the concentration of target compounds in biological

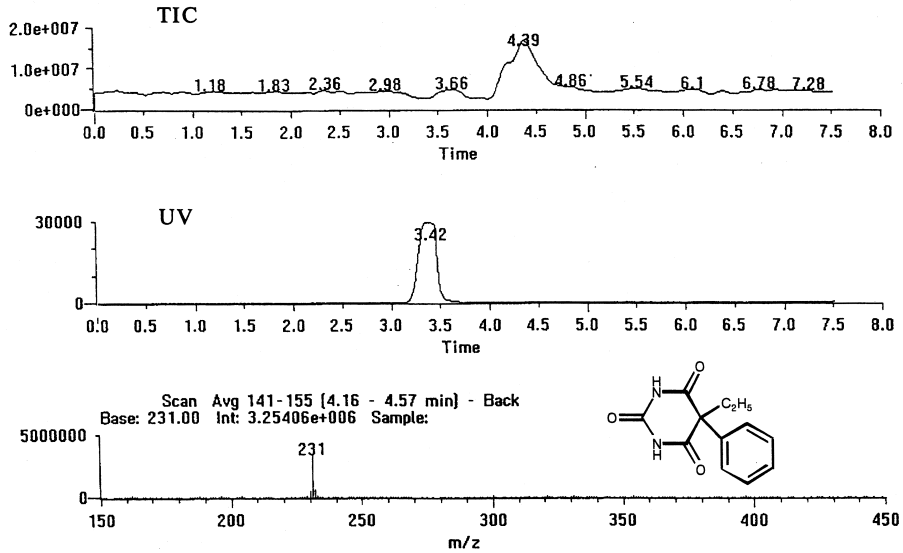

Fig. 6. Mass and UV chromatograms and the mass spectrum of phenobarbital in the negative-ion mode.

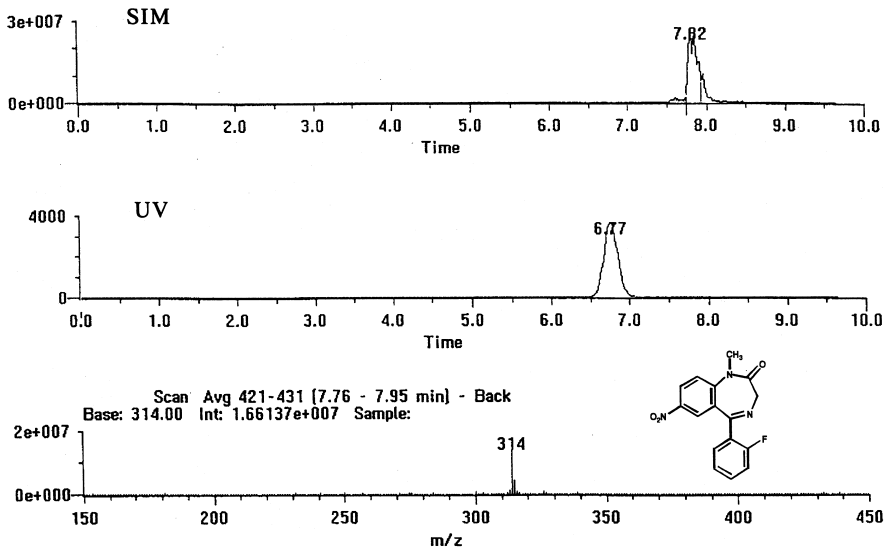

Fig. 7. Mass and UV chromatograms and the mass spectrum of flunitrazepam.

fluids using trapping column in the concentrating mode. A trace amount of ascorbic acid in rat tissue was determined using this method (the data will be report elsewhere).

The precision of the method was established from five assays using LC–MS with the desalting system. The mean values and relative standard deviations (R.S.D.s) are shown in Table 1. The R.S.D. values of the retention times were less than 1% and those of

Table 1
Precision of the LC–MS assay using desalting system

	Retention time (min)	R.S.D. (%)	Peak area[a] ($\cdot 10^4$)	R.S.D. (%)
Flumazenil[b]	5.33	0.32	113.2	5.01
Butorphanol[b]	14.03	0.39	192.5	3.21
Midazolam[c]	8.58	0.14	258.4	4.95

[a] Total ion chromatogram.
Eluent: [b] CH_3CN–NaH_2PO_4 (30:70); [c] CH_3CN–NaH_2PO_4 (40:60), flow-rate: 1.0 ml/min.

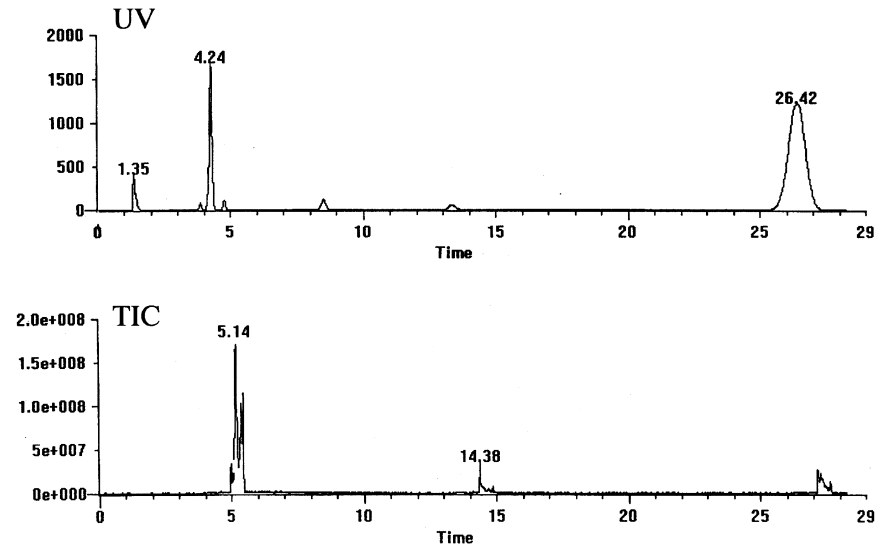

Fig. 8. Mass and UV chromatograms of an extract of human plasma spiked with a mixture of flumazenil, butorphanol and midazolam.

the peak areas on total ion chromatograms were less than 5%.

3.3. Plasma sample

Fig. 8 shows the mass and UV chromatograms of an extract of human plasma spiked with a mixture of flumazenil, butorphanol and midazolam. In the mass spectra shown in Fig. 9, the molecular ions, $[M+H]^+$ of flumazenil, butorphanol and midazolam were clearly observed at m/z 304, 328 and 326, respectively, as base peaks. There was no disturbance from plasma components.

3.4. Chiral separation

The assay of drug enantiomers in biological samples is very important, since the enantiomers of a drug often show different in vivo behavior and hence have different pharmacological activity [11].

Fig. 10 shows UV chromatograms of lorazepam on a Chiralpak AS and OD column under the conditions described in Section 2.4. The OD column is cellulose 3,5-dimethylphenylcarbamate, and AS is amylose *S*-methylbenzylcarbamate. The good resolution of lorazepam enantiomers was obtained on Chiralpak AS.

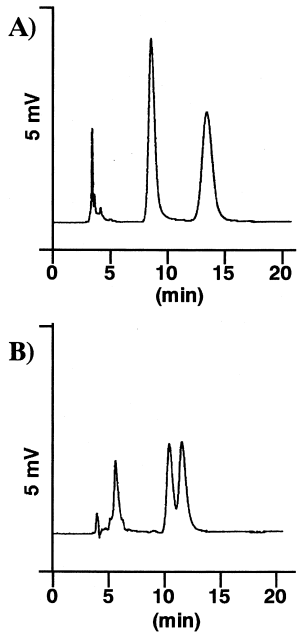

Fig. 10. UV chromatograms of lorazepam on Chiralpak AS (A) and OD (B) under the conditions described in Section 2.5.

Table 2 shows selectivity factor (α) and resolution (R_s) of lorazepam enantiomers on the chiral columns. R_s on Chiralpak AS was not less than 1 and higher than on OD.

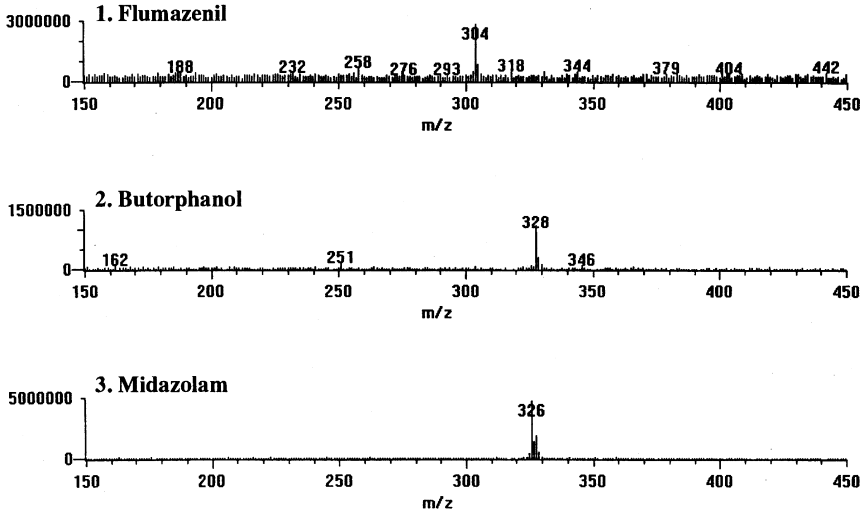

Fig. 9. Mass spectra of an extract of human plasma spiked with a mixture of flumazenil (1), butorphanol (2) and midazolam (3).

Table 2
Chromatographic parameters for the separation of lorazepam

Column	Mobile phase (v/v)	k'_1	k'_2	α	R_s
OD	*n*-Hexane–EtOH (50:50)	1.47	1.57	1.07	1.03
	n-Hexane–2-Propanol (50:50)	3.42	3.93	1.15	1.60
	n-Hexane–2-Propanol–EtOH (50:50:10)	1.78	1.97	1.11	1.38
AS	*n*-Hexane–EtOH (50:50)	1.61	2.46	1.52	4.32
	n-Hexane–2-Propanol–EtOH (50:50:10)	2.54	3.99	1.57	3.75

Capacity factor: $k'=t_r/t_0$, k'_1=capacity factor for first-eluted enantiomer.
Selectivity factor: $\alpha=k'_1/k'_2$.
Resolution: $R_s=2(t_{r2}-t_{r1})/(w_1+w_2)$ where w is the baseline peak width.

Fig. 11A and Fig. 12 show the mass chromatogram and the mass spectrum of lorazepam on the AS column. The molecular ion, $[M+H]^+$ was clearly observed at m/z 321 as a base peak. The peak at m/z 303 should be due to fragment ion $[(M+H)-H_2O]^+$. Fig. 11B shows the mass chromatogram of an extract of human plasma spiked with lorazepam. The well resolved chromatogram with chiral separation was obtained without any influence of endogenous compounds in plasma.

The chromatogram by the optical rotatory detector is shown in Fig. 13. The (−)-enantiomer of lorazepam is eluted faster than (+)-enantiomer on the AS column. The (+)-enantiomers of 3-substituted 1,4-benzodiazepines have been found to correspond to the *S*-enantiomers [12,13].

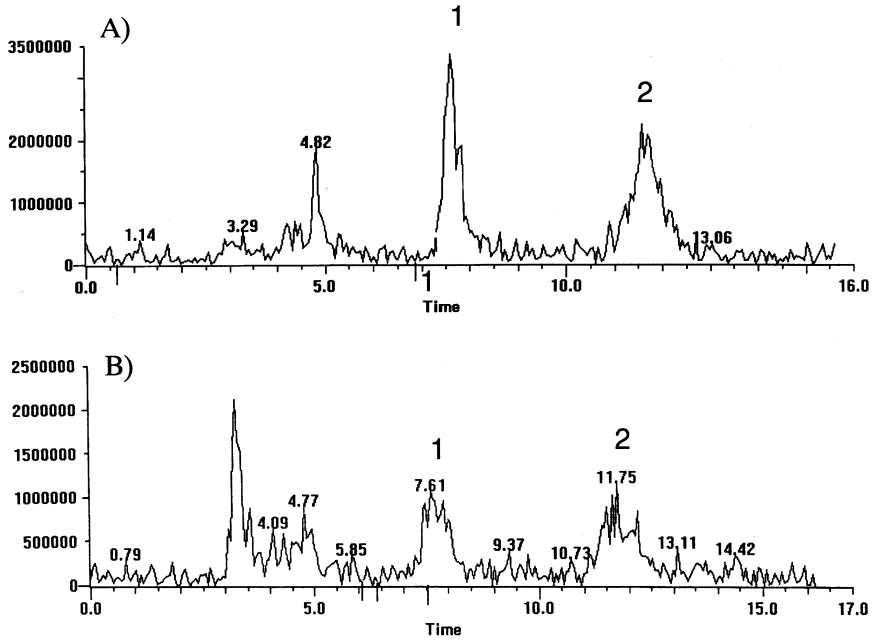

Fig. 11. (A) Mass chromatogram of lorazepam enantiomers on the AS column. (B) Mass chromatogram of an extract of human plasma spiked with lorazepam.

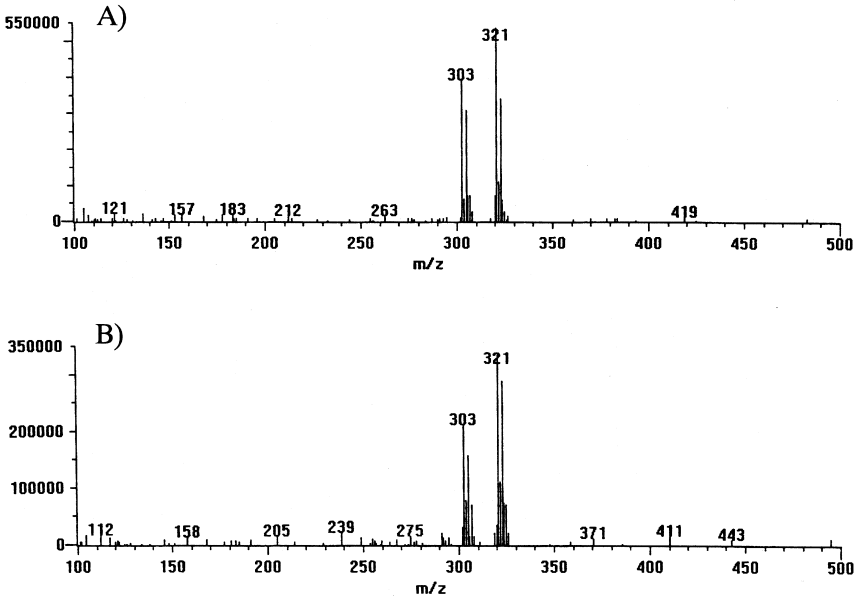

Fig. 12. Mass spectrum of lorazepam enantiomers on the AS column.

4. Conclusions

Knowledge of the pharmacokinetics of drugs should be necessary in clinical practice. LC–MS is a powerful tool for analysis of drugs and their metabolites. We used a column-switching desalting system in combination with LC–MS for analysis of sedatives and anaesthetics. In the analysis, phosphate buffer was completely removed with the trapping column and did not interfere with ionization of the drugs in MS. The present method is useful for the pharmacokinetic studies of drugs in biological samples including their enantiomers.

We were successful in enantiomeric separation and identification of lorazepam by optical rotatory detection and MS.

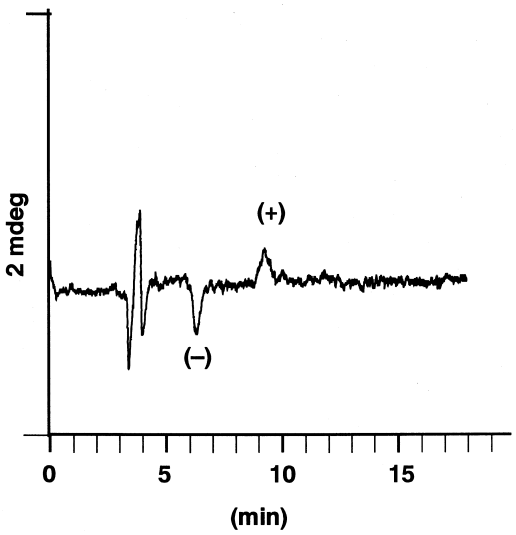

Fig. 13. The chromatograms obtained using optical rotation detection.

Acknowledgements

The authors thank Dr. Takashi Nishikawa of Kitasato University for valuable comments, and Ryoko Atsumi for technical assistance.

References

[1] H. Kanazawa, Y. Nagata, Y. Matsushima, N. Takai, H. Uchiyama, R. Nishimura, A. Takeuchi, J. Chromatogr. 631 (1993) 215.

[2] H. Kanazawa, R. Nishimura, N. Sasaki, A. Takeuchi, N. Takai, Y. Nagata, Y. Matsushima, Biomed. Chromatogr. 9 (1995) 188.
[3] L. Oliveros, C. Minguillon, C. Billaud, J. Pharm. Biomed. Anal. 10 (1992) 925.
[4] X.-L. Lu, S.K. Yang, J. Chromatogr. A 666 (1994) 249.
[5] N. Mano, Y. Oda, H. Ohe et al., J. Pharm. Pharm. Biomed. Anal. 12 (1994) 557.
[6] T. McDonnell, M.T. Kelly, M.R. Smyth, Anal. Lett. 26 (1993) 1547.
[7] R.M. Hanna, R.E. Borchard, S.L. Schmidt, J. Vet. Pharm. Therap. 11 (1988) 84.
[8] L.L. Needham, M.M. Kochhar, J. Chromatogr. 114 (1975) 220.
[9] A. Nolan, J. Reid et al., Vet. Sci. 60 (1996) 262.
[10] J.X. de Vries, J. Rudi, I. Walter-Sack, Bio. Chromatogr. 4 (1990) 28.
[11] K. Chan, R.D.M. Jones, J. Chromatogr. 619 (1993) 154.
[12] A. Corbella, P. Gariboldi, G. Jommi, A. Forgione, F. Marcucci, P. Martelli, E. Mussini, F. Mauri, J. Chem. Soc., Chem. Commun. XX (1973) 721.
[13] W.H. Pirkle, A. Tsipouras, J. Chromatogr. 291 (1993) 291.