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Mass Fragmentographic Determination of 1-Piperidino-2,4′-dimethylpropiophenone(Mydocalm) by Use of 1:1 Mixture Technique

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Artificially created cluster technique has been used for the quantitative determination of serum levels of 1-piperidino-2,4′-dimethylpropiophenone (Mydocalm). A mixture of unlabeled drug and stable isotope labeled drug (Mydocalm-^{15}N) was orally administered for identification of Mydocalm. Decadeuterium labeled Mydocalm (Mydocalm-d_{10}) was prepared and used as an internal standard and carrier for determination. A 100 mg dose of Mydocalm has been given orally to 3 subjects.

Introduction

1-Piperidino-2,4′-dimethylpropiophenone(Mydocalm) has been found to be effective as a central acting muscle relaxtant by Pórszász, *et al.*[2] The clinical efficiency of Mydocalm has been recognized also in Japan. On the other hand, it is required to develope a specific, sensitive and accurate analytical method for the investigation of drug pharmacodynamics and metabolism.

In the previous work,[3] a microdetermination method for Mydocalm in human blood has been devised by using gas chromatograph equipped with electron capture detector (ECD). The method involves conversion of Mydocalm to its pentafluorobenzyl derivative, which is extremely sensitive to ECD, with pentafluorobenzylmagnesium bromide. This technique is useful for enhancing electron affinity of carbonyl compounds such as alkylphenone-type drugs. However, it requires a tedious operation to eliminate the biological substances and excessive reagent, and moreover the reagent should be prepared freshly in every use for this technique.

For the purpose of improving such disadvantages to gas chromatographic analysis, mass fragmentography and mass chromatography have been employed; especially, these techniques in combination with internal standard labeled with stable isotope have recently utilized for quantitative determination of biologically important substances or drugs of interest.[4] A great many papers using this internal standard technique for the quantitative determination of drugs and biological substances by mass fragmentography have been reported as reviewed by Gordon and Frigerio.[5]

On the other hand, for the complete discrimination of trace amount of interested drugs from a great quantity of endogenous substances in biological fluids, mass fragmentography with one to one intensity ratio technique has attracted attention in the field of clinical pharmacology.[6]

1) Location: *3-31, Shimo, Kita-ku, Tokyo, Japan.*
2) J. Pórszász, K. Nador, K.G. Pórszász, and T. Barankay, *Arzneimittel-Forschung*, **11**, 257 (1961).
3) M. Ishibashi, G. Idzu, and H. Miyazaki, 92nd Annual Meeting of Pharmaceutical Society of Japan, Osaka, April, 1972.
4) B. Samuelsson, M. Hamberg, and C.C. Sweeley, *Anal. Biochem.*, **38**, 301 (1971).
5) A.E. Gordon and A. Frigerio, *J. Chromatog.*, **73**, 401 (1972).
6) D.R. Knapp, T.E. Gaffney, and R.E. McMahon, *Biochem. Pharmacol.*, **21**, 425 (1972).

In the previous report,[7] we have applied this technique to the analysis of biological substances. The artificially created ion cluster was prepared with a mixture of protium and deuterium labeled reagents (1:1) in the process of the derivatization. Peaks monitored with the ion cluster were observed as a single peak; the objective peaks should be discriminated easily from other peaks. Therefore, the technique is of great use for insuring the reliability of analytical result.

For improving an accuracy of the microdetermination method by this new identification technique, it is indispensable to use an isotope-labeled internal standard because it enables to compensate the losses in the processes of extraction, clean-up and derivatization.

In the present paper, the stable isotope-labeled compounds are used as a marker in ion cluster technique and also an internal standard for the microdetermination of Mydocalm.

Experimentals

Mass Spectrometry and Mass Fragmentography——LKB-9000s GC-MS systems equipped with multiple ion detectors (LKB-9060 and 9066) and data processing system were employed. The column was 2 m × 2.5 mm glass coil with 3% OV-3 (Ohio Valley Co., U.S.A.) on Chromosorb W-HP 80—100 mesh (Applied Science Lab., U.S.A.).

The temperature of column oven was maintained at 190°. The flow rate of carrier gas (Helium) was 30 ml/min. The temperature of injection port and separator was 230°, and ionization source was kept at 250°. Accelerating voltage was 3.5 kV. The ionization energy and trap current were 20 eV and 60 μA, respectively.

Samples and Reagents——All the reagents and solvents used in this research were of analytical grade and were used without further purification. Diazomethane was prepared from *p*-toluenesulfonyl-N-methyl-N-nitrosoamide.

Hydroxylamine-^{15}N hydrochloride was purchased from Daiichi Pure Chemicals Co. and Merck sharp and Dohme (Japan) Co., and their isotope purities were 52%. Mydocalm used as an standard material was obtained by recrystalization.

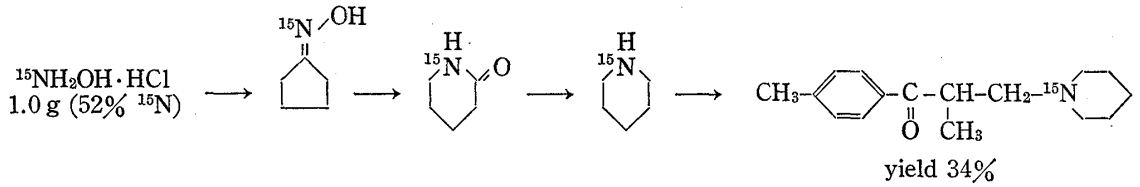

Fig. 1. Synthetic Route of Labeled Mydocalm with Nitrogen-15

The synthesis of 1-piperidino[^{15}N]-2,4′-dimethylpropiophenone (Mydocalm-^{15}N) was summerized in Figure 1. Hydroxylamine-^{15}N was converted to cyclopentanone oxime. Conversion to δ-valerolactam-^{15}N was followed by Beckmann rearrangement. δ-Valerolactam-^{15}N was reduced to piperidine-^{15}N hydrochloride with $LiAlH_4$. Mydocalm-^{15}N was synthesized by Mannich reaction using 4′-methyl-propiophenone, paraformaldehyde and piperidine-^{15}N hydrochloride.

1-Piperidino[d_{10}]-2,4′-dimethylpropiophenone (Mydocalm-d_{10}) was prepared according to the above mentioned method using decadeuteropiperidine hydrochloride which was obtained by catalytic hydrogenation of perdeuteropyridine with deuterium gas.

A mixture for administration was prepared by diluting Mydocalm-^{15}N with non-labeled Mydocalm until both ion intensities of *m/e* value 98 and 99 became almost equal.

Procedure——The volunteers were given with a single oral dose of 100 mg of the mixture of Mydocalm and Mydocalm-^{15}N. Blood specimens were taken at 30 minutes after administration, and 1, 2, 3, 4, and 7 hours.

To 1.0 ml of serum in 50 ml centrifuging tube, 1.0 ml of internal standard solution (1.0 μg Mydocalm-d_{10}/ml), 1 ml of water and 0.1 ml of conc. ammonia water were added and mixed. Then, 30 ml of ethanol was added. The tube was shaken well by hand and allowed to stand for 30 minutes, and then centrifuged for 10 minutes at 3000 rpm. The supernatant was evaporated to dryness below 25° under reduced pressure. The residue was added with 5 ml of benzene and 5 ml of saturated potassium carbonate solution, shaken well and transferred into a separatory funnel. This extraction procedure was repeated twice. The benzene layer was separated and washed with water until the washing did not colour a litmus paper blue. The

7) M. Inoue, M. Ito, M. Ishibashi, and H. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), 22, 1949 (1974).

benzene layer was shaken well with 5 ml of 0.01N HCl. The acid layer was transferred into a 10 ml glass stoppered tube, and added with 0.5 ml of saturated potassium carbonate solution and 1 ml of *n*-hexane. The tube was shaken for 2 minutes and then centrifuged for 10 minutes at 3000 rpm. The *n*-hexane layer was transferred into an another tube and dried over anhydrous potassium carbonate. This hexane solution was subjected to a gas chromatograph–mass spectrometer.

Results and Disccusion

Preparation of Mixture of Drug and Stable Isotope Labeled Drug for Administration

Stable isotope labeled compounds were used for discrimination between the metabolites of the administered drug and endogenous substances.[8] Such a technique is widely utilized in the field of drug metabolism research.[6]

By combination with specificity of the mass fragmentography and one to one ion intensity cluster technique which is based on the monitoring of the ions created with an equimolar mixture of non-labeled and stable isotope labeled drugs, the analytical results have been obtained with high specificity and reliability.[9]

However, the use of deuterium labeled compounds for the study of drug metabolism offers, in general, several problems concerning biological isotope effects such as delay of absorption[10] and protium-deuterium rearrangement represented with NIH-shift.[11] Also, it has been known as one of the physical isotope effects that gas chromatographic retention time of the deuterated compound is shorter than that of non-labeled compound as reported for the cases of sugar,[12] amino acid,[13] fatty acid,[14] and perdeuterated trimethylsilyl derivative.[15]

On the contrary, the physical property of ^{13}C-labeled compound in gas chromatography has been reported to be equal to that of non-labeled compound.[16] Then, a mixture of Mydocalm, Mydocalm-^{15}N and Mydocalm-d_{10} was subjected to gas chromatograph–mass spectrometer. As shown in Figure 2, the retention time of Mydocalm agrees completely with that of Mydocalm-^{15}N, while Mydocalm-d_{10} is eluted faster than Mydocalm itself. Therefore the difference can be ascribed to the physical isotope effects of deuterated compound. Each peak of Mydocalm and Mydocalm-^{15}N is recorded just as a single peak on mass fragmentogram. In other

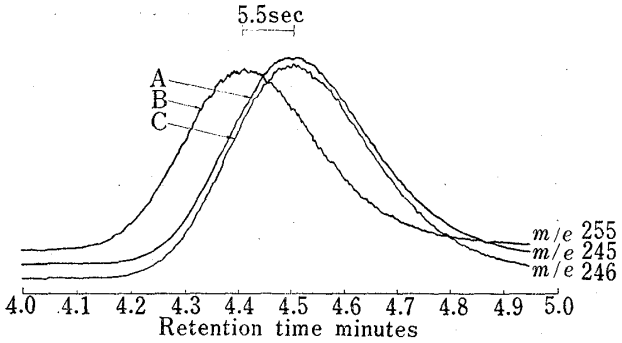

Fig. 2. Recording of Molecular Ions of Mydocalm(A), M 245, Decadeuterium Labeled Mydocalm (B), M 255 and Mydocalm-^{15}N(C), M 246

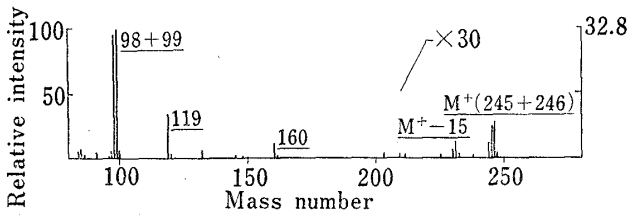

Fig. 3. Mass Spectrum of The Mixture of Mydocalm and Mydocalm-^{15}N

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10) T.B. Vree, J.P.M.C. Gorgels, A. Th. J.M. Muskens, and J.M. Van Rossum, *Clin. Chim. Acta*, **34**, 333 (1971).
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14) J.A. McCloskey, A.M. Lawson, and F.A.J.M. Leemans, *Chem. Commun.*, 285 (1967).
15) G.R. Waller, S.D. Sastry, and K. Kinneberg, *J. Chromatog. Sci.*, **7**, 577 (1969).
16) W.J.A. VandenHeuvel and J.S. Cohen, *Biochem. Biophys. Acta*, **208**, 251 (1970).

words, this fact means that the peak of Mydocalm can be identified easily by looking for the peaks which keep equal peak height at the *m/e* values 98 and 99.

Then, Mydocalm-^{15}N which might require no consideration concerning above mentioned isotope effects was adopted as a tracer in the ion cluster technique. Mydocalm-^{15}N showed a mass spectrum identified to authentic Mydocalm except the shifts of the molecular ion from *m/e* value 245 to 246 and base ion from 98 to 99, respectively.

The mixture of Mydocalm and Mydocalm-^{15}N for administration was prepared by diluting Mydocalm-^{15}N with non-labeled Mydocalm until each of base ions (*m/e* value 98 and 99) appeared equal intensity on the mass spectrum. Mass spectrum of the mixture was shown in Figure 3. The ion intensity of *m/e* value 99 should be contributed theoretically to the natural isotopic abundance of carbon-13 and nitrogen-15 in the non-labeled Mydocalm at the rate of 6.98% (1.1%×6+0.38%) for $C_6H_{12}N$. Therefore, it is preferable for utilizing this technique that the mixture of non-labeled drug and drug with stable isotope of nitrogen-15 should be prepared not by mixing equimolar quantity of them[6] but by making equal both ion intensities of *m/e* value of 98 and 99 with non-labeled Mydocalm. For identifying Mydocalm by this technique, it is necessary to investigate whether the ratio of peak heights agrees with the definite ratio estimated before administration.

Judging from this points, the purity of stable isotope labeled compounds may be of secondary importance and the first importance is to measure exactly the intensity ratio of isotopically created ion cluster.

Thus, this technique provides to eliminate the necessity of multi-labeling to drug or biological substances of interest, and so single labeled compounds with nitrogen-15 or carbon-13 can be used easily because they have neither biological nor physical isotope effect and can be synthesized more conveniently than these multi-labeled compounds.

Calibration Curve

The limit of detection by this technique was 0.01 microgram per 1 milliliter of serum, and 0.05 microgram of Mydocalm per 1 milliliter of serum was detectable with *S/N* ratio of 3:1. The calibration curve for Mydocalm, obtained by plotting peak area ratio of Mydocalm to Mydocalm-d_{10} as an internal standard against known amounts of Mydocalm added to blood, was illustrated in Figure 4. There was linear relationship between the ratio of peak areas and the amounts of Mydocalm in the range of 0.05 to 1.0 microgram per 1 milliliter of serum.

Internal Standard

Stable isotope labeled compounds have been widely used as an internal standard and a carrier for the determination of xenobiotics[17,18] and biological substances,[19] because these stable isotope labeled compounds enable to compensate the losses in extraction and purification steps and also to enhance the sensitivity by reducing the absorption in GC column. Thus, the use of them provides a convenient and accurate method for quantitative determination of extremely small amounts of the objective compounds.

In our investigation, multi-deuterated Mydocalm was used as the internal standard and the carrier because it can be synthesized more easily and cheaply than carbon-13 and/or nitrogen-15 labeled Mydocalm.

Mass spectrum and mass fragmentation of Mydocalm are shown in Figure 5 and 6. Deuterium labeling onto piperidine ring was carried out in this experiment because fragment ion of $CH_2{=}\overset{+}{N}H\rangle$ (*m/e* value 98) appeared as base ion of Mydocalm on its mass spectrum. Mydocalm-d_{10} gave the same spectrum to that of non-labeled form except a shift of 10 mass

17) O. Borgå, L. Palmér, A. Linnarsson, and B. Holmstedt, *Anal. Letters*, **4**, 837 (1971).
18) T.E. Gaffney, C-G. Hammar, B. Holmstedt, and R.E. McMahon, *Anal. Chem.*, **43**, 307 (1971).
19) U. Axen, K. Gréen, D. Hörlin, and B. Samuelsson, *Biochem. Biophys. Res. Commun.*, **45**, 519 (1971).

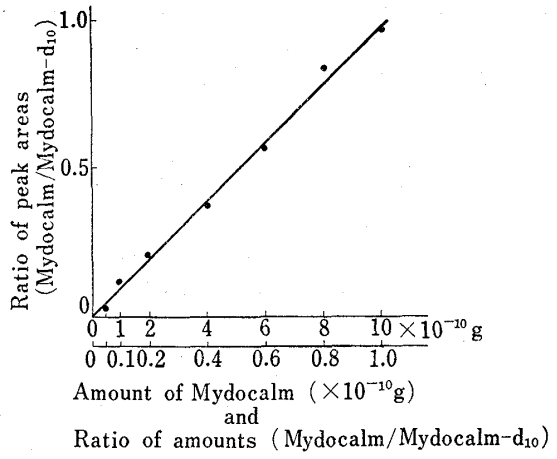

Fig. 4. Calibration Curve of Mydocalm

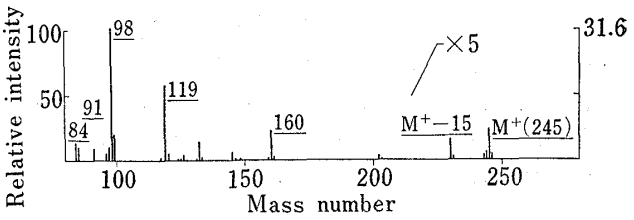

Fig. 5. Mass Spectrum of Mydocalm

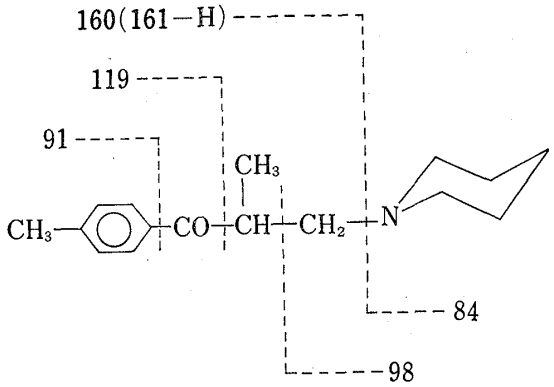

Fig. 6. Mass Fragmentation of Mydocalm

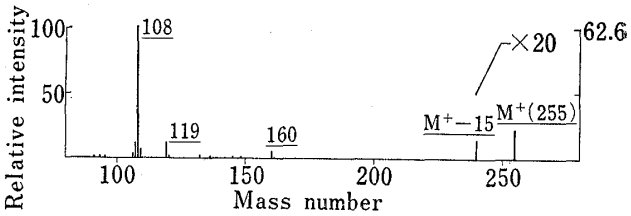

Fig. 7. Mass Spectrum of Mydocalm-d_{19} prepared as Internal Standard

units in some fragment ions *i.e.*, molecular ion from *m/e* value 245 to 255 and base ion from 98 to 108 (Figure 7). Retention time of Mydocalm-d_{10} is slightly shorter than that of non-labeled one by the influence of a physical isotope effect of deuterium as mentioned above.

Mass spectrometric analysis of deuterium content in Mydocalm-d_{10} used as an internal standard is shown in Table I. This result suggested that this internal standard was a mixture of multi-deuterated compounds and the isotopical purity was calculated approximately 90%.

TABLE I. Mass Spectrometric Analysis of Deuterium Content in Internal Standard

m/e	Relative intensity	Deuterium content (%)
98	—	less than 0.1
99	—	less than 0.1
100	—	less than 0.1
101	—	less than 0.1
102	—	less than 0.1
103	0.4	0.4
104	0.4	0.4
105	0.6	0.6
106	3.8	3.8
107	13.0	12.8
108	100.0	100.0
109	7.6	0.7

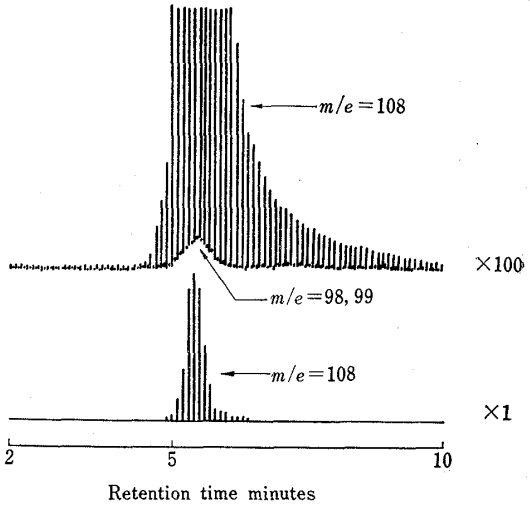

Fig. 8. Recording of Ion Intensities at *m/e* Value 98, 99 and 108 after Injection of Mydocalm/Mydocalm-d_{10} in a Ratio of 1:1000

For determining accurately the contents of Mydocalm-d_0 and -d_1 in this internal standard, the mixture was prepared by adding non-labeled Mydocalm to this internal standard in the ratio of 1: 1000 and analyzed by mass fragmentography. The recording of ion intensities at *m/e* value 98, 99 $\left(CH_2=\overset{+}{\overset{15}{N}}H\right)$ and 108 $\left(CH_2=\overset{+}{N}D\right)$ is shown in Figure 8. The ratio of peak areas of each ion indicates that d_0- and d_1-compounds in the internal standard are contained less than 0.2% respectively.

Therefore, these facts have indicated obviously that it is permissible to use Mydocalm-d_{10} as an internal standard and a carrier if it is added less than 50 times by weight of Mydocalm in the samples.

Extraction and Clean up Methods

Many methods for isolation and purification of drugs from blood and other biological fluids have been reported.

As Mydocalm seems to be unstable in acidic media,[20] it is not suitable for a clean-up procedure to deproteinize with strong acids such as trichloroacetic acid. For this purpose, alcohol dilution technique developed by Sjövall, *et al.*[21] was very profitable to yield a good result. Further purification could be carried out by utilizing its basic property.

The absolute recovery through this procedure gave near 85% for 0.1 microgram Mydocalm per 1 milliliter serum.

Accuracy

Accuracy of mass fragmentographic determination for serum levels of Mydocalm was studied. One hundred nanograms of Mydocalm and ten-fold amount of internal standard were added to serum. Mydocalm was extracted, purified and analyzed in the same manner as described in experimentals. The analytical data and recoveries are shown in Table II.

Table II. Recovery of Added Mydocalm

Added amount (μg/ml)	Found amount (μg/ml)	Recovery (%)	Mean±S.D. (μg/ml)
0.100	0.107	107	0.0986±0.0078
	0.102	102	
	0.089	89	
	0.092	92	
	0.103	103	

Coefficient of variation of these recoveries gave 7.8% for 0.1 microgram Mydocalm per 1 milliliter serum. As the error by the losses of Mydocalm in extraction and clean-up operations could be compensated by use of the internal standard labeled with stable isotope, most of errors in this determination seems due to the operation by mass fragmentographic analysis.

Quantitative Analysis for Serum Level Concentration of Mydocalm

Each base ion of Mydocalm, Mydocalm-^{15}N and Mydocalm-d_{10}, *m/e* value 98, 99, and 108, were used for quantitative analysis. Although the ion intensities of *m/e* value 98 and 99 have kept the rate of 1: 1, the final correction was wade mechanically by use of peak matching operation.[22] Thus, peaks corresponding to Mydocalm could be identified by the informations concerning the fragment ions and its relative intensities in combination with the gas chromatographic retention time.

20) E. Suenaga, H. Murakami, and M. Omae, *Yakuzaigaku*, **24**, 63 (1964).
21) P. Eneroth and J. Sjövall, in P.P. Nair and D. Kritchevsky (Editors), "The Bile Acids," Vol. I, Plenum Press, New York, 1971, p. 121.
22) C-G. Hammar and R. Hessling, *Anal. Chem.*, **43**, 298 (1971).

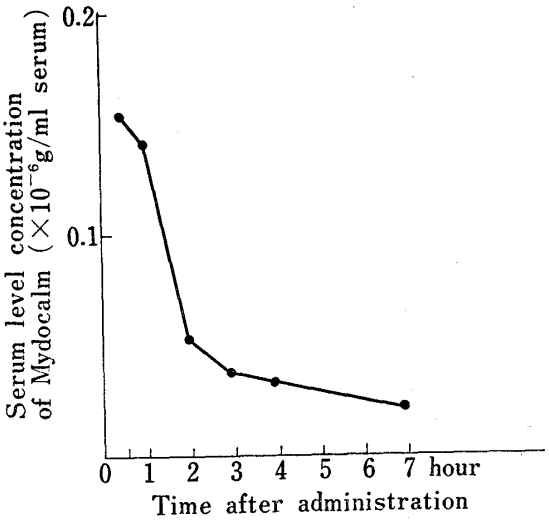

Fig. 9. Serum Lebel Concentration of Mydocalm in Three Normal Adult Subjects following Single 0.10 gram Dose

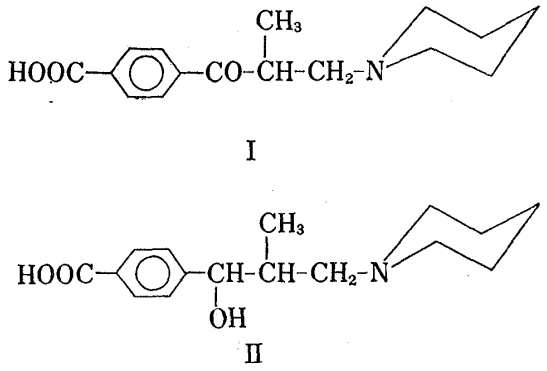

Fig. 10. Major Metabolites of Mydocalm

I: 3-piperidino-2-methyl-1-(4'-carboxyphenyl)-1-propanone
II: 3-piperidino-2-methyl-1-(4'-carboxyphenyl)-1-propanol

Entire agreements peak shapes and peak heights in this technique could verify that the objective peaks have no contaminant with biological substances, and it enables accurate analytical results without any overestimation. Moreover, the most striking observation in the present study is that any isotope effect concerning drug absorption and excretion could not be observed between Mydocalm and ^{15}N-labeled Mydocalm.

The results for the serum level concentration of Mydocalm are shown in Figure 9. The intact was found out less than 0.1% of dose in 24 hour urine.

On the other hand, it has been reported that most of Mydocalm was excreted in the forms of the metabolite I and II (Figure 10) among 13 known metabolites.[23] Determination of these metabolites will be reported elsewhere.

Conclusion

Knapp, *et al.*[8] have been recommended that labeled fragment ions to be used in artificially created ion cluster technique should be shifted from the non-labeled ones by more than one mass unit in order to avoid the interference of natural isotopes. However, by adjusting the blend ratio of drugs and by peak matching operation, one to one ion cluster prepared with M and M+1 doublet will be quite capable of satisfying the conditions necessary for investigation in the field of biological chemistry. Moreover, the present technique made it possible to use conveniently single labeled compounds (M+1) such as Mydocalm-^{15}N for the study of pharmacodynamics and to eliminate the consideration about biological and physical isotope effects.

Acknowledgement The authors are grateful to W. Tanaka, Nippon Kayaku Co., for his encouragements and valuable suggestions through this work.

23) H. Miyazaki, M. Ishibashi, H. Takayama, H. Abuki, G. Idzu, N. Morishita, and M. Ando, The 4th Symposium on Drug Metabolism and action, Sendai, Japan, September, 1972.