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DETERMINATION OF DEOXYSPERGUALIN BY GAS CHROMATOGRAPHY–SELECTED-ION MONITORING

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SUMMARY

A gas chromatographic–selected-ion monitoring method was developed for the sensitive and specific determination of deoxyspergualin in dog plasma using a capillary column and a C_8-amide homologue of deoxyspergualin as an internal standard. Extraction and purification of deoxyspergualin from its constituents, 7-guanidinoheptanamide and glyoxylylspermidine, and its possible metabolites was achieved by using CM-Sephadex C-25 column chromatography with stepwise elution with sodium chloride solution. Treatment of deoxyspergualin with acetylacetone resulted in the formation of the volatile pyrimidine derivative of 7-guanidinoheptanamide accompanying hydrolytic cleavage in the α-hydroxyglycine moiety of deoxyspergualin. Deoxyspergualin could be determined in a concentration of 10 ng/ml in plasma. The method was applied to the determination of deoxyspergualin in plasma during and after intravenous infusion into dogs.

INTRODUCTION

Deoxyspergualin (1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-10,13-dione; NKT-01) is an antitumour agent with stronger activity against several animal tumour model systems, including L-1210 and P-388 murine leu-

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kaemia, than the natural spergualin which was discovered in culture filtrates of *Bacillus laterosporus* [1–4]. These results suggested that deoxyspergualin may be a promising agent for therapy of human leukaemia.

To clarify the relationship between its antitumour activity and pharmacokinetics as a therapeutic agent, it is necessary to establish a practical method for the determination of trace amounts of deoxyspergualin in biological specimens. In order to develop a sensitive and selective analytical method for deoxyspergualin, we introduced gas chromatography–selected-ion monitoring (GC–SIM), which has been used as a powerful tool for the determination of trace amounts of drugs and biologically important materials after conversion of deoxyspergualin into volatile and thermostable derivatives. This paper deals with the effective extraction of deoxyspergualin from biological fluids and the selection of suitable derivatives for GC–SIM. Application of the method to the determination of deoxyspergualin in dog plasma was also investigated.

EXPERIMENTAL

Samples and reagents

Deoxyspergualin, 7-guanidinoheptanamide and glyoxylylspermidine were obtained from Takara Shuzo (Shiga, Japan), methoxylamine hydrochloride and acetylacetone from Tokyo Kasei Kogyo (Tokyo, Japan), *n*-butoxylamine hydrochloride from Gasukuro Kogyo (Tokyo, Japan) and ethoxylamine hydrochloride from Eastman Kodak (New York, NY, U.S.A.). CM-Sephadex C-25 and Sephadex LH-20 were purchased from Pharmacia (Uppsala, Sweden), activated aluminum oxide (basic) from E. Merck (Darmstadt, F.R.G.) and Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.). Other solvents and reagents were of the highest grade available. C_8-amide-deoxyspergualin (1-amino-20-guanidino-11-hydroxy-4,9,12-triazaeicosane-10,13-dione), used as an internal standard, was synthetized in our laboratories according to the method of Umeda et al. [5].

Capillary gas chromatography

A Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector was employed. An open-tubular capillary column (Ultra 2) (25 m×0.3 mm I.D.) (Hewlett-Packard) was used. Helium was used as the carrier gas and make up gas. An all-glass Van den Berg-type solventless injector [6] was used for sample injection. The temperature of the injection port and detector was kept at 320°C and that of the column oven at 260°C.

Gas chromatography–mass spectrometry (GC–MS)

A Hitachi M-80B gas chromatograph–mass spectrometer equipped with an electron-impact ionization source and a data processing system was employed. The column was an Ultra 2 open-tubular capillary (25 m×0.3 mm I.D.). A Van den Berg-type solventless injector was used for sample injection. An inlet pressure of 0.1 kg/cm^2 produced a linear gas velocity of 25 cm/s. The temperatures of the column oven, ion source and injector were 260, 220 and 320°C, respectively.

The ionization energy and accelerating voltage were 20 eV and 3.5 kV, respectively. SIM was carried out under an MS resolution of 3000 ($M/\Delta M$) by detecting the mass which corresponded to a particular elemental composition.

Extraction and purification of deoxyspergualin from dog plasma

After addition of an internal standard (C_8-amide-deoxyspergualin, 5 μg) to dog plasma (1 ml), the resulting mixture was diluted with distilled water (10 ml) and then transferred on to a CM-Sephadex C-25 column (5 cm×0.8 cm I.D.). The column was washed with 0.3 *M* sodium chloride solution (10 ml), and deoxyspergualin was eluted with 0.4 *M* sodium chloride solution (10 ml). Excess sodium chloride was removed by means of a Sep-Pak C_{18} cartridge with methanol as eluting solvent. After evaporation of methanol, the residue was dissolved in 90% ethanol (0.2 ml) and transferred on to a Sephadex LH-20 column (5 cm×0.8 cm I.D.), then eluted with 90% ethanol (0.8 ml). The eluate was evaporated to dryness and the residue obtained was submitted to derivatization.

Derivatization

To the residue obtained from plasma as described above was added water–ethanol–acetylacetone–triethylamine (1:2:2:1) (0.2 ml) [7] and the resulting mixture was heated at 80°C for 2 h. After evaporation of excess of the reagent under a stream of nitrogen, the residue was dissolved in benzene. The resulting solution was transferred on to an alumina (basic) column (2 cm×0.8 cm I.D.). The column was washed with ethyl acetate (10 ml) and the pyrimidine derivative obtained was eluted with ethyl acetate–methanol (95:5) (10 ml). After evaporation of the solvent the residue was dissolved in ethyl acetate (0.2 ml) and then submitted to GC–SIM.

RESULTS AND DISCUSSION

For the specific determination of deoxyspergualin in biological specimens by GC–SIM, it is essential to extract and purify deoxyspergualin selectively. An investigation of the extraction, separation and purification of deoxyspergualin from 7-guanidinoheptanamide and glyoxylylspermidine and other plasma components was carried out by CM-Sephadex C-25 column chromatography. Fig. 1 shows the separation of deoxyspergualin and its related components. As shown, deoxyspergualin was separated completely from 7-guanidinoheptanamide and glyoxylylspermidine by CM-Sephadex C-25 column chromatography using stepwise elution with sodium chloride solution. Further, preliminary experiments using radioisotope-labelled deoxyspergualin revealed that none of metabolites of deoxyspergualin in rat and dog was recognized in the 0.4 *M* sodium chloride fraction. These results suggest that the expected extraction and purification of deoxyspergualin from 7-guanidinoheptanamide and glyoxylylspermidine, and possible deoxyspergualin metabolites co-existing in biological specimens of rat and dog, was achieved by means of CM-Sepadex C-25 column chromatography.

Treatment of deoxyspergualin with acetylacetone in aqueous solution in the presence of triethylamine [7,8] resulted in the formation of a pyrimidine deriv-

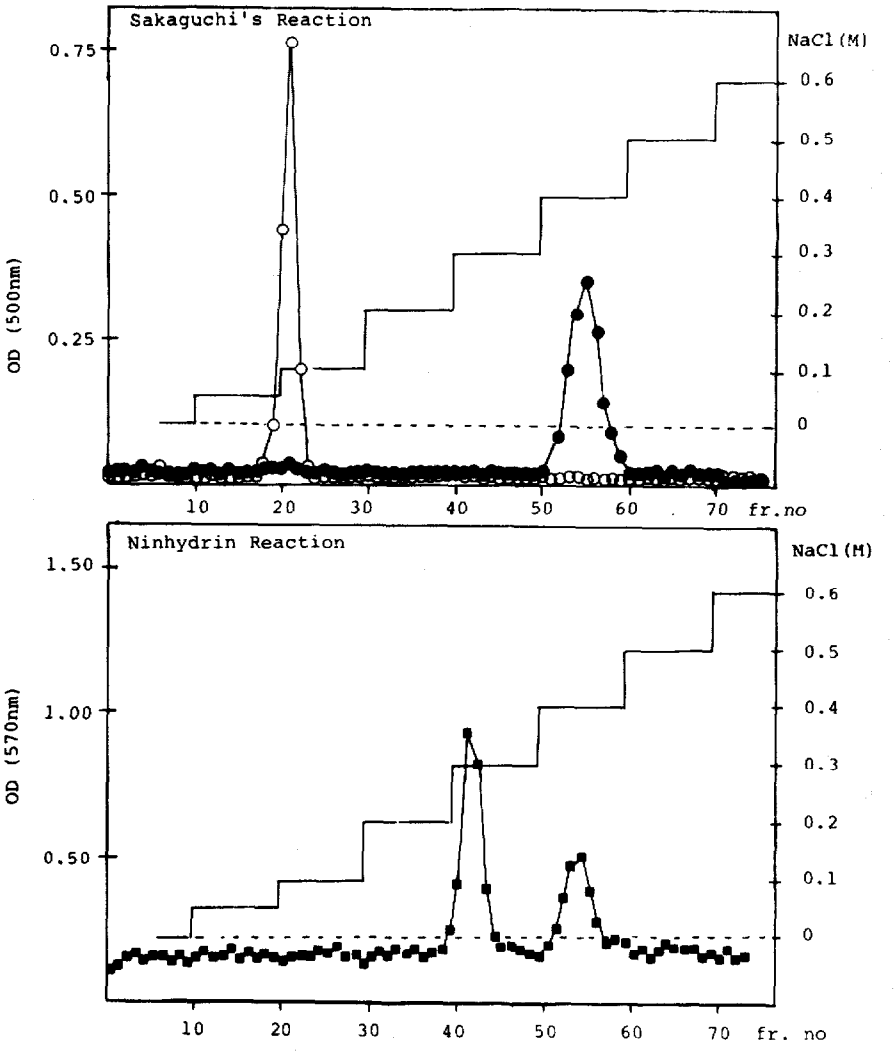

Fig. 1. Separation of deoxyspergualin and related compounds using CM-Sephadex C-25 column chromatography. Deoxyspergualin, 7-guanidinoheptanamide and glyoxylylspermidine were applied to a 5 cm×0.8 cm I.D. column and elution was carried out with sodium chloride solution of concentration 0.1–0.6 *M*, 1-ml fractions being collected. The absorbance at 570 nm (■) (ninhydrin reaction) and 500 nm (●, ○) (Sakaguchi's reaction) were measured in every fraction after coloration. ●, Deoxyspergualin; ○, 7-guanidinoheptanamide; ■, glyoxylylspermidine.

ative of 7-guanidinoheptanamide accompanying the hydrolytic cleavage of the C-11–N-12 bond in the α-hydroxyglycine moiety. When the reaction of deoxyspergualin with acetylacetone was examined by GC, a higher yield of the desired derivative in a short time was observed at 80 °C rather than 60 °C. Fig. 2a shows the gas chromatogram of the product of the reaction of deoxyspergualin with acetylacetone at 80 °C for 2 h. GC revealed that the reaction product obtained exhibited a well shaped single peak with a methylene unit value of 23.4, indicating that the

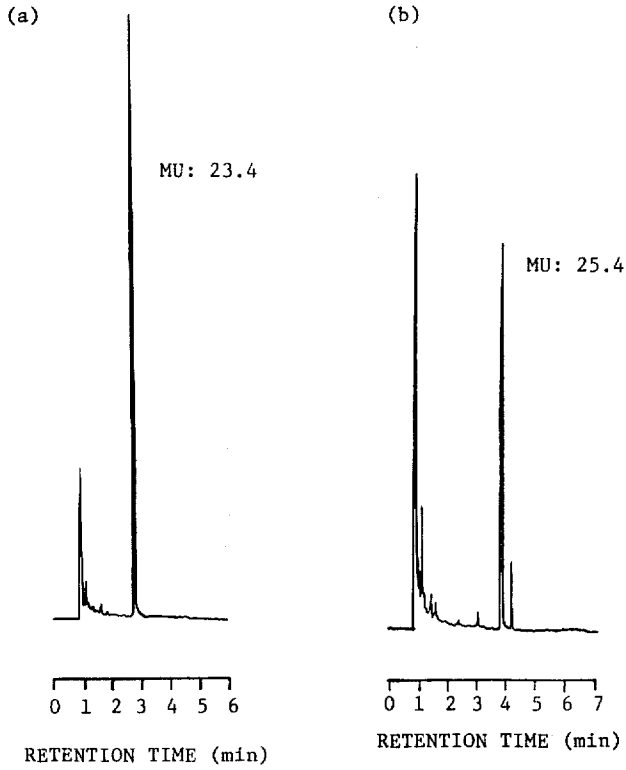

Fig. 2. Gas chromatograms of the products of the reaction of deoxyspergualin with acetylacetone at 80 °C for 2 h (a) and deoxyspergualin with *n*-butoxylamine hydrochloride and subsequent esterification with trifluoroacetic anhydride (b). MU = methylene units.

derivatization of deoxyspergualin with acetylacetone proceeded smoothly and quantitatively.

Fig. 3a shows the mass spectrum of the pyrimidine derivative of 7-guanidinoheptanamide derived from deoxyspergualin by quantitative hydrolytic cleavage. The appearance of a molecular ion at m/z 250 was very informative in confirming the formation of the pyrimidine derivative from deoxyspergualin. The mass spectrum was also characterized by ions of m/z 192, 164, 150 and 136, which were formed by the loss of $-CH_2CONH_2$, $-(CH_2)_3CONH_2$, $-(CH_2)_4CONH_2$ and $-(CH_2)_5CONH_2$, respectively, from the molecular ion. The GC and MS behaviour of the pyrimidine derivative obtained from deoxyspergualin was identical with that of the corresponding derivative obtained from authentic 7-guanidinoheptanamide.

As mentioned above, the C-11–N-12 bond in the α-hydroxyglycine moiety of the deoxyspergualin molecule was easily cleaved and resulted in the formation of the corresponding amide and glyoxylyl moieties under basic conditions during the pyrimidine cyclization process. Fig. 2b shows the gas chromatogram of the reaction product of deoxyspergualin with *n*-butoxylamine hydrochloride and subsequent acylation with trifluoroacetic (TFA) anhydride [9]. Treatment of deoxyspergualin with *n*-butoxylamine hydrochloride in pyridine solution also re-

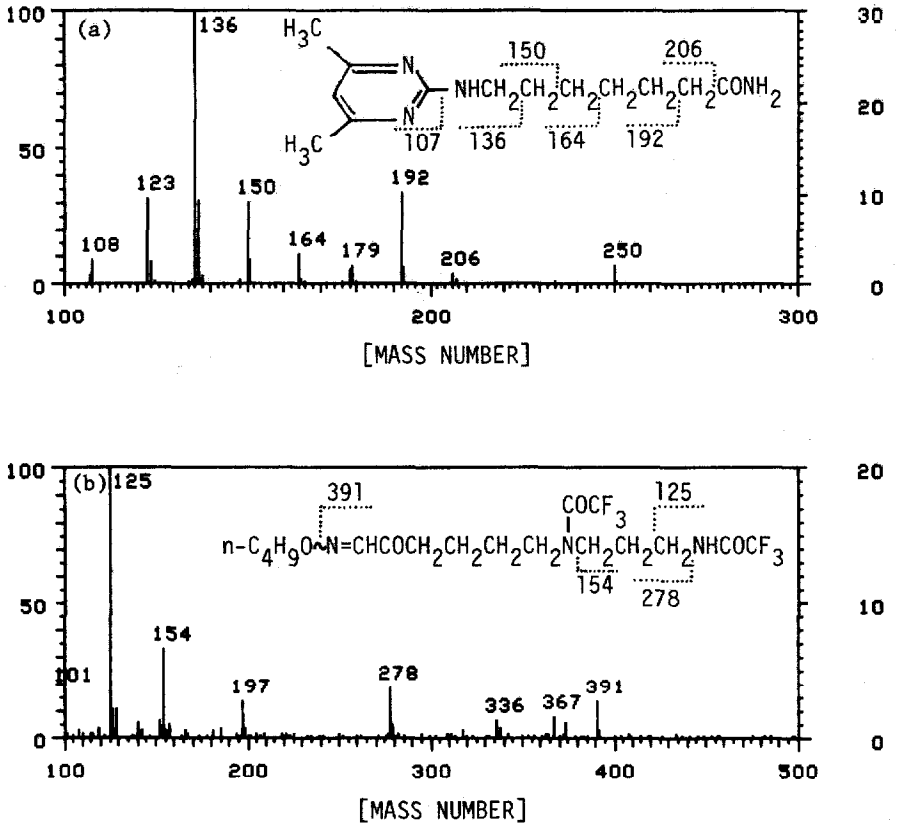

Fig. 3. Mass spectra of the pyrimidine derivative of 7-guanidinoheptanamide derived from deoxyspergualin with acetylacetone (a) and the major isomer of the *n*-BO-TFA derivative of glyoxylylspermidine derived from deoxyspergualin with *n*-butoxylyamine hydrochloride and subsequent esterification with trifluoroacetic anhydride (b).

sulted in the formation of the *n*-butyloxime derivative of glyoxylylspermidine (11-amino-1,1-dihydroxy-3,8-diazaundecan-2-one) accompanying the hydrolytic cleavage of the C-11–N-12 bond in the α-hydroxyglycine moiety. GC revealed that the reaction product of deoxyspergualin with *n*-butoxylamine hydrochloride provided well resolved doublet peaks when analysis was based on the use of an Ultra 2 capillary column. The observation of two well resolved peaks on the gas chromatogram may be attributed to the formation of *syn*- and *anti*-oxime isomers. The retention times of the oxime derivatives of glyoxylylspermidine TFA amide increase in the order methyloxime (MO) < ethyloxime (EO) < *n*-butyloxime (nBO) and the methylene unit values of the major isomer were 22.8, 23.4 and 25.4, respectively. The *syn* and *anti* isomers of the corresponding MO and EO derivatives were also resolved under the same GC conditions.

Fig. 3b shows the mass spectrum of the major isomer of the nBO–TFA derivative of glyoxylylspermidine derived from deoxyspergualin. The molecular ion easily lost the *n*-butyloxy moiety to give the characteristic ion of $[M-73]^+$ at *m/z* 391. The loss of the trifluoroacetyl moiety from the ion of *m/z* 391 gave rise

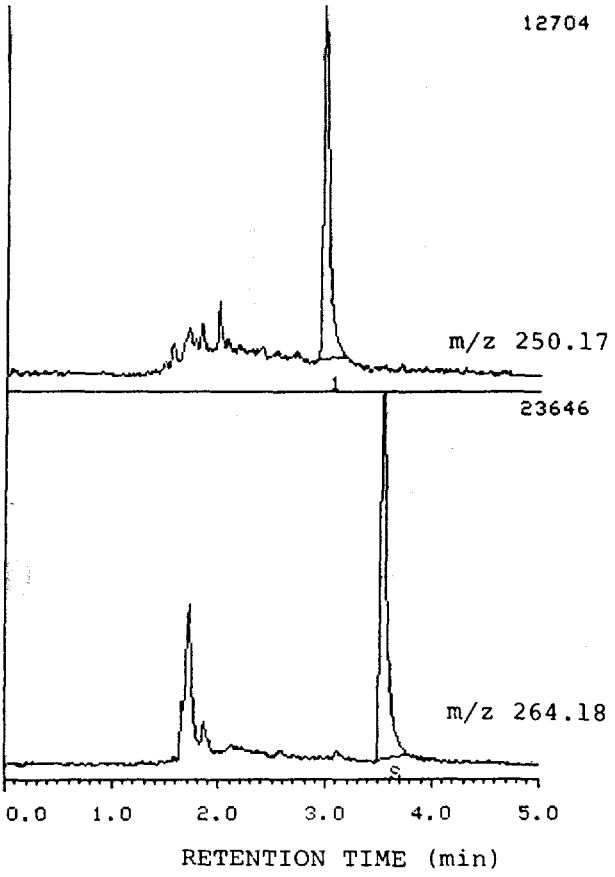

Fig. 4. Typical selected-ion recordings obtained by analysis of a 1-μl aliquot of dog plasma extract which contained 0.5 ng of deoxyspergualin and 1 ng of C_8-amide-deoxyspergualin under an MS resolution of 3000.

TABLE I

RECOVERY OF DEOXYSPERGUALIN ADDED TO DOG PLASMA

Added (μg/ml)	Measured (μg/ml)	Recovery (mean $\pm$ S.D.) (%)	Coefficient of variation (%)
0.2	0.188	103.1 $\pm$ 6.1	5.9
	0.206		
	0.209		
	0.222		
	0.206		
1.0	0.991	98.8 $\pm$ 2.1	2.1
	1.000		
	1.010		
	0.985		
	0.954		

TABLE II

STATISTICAL ANALYSIS OF VARIANCE

SS = residual sum of squares; f = number of degrees of freedom; mf = unbiased variance; F_0 = observed value following F distribution variance ratio.

Source	SS	f	mf	F_0
Sample preparation	46.22	1	46.22	2.24
Error	165.22	8	20.56	
Total	211.44	9		

$F(0.05) = 5.23 > F_0$

to the ion at m/z 278 with high relative intensity. The mass spectrum was dominated by the ions at m/z 125 and 154, which were presumably formed by the successive elimination of $-CH_2NHCOCF_3$ and $-(CH_2)_3NHCOCF_3$ with proton transfer. The mass spectral pattern of the minor isomer was similar to that of the major isomer for the individual oxime derivative.

In order to examine the applicability of the individual derivatives to the determination of deoxyspergualin, GC–SIM was carried out using the characteristic ion of m/z 250 ($[M]^{+\cdot}$) for the pyrimidine derivative of the 7-guanidinoheptanamide moiety or m/z 391 ($[M-nBO]^+$) for the nBO–TFA derivative of the

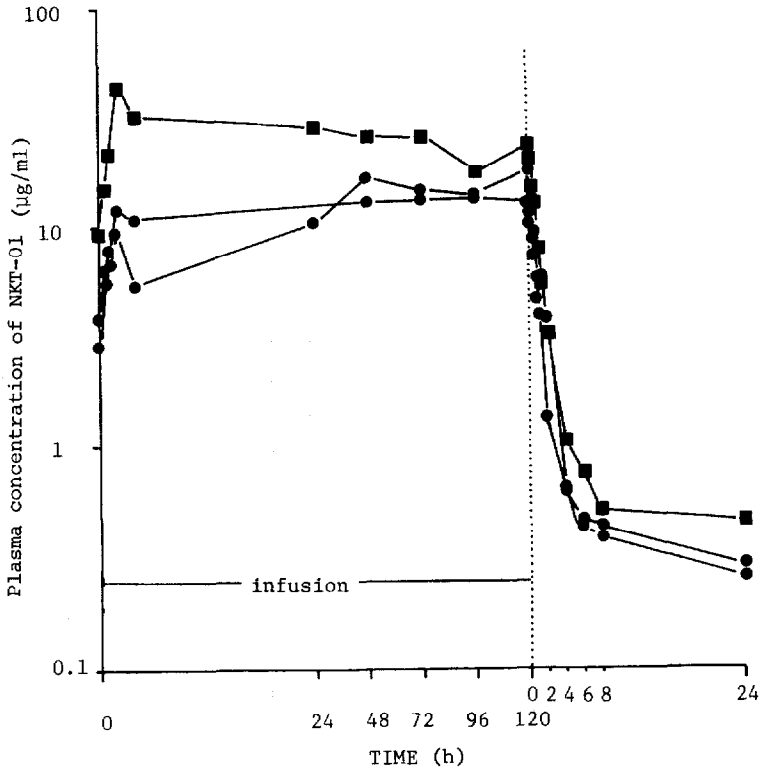

Fig. 5. Plasma levels of deoxyspergualin during and after intravenous infusion of deoxyspergualin into male beagle dogs at doses of 4 mg/kg·h (●) and 8 mg/kg·h (■) for 120 h.

glyoxylylspermidine moiety. Although both derivatives of deoxyspergualin constituents provided high sensitivity in SIM, we selected the pyrimidine derivative of 7-guanidinoheptanamide moiety for the determination of deoxyspergualin owing to the ease of obtaining an internal standard and the simplicity and reproducibility of the derivatization process.

For the determination of deoxyspergualin by the present method, a calibration graph was constructed for deoxyspergualin using C_8-amide-deoxyspergualin as an internal standard. A linear relationship between the peak-area ratio and weight ratio of deoxyspergualin and its internal standard was observed over the range of 10–1000 ng/ml in plasma. Fig. 4 shows the typical selected-ion recording obtained by analysing a 1-μl aliquot of dog plasma extract which contained 0.5 ng of deoxyspergualin and 1 ng of C_8-amide-deoxyspergualin under an MS resolution of 3000. It can be seen that the selected-ion recording was obtained with a high signal-to-noise ratio when monitoring the ions of m/z 250.17 for deoxyspergualin and m/z 264.18 for C_8-amide-deoxyspergualin, indicating that the interfering substances present in the extract obtained from plasma were eliminated completely by the present clean-up procedure. The pyrimidine derivative of 7-guanidinoheptanamide derived from 100 pg of deoxyspergualin (injected amount) was detected by SIM with a signal-to-noise ratio of 5 when monitoring the ion of m/z 250.17.

The accuracy of the method was assessed by measuring the recovery of deoxyspergualin added to dog plasma. A known amount of deoxyspergualin was added to drug-free dog plasma and then determined according to the above method. The results are shown in Tables I and II. The coefficient of variation was calculated to be 5.9 and 2.1% for deoxyspergualin concentrations of 0.2 and 1 μg/ml, respectively. The recoveries for two series of spiked plasma were 103.1% (0.2 μg/ml) and 98.8% (1 μg/ml), and there was no significant difference among the drug levels. The method was applied to the determination of deoxyspergualin in beagle dog plasma. Fig. 5 shows the plasma levels of deoxyspergualin during and after intravenous infusion of deoxyspergualin in a dose of 4 or 8 mg/kg·h for 120 h into three male beagle dogs. The plasma levels of deoxyspergualin reached ca. 13–18 μg/ml (4 mg/kg·h) and ca. 24 μg/ml (8 mg/kg·h), respectively, at the end of infusion. Rapid elimination of deoxyspergualin from plasma was observed with three distinct phases and mean half-lives for α, β and γ phases of 3 min, 50 min and 30 h, respectively. The short half-lives of the initial phase indicated rapid absorption, distribution and metabolism of deoxyspergualin in dogs.

CONCLUSION

Capillary column, GC–SIM with the use of the pyrimidine derivative for the 7-guanidinoheptanamide moiety and CM-Sephadex C-25 column chromatography enabled deoxyspergualin in dog plasma to be determined rapidly with sufficient sensitivity and selectivity without any interference from endogenous substances and possible metabolites. The method may be very useful for the elucidation of the pharmacokinetics of deoxyspergualin in humans.

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