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Note

Determination of 22,23-dihydroavermectin B_{1a} in dog plasma using solid-phase extraction and high-performance liquid chromatography

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Ivermectin belongs to a group of broad-spectrum antiparasitic agents that have been widely used in the treatment of endo- and ectoparasites in sheep, horses and cattle. Ivermectin is a mixture of homologues comprising not less than 80% of 22,23-dihydroavermactin B_{1a} and not more than 20% of 22,23-dihydroavermectin B_{1b}. The structures of these compounds are shown in Fig. 1. Effective dosage levels of ivermectin in all species are very low, so that its plasma concentrations are correspondingly low. In order to study the pharmacokinetics of ivermectin, a sensitive and specific assay method for 22,23-dihydroavermectin B_{1a} is therefore necessary.

High-performance liquid chromatographic (HPLC) methods with fluorescence detection for the assay of 22,23-dihydroavermectin B_{1a} in plasma were reported by Tolan et al. [1] and Tway et al. [2] and later HPLC with ultraviolet photometric detection by Pivnichny et al. [3]. However, the three-fold extraction of 22,23-dihydroavermectin B_{1a} from plasma with ethyl acetate in these methods was difficult owing to the large solvent volume requirements, the effect of interferences in the plasma on the derivatization, emulsion formation and erratic recoveries. Therefore, we have developed a solid-phase extraction method that extracts 22,23-dihydroavermectin B_{1a} efficiently from the plasma of dogs. The

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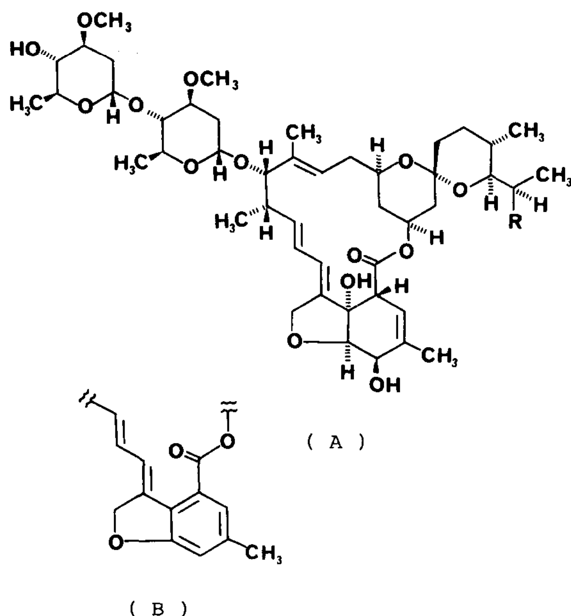

Fig. 1. Structures of (A) 22,23-dihydroavermectins and (B) the fluorescent derivative used for their determination; $R=C_2H_5$ for 22,23-dihydroavermectin B_{1a} and $R=CH_3$ for 22,23-dihydroavermectin B_{1b}.

method is described here and its superiority over the ethyl acetate extraction method is demonstrated.

EXPERIMENTAL

Materials

Ivermectin and Cardomec tablets 68® were provided by Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.). The purity of 22,23-dihydroavermectin B_{1a} reference standard was confirmed by reversed-phase HPLC to be 95%. Baker-10 SPE $C_{18}^{®}$ and Bond-Elut SI® columns were obtained from Baker (Phillipsburg, NJ, U.S.A.) and Analytichem International (Harbor City, CA, U.S.A.), respectively. The derivatizing reagent for imidazole was 1-methylimidazole–acetic anhydride–dimethylformamide (2:3:9). HPLC-grade methanol and acetonitrile were purchased from Wako (Osaka, Japan). Other reagents and chemicals were of analytical-reagent grade.

HPLC instrumentation

The chromatographic system consisted of a Shimadzu Model LC-4A high-performance liquid chromatograph (Shimadzu, Osaka, Japan) and a Shimadzu Model RF-510 digital fluorescence spectrophotometer using excitation and emission wavelengths of 364 and 440 nm, respectively. A stainless-steel column (15 cm×4.6 mm I.D.) packed with 5-μm Zorbax ODS (DuPont, Wilmington, DE,

U.S.A.) was used at 40 °C. Methanol–acetonitile–water (15:10:1) was employed as the mobile phase at a flow-rate of 1.8 ml/min.

Analytical procedure

Plasma samples (2 ml) were placed in a centrifuge tube, diluted with 2 ml of distilled water in an ice-bath and deproteinized with 0.6 ml of 30% trichloroacetic acid. The deproteinized solution was dissolved in 12 ml of 0.2 *M* phosphate buffer solution (pH 7.0). The solution was passed through a Baker-10 SPE C_{18} column at room temperature, then rinsed first with 7 ml of methanol followed by 7 ml of distilled water. The centrifuge tube was rinsed with 3 ml of 0.2 *M* phosphate buffer solution (pH 7.0), which was also applied to the Baker-10 SPE C_{18} column. After interferences from the plasma had been removed by washing three times with 7 ml of distilled water, 22,23-dihydroavermectin B_{1a} was eluted with 5 ml of methanol. The solvent was evaporated in a centrifugal evaporator (Yamato Model RD-21) at 50 °C and the residue was dissolved in derivatizing reagent for imidazole. The tube was sealed and derivatization was carried out by heating for 1 h in an oil-bath at 95–100 °C. After cooling, 1 ml of chloroform was added and mixed, then the solution was applied a Bond-Elut SI column at room temperature, rinsed three times with 2 ml of chloroform and the fluorescent derivative of 22,23-dihydroavermectin B_{1a} was eluted with 4 ml of chloroform. The chloroform was evaporated in the centrifugal evaporator at 50 °C, the residue was dissolved in 100 μl of methanol and 25 μl of the resulting solution were injected into the HPLC system.

Extraction assay

The ethyl acetate extracts of 22,23-dihydroavermectin B_{1a} from dog plasma were assayed according to the procedure of Tway et al. [2].

Animal experiments

Ten male beagle dogs (body weight 9.2–12.2 kg) were fasted for one night and each received 272 μg of invermectin (four Cardomec tablets 68) with 60 ml of water.

Blood samples were withdrawn into heparinized tubes at 1, 2, 3, 5, 7, 9, 24 and 48 h after oral administration. They were centrifuged immediately to separate the plasma and stored at −20 °C until taken for analysis.

RESULT AND DISCUSSION

Solid-phase extraction and isolation

Because of the binding of the 22,23-dihydroavermectin B_{1a} to plasma components, the plasma samples was deproteinized with 30% trichloroacetic acid in an ice-bath and subsequently subjected to solid-phase extraction using a Baker-10 SPE C_{18} column. After oral administration to beagle dogs, the plasma concentrations of 22,23-dihydroavermectin B_{1a} obtained by the extraction method as described above were approximately equal to those given by the liquid–liquid extraction method, as shown in Table I. The precision of the solid-phase extrac-

TABLE I

RECOVERY OF 22,23-DIHYDROAVERMECTIN B_{1a} FROM DOG PLASMA SAMPLES BY TWO EXTRACTION METHODS

Each value represents the mean ± S.D. for three samples.

Sample	Liquid–liquid extraction method		Solid-phase extraction method	
	Plasma concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Plasma concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
A	2.35 ± 0.61	26.0	1.75 ± 0.11	6.3
B	10.15 ± 0.71	7.0	9.38 ± 0.48	5.1

tion method, expressed as coefficients of variation, were 6.3% at 1.75 ng/ml and 5.1% at 9.38 ng/ml. These variations were smaller than that for the liquid–liquid extraction method. A comparison of the solid-phase and liquid–liquid extraction methods clearly demonstrated the superiority of the former method with respect to simplicity and speed of extraction.

Plasma interference

Fig. 2 shows the chromatograms resulting from the derivatization of 22,23-dihydroavermectin B_{1a}. Each chromatogram shows (A) dog plasma spiked with a known amount of 22,23-dihydroavermectin B_{1a}, (B) drug-free dog plasma and (C) plasma from a dog after oral administration of ivermectin. The retention time of the fluorescent derivative of 22,23-dihydroavermectin B_{1a} was 8.6 min. It can be seen that the plasma components did not interfere in the determination of 22,23-dihydroavermectin B_{1a}.

Calibration graph

Standard samples were prepared with drug-free dog plasma by adding 22,23-dihydroavermectin B_{1a} at concentrations ranging from 0.54 to 20.07 ng/ml. Good linearity was obtained for 22,23-dihydroavermectin B_{1a} over this range. Under these conditions, 22,23-dihydroavermectin B_{1a} in plasma was detectable at a concentration as low as 0.54 ng/ml. The calibration graph was prepared daily.

Recovery and reproducibility

Various spiked plasma samples were prepared and analysed five times. Table II shows the within-day reproducibility for five different plasma concentrations of 22,23-dihydroavermectin B_{1a}. The mean overall recovery was 97.8% with a coefficient of variation of 3.8%.

Application to biological samples

We applied the proposed method to the determination of plasma concentrations of 22,23-dihydroavermectin B_{1a} after a single oral dose to beagle dogs. Fig. 3 shows the mean plasma concentration–time profiles of 22,23-dihydroavermectin B_{1a} after administration and Table III gives the pharmacokinetic parameters

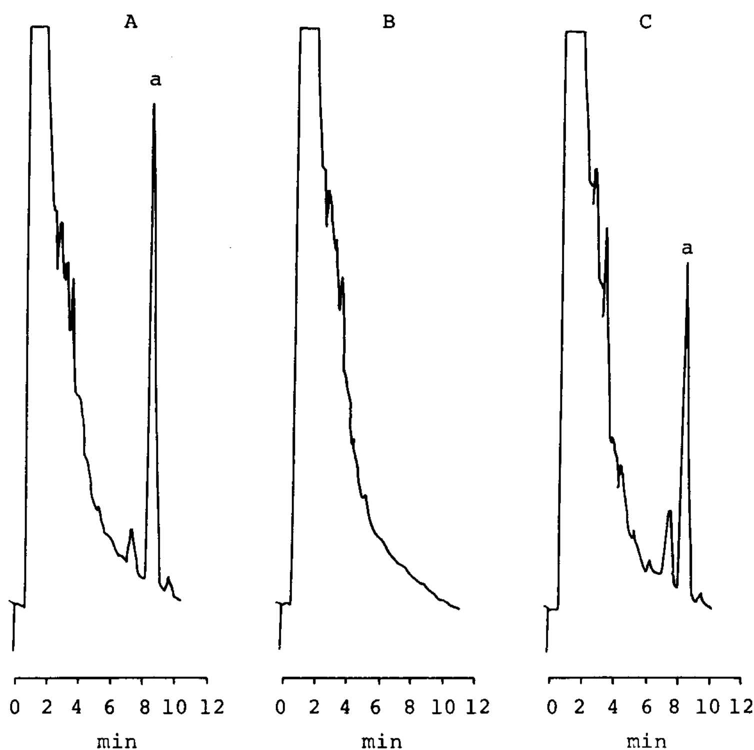

Fig. 2. HPLC profiles of (A) drug-free dog plasma spiked with 20.07 ng/ml 22,23-dihydroavermectin B_{1a}, (B) drug-free dog plasma and (C) a dog plasma sample collected 3 h after oral administration of 272 μg of ivermectin. Peak a = fluorescent derivative of 22,23-dihydroavermectin B_{1a}.

calculated from Fig. 3. The maximum plasma concentration of 19 ng/ml was attained 3 h after administration. Thereafter the plasma level declined biphasically with half-lives of 4.3 and 37.1 h.

CONCLUSION

The method described here is sufficiently simple, rapid and sensitive for the determination of 22,23-dihydroavermectin B_{1a} in dog plasma. Therefore, it can be used for bioavailability studies of ivermectin.

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF 22,23-DIHYDROAVERMECTIN B_{1a} IN SPIKED DOG PLASMA ($n=5$)

Amount spiked into plasma (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Mean recovery (%)
0.54	0.52 ± 0.01	2.7	96.4
3.48	3.36 ± 0.12	3.6	96.6
8.03	7.79 ± 0.41	5.3	97.1
12.04	11.85 ± 0.54	4.6	98.4
20.07	20.26 ± 0.52	2.6	100.5

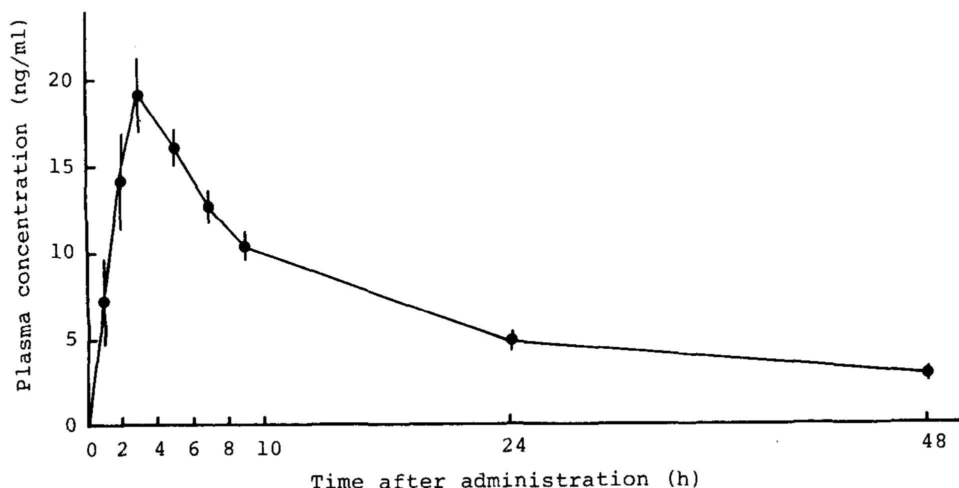

Fig. 3. Mean plasma concentrations of 22,23-dihydroavermectin B_{1a} in ten beagle dogs following a single oral administration of Cardomec tablets 68 containing 272 μg as ivermectin. Each point represents the mean ±standard error for ten dogs.

TABLE III

PHARMACOKINETIC PARAMETERS OF 22,23-DIHYDROAVERMECTIN B_{1a} CALCULATED FROM THE MEAN DOG PLASMA CONCENTRATION-TIME PROFILE

Parameter	Estimate
α (h^{-1})	$1.62 \cdot 10^{-1}$
$t_{1/2\alpha}$ (h)	4.3
β (h^{-1})	$1.87 \cdot 10^{-2}$
$t_{1/2\beta}$ (h)	37.1
C_{max} (ng/ml)	19.1
T_{max} (h)	3

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