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Quantitative Determination of Nifedipine and Its Metabolite in Hamster Plasma by Radio-Gas Chromatography

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A specific radio-gas chromatographic method for the quantitative analysis of [^{14}C]nifedipine and its metabolite in hamster plasma is described. Nanogram amounts of nifedipine could be precisely analyzed without any thermal decomposition using a column packing of 1.5% OV-1. The usefulness of this method was demonstrated by tracing the time course of plasma concentration of nifedipine and its pyridine metabolite following intravenous administration to hamsters. Nifedipine plasma levels as low as 5 ng/ml could be measured by using the labeled compound synthesized in the present study.

Keywords——[^{14}C]nifedipine; hamster; radio-gas chromatography; thermal decomposition; intravenous administration

Introduction

Nifedipine, dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate (NP) is a potent coronary vasodilator widely used for treatment of angina pectoris.[1] Gas-liquid chromatography (GLC) with electron capture detection (ECD) is generally used for microanalyses of NP in biological fluids.[1] However, several papers have reported that NP decomposes under the GLC conditions employed, and the validity of applying GLC for the analysis is questionable. Although the disposition of this drug has been studied in detail, the tissue distribution of NP itself as a potent form is still unknown.

A synchronized accumulating radio-gas chromatography system with a mutual anti-coincidence guard (radio-GLC), for improving detection efficiency without sacrificing resolution, was previously developed at our laboratory.[2] We thus conducted a tissue distribution study of NP by tracer techniques with radio-GLC. A new radio-gas chromatographic method for determining plasma concentration of NP and its metabolite is described.

Experimental

All melting points (mp) were recorded on a Yamato MP-1 melting point apparatus and are uncorrected. Spectral data were obtained using the following instruments: mass spectra (MS) on a Hitachi M-80, proton nuclear magnetic resonance (^{1}H-NMR) on a Varian EM-390 (90MHz; internal standard, tetramethylsilane), carbon-13 nuclear magnetic resonance (^{13}C-NMR) on a Brucker AM-400 (400 MHz), and infrared (IR) spectra on a JASCO IRA-1. NP was kept in brown glass vessels under pure yellow fluorescent light. Radioactive samples were dissolved in 10 ml of toluene base scintillation cocktail and radioactivity was determined with an Aloka LSC 903 liquid scintillation counter. All injections into the GLC column were made using a 10-μl Hamilton microsyringe.

Radioactive Samples and Reagents——[^{14}C]Methyl iodide (specific activity, 56 mCi/mmol; radiochemical purity, >98%) and *n*-[1-^{14}C]hexadecane (specific activity, 61 mCi/mmol; radiochemical purity, >97%) were purchased from the Radiochemical Centre (Amersham, U.K.). [^{14}C]Hexadecane purchased was diluted by mixing it with nonlabeled hexadecane to give a specific activity of 2—3 mCi/mmol and used as a cyclohexane solution (*ca.* 0.3 nCi/μl). NP,[3]

dimethyl 2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinediacarboxylate (NPO-NO),[4] dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate (NPO)[5] and 6-methoxycarbonyl-5-methyl-7-(2-nitrophenyl)-4-azaphthalide (M-III)[6] were synthesized according to the reported procedures. All reagents were purchased from Wako (Tokyo, Japan) and were of analytical-reagent grade.

Synthesis of 1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic Acid——One-molar boron trichloride[7] in dichloromethane (23 ml) was added to a solution of NP (1.0 g) in 6 ml of dichloromethane in an ice bath followed by stirring for 1.5 h at room temperature. The reaction mixture was diluted with 200 ml of ice-cold water and extracted twice with 150 ml of ethyl acetate. The combined extract was dried over magnesium sulfate and evaporated to dryness under reduced pressure. The residue was triturated with 20 ml of chloroform and a precipitated yellowish powder was collected by filtration. The powder was recrystallized from ethyl acetate to afford yellow needles (100 mg), mp 182—183 °C (dec.). IR $^{KBr}cm^{-1}$: 1670 (C=O) 1215 (C-O). ^{1}H-NMR (DMSO-d_6): 2.22 (6H, s, CH_3), 5.53 (1H, s, –CH–), 7.12—7.72 (4H, aromatic protons), 8.63 (1H, br s, NH). ^{13}C-NMR (DMSO-d_6): 18.8 (CH_3), 35.3 (C4), 102.5 (C2), 124.2, 128.0, 131.5, 133.6 (C3′, C4′, C5′, C6′), 143.0 (C1′), 146.3 (C3, C5), 148.3 (C2′), 169.3 (COOH). The signals at 102.5, 143.0, 146.3, 148.3 and 169.3 disappeared in distortionless enhancement by polarization transfer (DEPT). Secondary ion mass spectrometry (SIMS) *m/z*: 319 (M+1). *Anal.* Calcd for $C_{15}H_{14}N_2O_6$: C, 56.60, H, 4.43, N, 8.80. Found: C, 56.31, H, 4.53, N, 8.59.

Synthesis of [O-Methyl-^{14}C]NP——Anhydrous potassium carbonate (18.4 mg) was added to 0.88 ml of a solution of 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid (14 mg) in dimethylformamide. [^{14}C]Methyl iodide (5 mCi) was trapped through a vacuum line in the solution which was subsequently stirred for 4 h at room temperature. Methyl iodide (60 μl) was trapped in the same manner in the reaction mixture followed by stirring for 1 h at room temperature. The reaction mixture was diluted with 20 ml of water and extracted with chloroform (40 and 20 ml). The combined extract was dried over magnesium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in chloroform and subjected to thin-layer chromatography (TLC) on a Kieselgel 60 F_{254} plate (0.25 mm thickness; E. Merck, Darmstadt, G. F. R.). The TLC plate was developed with chloroform–methanol (50 : 1, v/v) and the yellow zone corresponding to standard NP with an *Rf* value of 0.38 was scraped off. [O-Methyl-^{14}C]NP ([^{14}C]NP) was eluted with chloroform and the eluates were filtered through a membrane filter (regenerated cellulose; 0.45 μm pore size; Advantec Toyo Co., Ltd.). The filtrate was evaporated to dryness under reduced pressure to obtain 13.5 mg of [^{14}C]NP with a specific activity of 112.1 mCi/mmol as determined by radio-high-performance liquid chromatography (HPLC).[8] Radiochemical purity exceeded 99% as calculated by radio-TLC and radio HPLC. The [^{14}C]NP was used without dilution by non-labeled NP as a benzene or methanol solution, unless otherwise stated.

Radio-GLC System——The apparatus previously reported[2] (Shimadzu GC-6AM gas chromatograph coupled with an Aloka RGC-212 equipped with a mutual anti-coincidence guard) was used. The carrier (nitrogen) and counting gas (methane) flow-rates were 50 and 250 ml/min, respectively. The sampling time was thus set at 6 s. A glass column (1 m × 3 mm i.d.) was packed with 1.5% OV-17 or OV-1 on Shimalite W (AW-DMCS, 80—100 mesh). The combustion tube was packed with 10 g of copper oxide wire (dimensions *ca.* 5 × 1 mm) and heated to 800 °C. The gate width of the mutual anti-coincidence circuit was set at 100 μs. GC peak yields were calibrated by using [^{14}C]hexadecane according to the method in our previous paper.[9]

Animal Experiments——Male golden hamsters (109—116 g body weight) were anesthetized with ether and treated intravenously (right jugular vein) with a single dose of [^{14}C]NP dissolved in *ca.* 150 μl of polyethylene glycol 400 (63.5 μCi/kg). While the animals were anesthetized, blood samples of about 2 ml were obtained from the inferior vena cava at 5 min or 1 h following injection for use in experiments to determine precision, and 300 μl blood samples were taken from the left jugular vein at 2, 5, 10, 30 min, 1, 2, 4 and 6 h following injection for experiments to determine the concentrations of NP and its metabolite in the blood. Plasma was obtained by centrifuging the blood at 950 ***g*** for 10 min.

Sample Preparation for Radio-GLC Analysis——A plasma sample (100 μl) was placed in a 2-ml brown sample tube, and 5 μg of NP and 0.2 ml of borate standard buffer (pH 9.18) were added. The mixture was extracted with 0.5 and 0.3 ml of ethyl acetate, followed by centrifugation in each case (*ca.* 600 ***g*** for 5 min). The organic layer was collected into a 1-ml small brown sample tube with a Pasteur disposable pipet and evaporated to dryness under a nitrogen stream. The sample was injected into the radio-GLC column.

Extraction Recoveries——A solution of [^{14}C]NP in benzene (1.53 nCi/μl; 4 μl) was placed in a 2-ml brown sample tube, followed by flushing to dryness with nitrogen, Control plasma (100 μl) was then added, and the tube was vortexed for 1 min. All samples were prepared according to the above procedures. The radioactivities of 5 μl aliquots were measured to calculate recovery yields.

Thermal Decomposition of NP——[^{14}C]NP methanol solution (2.13 nCi/μl; 4 μl) and [^{14}C]NP (172.3 μCi/mmol) methanol solution (4.98 nCi/μl; 1 μl) were injected into an OV-17 glass column at column oven temperature of 230 °C and an injection port temperature of 250 °C, or a column oven temperature of 250 °C, and an injection port temperature of 270 °C. [^{14}C]NP methanol solution (0.25 nCi/μl; 4 μl) was injected into an OV-1 glass column at column oven temperatures of 200, 240 and 280 °C and an injection port temperature of 300 °C.

Results and Discussion

Use of radio-GLC offers the following advantages. First, it permits estimation of the extents of derivatization, column adsorption and thermal decomposition. So far, this has been virtually impossible but now, such estimation can be done quite easily by calculating the GC peak yields[9] of substances under consideration. GC peak yield is the percentage of the amount of an injected substance that reaches the detection system (peak radioactivity ÷ injection radioactivity × 100). Secondly, only labeled compounds in complex mixtures of predominantly unlabeled components are detected and an unchanged drug and its metabolites can be determined separately. Even with these advantages, the present method has so far been given only limited application to drug metabolism studies. In particular, a few examples have been reported of its use for quantitative determinations.[10]

A scheme of photodegradation and biotransformation of NP is shown in Fig. 1.[11] NP in solution is very photolabile. Daylight-induced decomposition to the nitrosopyridine derivative (NPO-NO) and ultraviolet (UV)-induced decomposition to the nitropyridine derivative (NPO) have been reported. In addition, NP is known to be thermally unstable. Whether NP decomposes under gas-chromatographic conditions is a matter that has been frequently discussed. Jakobsen *et al.*,[12] Testa *et al.*[13] and Dokladalova *et al.*[14] found NP to be thermally stable. Kondo *et al.*[6] reported that nanogram amounts of NP partially decompose to NPO under the chromatographic conditions reported by Jakobsen *et al.*[12]

In general GLC with ECD, flame ionization detection (FID), thermal conductivity detection and the like, it is difficult to determine whether thermal decomposition actually occurs, due to the concealment by the solvent peak and baseline of the minor peaks of thermal decomposition products. In contrast, radio-GLC makes possible elucidation of the extent of thermal decomposition, being capable of detecting only labeled substances. In the present study, an attempt was made to find conditions that would suppress the thermal decomposition of NP so that it could be analyzed with good reproducibility, using radio-GLC, in view of its advantages.

Silicone polymer column packing is generally used for the GLC analysis of NP. [^{14}C]NP,

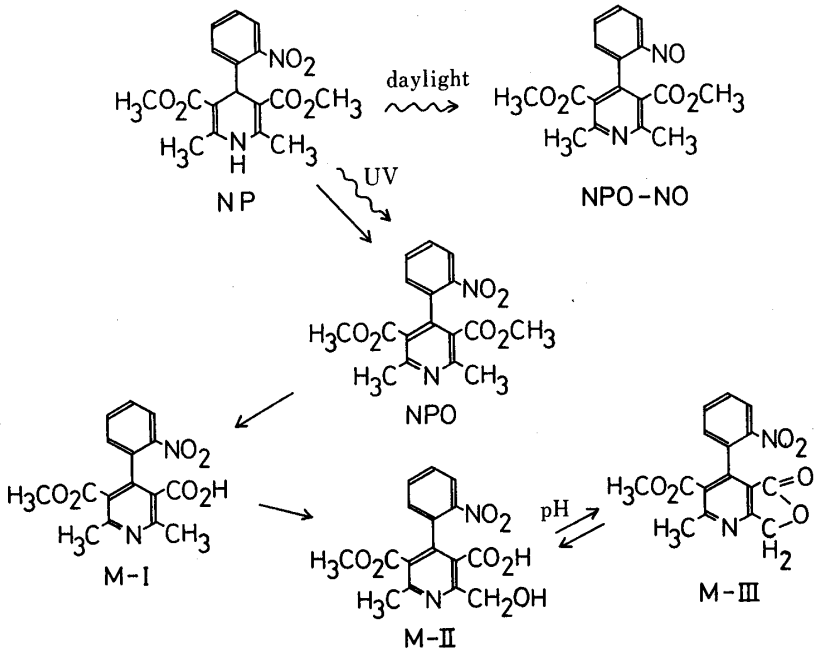

Fig. 1. Photodegradation and Biotransformation Scheme

⇝, photodegradation; →, biotransformation.

8.52 nCi (26 ng), was thus injected into an OV-17 glass column for radio-GLC. As shown in Fig. 2, the [^{14}C]NP peak appeared at a retention time (t_R) of 9 min in the case of panel A and at a t_R of 4.5 min in the case of panel B. The peak of the thermal decomposition product appeared within 1 min following injection in both cases. This peak was judged to represent an unknown product, since the t_R of NPO, reported to be a thermal decomposition product formed during GLC,[6,11,14] was about 3.3 min for panel A. The background (BKG) between NP and thermal decomposition peaks was higher than the original level. GC peak yields of the thermal decomposition product and NP and the value obtained by dividing the radioactivity of the BKG elevation region by injected radioactivity, were 11.0, 63.6 and 14.7% in A, and

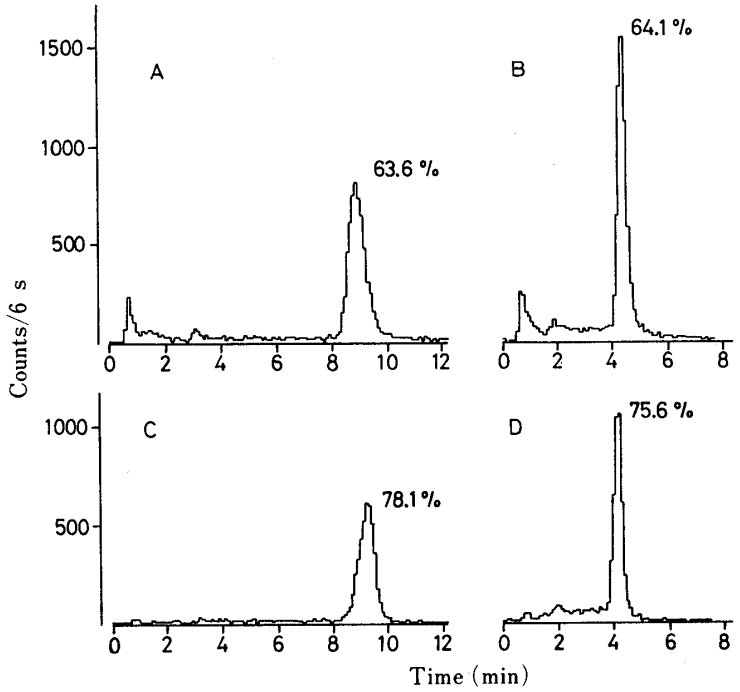

Fig. 2. Thermal Decomposition of NP on a 1-m Long 1.5% OV-17 Glass Column

GC peak yield of NP; sample size for A and B, 26 ng (8.52 nCi); for C and D, 10 μg (4.98 nCi); column oven temperature for A and C, 230 °C; for B and D, 250 °C; injection port temperature for A and C, 250 °C; for B and D, 270 °C.

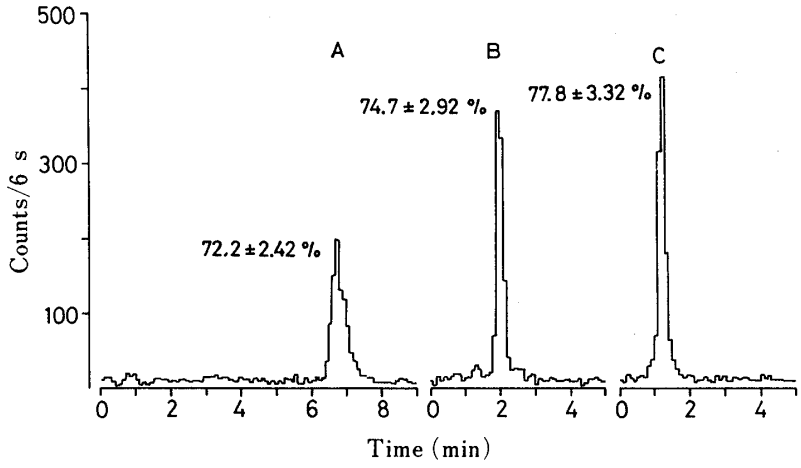

Fig. 3. Radio-Gas Chromatograms of [^{14}C]NP on a 1-m Long 1.5% OV-1 Glass Column

GC peak yield of NP (mean ± S.D., $n=4$); sample size, 3.1 ng (1.0 nCi); column oven temperature for A, 200 °C; B, 240 °C; C, 280 °C; injection port temperature, 300 °C.

11.9, 64.1 and 19.8% in B, respectively.

In radioisotope tracer methods, it is possible to add large amounts of the corresponding unlabeled substance as a carrier to minimize loss of labeled substances due to decomposition and adsorption during analysis. [^{14}C]NP (4.98 nCi) in the amount of 10 μg was thus injected into the glass column. As shown in panel C, thermal decomposition under the same GLC conditions as in A was prevented for the most part. However, in the case of panel D, the BKG elevation region became remarkably apparent under the same conditions as in B. It is thus evident that thermal decomposition in the OV-17 glass column can be suppressed to some extent by a large amount of carrier. However, this should be of limited use since the range of conditions available for GLC is restricted. The GC peak yields of NP in C and D were increased more than 10% compared to those in A and B. This may possibly be due to suppression of the thermal decomposition peak following addition of the carrier. This is the first report to indicate carrier effects on thermal decomposition in a GLC column.

Radio-GLC was performed using an OV-1 glass column. [^{14}C]NP (1.0 nCi, 3.1 ng) was injected at several column oven temperatures while maintaining the injection port temperature at 300°C. It is evident from Fig. 3 that no thermally decomposed product appeared on the chromatograms, and good reproducibility was obtained at all temperatures. OV-1 thus appears suitable for analysis of nanogram amounts of NP. In the following experiments, [^{14}C]NP in hamster plasma was analyzed on OV-1.

First, GLC conditions were determined for separating NP and its metabolites and photodegradation products. M-I and M-II (Fig. 1) are considered not to be recovered in the organic layer when plasma samples are treated under alkaline conditions, owing to the chemical structures of these two metabolites. A mixture of NP, NPO-NO, NPO and M-III was injected into GLC at several column oven temperatures and each substance was detected by FID. Good separation was attained at a temperature of 200 °C as shown in Fig. 4. Under usual GLC conditions, broadening of the radioactive peaks was a little less than 1.5 times that noted for the FID peaks. It was thus concluded that sufficient separation was achieved in radio-GLC under the conditions of Fig. 4 in spite of peak broadening. These conditions were

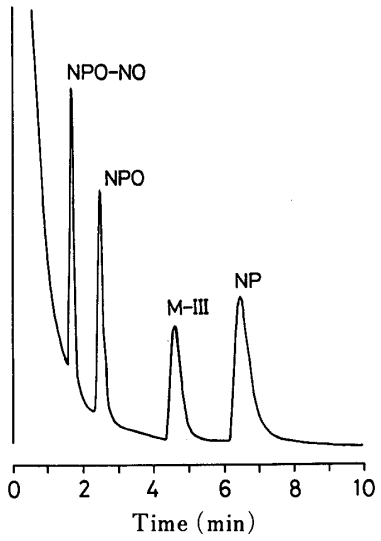

Fig. 4. FID Traces of NP and Metabolites and Photodegradation Products

Column, 1-m long 1.5% OV-1 glass column; column oven temperature, 200 °C; injection port temperature, 220 °C; sample size for NP, *ca.* 1 μg; other substances, *ca.* 500 ng.

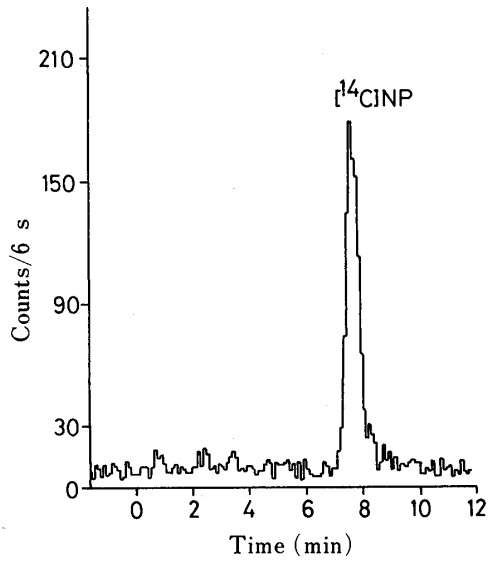

Fig. 5. Typical Radio-Gas Chromatograms of an Extract of Hamster Blank Plasma Spiked with [^{14}C]NP

Samples prepared in experiments on extraction recovery were injected into the radio-GLC column. GLC conditions were as in Fig. 4.

thus used in the following experiments. It should be difficult to detect NPO-NO by ECD, since not only does NPO-NO have poor electron-capturing ability but also solvent peaks hinder detection. In contrast, by radio-GLC, it is possible to analyze NP and know at any time whether NPO-NO is formed by photodegradation. This is because of its equal detection efficiency for NP and substances produced from it.

No calibration curve is required for determining labeled substances in biological samples by radio-GLC. Such determination is possible only if the GC peak yields and recovery yields of the substances under consideration are known. GC peak yields actually fluctuate to some extent for unknown reasons, even if the injected substance and GLC conditions are unchanged. For this reason, [^{14}C]NP of known radioactivity was injected into the column to determine GC peak yields in each and every experiment.

The absolute extraction recovery of [^{14}C]NP from spiked hamster plasma was $84.7 \pm 1.50\%$ ($n=5$). A carrier was added to prevent losses of [^{14}C]NP by decomposition and adsorption during treatment of the plasma samples.

To examine the reproducibility of [^{14}C]NP analysis, [^{14}C]NP was injected intravenously into hamsters, and plasma samples were obtained at 5 min or 1 h after injection, followed by radio-GLC. A peak eluting earlier than [^{14}C]NP was observed and identified as [^{14}C]NPO from its t_R. Raemsch and Sommer[15] reported a considerable amount of NPO to be formed in stock NP solutions and plasma samples containing pure NP stored at room temperature for several hours. They considered NPO in the plasma samples to be at least partially artifact. To determine whether NPO was formed artificially in our procedures, the samples prepared in the extraction recovery experiments were injected into the radio-GLC column. A typical radio-gas chromatogram obtained is shown in Fig. 5. A single peak of [^{14}C]NP was obtained and no decomposition product was recognized. [^{14}C]NPO on the chromatograms was thus ascribed to metabolism of [^{14}C]NP, and the present method is considered to be reliable. NPO has generally been reported to be almost absent in human[16,17] and rat blood[13] following

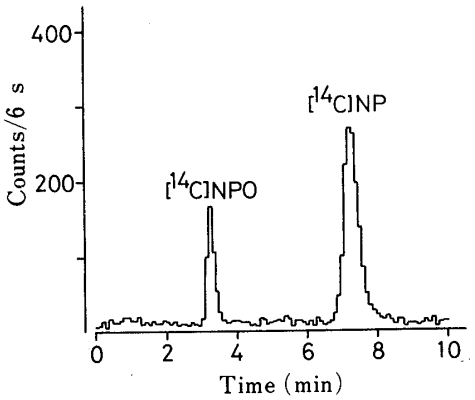

Fig. 6. Typical Radio-Gas Chromatograms of an Extract of Hamster Plasma Sample at 10 min Following Intravenous Administration of [^{14}C]NP (63.5 μCi/kg)

GLC conditions were as in Fig. 4.

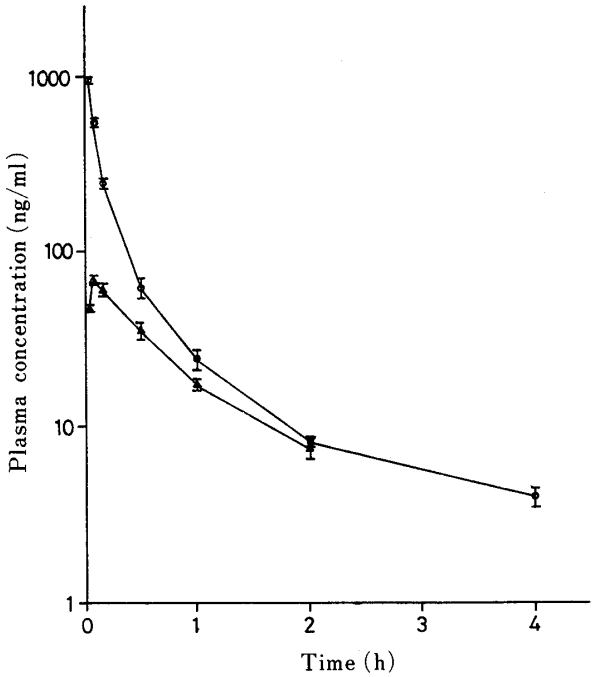

Fig. 7. Plasma Concentrations of NP and NPO as a Function of Time after Intravenous Administration of [^{14}C]NP (63.5 μCi/kg) in Male Hamsters (Mean $\pm$ S.E.M., $n=4$)

○, NP; △, NPO.

TABLE I. Reproducibility of Analyses of [^{14}C]NP and [^{14}C]NPO in Hamster Plasma

	Counts/6 s			
	5 min		1 h	
	[^{14}C]NP	[^{14}C]NPO	[^{14}C]NP	[^{14}C]NPO
	5310	618	100	87
	5243	638	83	86
	5078	614	82	78
	5086	600	92	94
	4840	564	98	72
	4594	594	112	76
			131	88
Mean	5025	605	100	83
S.D.	243	23	16	7
C.V. (%)	4.9	3.8	16.0	8.4

A hamster plasma sample was separated from blood taken at 5 min or 1 h after intravenous administration of [^{14}C]NP (63.5 μCi/kg). Several aliquots (100 μl) were taken from each plasma sample and treated as described in the experimental section.

intravenous administration of NP. Hamsters were found to show a remarkable species difference in this regard. Table I shows the reproducibility of analysis of [^{14}C]NP and [^{14}C]NPO in the plasma samples obtained at 5 min or 1 h after intravenous administration.

[^{14}C]NP was injected intravenously into four hamsters to study the time courses of the plasma concentrations of NP and NPO. Figure 6 shows typical radio-gas chromatograms at 10 min following intravenous administration. Figure 7 shows plasma decay curves. Plasma NP could be detected at levels as low as 5 ng/ml. Several papers[5,11,13] have reported the detection limits of NP to be 1—5 ng/ml using GLC with ECD. The present method has sensitivity comparable to those of the previous papers. Plasma NPO concentration was calculated, assuming its GC peak yield and recovery yield to be equal to those of NP. NPO could first be detected in blood at 2 min and reached a maximum plasma concentration at 5 to 10 min following injection. It then disappeared from the blood at approximately the same rate as NP.

The present data demonstrate radio-GLC to be useful for determining suitable conditions for analyzing substances by GLC. NP and NPO concentrations in hamster plasma could be determined precisely by radio-GLC. The present method may find application in the determination of NP not only in plasma but in various tissues as well.

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