A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD OF ANALYSIS OF 4'-*O*-TETRAHYDROPYRANYLADRIAMYCIN AND THEIR METABOLITES IN BIOLOGICAL SAMPLES

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A method for measuring 4'-*O*-tetrahydropyranyladriamycin (THP) and its metabolites in biological samples are described. By reversed-phase high performance liquid chromatography using fluorescence detection, THP and its metabolites were all separated on a single chromatogram within 18 minutes. A linear calibration curve was obtained up to 2,000 ng/ml of THP in plasma. The recovery of THP in the analysis was more than 95% above 5 ng/ml and 87.1% even at 1.25 ng/ml. Thus the lower limit was 1.25 ng/ml in biological samples. Blood levels and urinary excretion in mice and dogs were satisfactory measured by this analytical method.

4'-*O*-Tetrahydropyranyladriamycin (THP) which exhibits a strong antitumor activity against L1210[1] and other mouse tumors,[2] has been studies clinically. THP is less liable to cause alopecia than is adriamycin (ADM) and clinical studied have suggested its potential usefulness in the treatment of lymphoma, ovarian carcinoma, mesothelioma, *etc.*[3,4] THP, ADM, aclacinomycin and other anthracycline are metabolized by aldo-keto reductase, cytochrome P450 reductase or hydrolase *etc.*[5,6] Adriamycin and aclacinomycin has been shown to yield many active and inactive metabolites *in vivo*.[7,8] Therefore a precise analytical method for analysis of these anthracyclines in plasma, tissues and cells is desirable.

In this paper we report a high performance liquid chromatographic method and its application for analysis of THP and its metabolites in biological materials.

Materials and Methods

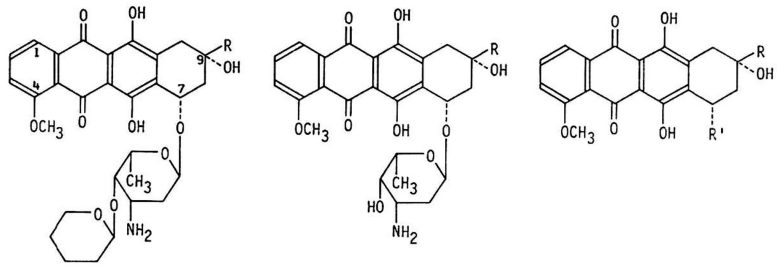
Reagents

Acetonitrile (liquid chromatography grade, Wako Pure Chemicals, Osaka, Japan) was used without further purification. Other reagents were purchased from commercial sources. Deionized-distilled water was used for preparation of the mobile phase solution. Daunomycin (DM) was obtained from Meiji Seika Ltd. and adriamycin (ADM), from Kyowa Hakko Co. Ltd. THP, 13-dihydro-4'-*O*-tetrahydropyranyladriamycin (THP-OH), 13-dihydroadriamycin (ADM-OH), 7-deoxy-13-dihydroadriamycinone (7H-ADn-OH), 7-deoxyadriamycinone (7H-ADn), adriamycinone (ADn) and 13-dihydroadriamycinone (ADn-OH) were prepared in our laboratory. These are listed in Fig. 1.

High Performance Liquid Chromatography

A liquid chromatograph (Shimadzu Model LC3A, Kyoto, Japan) equipped with a syringe-loading

Fig. 1. Structures of THP and its metabolites.



R=–C(=O)CH$_2$OH	4′-*O*-Tetrahydropyranyl-adriamycin (THP)	Adriamycin (ADM)	R′=H 7-Deoxyadriamycinone (7H-ADn) R′=OH Adriamycinone (ADn)
R=–CH(OH)CH$_2$OH	4′-*O*-Tetrahydropyranyl-adriamycinol (THP-OH)	Adriamycinol (ADM-OH)	R′=H 7-Deoxy-13-dihydro-adriamycinone (7H-ADn-OH) R′=OH 13-Dihydroadriamycinone (ADn-OH)

sample injection valve (Shimadzu SIL-1A) fitted with an appropriate sample loop was employed. A column consisted of reversed-phase μBondapak alkyl phenyl (30 cm × 3.9 mm i.d., Waters Assoc., USA) was used. The eluent was monitored by a variable-wavelength fluorescence detector (Shimadzu Model RF500LC): the excitation wavelength was 470 nm with detection at 550 nm. The profiles were recorded by an intelligent integrator (Shimadzu Chromatopak CR1A). The mobile phase was consisted of 35% acetonitrile and 65% ammonia - formate buffer (0.035 M, pH 3.0, v/v). The analysis was performed at ambient temperature (normally 20~24°C) and at a flow rate of 1.3 ml/minute.

Internal Standard

Daunomycin (DM) was used as the internal standard. The internal standard solution was prepared by dissolving DM in methanol at 50 ng/ml.

Standard Solution

Standard solutions of THP and its metabolites (Fig. 1) were prepared by dissolving in methanol and diluting with a mixture of acetonitrile and water (35 : 65, v/v) at concentrations of 2, 4, 8, 10, 20, 30, 40, 1,000, 1,500, 2,000 ng/ml.

Extraction Procedure

One ml of plasma or biological fluids was mixed with 3 ml of ammonia - ammonium chloride buffer (0.1 M, pH 9.0) in a 50-ml glass test tube with a stopper. After adjusting the pH exactly to 9.0 by addition of a few drops of 0.2 M sodium hydroxide, it was shaken with 12 ml of chloroform - methanol mixture (2: 1, v/v) for 2 minutes on a mechanical shaker (100~120 rpm). After centrifugation at 3,000 rpm for 10 minutes, the organic layer was transferred to a test tube. The residual water layer and precipitants, if any, were adjusted again to pH 9.0 and were subjected to re-extraction with 8 ml of chloroform. After centrifugation, the organic layer was combined with the first organic layer. The combined extract was concentrated at below 30°C *in vacuo* (Taiyo Concentrator Model TC-8). The dried sample was dissolved in 2 ml of chloroform - methanol and supplemented with 1 ml of an internal standard solution (DM, 50 ng), and evaporated to dryness. All samples were stored at −20°C under protection from light until used.

Preparation of Plasma and Urine Samples

Mongrel dogs (7~8.5 kg) were given 1.5 mg/kg of THP intravenously. Blood samples (3 ml) were collected from the cephalic vein by using a containing 20 unit of heparin at 5, 30, 60 minutes, 2, 8, and 24 hours after the injection and thereafter centrifuged to separate plasma from blood cells. Urines were collected after 24 hours and 72 hours.

Mice (*dd*Y, male, 23~26 g) were given 5 mg/kg of THP intravenously. Mice were anesthetized

with ether and blood samples were collected through inferior vena cava from 5 minutes to 24 hours as described for mongrel dogs.

Results

High Performance Liquid Chromatography

The concentration of acetonitrile in the mobile phase influenced the retention times of THP and other test compounds. Fig. 2 indicates the relationship between the concentration of acetonitrile and the retention times of THP, ADM, 7H-ADn and 7H-ADn-OH. When the concentration was higher than 40%, ADM, 7H-ADn and 7H-ADn-OH were eluted close to each other. At concentration below 30%, on the contrary, the elution of these compounds was too prolonged. For a good separation and reasonably quick operation, 35% was selected.

The formate buffer concentration also produced an effect on the retention time. Fig. 3 indicates the effect of buffer concentration on the retention times of THP, ADM, DM, 7H-ADn, 7H-ADn-OH or ADM-OH with 35% acetonitrile. At higher buffer concentrations, THP, ADM, DM and ADM-OH were eluted more quickly, while the retention time of aglycones was not affected.

With a higher pH of the buffer solution, the elution of the glycosides, including THP, was faster. Above pH 3, however, the peak of ADn-OH and/or ADM-OH overlapped with those of some constituents of plasma, but the retention times of these materials were not affected by pH. Therefore we selected pH 3.0 for the formate ammonia buffer solution.

Fig. 4 indicates a HPLC elution profile of THP and others dissolved in a mixture of acetonitrile and water (35: 65, v/v). DM was added as an internal standard of the procedure. THP, ADM, THP-OH, ADM-OH, 7H-ADn, 7H-ADn-OH, ADn and ADn-OH were well separated from each other, retention times in minute being 4.1 for ADn-OH, 4.8 for ADM-OH, 5.5 for 7H-ADn-OH, 5.9 for ADn, 6.8 for ADM, 8.8 for 7H-ADn, 9.5 for THP-OH, 11.5 for DM and 16.2 for THP.

Calibration

The calibration curves for THP were obtained in a series of experiments, with varying amounts of THP ranging from 2 to 2,000 ng/ml and fixing the concentration of internal standard (DM at 25 ng/ml). These results are shown in Fig. 5. The curves were linear over a range from 0 to 2,000 ng/ml both in the peak height and in the peak area. Calibration by the peak height was more accurate than the peak area when THP and its metabolites were analyzed at the same time, because the retention times

Fig. 2. Effect of acetonitrile concentration on the retention times of THP, ADM, 7H-ADn-OH and 7H-ADn.

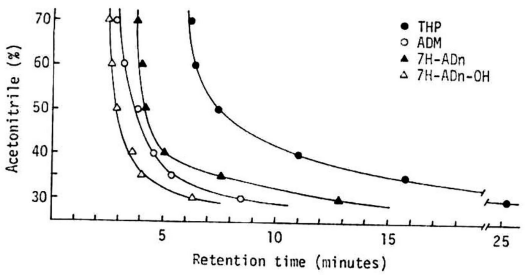


Fig. 3. Effect of buffer concentration on the retention times of THP and its metabolites.

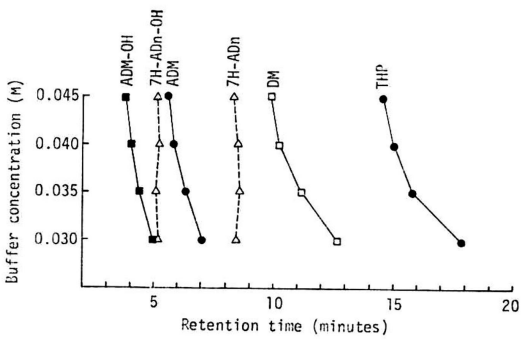


Fig. 4. Typical HPLC elution pattern for a standard mixture of THP and its metabolites.
Column; μBondapak alkyl phenyl.
Mobile phase; acetonitrile - ammonia formate buffer (0.035 M, pH 3.0), 35: 65 (v/v).
Detection; exciting 470 nm, emission 550 nm.
Sensitivity; range 100, gain 8.

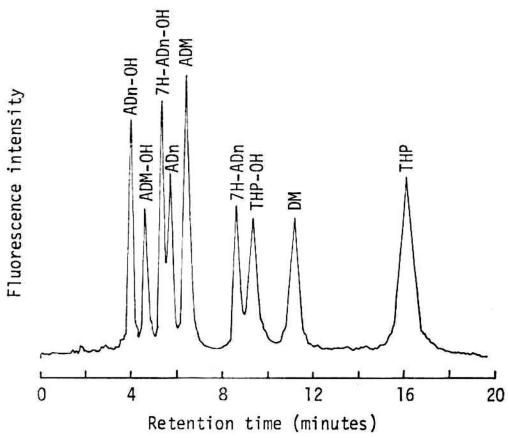


Fig. 5. Calibration curves of THP in a standard solution.
a) (peak height of THP)/(peak height of DM (25 ng))
b) (peak area of THP)/(peak area of DM (25 ng))

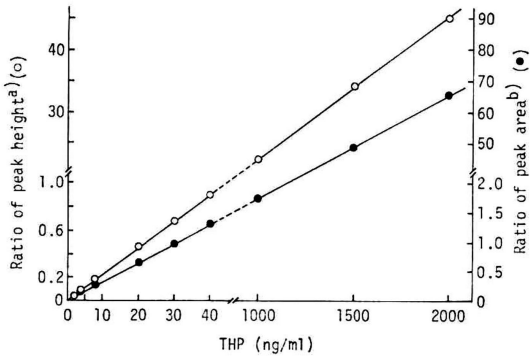


Table 1. Recovery and reproducibility of the determination of THP and its metabolites in dog plasma.

Concentration (ng/ml)	THP		ADM		7H-ADn-OH	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
200	98.9	1.2	97.3	2.2	98.9	1.2
100	98.7	1.0	96.6	2.7	99.0	1.7
50	95.6	5.2	96.2	2.3	98.3	1.7
20	101.0	2.3	95.7	4.3	99.8	2.4
10	97.0	2.9	95.2	2.7	102.3	3.2
5	98.2	5.9	92.8	6.1	101.7	2.6
2.5	94.3	4.5	86.3	5.2	98.3	3.8
1.25	87.1	6.8	82.5	4.8	97.1	4.9

C.V.: Coefficient of variation (n=5).

of compounds other than THP were close to each other. The detection limits of THP, based on a signal to noise ratio (3: 1), was 0.5 ng. Evaluation of the amount of THP and its metabolites in biological samples was based on the peak height ratio to DM (internal standard), and the following calibration formula was used:

$$X=50\times F\times A/B$$

where X is the concentration of THP or its metabolites in ng/ml, A is the peak height of THP of its metabolites, B is the peak height of DM.

The factor F of each compounds was determined by chromatography of THP and metabolites at known concentration, using DM as the internal standard. F was calculated by dividing the peak height of DM by that of THP or metabolites at the same concentrations.

Recovery and Biological Samples

For the analysis of THP and metabolites in biological samples, they were first extracted. THP was well transferred to organic layer at pH 8.0 but ADM was not. Recovery of THP from dog plasma

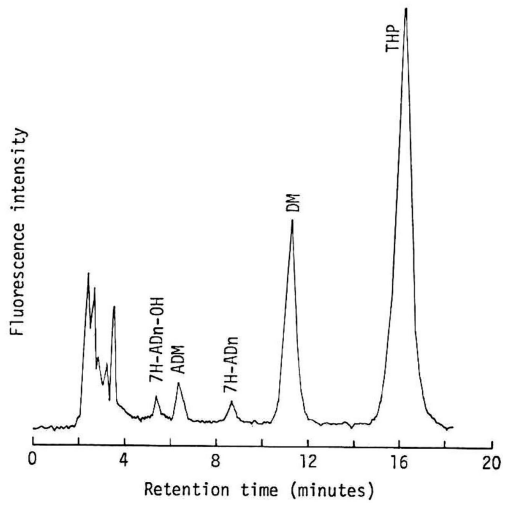
Table 2. Plasma levels of THP and its metabolites in mice (5 mg/kg, i.v.).

	THP	ADM	7H-ADn-OH	7H-ADn
5 minutes	707	26.5	0	3.1
30 minutes	280	8.2	2.8	2.7
60 minutes	166	7.3	12.9	4.8
2 hours	121	6.6	10.8	5.1
8 hours	32.4	3.4	4.3	3.1
24 hours	3.8	tr	1.5	1.3

(ng/ml, n=5).

Fig. 6. HPLC chromatogram of plasma from a mouse 30 minutes after intravenous injection of THP (5 mg/kg).

Chromatographic conditions were the same as in Fig. 4.



at pH 8.0 was 97.8% but that of ADM, 72.8%, although at pH 9.0, that of THP and ADM was 97.6% and 95.2% respectively, so we set the pH at 9.0 during the chloroform - methanol extraction procedure. The artificial formation of ADM from THP during this extraction procedure (at pH 9.0) was less than 1.2%.

The recoveries of THP, ADM, and 7H-ADn-OH in the procedure including HPLC from dog plasma are shown in Table 1. In this table, the coefficients of variation for THP, ADM and 7H-ADn-OH in dog plasma added at a concentration from 1.25 to 200 ng/ml are shown. The recoveries were more than 95% above 5 ng/ml of these compounds except for 92.8% for ADM at 5 ng/ml. Even at 1.25 ng/ml, THP was well recovered (87.1%). Coefficients of variation were all below 6.8% in these compounds. The lower limit of the analysis for THP was 1.25 ng/ml.

Table 2 gives the concentrations of THP and its metabolites in plasma of mice which received 5 mg/kg of THP intravenously. Fig. 6 indicates the chromatogram of HPLC for the mouse plasma sample obtained 30 minutes after injection. Table 3 gives the plasma levels and urinary excretion of THP and its metabolites in dogs received 1.5 mg/kg of THP, intravenously. THP disappeared very rapidly from the plasma in each animal and ADM, 7H-ADn-OH and 7H-ADn were detected at 0.5~8 hours after injection. As shown in Table 3, THP, THP-OH, ADM, ADM-OH, 7H-ADn and 7H-ADn-OH were detected in dog urine.

Table 3. Plasma levels and urinary excretion of THP and its metabolites in dogs (1.5 mg/kg, i.v., n=2).

	THP	ADM	THP-OH	ADM-OH	7H-ADn-OH	7H-ADn
Plasma levels (ng/ml)						
5 minutes	109	26.3	—*	—	3.1	2.5
30 minutes	54	1.8	—	—	1.3	tr
60 minutes	40	1.9	—	—	1.0	tr
2 hours	31.6	1.8	—	—	4.6	0.7
8 hours	15.3	—	—	—	1.7	—
24 hours	—	—	—	—	1.3	—
Urinary excretion (μg)						
0~24 hours	54.9	10.9	7.2	—	—	3.0
24~72 hours	6.3	12.4	1.9	1.6	0.6	8.9

* Not detected.

Discussion

ISRAEL *et al.*,[9] PIERCE and JATLOW,[10] and EKOSBORG *et al.*[11] have reported reversed-phase high performance liquid chromatographic analyses of ADM and its metabolites in plasma. OGASAWARA *et al.*[12,13] have published the results of the determinaton of aclacinomycin and its metabolites by normal and reversed-phase liquid chromatography.

In this paper, we reported an efficient reversed-phase high performance liquid chromatography using fluorescence detection. THP and seven metabolites in biological samples were quantitatively separeted at one time.

THP and the metabolites were extracted from biological samples at pH 9.0, at which they were almost completely recovered. Although THP is labile to prolonged treatment at pH 3.0, the pH value of the elution buffer in mobile phase, with a short retention time of 16.2 minutes, there was almostly no effect during the analysis.

In biological samples such as plasma, there were unknown substances giving anthracycline-like fluorescence at 550 nm that were extracted together with THP and the metabolites. These contaminants were eluted at the begining of the chromatography with retention times being less than 4 minutes and the retention times did not vary with pH, therefore we set up delayed elution conditions for metabolites by regulating the pH value of the elution solvent to avoid overlapping.

The whole chromatographic elution process is completed in 18 minutes. Retention times of THP and its metabolites are affected by acetonitrile concentration, ionic strength and pH. Especially, with variations of the ionic strength, glycosides and aglycones move in a different way. With increased ionic strength, glycosides migrated faster, while the migration of aglycones did not change. We studied all conditions necessary for the assay method by which THP and its metabolites can be quantitatively determined on a single chromatographic run.

The blood levels of THP and its metabolites in mice and dogs were determined with a satisfactory accuracy. Tissue levels which we will report elsewhere, can also be determined accuracy. Biodynamic and pharmacokinetic studies of THP in experimental animals, as well as in clinical trial will be reported elsewhere.[14]

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