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Simultaneous determinations of anthracycline antibiotics by high performance liquid chromatography coupled with radial-flow electrochemical cell

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고성능 액체 크로마토그래피/방사흐름 전기화학전지를 이용한 안트라사이클린계 항생제의 동시 정량

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요 약: 방사흐름 전지를 연결한 고성능 액체 크로마토그래프를 사용하여 독소루비신, 에피루비신, 노갈라마이신, 다우노루비신 및 아이다루비신을 동시에 정량 할 수 있는 역상 크로마토그래피법을 개발하였다. 안트라사이클린계 항생제들은 이동상 용매 중에서 은/염화은 (0.01 M NaCl) 기준전극에 대하여 확산전류를 나타내는 –0.74 V에서 검출되었다. 부피 흐름속도 (V_f)를 1.0 mL/min로 고정하였을 때 독소루비신, 에피루비신, 다우노루비신 및 아이다루비신은 각각 6.4 분, 7.4분, 12.7분 및 18.4분의 머무름 시간 (t_r)에서 나타났으며, V_f 0.6 mL/min 에서는 독소루비신, 에피루비신, 노갈라마이신, 다우노루비신 및 아이다루비신의 t_r은 각각 9.9분, 11.5분, 13.5분, 19.6분 및 28.7분에서 나타났다. 주입된 각각의 안트라사이클린계 항생제의 농도 (2.40×10^{-7} M~1.42×10^{-5} M)에 대하여 봉우리 면적 (전하)를 도시하였을 때 상관계수의 제곱 (R^2)은 0.999 이상으로 직선 성이 우수하였다. 다섯 가지 안트라사이클린계 항생제의 검출한계는 1.0×10^{-8} M~1.5×10^{-7} M이었으며 정밀도는 0.7 μM 이하의 농도를 제외하고는 상대 표준편차3 % (1.00×10^{-6} M~1.42×10^{-5} M) 미만이었다. 사람의 혈청 중에 포함된 1.00×10^{-5} M 에피루비신, 0.48×10^{-5} M 노갈라마이신 및 1.52×10^{-5} M 다우노루비신을 C_{18} 카트리지로 고상 추출하였을 때 회수율은 각각 97%, 100 % 및 90 % 이었다.

Abstract: The analytical method of HPLC with the radial-flow electrochemical cell (RFEC) has been developed to determine doxorubicin, epirubicin, nogalamycin, daunorubicin and idarubicin simultaneously by employing a reversed-phase chromatography. Anthracyclines were detected at -0.74 V vs. a Ag/AgCl (0.01 M NaCl) reference electrode, a potential of diffusion current plateau in the mobile phase. At a V_f of 1.0 mL/min doxorubicin, epirubicin, daunorubicin and idarubicin appeared at a retention time (t_r) of 6.4 min, 7.4 min, 12.7 min and 18.4 min, respectively, while at a V_f of 0.6 mL/min, doxorubicin, epirubicin, nogalamycin, daunorubicin and idarubicin appeared at a t_r of 9.9 min, 11.5 min, 13.5 min, 19.6 min and 28.7 min, respectively. The linearity

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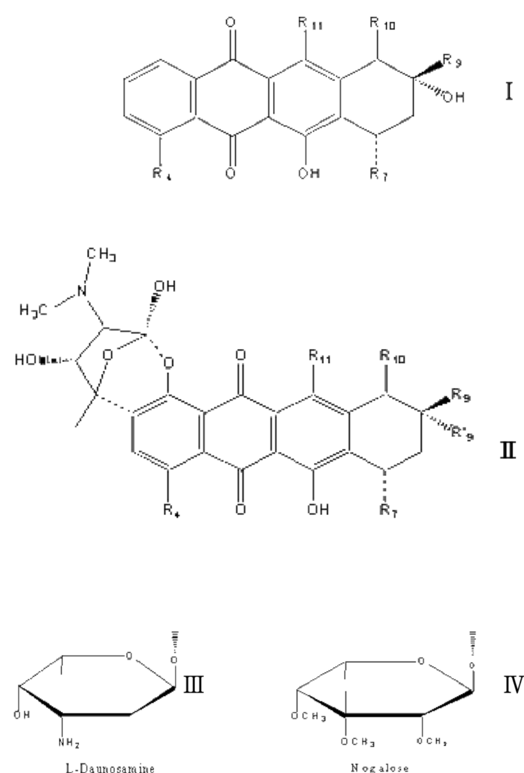
between each anthracycline injected (2.40×10^{-7} M~1.42×10^{-5} M) and peak area (charge) was excellent with the square of the correlation coefficient (R^2) higher than 0.999. The detection limits were 1.0×10^{-8} M~1.5×10^{-7} M for the five anthracyclines. Within-day precision for the five anthracyclines were in reasonable relative standard deviations less than 3 % (1.00×10^{-6} M~1.42×10^{-5} M) except the lower concentrations less than 0.7 μM. Solid phase extractions of 1.00×10^{-5} M epirubicin, 0.48×10^{-5} M nogalamycin and 1.52×10^{-5} M daunorubicin from human serum with a C_{18} cartridge resulted in 97 %, 100 % and 90 % of recoveries, respectively.

Key words: HPLC, radial-flow electrochemical cell, anthracycline antibiotics

1. Introduction

Among the antineoplastic anthracycline antibiotics, doxorubicin, epirubicin, daunorubicin and idarubicin have been widely used in clinical practice, although nogalamycin from *Streptomyces nogalator* has been withdrawn from the clinical trials for its toxicity. The cardiotoxicity of these anticancer agents is considerably reduced avoiding high peaks in the plasma concentration of the drugs with administration by continuous infusion or using a liposomal encapsulation that releases the drug very slowly.[1] Doxorubicin is produced by *Streptomyces peucetius var. caesius* and its derivative is epirubicin (4'-epidoxorubicin). Idarubicin is a semi-synthetic antibiotic of daunorubicin obtained from *Streptomyces peucetius*. A sensitive and reliable analytical technique is needed in monitoring the plasma concentrations of anthracyclines as well as in monitoring the manufacturing process to differentiate the synthesized antibiotics from the natural ones.

The spectrophotometric measurement at 495 nm was employed for the official assay of doxorubicin and the high performance liquid chromatography (HPLC) method with UV detection at 254 nm was officially used to determine daunorubicin, epirubicin and idarubicin.[2] However, there is no consistency in the stationary and mobile phases for the anthracyclines in the HPLC/UV method. The other official assay methods listed on USP, EP and BP also use the HPLC method detecting at 254 nm. The analytical methods of anthracyclines published in the literature include HPLC methods employing scanning fluorescence detector,[3] electrochemical detector (EC),[4] mass spectrometer[5] and capillary zone electrophoresis (CE) with amperometric detection.[6] The quantity of charge was measured at an applied potential of -0.30



Drug	S.	R_4	R_9	R_9'	R_7	R_{10}	R_{11}
Dau.	I	OCH_3	$-C(=O)CH_3$	OH	III	H	OH
Dox.	I	OCH_3	$-C(=O)CH_2OH$	OH	III	H	OH
Ida.	I	H	$-C(=O)CH_3$	OH	III	H	OH
Epi.	I	OCH_3	$-C(=O)CH_2OH$	OH	III	H	OH
Nog.	II	OH	OH	CH_3	IV	CO_2CH_3	H

Fig. 1. Chemical structures of anthracyclines.

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V vs. a palladium reference electrode in the HPLC-EC method due to the reduction of quinone moiety in the aglycones of epirubicin, doxorubicin and their metabolites,[4] while current was measured with a potential of +0.95 V vs. Ag/AgCl (3 M KCl) in the CE-EC method owing to the oxidation of two phenolic hydroxyls in the aglycone of daunorubicin.[6]

The chemical structures of anthracycline antibiotics as shown in *Fig.* 1 consist of the sugar linked to the anthraquinone aglycone, which commonly contains the electrochemically reducible quinone moieties. Protons were involved in the reduction of doxorubicin at the H^+/e^- ratio of one.[7] In the present study, a radial-flow electrochemical cell (RFEC) coupled to HPLC was employed to develop the analytical procedure which can be applied to monitor the manufacturing process of epirubicin from doxorubicin, or idarubicin from daunorubicin as well as to determine doxorubicin, epirubicin, daunorubicin and/or idarubicin in human serum with nogalamycin as the internal standard using the identical stationary and mobile phases.

2. Experimental

2.1. Chromatographic system

The HPLC system consisted of a solvent delivery pump (Younglin, SP930D, Korea), an injection valve (Rheodyne, 7725-018, U.S.A.), a 4.6 × 250 mm, 5C18-AR column (Waters, Code No. 378-62, U.S.A.), an amperometric detector (BAS, LC-4C, U.S.A.) equipped with a radial-flow electrochemical cell (BAS, MF-1003, U.S.A.) and a computer system using Autochrowin version 2.0 plus software. The chromatographic experiments were performed at the room temperature of 29.5°C. The RFEC was composed of a glassy carbon (dia.; 3 mm) unijet working electrode (area=0.07065 cm^2), a Ag/AgCl reference electrode and a stainless steel auxiliary electrode. The reference electrode located in the outlet channel of RFEC was prepared by dropping AgCl coating solution on the surface of silver and drying it at room temperature. The chloride concentration of the reference electrode was fixed by adding 10 mM NaCl into the mobile phase.

2.2. Chemicals and solutions

Daunorubicin hydrochloride ($C_{27}H_{29}NO_{10}\cdot HCl$; 95%), doxorubicin hydrochloride ($C_{27}H_{29}NO_{11}\cdot HCl$; 97%) and epirubicin hydrochloride ($C_{27}H_{29}NO_{11}\cdot HCl$; 97%) were kindly supplied by Dong A Pharmaceutical Company in Korea. Idarubicin hydrochloride ($C_{26}H_{27}NO_9\cdot HCl$) and nogalamycin ($C_{39}H_{49}NO_{16}\cdot HCl$; 95 %) were purchased from Sigma Company (U.S.A.). A 0.05 M citrate buffer of pH 3.0 was prepared by dissolving citric acid monohydrate (Sigma-Aldrich Co., U.S.A.) in pure water, subsequently adjusting the pH value with ammonia water (99.99%; Aldrich Co., U.S.A.). The mobile phase contained 0.05 % triethylamine (Sigma Co., U.S.A.) and 10 mM NaCl (99.5 %; Junsei Co., Japan) in the mixture consisted of 0.05 M citrate buffer of pH 3.0 and acetonitrile at the volume ratio of 7 to 3. The anthracycline antibiotics stock solutions were prepared by dissolving each antibiotic (0.4~0.85 mg) in 1 mL of methanol and were stored at –20°C. The standard antibiotic solutions were made by diluting each stock solution with mobile phase in the concentration range between 5.0×10^{-7} M and 1.0×10^{-5} M.

2.3. Procedure for the recovery of anthracyclines from human serum

Human serum sample was prepared by adding anthracyclines into 1.00 mL of human serum purchased from Sigma company (U.S.A.) to contain epirubicin, nogalamycin and daunorubicin at the concentration of 1.00×10^{-5} M, 0.48×10^{-5} M and 1.52×10^{-5} M, respectively. The serum sample added with anthracyclines was injected into the C_{18} cartridge (LiChrolut RP-18E; 40-63 μm, Merck Co., U.S.A.) washed with 3 mL of methanol (J. T. Baker, U.S.A.) and 6 mL of pure water in advance. The serum loaded C_{18} cartridge was washed with 0.5 mL of pure water and then was eluted into an eppendorf tube with 1.00 mL of methanol and dichloromethane (99.9%; Sigma-Aldrich, U.S.A.) at the volume ratio of 4 to 1. The internal standard doxorubicin added into 1.00 mL of eluate to make concentration of 2.84

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$\times 10^{-5}$ M, successively followed by filtration with a syringe PVDF filter (0.45 μm, 25 mm, Waters, U.S.A.). Two hundred μL of the filtrate was injected into the chromatographic system three times. Meanwhile, 1.00 mL of standard solution which containes 1.00×10^{-5} M epirubicin, 0.48×10^{-5} M nogalamycin, 1.52×10^{-5} M daunorubicin and 2.84×10^{-5} M doxorubicin was prepared by dissolving each anthracycline in the mobile phase. Two hundred μL of the standard solution was injected into the chromatographic system three times in order to find recovery of anthracyclines from human serum.

3. Results and Discussion

3.1. Voltammograms of doxorubicin, daunorubicin and epirubicin

The chromatographic system omitting a stationary column was employed in the voltammetric studies of the anthracyclines. Two hundred μL of each standard antibiotic solution was injected into the Rheodyne injection valve while the mobile phase was flowing through the RFEC at a volume flow rate of 1.0 mL/min (Flow injection analysis; FIA). The peak currents were measured at each applied potential.

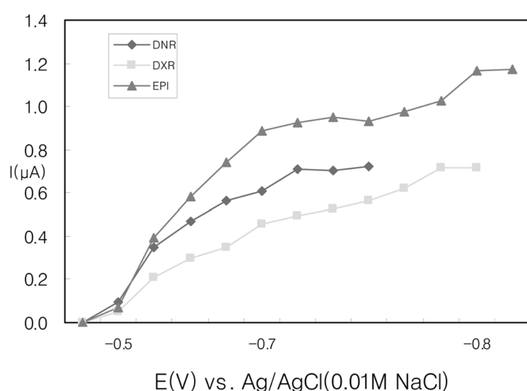

Fig. 2. Voltammograms of daunorubicin, doxorubicin and epirubicin in the mobile phase.
mobile phase: pH 3.0, 0.05 M citrate buffer-acetonitrile (7:3, V/V) containing 0.05 % triethylamine and 10 mM NaCl. daunorubicin: 0.94×10^{-5} M, doxorubicin & epirubicin: 0.97×10^{-5} M. RFEC: glassy carbon unijet working electrode, Ag/AgCl (10 mM NaCl) reference electrode, stainless steel auxiliary electrode. V_f: 1.0 mL/min, inj. vol.: 200 μL, temp.: 29.5°C.

Fig. 2 shows the current-potential curves of 0.94×10^{-5} M daunorubicin, 0.97×10^{-5} M doxorubicin and epirubicin in the mobile phase (0.05% triethylamine and 10 mM NaCl in the mixture of 0.05 M citrate buffer of pH 3.0/acetonitrile (7:3 V/V)). These anthracyclines reached the diffusion current plateau at –0.74 V vs. a Ag/AgCl (0.010 M Cl^-). Since the potentials of the Ag/AgCl reference electrode depend on the chloride concentration, using a Ag/AgCl (0.010 M Cl^-) as the reference electrode will shift the potential by -0.146 V comparing with using a Ag/AgCl (3.0 M Cl^-) electrode. In the cyclic voltammetric study employing a gassy carbon disk working electrode and a Ag/AgCl (sat. Cl^-) reference electrode, the peak currents of daunorubicin and doxorubicin in 0.10 M KCl/0.010 M HCl appeared at –0.4 V[9], that describes the reasonable diffusion current plateau of the anthracyclines in the present mobile phase.

3.2. Separations of anthracycline antibiotics by HPLC-RFEC

The chromatographic system with a C_{18} column (4.6×250 mm, 5C18-AR column Waters, U.S.A.) was used for the separations of anthracycline antibiotics. Two hundred μL of four anthracycline mixture (1.42×10^{-5} M doxorubicin, 1.00×10^{-5} M epirubicin, 1.01×10^{-5} M daunorubicin, 1.12×10^{-5} M idarubicin) was injected into the Rheodyne injector, while the mobile phase was flowing through the chromatographic system at the volume flow rate (V_f) of 1.0 mL/min and the currents flowing between the glassy carbon working electrode and the counter electrode were measured at the applied potential of –0.74 V. The retention times (t_r) of anthracyclines at temperature of 29.5°C and V_f of 1.0 mL/min were as follows (*Fig.* 3(a)); doxorubicin at 6.4 min, epirubicin at 7.4 min, daunorubicin at 12.7 min and idarubicin at 18.4 min. This procedure can be employed to monitor synthesis of epirubicin from doxorubicin or synthesis of idarubicin from daunorubicin. When nogalamycin (0.48×10^{-5} M) was added to the above anthracycline mixture and V_f was adjusted to 0.6 mL/min, each anthracycline appeared at the following t_r with temperature of 29.5°C; doxorubicin at 9.9

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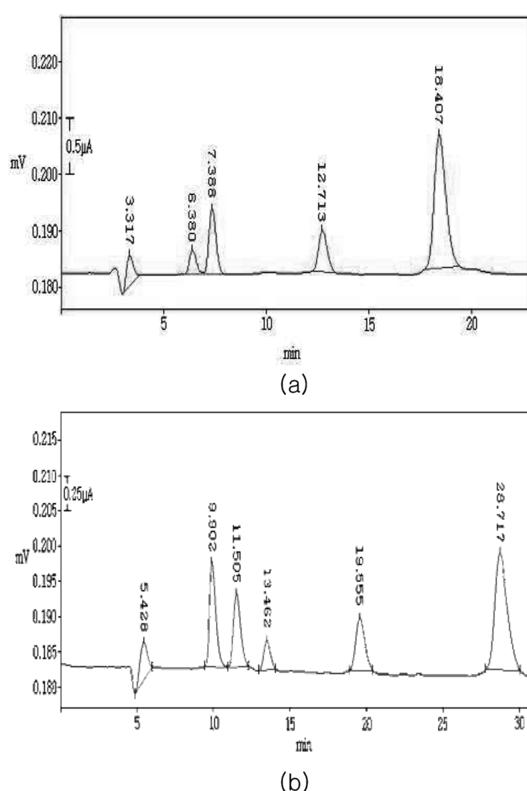

Fig. 3. Separation chromatograms of anthracycline antibiotics. mobile phase: pH 3.0, 0.05 M citrate buffer-acetonitrile (7:3,V/V) containing 0.05% triethylamine and 10 mM NaCl. stationary phase: $5C_{18}$-AR 4.6×250 mm column. RFEC: glassy carbon unijet working electrode, Ag/AgCl (10 mM NaCl) reference electrode, stainless steel auxiliary electrode. inj. vol.: 200 μL, temp.: 29.5°C. (a) V_f: 1.0 mL/min, doxorubicin at 6.4 min, epirubicin at 7.4 min, daunorubicin at 12.7 min and idarubicin at 18.4 min (b) V_f: 0.6 mL/min, doxorubicin at 9.9 min, epirubicin at 11.5 min, nogalamycin at 13.5 min, daunorubicin at 19.6 min, idarubicin at 28.7 min.

min, epirubicin at 11.5 min, nogalamycin at 13.5 min, daunorubicin at 19.6 min and idarubicin at 28.7 min as shown in *Fig.* 3(b).

When the composition of the mobile phase (citrate buffer/acetonitrile; 7/3 V/V) was changed to the volume ratio of citrate buffer and acetonitrile at 6:4, doxorubicin and its enantiomer epirubicin were not separated each other. When the V_f was adjusted to 1.3 mL/min without changing the composition of the mobile phase, peaks of doxorubicin and epirubicin were not completely resolved with a little overlapping. Chromatographic conditions for determining five anthracyclines were fixed for the following experiments as follows; mobile phase: 0.05 M citrate buffer of pH 3.0/acetonitrile (7:3,V/V) containing 0.05 % triethylamine and 10 mM NaCl, stationary phase: $5C_{18}$-AR 4.6×250 mm column, V_f: 0.6 mL/min, temperature; 29.5°C and Eapp: –0.74 V.

3.3. Calibrations and detection limits of anthracycline antibiotics

Standard solutions containing five anthracyclines were prepared using the mobile phase in the concentration range between 5.0×10^{-7} M and 1.0×10^{-5} M. Two hundred μL of each standard solution was injected into the chromatographic system at the above conditions described. Each peak area, corresponding to the electric charge, of doxorubicin, epirubicin, nogalamycin, daunorubicin and idarubicin was measured three times for each concentration. *Table* 1 shows average peak area of three measurements for the concentration of each antibiotic injected. The linearity between each anthracycline injected (2.40×10^{-7} M~1.42×10^{-5} M) and peak area (charge) was excellent with the square of the correlation coefficient (R^2) higher than 0.999. The calibration factor (m) which is the slope of the linear plot varied among the anthracycline antibiotics as shown in *Table* 1. The relative detector responses (F) were calculated as 1.14 for doxorubicin, 1.20 for epirubicin, 1.20 for daunorubicin and 3.39 for idarubicin when the detector response for nogalamycin was fixed as 1.00. The detection limits of the five anthracyclines were 1.0×10^{-8} M~1.5×10^{-7} M based on the calculation of 3 times standard deviation divided by calibration factor (3s.d./m).[8] Nogalamycin can be employed as the internal standard for analyzing four other anthracycline antibiotics in the biological fluid samples, which are clinically widely used.

3.4. Precision studies

As shown in *Table* 1, within-day precision studies for the five anthracycline antibiotics resulted in reasonable relative standard deviations less than 3% (1.00×10^{-6} M ~ 1.42×10^{-5} M) except the lower

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Table 1. Calibrations of anthracycline antibiotics by HPLC-RFEC

doxorubicin		epirubicin		nogalamycin		daunorubicin		idarubicin	
conc. (μM)	ave.± s.d. q(μC)	conc. (μM)	ave.± s.d. q(μC)	conc. (μM)	ave.± s.d. q(μC)	conc. (μM)	ave.± s.d. q(μC)	conc. (μM)	ave.±s.d. q(μC)
14.2	21.9± 0.20	10.0	16.2± 0.15	4.80	6.61± 0.04	10.1	16.4± 0.15	11.2	51.2± 0.23
7.11	10.7± 0.00	5.00	8.49± 0.08	2.40	3.23± 0.08	5.05	8.19± 0.03	5.62	25.4± 0.17
1.42	1.93± 0.01	1.00	1.54± 0.04	0.48	0.77± 0.04	1.01	1.56± 0.04	1.12	4.69± 0.14
0.711	1.06± 0.08	0.500	0.84± 0.03	0.24	0.33± 0.02	0.505	0.71± 0.04	0.562	2.17± 0.02
R^2	0.9998	R^2	0.9992	R^2	0.9994	R^2	1.000	R^2	1.000
m	1.55	m	1.63	m	1.36	m	1.63	m	4.61
F	1.14	F	1.20	F	1.00	F	1.20	F	3.39
3s.d./m	0.15	3s.d./m	0.06	3s.d./m	0.04	3s.d./m	0.07	3s.d./m	0.01

R^2; the square of the correlation coefficient. m; calibration factor (μC/μM), F; detector response factor, 3s.d./m; detection limit (mM)

Table 2. Inter-day precision studies of anthracyclines

anthracyclines	1st day*	5th day*	% (5th day/1st day)
epirubicin	0.35	0.35	100
daunorubicin	0.38	0.37	97.4

*; peak area ratio of anthracycline over the internal standard, doxorubicin
V_f: 0.6 mL/min Temp.: 29.5°C

concentrations of anthracyclines less than 0.7 μM. Inter-day precision studies of 5 days interval were performed for epirubicin and daunorubicin at the concentrations of 1.00×10^{-5} M by using 2.84×10^{-5} M doxorubicin as the internal standard. The peak area ratios of epirubicin and daunorubicin over the internal standard were calculated in *Table* 2. Epirubicin was found as quite stable, while daunorubicin deteriorated by 2.4%.

3.5. Recovery of anthracyclines from human serum by solid phase extraction

The human serum sample containing anthracyclines and the standard anthracycline solution prepared according to the procedure in the experimental section were analyzed by HPLC-RFEC. The peak areas of epirubicin, nogalamycin and daunorubicin were compared with that of doxorubicin in the chromatograms of the serum sample as well as those of the standard solution as listed in *Table* 3. Recoveries of 1.00×10^{-5} M epirubicin, 0.48×10^{-5} M nogalamycin and 1.52×10^{-5} M daunorubicin were 97 %, 100 % and 90 %, respectively.

Acknowledgments

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Table 3. Recovery of anthracyclines from human serum

anthracyclines	standard*	serum*	recovery
1.00×10^{-5} M epirubicin	0.33± 0.01	0.32± 0.02	0.97± 0.07
0.48×10^{-5} M nogalamycin	0.07± 0.01	0.08± 0.007	1.00± 0.09
1.52×10^{-5} M daunorubicin	0.40± 0.02	0.36± 0.03	0.90± 0.09

*; peak area ratio of anthracycline over the internal standard, 2.84×10^{-5} M doxorubicin
V_f: 0.8 mL/min Temp.: 29.5 °C

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