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Journal of Chromatography A, 1076 (2005) 207–211
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Preparative counter-current chromatography purification of valrubicin (AD-32) from crude synthetic drug using upright coil planet centrifuge with four multilayer coils connected in series

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Received 21 September 2004; received in revised form 5 April 2005; accepted 14 April 2005

Abstract

Preparative counter-current chromatography (CCC) purification of valrubicin (AD-32) from crude synthetic drug has been successfully performed for the first time using upright coil planet centrifuge with four multilayer coils connected in series with 1600 ml capacity. The two-phase system used was composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water at an optimized volume ratio of 1:1:8:6:1. Target compound (1.2 g) with a purity of 99.88% was obtained from 1.5 g of crude synthetic drug with a purity 95.49% based on HPLC peak area percentage. Identification of the target compound was performed by electrospray ionization mass spectrometry, one- and two-dimensional nuclear magnetic resonance.

Keywords: Valrubicin; AD-32; Adriamycin; Counter-current chromatography; Synthetic drug

1. Introduction

Valrubicin (AD-32) is an *N*-trifluoroacetyl, 14-valerate derivate of the anthracycline adriamycin (ADR) and daunorubicin [1,2]. Unlike ADR, AD-32 is a lipophilic compound and rapidly traverses cell membranes and accumulates in the cytoplasm [3]. Its most interesting feature is the lack of capacity to intercalate with DNA [4,5]. In preclinical and clinical studies AD-32 was shown to be superior to the parent compound in several areas, including toxicity and antitumor efficacy [6–14]. The drug possesses potential antitumor activity against urinary bladder cancer and other tumors. It is also associated with significantly less toxicity, including less contact toxicity and no cardiotoxicity in human subjects. Futhermore a six-week course of AD-32 has proved effective in ablating a marker tumor [15].

In view of the potential therapeutic values, the synthesis and purification of AD-32 and its analogues have been of much interest to pharmaceutical chemists. Because AD-32 is a semisynthetic derivative of ADR or daunorubicin, the crude synthetic drug contained several impurities so that it should be further to purify for preclinical and clinical chemotheraphy. The preparative purification of AD-32 from crude synthetic product is tedious and usually requires multiple chromatography steps, such as column chromatography (CC) and thin-layer chromatography (TLC) [16–18]. To obtain high pure AD-32 is difficult because of its unstable properties and irreversible adsorption on solid support column chromatography. Counter-current chromatography (CCC) is a unique liquid–liquid partition chromatography without use of solid support matrix [19]. Therefore, it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination. The method has been successfully applied to the analysis and separation of various natural and synthetic products [20–22]. Recently, we have developed a versatile type-J CCC with four upright multilayer coil columns arranged symmetrically around the centrifuge axis [23]. Our primary experiments have demonstrated the

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doi:10.1016/j.chroma.2005.04.027

upright CCC apparatus is very useful for large-scale isolation and purification of crude extracts of natural products or synthetic compounds. So far, no report has been published on the use of CCC for the purification of AD-32. The purpose of this study, therefore, is to develop a CCC method for the preparative purification of AD-32 from crude synthetic drug.

2. Experimental

2.1. Apparatus

The CCC isolation and purification of AD-32 from crude synthetic drug was performed by upright coil planet centrifuge with four multilayer coils connected in series. Its design principle and dimensions were described in detail in the literature [23]. The upright CCC apparatus holds four identical multilayer coil columns and four same counter-rotating hollow tube supports in the alternate symmetrical positions around the rotary frame to maintain perfect balance of centrifuge system without the use of a counterweight. The unique gear arrangement on the rotary frame establishes a twist-free mechanism of the flow tubes so that continuous elution can be performed without the use of rotary seal. Each separation column was made by winding a single piece of 4 mm I.D. and 1 mm wall thickness polytetrafluroethylene (PTFE) tubing directly onto the holder hub of 5 cm diameter forming three layers of right-handed and left-handed coils alternating in each layer between a pair of flanges spaced 35 cm apart. The β value (ratio of helical radius of the coil and revolution radius) of the multilayer coil varies from 0.28 at the internal terminal to 0.48 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge, $R = 9$ cm). The four multilayer coils are connected in series on the rotary frame using a flow tube (PTFE, 1.6 mm I.D. and 0.7 mm wall thickness) to give a total capacity of 1600 ml in such a manner that the external terminal of the first column joins the internal terminal of the second column and, similarly, the external terminal of the second column joins the internal terminal of the third column. In this way all multilayer coils are subjected to the identical elution modes.

The apparatus can be operated up to maximum speed of 800 rpm with a speed Sunwind control unit (Shenduo Electric Corp., Shanghai, China) and up to 60 °C with a temperature control unit. In addition, this CCC system is equipped with a Type-J-W metering pump (Zhijiang Petroleum Equipment, Hangzhou, China), a HD-9704 UV spectrometer operating at 254 nm and 280 nm, Shimadzu C-R1B Chomatopac recorder, BSZ-100 fraction collector, a sample injection valve with a 30 ml sample loop and NT2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China).

The high-performance liquid chromatography (HPLC) system used was Agilent 1100 system including G1312A BinPump, G1314A variable-wavelength detector (VWD), a model 7725 injection valve with 20 μl loop, a PT100 column oven and Agilent ShemStation for LC.

2.2. Reagents

All organic solvents used for CCC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany.

The crude synthetic drug was kindly obtained from Zhejiang Biopharm. Co., Hangzhou, China. It is synthesized from the reported reactions as shown in Fig. 1 [18].

2.3. Preparation of two-phase solvent system and sample solutions

The two-phase solvent system used was composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–chloroform–methanol–water at various volume ratios, i.e., 1:0:6:4:1, 0:1:6:4:1, and 1:1:8:6:1. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. For the present preparative CCC separation, the total volume of the prepared two phases each time is 6 l.

The sample solutions were prepared by dissolving the crude synthetic drug in a solvent mixture consisting of equal volumes of both upper and lower phases at suitable concentration according to the preparative scale of CCC separation.

2.4. Separation procedure

Preparative CCC was performed as follows: the four upright multilayer coil columns connected in series were first entirely filled with upper phase as stationary phase, and then the sample solution was injected through the sample port and the lower phase as a mobile phase was pumped in head-to-tail elution mode at flow rate of 7 ml min^{-1} while the column was rotated at 500 rpm. The effluent was monitored on-line at 280 nm and automatically collected in 20 ml test tube per 3 min using a BSZ-100 fraction collector. Peak fractions were collected according to the elution profile and HPLC detection.

2.5. HPLC analysis and identification of CCC peak fractions

The crude sample and CCC peak fractions were analyzed by HPLC. The analyses were performed with an YMC-Pack ODS-A column [150 mm × 4.6 mm I.D., 5 μm (particle size), 120 Å (pore size)]. The mobile phase was acetonitrile–0.015 M orthophosphoric acid (70:30, v/v). The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 254 nm.

Identification of the CCC peak fraction was carried out by mass spectrometry (MS) on Bruker Esquire 3000 plus

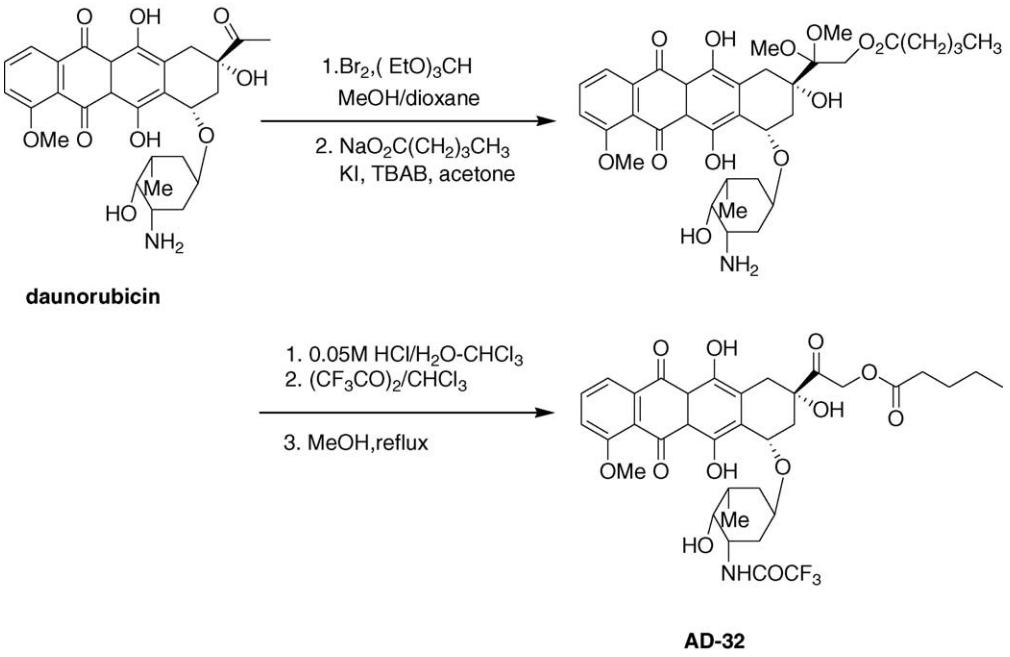

Fig. 1. Synthesis of AD-32 [18].

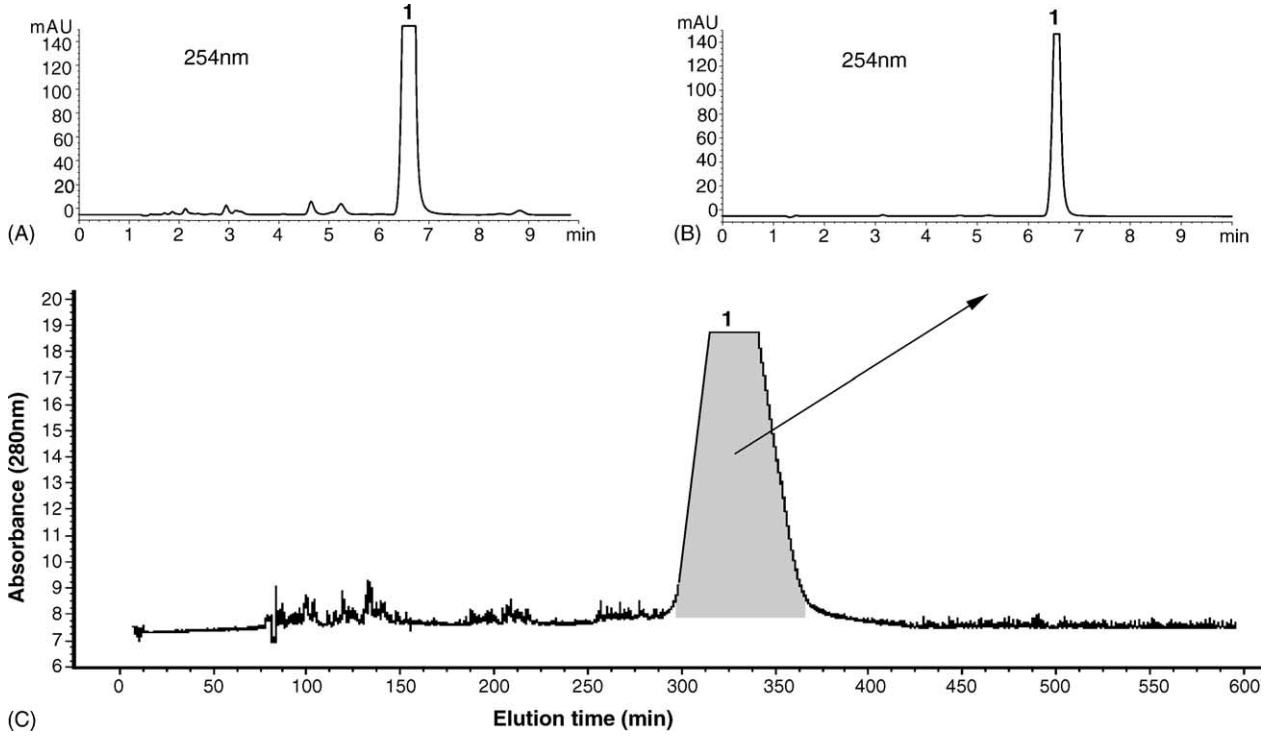

Fig. 2. Chromatograms of (A, B) HPLC analyses and (C) CCC separation of AD-32; (A, C) crude sample, (B), purified AD-32; peak **1**, AD-32. CCC conditions: column, PTFE tube of 4.0 mm I.D. with the total capacity of 1600 ml; rotary speed, 500 rpm; column temperature, 35 °C; solvent system, light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v); mobile phase, lower phase; elution mode, head-to-tail; flow rate, 7 ml min^{-1}; detection, 280 nm; sample size, 1.5 g crude AD-32 in 5 ml upper phase and 5 ml lower phase; retention of the stationary phase, 76.9%. HPLC conditions: column, YMC-PACK ODS-A column (150 mm × 4.6 mm I.D., 5 μm, 120 Å); column temperature, 25 °C; mobile phase, acetonitrile–0.015 M orthophosphoric acid (70:30, v/v); flow-rate, 1.0 ml min^{-1}; detection, 254 nm. Purity (A) 95.49%, (B) 99.88%.

spectrometer, one- and two-dimensional NMR spectrometry on a Bruker Advanced DMX 500 NMR spectrometer. NMR experiments were performed with dimethyl sulphoxide (DMSO) as solvent and TMS as internal standard, and operated at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

3. Results and discussion

The crude synthetic AD-32 drug was first analyzed by HPLC. As shown in Fig. 2A, the analysis indicates several impurities were contained and the content of AD-32 in crude sample is about 95.49% based on HPLC peak area percentage.

Successful CCC separation depends on the correct of the solvent system. Generally speaking, the two-phase solvent system should satisfy the following requirements: (1) no decomposition or denaturation of the sample; (2) sufficient sample solubility; (3) suitable partition coefficient values; (4) satisfactory retention of the stationary phase [21]. Due to the lipophilic property and poor water solubility of AD-32, the two-phase solvents with medium polarity composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water at various volume ratios were used as start solvents. In order to achieve an efficient resolution of target compound, 500 mg each time of crude sample dissolved in 5 ml of upper phase and 5 ml

Table 1
One- and two-dimensional NMR data of AD-32 (in DMSO)

Atom	δ_C (ppm)	DEPT[a]	HMQC δ_H (ppm)	HMBC correlation with	^{1}H–^{1}H COSY correlation with
1	70.67	CH	4.88 (1H, s)	H-1′, 2-$H_{eq.}$	2-$H_{ax.}$
2	36.97	CH_2	2.00 (1H, d, J = 14.26 Hz, 2-$H_{ax.}$) 2.34 (1H, d, J = 14.18 Hz, $H_{eq.}$)	3-OH	2-$H_{eq.}$, H-1 2-$H_{ax.}$
3	76.07	q[b]		3-OH, 4-$H_{eq.}$, 4-$H_{ax.}$, 2-$H_{eq.}$	
4	32.63	CH_2	2.76 (1H, d, J = 14.26 Hz, 4-$H_{ax.}$) 3.00 (1H, d, J = 18.15 Hz, 4-$H_{eq.}$)	3-OH, H-2-$H_{eq.}$	4-$H_{eq.}$ 4-$H_{ax.}$
4a	134.68	q		5-OH, 4-$H_{ax.}$,4-$H_{eq.}$	
5	155.47	q		5-OH, 4-$H_{eq.}$	
5a	111.40	q		5-OH	
6	187.04	q		H-7	
6a	135.37	q		H-7	
7	119.83	CH	7.79 (1H, m)	H-9	H-8
8	137.04	CH	7.81 (1H, d, J = 7.35 Hz)		H-9
9	120.50	CH	7.52 (1H, d, J = 8.20 Hz)	H-7, H-8	H-8
10	161.64	q		H-13, H-7, H-8	
10a	120.74	q			
11	187.19	q			
11a	111.52	q		12-OH	
12	157.03	q		12-OH	
12a	136.14	q		12-OH, 4-$H_{eq.}$, 4-$H_{ax.}$, 2-$H_{eq.}$	
13	57.42	CH	3.92 (3H, s)		
14	208.98	q		3-OH, 15 $H_{ax.}$, 15 $H_{eq.}$	
15	66.44	CH_3	5.16 (1H, d, J = 17.81 Hz, 15-$H_{ax.}$) 5.27 (1H, d, J = 17.88 Hz, 15-$H_{eq.}$)		15-$H_{eq.}$ 15-$H_{ax.}$
16	173.40	q		H-17, H-18, 15-$H_{ax.}$, 15-$H_{eq.}$	
17	33.87	CH_2	2.42 (2H, t, J = 7.29 Hz)	H-18	H-18
18	27.57	CH_2	1.57 (2H, M)	H-20, H-17	H-19, H-17
19	22.52	CH_2	1.36 (2H, q, J = 7.52 Hz)	H-20, H-17, H-18	H-18, H-20
20	14.60	CH_3	0.9 (3H, t, J = 7.32 Hz)	H-19, H-18	H-19
1′	101.15	CH	5.25 (1H, s)	2′-$H_{ax.}$	
2′	29.73	CH_2	1.49 (1H, d, J = 14.26 Hz, 2′-$H_{ax.}$) 2.10 (1H, dd, J = 9.84, 12.89 Hz, 2′-$H_{eq.}$)	H-4′	2′-$H_{eq.}$ 2′-$H_{ax.}$, H-3′
3′	47.96	CH	4.05 (1H, t, J = 6.41, 6.19 Hz)	2′-$H_{ax.}$, 2′–$H_{eq.}$, H-4′, H-1′	7′-NH, 2′–$H_{eq.}$, 2′-$H_{ax.}$
4′	67.98	CH	3.53 (1H, s)	2′-$H_{ax.}$, H-6′	4′-OH
5′	67.38	CH	4.28 (1H, t, J = 5.94, 6.51 Hz)	H-6′, H-1′	H-6′
6′	17.79	CH_3	1.16 (1H, d, J = 6.35 Hz)		H-5′
8′	156.75	q			
9′	116.79	q			
4′-OH			5.01 (1H, s)		H-4′
7′-NH			9.11 (1H, d, J = 7.35 Hz)		H-3′

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

of lower phase were used and the two-phase solvent systems with volume ratios, i.e., 1:0:6:4:1, 0:1:6:4:1, and 1:1:8:6:1, have been examined using the present CCC apparatus. The result indicated that using the solvents system with the volume ratio of 1:1:8:6:1, the high retention of stationary phase (up to 75%) and high purity of AD-32 (up to 99.5%) are easier to achieve in one-step separation as compared with other solvent systems. Therefore, the optimized solvent system with volume ratio of 1:1:8:6:1 was used for large-scale purification of AD-32.

Fig. 2C shows the preparative CCC separation of 1.5 g of the crude synthetic AD-32 sample using the solvent system composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v). Peak fractions were collected according to the elution profile and HPLC detection. As a result, 1.2 g AD-32 with 99.88% purity was obtained. The HPLC analysis of purified AD-32 is shown in Fig. 2B. Its negative ESI-MS spectrum showed the characteristic ion at *m*/*z* 722 due to $[M-H]^-$ corresponding to the molecular formula $C_{34}H_{36}F_3NO_3$. Its one- and two-dimensional NMR data are shown in Table 1, which identified the chemical structure of AD-32 (Fig. 2).

In conclusion, large-scale preparative counter-current chromatography purification of AD-32 from the crude synthetic drug was successfully performed using upright coil planet centrifuge which holds four identical multi-layer coil columns in the symmetrical positions around the centrifuge axis, and yielded 1.2 g AD-32 at 99.88% purity from 1.5 g of crude synthetic sample in one-step separation. The present study indicated that the CCC apparatus is very practical for the preparative separation of AD-32 from crude synthetic drug. However, a drawback to the method is tetrachloromethane, a toxic solvent.

Acknowledgements

The authors gratefully acknowledge the Natural Science Foundation of China (20375036) and Zhejiang Province (RC0042, Y304118).

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