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齐墩果醇酸对化学物质致小鼠急性肝损伤的保肝作用

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目的：评价齐墩果醇酸(OA)对急性肝损伤的保肝作用. **方法**：小鼠 sc OA 200 μmol·kg⁻¹三天，然后给予肝毒物. 通过病理组织学观察及测定血清丙氨酸转氨酶和艾杜糖醇脱氢酶活性来估价肝损伤. **结果**：OA 能明显减轻四氯化碳，溴苯，醋氨酚，速尿，硫代乙酰胺，鬼笔毒环肽，秋水仙硷，氯化镉，*D*−半乳糖胺和内毒素等所致小鼠急性坏死性肝损伤，降低这些肝毒物所引起的血清转氨酶和艾杜糖醇脱氢酶的升高，但对氯仿，二甲亚硝氨，鹅膏菌素和烯丙醇的毒性无作用. **结论**：OA 能减轻多种化学物质(但并非全部)引起的肝损伤. 其保肝机制可能是多方面的.

关键词 齐墩果醇酸；肝；四氯化碳中毒；醋氨酚；溴化苯类；鬼笔毒环肽；镉中毒；半乳糖胺；内毒素；硫代乙酰胺

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Simultaneous determination of *N*,*N*-di(*n*-butyl)doxorubicin-14-valerate and its 8 urinary metabolites by HPLC

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AIM: To develop an HPLC assay for simultaneous determination of AD202 and its metabolites and to examine metabolites of AD202 in urine of rats. **METHODS**: Reverse-phase HPLC with fluorescence detection and gradient elutionusing a Waters Chromatograph equipped with a 710 B WIFP autosampler, a power Mate SX plus computer, C_{18} Nova-pak™ (4 μm) 5 mm×10 cm radial compression column connected with a guard micro-column, and a Waters 991 photodiode array detector for online observation of UV spectrum of related fraction. **RESULTS**: Detection limit was 1−3 ng for AD202 and 1−3 ng for its metabolites per injection. After iv AD202 20 mg·kg⁻¹, only 4.9 % dose as total anthracycline fluorescence signal was recovered in urine over 72 h. The predominant urinary metabolites were AD285 (desacyl AD202) and AD284 (*N*-mono-debutyl AD285). Six minor metabolites including aglycones and 13-keto reductive product were identified and 3 as-yet unknown metabolites were seen. Enzymatic and acid-hydrolysis failed to reveal the presence of glucuronides in urine. **CONCLUSION**: The sample analysis techniques developed in this study proved to

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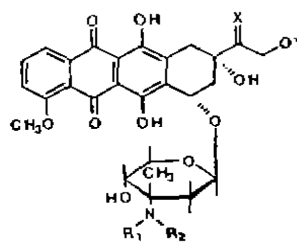
be very efficient, sensitive, and specific, a total of 11 compounds achieved resolution with detection limit of 2 ng and no interference from matrix. Urine sample can be injected directly into chromatograph without any extraction, making sample analysis simple and time-saving.

KEY WORDS *N*,*N*-di(*n*-butyl)doxorubicin-14-valerate; high pressure liquid chromatography; fluorescence; pharmacokinetics

Since doxorubicin (Dox, adriamycin), an anthracycline antineoplastic drug, was introduced into clinic use, there has been a great interest in search for Dox analogs having high therapeutic index.

N,*N*-Di(*n*-butyl)doxorubicin-14-valerate (AD202) is one of lipophilic *N*-alkyl-substituted Dox congeners synthesized in our laboratory, which showed therapeutic superiority to Dox *in vivo* murine tumor system and ability to bypass multidrug resistance *in vitro*[1,2]. But its quantitation and metabolic fate have not been reported to date. This study was undertaken (1) to develop an HPLC assay for simultaneous determination of AD202 and its metabolites and (2) to examine metabolites of AD202 in urine of rats. the chemical structures of AD202 and possible major biotransformation products are presented in Fig 1.



	X	Y	R_1	R_2
AD202	O	$OC(CH_2)_3CH_3$	*n*-Butyl	*n*-Butyl
AD194	O	$OC(CH_2)_3CH_3$	*n*-Butyl	H
AD284	O	H	*n*-Butyl	H
AD285	O	H	*n*-Butyl	*n*-Butyl
AD294	H, OH	H	*n*-Butyl	H
AD295	H, OH	H	*n*-Butyl	*n*-Butyl
Doxorubicin	O	H	H	H

Fig 1. AD 202 and possible major metabolites.

MATERIALS AND METHODS

Chemicals and enzymes AD202, Dox, *N*-mono (*n*-butyl)-doxorubicin-14-valerate (AD194), *N*-mono (*n*-butyl)-doxorubicin (AD284), *N*,*N*-di(*n*-butyl)-doxorubicin (AD285), *N*-mono (*n*-butyl)-13-dihydro-doxorubicin (AD294), *N*,*N*-di(*n*-butyl)-13-dihydro-doxorubicin (AD295), Adrimycinone (AD8), and 7-deoxy-13-dihydro-adriamycinone (AD151) were synthesized and used as their corresponding hydrochloride salts in our lab (>99 % chemical purity by reverse phase HPLC). For iv injection, AD202 was dissolved in appropriate volume of NCI Diluent 12 [cremophor EL (polyethoxylated castor oil); ethanol, 1:1 by volume; Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda MD] and diluted with 4 volumes of 0.9 % saline. β-Glucuronidase (type B-1, 624 000 units·g^{-1} solid) was purchased from Sigma Chemical Co (St Louis MO). Other chemical reagents are of analytical grade.

Drug administration and sampling procedures Sprague-Dawley rats, ♀, $n=7$, weighing $257 \pm s$ 42 g under momentary methoxyfluorane anesthesia were injected AD202 (20 mg·kg^{-1}) as single bolus via caudal vein and then returned to individual metabolic cages. At scheduled times urine samples were collected and immediately put in freezer at −70 ℃ pending analysis.

Urine analysis Urine samples (usually 10 μL) were directly injected into the column after centrifugation (1100×*g*, 10 min) without any extraction. In addition, where appropriate, selected urine samples were diluted with distilled water or subjected to bulk extraction with solid phase C_{18} Sep-Paks™ (Waters Associates, Milford MA) to enrich the minor metabolites.

The separation of AD202 and its metabolites in the urine were achieved by reverse-phase HPLC using a Waters Chromatograph equipped with a 710 B WIFP autosampler (Waters Associates), a power Mate SX plus computer, C_{18} Nova-pak™4 μm 5 mm×10 cm ra-

dial compression column (Waters Associates) connected with a guard micro-column (7 μm particles 3.2 mm ×15 mm Milford, MA), and a Waters 991 Photodiode array detector for on-line observation of UV spectrum of related fraction. The mobile phase composed of pH 4.0 ammonium formate buffer 0.05 mol·L^{-1} (A) and acetonitrile (B) at a flow rate of 1 mL·min^{-1}, consisting initially of 70 % A/30 % B and then changed in a linear gradient manner over 5 min to a composition of 20 % A/80 % B. This composition was maintained for 5 min, then returned back to initial condition (70 % A/30 % B, total run time 15 min) in linear gradient manner. The eluate was monitored by a flow fluorometer (Model FS 970, Schoeffel Instruments, Ramsay NJ) set at λ_{ex} 482 nm and equipped with a λ_{em} 550 nm cut-off filter. Each run of determination of urine samples was accompanied by 5 different concentrations of AD202 standard. AD202 and metabolites were quantified by external standard method, ie, reference to the calibration curve constructed daily for AD202 by plotting peak area *vs* concentration of AD202. The amount of metabolites were expressed as equivalents of AD202.

Fluoresence signals of AD202 and metabolites were identified by their retention time (t_r) relative to authentic standards and by co-chromatography of urine samples with reference compounds, and where necessary were further confirmed by normal phase HPLC of urine or eluate collected from reverse-phase HPLC and subsequently enriched by solid phase extraction on Sep-Pak column.

The normal-phase HPLC was performed on a Waters chromatograph equipped with a model 660 solvent programmer, model 745 Data Module and a Partisil PXS10/25 PAC Waters column. Mobile phase consisted of $CHCl_3$(C) and $CHCl_3$-MeOH-glacial acetic acid-water (850/150/50/15 by volume, D), delivered at flow rate of 2.5 mL·min^{-1} in linear gradient manner, ie, initially 90 % C/10 % D, at 10 min 0 % C /100 % D, and thereafter isocratically delivered until 15 min.

Enzymatic and acidic detection of conjugates Urine samples 300 μL following alkalinization with 1 mL pH 8.5 Tris buffer 0.05 mol·L^{-1} was extracted twice with 3 volumes of EtoAc/propanol (9/1). The resulting aqueous phase was incubated[3] with 0.5 mg β-glucuronidase (100 units·mL^{-1} final) at pH 5.2, at 37℃ for 0.5, 4, 8, 22, and 48 h or with HCl 0.1 mol·L^{-1} in methanol at 100 C for 48 h. Appropriate incubation without added enzyme and HCl was carried out as control. The supernatants after centrifugation of incubation product were subjected to HPLC analysis.

RESULTS

On the reverse phase chromatograms AD202 and its 8 expected metabolites Dox, AD294, AD151, AD8, AD284, AD295, AD285, and AD194 yielded good separation from each other except 2 aglycones AD8 and AD151 which were extremely similar in chemical structures. AD202 and its metabolites were free of matrix interference(Fig 2).

The normal phase chromatogram were fully resolved with retention times of 3.10, 5.07, 6.12, 7.44, 8.73, 9.43, 10.95, and 12.77 min for AD8, AD151, AD194, AD285, AD284, AD295, AD294, and Dox, respectively.

The detection limits of the assay were found to be 2 ng per injection of AD202 and 1-3 ng per injection of related metabolites at signal-to-noise ratio of 3. A good linearity ($r > 0.998$) was obtained in the range of 2-500 ng (2, 10, 50, 200, and 500 ng; $n = 3$ each) for AD202 and metabolites. By calculating the ratio of peak area obtained for urine sample spiked with standards to those acquired for equal amounts of respective standards in methanol the recoveries were found to be 100.2±3.0, 98.9±2.8, 99.5±3.2, 96.8±3.5 % for 10, 50, 200, 500 ng AD202, respectively, and >95 % for metabolites. The intraday coefficiency of variation (CV) ranged from 1.5 % to 7.0 % at injections of 10, 50, 200, and 500 ng of AD202 (5 determinations each), indicating a high reproducibility of this method.

Urinary drug elimination was only 4.9±1.6 % of the injected dose over 72 h on the total anthracycline fluorescence basis (Fig 3).

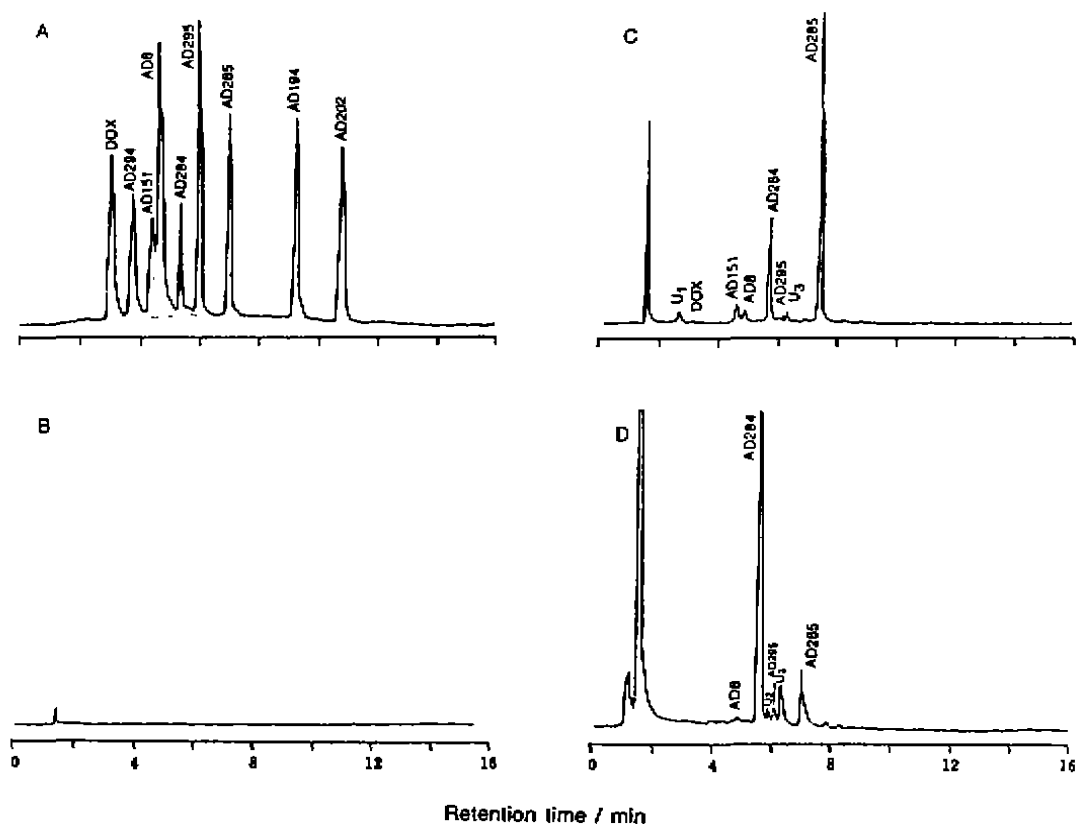


Fig 2. Reverse-phase HPLC chromatograms. A) standards in MeOH corresponding to 200 ng of hydrochloride form each. B) Rat blank urine sample (100 μL). C&D) Rat urine samples taken during 0—4 h and 24—36 h, respectively after iv AD202 20 mg·kg^{-1}.

AD202 was undetectable in urine from 6 out of 7 rats. Only one rat showed AD202 excretion (0.002 %) in urine only in the earliest sampling time (4 h). Predominant metabolites in urine were AD285 and AD284 (Fig 2 C, D, and Fig 3) accounting for 2.2±0.7 and 1.9±0.7 % of injected dose or 45±13 and 39±14 % of total metabolites excreted in urine, respectively. Other minor metabolites included aglycones (AD151 and AD8), AD295, AD294, and Dox representing 0.24±0.08, 0.15±0.07, 0.04±0.05, and 0.04±0.05 % of dose. Three as-yet uncharacterized metabolites (U1, U2, U3) with t_r of 2.78, 6.25, 6.68 min, accounting for 0.32±0.24 % of dose were seen.

The maximum elimination rate of total anthracyclines, AD285, and AD284 reached at 8, 8, and 12 h respectively, subsequently falling off sharply, and AD284 excreted in urine in a lower rate than AD285 in early 12 h. Thereafter AD284 excretion rate exceeded that of AD285 (Fig 2C and D) where the ratio of AD285 to AD284 reduced from 3:1 at 0-4 h to 1:4 at 24-36 h. By calculation[4] the excretion rate constant (and half-life) of total anthracyclines, AD285, and AD284 were 0.046±0.011 h^{-1}(15±4 h), 0.036±0.010 h^{-1}(19±5 h), and 0.050±0.012 h^{-1}(13.9±2.8 h).

The urine sample contained a high polar

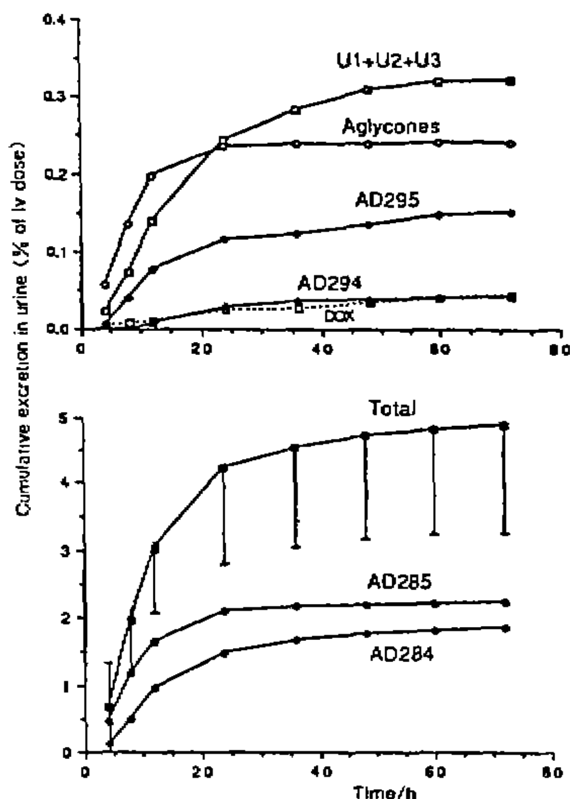


Fig 3. Cumulative urine excretion of AD202 metabolites over 72 h after iv AD202 20 mg·kg^{-1}.

product of which t_r(1.78 min) on the reverse-phase HPLC system was shorter than Dox (t_r 3.28)(Fig 2C, D). This product was not extractable with EtoAc/Propanol and thus resided in the aqueous phase. After enzymatic and acidic hydrolysis of aqueous phase, no differences in chromatograms between the treated and control samples were seen, indicating that there were no conjugates of anthracyclines in the urine. This was further supported by spectrogram determined by photodiode array detector. Urinary polar chromatographic peak (t_r1.78 min) was lacking in peak absorbance at 485 nm which is a characteristic of AD202 and other anthracyclines (Fig 4).

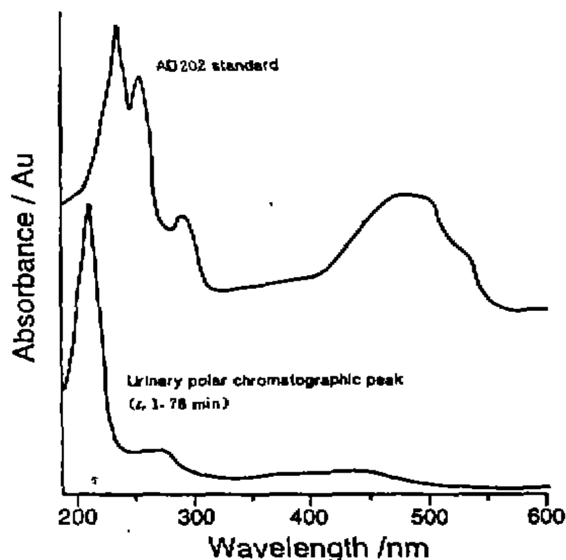


Fig 4. Spectrogram on-line determined by photodiode array detector.

DISCUSSION

The sample analysis techniques developed in this study proved to be very efficient, sensitive, and specific, a total of 11 compounds achieved resolution with detection limit of 1—3 ng per injection and no interference from matrix. The isocratic elution and UV detection were tried, but failed to succeed in separation of metabolites and elimination of interference from endogenous substances. With use of fluorescence detector and gradient elution, urine sample can be injected directly into chromatograph without any extraction, making sample analysis simple and time-saving.

No or extremely negligible quantity of parent drug were seen in urine but as many as 8 fluorescent metabolites were detected indicating that AD202 underwent extensive biotransformation. Based on the detected metabolic products, the AD202 metabolism is most likely to involve the following at least 4 different reactions (in order of importance):

(1) hydrolysis of ester group to desacyl-AD202 ie AD285 (2) N-debutylization of aminosugar moiety giving rise to mono and di-debutyl products, eg, AD284 and Dox, respectively. (3) deglycosidation, yielding aglycones such as AD8 (adriamycinone) and AD151 (7-deoxy-13-dihydro-adriamycinone) (4) reduction of C-13 keto group to a secondary alcoholic group, the product being AD295. No AD194 was detected in urine, it follows that the ester-hydrolysis outweighs by far greatly debutylization due to abundant esterase in plasma and other tissues. Only about 4 % of administered dose were recovered in urine as anthracycline-related metabolites. This implies that AD202 may have other excretory routes such as biliary excretion, which has proved to be principally responsible for elimination of Dox and other Dox-related anthracyclines, and/or metabolic pathways such as anthracycline ring cleavage to non-anthracycline metabolites without fluorenscene[5].

Contrary to AD201 〔N,N-di (n-propyl) doxorubicin-14-valerate〕 but in common with AD198 (N-benzyldoxorubicin-14-valerate)[6,7], D202 does not elicit any glucuronide conjugate. This may be related to steric hindrance effect of the amino nitrogen substituent. However, all three of Dox analogs are subjected to sequential N-dealkylation, in this manner removing any hindrance. To clarify this metabolic difference, much more work need to be done.

It is evident that urine samples contained a more polar material than Dox. Our preliminary analysis demonstrated that this unknown product was neither conjugate no anthracycline-related metabolites. It seemed resonable to assume that the product may be an endogenous substance which was formed and excreted in urine under effect of xenobiotic AD202.

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应用HPLC同时测定大鼠尿中N,N-二(正丁基)阿霉素-14-戊酸酯及其8种代谢产物

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目的: 同时测定大鼠尿中N,N-二(正丁基)阿霉素-14-戊酸酯及其8种代谢产物 **方法**: 建立了一种反相高压液相色谱法，大鼠iv 20 mg·kg^{-1}原药后，其尿直接进样. 梯度洗脱，荧光检测. **结果**: 原药最低检出量2 ng，代谢物1－3 ng. 被检物不受尿成分干扰. 72 h尿中总蒽环荧光信号仅为剂量的4.9 %，其中主要为脱酰基以及N-脱丁基代谢物. 6种次要代谢物包括苷元以及13－酮基还原性代谢物等，但未检出葡萄糖醛酸结合物. **结论**: 本法简便易行，灵敏度高，特异性强。

关键词 N,N-二(正丁基)阿霉素-14-戊酸酯; 高压液相色谱法; 荧光; 药物动力学

阿霉素 戊酸酯 正丁基