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Simultaneous high-performance liquid chromatographic determination of a glucuronyl prodrug of doxorubicin, doxorubicin and its metabolites in human lung tissue

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Abstract

A rapid and sensitive method was developed for the simultaneous determination of the new doxorubicin glucuronide prodrug HMR 1826, the parent drug doxorubicin and its metabolites in human lung tissue samples. Homogenization of frozen tissue samples with the micro-dismembrator was followed by a silver nitrate precipitation step. By removing the exceeding silver ions with sodium chloride further purification steps could be omitted. Compounds were separated by isocratic high-performance liquid chromatography on a LiChrospher 100 RP18 column and a mobile phase consisting of citric acid buffer–acetonitrile–methanol–tetrahydrofuran within 30 min and quantified with fluorescence detection. The method showed good recoveries for all compounds (86–99%) and a linear calibration range of 20 ng/g–80 μg/g for doxorubicin and 1–600 μg/g for HMR 1826.

Keywords: Doxorubicin

1. Introduction

Doxorubicin (Dox) is an anthracycline antibiotic agent widely used in the treatment of cancer [1]. The major dose limiting factor of Dox, however, is cardiotoxicity [2]. One approach to overcome this limitation is the application of a nontoxic prodrug which is activated by an enzyme occurring at enhanced levels in tumor tissue or by antibody directed enzyme prodrug therapy (ADEPT) [3]. *N*-[4-β-Glucuronyl-3-nitro-benzyloxycarbonyl]doxorubicin (HMR 1826) (Fig. 1) is such an inactive prodrug which requires bioactivation by β-glucuronidase. The resulting doxorubicin-spacer derivative is instable at physiological pH and decomposes to free Dox. In man Dox is metabolized to several compounds (Fig. 1) but beside the parent compound only doxorubicinol exerts cytostatic activity [4]. To date the role of the other metabolites is poorly understood but lipid peroxidation and some of the side effects are discussed to be mediated by metabolites of Dox [5]. Several methods for determination of Dox and its metabolites in plasma or in tissues have been reported in the literature [6–13] but to our knowledge no validated and standardized

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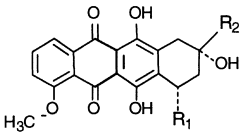


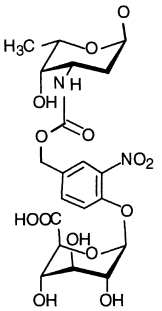
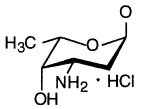
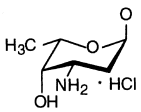
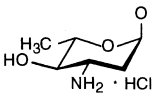
Compound	R_1	R_2
N-[4-ß-glucuronyl-3-nitro-benzyl-oxycarbonyl]doxorubicin (HMR 1826)		
Doxorubicin * HCl (Dox)		
Doxorubicinol * HCl (Doxol)		
Doxorubicin-aglycone (Dox-one)	OH	
Doxorubicinol-aglycone (Doxol-one)	OH	
Doxorubicin-desoxyaglycone (Dox-done)	H	
Doxorubicinol-desoxyaglycone (Doxol-done)	H	
Epirubicin * HCl (Epi)		

Fig. 1. Structures of the prodrug HMR 1826, Dox and its metabolites and abbreviations used in Section 1.

method is described for the simultaneous analysis of HMR 1826, Dox and its metabolites.

In order to assess the selectivity of prodrug therapy using HMR 1826 concomitant quantification of the prodrug and its metabolites is a crucial issue. Moreover, vascularization of tumors is highly variable [14] thus the access of prodrug to the enzyme is a limiting factor for bioactivation. Therefore, an exact quantification of HMR 1826 is pivotal to determine the extent of Dox liberation by β-glucuronidase-mediated cleavage. Because of the high HMR 1826 doses that may be used in vivo [15] which lead to rather high tissue concentrations it is not trivial to quantitate the prodrug concentration simultaneously together with low Dox and metabolite levels. Moreover, most methods reported so far are hampered either by low drug recovery, or low sensitivity [8–10,13], or time consuming procedures involving one or more solid-phase extraction steps [7]. We therefore established a very sensitive and fast method for the simultaneous determination of low concentrations of Dox and its metabolites in presence of high concentrations of HMR 1826. The combination of the advantages of an effective tissue homogenization method and a very sensitive fluorescence detection resulted in high recoveries of the analyzed compounds with no need for a solid-phase extraction step. The method was applied to the quantification of Dox derivatives in an isolated human lung tumor model [16] perfused with Dox or the prodrug HMR 1826.

2. Experimental

2.1. Chemicals

All solvents used were of HPLC quality; chemicals were of analytical grade. Dox, Dox-one, Dox-done, Doxol, Doxol-one, Doxol-done and Epi (for structures see Fig. 1) were gifts from Pharmacia, Farmitalia (Freiburg, Germany). HMR 1826 was synthesized according to Jaquesy et al. [17]. Ascorbic acid, silver nitrate and sodium chloride were obtained from Merck (Darmstadt, Germany), D-saccharic acid 1,4-lactone monohydrate and triethylamine were supplied by Sigma (Deisenhofen, Germany).

2.2. Tissue

Samples of tumor and normal lung were obtained after isolated perfusion of human lobe preparations [16] with perfusion buffer consisting of Hank's buffer with 5% bovine serum albumin and various concentrations of Dox or HMR 1826, respectively. Swine lung was from a local slaughter house. Samples were immediately frozen in liquid nitrogen and stored at −80°C.

2.3. Sample homogenization

Deep frozen samples were homogenized by using a Micro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany) with a PTFE shaking flask (volume 5 ml) and a chromium steel ball (10 mm diameter) at 2300 rpm for 2 min. The frozen tissue powder was transferred into polypropylene cups without thawing and stored at −80°C.

2.4. Sample preparation

A 30–100 mg amount of frozen tissue powder was suspended in 400 μl 50 m*M* ascorbic acid buffer pH 4.5 containing 2 m*M* D-saccharic acid 1,4-lactone to inhibit β-glucuronidase activity. A 50-μl volume of a solution of 10 μg/ml epirubicin hydrochloride in water was added as an internal standard. Protein and DNA were denatured by adding 50 μl of 3 *M* $AgNO_3$ and by mixing the resulting suspension for 10 min at room temperature. The excess of silver ions was precipitated with 50 μl of 3 *M* NaCl. After adding 1.25 ml acetonitrile–methanol (2:1, v/v) the suspension was mixed for 10 min at room temperature and afterwards separated by centrifugation (11 000 *g*, 5 min). A 50-μl aliquot of the clear supernatant was analyzed by high-performance liquid chromatography (HPLC).

2.5. Instrumentation and HPLC conditions

The HPLC-system consisted of a 110 B solvent delivery pump (Beckman Instruments, Munich, Germany) an autoinjector ASil 9 (Shimadzu, Kyoto, Japan), a column thermostat (Bischoff Analysen Technik, Leonberg, Germany) operated at 6°C, a 250 mm×4.6 mm I.D. column packed with LiChrospher

100 RP18 5 μm (Merck, Darmstadt, Germany) with a 40 mm pre-column packed with Polygosil C_{18} 10 μm, and a programmable fluorescence detector FP 920 (Jasco, Gross-Umstadt, Germany).

Separation was performed with 20 m*M* citric acid+0.14% triethylamine pH 2.4 (by addition of 1 *M* sulfuric acid)–acetonitrile–methanol–tetrahydrofuran (100:50:25:5, v/v/v/v) at a flow-rate of 1.0 ml/min. The analytes were detected with an excitation wavelength of 490 nm and an emission wavelength of 590 nm, (band width 18 nm), respectively. The gain was reduced prior to the elution time of HMR 1826 to 10% of the initial value.

2.6. Calibration and standardization

Stock standard solutions of HMR 1826, Dox and the metabolites were prepared by solving in water or dimethylsulfoxide (aglycones) at 1 mg/ml. Working solutions were prepared from these stock solutions in water or methanol. All standard solutions were stored at −30°C until use. Calibration samples and quality controls were prepared by adding different amounts of stock solutions to 100 mg of swine lung that was homogenized by using a micro-dismembrator. These samples were suspended in ascorbic acid buffer and treated as described above. Calibration curves were obtained by plotting the peak area ratios of the compounds and the internal standard epirubicin hydrochloride against the substance concentration added.

2.7. Assay validation

To determine the accuracy and variability of the assay various amounts of a mix of Dox, Dox-one, Dox-done, Doxol, Doxol-one and Doxol-done, or HMR 1826 alone were added to swine lung homogenates. These mixtures were analyzed alone (accuracy) or together with the samples in every series of experiments as quality controls (variability).

The recoveries were determined by comparing the peak areas of samples prepared from stock solutions and swine lung homogenates after extraction with direct dilution by adding mobile phase to the stock solutions to obtain the same end volume.

3. Results and discussion

The method described in this paper allows the highly sensitive quantification of Dox and its metabolites in small tissue samples in presence of an excess of the prodrug HMR 1826. This was achieved by the combination of a very sensitive fluorescence detector with a highly efficient sample preparation method.

The critical point in determination of Dox and its metabolites in tissue samples is the homogenization and extraction procedure. Neither homogenizing with Ultra-turrax [11] (30 000 rpm for 1 min in phosphate buffer) nor grinding with a glass potter gave satisfying results in particular with small sample volumes of lung tissue. Both methods led to a loss of tissue which was not properly homogenized. Using the micro-dismembrator for homogenization and an $AgNO_3$ precipitation step in order to liberate intercalated Dox and Doxol we obtained highly reproducible recoveries of 86–99% even at low concentrations added (Table 1). In contrast to most methods reported in literature [8–12] we could perform the sample preparation without any column extraction or

Table 1
Recovery: the peak areas of samples prepared from stock solutions and swine lung homogenates after extraction (n=4) are compared with direct dilutions by adding mobile phase to the stock solutions to obtain the same final volume (n=3)

Concentration added (μg/g)	Recovery (mean±C.V.) (%)						
	Dox	HMR 1826	Doxol	Doxol-one	Doxol-done	Dox-one	Dox-done
0.05	89.8±9.6		88.5±3.1	94.4±3.4	95.5±3.4	98.9±2.1	97.5±5.2
1	93.1±11.9	90.9±14.3	96.8±7.4	97.3±4.6	97.3±4.6	96.5±4.9	96.7±4.8
10		86.1±2.1					
20	94.3±6.8						
300		87.6±1.1					

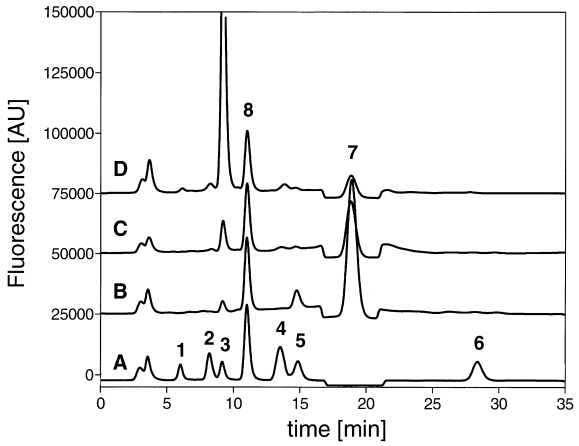


Fig. 2. Chromatogram of swine lung tissue spiked with 0.5 μg/g of Dox and its metabolites (A), swine lung tissue spiked with 300 μg/g HMR 1826 which contained 0.15% of Dox and Dox-one in addition (B), a human lung tissue sample (C), and a human tumor tissue sample (D) from a perfusion experiment with 400 μg/ml HMR 1826 (1=Doxol, 2=Doxol-one, 3=Dox, 4=Doxol-done, 5=Dox-one, 6=Dox-done, 7=HMR 1826, 8=internal standard Epi).

evaporation step. Furthermore sample clean-up was good enough to allow more than 500 samples to be analyzed with the same analytical column.

Dox, Doxol, Doxol-one, Doxol-done, Dox-one, Dox-done, HMR 1826 and the internal standard epirubicin were readily separated with retention times of 9.2, 6.0, 8.1, 13.5, 14.8, 28.3, 18.8 and 11.0 min, respectively (Fig. 2). Among the HPLC methods published so far, there are only few which achieve separation of Dox-one and Doxol-done [6,7]. Since the separation of these compounds can be improved by adding tetrahydrofuran especially at low temperature and the retention time of HMR 1826 is influenced by the pH of the mobile phase we developed a new mobile phase containing tetrahydrofuran at a pH of 2.4 resulting in a good resolution of Dox-one and Doxol-done in presence of high concentrations of HMR 1826 (Fig. 2). The problem of quantification of about 100-fold higher prodrug concentrations compared to Dox concentrations was circumvented by reducing the sensitivity of the fluorescence detector prior elution of HMR 1826.

Standardization was carried out with swine lung homogenate instead of human lung homogenate in the presence of Dox, HMR 1826, each of the Dox metabolites and Epi as internal standard. Calibration ranges were 0.02–80 μg/g of Dox, 0.02–5 μg/g of a mixture of metabolites and 1–500 μg/g of HMR 1826. Our method showed good linearity over the entire concentration range (Table 2). Even 0.01 μg/g could be detected with a signal-to-noise ratio higher than 5. Reproducibility was tested by repeated analyses of swine lung homogenates spiked with different known amounts of Dox, HMR 1826 and the metabolites. Table 3 displays the intra-assay and inter-assay reproducibility. Inter-assay variability was less than 8% except for the limit of quantitation of HMR 1826 where it averages 14%.

The suitability of the method described was demonstrated by measuring the levels of Dox, of HMR 1826 and of the metabolites in human lung tissue. Following lobectomy or pneumectomy for bronchial carcinoma tissue samples were obtained after perfusion of the isolated, ventilated human lung lobe containing a tumor with Dox or HMR 1826. No Doxol, the main metabolite in humans [18] was found in lung tissue after perfusion with Dox.

Table 2
Calibration curve parameters for the determination of HMR 1826, Dox and its metabolites in tissue

Analyte	Calibration range (μg/g)	$\frac{\text{Area analyte}}{\text{Area internal standard}} = (\text{concentration})a + b$		
		$a \pm$S.D. [$(\mu g/g)^{-1}$]	$b \pm$S.D.	r
Dox	0.02–80	0.2276±0.0006	−0.0237±0.0209	0.9999
HMR 1826	1–500	0.0121±0.0002	−0.0388±0.0409	0.9993
Doxol	0.02–5	0.2014±0.0013	−0.0097±0.0031	0.9999
Doxol-one	0.02–5	0.4003±0.0031	−0.0107±0.0073	0.9998
Doxol-done	0.02–5	0.7467±0.0056	−0.0238±0.0134	0.9998
Dox-one	0.02–5	0.4416±0.0052	−0.0205±0.0124	0.9995
Dox-done	0.02–5	0.5020±0.0049	−0.0171±0.0118	0.9996

Table 3
Intra-assay and inter-assay precision for the determination of HMR 1826, Dox and its metabolites

Concentration of analyte added	Intra-assay		Inter-assay	
	Found mean (μg/g) (bias, C.V.)	*n*	Found mean (μg/g) (bias, C.V.)	*n*
0.05 μg/g Dox	0.049 (−2%, 8%)	5	0.049 (−1%, 2%)	7
1 μg/g Dox	0.937 (−6%, 6%)	5	0.994 (−1%, 4%)	9
20 μg/g Dox	20.69 (3%, 2%)	5	20.86 (4%, 3%)	6
1 μg/g HMR 1826	0.812 (−19%, 11%)	5	0.905 (9%, 14%)	6
10 μg/g HMR 1826	10.14 (1%, 5%)	5	10.17 (2%, 7%)	6
300 μg/g HMR 1826	313.9 (5%, 1%)	5	320.5 (7%, 4%)	5
0.05 μg/g Doxol	0.044 (−12%, 8%)	4	0.048 (4%, 4%)	6
1 μg/g Doxol	1.096 (10%, 2%)	5	0.986 (1%, 4%)	10
0.05 μg/g Doxol-one	0.047 (−6%, 8%)	4	0.044 (12%, 8%)	7
1 μg/g Doxol-one	1.001 (0%, 1%)	5	0.990 (1%, 2%)	10
0.05 μg/g Doxol-done	0.047 (−7%, 12%)	4	0.044 (12%, 8%)	7
1 μg/g Doxol-done	1.008 (1%, 1%)	5	1.061 (6%, 8%)	10
0.05 μg/g Dox-one	0.054 (8%, 11%)	4	0.044 (12%, 6%)	6
1 μg/g Dox-one	0.983 (−2%, 1%)	5	1.005 (1%, 4%)	9
0.05 μg/g Dox-done	0.051 (10%, 12%)	4	0.053 (6%, 8%)	7
1 μg/g Dox-done	0.982 (−2%, 1%)	5	1.022 (2%, 7%)	10

Although the NADH-dependent reductase responsible for the formation of Doxol is present in all cells it exhibits its main activity in erythrocytes, liver and kidney [19]. The lung parenchyma seems not to participate in the metabolism of Dox. In some tumor tissue traces of Dox metabolites such as Doxol and Doxol-done were detectable after perfusion with HMR 1826 (Fig. 2D). Concentrations of these metabolites, however, were less than 1% of the concentration of Dox.

After performing a perfusion with 400 μg/ml HMR 1826, tissue samples were homogenized and extracted in presence of D-saccharic acid 1,4-lactone, a competitive inhibitor of β-glucuronidase, in order to prevent post-perfusion cleavage of HMR 1826. Dox liberated by β-glucuronidase action during perfusion could be readily separated and quantified in presence of HMR 1826 despite of the high tissue concentration of the latter compound (Fig. 2B,C). In some cases Dox concentration in tumor tissue was higher than Dox concentration in corresponding normal lung tissue although the tumor tissue showed a lower concentration of HMR 1826 (Fig. 2C,D). This indicated a higher β-glucuronidase activity which in turn was capable to cleave HMR 1826 thereby liberating Dox. The tumor concentration of Dox cleaved from the prodrug was about 7-fold higher (14.04 μg/g tissue) than the tumor concentrations obtained by perfusion with Dox alone (1.78 μg/g), suggesting a better tumor selectivity of prodrug due to an elevated tumor β-glucuronidase level in the tumor sample used for perfusion [20].

Our data demonstrate that the HPLC assay described is suitable for sensitive quantification of Dox, its metabolites and the glucuronyl-prodrug of Dox HMR 1826 in lung tissue. It may be a valuable tool for lung perfusion experiments as well as for clinical trials in order to determine the pharmacodynamic and pharmacokinetic properties of the Dox prodrug compared to Dox.

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