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A simple HPLC method using a microbore column for the analysis of doxorubicin

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Abstract

Doxorubicin is one of the most potent anti-tumor agents generally used in the treatment of bone cancer. A simple and sensitive HPLC method was developed and validated for the assay of doxorubicin. The method used a C_{18} Luna microbore column (50 × 1 mm) with a fluorescent detector (505 nm Ex. and 550 nm Em.). The mobile phase consisted of water–acetonitrile–acetic acid (80:19:1, v/v/v, pH 3.0) and the flow rate was 0.1 ml min^{-1}. Daunomycin was used as the internal standard. This isocratic system required a 10-min run-time, giving a detection limit of 0.02 ng (0.035 pmol per injection). Standard curves were linear over the concentration range of 0.01–0.1 μg ml^{-1}. Relative standard deviations (R.S.D.) for the within-day, day-to-day precision, and the accuracy measurement for the assay were less than 4.0, 3.2, and 4.1%, respectively. This HPLC method was used to study the in vitro release characteristics of doxorubicin from implantable drug delivery system.

Keywords: Doxorubicin; Daunomycin; Microbore column

1. Introduction

Doxorubicin (Fig. 1) is an anthracycline cytostatic antibiotic with the widest antineoplastic spectrum. It is highly effective in the treatment of soft tissue and bone sarcoma [1,2]. The structure of doxorubicin consists of a teracyclic quinoid aglycone (doxorubicinone), with an endogenous fluorescence, and an amino sugar (daunosamine) [3]. Several HPLC methods have been reported for the assay of doxorubicin and its metabolites in vitro and in vivo [4–10]. Most of these methods used conventional reversed phase HPLC column

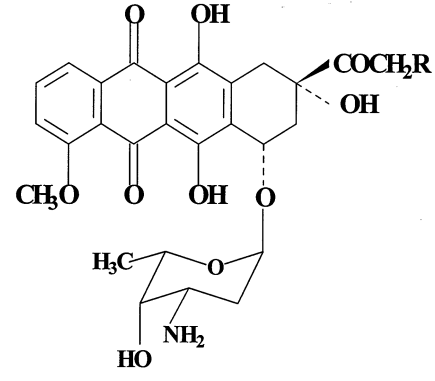

Fig. 1. Structure of doxorubicin (R = OH) and daunomycin (R = H).

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requiring expensive mobile phase systems, longer run-times, higher analyzing temperature, or even gradient systems. A review on the analysis of anthracycline anti-tumor drugs in tissues and body fluids using liquid chromatographic procedures is described else where [11]. Rossi et al. [12] have reported the use of a narrow-bore (2 mm ID) column for the analysis of doxorubicin. However, microbore columns have some distinct advantages over the conventional reversed phase columns and are therefore gaining popularity in pharmaceutical analysis [13]. The major advantages of microbore columns include: rapid equilibration, enhanced sensitivity and resolution, reduced solvent consumption and waste generation as compared to conventional packed columns [14]. The objective of this study was to develop and validate a rapid and sensitive HPLC method for the assay of doxorubicin in aqueous solution using a microbore column. This method was also used to study the in vitro release characteristics of doxorubicin from implantable drug delivery system designed in our laboratory for the treatment of bone cancer.

2. Experimental

2.1. Materials

Doxorubicin hydrochloride (Sigma, St. Louis, MO, USA); Daunomycin hydrochloride (Fluka, Milwaukee, WI, USA); acetonitrile, water, methanol (HPLC grade), and hydrogen chloride (Fisher Scientific, Fairlawn, NJ, USA); glacial acetic acid (Mallinckrodt, Paris, KY, USA); absorbable gelatin powder (Gelfoam®, Upjohn, Kalamazoo, MI, USA) were used as received.

2.2. Chromatography

The HPLC system consisted of the Solvent Delivery Module LC-10AT (Shimadzu Corporation, Kyoto, Japan), a manual injector (20 μl loop, Rheodyne, Cotati, CA, USA), a Waters 470-Fluorescent Detector, and a Waters 745 Data Module (Waters Chromatography Division, Milfold, MA, USA). The separation was carried out on a 50×1 mm I.D. (5 μ) reversed phase C_{18} Luna column, (Phenomenex, Torrance, CA, USA). The mobile phase consisted of water:acetonitrile:acetic acid (80:19:1, v/v/v) with an apparent pH of 3.0. The flow rate was maintained at 0.1 ml min^{-1}. Daunomycin was used as the internal standard. The effluents were monitored at $\lambda_{Ex} = 505$ nm and $\lambda_{Em} = 550$ nm. All chromatographic analyses were performed at room temperature (25°C).

2.3. Standard solutions

Doxorubicin (0.01–0.10 μg ml^{-1}) and daunomycin (0.08 μg ml^{-1}) were prepared in the mobile phase. Doxorubicin (0.28 mg) was dissolved in 100 ml mobile phase in a volumetric flask. Various standard solutions were then prepared from this stock solution after adequate dilution with mobile phase. Daunomycin (0.13 mg) was dissolved in 100 ml mobile phase to make the stock solution. The standard solution of daunomycin was prepared by diluting 3.0 ml of the stock solution in a 50 ml volumetric flask with the mobile phase.

2.4. Sample preparation

To 900 μl of doxorubicin standard solutions, or solution to be analysed for the drug content, 100 μl of internal standard solution was added and vortexed for 10 s. This mixture (20 μl) was injected directly to the HPLC.

2.5. Quantitation

The ratios of the peak heights of doxorubicin to that of the internal standard were calculated. The unknown doxorubicin concentration was determined from the regression equation relating the peak-height ratios (PHR) of the standards to their nominal concentrations.

2.6. Formulation of the implants

Our laboratory was involved in the development of implantable drug delivery systems containing doxorubicin used for the treatment of

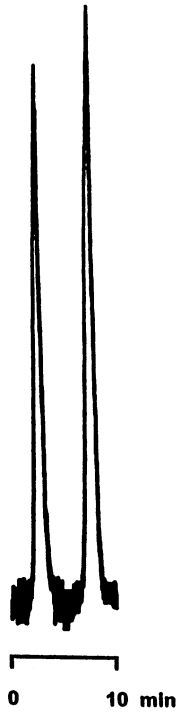

Fig. 2. Representative chromatograms of doxorubicin (retention time is 2.7 min) and daunomycin (retention time is 6.8 min).

bone cancer. One-part gelatin (Gelfoam®), two-part water, and doxorubicin were mixed in a glass beaker and forced through an one ml plastic syringe to form the cylindrical implant (12 mm long, 3.5 mm diameter). The implants were kept in a desiccator overnight prior to the release study. The drug load was kept between 0.01 and 0.1% (w/w).

2.7. In vitro release study

The in vitro release of doxorubicin from the implants was carried out in a tightly closed 50 ml Erlenmeyer flask. The weighed implants were kept in 40 ml of Tris buffer (pH 7.4). The flasks were shaken (150 rpm) at 37°C by an environmental shaker (Lab-Line Instrument, Melrose Park, IL, USA). One ml of the release medium was collected at each time interval (0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3.5, 4.5, 5.5, 7.5, 9.5, and 24 h), and replaced with 1 ml of fresh buffer. In vitro release studies were carried out in triplicates. The doxorubicin content in the release medium was determined by HPLC.

2.8. Determination of the drug load in the implant

A fixed weight of doxorubicin implant was dispersed in 2.0 ml of mobile phase and sonicated for 2 h. The sample was filtered through a 0.45

Table 1
Within-day and day-to-day analytical precision of doxorubicin

Within-day[a]			Day-to-day[b]	
Concentration (μg ml^{-1})	Mean peak-height ratio[c]	R.S.D. (%)	Mean peak-height ratio[d]	R.S.D. (%)
0	0	–	0	–
0.01	0.83	4.0	0.84	2.2
0.03	2.61	2.2	2.63	2.2
0.05	4.15	3.9	4.25	3.2
0.08	6.67	2.3	6.63	1.3
0.10	8.66	2.0	8.66	2.0
Slope	84.8 ± 1.9	2.2	84.6 ± 1.6	1.9

[a] Analyzed on the same day.
[b] Analyzed on 6 different days within a period of 28 days.
[c] Mean, $n = 4$.
[d] Mean, $n = 6$.

Table 2
Accuracy in the analysis of doxorubicin in quality control samples

Actual conc. (μg ml^{-1})	Measuredconc. (μg ml^{-1})	Accuracy[b]	R.S.D.[a] (%)
0.020	0.020 ± 0.0008	98.09 ± 4.02	4.1
0.070	0.070 ± 0.0011	99.20 ± 1.51	1.5
0.090	0.090 ± 0.0033	98.66 ± 3.58	3.6

[a] Mean ± S.D., $n = 6$.
[b] Accuracy = (measured conc./actual conc.) × 100.

μm syringe filter. The doxorubicin content in the filtrate was then determined by HPLC.

3. Results and discussion

3.1. Validation of the HPLC method for the analysis of doxorubicin

Doxorubicin is stable at a pH range of 3.0–6.5 [15]. The degradation of doxorubicin is dependent on temperature, pH, buffer type, and light. It has been reported to be adsorbed to many materials [3]. A simple, sensitive, and solvent saving HPLC method was developed and validated. The strategy was to improve previous methods by enhancing the sensitivity (enhancing minimum detectable mass) as well as decreasing the run-time and flow rate to save the expensive mobile phase. The implantable drug delivery system design also requires a method that can be used on line to analyze the limited sample size of analyte generated from microdialysis sampling technique. Most importantly, reduction of chemical and toxic waste during analysis of these highly cytotoxic drugs was also taken into consideration during method development. Therefore, a Luna C_{18} reversed phase microbore column was used for the separation. This column (50 × 1 mm I.D., 5μ) required a flow rate of only 0.1 ml min^{-1}. The retention times for doxorubicin and the internal standard (daunomycin), were 2.7 and 6.8 min, respectively, with a baseline-to-baseline separation (Fig. 2). The run-time was 10 min. The apparent pH of the mobile phase was 3.0, which was an appropriate pH for the stability of both the analytes and the column.

3.1.1. Linearity

The standard curves were linear over the concentration range of 0.01–0.10 μg ml^{-1}. The equation of the standard curve relating the peak-height ratio (P) to the doxorubicin concentration (C in μg ml^{-1}) in this range was: $P = 84.58C - 0.002$, $r^2 > 0.999$.

3.1.2. Precision

Within-day precision was determined by analysis of four different standard curves on the same day. Day-to-day precision was determined by the analysis of the same solutions on six different days during a period of 28 days. During this period, the solutions were stored under 4°C and wrapped with aluminum foil. Within-day and day-to-day precision, relative standard deviation (R.S.D.) values ranged from 2.0 to 4.0% and 1.3 to 3.2%, respectively (Table 1).

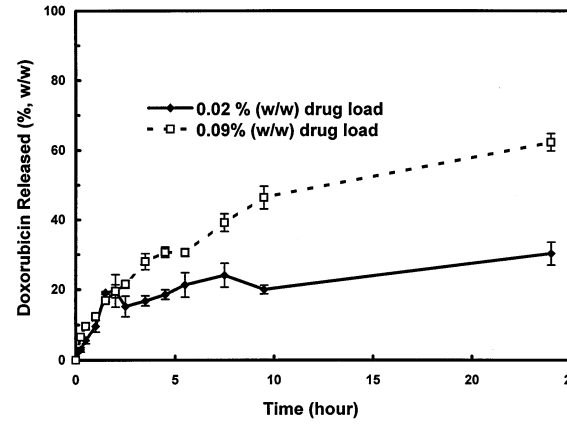

Fig. 3. The effect of drug load on the release of doxorubicin from an implantable drug delivery system.

3.1.3. Accuracy

Three quality control samples were also stored under the same condition as the standard solutions over a period of 28 days. These samples were analyzed six times during this period and the accuracy of the assay was determined by comparing the measured concentration to its nominal value (Table 2). The R.S.D. ranged from 1.5 to 3.6%.

3.1.4. Sensitivity

The lowest limit of reliable assay measurement criteria described by Oppenheimer et al. [16] was used to determine the sensitivity parameters. Seven different standard curves were used in this calculation. The critical level is defined as the assay response above which an observed response is reliably recognized as detectable. This value is also considered as the threshold value, defining detection. If the measured value exceed this value then the presence of analyte is detected, otherwise it is not. This was 0.0008 ± 0.001 μg ml^{-1} (mean ± S.D.). The detection level is the actual net response which may a priori be expected to lead to detection. This is the least value of the true concentration that is 'nearly sure' to produce a measured value that results in detection [17]. This was 0.0015 ± 0.0002 μg ml^{-1} (mean ± S.D.). The determination level is the concentration at which the measurement precision will be satisfactory for quantitative determination was 0.0042 ± 0.0006 μg ml^{-1} (mean ± S.D.) for a level of precision of 10% R.S.D.

3.2. Application of the HPLC method

3.2.1. In vitro release of doxorubicin from the implants

Doxorubicin has been proved to be highly effective against osteogenic sarcoma. However, the problems associated with parenteral administration of doxorubicin for the treatment of bone cancer are: (i) systemic cardiac toxicity caused by the high levels of doxorubicin; (ii) the drug concentration at cancerous site is likely to be low because bone is a moderately perfused organ; (iii) the narrow therapeutic range of doxorubicin does not permit substantial increases in the dose administrated. Therefore, local and targeted delivery of antitumor agent is an effective means of minimizing the problems occurred during the conventional administration of anticancer drug in the treatment of osteogenic sarcoma [2].

Gelfoam is the absorbable sterile gelatin powder intended for application to bleeding surfaces. Gelatin is a macromolecular protein which is inert and biodegradable. In this study, Gelfoam was used as the matrix material for fabrication of the implant. Tris buffer was used as the release medium since doxorubicin is more stable in Tris buffer at 37°C as compared to other buffer systems [18]. The doxorubicin content from the released medium and the drug load was determined by the HPLC method mentioned above. No interfering peak was detected during the analysis of samples collected from the in vitro release studies of the implants. The effect of drug load on the in vitro release of doxorubicin from the implant is shown in Fig. 3. As expected, an increase in drug load (from 0.02 to 0.09%, w/w) in the formulation increased the release rate of the drug.

4. Conclusions

An accurate, simple, and sensitive HPLC method using a microbore column was developed and validated for the analysis of doxorubicin in aqueous solution. The method has been successfully used in the evaluation of the in vitro release characteristics of doxorubicin from the implantable drug delivery system and also in the determination of drug load in the implants.

Acknowledgements

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