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Short communication

Simultaneous liquid chromatographic determination of doxorubicin and its major metabolite doxorubicinol in parrot plasma

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Abstract

A new microscale method is reported for the determination of doxorubicin and its active metabolite, doxorubicinol, in parrot plasma. Sample workup involved acetonitrile protein precipitation, ethyl acetate extraction, followed by back extraction into HCl. Separations were achieved on a phenyl-hexyl column at 30 °C using acetonitrile (17%, v/v) in 0.01 M orthophosphoric acid (83%, v/v) delivered via a linear flow program. Fluorometric detection wavelengths were 235 nm (excitation) and 550 nm (emission). Calibration plots were linear ($r^2 > 0.999$), and recoveries were 71–87% from 20 to 400 ng/mL. Assay imprecision was ≤8.5% and inaccuracy was ≤6.3%. The limit of quantification was 25 ng/mL.

Keywords: Doxorubicin; Doxorubicinol; Parrot; Liquid chromatography

1. Introduction

Doxorubicin is used widely as a part of cytotoxic chemotherapy regimens against solid and haematological cancers in humans but also in animals and, more recently, in birds with solid tumours [1]. The current approaches to using doxorubicin in avian chemotherapy are largely empirical and have been hindered by a lack of pharmacokinetic data to guide dosage regimens. There is a growing body of evidence to suggest that the primary ^{13}C hydroxy metabolite, doxorubicinol (Fig. 1) is also cytotoxic and may be responsible for some of the major adverse effects, especially on the heart [2]. Therefore, there is a strong case for studying the pharmacokinetics of doxorubicin and doxorubicinol concurrently. To obtain such data in birds which have a small body mass and blood volume is difficult because of severe restrictions on the frequency of sampling and the total amount of blood which can be drawn during a dosing interval. As a precursor to our pharmacokinetic studies of doxorubicin and doxorubicinol in the sulphur crested cockatoo (*Cacatua galerita*) several published assay methods were applied [3–9], but these were mostly unsatisfactory because of one or more of the following factors: unacceptably large sample volumes, interference from endogenous substance peaks, doxorubicinol was not assayed concurrently, the presence of an insoluble residue of unknown composition following organic solvent extraction.

Accordingly, a simple, robust HPLC method was developed which was precise and accurate and, with the use of fluorescence detection, was sufficiently selective and sensitive for the simultaneous determination of doxorubicin and doxorubicinol following doxorubicin dosage regimens currently used to treat parrots with solid tumours.

2. Materials and methods

2.1. Drugs and reagents

Doxorubicin hydrochloride was obtained from Pharmacia & Upjohn (via Robert Koch, 1.2, 20152 Milan, Italy). Daunorubicin (internal standard), was obtained from a commercial product of daunorubicin hydrochloride for injection (daunorubicin

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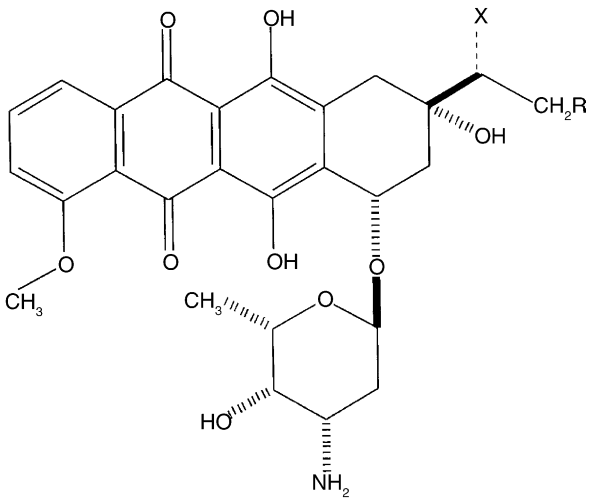

Doxorubicin:	R: -OH X: =O
Doxorubicinol:	R: -OH X: -OH
Daunorubicin: (internal standard)	R: -H X: =O

Fig. 1. Structures of doxorubicin, doxorubicinol and daunorubicin (internal standard).

HCl USP, 2.14 mg/mL and sodium chloride BP in Water for Injections BP; Pharmacia & Upjohn (Perth) Pty Ltd., Bentley, WA, Australia). Hydrochloric acid (HCl; EM Science, Gibbstown, NJ, USA) and orthophosphoric acid (AJAX Chemicals, Sydney, NSW, Australia) were analytical reagent grade. Acetonitrile, ethyl acetate and methanol were HPLC grade (EM Science, Gibbstown, NJ, USA). Reagent grade water (18 MΩ, Milli-Q Synthesis ultrapure water purification system, Millipore, Billerica, MA, USA) was used throughout. Doxorubicinol, the putative active metabolite of doxorubicin was synthesised as described below.

2.2. Synthesis of doxorubicinol

Sodium borohydride (22 mg, 580 mmol) was added to a stirred, chilled (0 °C) solution of doxorubicin hydrochloride (218 mg, 376 mmol) in methanol (100 mL). The solution immediately changed color from red to orange. The ice-bath was removed and the solution was stirred for 10 min, then acetic acid (0.5 mL) was added. Stirring was continued overnight with the flask exposed to air by which time the color had changed to deep red. The methanol was evaporated, then the residue was loaded onto a short silica column and then eluted with chloroform/methanol/acetic acid/water (40:10:7:3) until the red coloration had disappeared from the column. The filtrate was evaporated and purified by reverse-phase HPLC (0–90% CH_3CN in H_2O; 0.1% CF_3CO_2H), and the appropriate fraction of the eluent was lyophilised to give the product (1:1 mixture of diastereoisomers) as a deep red, amorphous solid. This compound exhibited ^1H NMR, ^{13}C NMR, and mass spectra data consistent with the proposed structure.

2.3. Instrumentation

The HPLC system consisted of an LC-10AS solvent delivery unit and RF-551 spectrofluorometric detector (Shimadzu Corporation, Kyoto, Japan), and Millipore-Waters (Milford, MA, USA) Model 712 WISP automatic sampler, temperature control module and column heater. Peaks were obtained on a strip-chart recorder (Model BD 111, Kipp and Zonen, Delft, The Netherlands).

2.4. Standards and controls

Stock aqueous solutions of doxorubicin and doxorubicinol were prepared and subdivided into 10 μL portions for storage at −80 °C in Eppendorf tubes (2 mL). On the day of assay, drug-free plasma (90 μL) was added to the thawed stock solutions to produce standards containing 20, 40, 100, 200 and 400 μg/L of doxorubicin and doxorubicinol base. Likewise, seeded controls containing 25 and 250 μg/L of each analyte were prepared independently. An aqueous solution of the internal standard, daunorubicin (80 ng in 20 μL) was added to each 100 μL standard, control or unknown sample.

2.5. Sample workup

To each tube containing 100 μL of plasma (standard, control or unknown sample) was added acetonitrile (200 μL) followed by vortex-mixing (30 s) and centrifugation (9300 × *g*, 5 min). The supernatant was transferred to a teflon-lined screw-cap Pyrex centrifuge tube (10 mL, 100 mm × 13 mm), ethyl acetate (2 mL) was added and the tube was vortex-mixed (30 s) and centrifuged (900 × *g*, 15 min). The supernatant was transferred to another Pyrex centrifuge tube (10 mL, 100 mm × 13 mm), 0.05 M HCl (100 μL) was added and the contents were vortex-mixed (30 s) and centrifuged (900 × *g*, 15 min). The upper (ethyl acetate) layer was gently aspirated to waste using mild suction through a Pasteur pipette, and the acidic phase was transferred to amber auto-sampler vials containing low volume glass inserts.

2.6. High performance liquid chromatography

Reverse-phase separations were achieved on a Luna™ phenyl hexyl column (100 mm × 4.6 mm i.d.) containing smooth, spherical 5 μm particles (Phenomenex USA, Torrance, CA, USA) at 30 °C using a mobile phase of 17% (v/v) acetonitrile and 83% (v/v) orthophosphoric acid (0.01 M). A programmed flow-rate of 1.5 mL/min (0.0–5.5 min), increased linearly to 2.3 mL/min (5.5–6.5 min), then maintained at 2.3 mL/min (6.5–20 min) was employed. The retention times for doxorubicinol, doxorubicin and daunorubicin were 4.3, 7.5, and 17 min, respectively. The best detector response was obtained using excitation and emission wavelengths of 235 and 550 nm,

respectively. The strip-chart recorder was set to 10 mV full-scale deflection and a speed of 2 mm/min.

2.7. Quantification

For both doxorubicin and doxorubicinol the peak height ratio (analyte to internal standard) was regressed on concentration using univariate, unweighted linear least squares analysis. Unknowns were estimated by inverse prediction assuming no variance in the independent variable (concentration).

The estimated concentrations of the seeded controls were used to determine the imprecision and inaccuracy of the assay. A drug-free sample of the matrix being analysed (matrix blank) was included in each assay run.

2.8. Recovery

The absolute recovery of doxorubicin and doxorubicinol was determined from triplicate analyses at each of three concentrations within the range of the standard curve (20, 250 and 400 μg/L). Internal standard (daunorubicin, 800 μg/L) was included in each extracted sample, thus permitting concomitant estimation of its recovery at each concentration.

2.9. Stability

The stability of doxorubicin and doxorubicinol in plasma at −80 °C over 18 months was estimated in triplicate from samples of 200 μg/L of doxorubicin and doxorubicinol in parrot plasma. The stability of doxorubicin, doxorubicinol and daunorubicin in post-extraction conditions was assessed at 4 °C (refrigerator temperature) and at 27 °C (auto-sampler temperature) in 0.05 M HCl. To do this, solutions containing 25 μg/L or 750 μg/L of doxorubicin and doxorubicinol, each with daunorubicin (200 μg/L) added, were prepared in 0.05 M HCl. Portions (100 μL) were transferred to low-volume inserts within amber auto-sampler vials sealed with Parafilm™ and stored. Stability was assessed by comparing peak heights (mean of triplicate samples), as well as the appearance of any new peaks in the chromatograms that may have indicated the formation of degradation products.

3. Results and discussion

3.1. Chromatography

All three peaks of interest were sharp, symmetrical and baseline resolved with no interference from co-eluting substances. Chromatograms from the analysis of blank parrot plasma, low (20 μg/L) and high (400 μg/L) standards, and a sample drawn 30 min after starting an infusion of 2 mg/kg doxorubicin hydrochloride are shown in Fig. 2. Without the flow program, the retention times of doxorubicinol, doxorubicin and daunorubicin were 4.3, 8.3 and 22.8 min, respectively, at 1.5 mL/min. The isocratic, flow-rate program was useful in the optimisation of the separation without the need to use gradient chromatography which requires relatively long equilibration times. If doxorubicinol is not to be assayed then the run time of 18 min can be reduced by increasing the proportion of acetonitrile in the mobile phase and/or altering the flow-rate program appropriately. The HCl back-extraction step was mandatory to obtain clean chromatograms, but it was very important that the ethyl acetate was completely aspirated otherwise there was a variable and marked reduction in the peak height of doxorubicinol. Attempts to withdraw the lower HCl phase up through the ethyl acetate phase with a pipette or syringe (with or without expulsion of an air pocket) were unsuccessful. The reason for this behaviour remains speculative and could have involved a “micro-phase” of metabolite in ethyl acetate/HCl solution which was trapped on the column after injection. Interestingly, the heights of the doxorubicin and daunorubicin peaks were largely unaffected.

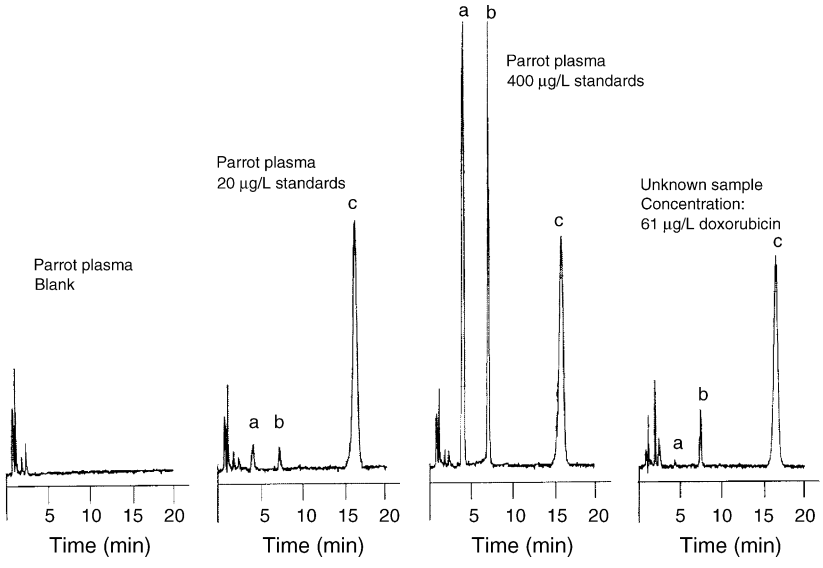

Fig. 2. Chromatograms of extracts from blank parrot plasma, standards and a sample drawn 30 min after the start of a 20 min intravenous infusion of 2 mg/kg doxorubicin to a sulphur crested cockatoo: (a) doxorubicinol, (b) doxorubicin, (c) daunorubicin.

Table 1
Assay performance assessment: imprecision and inaccuracy data ($n = 6$)

Concentration (μg/L)	Imprecision (CV%)[a]		Inaccuracy (%)[b]	
	Doxorubicin	Doxorubicinol	Doxorubicin	Doxorubicinol
25	8.5	7.3	6.3	6.0
250	1.8	3.4	1.1	1.6

[a] Coefficient of variation.
[b] Percent deviation from weighed-in amounts.

3.2. Assay performance

The mean (±S.D.) regression equations ($n = 6$) for doxorubicin and doxorubicinol were: $y = 0.0053\ (\pm 0.0003)x - 0.0106\ (\pm 0.0173)$; $y = 0.0052\ (\pm 0.0003)x - 0.0017\ (\pm 0.0141)$, respectively. The correlation coefficient (r) exceeded 0.999 in all cases. The limit of detection (LOD), defined as a peak height of three times the baseline noise, was 6 μg/L for both analytes. Table 1 shows the assay imprecision and inaccuracy. Based on these results the lower limit of quantification (LOQ) was set a priori at 25 μg/L for both doxorubicin and doxorubicinol. The absolute assay recovery ($n = 9$) was 71–86% for doxorubicin, and 77–87% for doxorubicinol, and 48–50% for daunorubicin. There was no decrease in peak height for either doxorubicin or doxorubicinol at 25 and 750 μg/L, or for daunorubicin (200 μg/L) when stored in 0.05 M HCl at 4 °C over 72 h. Likewise, there was no change in peak height after 4 h at ∼27 °C in the WISP auto-sampler compartment. However, after 24 h there were minor changes in the peak heights of doxorubicin (−3.3%), doxorubicinol (−2.7%) and daunorubicin (−1.3%), and at 750 μg/L two new peaks appeared with retention times of 16 min and 32 min. Thus, the maximum time allowed for storage in the autosampler was 4 h. There was no decrease in peak height following 18 months storage of doxorubicin and doxorubicinol (200 μg/L) in plasma at −80 °C. Because of the extremely limited supplies of blank parrot plasma it was convenient to substitute human plasma since calibration curves using both matrices were virtually superimposable.

3.3. Applicability of the method

Fig. 3 shows a plot of plasma doxorubicin and doxorubicinol concentrations measured at various times during and after a 20 min intravenous infusion of doxorubicin hydrochloride (2 mg/kg) to a sulphur crested cockatoo weighing 657 g. The four samples above 400 μg/L were assayed after dilution with drug-free plasma. Quantifiable doxorubicinol concentrations appeared within 15 min but were much lower than the corresponding doxorubicin concentrations and reached a peak of only ∼5% of the parent drug. These data and the results of other experiments involving administration of doxorubicinol alone (unpublished data) indicate that this metabolite has formation rate-limited pharmacokinetics. The data in Fig. 3 were analysed using established non-parametric methods. The pharmacokinetic results for this bird were: clearance, 2.46 L/h; volume of distribution, 240 mL; terminal phase half-life, 2.38 h; mean residence time, 5.89 min; area-under-the-curve, 520 μg/L h.

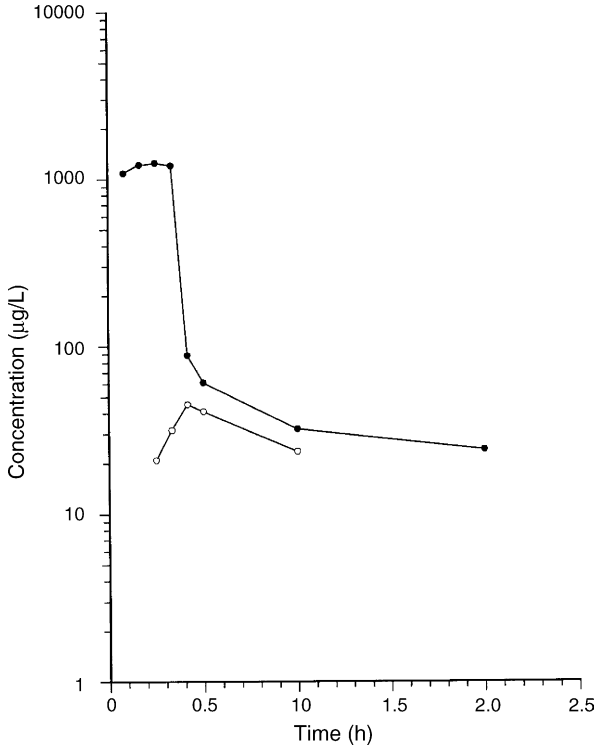

Fig. 3. Plasma doxorubicin (●) and doxorubicinol (○) concentrations measured at various times during and after a 20 min intravenous infusion of doxorubicin hydrochloride (2 mg/kg) to a sulphur crested cockatoo.

Our studies of the pharmacokinetics of doxorubicin and doxorubicinol in parrots are described more fully elsewhere [10].

References

[1] L.J. Filippich, B.G. Charles, Vet. Clin. Exot. Anim. 7 (2004) 821.
[2] P.S. Mushlin, B.J. Cusack, R.J. Boucek, T. Andrejuk, X. Li, R.D. Olson, Br. J. Pharmacol. 110 (1993) 975.
[3] A. Andersen, D.J. Warren, L. Slordal, Ther. Drug. Monit. 15 (1993) 455.
[4] S. Fogli, R. Danesi, F. Innocenti, A. Di Paolo, G. Bocci, C. Barbara, et al., Ther. Drug. Monit. 21 (1999) 367.
[5] R.F. Greene, J.M. Collins, J.F. Jenkins, J.L. Speyer, C.E. Myers, Cancer Res. 43 (1983) 3417.
[6] P. Zhao, A.K. Dash, J. Pharm. Biomed. Anal. 20 (1999) 543.
[7] L. Alvarez-Cedron, M.L. Sayalero, J.M. Lanao, J. Chromatogr. B Biomed. Sci. Appl. 721 (1999) 271.
[8] J.H. Beijnen, P.L. Meenhorst, R. van-Grijn, M. Fromme, H. Rosing, W.J. Underberg, J. Pharm. Biomed. Anal. 9 (1991) 995.
[9] D.E. Brenner, S. Galloway, J. Cooper, R. Noone, K.R. Hande, Cancer Chemother. Pharmacol. 14 (1985) 139.
[10] C.M. Gilbert, L.J. Filippich, B.G. Charles, Aust. Vet. J. 82 (2004) 769.