*Journal of Pharmaceutical & Biomedical Analysis*
Vol. 10, No. 5, pp. 343–348, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00


# A sensitive and reproducible HPLC assay for doxorubicin and pirarubicin

J.M. JACQUET,* M. GALTIER,† F. BRESSOLLE‡§ and J. JOURDAN*

* *Laboratoire Universitaire de Thérapeutique, Faculté de Médecine, CHRU Nîmes, Avenue du Pr. Debré, 30006 Nîmes Cedex, France*
† *Laboratoire de Pharmacocinétique, CHRU Nîmes, Avenue du Pr. Debré, 30006 Nîmes Cedex, France*
‡ *Département de Pharmacocinétique, Faculté de Pharmacie, Avenue Charles Flahault, 34060 Montpellier Cedex 1, France*

**Abstract**: A high-performance liquid chromatographic method with spectrofluorometric detection has been developed for the analysis of doxorubicin (DOX), pirarubicin (PIRA) and their metabolite, doxorubicinol, in plasma. The detection was performed at 480 nm for excitation, and 590 nm for emission. The proposed technique is selective, reliable, and sensitive. The limit of quantification was 2 ng $ml^{-1}$ for DOX and 5 ng $ml^{-1}$ for PIRA. The reproducibility of the analytical method through statistical coefficients is ~5%. The accuracy of the method is good; the relative error is <5%.

**Keywords**: *Doxorubicin; pirarubicin; doxorubicinol; HPLC method; biological fluids.*

## Introduction

After the discovery of the antineoplastic activity of anthracyclines [1], numerous methods for their detection and quantification in biological fluids have been developed. Since Finkel *et al.*'s work [2], fluorometry has been the prevailing means of detection. Fluorometric detection requires extraction of anthracyclines from biological samples. The characteristics of the assay, in terms of reproducibility and reliability, mostly depend on the quality of the extraction. Separation of the drug from its metabolites is now usually performed by HPLC [3], but the extraction procedures are still quite diversified. They are principally of two types: methods using an organic solvent of varying nature [4–7], or extraction by short chromatography on small C18 open columns [8–10].

In this work, a method for the simultaneous assay of doxorubicin (DOX), pirarubicin (PIRA) and their metabolite, doxorubicinol (DOXOL) is described. PIRA or 4′-*O*-(α-tetrahydropyranyl) adriamycin, is a derivative of DOX, which showed stronger effects than DOX in inhibiting mouse tumours [11] and lower cardiac toxicity [12]. DOX is one of the metabolites of PIRA.

## Experimental

### *Materials*

DOX, DOXOL, PIRA and daunorubicin (internal standard) were obtained from Roger Bellon (Paris, France). Stock solutions of DOX and PIRA (0.1 g $l^{-1}$ in absolute methanol) were diluted 5- and 50-fold with absolute methanol when appropriate while internal standard was diluted in HPLC mobile phase to 0.5 μg $ml^{-1}$ for solubility reasons.

All other chemicals were of analytical grade. Acetonitrile and absolute methanol were of HPLC grade (SDS, France) and were used without further purification.

All validation samples were prepared in reconstituted lyophilysed standard bovine serum (Biotrol-00 purchased from Biotrol, Paris, France) and in human plasma samples. Human blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes from each patient prior to the injection of DOX, then rapidly centrifuged.

### *Instrumentation*

Analysis by HPLC was performed using a model 302 pump with a Rheodyne syringe, a loading valve fitted with a 500-μl sample loop, a model 802 C manometric module, an auto-

§ Author to whom correspondence should be addressed.

matic sample injection system (model 231), all from Gilson (France), a stainless-steel column (250 × 4.6 mm i.d.; SFCC, Neuilly Plaisance, France) packed with Spherisorb phenyl (5 μm) and a guard column (10 × 4.6 mm i.d.) packed with Spherisorb phenyl (10 μm). The column effluent was monitored with a variable wavelength fluorescence detector (model LS-1, Perkin–Elmer, France) operated at 480 nm for excitation and 590 nm for emission.

*Chromatographic conditions*

The mobile phase, containing 30 parts acetonitrile and 70 parts citrate buffer (0.03 M) adjusted to pH 4 with formic acid, was degassed ultrasonically before use. Acetonitrile and the aqueous phase were filtered through a membrane filter (0.45 μm; Millipore, Molsheim, France). The oven temperature was 50°C, and the flow rate was 1.5 ml $min^{-1}$. The signal was recorded and the peak heights were determined with a LCI-100 Perkin–Elmer computing integrator (chart speed of 3 mm $min^{-1}$).

*Extraction procedure*

Plasma samples (0.5 ml) were pipetted into a 5 ml glass centrifuge tube. Internal standard solution (25 ng in 250 μl) was added. The solutions were extracted with 3 ml acetonitrile for 10 min. Then 100 mg NaCl were added to release anthracyclines in the organic phase and the tubes were shaken again for 5 min, after which time, the tubes were centrifuged at 995*g* for 15 min, and then kept at −20°C for 1 h in a freezer.

The supernatant was transferred into another glass tube and evaporated under nitrogen at 60°C. The residue was reconstituted into 250 μl of the mobile phase and 100 μl of this solution was injected into the chromatograph.

*Instrument calibration*

Calibration standards for control serum and plasma (0.5 ml) were prepared using concentrations of 10, 20, 50, 100, 150 and 200 ng $ml^{-1}$ for DOX and PIRA. The standard samples were prepared by adding appropriate volume of methanolic spiking solution of DOX and PIRA to Biotrol and of DOX to human plasma. The volume added was always smaller than or equal to 2% of total volume of the sample, so that the integrity of the serum or plasma was maintained.

These standards were extracted concurrently and in the same manner as the samples to be analysed.

*Data analysis*

The ratio of the peak height of DOX and PIRA to that of internal standard was used as the assay parameter. Peak height ratios were plotted against analyte concentration, and standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data.

The linearity of the method was confirmed using the classical statistical tests, that is, comparison of intercept with zero and correlation coefficients.

*Stability study*

Control serum samples were spiked with a standard solution of PIRA and DOX to provide concentrations of 5, 10, 50, 100, 150 and 200 ng $ml^{-1}$. For each concentration, three aliquots were immediately assayed according to the procedure given. The ambiant storage of the extracts in the mobile phase was checked after 2 and 15 h.

## Results

*Retention times*

Observed retention times were 6.45, 8.5 and 10.8 min for DOX, internal standard and PIRA, respectively (Fig. 1). The capacity factors ($k'$) were 0.998 for DOX, 1.73 for internal standard, and 2.48 for PIRA. The resolution between the following compounds DOX–internal standard, PIRA–internal standard were 5.5 and 5.8, respectively. There were no interfering peaks in control serum or plasma at the retention times of the respective analytes (Figs 1 and 2). An HPLC chromatogram of plasma sample 73 h after the beginning of a 96-h PIRA infusion (15 mg/24 h) to a patient is shown in Fig. 2.

*Linearity*

The peak height ratio of DOX and PIRA over the internal standard varied linearly with concentration over the range used (10–200 ng $ml^{-1}$). The correlation coefficients ($r$) for calibration curves were equal to or better than 0.995.

In standard bovine serum, the intraday average slopes of the fitted straight lines ($n = 6$) were 0.0247 ± 0.0003 (relative standard deviation, RSD = 1%) and 0.0099 ± 0.0001

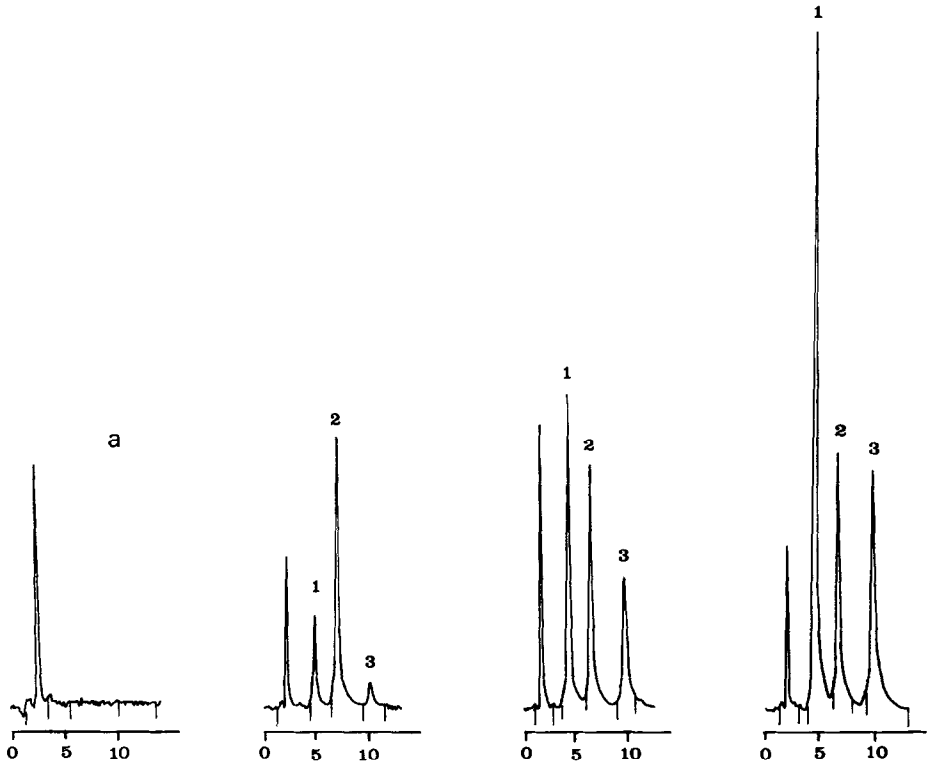


**Figure 1**
HPLC-chromatograms of blank serum (a) and of serum spiked with DOX (10, 50, 100 ng $ml^{-1}$) and PIRA (10, 50, 100 ng $ml^{-1}$). Peaks: 1, DOX; 2, internal standard; 3, PIRA. For chromatographic conditions, see text. Analysis: 16 mV FS.

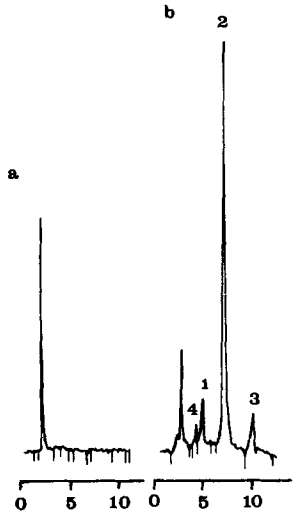


**Figure 2**
Representative chromatograms of blank plasma (a) and of a plasma sample (b) from a patient 73 h after the beginning of a 96-h pirarubicin infusion (15 mg/24 h). The levels were 1.5, 2.8 and 4.8 ng $ml^{-1}$ for DOXOL, DOX and PIRA, respectively. Peaks: 1, DOX; 2, internal standard; 3, PIRA; 4, DOXOL. For chromatographic conditions, see text.

(RSD = 1%) for DOX and PIRA, respectively, the mean intercepts of calibration curves were 0.054 and 0.007, respectively. Between-day RSD values of the slope ($n$ = 12) were 5.20% for DOX and 5.04% for PIRA.

In human plasma spiked with DOX ($n$ = 15), the results of the unweighted least-squares linear regression analysis were as follows: $r = 0.998 \pm 0.00159$ (RSD = 0.16%), slope = $0.0193 \pm 0.0012$ (RSD = 6.2%) and intercept = $0.046 \pm 0.033$; each calibration curve was obtained from a different patient in blank plasma taken prior to the drug administration.

The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. Furthermore, the response factors (RF = peak height ratio:concentration) were computed for DOX and PIRA for each point of calibration standards and for all calibration curves. The relative standard deviations (RSDs %) computed by dividing the standard deviation by the mean RF value, ranged from 4 to 6%. In addition, the mean values of these

response factors were always very close to the slope of the linear calibration curves.

*Precision and accuracy*

For each point of calibration standards, the concentrations were recalculated for the equation of the linear regression curves (experimental concentration). The per cent relative standard deviations (RSD %) are shown in Table 1.

The intraday and between-day precisions of the assay were assessed by performing 30 and six replicate analyses, respectively, of three standard solutions prepared in serum containing 10, 25 and 50 ng $ml^{-1}$. Results, expressed as a percentage of the theoretical concentrations with the relative error, are presented in Table 2 for DOX and in Table 3 for PIRA.

*Recovery*

The extraction efficiency (recovery) was determined by the extraction of serum and plasma prepared by spiking drug-free plasma with known amounts of drug (10, 50 and 200 ng $ml^{-1}$). After extraction, the internal standard was added. Each sample was determined in replicate.

Peak height ratios were compared with unextracted external standards. The unextracted standards were prepared by the addition of stock solutions of DOX, PIRA and internal standard to give concentrations equivalent to those of extracted standards. The mean recoveries were 72% for DOX (72.1 ± 8%, $n = 32$) and more than 80% for PIRA (85.1 ± 10.5%, $n = 26$) and internal standard (81.6 ± 4.8%, $n = 32$).

*Limit of quantification*

The limit of quantification was 2 ng $ml^{-1}$ for DOX and 5 ng $ml^{-1}$ for PIRA. At these levels, the analytical error ranged from 20 to 30%.

*Limit of detection*

The limit of detection which represents a signal noise ratio of 3:1 was 0.5 ng $ml^{-1}$ for DOX and 1 ng $ml^{-1}$ for PIRA.

*Stability*

The stability of DOX and PIRA in the autosampler was determined for each point of

**Table 1**
Accuracy of the method

| Sample concentration (ng $ml^{-1}$) | DOX RSD (%) | PIRA RSD (%) |
|---|---|---|
| 10 | 15 | 20 |
| 20 | 10 | 14 |
| 50 | 5 | 4.5 |
| 100–200 | <3 | <2 |

**Table 2**
Precision of the DOX plasma assay

| Sample concentration (ng $ml^{-1}$) | $n$ | Mean | RSD (%) | Mean recovery (%) | Relative error (%) |
|---|---|---|---|---|---|
| Within-day | | | | | |
| 10 | 30 | 10.2 | 4.55 | 102 | +2.0 |
| 25 | 30 | 26.3 | 3.71 | 105.2 | +5.2 |
| 50 | 30 | 52.5 | 3.40 | 105 | +5.0 |
| Between-day | | | | | |
| 10 | 6 | 10.6 | 8.85 | 106 | +6.0 |
| 25 | 6 | 25.3 | 8.96 | 101.2 | +1.2 |
| 50 | 6 | 50.6 | 6.90 | 101.2 | +1.2 |

**Table 3**
Precision of the PIRA plasma assay

| Sample concentration (ng $ml^{-1}$) | $n$ | Mean | RSD (%) | Mean recovery (%) | Relative error (%) |
|---|---|---|---|---|---|
| Within-day | | | | | |
| 10 | 30 | 10.9 | 5.36 | 109 | +9.0 |
| 25 | 30 | 26.3 | 4.66 | 105.2 | +5.2 |
| 50 | 30 | 46.1 | 4.11 | 92.2 | −7.8 |
| Between-day | | | | | |
| 10 | 6 | 10.8 | 7.07 | 108 | +8.0 |
| 25 | 6 | 25.2 | 6.49 | 100.8 | +0.8 |
| 50 | 6 | 49.7 | 5.48 | 99.4 | −0.6 |

**Table 4**
Stability of PIRA in the mobile phase*

| Sample concentration (ng ml$^{-1}$) | $t$ = 2 h | $t$ = 15 h |
|---|---|---|
| 10 | >95% | — |
| 20–200 | >98% | — |
| 10–20 | — | 80% |
| 50–100 | — | 85% |
| 150–200 | — | 88% |

*The concentrations were expressed in per cent compared with the concentrations at time zero.

calibration standards in the mobile phase. The concentrations were expressed in per cent compared with the concentrations at time zero. For DOX, no significant difference appeared between $t = 0$ and $t = 15$ h. For PIRA, the results are given in Table 4; in addition for all samples, at $t = 15$ h, a peak with the same retention time as DOX appeared on the HPLC-chromatograms.

## Discussion and Conclusion

The present HPLC method involves a rapid and automated assay for the determination of DOX. The main advantages of this method are the good reproducibility, accuracy and the ability to process a great number of tubes (up to 30–40 a day) simultaneously.

The stability of DOX at pH 4 in the HPLC mobile phase allows the automatization of the method. On the other hand, PIRA, is swiftly converted into DOX at ambient temperature (20–25°C), to prevent its breakdown outlined, the extracts in the mobile phase were immediately injected into the HPLC system.

The major metabolite of DOX in humans is doxorubicinol (DOXOL). Since both DOX and DOXOL have cytotoxic effects [13], it would be desirable to measure both of these compounds. The present method allows the quantification of DOXOL with good accuracy. With the chromatographic conditions used, observed retention time of DOXOL is 4.7 min ($k' = 0.831$). The resolution of DOXOL–DOX is equal to 4.01. This metabolite is quantified by the use of a corrective factor between the parent drug (DOX) concentrations and the metabolite concentrations. This factor is the mean ratio of the slopes of the calibration curves constructed for DOX and DOXOL. It ranges from 1.026 to 1.09 and averages 1.05, which is the value used.

*Applications in pharmacokinetic studies*

The technique proposed for HPLC determination of DOX, PIRA and DOXOL in plasma was found to be suitable for the analysis of all samples collected during pharmacokinetic study investigations in patients.

Figure 3 illustrates the concentration–time profile of PIRA, DOX and DOXOL in a patient dosed with 75 mg of PIRA by short i.v. infusion (5 min). Blood samples were collected over a 24-h period.

This method has been used to determine plasma levels of DOX and its major metabolite DOXOL in subjects undergoing treatment for breast cancer. Blood samples were collected over a 48-h period. DOX was given by short i.v. infusion (5 min) (dose range 25–72 mg m$^{-2}$) to 18 patients who underwent 3–7 successive courses of chemotherapy. Plasma concentration–time curves of DOX and DOXOL following intravenous administration of DOX

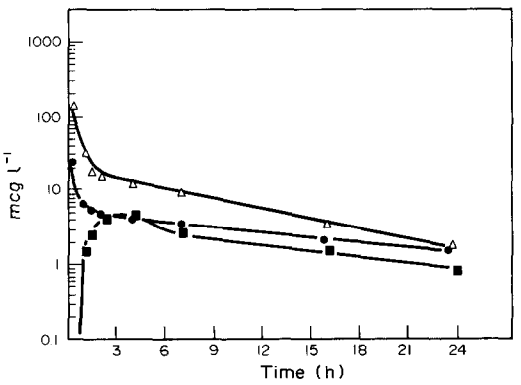


**Figure 3**
Semilogarithmic plot of concentrations of PIRA (△), DOX (●) and DOXOL (■) in plasma following short i.v. infusion (5 min) of 75 mg PIRA to a patient.

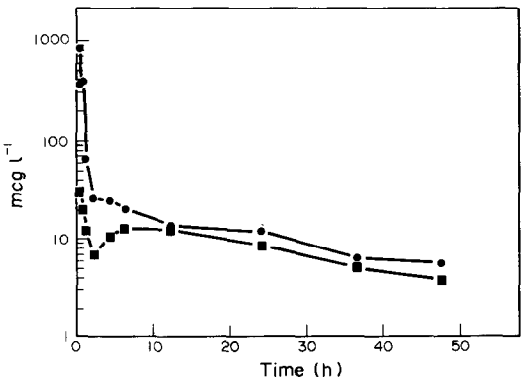


**Figure 4**
Semilogarithmic plot of concentrations of DOX (●) and DOXOL (■) in plasma following short i.v. infusion (5 min) of 90 mg DOX to a patient.

were consistent with an open three-compartment model for DOX and an open two-compartment model for DOXOL (Fig. 4). The mean apparent half-lives of elimination were 33 h for DOX and 42 h for DOXOL. For the parent drug, the steady state volume of distribution was 30 l $kg^{-1}$ and the total clearance 48 l $h^{-1}$. The ratio of the AUCs for DOXOL: DOX averaged 0.514 [14].

*Acknowledgement* — This work was supported by a grant from the Association pour la Recherche sur le Cancer (Villejuif, France).

## References

[1] F. Arcamone, *Doxorubicin. Anticancer Antibiotics.* Academic Press, New York (1981).
[2] J.M. Finkel, K.T. Knapp and L.T. Mulligan, *Cancer Chemother. Rep.* **53**, 159–164 (1969).
[3] J.H. Beijnen and W.J.W. Underberg, *J. Pharm. Biomed. Anal.* **6**, 677–686 (1988).
[4] K.K. Chan and P.A. Harris, *Res. Commun Chem. Pathol. Pharmacol.* **6**, 447–463 (1973).
[5] R.N. Pierce and P.I. Jatlow, *J. Chromatogr.* **164**, 471–478 (1979).
[6] J.E. Brown, P.A. Wilkinson and J.R. Brown, *J. Chromatogr.* **226**, 521–525 (1981).
[7] A.J. Quattrone and D.F. Ranney, *J. Anal. Toxicol.* **4**, 12–16 (1980).
[8] J. Robert, *J. Liq. Chromatogr.* **3**, 1561–1572 (1980).
[9] P.A. Maessen, K.B. Mross, H.M. Pinedo and W.J.F. Van der Vijgh, *J. Chromatogr.* **417**, 339–346 (1987).
[10] G. Degroot, B.C.A. Tepas and G. Storm, *J. Pharmaceut. Biomed. Anal.* **6**, 927–932 (1988).
[11] H. Umezawa, *Proc. Roy. Soc. Lond. (Biol.)* **217**, 357–376 (1987).
[12] D. Dantchev, M. Paintrand, M. Hayat, C. Bourut and G. Mathe, *J. Antibiot.* **32**, 1085–1086 (1979).
[13] R.N. Pierce and P.L. Jatlow, *J. Chromatogr.* **164**, 471–478 (1979).
[14] J.M. Jacquet, F. Bressolle, M. Galtier, M. Bourrier, D. Donadio, J. Jourdan and J.F. Rossi, *Cancer Chemother. Pharmacol.* **27**, 219–225 (1990).

*[Received for review 18 June 1991; revised manuscript received 28 October 1991]*