ELSEVIER

Journal of Chromatography B, 707 (1998) 219–225

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of epirubicin, doxorubicin and their principal metabolites in human plasma by high-performance liquid chromatography and electrochemical detection

R. Ricciarello[a], S. Pichini[a], R. Pacifici[a], I. Altieri[a], M. Pellegrini[a], A. Fattorossi[b], P. Zuccaro[a,*]

[a]*Clinical Biochemistry Department, Istituto Superiore di Sanità, V. le Regina Elena 299 00161, Rome, Italy*
[b]*Department of Obstetrics and Gynecology, Università Cattolica del Sacro Cuore, Roma, Italy*

Received 4 July 1997; received in revised form 13 November 1997; accepted 2 December 1997

Abstract

A high-performance liquid chromatographic method with electrochemical detection has been developed for the simultaneous determination of epirubicin, 13-*S*-dihydroepirubicin, doxorubicin and 13-*S*-dihydrodoxorubicin in human plasma. An aliquot of 200 μl plasma, spiked with internal standard, was extracted by solid-phase extraction using polymeric adsorbent columns. Chromatography was performed using a C_{18} reversed-phase column with a mobile phase consisting of water–acetonitrile (71:29, v/v) containing 0.05 *M* Na_2HPO_4 and 0.05% v/v triethylamine adjusted to pH 4.6 with citric acid. Linearity of the method was obtained in the concentration range of 1–500 ng/ml for all the analytes. Analytical recoveries of the analytes ranged from 89 to 93%. The assay can be used for the simultaneous determination of the four analytes, or for epirubicin and its metabolite or doxorubicin and its metabolite, using the other parent drug as an internal standard. The method was applied to analyze human plasma samples from patients treated with epirubicin using doxorubicin as an internal standard.

Keywords: Epirubicin; 13-*S*-Dihydroepirubicin; Doxorubicin; 13-*S*-Dihydrodoxorubicin

1. Introduction

Anthracyclines are a group of antitumor antibiotics widely used for the treatment of neoplastic diseases [1,2]. In particular, doxorubicin was the first drug of its class successfully applied to a variety of malignancies, such as leukemias, sarcomas, breast cancer and ovarian cancer [3]. However, this compound suffers from side effects such as myeloablation and cardiotoxicity [4–6].

Epirubicin (4′-epidoxorubicin) is one of a series of anthracyclines synthesized with the aim of finding a doxorubicin analog with a more favourable therapeutic index [7,8]. It differs from doxorubicin only in the spatial orientation of the 4′-moiety and it is being administered in advanced breast cancer and other malignancies, having shown activity against a broad range of tumors in experimental models [9,10]. Nonetheless, doxorubicin is still considered the most

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00610-5

effective drug of its class and continues to be in use worldwide [11]. Indeed, treatment protocols currently applied in our country still involve the use of doxorubicin as well as epirubicin as antitumor agents, although these two drugs are unlikely to be used concomitantly in clinical trials.

Both doxorubicin and epirubicin are extensively metabolized to their 13-dihydro derivatives: 13-*S*-dihydrodoxorubicin (doxorubicinol) and 13-*S*-dihydroepirubicin (epirubicinol) (Fig. 1) and, to a minor extent, to the 7-hydroxy and 7-deoxyaglycone metabolites [12]. Furthermore, unlike doxorubicin, epirubicin is conjugated with glucuronic acid [13,14].

Several chromatographic methods have been developed to determine anthracyclines and their metabolites in biological samples [7,15–19], but to our knowledge only one high-performance liquid chromatographic (HPLC) assay has been published, which determines epirubicin, doxorubicin and their metabolites in 1 ml serum using fluorescence detection [12].

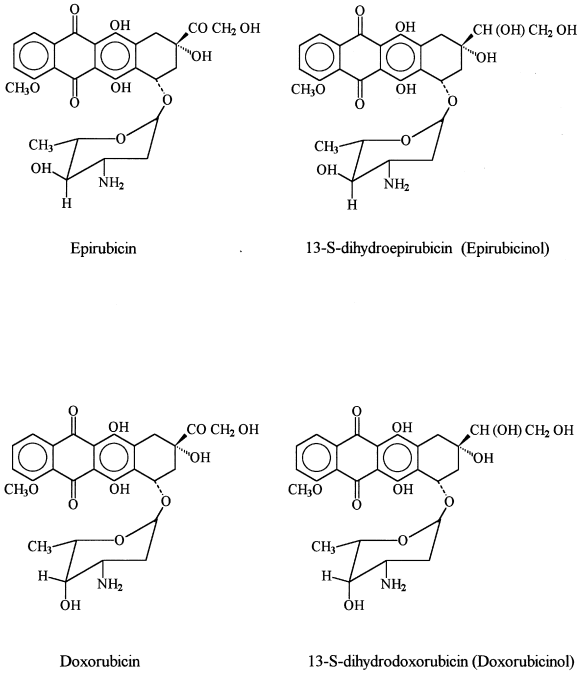

Fig. 1. Structures of epirubicin, doxorubicin and their principal metabolites.

The aim of our study was the development of a chromatographic method, with an alternative system of detection, which could allow a high sensitivity and reliability using small sample volumes. For this reason, an electrochemical detector composed of an amperometric electrode coupled with a coulometric electrode was applied to the simultaneous determination of epirubicin, doxorubicin and their metabolites, 13-*S*-dihydroepirubicin and 13-*S*-dihydrodoxorubicin, in human plasma.

Due to the fact that epirubicin and doxorubicin are unlikely to be administered concomitantly, this method can be used for a parent drug and its metabolite, using the other parent drug or the other metabolite as an internal standard. In our experience, the assay has been validated in a group of patients treated with epirubicin for ovarian cancer using doxorubicin as an internal standard.

2. Experimental

2.1. Chemicals

Epirubicin, 13-*S*-dihydroepirubicin HCl, doxorubicin and 13-*S*-dihydrodoxorubicin HCl were a gift from Carlo Erba (Milan, Italy). Oasis HLB solid-phase extraction (SPE) columns were obtained from Waters (Milan, Italy). Control blank plasma (lithium heparin) were purchased from Sigma (Milan, Italy). All reagents and solvents were analytical or HPLC grade.

2.2. Chromatographic equipment

The HPLC system used in this study consisted of a Series 410 BIO LC Pump (Perkin Elmer, Norwalk, CT, USA), a pulse damper LP-21 (SSI, State College, PA, USA), a Rheodyne Model 7125 injection valve (Rheodyne, Berkeley, CA, USA), a Coulochem II electrochemical detector (ESA, Belford, MA, USA) equipped with a Model 5014 high-performance analytical cell containing an enhanced response amperometric electrode coupled with a coulometric electrode with associated palladium reference electrodes. For ED data collection and calculations, a

PeNelson 900 Series interface and a Turbochrom Navigator (Perkin-Elmer) were used.

2.3. HPLC conditions

The chromatographic separation was performed with a 200×4.6 mm I.D. LiChrosorb RP 18 reversed-phase column (particle size 10 μm) (Hewlett-Packard, Milan, Italy). The isocratic mobile phase consisted of water–acetonitrile (71:29 v/v) containing 0.05 *M* Na_2HPO_4 and 0.05% v/v triethylamine. The pH of the final solution was adjusted to 4.6 with citric acid. The separation was performed at a flow-rate of 1.0 ml/min. During analysis the mobile phase was recycled. Run time was 25 min. The working parameters for the electrochemical detector were +400 mV for the first electrode and −300 mV for the second. The signals generated by the second electrode were used for the quantitation.

2.4. Standards and controls

Solutions of stock reference standards (1 mg/ml, 10 μg/ml, 1 μg/ml) were prepared in methanol and stored below 0°C. Aqueous dilutions were made fresh daily for each analysis. Aliquots of these standards were spiked into blank plasma and used throughout the entire sample preparation to create calibration curves and to determine SPE recoveries and intra- and inter-day variabilities.

2.5. Sample collection protocol

Blood samples (2–3 ml) were taken into lithium heparin from patients suffering from ovarian cancer and treated with epirubicin at Agostino Gemelli General Hospital (Rome, Italy). The drug was administered as an i.v. bolus at a dose of 90–120 mg/m^2. A sample was taken before treatment, while a second sample was taken about 30 min after the end of the treatment. Blood samples were centrifuged immediately and the plasma stored at −20°C until analysis.

2.6. Sample preparation

A 500-μl volume of the mobile phase diluted 1:3 with water was mixed with 200 μl plasma, and 100 μl doxorubicin (120 ng/ml in water) was added as an internal standard. This mixture was applied to a polymeric adsorbent SPE column. Before use, the columns were pretreated with 1 ml of mobile phase diluted 1:3 with water. The columns were washed with 1 ml mobile phase diluted 1:3 with water, then the compounds retained in the column were eluted with 600 μl of mobile phase diluted 1:1 with acetonitrile. A 20-μl aliquot of the resulting solution was injected into the HPLC system.

2.7. Stability studies

Stability studies were conducted in lithium–heparinized blank plasma spiked with 100 ng/ml of all the four analytes stored identically to patients samples at −20°C. Five samples were analyzed on the day of samples preparation (control samples) and then every 7 days for a 3-month period. Furthermore, some plasma samples from patients treated with epirubicin were randomly reanalyzed once a month for a 3-month period.

3. Results and discussion

The method reported here for the determination of epirubicin, doxorubicin and their principal metabolites employs HPLC with the use of amperometric–coulometric detection with two working electrodes. For the determination of optimum potentials for the two electrodes, current–voltage curves for the anthracyclines were investigated both in oxidative and in reductive screen mode.

Fig. 2 shows the hydrodynamic voltammograms of the four analytes both in oxidative and in reductive screen mode. It was decided to work in reductive screen mode, as in oxidation the chromatographic profile suffered severe interference from substances coming from the biological matrix having oxidation potentials similar to those of the analytes. For this reason, the first electrode was set at +400 mV in order to oxidise both the analytes and substances coming from the matrix, and the second electrode at −300 mV, which was the best compromise between a potential sufficient to completely

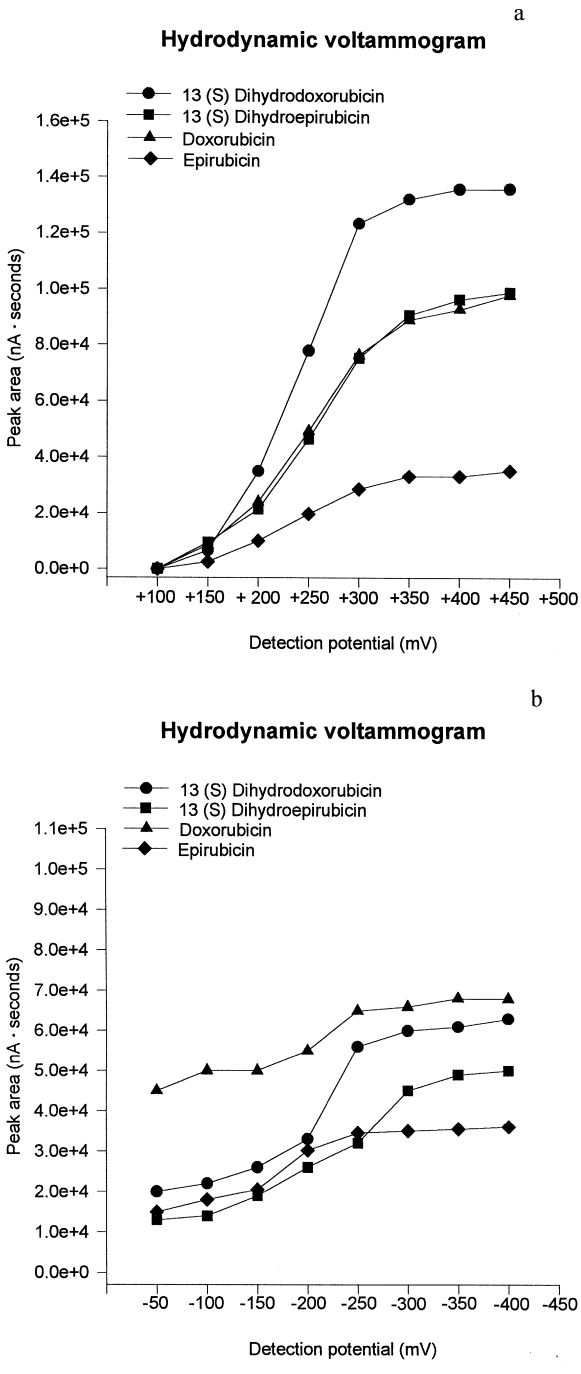

Fig. 2. Hydrodynamic voltammograms for standard substances obtained under the conditions described in the text. (a) Oxidative screen mode; (b) reductive screen mode.

reduce the anthracyclines and their metabolites in the absence of matrix interferents, which usually have higher reductive potentials.

Fig. 3 depicts the chromatogram of an extract of a plasma sample from a patient treated with 120 mg/m^2 epirubicin. Both epirubicin and its principal metabolite were present at concentrations of 29.8 ng/ml and 12.0 ng/ml, respectively.

The linearity of the method was evaluated on calibration curves of the peak area (y, nA s) versus the concentration of analytes (x, ng/ml) which were prepared and checked daily from blank plasma spiked with eight different concentrations of the

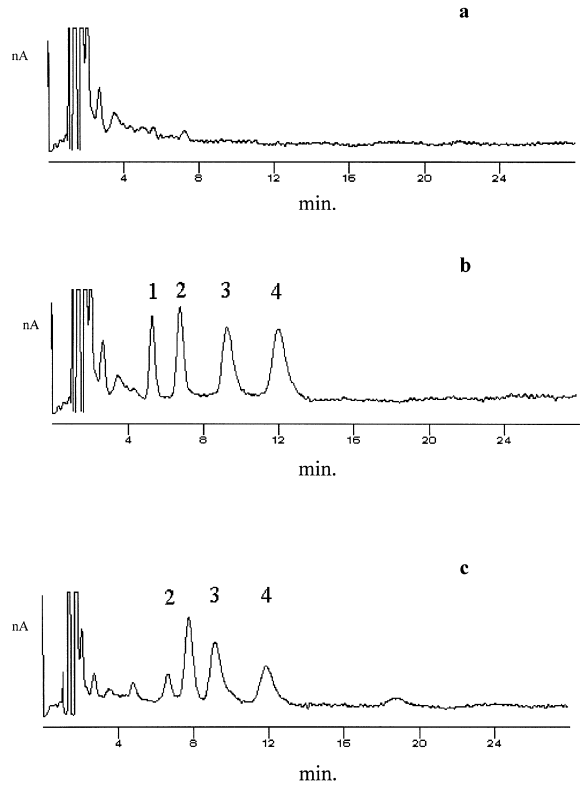

Fig. 3. (a) Chromatogram of an extract of blank plasma; (b) chromatogram of an extract of blank plasma spiked with 40 ng/ml each of 13-*S*-dihydrodoxorubicin (1), 13-*S*-dihydroepirubicin (2), epirubicin (3) and doxorubicin (4); (c) chromatogram of an extract of a plasma from a patient containing 12.0 ng/ml 13-*S*-dihydroepirubicin (2), 40 ng/ml doxorubicin as an I.S. (3) and 29.8 ng/ml epirubicin (4).

analytes and carried through the entire procedure. Calibration curves were linear over the range 1–500 ng/ml for all the analytes. The quantitation limits (signal-to-noise ratio of 3) and the calibration curve data are shown in Table 1.

The analytical recoveries of the compounds and the intra-day and inter-day variabilities were evaluated at three different concentrations on 6 days and are summarized in Table 2.

No indications of drug instability was found in blank plasma spiked with the four analytes as the amount of the analytes recovered in samples analyzed during a 3-month period did not statistically differ from control samples. The same results were obtained on reanalyzing some patients samples.

Using the methodology described above, plasma samples from female patients suffering from ovarian cancer and treated with 90–120 mg/m^2 epirubicin were analyzed. The results obtained (Table 3) were a preliminary attempt at drug monitoring and appeared to be in accordance with the few data reported in the literature [8,19]. Furthermore, these data are going to be coupled with the data from cytometric analysis of lymphocytes subsets of the patients in order to evaluate intracellular drug incorporation.

4. Conclusions

In conclusion, a new method employing high-performance liquid chromatography with electrochemical detection is proposed for the quantitative determination of epirubicin, 13-*S*-dihydroepirubicin, doxorubicin and 13-*S*-dihydrodoxorubicin in plasma samples. The analytical technique proposed can also

Table 1
Quantitation limit and linearity of the method

Compound	Quantitation limit (ng/ml)	Slope (*m*)	Intercept (*b*)	Correlation coefficient
Epirubicin	1	198.68	174.89	0.9986
		198.88	174.59	0.9990
		198.75	175.05	0.9985
		198.89	175.10	0.9981
Mean		198.80	174.90	0.9986
S.D.		0.1023	0.2298	0.0004
13-S-Dihydroepirubicin	1	428.88	115.20	0.9969
		428.55	114.89	0.9979
		428.99	115.00	0.9998
		428.39	115.21	0.9979
Mean		428.70	115.07	0.9981
S.D.		0.2799	0.1567	0.0012
Doxorubicin	1	424.04	314.52	0.9988
		424.16	315.01	0.9990
		424.26	314.71	0.9986
		423.98	314.50	0.9985
Mean		424.11	314.68	0.9987
S.D.		0.1249	0.2364	0.0002
13-S-Dihydrodoxorubicin	1	747.18	317.09	0.9993
		747.23	317.25	0.9996
		747.29	317.12	0.9997
		747.52	317.56	0.9997
Mean		747.30	317.25	0.9996
S.D.		0.1502	0.2149	0.0002

Table 2
Analytical recovery and precision of the method ($n=5$)

Concentration (ng/ml)	Recovery (mean±S.D.	R.S.D. (%)	
		Intra-day	Inter-day
Epirubicin			
1	88.2±3.9	4.3	4.7
5	88.6±3.1	3.5	3.8
100	90.2±2.9	3.2	3.8
500	92.3±2.8	3.0	3.6
13-S-Dihydroepirubicin			
1	93.0±4.7	5.0	5.3
5	93.0±4.2	4.5	4.6
100	93.5±4.0	4.2	4.4
500	94.2±4.0	4.2	4.0
Doxorubicin			
1	92.4±3.3	3.6	3.9
5	92.6±2.9	3.2	3.4
100	92.8±3.0	3.2	3.2
500	93.2±2.7	2.9	2.4
13-S-Dihydrodoxorubicin			
1	93.0±3.7	4.0	4.5
5	93.3±3.4	3.6	4.0
100	93.5±3.0	3.2	3.9
500	95.6±2.9	3.0	4.1

be applied to therapeutic drug monitoring of the parent drugs and their principal metabolites in combination with immune functions monitoring. Further studies are required to extend the methodology to the determination of 7-hydroxy, 7-deoxy-aglycone and glucuronide metabolites.

Table 3
Concentration of epirubicin and 13-*S*-dihydroepirubicin in plasma samples from treated patients

Subject (no.)	Drug treatment (mg/m^2)	Epirubicin (ng/ml)	13-*S*-Dihydroepirubicin (ng/ml)
1	120	30.4	15.9
2	120	36.6	19.7
3	120	29.8	12.0
4	120	11.6	19.5
5	100	15.2	8.6
6	100	14.8	5.7
7	100	28.8	15.8
8	100	22.3	16.1
9	90	13.0	12.0
10	90	15.0	5.0
11	90	14.0	18.0
12	90	11.2	9.5

References

[1] V.T. De Vita, in: V.T. De Vita, S. Hellmann, S.A. Rosemberg (Editors), Cancer principles and practice of oncology, Lippincott, Philadelphia, 1989, Ch. 18, p. 376.
[2] B.K. Sinha, P.M. Politi, in: H.L. Pinedo, D.L. Longo, B.A. Chebner (Editors), Cancer chemotherapy and biological response modifiers Annual 11, Elsevier, Amsterdam, 1990, p. 45.
[3] P.A.J. Speth, Q.G.C.M. van Hoesel, C. Haanen, Clin. Pharmacokinet. 15 (1988) 15.
[4] D.D. Von Hoff, M.W. Layard, P. Basa, H.L. Davis, A.L. Von Hoff, M. Rozencweig, F.M. Muggia, Ann. Int. Med. 91 (1979) 710.
[5] R.D. Olson, P.S. Mushlin, FASEB J. 4 (1990) 3076.
[6] E. Saltiel, W. McGuire, Western J. Med. 139 (1983) 332.
[7] G. Cassinelli, E. Configliacchi, S. Penco, G. Rivola, F. Arcamone, A. Pacciarini, L. Ferrari, Drug Metab. Dispos. 12 (1984) 506.
[8] J. Robert, Drugs 45 (1993) 20.
[9] F. Arcamone, S. Penco, A. Vigevani, S. Redaelli, G. Franchi, A. Di Marco, A.M. Casazza, T. Dasdia, F. Formelli, A. Necco, C. Soranzo, J. Med. Chem. 18 (1975) 703.
[10] A.M. Casazza, Cancer Treat. Rep. 63 (1979) 835.
[11] R.B. Weiss, Semin. Oncol. 19 (1992) 670.
[12] G. Nicholls, B.J. Clark, J.E. Brown, J. Pharm. Biomed. Anal. 10 (1992) 949.
[13] H. Weenen, J.M.S. Van Maanen, M.M. de Planque, J.G. McVie, H.M. Pinedo, Eur. J. Cancer Clin. Oncol. 20 (1984) 919.
[14] J.D.M. Robert, C. Granger, Cancer Chemother. Pharmacol. 27 (1990) 147.
[15] J.E. Brown, P.A. Wilkinson, J.R. Brown, J. Chromatogr. 226 (1981) 521.
[16] J. Robert, N.B. Bui, Ann. Oncol. 3 (1992) 651.
[17] A. Andersen, D.J. Warren, L. Slordal, Ther. Drug Monit. 15 (1993) 455.
[18] P.E. Deesen, B. Leyland-Jones, Drug Metab. Dispos. 12 (1984) 9.
[19] I.K. Barker, S.M. Crawford, A. Fell, J. Chromatogr. B 681 (1996) 323.