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Quantitation of adriamycin in plasma and urine: comparative study of radioimmunoassay and high-performance liquid chromatography methods

ROGER RAHMANI*[1], PHILIPPE GIL[2], MARIE MARTIN[1], ALAIN DURAND[2], JACQUES BARBET[3] and JEAN-PAUL CANO[2]

[1] *Groupe de Recherche sur l'Immunologie et la Pharmacologie des Anti-Cancéreux, 232 Bd de Sainte Marguerite, 13273 Marseille Cedex 9, France*
[2] *Laboratoire de Pharmacocinétique et Toxicocinétique INSERM SC No. 16, Faculté de Pharmacie, 27, Bd Jean Moulin, 13385 Marseille Cedex 5, France*
[3] *Centre d'Immunologie, INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9, France*

Abstract: The response of tumours to adriamycin, and the cardiotoxicity of the drug, may be related to its pharmacokinetics and plasma levels. Rapid and sensitive methods of adriamycin determination in plasma and urine samples are thus needed. A comparative study shows that high-performance liquid chromatography with fluorimetric detection is a reliable and specific method, but it is relatively slow and sometimes lacks sensitivity. A commercially-available radioimmunoassay kit is convenient, but there is a cross reaction with the major metabolite adriamycinol and unless the assay is combined with an extraction step, it gives erroneously high results.

Keywords: *Adriamycin; doxorubicin; pharmacokinetics; radioimmunoassay; high-performance liquid chromatography.*

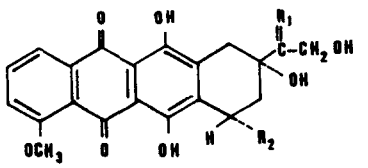
Introduction

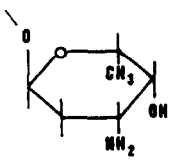
Adriamycin (ADR) is an anthracycline antibiotic which is effective in the treatment of several solid tumours and haematologic malignancies [1–6]. Its pharmacokinetic behaviour and metabolism in human are not fully understood. The present authors [7] and others [8–10] have reported large inter-individual variations and time-dependent kinetics in patients treated for various types of cancer. Accurate kinetic data are thus necessary for the further development of ADR therapy.

Several techniques have been used for ADR analysis. High-performance liquid chromatography (HPLC) with fluorometric detection [11–13] is a very specific and sensitive method for the quantification of ADR and of some of its metabolites (adriamycinol, (ADR-OH), adriamycin and adriamycinol-aglycones; Fig. 1) in extracts

* To whom correspondence should be addressed.

of plasma and urine. Radioimmunoassays (RIA) have been developed [14, 15] and a kit is sold by Diagnostic Biochemistry Inc. This method should be especially useful when ADR levels in biological fluids are low and when large scale clinical monitoring is undertaken. RIA is a very rapid method as many samples of plasma or urine can be studied simultaneously without preliminary extraction. Nevertheless, metabolites and endogenous compounds could interfere with RIA measurements and the specificity of this method must be assessed before it is used for pharmacokinetic and clinical studies. These problems were overcome by Langone *et al.* [16] who used RIA as a quantitation method after separation of ADR and its metabolites by HPLC. Adriamycin RIA [14] was compared with a total fluorescence assay by Bachur *et al.* [17] using paired samples from patients treated for malignancy, but both methods were relatively non-specific.





Daunosamine

	R_1	R_2	Cross Reactivity
Adriamycin	=O	Daunosamine	100 [a,b]
Adriamycinol	$\lesssim^{H}_{OH}$	Daunosamine	100 [a,b]
Adriamycin aglycone	=O	– OH	22 [a,b]
Adriamycinol aglycone	$\lesssim^{H}_{OH}$	– OH	13 [a]
Deoxyadriamycin aglycone	=O	– H	7 [b]
Deoxyadriamycinol aglycone	$\lesssim^{H}_{OH}$	– H	80 [b]

Figure 1
Chemical structures of ADR and its major metabolites, and the RIA cross-reactivities of each (a: present work; b: data from Diagnostic Biochemistry Inc.).

In the present study concentrations of ADR in plasma and urine samples from treated patients were measured by HPLC and by RIA. The first assays confirmed the observations of Piall *et al.* [18] who compared a similar ADR RIA kit with measurements performed with a tritiated ADR probe. Very strong non-specific interferences were found which were variable from one patient to another. In this work attempts were made to minimize such interferences and possible discrepancies between the HPLC and RIA methods were further investigated. The effects of ADR plasma and urine level errors on pharmacokinetic data were also studied.

Experimental

Reagents

ADR chlorhydrate, ADR-OH chlorhydrate and Daunorubicin chlorhydrate (DNR) were kindly supplied by Rhône Poulenc (Vitry, France), and ADR and ADR-OH aglycones by Roger Bellon (Neuilly, France).

All solvents were of analytical grade. The phosphate buffer (0.05 M) was made up by dissolving 18 g of disodium phosphate in a litre of distilled water. The formate buffer was a solution of 0.1% ammonia in distilled water adjusted to pH 4 with pure formic acid.

Adriamycin (Doxorubicin) (^{125}I) Radioimmunoassay kits were purchased from Diagnostic Biochemistry Inc. The kits were stored as directed in the instruction manual and used before their specific expiry dates. Dilutions of plasma and urine samples were made in phosphate buffer saline (pH 7.2) containing 1 g l^{-1} bovine serum albumin (Fraction V, Sigma).

Subjects

Three subjects with solid tumours were used in this comparative study. Diagnoses were oesophagus carcinoma, lymphoma and parotidian carcinoma for patients I, II and III, respectively. These patients received ADR for the first time and a concomitant therapy four days after ADR administration to avoid any interference during the pharmacokinetic analysis. No pathological values for hepatic and renal functions were found during the observation period.

Injections of ADR were performed as IV boluses for each patient at the dose of 30 mg m^{-2} (Adriblastine, Roger Bellon). The duration of the injection was measured exactly for pharmacokinetic analysis. Blood samples were collected in heparinized tubes at 0.083, 0.166, 0.333, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 36, 60 and 72 h after the end of the injection. The blood was centrifuged at 3000 rpm and the plasma was immediately frozen until analysis. Urine was completely collected for 96 h and analysed immediately after determination of the volume.

High-performance liquid chromatography

HPLC analyses of extracts from patients' plasma and urine samples were performed essentially as described in refs [19] and [20]. A Waters model 6000 A pump fitted with a Waters U6K injector was used. A microBondapak C_{18} (30 × 0.4 cm) column was connected to a Gilson fluorescence monitor. The excitation and emission wavelengths were 480 and 560 nm respectively. The mobile phase was a mixture of acetonitrile–formate buffer pH 4 (32:68 v/v) at a flow rate of 2 ml/min. Under these conditions, retention times were about 3, 4 and 8 min for ADR-OH, ADR and DNR respectively. The detection and integration of the peaks were performed by a Hewlett-Packard model 3390 A integrator. The limit of sensitivity of the technique was 5 ng ml^{-1} and the coefficient of variation 8.7% [19, 20].

Extraction procedures

Plasma. The extraction procedure for plasma samples was adapted from the method of Robert [19]. Plasma samples (0.1–0.3 ml) were extracted on a polyethylene cartridge filled with C_{18}-bonded silica (C18 SEP-PAK, Waters Associates, Milford). Pure methanol 3 ml, methanol–water (1:1 v/v) 3 ml, Na_2HPO_4 0.05 M (10 ml) and plasma (0–3 ml) containing known amounts of DNR (internal standard) were passed through the column

using a 10 ml glass syringe. The column was washed with phosphate buffer (3 ml) and the compounds were eluted with chloroform–methanol (2:1 v/v, 3 ml). The organic eluate was dried and the residue redissolved in 120 μl of mobile phase. After centrifugation, the supernatant (50 μl) was injected into the chromatograph.

Urine. Urine samples containing internal standard were adjusted to pH 8.5 with phosphate buffer and mixed with 10 ml of chloroform–methanol (4:1, v/v) for 5 min. The organic phase was evaporated at 45°C under a stream of nitrogen. The residue was redissolved in 120 μl of mobile phase and 50 μl aliquots were injected into the chromatograph.

RIA procedure

ADR plasma or urine concentrations were determined using a radioimmunoassay provided by Diagnostic Biochemistry Inc. The assays were performed by incubation for 1 h at 4°C of the mixture:radiolabel (100 μl), unknown or standard (100 μl), antiserum (500 μl). The standards (1, 2, 5 and 10 ng ml^{-1}) were prepared just before use from a stock solution of ADR (1 μg ml^{-1}) determined by UV spectrometry. Charcoal was used to separate bound from free ^{125}I ADR. The charcoal was centrifuged at 3000 rpm for 5 min and the supernatant aspirated and counted for 2 min on a Kontron MR 252 γ-Counter. The determinations of ADR levels in patient serum and urine samples were done by interpolation on the linearized standard curve (log logit). The mean of duplicate counts (B) was plotted as log (B-NSB)/(B_0-B), where NSB is the non-specific binding and B_0 the control binding, against the logarithm of the concentrations of standards. The slope (S) and intercept (I) of the least squares regression line were then computed and used for the calculation of unknown samples (log (C/dilution) = ((log (B-NSB)/(P_0-B)-I)/S), where P_0 is the control binding in the presence of 100 μl of a corresponding dilution of the patient's plasma or urine collected before ADR injection).

Pharmacokinetic analysis

The pharmacokinetic parameters have been compared using data corresponding to the sum of ADR and ADR-OH concentrations. The terminal half-lives of ADR kinetics were determined by using a semilogarithmic least square regression procedure on the experimental data points from 12 to 72 h. The area under the curve (AUC) was then calculated by the trapezoidal rule on all experimental points and extrapolated to infinity. Clearances were assessed according to the equation: clearance = dose/AUC.

Results

Specificity

The HPLC method allows the separate monitoring of unchanged ADR and some of its metabolites: ADR-OH, adriamycin and adriamycinol-aglycones. By contrast, the antisera in the RIA kit recognizes ADR and ADR-OH equally, whereas ADR and ADR-OH aglycones are only partially immunoreactive (22 and 13% cross-reactivity respectively, confirming the data for ADR aglycone provided with the RIA kit: 25% cross-reactivity). Deoxy-adriamycinol-aglycone and deoxy-adriamycin-aglycone cross-react to the extent of 80 and 7% respectively (Fig. 1).

HPLC studies of ADR elimination kinetics showed that only ADR-OH is found in large amounts in both the plasma and urine of treated patients. The other metabolites

never exceeded a few percent of the total ADR-related compounds in the samples ([13, 16] and unpublished observations by the present authors). The RIA method will thus in practice determine ADR + ADR-OH levels, the effect of other metabolites being very small. Subsequent comparisons between RIA and HPLC measurements therefore use RIA concentrations and the sum of the concentrations of ADR and ADR-OH obtained by HPLC analysis.

Interference in the RIA

There were strong plasma interferences in the RIA method, with 20–90% inhibition of radiolabelled ADR binding to antiserum for blood samples collected before IV bolus administration. These non-specific effects were minimized by diluting the samples at least 10 times in phosphate buffer containing 1 g l^{-1} BSA before assay. After dilution the non-specific binding was reduced to < 25%, except for a fourth patient where it was > 70%, thus preventing RIA measurements. This residual inhibition was taken into account in ADR + ADR-OH concentration estimation by using dilutions of the patient plasma collected before administration for the control binding measurement (Po) (see Experimental). When known quantities of ADR (2, 10, 20, 100, 200, 1000 ng/ml) were added to blank serum or plasma samples, the RIA results were reasonably close to the expected values (mean ratio = 1.10 with SD = 0.26, 12 values), provided non-specific inhibitions (24 and 18% respectively) were taken into account. The modified RIA procedure was thus applied to the analysis of plasma and urinary ADR levels after IV bolus administration in three patients, the results being compared to those for the same patients obtained by HPLC.

Plasma results. Figure 2 shows the plasma elimination of ADR + ADR-OH for patient I determined by RIA and HPLC. Even 72 h after injection, the RIA procedure permitted the measurement of ADR + ADR-OH levels, whereas with HPLC concentrations were below the limit of detection. When comparing RIA values with the corresponding HPLC ones, discrepancies were observed. For patients I and II, concentrations found using RIA were higher than those obtained by HPLC. The means of

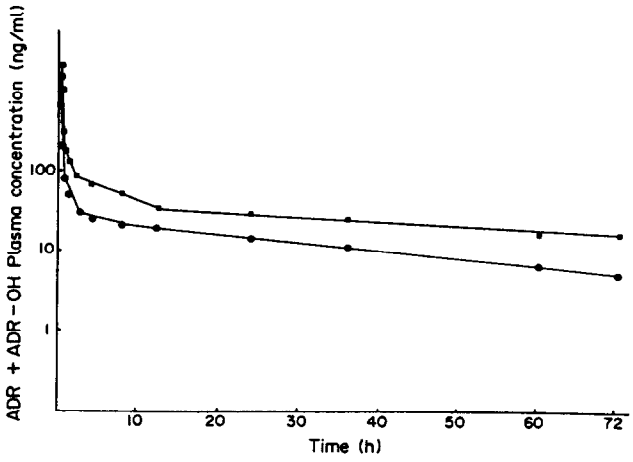


Figure 2
ADR concentrations for patient I determined in diluted plasma samples by RIA, taking into account residual non-specific inhibition. ADR and ADR-OH were also monitored by HPLC after SEP-PAK extraction. RIA (■) and HPLC (ADR + ADR-OH: ●).

the RIA/HPLC ratios (ADR + ADR-OH) were significantly different from 1 ($p < 0.001$, Table 1). The discrepancy was greatest at lower concentrations (i.e. at longer times after injection) as illustrated in Fig. 3. For patient III, the ratios were closer to 1 and there was little concentration dependence of the ratio.

Table 1
RIA and HPLC determinations of ADR + ADR-OH in plasma and urine samples from patients receiving ADR

Patient	Sample	Mean RIA/HPLC ratio (SD)	Slope of the regression line ratio versus log (ADR + ADR-OH) (SD)
I	Plasma	2.17 (0.55)	− 0.59 (0.14)
I	Urine	1.59 (0.73)	− 1.00 (0.25)
II	Plasma	2.34 (0.68)	− 0.69 (0.24)
III	Plasma	1.11 (0.20)	− 0.06 (0.08)

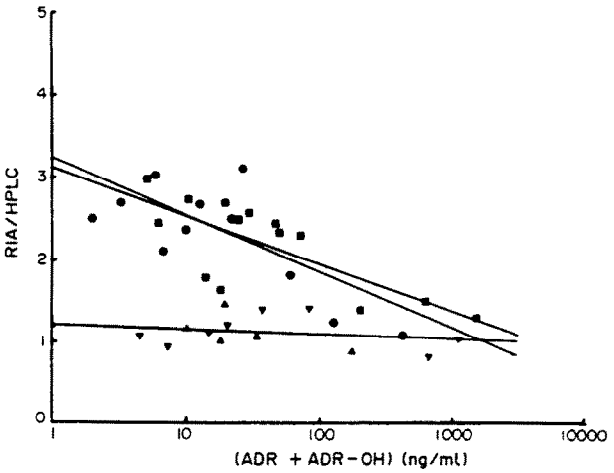


Figure 3
ADR + ADR-OH concentrations measured by RIA and by HPLC in plasma samples from three patients receiving ADR. The ratio of RIA and HPLC values for ADR + ADR-OH is plotted against the logarithm of (ADR + ADR-OH) determined by HPLC. (patient I: ■; II: ●; III: ▲).

To examine the origin of the discrepancies, RIA measurements of ADR + ADR-OH levels were performed after extraction on a SEP-PAK mini column as described for the HPLC method. The agreement between the results was much better (ratio, 1.32; SD, 0.85; 8 measurements). The measurement of SEP-PAK effluent buffer proved also that only small amounts of immunoreactive materials remained unextracted (mean 11.2%, 8 measurements).

The discrepancy observed for two patients using RIA and HPLC analysis could not be explained either by an RIA cross-reaction with a non-fluorescent metabolite not detected by HPLC, or by the incomplete extraction of ADR-related compounds. The HPLC extraction procedure was thus validated.

Urine results. The results of applying the two methods to urine samples were also compared. The RIA procedure used for plasma was also applied to urine samples. When plotting the cumulative dose (taking into account urine volume) versus time after administration, less discrepancy between the methods than that observed for plasma was found (Fig. 4), the RIA/HPLC ratio for the cumulated dose being 1.14. When results for each fraction were compared, this ratio was smaller than for the plasma from the same patient. However, the trend to increased differences at lower concentrations (i.e. longer time after injection) was maintained (Table 1).

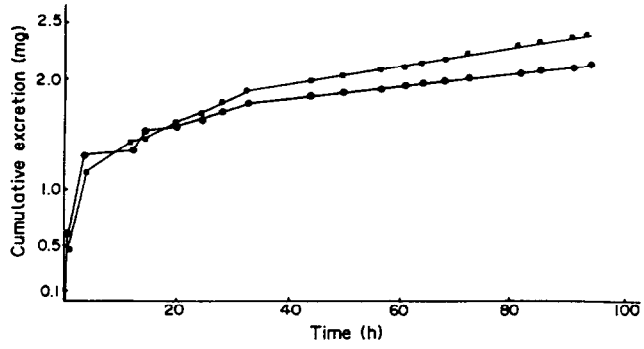


Figure 4
The cumulative urinary excretion of ADR + ADR-OH measured by HPLC and by RIA for patient I (RIA: ■; HPLC: ●).

Pharmacokinetic analysis

The pharmacokinetic parameters from the data obtained by each procedure were calculated (Table 2). The clearance and half-time of elimination for the sum ADR + ADR-OH obtained by the two methods for the same patient differed considerably. These discrepancies were sometimes larger than the inter-individual variations.

Table 2
Pharmacokinetic parameters after injection of ADR

	AUC (h.μg/ml)		Plasma clearance (l/h)		$T_{1/2}$ (h)	
	RIA	HPLC	RIA	HPLC	RIA	HPLC
I	2430	1136	14.4	34.3	47.9	31.6
II	708	245	61.3	147	17.8	12.6
III	880.5	830.5	44.9	47.2	23.1	26.4

Discussion

Plasma and urine ADR levels must be measured on very large numbers of samples from treated patients to study significant correlations between pharmacokinetic parameters and clinical responses or toxicities, especially since time-dependent kinetics were observed in patients given repeated injections [7–10]. A sensitive radioimmunoassay method for ADR was thus studied as a possible replacement for the fluorometric HPLC method.

The ADR RIA kit used is described as having a complete cross-reaction with ADR-OH and a partial cross-reaction (25 and 7%) with ADR and ADR-OH aglycones. The present study confirmed these results and, because only small amounts of aglycone metabolites were measured by HPLC [13, 16], a comparison was made between the RIA results and the sum of the ADR and ADR-OH concentrations determined by HPLC. This is an important limitation of the RIA procedure but, as ADR and ADR-OH are both active and potentially toxic compounds, the monitoring of this sum (ADR + ADR-OH) has some biological significance.

More disturbing were the strong plasma interferences in the immunoassay. When following the recommended procedure inhibition of binding in blood samples collected before ADR administration corresponded to 0.5–8 ng ml^{-1} ADR. Diluting the samples at least 10 times in phosphate buffer containing BSA was not an adequate safeguard because in one sample the residual inhibition was too strong. Similar effects were obtained with human serum from healthy donors provided by the Centre de Transfusion Sanguine in Marseilles. Recently, Piall *et al.* [18] reported large discrepancies (which could have similar origins) between results obtained with this RIA method and results obtained with the same antiserum but with tritiated ADR as the radioactive probe.

Despite the modification of the technique RIA values were higher than HPLC values for two patients out of three, the discrepancy being more obvious at longer times after the IV injection and not constant from one patient to another. When measurements were performed after plasma extraction on SEP-PAK minicolumns as described for the HPLC method, the agreement between the two methods was much better: the amounts of non-extracted immunoreactive materials were trivial. In urine, the discrepancies are smaller, but the RIA still gave significantly higher results. This study shows that the RIA kit cannot be applied to pharmacokinetic studies in body fluids unless preceded by an extraction of ADR-related compounds. The procedure then becomes time-consuming and, because of its lack of specificity with respect to ADR-OH, its value is not obvious.

To emphasize the errors that could arise in the evaluation of pharmacokinetic parameters, the clearance and half-time of elimination for the sum ADR + ADR-OH have been calculated from the data given by the two methods. The evaluation of the clearance could differ by a factor greater than two according to the method used: more seriously, this factor might vary from one patient to another. So inter-individual variations of pharmacokinetic parameters, that might be correlated with clinical or biological observations such as tumour responses or toxicities, could be completely misleading. This exemplifies the extreme difficulty encountered when radioimmunoassays are applied to the quantification of antitumour compounds in body fluids.

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