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High-performance liquid chromatographic analysis of idarubicin and fluorescent metabolites in biological fluids

Carlo M. Camaggi[1], Patrizia Carisi[1], Elena Strocchi[1], and Franco Pannuti[2]

[1] Department of Organic-Chemistry, University of Bologna, 4, Via Risorgimento, I-40 136 Bologna, Italy
[2] Division Oncology, M. Malpighi, Hospital, 15, Via Albertoni, Bologna, Italy

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Summary. A specific, sensitive, and reliable high-performance liquid chromatographic (HPLC) method for the determination of idarubicin (IDA) and its known fluorescent metabolites idarubicinol (IDAol) and 4-demethoxydaunomycinone (AG1) in biological fluids (human plasma and urine) was developed and tested. Plasma samples were solid-phase-extracted (C18 bonded silica cartridges). Complete separation of unchanged drugs and metabolites was achieved on a Cyanopropyl chromatographic column (25 cm × 4.6 mm inside diameter; particle size, 5 μm) using fluorescence detection (excitation wavelength, 470 nm; emission wavelength, 580 nm). Sensitivity was better than 0.2 ng/ml for all analytes; rates of recovery of unchanged drug and metabolites were better than 84.5% (IDA), 80.3% (IDAol), and 83.9% (AG1). The interassay coefficient of variation was 6.5% for IDA, 5.8% for IDAol, and 9.8% for AG1. Mean intra-assay precision was 4.6% for IDA, 5.9% for IDAol, and 5.0% for AG1 at sample concentrations of above 1 ng/ml and 12.1% for IDA, 10.8% for IDAol, and 14.1% for AG1 at sample concentrations of below 1 ng/ml.

Introduction

Idarubicin (4-demethoxydaunorubicin, IDA) is a new daunorubicin analogue endowed with higher biological activity and lower cardiotoxicity than the parent compound. Antitumor activity has been observed in clinical trials following both intravenous (i.v.) and oral (p.o.) administration [5]. Its main metabolite, idarubicinol (4-demethoxy-13-dihydrodaunorubicin, IDAol) also exhibits antineoplastic activity in experimental models [2]. We studied the comparative pharmacokinetics and metabolism of IDA in plasma samples obtained from 21 cancer patients who had been given 12 mg/m^2 as an i.v. bolus and 30 mg/m^2 orally according to a balanced crossover design. A new analytical procedure was developed that is capable of accurately determining unchanged drugs and known fluorescent metabolites within the concentration range typically observed in biological fluids at up to 168 h after drug administration.

Several high-performance liquid chromatographic (HPLC) assays for IDA have been described in the literature. The organic solvent extraction of plasma samples followed by counterextraction with 0.03 M phosphoric acid [8, 9] leads to the loss of anthracycline aglycones, which are not well back-extracted in the acidic phase. If the back-extraction is avoided [1, 3, 6, 7, 10] the chromatographic column life is shortened. The present technique is based on solid-phase extraction using C18 reversed-phase cartridges followed by HPLC separation and fluorimetric detection. Daghestani et al. [4] have previously described a similar extraction procedure but failed to report any validation data. In the present study, different chromatographic conditions were used and the detection limit was improved by 1 order of magnitude. We report the experimental details and the validation data of such an assay procedure.

Materials and methods

Materials. Compounds used as reference standards and daunorubicin used as the internal standard were obtained from Farmitalia: idarubicin hydrochloride (IDA, batches 5/83, L5/83, and 7005B686); idarubicinol (IDAol, batches PZ 4414/96 and GR 6682/76A); 4-demethoxydaunomycinone (AG1, batch 3/82); and daunorubicin (batches 500IF644 and RR18M001). The reagents were of analytical grade (Farmitalia-Carlo Erba, Milan) and were used without further purification; laboratory-grade distilled water was purified of residual ions and organic impurities using a Milli-Q Water System (Millipore S. A., Molsheim, France) and was filtered through a 0.25 μm membrane filter. All glassware used had to be first deactivated by treatment with 10% dimethyldichlorosilane in anhydrous toluene and then washed with absolute methanol; this process prevents drug complexation and degradation on the active catalytic centers (free SiOH groups) of the glassware.

Apparatus. Chromatographic analysis was performed using a Varian model 5000 liquid chromatograph equipped with a Perkin-Elmer 650-10LC fluorescence detector (excitation wavelength, 470 nm; emission wavelength, 580 nm; slit width: 20 nm) and a Supelcosil LC-CN chro-

matographic column (25 cm × 4.6 mm inside diameter; particle size, 5 μm). Peak area determinations and internal standard calculations were carried out using a Varian Vista 401 data system. Detector linearity was determined by injecting known amounts of IDA, IDAol, and AG1 into the chromatographic system under the operating conditions applied for the analysis. Chromatographic peaks of 1,000 area counts were recorded at signal-to-noise ratios of 5–7.5 and were considered to represent the system's detection limit.

Plasma extraction. For plasma extraction, 10–100 μl internal-standard stock solution (daunorubicin hydrochloride, 1 μg/ml in distilled water), 1 ml 10 mM phosphate buffer (pH 8) supplemented with 0.6 μM tetrabutylammonium bromide, and 1 ml methanol were added to 1 ml plasma samples. The solution was filtered through a 6-cm³ Bondelut C18 Disposable solid-phase column (number 607306; Analytichem International, Harbor City, Mich., USA) that had previously been washed with 3 ml methanol and 3 ml phosphate buffer: methanol (2:1, v/v). The cartridge was eluted first with 4 ml water: methanol (3:1, v/v, discharged) and then with 3 ml 0.03 M phosphoric acid in methanol. After the addition of 100 mcl 0.1 M KH_2PO_4, this solution was evaporated in a Buckler vortex evaporator under vacuum (50–5 torr) at 25° C. The residue (100–400 μl) was analyzed by HPLC (10- to 100-μl injections; the amount injected was chosen so as to maintain the peak area counts in the linear range of 1,000–300,000).

To maximize the sensitivity and linearity of the method, the amount of plasma extracted (and the amount of internal standard added to the solution) was selected as a function of the expected sample concentration. For processing of samples obtained after i. v. administration, 0.5 ml plasma was extracted for samples obtained over 0–15 min; 1 ml, for those obtained between 30 min and 12 h; and 2 ml, for those obtained at 24 h after treatment. After p.o. administration, 2 ml plasma was processed for samples obtained at 24 h after dosing. Validation data are not dependent on the initial plasma volumes.

Chromatographic analysis. Routine analyses were performed using two starting solutions as components of the mobile phase: component A, 78% KH_2PO_4(10 mM +22% CH_3CN; and component B, 30% [KH_2PO_4(10 mM) +H_3PO_4(0.006 M)]+70% CH_3CN. During the analysis, mobile-phase composition was linearly modified from A = 90% and B = 10% to A = 80% and B = 20% in 9 min. Identification of free drugs and metabolites in biological fluids was achieved by comparison of the samples with the authentic specimens obtained from Farmitalia. The retention times noted under various chromatographic conditions (pH and percentage of the organic modifier) and the identity of fluorescence spectra recorded after cessation of the mobile-phase flow during peak elution constituted the comparison criteria.

Quantitative analysis. Calibration of the analytical method was carried out as described above by analyzing blood-bank plasma samples (or urine samples) spiked with known amounts of free drugs, metabolites, and daunomycin hydrochloride as the internal standard. Calibration factors (Cf) were then computed as:

$$Cf_x = c_x \times A_{is}/c_{is} \times A_x,$$

where c_x represents the known concentration of compound x, c_{is} indicates the known concentration of internal standard (daunomycin hydrochloride) and A_x and A_{is} stand for the areas under chromatographic peaks relative to compound x and the internal standard. Each calibration was carried out at least in triplicate.

Quality control. In the application phase, a complete calibration run was performed each time a patient was treated p.o. or i.v. during the cross-over study. Day-to-day quality control was also accomplished by the random submission of blank and spiked plasma samples. The identity of retention times was controlled daily by the addition of known amounts of authentic specimens to the umknown mixture.

Results and discussion

Chromatograms and column efficiency

Plasma and urine samples obtained before treatment were free of interfering fluorescent compounds. Figure 1 illustrates typical chromatograms obtained during routine analysis of plasma samples from patients who had been treated with IDA. The theoretical plate number of the column that was recorded under these experimental conditions was between 7,092 and 16,050.

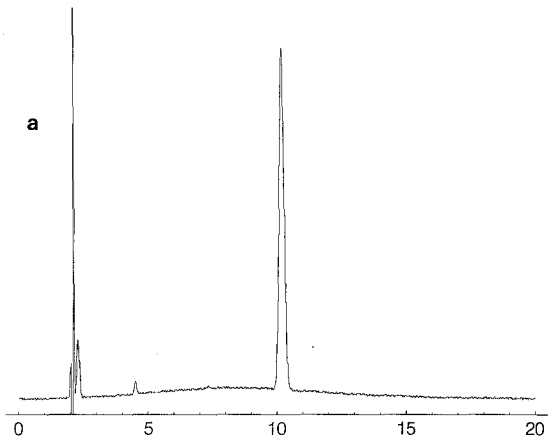
Detector linearity and minimal detectable concentration

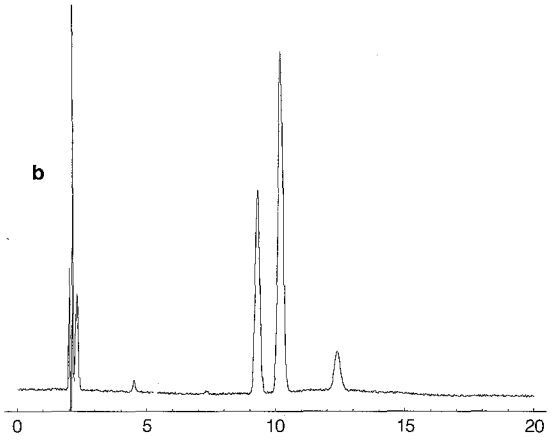
The detector response was found to be at least linear (linear regression, $r > 0.99$) for injections of absolute amounts of IDA ranging from 0.14 (area counts, 989) to 35.3 ng (area counts, 253,685), quantities of IDAol ranging from 0.17 (area counts, 1,668) to 42.3 ng (area counts, 379,676), and levels of AG1 ranging from 0.15 (area count, 1,025) to 38.95 ng (area count, 232,953). The actual amount of sample injected for both calibration and analytical runs was therefore selected so as to obtain peak area counts in the range of 1,000–250,000; chromatographic peaks of 1,000 area counts (corresponding to a signal-to-noise ratio of 5–7.5) were considered to represent the lowest detectable quantity.

Calibration and intra- and interassay precision

Using the described internal-standard procedure, we found a linear correlation between sample concentration, c_x, and $c_{is} \times A_x/A_{is}$, where c_x represents the amount of internal standard added to the sample, A_{is} indicates the area of chromatographic peak relative to the internal standard, and A_x stands for the area of chromatographic peak relative to the compound to be determined. As determined over 165 calibration runs, the resultant data are shown in Figs. 2a, 2b, and 2c for IDA, IDAol, and AG1, respectively. The slope of the regression lines (plotted bilogarithmically to highlight the low-concentration data points) represent the Cf values; the 95% confidence limits for the slope of each regression line are also shown. Point overlap was handled by cellulation; a single, larger circle represents several closely spaced experimental data points.

The overall interassay coefficient of variation (CV) in Cf values (for 45 independent determinations, each computed as the mean of at least 3 chromatographic runs of the same sample) was 6.5% for IDA, 5.8% for IDAol, and 9.8% for AG1. The mean intra-assay precision of the analytical procedure (computed over 32 distinct analytical runs, each carried out at least in triplicate) was 4.6% (CV) for IDA, 5.9% (CV) for IDAol and 5.0% (CV) for AG1 at sample concentrations of above 1 ng/ml and 12.1% for IDA, 10.8% for IDAol, and 14.1% for AG1 at sample concentrations of below 1 ng/ml (Fig. 3).





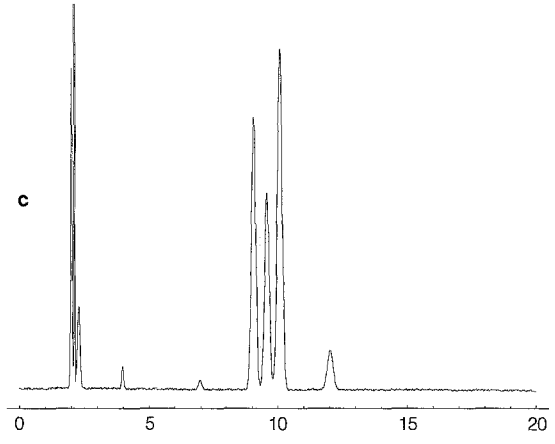
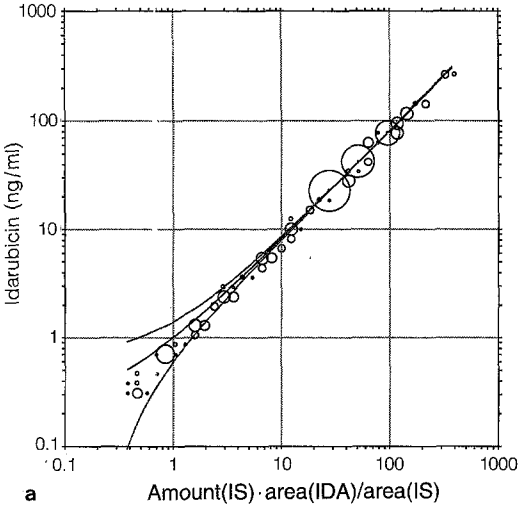
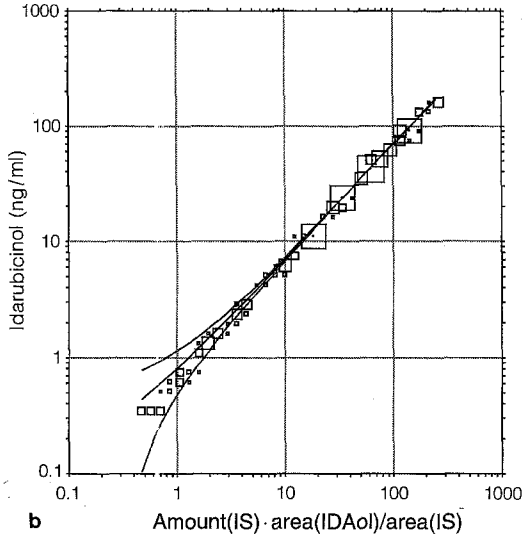


Fig. 1 a. Chromatogram of a plasma sample obtained from patient 82 prior to oral IDA treatment [10.3 min, daunomycin (internal standard, IS)]. **Fig. 1 b.** Chromatogram of a plasma sample obtained from the same patient at 12 h after i.v. IDA treatment [9.3 min, IDAol (11.5 ng/ml); 10.2 min, IS (31.7 ng/ml); 12.4 min, IDA (3.4 ng/ml)]. **Fig. 1 c.** Chromatogram of a plasma sample obtained from this patient at 12 h after oral IDA treatment [9.1 min, IDAol (12.0 ng/ml); 9.6 min, AG1 (15.1 ng/ml); 10.1 min, IS (25.9 ng/ml); 12.1 min, IDA (2.6 ng/ml)]





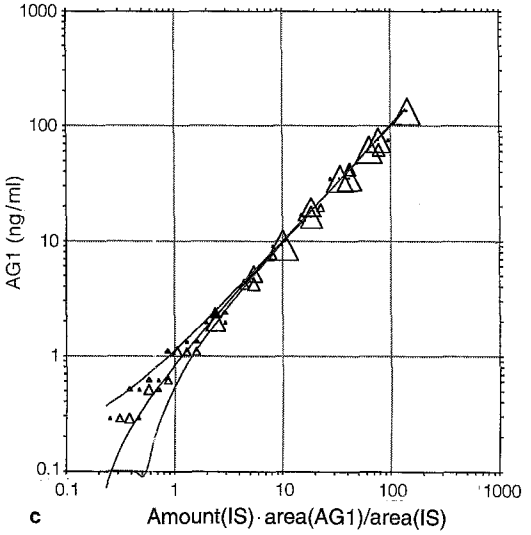


Fig. 2 a. Interassay precision for IDA, demonstrating, linearity over 165 calibration runs. Point overlap was handled by cellulation; the *single, larger circles* represent several closely spaced experimental data points. $y = 0.775+0.206x$, $r^2 = 0.995$. **Fig. 2 b.** Interassay precision for IDAol, showing linearity over 165 calibration runs. $y = 0.66x+0.12$, $r^2 = 0.995$. **Fig. 2 c.** Interassay precision for AG1, indicating linearity over 165 calibration runs. $y = 0.96x-0.15$, $r^2 = 0.995$

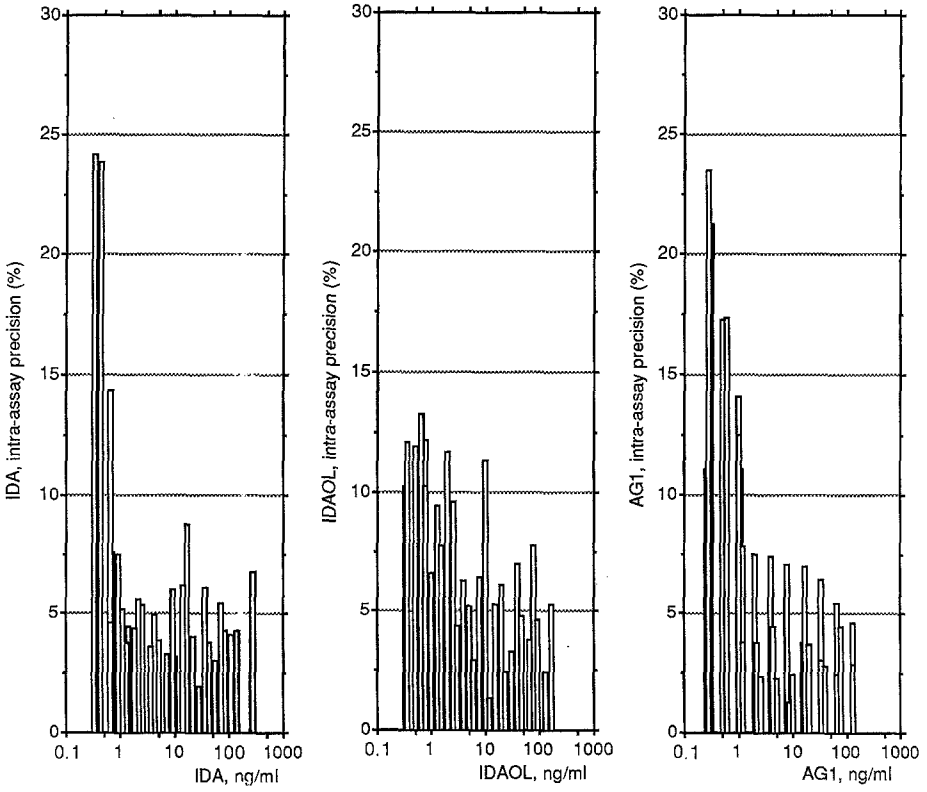


Fig. 3. Intra-assay precision of the analytical procedure as a function of the sample concentration as determined over 32 analytical runs, each carried out at least in triplicate

Recovery

Plasma samples spiked with known amounts of IDA, IDAol, and AG1 were divided into two parts (a and b) and processed as usual. The internal standard daunorubicin was added *prior to* extraction in sample a and *after* extraction in sample b. Recovery was then computed as:

$$\text{Recovery } (\%) = \frac{\text{Concentration in sample b}}{\text{Concentration in sample a}} \times 100.$$

The recovery of unchanged drug and metabolites averaged 91.5% for IDA (range, 84.5%–97.8%), 86.8% for IDAol (range, 80.3%–93.4%), and 91.7% for AG1 (range, 83.9%–99.1%).

Conclusions

The use of solid-phase extraction enables the separation of unchanged drugs and metabolites from plasma samples and their subsequent recovery in high yields. HPLC analysis with fluorimetric detection then allows the determination of IDA, IDAol, and AG1 with the specified selectivity, sensitivity, and precision.

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