Journal of Chromatography, 529 (1990) 359–368
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5307

Sensitive method for the determination of daunorubicin and all its known metabolites in plasma and heart by high-performance liquid chromatography with fluorescence detection

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(First received December 21st, 1989; revised manuscript received March 5th, 1990)

ABSTRACT

The cytostatic agent daunorubicin is effective against leukaemia. An important side-effect is cardiomyopathy, common to all anthracyclines. Since anthracycline metabolites are thought to contribute to the observed cardiotoxicity, a method for the quantitative determination of all metabolites in plasma as well as in tissues is needed as a basis for the further investigation of the correlation between toxicity and the amount of each metabolite formed. Using Sep-Pak C_{18} cartridges we were able to extract daunorubicin and its five metabolites, including the aglycones, with recoveries in the range 50–90%. Depending on the chemical properties of each metabolite, fluorescence detection following high-performance liquid chromatographic separation permitted detection limits as low as 0.2–0.9 n*M* in plasma and $0.8–3 \cdot 10^{-11}$ mol/g in tissue, at a signal-to-noise ratio of 2, which compare favourably with literature data. The method showed linearity in the ranges 1–250 n*M* in plasma and 0.04–4.0 nmol/g in tissue ($r \geq 0.998$). The accuracy, determined at 10 and 100 n*M* for plasma and at 0.1 and 1.0 nmol/g for tissue, was in the range 86–103 and 85–110% for plasma and tissue, respectively. The within-day and between-day repeatability values were acceptable (between 2 and 12%). Because of large inter-compound differences, separate calibration curves were used for each anthracycline. Application of the assay to the analysis of plasma and tissue samples of mice after intravenous injection of daunorubicin proved successful.

INTRODUCTION

In cancer chemotherapy, natural products play a very important role. The bacterial compound daunorubicin (D), discovered in 1957, was the first of a

0378-4347/90/$03.50

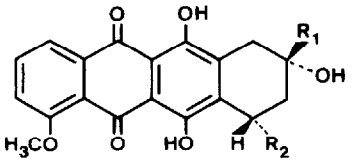
long series of so-called anthracyclines, most of them produced by mutants of the original strain. Nevertheless, D is still frequently used in the treatment of leukaemias. A dose-limitation for all anthracyclines is the cardiotoxicity that develops after repeated treatment [1].

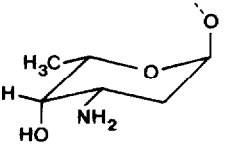
As a result of its less general usage, analysis of D has not been investigated as thoroughly as that of, for example, its analogue doxorubicin. The papers dealing with pharmacokinetics of D [1–5] mention only the 13-hydroxy metabolite daunorubicinol (Dol): hardly any attention is paid to the other metabolites that also appear in urine, namely the aglycones and the corresponding 7-deoxyaglycones (structures are shown in Table I). Although the latter do not possess a significant cytostatic activity, it is far from proved that the same holds for the cardiotoxicity. The aglycones are thought to arise from the semiquinone free radical, which is derived from the parent compound by reduction of the quinone structure present in the anthracycline molecule [2]. In this reduction step oxygen radicals and, subsequently, hydroxyl radicals are also formed, giving rise to lipid peroxidation and presumably cardiotoxicity. In the case of doxorubicin, there are strong indications that the 13-hydroxy metabolite is more cardiotoxic than the parent compound [3]. To determine whether there is a correlation between metabolite levels and cardiotoxicity, a sensitive and quantitative analysis in plasma as well as in tissue is required.

Of course, this makes higher demands upon the methodology than the anal-

TABLE I

STRUCTURES OF DAUNORUBICIN AND METABOLITES



Compound	Abbreviation	R_1	R_2	
Doxorubicin	A	$COCH_2OH$	–	
Daunorubicin	D	$COCH_3$	–	
Daunorubicinol	Dol	$CH(OH)CH_3$	–	
Daunorubicin aglycone	Don	$COCH_3$	OH	
Daunorubicinol aglycone	Dolon	$CH(OH)CH_3$	OH	
7-Deoxydaunorubicin aglycone	7-Don	$COCH_3$	H	
7-Deoxydaunorubicinol aglycone	7d-Dolon	$CH(OH)CH_3$	H	

ysis of only D and Dol, in which case a simple liquid–liquid extraction suffices [4–7]. This paper describes a sensitive method for the analysis of D and all its metabolites in both plasma and heart tissue, based on the analytical procedure reported for the analysis of doxorubicin and 4'-epidoxorubicin [8]. It consists of solid-phase extraction followed by isocratic separation by high-performance liquid chromatography (HPLC) with fluorescence detection.

EXPERIMENTAL

Materials

Daunorubicin and its metabolites, as well as doxorubicin, all analytically pure, were kindly provided by Farmitalia Carlo Erba (Milan, Italy). Acetonitrile, $AgNO_3$, NaH_2PO_4 and H_3PO_4 were obtained from Merck (Amsterdam, The Netherlands) and tetrahydrofuran (THF) and methanol from Baker (Deventer, The Netherlands). All reagents were of analytical grade, except THF, which was HPLC grade.

Stock solutions of D and its metabolites were prepared in methanol at 10^{-4} *M* and combined to yield an equimolar mixture of the compounds. From this mixture further dilutions in methanol were prepared, ranging from $5 \cdot 10^{-9}$ to $2.5 \cdot 10^{-6}$ *M*. All solutions were kept in polypropylene tubes and stored at −20°C.

C_{18} Sep-Pak cartridges (Waters-Millipore, Etten-Leur, The Netherlands) were equilibrated before use with 5 ml of methanol and 5 ml of water, according to the manufacturer's instructions.

Heparinized plasma was obtained from healthy volunteers; bovine heart served as blank cardiac tissue.

Sample preparation

Cardiac tissue was cut into pieces of ca. 10 mg, weighed, and subsequently homogenized by dismembration (1 min, 77 K) using a Mikrodismembrator II (Braun, Melsungen, F.R.G.). Immediately thereafter the tissue was suspended in a glucaric acid-1,4-lactone–glucose solution (3.0 g and 0.5 g per l distilled water, respectively, to prevent hydrolysis of D-glucuronides, should they be present [8]) to obtain a concentration of 100 mg of tissue (wet weight) per ml. Aliquots of 2 ml of homogenate were stored at −20°C.

Calibration samples were prepared in polypropylene tubes by evaporating (50°C, nitrogen) the solvent from 100 µl of the appropriate dilution of the mixture of D and metabolites, followed by the addition of 50 µl of internal standard dilution ($5 \cdot 10^{-7}$ *M*) and either 250 µl of homogenate (25 mg wet weight) or 1 ml of heparinized plasma, for the tissue and plasma analysis, respectively. In the case of plasma the mixture was vortexed briefly and applied to a C_{18} Sep-Pak cartridge, additionally pre-equilibrated with 5 ml of 0.02 *M* NaH_2PO_4, (pH 4)–acetonitrile (9:1, v/v) (solution A). The cartridge was

washed with 2 ml of solution A, dried with compressed air and eluted with 4 ml of methanol–THF (3:1, v/v) to desorb the anthracyclines. The effluent was evaporated to dryness (nitrogen, 50°C), and the residue was redissolved in 200 μl of mobile phase. For tissue, the spiked sample was mixed for 30 min, to allow the compounds to distribute over the binding sites. Next, 100 μl of 3 *M* $AgNO_3$, were added, again followed by mixing (10 min) (in order to release anthracyclines from biopolymer binding). Subsequently, the anthracyclines were extracted by shaking with 1 ml of THF–water (1:2, v/v) for 5 min. The extract was centrifuged (1500 *g*, 5 min), and the supernatant was applied to a C_{18} Sep-Pak cartridge. The cartridge was rinsed with 2 ml of water, dried with compressed air and eluted with 4 ml of methanol–THF (3:1). The pellet was extracted a second time using the same conditions and the same Sep-Pak cartridge, and the combined methanol–THF solutions were evaporated to dryness. The residue was processed as described above.

The solutions were transferred to polypropylene WISP vial inserts, which were centrifuged at 10 000 *g* (Eppendorf centrifuge) to prevent clogging of the analytical column by solid particles. An injection volume of 60 μl was used throughout.

Plasma and heart from a mouse injected intravenously with D (10 mg/kg) were collected and processed as previously described [8]. Samples were treated as described above, except that only internal standard was added.

Chromatography

Separation took place in a Waters-Millipore HPLC system, consisting of a WISP 710B injection system, a Model 6000A solvent-delivery system and a data module with system controller. A 20 (2×10)cm×4.6 mm I.D. 3 μm Microspher C_{18} analytical column (Chrompack, Middelburg, The Netherlands) was used, together with a 10 mm×2.0 mm I.D. Chromsep (Chrompack) guard column, slurry-packed with 5-μm Spherisorb ODS-2 material (Chrompack). A Merck-Hitachi (Amsterdam, The Netherlands) F1000 fluorescence detector, operated at 480 nm excitation and 580 nm emission wavelength, was used for peak monitoring. The mobile phase, 0.02 *M* NaH_2PO_4 (pH 4)–acetonitrile (1.35:1, v/v) filtered before use, was pumped at a flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

After comparison of the solid-phase clean-up procedure with Sep-Pak C_{18} cartridges with several liquid–liquid extraction procedures, the former was chosen because of a better general performance, considering the broad range of polarities of the anthracycline metabolites. The retention volume of the anthracyclines (when loading plasma or tissue extract) on a C_{18} Sep-Pak proved to be large enough to enable loading of 1-ml samples. An additional flushing step with 2 ml of water is sufficient to remove the majority of potentially in-

terfering substances present in the sample, while the anthracyclines are retained on the solid phase. (In the case of plasma samples, an acidic solution is used for equilibrating as well as washing, because breakthrough was seen at physiological pH values.) Subsequently, the compounds of interest can be desorbed by eluting the cartridge with THF–methanol (1:3). Elution with 4 ml turns out to be optimal, providing both sufficient desorption and a reasonably rapid evaporation step. THF was chosen over, for instance, chloroform, which is more effective in desorbing D and Dol, but the 7d-aglycones tend to be retained on the Sep-Pak. Increasing the strength of the organic solvent results in the desorption of too high an amount of lipid components, disturbing the chromatography. The present conditions should be seen as a compromise. Table II shows that the recovery obtained for the anthracyclines under investigation was in the range 50–90% for plasma and 50–65% for tissue. Although recoveries of D and Dol are lower than those mentioned in some other papers [4–7], in those cases the aglycones were not analysed. A paper by Andrews et al. [9], describing the analysis of D and all its metabolites, did not mention recovery values. However, it can also be seen that the lower recovery is not accompanied by a larger standard deviation (Table II).

The efficiency of the clean-up procedure is illustrated by the chromatogram of a blank human plasma extract depicted in Fig. 1b. It clearly shows the absence of interfering substances. In Fig. 1a a chromatogram of an extract of spiked plasma is presented to prove the quality of the separation, which compares favourably with ref. 9. All the components show baseline separation.

As to the separation of metabolites using HPLC, it should be mentioned that the resolution of the peak pair 7d-Dolon and Don was critically dependent on the column performance. Therefore, 3-μm column material was used, and two

TABLE II

RECOVERIES OF DAUNORUBICIN AND ITS METABOLITES FROM PLASMA AND HEART TISSUE AT TWO CONCENTRATION LEVELS

Compound[a]	Recovery (mean ± S.D., $n=6$) (%)			
	10 n*M*	100 n*M*	0.2 nmol/g	4.0 nmol/g
A (I.S.)		66 ± 4[b]		52 ± 3[b]
Dolon	88 ± 2	90 ± 2	65 ± 3	65 ± 3
Dol	50 ± 2	51 ± 2	58 ± 5	58 ± 2
7-Dolon	80 ± 3	70 ± 1	62 ± 3	59 ± 4
Don	77 ± 3	75 ± 2	54 ± 7	58 ± 3
D	53 ± 2	48 ± 2	55 ± 5	57 ± 3
7d-Don	71 ± 1	67 ± 3	51 ± 3	51 ± 3

[a]For abbreviations, see Table I.
[b]Plasma 25 n*M*, tissue 1 nmol/g.

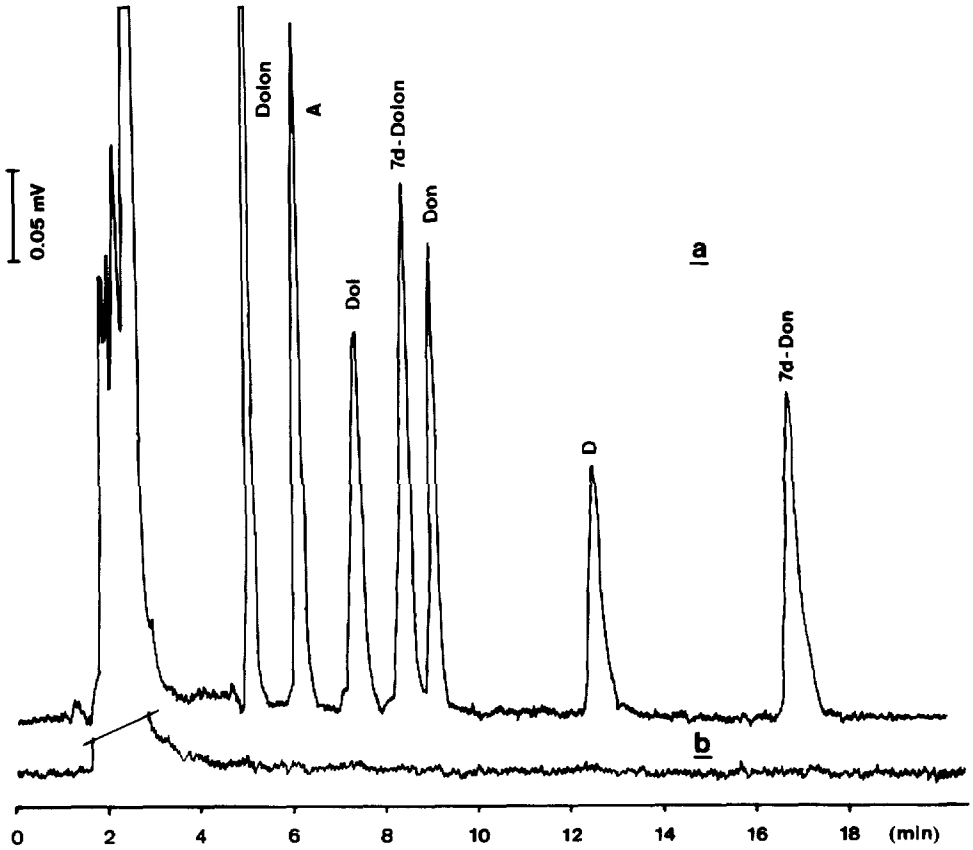


Fig. 1. Chromatograms of extracts of human plasma: (a) spiked with anthracyclines at 10 n*M* [internal standard (A) = 25 n*M*] and (b) blank. Chromatographic conditions: analytical column, 20 cm × 4.6 mm I.D., 3-μm Microspher C_{18}; guard column, 10 mm × 2.0 mm I.D. 5-μm Spherisorb ODS-2; mobile phase, 0.02 *M* NaH_2PO_4 (pH 4)–acetonitrile (1.35:1 v/v); flow-rate, 0.8 ml/min. Detection: fluorescence (480 nm excitation and 580 nm emission). For peak identification, see Table I.

10-cm cartridges were combined to provide the necessary plate number. Consequently, column back-pressure was increased and, to allow unattended operation, the flow-rate was set at 0.8 ml/min. The total analysis time was limited to 17 min by using a simple isocratic elution.

The application of the described method to pharmacokinetic studies is demonstrated by Fig. 2, which shows the chromatograms of plasma (A) and cardiac tissue (B) samples obtained 1 h after injection of D (10 mg/kg). Notice that the 7d-Don metabolite is not present in the samples, and that in heart tissue an unidentified component elutes a few minutes later. Both the plasma and tissue blanks show the absence of interfering peaks.

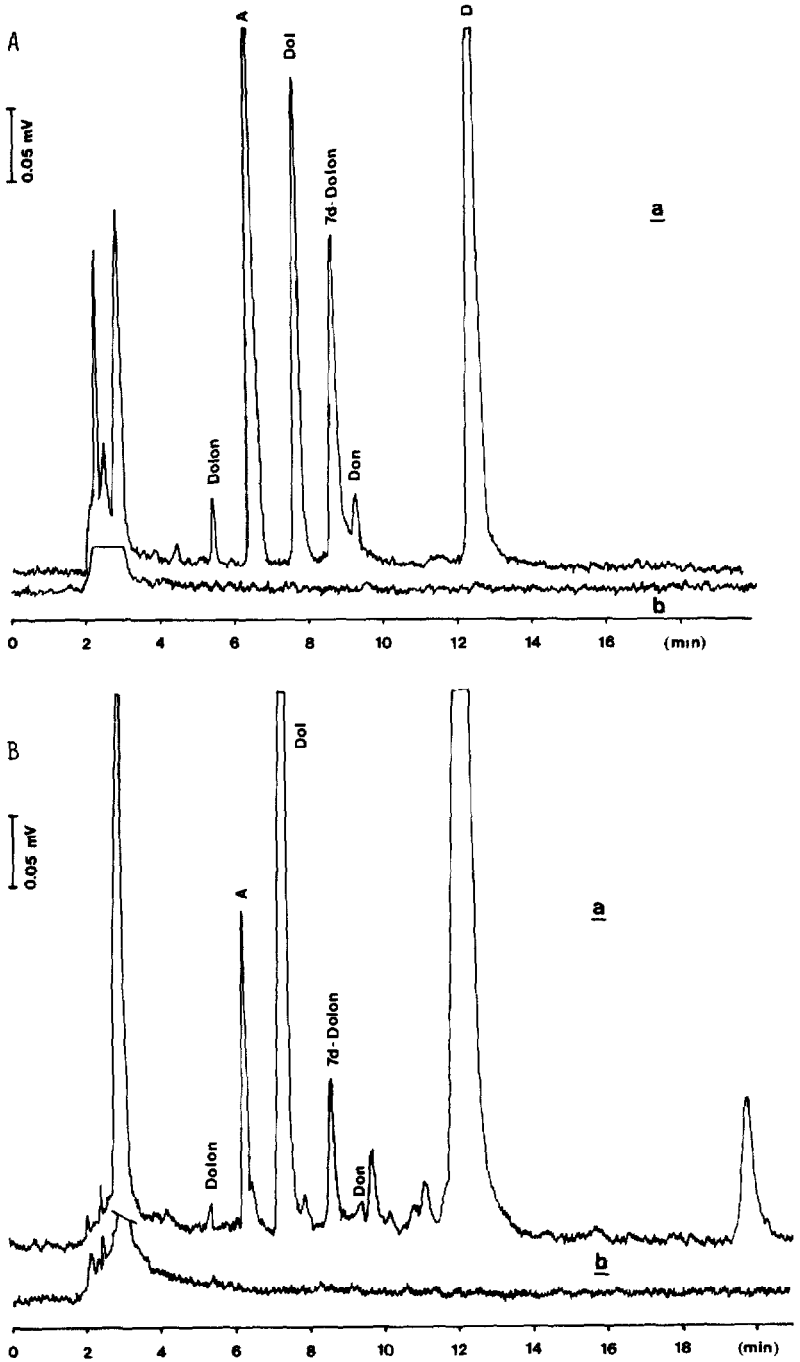


Fig. 2. Chromatograms of extracts of (A) diluted mouse plasma and (B) mouse heart: (a) 60 min after injection with 10 mg D per kg and (b) corresponding blanks. Concentration of A (internal standard) = 25 n*M* in A and 1 nmol/g wet weight in B. For chromatographic conditions, see Fig. 1; for peak identification, see Table I.

The efficient clean-up procedure and the selective and sensitive fluorescence monitoring mean that D and its metabolites can be detected at concentrations in the range 0.2–0.9 n*M* in plasma and 10–30 pmol/g wet weight in cardiac tissue (depending on the metabolite). These values are lower than those previously reported.

Calibration curves are obtained by plotting the ratio of the peak height of each compound versus the peak height of the internal standard against the concentration. Fig. 3 illustrates the wide range of slopes of the calibration curves for the individual compounds. These differences are a consequence of variations in fluorescence quantum yields, recoveries and peak shapes. In both plasma and tissue, calibration curves are linear ($r \geq 0.998$) over the concentration range tested (1–250 n*M* in plasma and 0.04–4.0 nmol/g in tissue). Table III lists within-day and between-day coefficients of variations (C.V.) of the slopes of the calibration lines, and it can be concluded that within-day and between-day repeatability for plasma are comparable (5.9–9.1 and 2.8–6.7%, respectively), whereas for tissue within-day values are somewhat better than between-day values (4.0–9.5 versus 6.3–12.2%). Therefore, calibration should always be performed daily. Accuracy values, determined using daily calibration, are reported in Table IV. It can be seen that the differences between observed and actual plasma concentrations are between 0 and 14% for the low spiking level and between 0 and 8% for the high level (in case of tissue these

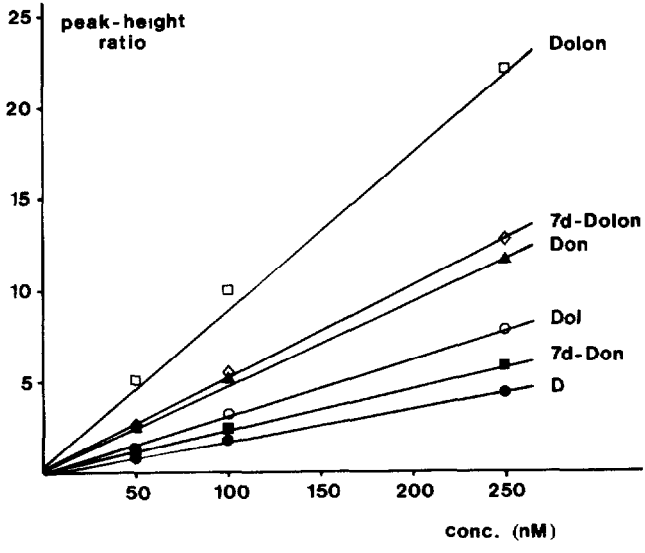


Fig. 3. Calibration curves (mean of within-day values, $n=2$) of D and its metabolites in human plasma. Values on *y*-axis are ratios of peak heights obtained by comparison with the internal standard A (25 n*M*). For abbreviations, see Table I.

TABLE III

WITHIN-DAY AND BETWEEN-DAY COEFFICIENTS OF VARIATION OF THE SLOPES OF CALIBRATION LINES

Compound[a]	Coefficient of variation (%)			
	Within-day ($n=6$)		Between-day ($n=6$)	
	Plasma	Tissue	Plasma	Tissue
Dolon	6.9	6.2	6.7	6.3
Dol	6.0	7.4	2.6	8.9
7d-Dolon	7.8	7.0	5.3	9.1
Don	7.9	4.0	5.5	8.3
D	5.9	9.5	2.8	10.8
7d-Don	9.1	7.9	6.1	12.2

[a]For abbreviations see, Table I.

TABLE IV

ACCURACY OF THE ASSAY FOR PLASMA AND TISSUE SAMPLES

Compound[a]	Percentage of spiked concentration			
	Plasma		Tissue	
	10 n*M*	100 n*M*	0.1 nmol/g wet weight	1.0 nmol/g wet weight
Dolon	94	103	102	91
Dol	86	99	95	100
7d-Dolon	100	102	95	89
Don	90	100	106	89
D	94	92	103	102
7d-Don	90	99	105	84

[a]For abbreviations, see Table I.

values are 2–6% and 0–16%). Using the same samples, within-day and between-day repeatability values were determined (Table V). These are acceptable, especially considering the rather extensive pretreatment of tissue samples.

It can be concluded that the method we have developed for the determination of daunorubicin and its metabolites in plasma and cardiac tissue offers an improvement over existing methods, especially because of its ability to determine all the metabolites with high sensitivity.

TABLE V

WITHIN-DAY AND BETWEEN-DAY COEFFICIENTS OF VARIATION FOR PLASMA AND TISSUE SAMPLES AT TWO CONCENTRATION LEVELS

Compound[a]	Coefficient of variation (%)							
	Within-day ($n=6$)				Between-day ($n=6$)			
	Plasma		Tissue		Plasma		Tissue	
	10 n*M*	100 n*M*	0.1 nmol/g	1.0 nmol/g	10 n*M*	100 n*M*	0.1 nmol/g	1.0 nmol/g
Dolon	4	5	9	9	7	6	7	8
Dol	5	3	3[b]	11	7	3	6	9
7d-Dolon	6	7	8	7	4	7	9	7
Don	4	5	11	6	6	7	9	7
D	3	2	5[b]	11	5	3	5	7
7d-Don	5	6	8	12	12	8	10	9

[a]For abbreviations, see Table I.
[b]After deletion of one outlier (outside mean ±2 S.D.).

ACKNOWLEDGEMENT

This study was supported by a grant from Farmitalia Carlo Erba (Milan, Italy).

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