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Development and validation of a chiral high-performance liquid chromatography assay for rogletimide and rogletimide-*N*-oxide isomers in plasma

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Abstract The purpose of the present study was to develop and validate a stereo-specific high-performance liquid chromatography (HPLC) assay for rogletimide (Rog) and rogletimide-*N*-oxide (Nox) isomers in plasma. The assay was performed with a chiral cellulose-[4-methylbenzoate]ester column (Chiracel OJ). Optimal separation was achieved isocratically with a mobile phase consisting of *n*-hexane/anhydrous ethanol (65/35, v/v) at a flow rate of 0.9 ml/min, with the column being thermostated at +35°C (UV detection at 257 nm). Under these conditions, retention times were approximately 17, 28, 31 and 76 min for R-Rog, S-Rog, R-Nox and S-Nox, respectively. S-aminoglutethimide (S-Ag) served as the internal standard (retention time 70 min). An extraction procedure from plasma samples was developed on Bond Elut RP8 500-mg cartridges; conditioning was performed with 5 ml methanol and 5 ml water, after which 1 ml plasma that had previously been spiked with 5 μ*M* S-Ag was applied. Washing was done with 6 ml water and elution, with 4 ml methanol. After evaporation to dryness, residues were dissolved in 400 μl anhydrous ethanol and 12–48 μl was injected onto the HPLC system. Blank plasma from healthy donors showed the random presence of a small interference eluting at the retention time of R-Rog, precluding the accurate quantification of R-Rog concentrations below 2.5 μ*M*. Reproducibility assays demonstrated the need to use an internal standard. Taking into account the internal standard, at 2.5 μ*M* the intra- and inter-assay coefficients of variation were 10.5% and 21.0% for R-Rog, 5.5% and 8.7% for S-Rog, 7.6% and 20.8% for R-Nox and 11.7% and 6.4% for S-Nox, respectively. The detection limit was 2.5 μ*M* for R-Rog, 0.5 μ*M* for S-Rog, 0.25 μ*M* for R-Nox and 0.5 μ*M* for S-Nox. Linearity was satisfactory at concentrations ranging from 2.5 to 10 μ*M* for R-Rog, from 0.5 to 10 μ*M* for S-Rog, from 0.25 to 2.5 μ*M* for R-Nox and from 0.50 to 2.5 μ*M* for S-Nox. This assay was applied to plasma obtained from rogletimide-treated breast cancer patients receiving conventional oral doses and demonstrated its feasibility with regard to sensitivity. The preliminary pharmacokinetic results reported herein suggest for the first time that both the R-Rog and S-Rog isomers are metabolized into rogletimide-*N*-oxide.

Keywords Rogletimide · Aromatase inhibitors · Stereoisomers · Pharmacokinetics · Chiral high-performance liquid chromatography

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Introduction

Hormonal manipulation constitutes an effective treatment modality for breast cancer. Aromatase inhibition is one of the current approaches for treating hormone-dependent breast cancer in post-menopausal patients [10]. Aminoglutethimide (Ag) represents the leading compound of the aromatase-inhibitor family [5]. This drug inhibits P450 enzymes by interacting with the heme iron center and may therefore inhibit Cyt P450 enzymes other than aromatase, such as those involved in production of aldosterone and cortisol [4]. Analogs of Ag have been developed and among them, rogletimide (Rog), (Fig. 1) appears less toxic in animal models and more specific than Ag [14]. Hormonal treatment by Rog has reportedly shown promising activity in the clinical setting [6]. Like Ag, Rog exhibits one assymetric carbon and is used as the racemic mixture (50/50 for the R and S isomers, respectively). It has been established that the R isomer possesses approximately 20

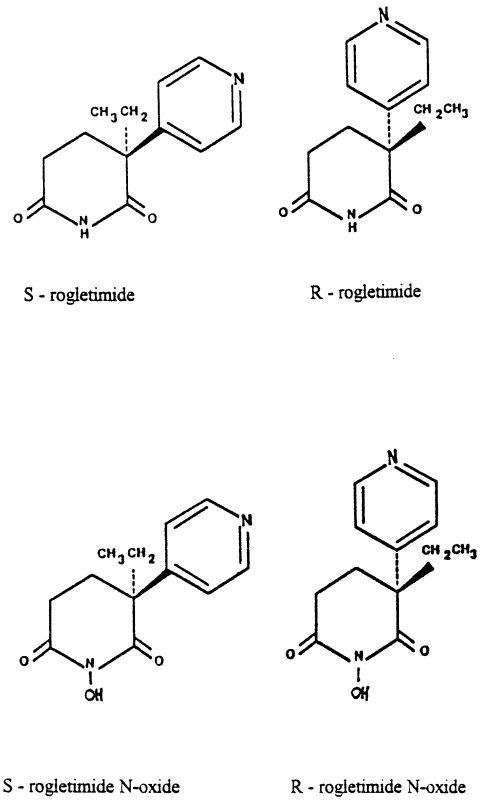

Fig. 1 Structure of rogletimide and rogletimide-*N*-oxide isomers

times more aromatase-inhibitory activity than the S isomer. Thus far, non-stereo-specific pharmacokinetics studies have been performed in humans using a reverse-phase high-performance liquid-chromatography (HPLC) assay with previous solid-phase extraction [11]. These studies have shown dose-dependent pharmacokinetics for Rog, with the plasma half-life of the parent compound increasing with increasing single doses of Rog. Rogletimide-*N*-oxide (Nox; Fig. 1) has been shown to be the major circulating metabolite and it is suggested that Rog induces its own metabolism [7, 11]. Recently, a successful separation of pure R and S isomers of Rog has been performed on a chiral HPLC column [1]. However, a stereo-specific pharmacokinetics study of Rog has previously never been performed.

The aim of the present study was to develop and validate a chiral HPLC assay for Rog isomers and Nox isomers in plasma and to apply this assay to plasma from treated patients.

Materials and methods

Chemicals

Rog racemate (100% purity) was obtained from Ganes Chemicals Inc. (Carlstadt, N.J., USA). Nox racemate was provided by Dr. B.P. Haynes (Cancer Research Campaign Laboratory, Sutton, UK). Ag racemate was provided by Sigma (St. Quentin Fallavier, France). R-Rog (purity $>98.5\%$), S-Rog (purity $>98.5\%$), R-Nox (purity 99%) and S-Nox (purity 98.7%) were provided by USB Pharma (Dr. Stedman, Watford, UK). R-Ag and S-Ag were obtained from Chiros. Pure solutions (10^{-3} *M*) of each compound were prepared in anhydrous ethanol and stored at $-20°C$ as aliquots.

HPLC solvents were of the highest purity available; *n*-hexane (reference 9304) and isopropanol (reference 8175) were obtained from J.T. Baker (Deventer, Holland). Anhydrous ethanol (reference 412522) was provided by Carlo Erba (Milano, Italy). Double-distilled water was obtained from Laboratoire Aguettant (Lyon, France), methanol (HPLC quality) and diethylether (normapur quality) were supplied by Prolabo (Paris, France), and acetonitrile (proanalysis quality) and dichloromethane (proanalysis quality) were obtained from Merck (Darmstadt, Germany). All other chemicals were provided by Sigma.

HPLC system

The HPLC system consisted of two pumps (Millipore Waters model 510) connected to an automated gradient controller (Millipore Waters model 680), an automated temperature-regulated injector (Millipore Waters model 717) fixed at 6°C, a column heater (Fiatron model TC-50), a UV detector (Millipore Waters model 484) and an integrator (Hewlett Packard model 3390 A). Integration parameters included a chart speed of 0.1 cm/min and a peak width of 0.64 min. Calculations were done according to peak height.

The HPLC column (chiracel OJ; cellulose tris-[4 methylbenzoate]ester, 10 μm, 250×4.6 mm) and precolumn (chiracel OJ, 50×4.6 mm) were obtained from Daicel (Tokyo, Japan). Since this chiracel column had special requirements (non-compatibility with water, acetonitrile and methanol), the whole HPLC system was flushed with isopropanol before connection of the column, and particular attention was paid to the column conditioning. The column was stored under 100% *n*-hexane and each mobile phase tested was progressively applied by performance of a linear gradient from 100% *n*-hexane to 100% mobile phase; the duration of the gradient was approximately 30 min for a 5% change in the mobile phase composition. In addition, the flow rate was also progressively modified using a linear gradient programme (1 min for a 0.1-ml/min flow rate). Also, the temperature of the column was applied in two steps (30°C for 30 min and then 35°C). All injected samples were in anhydrous ethanol (injected volumes between 12 and 60 μl). Under these conditions the stability of the column performance was very satisfactory (up to 200 injections/column).

Development of the assay

Detection

UV spectra of RS-Rog and RS-Nox were obtained from 180 to 340 nm (10^{-4} *M* solutions in isopropanol and/or *n*-hexane). UV spectra were not influenced by the solvent used. Values of λ max were 257 and 274 nm for RS-Rog and RS-Nox, respectively. At the same molarity (10^{-4} *M*) the maximal absorbance recorded for RS-Nox at 274 nm was 6 times that recorded for RS-Rog at 257 nm (10^{-4} *M*). Since the limitation in UV sensitivity appeared to be linked to RS-Rog, we chose to work at the RS-Rog λ max, i.e at 257 nm.

Optimisation of the HPLC separation

We first applied the HPLC conditions described by Aboul-Enein et al. [1] for the separation of R-Rog and S-Rog and then improved

them so as to separate Rog isomers from Nox isomers. In a last step we introduced Ag isomers so as to test one of them as an internal standard. We first tested a hexane/isopropanol mobile phase with the proportion of isopropanol ranging from 35% to 98%. With 35% isopropanol (0.7 ml/min, column thermostated at 40°C) a satisfactory resolution was achieved but retention times were too long (total run time around 120 min). A mobile phase consisting of hexane/ethanol was then studied with the proportion of ethanol varying from 20% to 35%. A satisfactory compromise (peak separation/duration of the analysis) was obtained with 35% ethanol. The importance of the column temperature was also checked (from 25° to 40°C) and the optimal temperature was set at 35°C. The last step was to test Ag as a possible internal standard that would be compatible with this HPLC condition since the Ag structure is close to that of Rog and Ag also absorbs at 257 nm. The injection of RS-Rog, RS-Nox and RS-Ag in mixture revealed that R-Nox and R-Ag co-eluted; we thus chose pure S-Ag as the potential internal standard.

The optimal HPLC conditions for separation of R-Rog, S-Rog, R-Nox, S-Nox and S-Ag (IS) were thus as follows: precolumn (non-thermostated) plus column Chiracel OJ thermostated at 35°C, mobile phase hexane/ethanol (65/35, v/v) run at 0.9 ml/min, detection at 257 nm. Under these conditions the column pressure was located between 370 and 400 psi and the total run time was 90 min. The chromatography characteristics are presented in Table 1 and show satisfactory resolution factors (Rs > 1.35) between the different compounds.

Optimisation of the extraction procedure from plasma samples

We compared several liquid-solid and liquid-liquid extraction procedures previously described for Rog[11] and Ag [2, 15–17] and then modified them so as to improve the specificity and recovery. As concerns liquid-liquid extractions, we tested dichloromethane and diethylether/dichloromethane (2/1). None of these organic phases allowed Nox to be quantitatively extracted. Solid-phase extractions were performed on a Vac Elut SPS 24 station connected to a vacuum pump. Extraction cartridges were not allowed to dry out before application of the plasma samples or before washing steps. All steps were performed at a low elution speed to improve the liquid-solid interactions.

Following the procedures of previous investigators [11], we first compared RP8 cartridges (Varian reference 1210–2002) and RP18 cartridges (Varian reference 1210–2028). Elution was achieved with

Table 1 Characteristics of the HPLC assay used in the present study

		R-Rog	S-Rog	R-Nox	S-Nox	IS
Chromatographic characteristics[a]:						
Retention time (min)		16–17	28–29	31–32	76–80	70–72
Retention factor (K)		4.0	7.4	8.7	22.7	20.0
Detection limit[b]		2.5 μ*M*	0.5 μ*M*	0.25 μ*M*	0.5 μ*M*	–
Sensitivity limit[c]		0.10 μ*M*	0.15 μ*M*	0.05 μ*M*	0.10 μ*M*	–
Linearity:						
Range		2.5–10 μ*M*	0.5–10 μ*M*	0.25–2.5 μ*M*	0.5–2.5 μ*M*	–
Number of calibration curve points		4	6	5	4	–
Coefficient of correlation without IS[d]		0.9860	0.9997	0.9886	0.9903	–
Coefficient of corelation with IS[e]		0.9895	0.9990	0.9872	0.9882	–
Recovery (%)[f]:						
At 2.5 μ*M*		74 ± 11	64 ± 5	44 ± 4	34 ± 6	–
At 5 μ*M*		71 ± 10	69 ± 13	28 ± 7	22 ± 5	81 ± 5
Intra-assay reproducibility:						
Spiked plasma 2.5 μ*M*	without IS[g]	17.2%	7.3%	9.9%	16.4%	8.6%
	with IS[h]	10.5%	5.5%	7.6%	11.7%	–
Spiked plasma 5 μ*M*	without IS[g]	14.4%	13.7%	24.5	22.6%	10.0%
	with IS[h]	4.8%	7.6%	12.4%	11.9%	–
Inter-assay reproducibility:						
Spiked plasma 2.5 μ*M*	without IS[g]	37.3%	35.0%	30.9%	26.4%	37.2%
	with IS[h]	21.0%	8.7%	20.6%	6.4%	–
Spiked plasma 5 μ*M*	without IS[g]	31.2%	36.4%	34.4%	38.2%	35.4%
	with IS[h]	10.5%	12.3%	22.2%	18.5%	–

IS, internal standard

[a]$K = (Rt–Rto)/Rto$, where Rt is the retention time of the peak of interest and Rto is the retention time of the dead volume. Resolution factor $Rs = 2(Rt2\text{-}Rt1)/(W2 + W1)$, where $Rt2$ and $Rt1$ are the retention times of the second and first peaks, respectively, and $W2$ and $W1$ are the peak widths of the second and first peaks, respectively. Rs values were 5.33 between R-Rog and S-Rog, 1.38 between S-Rog and R-Nox, 7.90 between R-Nox and IS and 1.36 between IS and S-Nox

[b]The detection limit was defined as the lowest plasma concentration detectable, i.e. corresponding to 3 times the bacground signal in optimal conditions

[c]The sensitivity limit was defined as the lowest variation in concentation giving a significant variation in the signal

[d]Correlation coefficient of the linear regression of the concentration as a function of the peak height, including the blank plasma. Mean coefficients of 2 calibration curves are presented

[e]Correlation coefficient of the linear regression of the concentration as a function of the peak height/IS peak-height ratio, including the blank plasma. Mean coefficients of 2 calibration curves are presented

[f]Mean values ± SD calculated on 2 series of 8 samples

[g]Coefficient of variation calculated on crude peak heights

[h]Coefficient of variation calculated on peak heights divided by the IS peak height; 8 and 7 samples were tested for intra- and inter-assay reproducibility, respectively

pure methanol or pure acetonitrile. Nox isomers were found to be quantitatively extracted on RP8 cartidges only. We then optimised the applied volume of plasma (0.5–1 ml), the rinsing volume (between 1 and 2 ml water) and the elution volume (1–2 ml methanol) on RP8 100-mg cartidges. The best recoveries were obtained with the higher cartridge-volume/plasma-volume ratio. Thus, RP8 500-mg cartridges (Varian references 1210–2053) were preferred. The optimal extraction procedure was thus performed on Bond Elut RP8 500-mg cartridges as follows: plasma samples were spiked with S-Ag as the internal standard at a 5 μ*M* final concentration; conditioning was done with 5 ml methanol and 5 ml water; 1 ml plasma was applied and washing was performed with 6 ml water; and 4 ml methanol was used for the elution. The eluates were then evaporated to dryness at 37°C under a stream of nitrogen and the dry residues were dissolved in 400 μl absolute ethanol and centrifuged at 4°C for 10 min (2500 rpm). Samples were thus concentrated by a factor of 2.5 as compared with the initial plasma concentration. A volume of 12–48 μl was injected onto the HPLC system.

Results

Specificity of the assay

Figure 2 shows the chromatography profiles obtained for pure standards, blank plasma, spiked plasma and plasma from Rog-treated patients. Retention times for R-Rog, S-Rog, R-Nox and S-Nox were 16.5, 28, 30.9, 76.3 min, respectively. The internal standard (IS) eluted at 70.3 min. The total run time was 90 min. Blank-plasma HPLC profiles did not show any interference peak with S-Rog, R-Nox, S-Nox or IS. However, a random minor interference peak with R-Rog was observed in some cases. The mean height of this interference peak comprised between 2 and 5 times the background noise.

Detection limit and sensitivity of the assay

The limit of detection was defined as the lowest plasma concentration detected, i.e. corresponding to 3 times the background signal in optimal conditions. Detection limits were thus 0.5, 0.25 and 0.5 μ*M* for S-Rog, R-Nox and S-Nox, respectively. For R-Rog we considered 2.5 μ*M* to be the lowest concentration accurately detectable due to the random presence of the small interference at the R-Rog retention time. The sensitivity of the assay was defined as the lowest variation in concentration giving a significant variation in the signal. The sensitivity was thus 0.10, 0.15, 0.05 and 0.10 μ*M* for R-Rog, S-Rog, R-Nox and S-Nox, respectively.

Linearity of the assay

Calibration curves were tested from a 0.10 μ*M* up to a 25 μ*M* (0.10, 0.25, 0.50, 1, 2.5, 5, 10 and 25 μ*M*) final concentration of R-Rog, S-Rog, R-Nox and S-Nox. Figure 3 illustrates the plot of measured concentrations (mean values ± SD) versus theoretical concentrations for each compound tested. These curves show a saturation in the extraction occurring above 10 μ*M* for the R-Rog and S-Rog isomers and above 5 μ*M* for the R-Nox and S-Nox isomers. On these bases the ranges of linearity were 2.5–10 μ*M* for R-Rog, 0.5–10 μ*M* for S-Rog, 0.25–2.5 μ*M* for R-Nox and 0.5–2.5 μ*M* for S-Nox. The coefficients of correlation within these ranges were very satisfactory (Table 1).

Reproducibility

Table 1 shows that the correction by the IS markedly decreased the variability of the intra- and inter-assay variability, suggesting that much of the assay variability was due to the extraction process. With the use of the IS, the intra-assay reproducibility was very satisfactory [coefficient of variation (CV) below 12%]. The inter-assay reproducibility was performed on seven in-

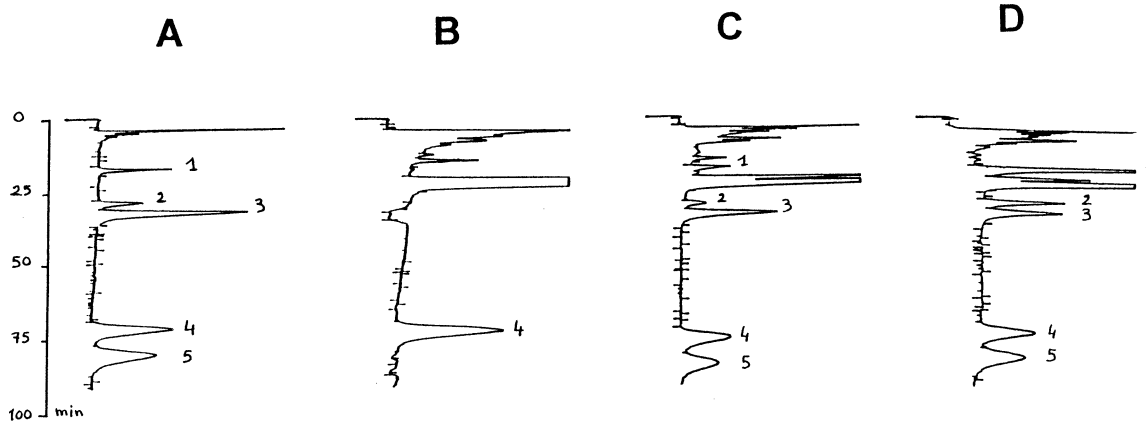

Fig. 2A–D Examples of chromatography profiles. **A** injection of 30 μl pure RS-Rog (10 μ*M* of the racemate), RS-Nox (10 μ*M* of the racemate) and internal standard (10 μ*M*). **B** Injection of 48 μl blank plasma. **C** Injection of 24 μl spiked plasma containing 2.5 μ*M* of each isomer. **D** Injection of 24 μl plasma obtained from a Rog-treated patient (patient B, 10 h). *Peaks 1, 2, 3, 4 and 5* correspond to R-Rog, S-Rog, R-Nox, IS and S-Nox, respectively

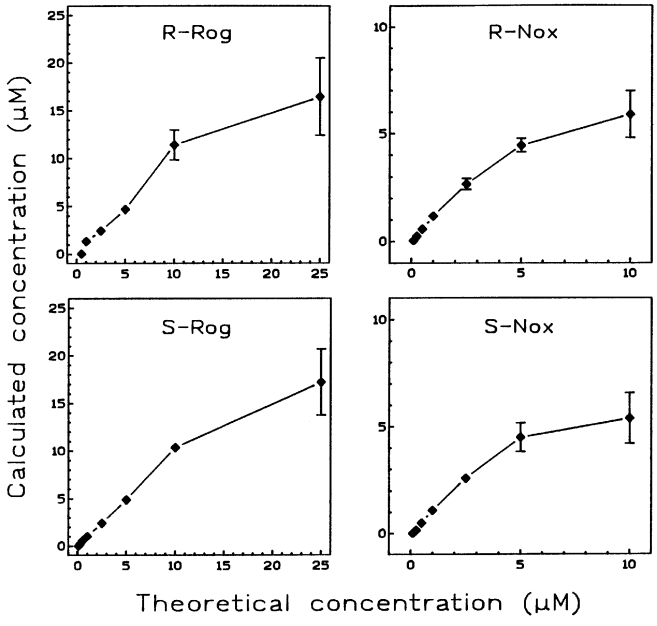

Fig. 3. Plot of measured concentrations (mean values ± SD determined on 3 spiked plasma samples from independent series) versus theoretical concentrations for each compound tested

dependent series using aliquots of spiked plasma containing either 2.5 or 5 μ*M* of each isomer. Again, the use of the IS to correct the results significantly reduced the inter-assay variability and led to CVs of between 6% and 22%. In total, the above-mentioned results demonstrate the necessity to use an IS. In addition, it appears that S-Ag is an adequate IS for Rog and Nox isomer analysis.

Recovery

On the basis of the above-mentioned analyses, recoveries were calculated, and the results are presented in Table 1. At 2.5 μ*M* the recovery of Nox isomers (34–44%) was lower than that of Rog isomers (64–74%). According to the saturation phenomenon previously observed for Nox isomers above 2.5 μ*M* (Fig. 3), recoveries of Nox isomers were significantly decreased at 5 μ*M* (22–28%), whereas recoveries of Rog isomers at 5 μ*M* (69–71%) were similar to those reported at 2.5 μ*M*.

Clinical application of the assay

The validated assay described above was applied to plasma obtained at steady state from Rog-treated patients receiving conventional doses. Two breast cancer patients were investigated, one receiving 400 mg and the other receiving 200 mg Rog twice daily per os. The morning intake was scheduled for around 8 a.m. Results are given in Table 2. Of the 24 measured samples,

Table 2 Plasma concentrations determined in Rog-treated patients (*ND* Not detectable)

	R-Rog (μ*M*)	S-Rog (μ*M*)	R-Nox (μ*M*)	S-Nox (μ*M*)
Patient A:				
15 h	11.9	8.8	5.2	6.2
Patient B:				
8 h[a]	ND	0.8	1.9	2.3
9 h	10.1	4.5	2.2	2.9
10 h	11.2	7.6	2.0	3.2
12 h	7.5	4.6	2.1	3.4
16 h	3.7	1.8	2.5	3.2

[a]Just before the morning intake

1 plasma sample was below the detection limit (patient B, 8 h, for R-Rog). For patient A the plasma was diluted 1:2 to bring it within the range of linearity of the assay for Nox isomers. This first chiral analysis of plasma from Rog-treated patients shows that both R-Rog and S-Rog isomers are metabolized into their respective rogletimide N-oxide isomers. Plasma concentrations of R-Rog seemed to be higher than those of S-Rog. The maximal concentrations of R-Rog and S-Rog seemed to occur at similar times, around 2 h after the oral intake. For patient B the half-lives of R-Rog and S-Rog as calculated between 9 and 16 h were 4.8 and 5.3 h, respectively. S-Nox concentrations were 20–60% higher than those of R-Nox.

Discussion

As previously stressed by Ariens [3], neglecting the stereochemical aspect of drug action, including pharmacokinetic behaviour, may limit the significance of many pharmacology investigations. The first data illustrating potential pharmacodynamic differences between enantiomers came from Louis Pasteur, who noted that the enantiomers of arginine tasted differently (sweet/insipid) [13]. Enzymes such as mixed-function oxidases, epoxide hydrolases, esterases, glutathione transferases and glucuronyl transferases exhibit stereoselective action in drug metabolism [12]. In the consideration of drugs used in the oncology field, stereochemical differences in metabolism have been particularly well studied for ifosfamide and leucovorin [18]. For the latter drug, after having developed a specific chiral HPLC method [9], we have contributed to highlighting the stereospecific metabolism of the drug into its main circulating active metabolite, 5-methyltetrahydrofolate [8]. During preliminary clinical studies with Rog, pharmacokinetics investigations showed that the drug induced its own metabolism since, during repeated dosing, both the Michaelis constant (K_m) and the peak volume (V_{max}) were increased [11]. Accordingly, another study showed that repeated treatment with Rog increased the area under the curve of the main

metabolite Nox by more than 40% in all patients [7]. Since Rog is used as a racemic mixture and taking into account the importance of drug metabolism, it appears necessary that the stereo-specific drug pharmacokinetics of both Rog and Nox be clearly identified.

We have developed and validated an original stereo-specific HPLC assay for quantification of Nox isomers in plasma. Optimal separation was achieved on a Chiracel OJ column thermostated at 35°C with a mobile phase consisting of *n*-hexane/anhydrous ethanol (65/35, v/v) at a flow rate of 0.9 ml/min (UV detection at 257 nm). Under these conditions the total run time was 90 min. The simplicity of this HPLC procedure guarantees its routine use in the clinical setting. With the exception of a minor interference occurring at the retention time of R-Rog, no other interference with endogenous compounds was observed. The reproducibility results demonstrate the need to use an internal standard. This assay was applied to plasma obtained at steady state from Rog-treated breast cancer patients receiving conventional oral doses and demonstrated its feasibility with regard to sensitivity. The very preliminary pharmacokinetic results presented herein suggest for the first time that both R-Rog and S-Rog isomers are metabolized into their respective Nox isomers. S-Nox concentrations appear to be 20–60% higher than those of R-Nox. Further stereo-specific investigations of Rog pharmacokinetics are needed to confirm these very preliminary data, and the presently developed method could prove to be a useful tool.

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