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High-performance liquid chromatographic assay of malagashanine in rat plasma and urine and its pharmacokinetic application

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Abstract

A reversed-phase HPLC method was developed for quantitative analysis of malagashanine in rat plasma and urine. Malagashanine and internal standard were extracted from alkalinized rat plasma. Urine analysis was performed by direct injection onto the HPLC system. Acetonitrile–aqueous 25 m*M* sodium acetate solution at pH 6.25 (45:55, v/v) was used as the mobile phase. The eluate was monitored by using UV detection at 250 nm. The assay was linear within the concentration range of 10–1000 ng/ml. Both intra- and inter-day accuracy and precision were within acceptable limits. The method was applied to study the pharmacokinetics of malagashanine in rats.

Keywords: Malagashanine

1. Introduction

Malagashanine and strychnobrasiline (Fig. 1) are two alkaloids isolated from the stem bark of Madagascan *Strychnos* species traditionally used as chloroquine adjuvants in the treatment of chronic malaria [1,2]. These compounds are devoid of intrinsic antiplasmodial activity but display a marked in vitro and in vivo chloroquine-potentiating action against chloroquine-resistant strains of *Plasmodium falciparum* [3]. Preliminary pharmacological and toxicological investigations have already been carried out and encouraging results were obtained with a decoction of *Strychnos myrtoides* in an open clinical trial on malaria patients in Madagascar [4]. Investigation of the pharmacokinetic characteristics of malagashanine, one of the major active molecules present in the stem bark of *Strychnos myrtoides*, is extremely important for the further development of this promising chloroquine adjuvant.

The objective of this work, therefore, was to develop a sensitive and specific high-performance liquid chromatography (HPLC) assay to quantify malagashanine in plasma and urine of rats and to

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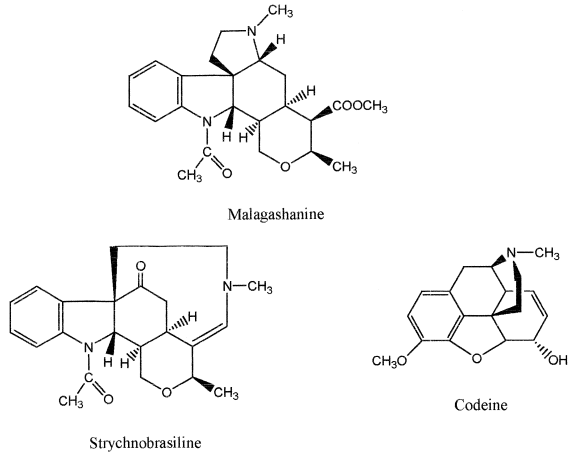

Fig. 1. Chemical structures of malagashanine and internal standards (strychnobrasiline and codeine).

show its applicability to pharmacokinetic studies in the rat.

2. Experimental

2.1. Reagents and chemicals

Malagashanine and strychnobrasiline (Fig. 1) were extracted from stem bark of *Strychnos myrtoides* Gilg & Busse (Loganiaceae) as described previously. Codeine was purchased from Certa (S.A. Medeva Pharma, Braine-l'Alleud, Belgium). Acetonitrile and diethyl ether were of HPLC grade; acetic acid, sodium acetate were of analytical grade (Merck, Darmstadt, Germany). Water was daily obtained from a Milli-Q water purification system (Millipore, Molsheim, France). A 1-mg amount of malagashanine or strychnobrasiline powder was dissolved in 20 μl of 2% acetic acid and the volume of stock solution was adjusted to 100 ml with water (10 μg/ml). A stock solution of codeine (100 μg/ml) was prepared by dissolving 1 mg in methanol–water (1:9, v/v). Stock solutions were stored at +4°C. Under these conditions, they were stable for at least 3 months. Working solutions of 1 μg/ml of internal standard (strychnobrasiline or codeine) were prepared in water.

2.2. Chromatography

The HPLC system consisted of a 7125 Rheodyne injector with a 100-μl loop (Cotati, CA, USA), a Kontron 420-Model pump and a Kontron 433-Model UV detector (Milan, Italy). Chromatograms were processed by a PC Integration Pack (Kontron) data system. Chromatographic separations were performed on a LiChroCart/LiChrospher 60 RP8-select B column (250×4 mm I.D., 5 μm particle size, Merck). A guard column was used (MOS 5 μm, Hypersil-Shandon, Cheshire, UK) to prevent obstruction by minute particles. A prefiltered and degassed mobile phase consisting of 25 m*M* aqueous sodium acetate solution (adjusted to pH 6.25 with concentrated acetic acid)–acetonitrile (55:45, v/v) was delivered to the column at a flow-rate of 1 ml/min and the eluate was monitored at 250 nm.

2.3. Preparation of analytical standards

Working solutions of malagashanine (in distilled water) were prepared from the stock solution. Plasma and urine standards were freshly prepared by transferring a small aliquot of the aqueous working solution of malagashanine to a clean tube and by adding blank rat plasma or urine. The following series of malagashanine standards in plasma and urine were thus prepared: 10, 25, 50, 100, 250, 500 and 1000 ng/ml and 50, 100, 250, 500, 750 and 1000 ng/ml, respectively.

2.4. Sample preparation

After transferring a 100-μl aliquot of plasma standard or plasma sample (obtained after i.v. bolus injection of malagashanine to rats) to a screw-capped glass tube, the following were added: internal standard working solution (50 μl of strychnobrasiline or 100 μl of codeine), 150 μl of a 4 *M* NaOH solution and 1 ml of organic solvent (diethyl ether when strychnobrasiline was used as internal standard or cyclohexane in the case of codeine). Tubes were vortex-mixed (1 min) and subsequently centrifuged at 4000 *g* for 10 min at 4°C. The supernatant was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 110 μl of the mobile phase and 100 μl

was injected into the HPLC system. Urine standards and diluted urine samples, collected following i.v. bolus injection of malagashanine to rats, were centrifuged before injection (100 μl) into the HPLC system.

2.5. Assay validation

Calibration curves in the range of 10 to 1000 ng/ml plasma or 50 to 1000 ng/ml urine were constructed based on the peak area ratio of malagashanine to internal standard. Least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. Inter-day accuracy and precision of the analytical method were determined based on the results of calibration curves run on several different days. Intra-day precision and accuracy were determined based on five replicate extractions carried out on aliquots of plasma (10, 100 and 1000 ng/ml) and urine standards (100, 500 and 1000 ng/ml) analyzed the same day. Recoveries of malagashanine and internal standard were measured by comparing the chromatographic peak areas of plasma standards following extraction to those obtained from direct injection of solutions of both compounds in the mobile phase. Recoveries, after plasma extraction, were determined at concentrations of 10, 100 and 1000 ng/ml for malagashanine, at 500 ng/ml for strychnobrasiline ($n=3$) and 1000 ng/ml for codeine ($n=5$).

2.6. Pharmacokinetic study

2.6.1. Animals

Pathogen-free male Wistar rats were purchased from Iffa Credo (l'Arbresle, France). Rats were housed in plastic cages on corn-cob bedding in a temperature controlled room (20±2°C) with a 12 h light/dark cycle, and had free access to food (A03, UAR, France) and water for 1 week before the start of the study. Rats weighing 250±10 g were anesthetized with a mixture of 4 mg/kg droperidol and 0.08 mg/kg fentanyl (Thalamonal, Janssen, Beerse, Belgium) injected subcutaneously and both jugular veins were cannulated. After surgery, rats were placed individually in metabolic cages and were allowed all night to recover from anesthesia. During that period and throughout the entire pharmacokinetic experiment they were fasted but had free access to water.

Each rat received an intravenous (i.v.) bolus dose of malagashanine (5 mg/kg) dissolved in isotonic saline through one of the jugular cannulae. Blood samples (200 μl) were drawn through the other jugular cannula just prior to (blank) and at 5, 10, 20, 30, 45, 60, 90 and 120 min following malagashanine administration. Blood samples were centrifuged and the plasma samples were immediately analyzed. Urine was collected before (blank) and from 0–2, 2–10 and 10–24 h following drug administration and stored at −20°C until analysis.

2.6.2. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by using a non-compartmental approach [5,6]. The terminal plasma half-life of malagashanine ($t_{1/2_z}$) was determined from the slope of the terminal linear portion of the log malagashanine plasma concentration–time profiles. Plasma clearance (CL) and distribution volume (Vss) were calculated as $\text{dose}_{\text{i.v.}}/\text{AUC}$ and $\text{dose}_{\text{i.v.}}\cdot(\text{AUMC})/(\text{AUC})^2$, respectively, where AUC represents the area under the malagashanine plasma concentration–time curve from 0 to ∞ and AUMC is the area under the first moment curve from 0 to ∞. The mean residence time (MRT) of malagashanine was calculated as the ratio of AUMC to AUC. The fraction excreted unchanged (fe) was calculated by dividing the total amount of malagashanine recovered in urine by the i.v. administered dose.

3. Results

HPLC chromatograms of rat plasma and urine samples are shown in Fig. 2. Plasma extracts and diluted urine did not show interfering peaks from endogenous substances. Retention times for strychnobrasiline and malagashanine were approximately 6.5 and 9.5 min, respectively. Recoveries of malagashanine from rat plasma were 95.8±0.8, 92.6±3.2 and 88.2±7.5% (mean±SD, $n=3$) at 10, 25 and 1000 ng/ml, respectively. The extraction recovery of strychnobrasiline, determined at 500 ng/ml (the concentration at which it was added to the plasma samples as internal standard), was

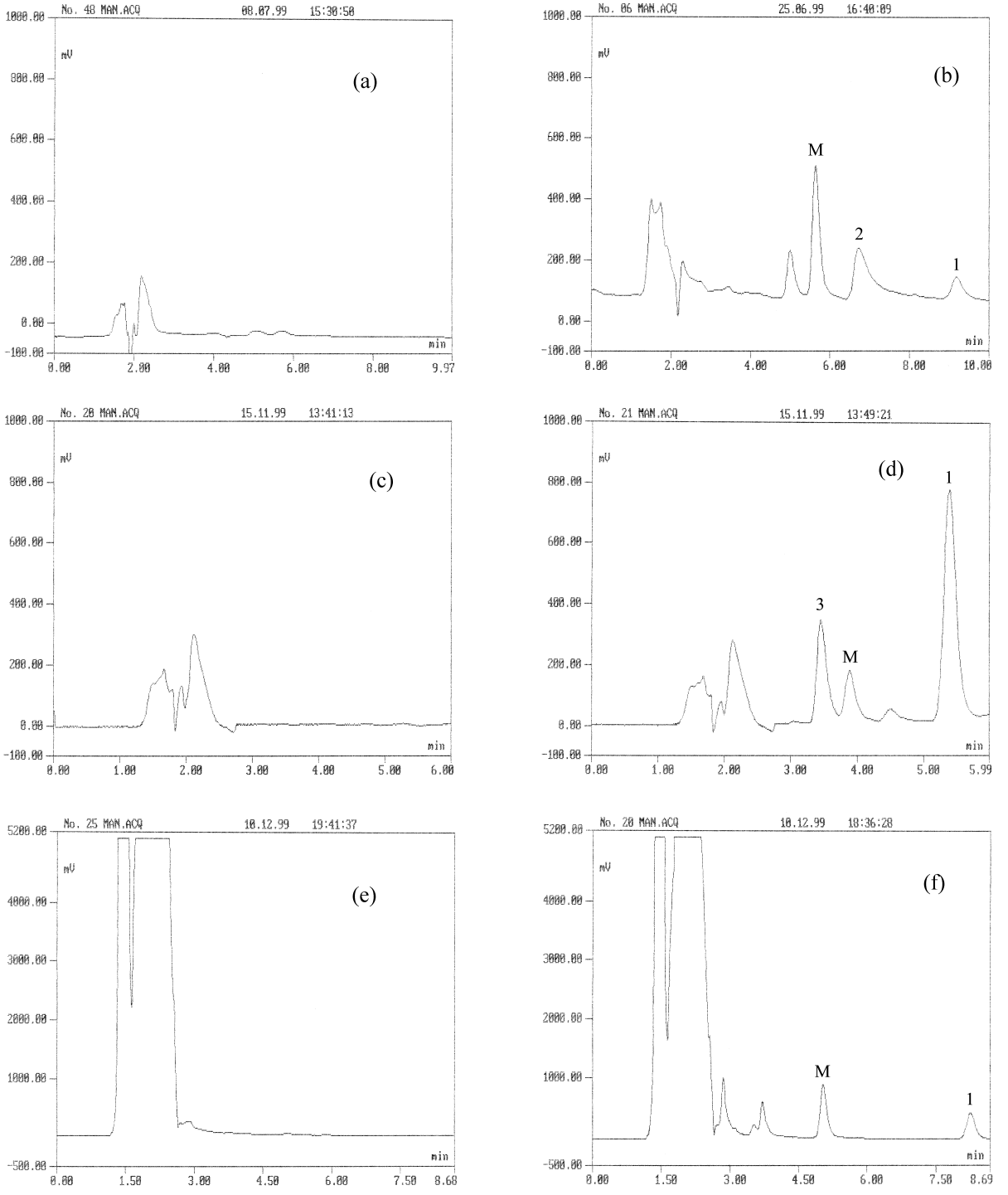

Fig. 2. Chromatograms of (A) blank rat plasma and (B) rat plasma spiked with strychnobrasiline obtained from blood withdrawn 30 min following i.v. injection of 5 mg/kg malagashanine, (C) blank rat plasma and (D) rat plasma spiked with codeine, malagashanine and the unidentified metabolite, (E) pre-dose urine and (F) urine sample collected from 0 to 2 h following i.v. injection of 5 mg/kg malagashanine (1=malagashanine, 2=strychnobrasiline, 3=codeine, M=unidentified metabolite).

Table 1
Precision and accuracy of the HPLC assay for malagashanine in rat plasma using strychnobrasiline as internal standard[a]

	Standard plasma concentration (ng/ml)	Calculated plasma concentration (ng/ml) (mean±SD)	CV (%)	RSD (%)
Inter-day (n=6)	10	11.5±0.6	5.3	+15
	25	26.4±1.6	5.9	+5.6
	50	53.3±1.8	3.4	+6.6
	100	97.8±3.1	3.2	−2.2
	250	266.3±7.9	3.0	+6.5
	500	464.2±18.1	3.9	−7.2
	1000	1013.9±41.5	4.1	+1.4
Intra-day (n=5)	10	8.7±0.2	2.4	−13.6
	100	109.8±8.7	7.9	+9.8
	1000	1040.6±28.2	2.7	+4.1

[a] SD=Standard deviation; CV=coefficient of variation; RSD=relative standard deviation [(found−nominal)/nominal].

90.8±7.0% (mean±SD, n=3). Calibration curves for malagashanine were linear within the studied range, i.e., 10–1000 ng/ml. The coefficient of variation of the slopes of calibration curves (n=5) was 6.9%. Intercepts were close to zero. The intra- and inter-day precisions of the malagashanine assay in plasma were acceptable as indicated by the coefficients of variation, which were always smaller than 10% (Table 1). In addition, the intra- and inter-day accuracies of the plasma assay were always within 10% of the nominal value, except for the smallest standard (i.e., 10 ng/ml) for which relative errors of +15% (inter-day) and −13.6% (intra-day) were found (Table 1). For the quantification of malagashanine in urine, sample preparation was extremely simple and the precision and accuracy parameters were good (Table 2).

Initially, strychnobrasiline, an alkaloid related to malagashanine was used as internal standard. However, because of the restricted commercial availability of this compound, codeine was subsequently selected as an appropriate internal standard. When codeine was used as internal standard, diethyl ether was replaced by cyclohexane as extraction solvent and the pH of the mobile phase was adjusted to 4.5 instead of 6.25. Under these conditions, retention times of codeine and malagashanine were 3.5 and 5.4 min, respectively. Calibration curves were linear from 25 and 1000 ng/ml and correlation coefficients were higher than 0.999. The results of the validation of the HPLC assay for malagashanine in plasma using codeine as internal standard are summarized in Table 3. Recoveries of malagashanine from rat plasma were 93.5±5.5, and 94.8±2.1% (mean±SD,

Table 2
Precision and accuracy of the HPLC assay for malagashanine in rat urine[a]

	Standard urine concentration (ng/ml)	Calculated urine concentration (ng/ml) (mean±SD)	CV (%)	RSD (%)
Inter-day (n=6)	50	56.2±4.9	14.2	+12.3
	100	102.2±8.4	8.2	+2.2
	250	240.9±7.4	3.1	−3.6
	500	499.3±11.6	2.3	−0.1
	750	741.2±23.9	3.2	−1.2
	1000	1008.7±30.1	2.9	−0.9
Intra-day (n=6)	100	90.4±9.8	10.8	−9.6
	500	496.2±6.2	1.2	−0.7
	1000	988.1±6.3	0.6	−1.2

[a] SD=Standard deviation; CV=coefficient of variation; RSD=relative standard deviation [(found−nominal)/nominal].

Table 3
Precision and accuracy of the HPLC assay for malagashanine in rat plasma using codeine as internal standard[a]

	Standard plasma concentration (ng/ml)	Calculated plasma concentration (ng/ml) (mean±SD)	CV (%)	RSD (%)
Inter-day (n=6)	25	22.1±3.6	16.4	−11.2
	50	46.4±3.8	8.3	−7.1
	100	103.6±10.8	10.4	+3.6
	250	231.8±24.9	10.7	−7.2
	500	510.0±34.5	6.7	+2.0
	1000	993.6±21.4	2.1	−0.6
Intra-day (n=3)	25	21.8±1.8	8.4	−12.5
	50	44.4±3. 6	8.1	−11.0
	100	97.7±9.1	9.3	−2.2
	250	260.5±16.6	6.3	+4.2
	500	487.4±42.6	8.7	−2.5
	1000	1077.8±77.5	7.1	+7.7

[a] SD=Standard deviation; CV=coefficient of variation; RSD=relative standard deviation [(found−nominal)/nominal].

n=5) at 100 and 1000 ng/ml, respectively. The extraction recovery of codeine, determined at 1000 ng/ml (the concentration at which it was added to the plasma samples as internal standard), was 73.8±5.0% (mean±SD, n=5).

The HPLC method, using strychnobrasiline as internal standard, was used to carry out some preliminary studies on the pharmacokinetics of malagashanine in rats. Fig. 3 shows a semi-logarithmic of malagashanine plasma concentration–time profile following i.v. bolus administration of this compound (5 mg/kg) to a rat. The malagashanine plasma concentrations declined biphasically as a function of time after administration. Approximately 2 h following i.v. administration, malagashanine plasma concentrations approached the limit of quantification. The most important pharmacokinetic parameters of malagashanine were calculated and the mean values are summarized in Table 4.

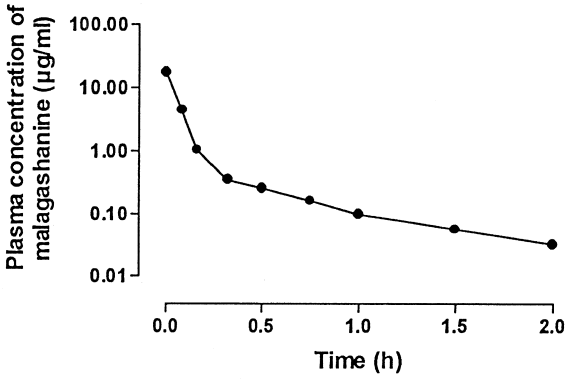

Fig. 3. Semi-logarithmic malagashanine plasma concentration–time profile following i.v. bolus administration of 5 mg/kg malagashanine to a rat.

4. Discussion

Malagashanine is an interesting natural compound isolated from *Strychnos myrtoides* with a proven in vitro and in vivo chloroquine-potentiating action against chloroquine-resistant strains of *Plasmodium falciparum* [1,2]. Its resistance-reversing activity was discovered as a scientific follow-up of the traditional use of three Malagasy *Strychnos* species in the treatment of chronic malaria. It is therefore very important for the further development of this promising chloroquine adjuvant to study its pharmacokinetic behavior.

Table 4
Mean (±SD) pharmacokinetic parameters of malagashanine following i.v. bolus injection of 5 mg/kg to four rats[a]

Parameter	Mean±SD
CL (ml/min)	13.7±1.1
Vss (l)	0.58±0.02
$t_{1/2_z}$ (h)	0.49±0.02
MRT (h)	0.29±0.02
fe×100 (% of i.v. dose)	1.6±0.27

[a] SD=Standard deviation.

The chromatographic conditions used to quantify malagashanine in rat plasma and urine are based on a modification of the method developed by Frédérich et al. [7] to determine a number of usambarane alkaloids present in the roots of *Strychnos usambarensis*. The proposed method is relatively simple and a large number of samples can be processed in 1 day. The influence of the chromatographic parameters on the retention time was investigated by varying the pH of buffer solution and the proportion of acetonitrile in the mobile phase. The chromatographic conditions selected with strychnobrasiline as internal standard resulted in a good separation between malagashanine, the internal standard and an unidentified metabolite in a run time of less than 10 min. The HPLC conditions (composition of mobile phase, pH of aqueous buffer, extraction solvent) were slightly modified when codeine was used as internal standard. The validation parameters for inter- and intra-day precision and accuracy are in accordance with the guidelines formulated by a group of experts during the 1990 conference on ''Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies'' [8].

The developed method was used to characterize the pharmacokinetics of 5 mg/kg malagashanine following an i.v. bolus injection. The results of these preliminary pharmacokinetic studies demonstrate that the elimination of malagashanine is fast: its plasma clearance (13.6 ± 1.1 ml/min) is much higher than liver plasma flow, which is approximately 4.7 ml/min in a 500 g rat [9]. This means that extrahepatic elimination mechanisms may significantly contribute to the overall elimination of malagashanine. Urinary excretion data (fe, Table 3) show that renal excretion of unchanged malagashanine is very small. Consequently, metabolism probably accounts for most of the elimination of malagashanine. A peak with retention time between 5 and 6 min (Fig. 2) was observed in the plasma samples of rats that had received malagashanine (method with strychnobrasiline as internal standard). This peak was also found in urine of rats treated with malagashanine. Since this peak was never found in blank samples, it most likely represents a major metabolite of malagashanine. For further pharmacokinetic studies of malagashanine, it is important to identify this metabolite.

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References

[1] P. Rasoanaivo, A. Petitjean, S. Ratsimamanga-Urverg, A. Rakoto-Ratsimamanga, J. Ethnopharmacol. 37 (1992) 117.
[2] P. Rasoanaivo, S. Ratsimamanga-Urverg, F. Frappier, Cur. Med. Chem. 3 (1996) 1.
[3] P. Rasoanaivo, S. Ratsimamanga-Urverg, R. Milijaona, H. Rafatro, C. Galeffi, M. Nicoletti, Planta Med. 60 (1994) 13.
[4] A. Ramialiharisoa, J. Ranaivoravo, S. Ratsimamanga-Urverg, P. Rasoanaivo, A. Rakoto-Ratsimamanga, Rev. Méd. Pharm. Afr. 8 (1994) 123.
[5] M. Gibaldi, D. Perrier, in: Pharmacokinetics, Marcel Dekker, New York, 1982, p. 409.
[6] L.Z. Benet, R.L. Galeazzi, J. Pharm. Sci. 68 (1979) 1071.
[7] M. Frédérich, M. Tits, L. Angenot, Phytochem. Anal. 9 (1998) 63.
[8] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Visnawathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.
[9] W.A. Colburn, J. Clin. Pharmacol. 28 (1988) 673.