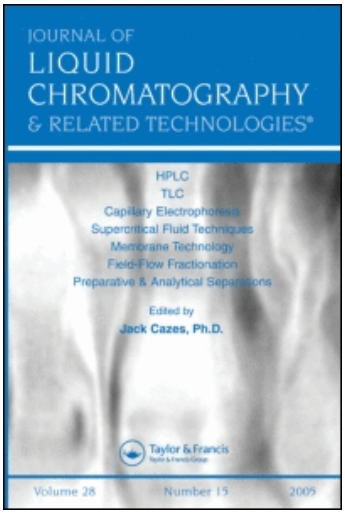

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AROTINOLOL AND AC 623, ITS MAIN METABOLITE, IN BIOLOGICAL SAMPLES

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ABSTRACT

Arotinolol and its main metabolite, AC 623, both antihypertensive agents with β blocking properties were simultaneously determined in human plasma and urine, using solid-liquid extraction and reversed phase HPLC with fluorimetric detection ; U.V. detection could be added as a further control of the specificity of the method.

Extraction yields were satisfactory and stable. Calibration curves in plasma of 0 to 100 ng/ml Arotinolol and 0 to 10 ng/ml AC 623 were used ; in urine concentrations of 0 to 1000 ng/ml of both drugs were quantified. Concentrations of 2 ng/ml Arotinolol and 1 ng/ml AC 623 in plasma and of 20 ng/ml of both drugs in urine could be quantified with good accuracy.

Repeatability (intra-day ; n = 6) was studied at 3 concentrations covering the standard curve ranges. Reproducibility was estimated from calibration curves (10 in plasma and in urine) carried out during a pharmacokinetic study in man. Specificity

was verified in general and with regards to another metabolite present in man.

The method is shown to be well adapted for accurate determination of Arotinolol and its main metabolite in biological samples during pharmacokinetic and clinical studies.

INTRODUCTION

Arotinolol (5-[2-[(3-tertiary butylamino-2-hydroxypropyl) thio]-4-thiazolyl]-2-thiophenecarboxamide hydrochloric acid) and its main metabolite, AC 623 (5-[2-(3-tertiarybutylamino-2-hydroxypropyl)thio-4-thiazolyl]-2-thiophenecarboxylic acid hydrochloride), have been studied in Japan in animal and man.

Arotinolol possesses α and β blocking properties (1, 2, 3, 4). This compound has been launched in Japan as Almarl^{R} by Sumitomo for the oral treatment of angina pectoris, tachy-arrythmias and essential hypertension (5).

Pharmacokinetic studies have been worked out in Japan in animal and man.

The aim of this work is to supply a suitable and sensitive analytical method for determining the racemic form of both compounds (Arotinolol and its acidic metabolite) in biological samples resulting from the pharmacokinetic and clinical studies in caucasian and asian subjects. The knowledge of the behaviour of the drug in caucasian and european subjects is necessary to obtain agreement for marketing of the drug in Europe.

MATERIALS AND METHOD

Materials

Arotinolol and AC 623 racemates were purchased from SUMITOMO (Japan), while Alpiropride, used as internal standard, was purchased from Laboratoires DELAGRANGE (France).

Acetonitrile, methanol and chloroform for fluorimetry and RPE triethylamine were supplied by CARLO ERBA ; diethylether for analysis and n-hexane for HPLC were supplied by FISONS.

Solutions to prepare the phosphate buffer were supplied by MERCK and tetrabutylammonium chloride was supplied by KODAK. Water for HPLC was obtained with a milli-Q system (MILLIPORE).

Standard Solutions

Stock solutions in methanol were prepared as a mixture of Arotinolol and AC 623 at concentrations of 0.1 mg/ml Arotinolol and 0.025 mg/ml AC 623 (solution 1) and at 0.1 mg/ml Arotinolol and 0.1 mg/ml AC 623 (solution 2). Standard solutions in water were obtained by a ten fold dilution of the stock solutions.

A stock solution in methanol at 0.25 mg/ml Alpiropride was prepared and diluted fifty fold with water to obtain the standard solution (5 ug/ml).

Calibration Curves and Spikes

For plasma (1 ml), standard curves containing 2, 4, 10, 20, 40 and 60 ng/ml Arotinolol and 0.5, 1, 2.5, 5, 10 and 15 ng/ml AC 623 and 250 ng/ml internal standard were prepared by spiking blank plasma.

For urine (100 ul) standard curves containing 20, 40, 100, 200, 400, 600 and 1000 ng/ml Arotinolol and AC 623 with 500 ng/ml internal standard were obtained by spiking blank urine.

Plasma spiked concomitantly with Arotinolol and AC 623 at concentrations of 4, 40 ng/ml and 1 and 10 ng/ml respectively were analysed as control samples (spikes) with each series of determinations, together with the calibration curve and blanks.

Urine spiked concomitantly with Arotinolol and AC 623 at concentrations of 40, 200, 600 ng/ml for each drug were used as control samples for determination in urine.

Instruments

Solid phase extraction was carried out on a Baker-SPE-21 extractor with teflon screens.

High performance liquid chromatography was performed with an automatic sampler (GILSON 231), a pump (GILSON 303) with a pulsation dumper (GILSON 802 C) and a dilutor (GILSON 401). Fluorimetric (SHIMADZU RF 535) and ultraviolet (SHIMADZU SPD-6A) detection was recorded by an integrator (SHIMADZU CR4A).

Sample Preparation

Simultaneous extraction of Arotinolol and AC 623 from plasma or urine was performed by solid phase extraction using C18 Baker-bond columns of 1 ml capacity.

Plasma (1 ml), to which Alpiropride was added as internal standard (50 ul of the 5 ug/ml standard solution) was placed on the columns (rinsed before use with methanol).

Pure or diluted urine (100 ul) was mixed with 250 ul blank plasma, and 100 ul of the Alpiropride internal standard solution (5 ug/ml) ; the mixture was placed on the columns.

Washing occured with water (3 X 1 ml) and a mixture of diethylether / n-hexane (50/50 ; V/V) (3 X 1 ml).

Elution of the drugs from the columns was performed with a mixture chloroform / triethylamine (90/10 ; V/V) (2 X 1 ml). The organic phase was evaporated under vacuum ; the residue was taken up by 150 ul of mobile phase for HPLC and 100 ul were analysed.

Chromatographic Conditions

Samples (100 ul) were injected with an automatic injector into the HPLC-system. Chromatographic separation occured on an ODS hypersil column (5 um ; 4.6 mm X 25 cm) with a C18 guard column (7 um ; 4.6 X 1.5 cm) at 25°C using a mixture acetonitrile / methanol / phosphate buffer 1/15 M at pH = 5.6 containing 6.10^{-4} M tetrabutylammonium chloride (150/150/900) at a flow rate of 1.2 ml/min.

Fluorimetric detection was realized with a excitation of 310 nm and an emission of 395 nm, and could be coupled with UV detection at 310 nm to ensure for further specificity control.

Chromatograms were recorded and peak height ratios of drugs and internal standard were calculated.

Quantification

Concentrations are determined by linear regression analysis of the standard curve peak height ratios and concentrations spiked.

RESULTS AND DISCUSSION

Retention times

They were respectively for Arotinolol, AC 623 and the internal standard 10.0, 8.0 and 4.7 min. Another metabolite of Arotinolol extracted under the same conditions appeared at 6.6 min.

Blank plasma and urine did not show any interferences with these compounds. Typical chromatograms are presented in figures 1 to 4.

Typical Chromatograms in plasma and Urine

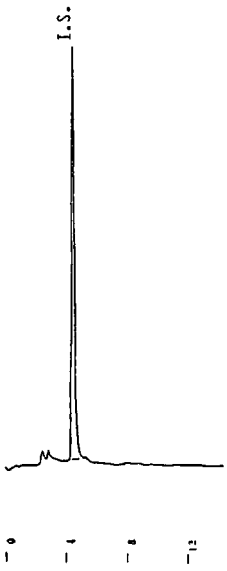

Fig 1 : 0 ng Arotinolol
0 ng AC 623
in plasma

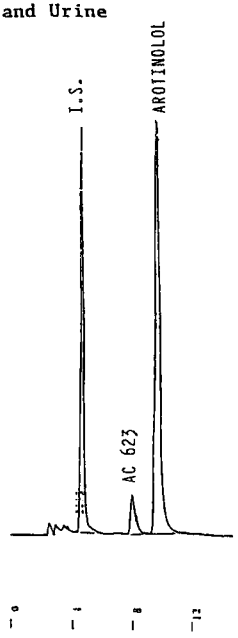

Fig 2 : 20 ng Arotinolol
5 ng AC 623
in plasma

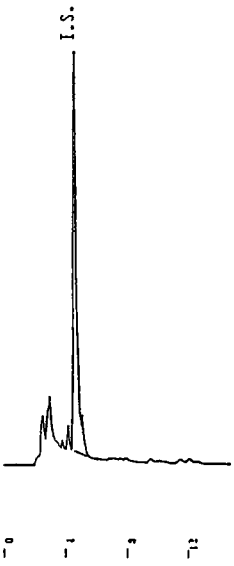

Fig 3 : 0 ng Arotinolol
0 ng AC 623
in urine

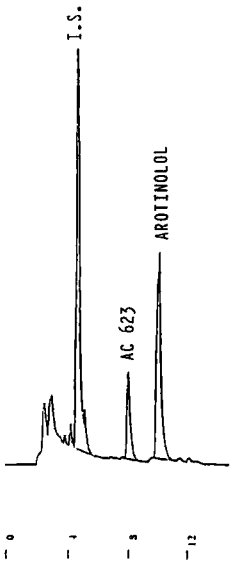

Fig 4 : 20 ng Arotinolol
20 ng AC 623
in urine

TABLE 1

Extraction Yields Using Simultaneous Solid Phase Extraction (n = 4)

Biological Samples Drug	Plasma Conc. (ng/ml)	Extraction yield (%)	CV (%)	Urine Conc. (ng/ml)	Extraction yield (%)	CV (%)
Arotinolol	4	72	6	20	94	2
	40	73	4	400	93	1
	100	76	6	1000	79	3
AC 623	1	71	8	20	69	2
	10	57	12	400	74	1
	25	64	9	1000	66	7
Internal Standard	250	96 to 99	2 to 3	5000	77	1 to 2

Extraction Yields

They are constant for plasma and urine ; coefficients of variation (CV) (n = 4) never exceed 12 %. They are presented in table 1.

Linearity

Regression analysis between peak height ratios and concentrations in calibration curves show good linearity for the concentration range studied (table 2 and 3, and figures 5 to 8).

TABLE 2

Regression Analysis of Calibration Curves (y = Bx + A) in Plasma for 1 ml Extracted (m ± SD or range)

Drug	Arotinolol	AC 623
Concentration range (ng/ml)	0 - 60	0 - 15
n	10	34
Slope(B)	0.05384 ± 0.00249	0.01631 ± 0.00184
Intercept (A)	-0.01806 ± 0.01731	0.00246 ± 0.00537
Corr. Coef.	0.99729 - 0.99993	0.99510 - 0.99965

TABLE 3

Regression Analysis of Calibration Curves (y = Bx + A) in Urine for 100 ul Extracted (m ± SD or range)

Drug	Arotinolol	AC 623
Concentration range (ng/ml)	0 - 1000	0 - 1000
n	11	11
Slope (B)	0.02420 ± 0.00177	0.00941 ± 0.00075
Intercept (A)	0.0216 ± 0.0152	0.00466 ± 0.00616
Corr. Coef. (r)	0.99820 - 0.9996	0.9932 - 0.9998

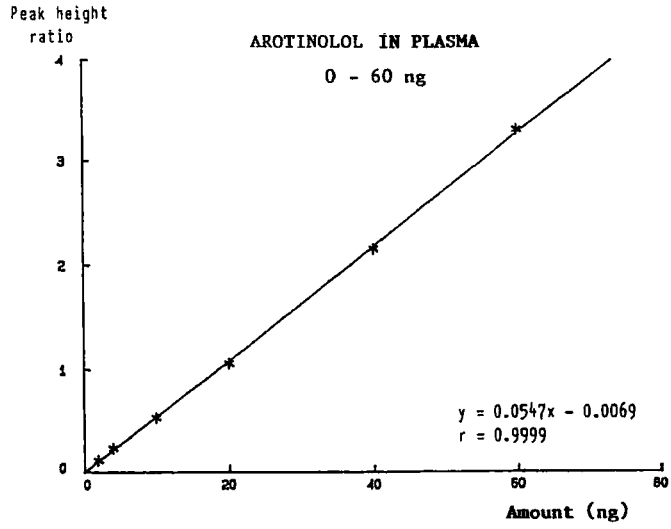

Figure 5 : Calibration curve for Arotinolol in plasma ; 1 ml extracted

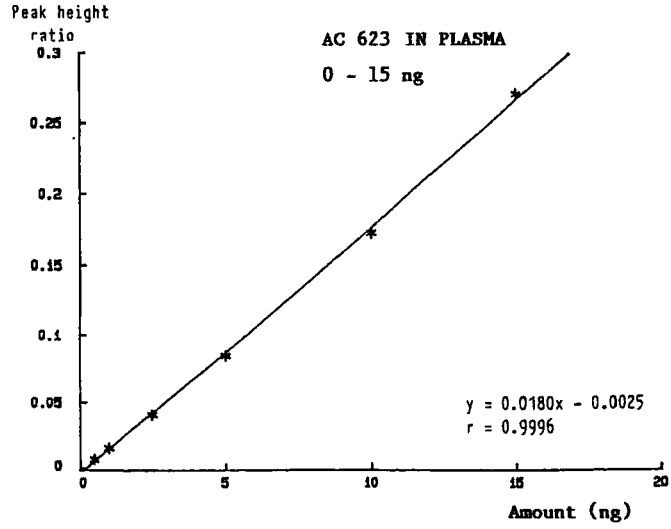

Figure 6 : Calibration curve for AC 623 in plasma ; 1 ml extracted

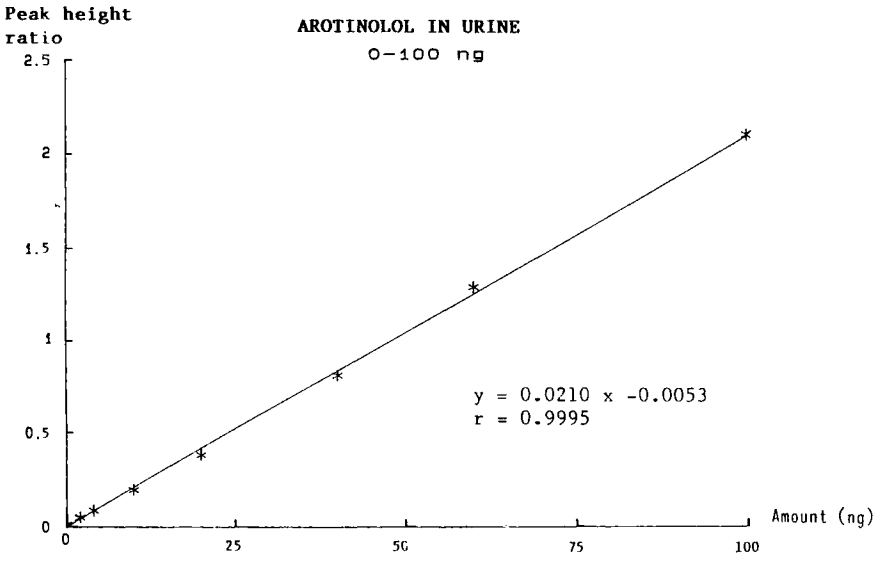

Figure 7 : Calibration curve for Arotinolol in urine ; 100 ul extracted

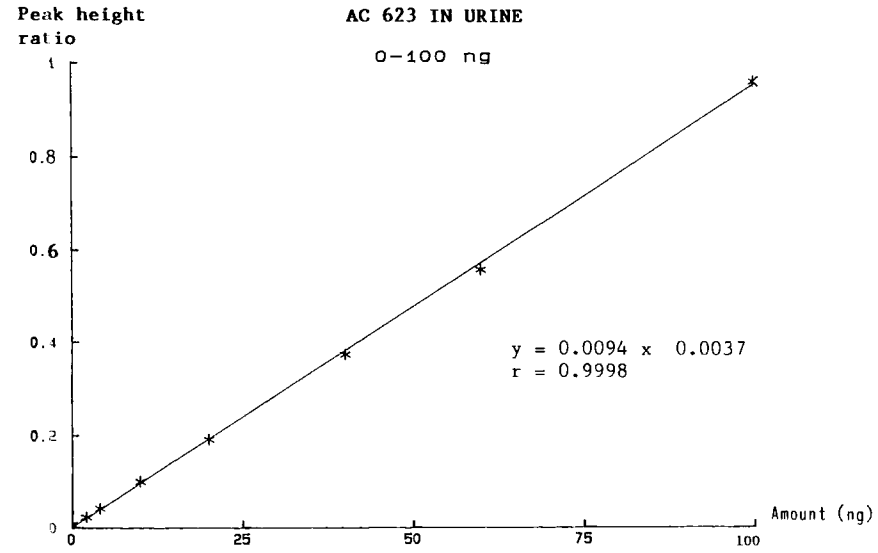

Figure 8 : Calibration curve for AC 623 in urine ; 100 ul extracted

Accuracy

Accuracy estimated from the calibration curve concentrations in plasma was very good, mean relative errors for each concentration did not exceed 10 % for 2 ng/ml and 6 % for the higher concentrations of Arotinolol and AC 623, but mean relative error of 17 % was observed for 0.5 ng/ml AC 623.

In urine, mean relative errors did not exceed 10 % for 100 ng/ml and higher concentrations of Arotinolol and AC 623 ; but a mean relative error of about 30 % was found for 20 ng/ml of both drugs.

Spiked plasma analysis over 10 days showed mean relative errors not exceeding 10 % for Arotinolol (4 and 40 ng/ml) and for AC 623 (1 and 10 ng/ml).

Spiked urine analysed over 9 days showed mean relative errors ranging between 6 and 13 %.

Detection Limit

Detection limits in plasma fixed at four times the background noise, are 0.11 ng/ml for Arotinolol and 0.25 ng/ml for AC 623 ; in urine, they are respectively 11 ng/ml and 2.5 ng/ml.

Amounts evaluated with sufficient accuracy for both drugs, using the standard curves described, are in plasma 2 ng/ml Arotinolol and 1 ng/ml AC 623, and in urine 20 ng/ml of both drugs. Nevertheless, lower concentrations of Arotinolol can be quantified, using appropriate standard curves.

Precision

Repeatability (intraday) estimated from 6 analyses in plasma containing respectively 4, 20 and 60 ng/ml Arotinolol, and 1, 5

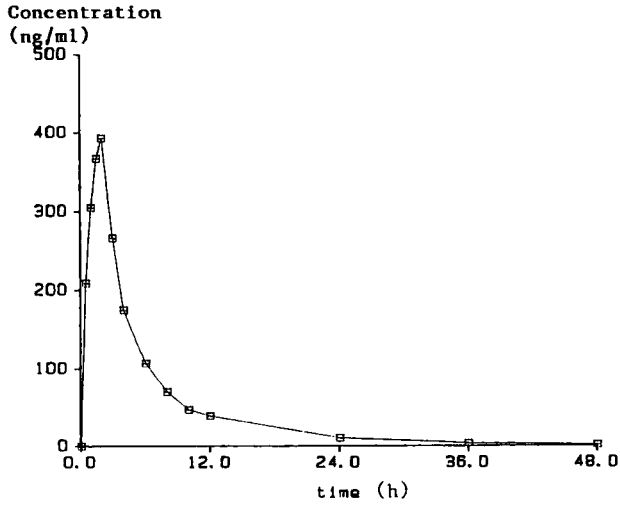

Figure 9 : Arotinolol plasma concentrations observed in subject 3 after 20 mg dose of arotinolol (capsules)

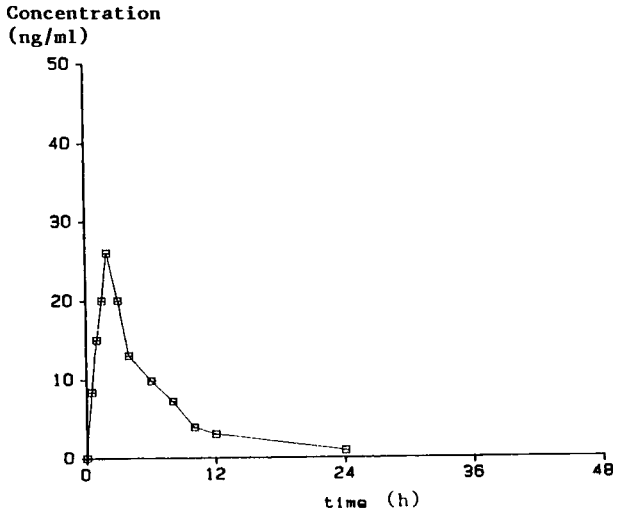

Figure 10 : AC 623 plasma concentration observed in subject 3 after 20 mg dose of arotinolol (capsules)

and 15 ng/ml AC 623 and in urine containing 40, 200 and 600 ng/ml of both drugs, gave variation coefficients not exceeding 10 %.

Reproducibility (interday) estimated from standard curve concentrations showed for Arotinolol in plasma (n = 10) coefficients of variation not exceeding 11 % and in urine (n = 11), not exceeding 10 % except at 20 ng/ml (32 %).

For AC 623, variation coefficients were less than 10 % for concentrations above 2.5 ng/ml in plasma, but reached 17 % at 0.5 and 1 ng/ml. In urine, they were below 10 % at concentrations superior to 100 ng/ml, but more important at 20 and 40 ng/ml.

Variation coefficients (over 10 days) for control samples in plasma, were less than or equal to 10 % for Arotinolol (4 and 40 ng/ml) and were 36 % and 14 % for AC 623 at 1 and 10 ng/ml. In urine (over 8 days) they did not exceed 10 % for Arotinolol and 16 % for AC 623 at 40, 200 and 600 ng/ml concentrations.

Specificity

The specificity of the analytical method for the internal standard, Arotinolol and AC 623 could be confirmed by the absence of any interfering endogeneous compound in the blank biological samples and from the constant ratios between UV and fluorimetric response determined for Arotinolol, AC 623 and the internal standard in the 6 standard curve concentrations (CV did not exceed 6 %). These same ratios were found for the three compounds over the 0 - 48 h kinetic profiles of the subjects studied (figures 9 and 10).

CONCLUSION

The high performance liquid chromatographic method elaborated for determining Arotinolol and 623 in biological samples is

well adapted for routine analysis and shows suitable characteristics to ensure the quantification of both drugs in pharmacokinetic and clinical studies in man.

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