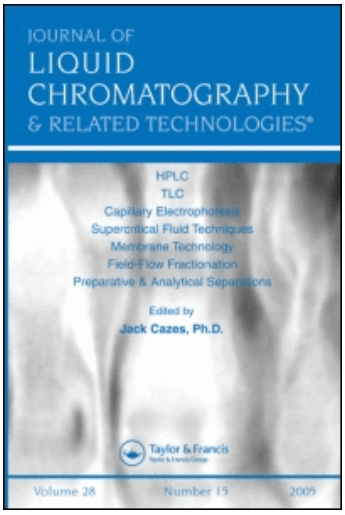

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE OPTICAL ISOMERS OF AROTINOLOL AND AC 623, ITS MAIN METABOLITE, IN BIOLOGICAL SAMPLES

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ABSTRACT

Arotinolol and its main metabolite, AC 623, both antihypertensive agents with β blocking properties could be separated simultaneously into their two enantiomers and determined in plasma and urine using a solid phase extraction in presence of an internal standard and HPLC using a chiral counterion (Z-glycyl-L-proline) followed by fluorimetric detection.

Extraction yields are satisfactory and reproducible. Calibration curves in plasma showed good linearity at levels from 75 to 375 ng/ml Arotinolol (97 % R (-) and 3 % S(+) and from 5 to 25 ng/ml AC 623 (80 % R(-) and 20 % S(+)).

Absolute amounts of each enantiomer could be calculated from the plasma standard curves, whereas percentages of each enantiomer were estimated from spiked plasma and urine. Both enantiomers of Arotinolol or AC 623 gave similar analytical response. They

could be related to total amounts of drugs dosed using non-chiral HPLC (10).

Accuracy was estimated from plasma calibration curves (R.E. < 10 % for Arotinolol enantiomers ; < 20 % for AC 623 enantiomers), and spikes (mean R.E. < 7 % for Arotinolol enantiomers ; < 24 % for AC 623 enantiomers).

Precision defined by the coefficient of variation (C.V. %) over several analysis in plasma and urine showed a C.V. inferior to 10 % for Arotinolol enantiomers, but reaching sometimes 40 % for AC 623 enantiomers.

The poorer accuracy and precision on AC 623 analysis was essentially due to the chromatography of the drug and to the lower levels analysed in comparison with Arotinolol.

The detection limit of the method used for plasma and urine analysis was 2 ng/ml of each enantiomer (R(-) and S(+)) of Arotinolol and AC 623.

This method could succesfully be applied for quantification of Arotinolol and AC 623 enantiomers in plasma and urine.

INTRODUCTION

Arotinolol (5-[2-[3-tertiarybutylamino-2-hydroxypropyl) thio]-4-thiazolyl]-2-thiophenecarboxamide hydrochloridric 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 and are being completed in France in order to obtain agreement for marketing of the drug in Europe.

Sensitive non-chiral high performance liquid chromatography has been used for quantifying Arotinolol and AC 623 in biological samples. Separation of the enantiomers of both compounds has been obtained using either non-chiral chromatography on diol lichro-

sorb column by ion pair diastereoisomer formation with (+)camphor-10-sulphonic acid, or chiral chromatography on a (R)-N-(3,5 dinitrobenzoyl)phenylglycin-aminopropylsilica (6).

This paper describes a reliable and sensitive method for the stereoselective quantification of Arotinolol and AC 623 in the same biological sample (plasma or urine), based on non chiral HPLC on a diol lichrosorb column using Z-glycyl-L-proline as chiral counter ion for ion pair diastereoisomer formation.

MATERIAL AND METHODS

Arotinolol (S(+) and R(-)) and AC 623 (S(+) and R(-)) were purchased from Sumitomo (Japan), while Alpiropride, used as internal standard, was purchased from Laboratoires DELAGRANGE (France).

Methanol for fluorimetry and HPLC, chloroform for fluorimetry and triethylamine RPE grade were supplied by Carlo-Erba ; diethylether, triethylamine and n-hexane for HPLC were supplied by Fisons, while diethylether for analysis was supplied by Merck.

Dichloromethane for HPLC was obtained from Rathburn and Z-glycyl-L-proline (ZGP) was supplied by Fluka.

Water for HPLC was obtained with a milli-Q system (Millipore).

Standard Solutions

Stock solutions in methanol were prepared for each of the two enantiomers of Arotinolol and AC 623 at a concentration of 1 mg/ml ; standard solutions in water were obtained by a ten-fold dilution of the standard solutions (100 ug/ml).

Since a few plasma and urine assays realized before hand showed a rather constant percentage of each enantiomer, standard solution mixtures of the four compounds (Arotinolol R(-) and S(+) and AC 623 R(-) and S(+)) were prepared taking into account the concentration of drugs and the percentage of each enantiomer expected in the biological samples.

A solution (P1) was prepared by mixing appropriate amounts of the standard solutions to obtain 7500 ng/ml Arotinolol (97 % R(-) and 3 % S(+)) and 500 ng/ml AC 623 (80 % R(-) and 20 % S(+)).

This solution (P1) was further diluted with water to concentrations of 6000 ng/ml Arotinolol and 400 ng/ml AC 623 (P2) 3000 ng/ml Arotinolol and 200 ng/ml AC 623 (P3) and 1500 ng/ml Arotinolol and 100 ng/ml AC 623 (P4), these solutions containing the same enantiomeric ratios as the solution (P1).

A solution (U1) was prepared by mixing appropriate amounts of the standard solutions to obtain 4000 ng/ml Arotinolol (70 % R(-) and 30 % S(+)) and 4000 ng/ml AC 623 (60 % R(-) and 40 % R(+)).

The solution (U1) was further diluted with water to concentrations of 2000 ng/ml Arotinolol and AC 623 (U2) and 1000 ng/ml Arotinolol and AC 623 (U3), in the same enantiomeric ratios as the solution (U1).

A stock solution in methanol at 50 ug/ml Alpiropride was diluted ten-fold with water to obtain a standard solution of 5 ug/ml Alpiropride (I.S.).

Calibration Curves and Spikes

For plasma (1 ml), standard curves containing 75, 150, 300 and 375 ng/ml Arotinolol (97 % R(-) and 3 % S(+)) and 5, 10, 20 and 25 ng/ml AC 623 (80 % R(-) and 20 % S(+)) were prepared by

spiking blank plasma with the (P) standard solutions and analysed with 500 ng/ml internal standard.

For urine (100 ul), spikes containing 0.5, 1 and 2 ug/ml Arotinolol (70 % R(-) and 30 % S(+)), and AC 623 (60 % R(-) and 40 % S(+)) were prepared by spiking blank urine with the (U) standard solutions and analysed with 0.5 ug/ml internal standard.

Spiked plasma with 300 ng/ml Arotinolol (97 % R(-) and 3 % S(+)) and 20 ng/ml AC 623 (80 % R(-) and 20 % S(+)) were analysed as "spikes" with each series of determinations, together with the calibration curve and blank.

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 detection (SHIMADZU RF 535) was recorded by an integrator (IBM-PC-AT) provided with a printer (HEWLETT PACKARD think jet).

Sample Preparation

Simultaneous extraction of Arotinolol and AC 623 from plasma or urine was performed by solid phase extraction using C18 Bakerbond 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 diol-lichrosorb column (5 um ; 4.6 mm X 20 cm) with a diol guard column (7 um ; 4.6 mm X 1.5 cm) at 25°C using a mixture dichloromethane containing 10 mM Z-glycyl-L-proline / anhydric methanol (1000/10) at a flow rate of 2 ml/min.

Fluorimetric detection was realized at 320 nm excitation and 425 nm emission wavelengths.

Chromatograms were recorded over 60 minutes.

For Arotinolol, peak areas were always measured ; for AC 623, peak heights were measured in plasma, and peak areas in urine.

Quantification

Each enantiomer in plasma was quantified directly using linear regression analysis of the plasma standard curves.

On the other hand, the enantiomers of Arotinolol and AC 623 could be expressed as percentages of the enantiomeric mixture R(-)

and S(+) present in the biological sample. For this purpose relation between the chromatographic response on the amount of each enantiomer was established using the analytical response factor for each enantiomer as well as the ratio of this response factor.

The percentages of each enantiomer could be further directly related to amounts or concentrations of each of them, having the total amounts of the enantiomeric mixture from non-chiral HPLC analysis.

RESULTS AND DISCUSSION

Retention Times

Retention times remained rather constant under the same chromatographic conditions, but they varied substantially as a function of batch and age of the column.

For one typical chromatogram, retention times were respectively for Arotinolol R(-) 12 min, Arotinolol S(+) 15 min, AC 623 R(-) 25.5 min, AC 623 S(+) 31.5 min, and for the internal standard, 7.5 min. The enantiomers of another metabolite of Arotinolol, extracted under the same conditions, appeared at 18 and 20 min.

Specificity

The specificity of the analytical method was verified for the internal standard, and for R(-) and S(+) enantiomers of Arotinolol and AC 623 in biological samples (blank plasma and urine).

Typical chromatograms in plasma

Figure 1 : Blank plasma

Figure 2 : Blank plasma spiked with : 300 ng/ml Arotinolol (97 % R(-) and 3 % S(+)) 20 ng/ml AC 623 (80 % R(-) and 20 % S(+)) 500 ng/ml I.S.

Figure 3 : Plasma sample analysis after oral administration of 15 mg Arotinolol racemate to a healthy caucasian volunteer (3 h after intake)

Typical chromatograms in urine

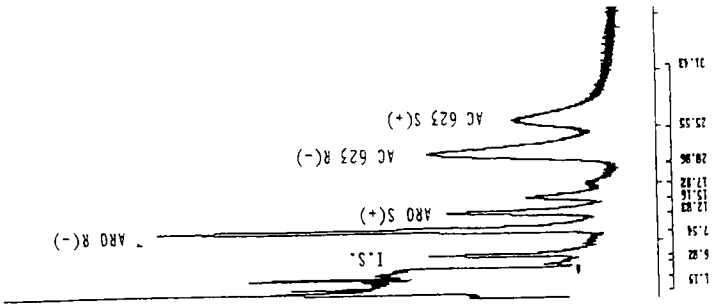

Figure 6 : Urine sample analysis after oral administration of 10 mg Arotinolol racemate to a healthy caucasian volunteer (0-12 h collection)

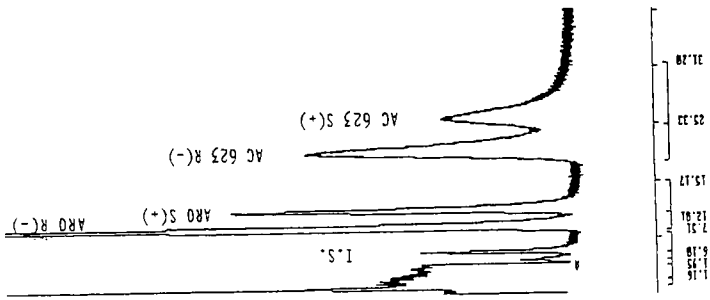

Figure 5 : Blank urine (100 µl) spiked with :
100 ng Arotinolol (70 % R(-) and 30 % S(+))
100 ng AC 623 (60 % R(-) and 40 % S(+))
500 ng I.S.

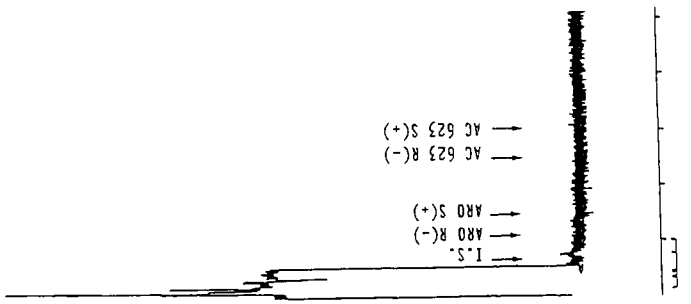

Figure 4 : Blank urine

TABLE 1

Extraction Yields of Arotinolol and AC 623 Racemate 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

Blank plasma and urine did not show any interferences with Arotinolol, AC 623, or with the other metabolite. Typical chromatograms are presented in figures 1 to 6.

Extraction Yields

Extraction yields of Arotinolol, AC 623 remained stable ; the coefficient of variation (CV) for n = 4 didn't exceed 12 %. The internal standard is almost totally extracted. Results are presented in table 1.

TABLE 2

Regression Analysis of Calibration Curves (y = Bx + A) in Plasma (n = 10 ; m ± SD or range)

Drug	Arotinolol		AC 623	
	R(-)	S(+)	R(-)	S(+)
Total Concentration range	75 - 375		5 - 25	
n	10		10	
Enantiomer (%)	97	3	80	20
Slope (B)	0.064 ± 0.007	0.052 ± 0.009	0.042 ± 0.032	0.027 ± 0.009
Intercept (A)	-0.261 ± 0.776	0.036 ± 0.038	-0.23 ± 0.062	0.011 ± 0.019
Correlation coefficient (r)	0.9983 - 1.0000	0.9979 - 1.0000	0.9902 - 0.9999	0.9880 - 0.9998

R(-) and S(+) enantiomers of each drug are extracted to the same extent, as could be concluded from the percentages (50 %) of each enantiomer recovered by HPLC after extraction of the racemate.

Moreover analysis of spiked plasma and urine containing variable amounts of each enantiomer showed recovery of the theoretical enantiomer.

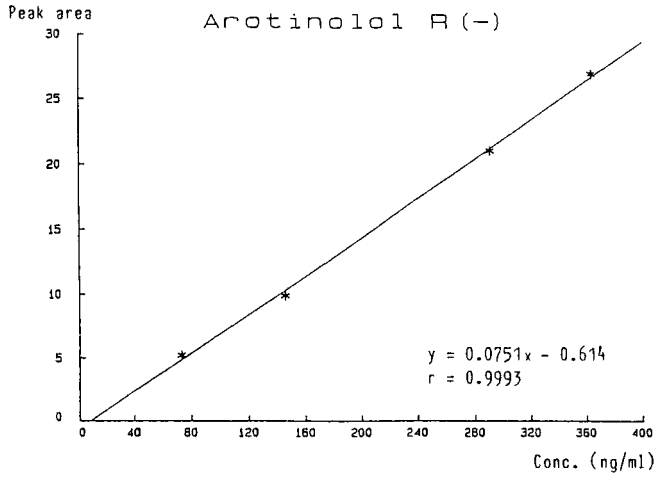

Figure 7 : Calibration curve of Arotinolol R(-) in plasma (2nd June 1989)

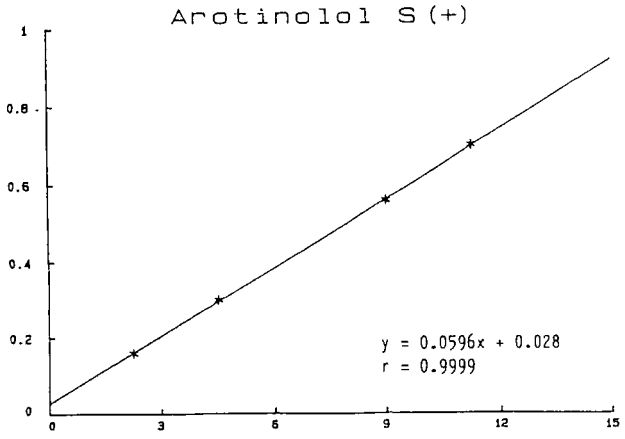

Figure 8 : Calibration curve of Arotinolol S(+) in plasma (2nd JUne 1989)

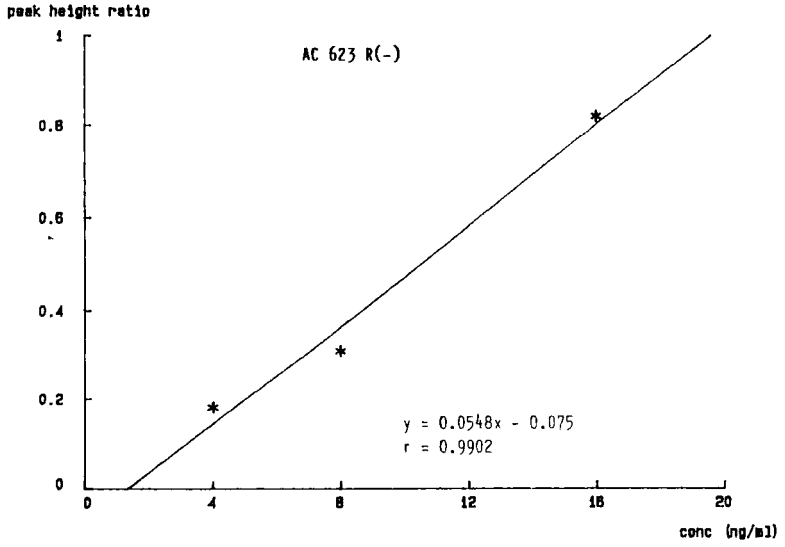

Figure 9 : Calibration curve of AC 623 R(-) in plasma (2nd June 1989)

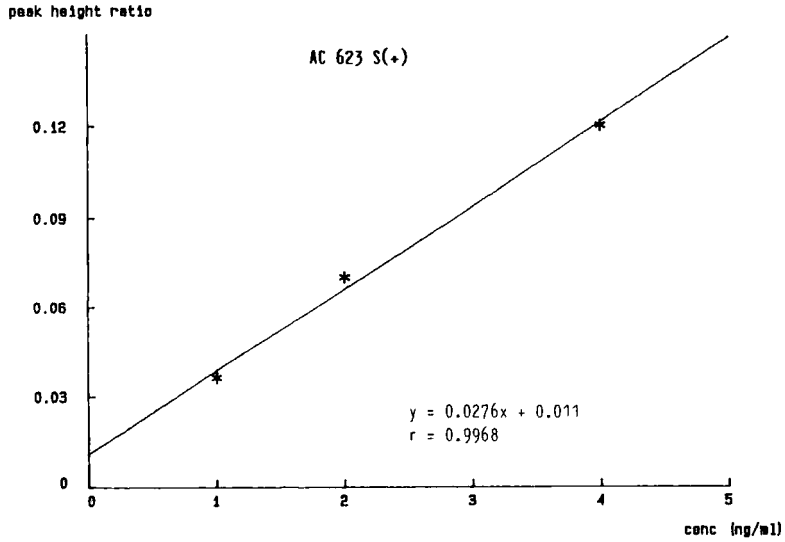

Figure 10 : Calibration curve of AC 623 S(+) in plasma (2nd June 1989)

Linearity

Calibration curves (n = 10) for Arotinolol and AC 623 in plasma contained total amounts of Arotinolol ranging from 75 to 375 ng/ml (97 % R(-) and 3 % S(+)) and of AC 623 ranging from 5 to 25 ng/ml (80 % R(-) and 20 % S(+)).

Regression analysis shows good linearity in the concentration range studied (table 2).

Typical calibration curves for R(-) and S(+) Arotinolol and for R(-) and S(+) AC 623 are presented in figures 7 and 10.

Analytical Response Factor (ARF)

The analytical response factor per unit of each enantiomer (ARF), being the ratio of the peak signal (height or area) to the amount of enantiomer analysed was very similar for both enantiomers of Arotinolol or AC 623 and remained quite constant over the concentration range studied.

In plasma, the mean ratio (m ± SD) ARF(-)/ARF(+) calculated from 12 calibrations curves containing each four concentrations was 1.05 ± 0.23 for Arotinolol and 1.17 ± 0.49 for AC 623.

In urine, the mean ratio (m ± SD) ARF(-)/ARF(+) calculated from 5 spikes at different concentrations was 0.92 ± 0.05 for Arotinolol and 1.30 ± 0.06 for AC 623.

Under these conditions, the analytical response could be closely related to the amount of each enantiomer present in the biological sample.

Accuracy

Accuracy estimated from the plasma standard curves was satisfactory.

Relative errors calculated for each concentration never exceeded 10 % for Arotinolol and 20 % for AC 623 enantiomers, even for the lowest concentration studied (i.e. 2.25 ng/ml Arotinolol S(+) and 1 ng/ml AC 623 S(+)).

These results were confirmed in plasma spikes. Mean relative errors (n = 10) were 7 % for 9 ng/ml Arotinolol S(+) and 4 % for 291 ng/ml Arotinolol R(-). They were 17 % for 4 ng/ml AC 623 S(+) and 24 % for 16 ng/ml AC 623 R(-).

Detection Limit

The detection limit for each enantiomer as the pure substance was 1 ng ; which corresponded to a limit of 2 ng/ml, when applied to the extraction from plasma or urine.

This limit implied that percentages of each enantiomer could be evaluated at plasma levels superior to 70 ng/ml of Arotinolol and superior to 10 ng/ml AC 623 for mixtures of 97 % R(-) and 3 % S(+) Arotinolol and of 80 % R(-) and 20 % S(+) AC 623.

In urine samples, percentages could be evaluated at levels superior to 7 ng/ml Arotinolol and superior to 5 ng/ml AC 623 for mixtures of 70 % R(-) and 30 % S(+) Arotinolol and of 60 % R(-) and 40 % S(+) AC 623.

Precision

Reproducibility (interday) estimated from calibration curves (n = 10) concentrations in plasma showed coefficients of variation (C.V.) never exceeding 5 % for Arotinolol R(-) and S(+) and never exceeding 15 % for AC 623 R(-) and S(+).

In plasma spikes (n = 10 for each concentration), the coefficient of variation was 5 % for 291 ng/ml Arotinolol R(-)

and 7 % for 9 ng/ml Arotinolol S(+), but reached 32 % for 16 ng/ml AC 623 R(-) and 23 % for 4 ng/ml AC 623 S(+).

The poorer precision for AC 623 is in general essentially due to the smaller amounts analysed and the peak shape for this drug.

In urine spikes studied over two days at different concentrations (50, 100, 200 and 250 ng/ml of Arotinolol and AC 623), the coefficient of variation (C.V. ; n = 5) on the percentages of each enantiomer calculated were 1.8 % for 70 % R(-) and 30 % S(+) Arotinolol and 1.6 % for 60 % R(-) and 40 % S(+) AC 623.

CONCLUSION

This high performance liquid chromatographic method for the enantiomeric separation of Arotinolol and AC 623 in biological samples permitted to quantify amounts of each enantiomers in plasma and to determine the percentage of each R(-) and S(+) form, with respect to the total amount of drug present.

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