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Optimization and validation of an analytical procedure by high-performance liquid chromatography for the quantification of peroxisomicines and isoperoxisomicines

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Abstract

Peroxisomicine A1 is a potential antineoplastic substance extracted from plants of the genus *Karwinskia*. An RP-HPLC–DAD method was developed and validated for the separation and quantification of four isomers of this compound. These isomers coelute in the preparative procedure and are present at a proportion ranging between 3 and 5% in the peroxisomicine A1 purified in the laboratory. The desirability coefficient of the method described here was enhanced 140% with respect to the previously reported method.

Keywords: Peroxisomicines; Isoperoxisomicines

1. Introduction

Peroxisomicine A1 (PA1, Fig. 1a) is a dimeric hydroxyanthracenone isolated from the semipolar extracts obtained from plants of the genus *Karwinskia* [1,2]. It has been demonstrated that this substance exhibits selective toxicity upon human neoplastic cells from lung, liver and colon [3]. It produces also, at non-lethal doses, irreversible and selective damage of yeast peroxisomes in vivo.

The presence of other dimeric hydroxyanthracenones in the semipolar extracts of these plants was previously reported [4]. More recently, two stereoisomers and two positional isomers were isolated and identified [5] and were named as peroxisomicine A2 (PA2, Fig. 1b), peroxisomicine A3 (PA3, Fig. 1c), isoperoxisomicine A1 (IsoPA1, Fig. 1d) and isoperoxisomicine A2 (IsoPA1, Fig. 1e).

Studies concerning the mechanism of action of PA1 need a reproducible and sensitive analytical methodology to analyze the composition of samples obtained from different tissues and biological fluids. HPLC methods using reverse phase (C_{18}) were

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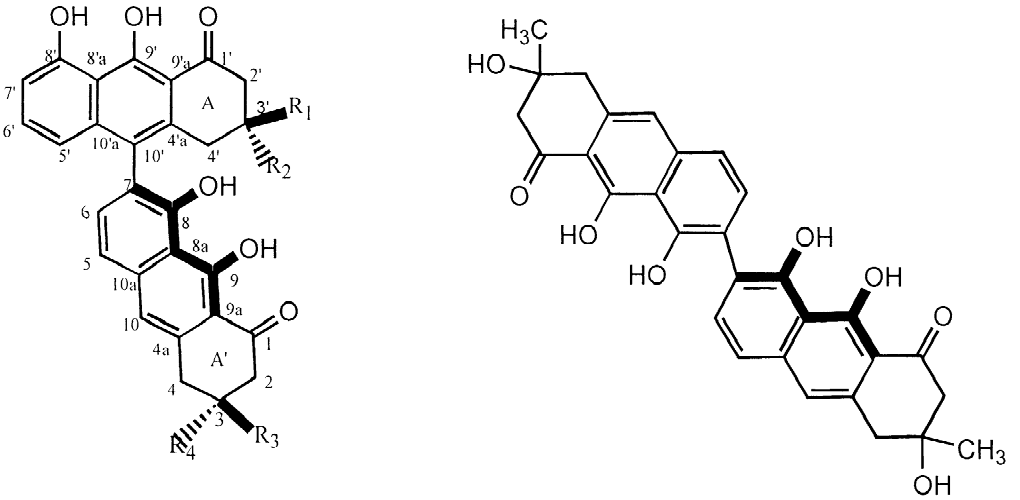

	R_1	R_2	R_3	R_4	
1	Me	OH	OH	Me	**4, 5**
2	Me	OH	Me	OH	
3	OH	Me	OH (Me)	Me (OH)	

Fig. 1. Structure of peroxisomicines and isoperoxisomicines.

developed and published earlier [6,7]. Repeatability, accuracy and sensitivity in the determination of PA1 were improved compared to the previous TLC method employed [8]. Nevertheless, when taking into account the presence of the isomers, the separation achieved was not optimal (Fig. 2a).

Currently, the peroxisomicine A1 obtained in the preparative laboratory contains between 3 and 5% of a mixture of PA3, IsoPA1 and IsoPA1 as impurities. For this reason, as the method previously reported was validated only for the quantification of PA1, it was considered necessary to develop an accurate method to quantify its isomers, in order to know exactly the characteristics of the substance used in biological tests.

Taking the above into account, analytical separation by means of HPLC of all the above-mentioned isomers is reported here. The method developed was validated with parameters such as precision, linearity, selectivity and detection and quantitation limits.

2. Experimental

2.1. Reagents

Acetonitrile, MeOH, HOAc and H_2O were HPLC grade from Merck (Darmstadt, Germany). Peroxisomicine A1, A2 and A3 and isoperoxisomicine A1 and A2 were isolated and purified as previously described [2,5]. The identity and purity of all of them are documented by means of HPLC–DAD, UV and NMR spectra. A stock solution of each compound was obtained by dissolving the pure compound in MeOH (1 mg/ml); the solution was stored at 4 °C and used within a week. An aliquot (5 μl) of each standard was injected.

2.2. Chromatographic separation

The HPLC system used was a Hewlett-Packard HP-1090 Series II/L with DAD detector and auto-

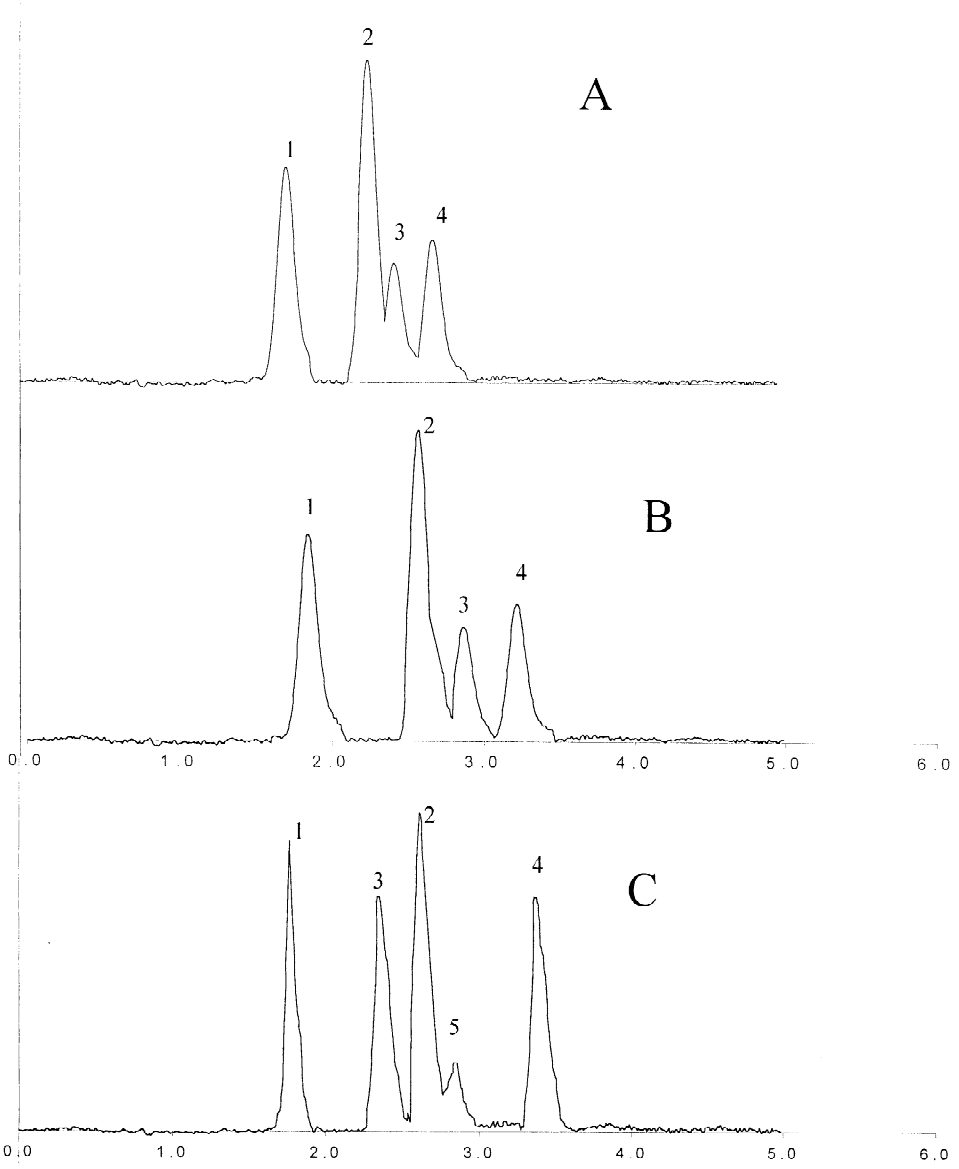

Fig. 2. Chromatograms obtained using eluents (A) MeOH and (B) acetonitrile–H_2O–HOAc (30:70:1.6). Peak assignation: 1, PA2; 2, PA1; 3, isoPA1; 4, PA3; 5, isoPA2: (a) pre-established method, 5 μm column; flow-rate, 0.4 ml/min; (b) optimized conditions, 5 μm column; flow-rate, 0.4 ml/min; (c) optimized conditions as in (b) but in 3 μm column.

injector. Optimization of the analytical separation was achieved starting from the method previously described [6]. Reverse-phase ODS-Hypersil columns (100×2.1 mm) with particle size of 3 and 5 μm were used; a gradient with different proportions of (A) MeOH and (B) acetonitrile–H_2O–HOAc (30:70:1.6) and different flow-rates were employed (Table 1). The flow-rate was 0.4 ml/min. All mobile phases were filtered prior to use through a Millipore HVLP membrane filter (0.45 μm). Data were collected and analyzed in a HPLC-3D ChemStation, DOS Series. For each experiment, retention (k'), separation (α) and resolution (R) parameters were calculated. In order to calculate k', t_0 was obtained using a solution

Table 1
Optimized chromatographic conditions

Time (min)	% A	% B	Flow rate (ml/min)
6	60	40	0.4
9	100	0	0.4
11	100	0	0.4
13	60	40	0.4

A, MeOH; B, acetonitrile–H_2O–HOAc (30:70:1.6).

of acetone as recommended in the literature [9]. Each datum obtained was analyzed using a method for optimization of multiple response systems [10,11]. In accordance to this method, a desirability index for each chromatogram was calculated at a common scale.

The optimized method was validated with the following parameters.

2.2.1. System precision

By means of variations in t_R, peak area and height at 410 nm after six consecutive injections from a methanolic solution containing 15 ng of injected mass of each compound.

2.2.2. Method precision

By means of variation in peak areas and height for all the compounds under examination after extraction of four samples from ground fruit of *Karwinskia parvifolia*.

2.2.3. Linearity

Determined at three different wavelengths, namely 269, 310 and 410 nm in the interval of 5 to 200 ng of injected mass for each compound.

2.2.4. Detection and quantitation limits

D_L and Q_L were calculated based on the standard deviation of the response and the slope

$$D_L = 3\sigma/s \quad Q_L = 10\sigma/s$$

where σ is the standard deviation of the response and s is the slope of the calibration curve.

The calculation of σ was accomplished in two ways.

(a) Based on the standard deviation of the blank (method recommended by IUPAC [12,13]).
(b) Based on the calibration curve. In this case the standard deviation of *y*-intercepts of regression lines were used [13].

2.2.5. Robustness

An experimental design for seven variables was used [14]. Variables under consideration were: amount of acid, amount of acetonitrile, flow-rate, time of preparation–injection of the samples, particle diameter, column temperature and experimental bandwidth. Values assayed are shown in Table 2.

Results were calculated using the following equation:

if $|Vt_R - vt_R| > s2^{1/2}$, the difference is significant

where Vt_R and vt_R are the mean of the retention times obtained from the high and low values defined for each variable, respectively (Table 2).

2.2.6. Selectivity

Spectral purity of the standards used was checked by means of the DAD at seven different points in the

Table 2
Experimental design used in the robustness study

Variable	Experiments								
	0[a]	1	2	3	4	5	6	7	8
Amount of acid (%)	1.6	3.2	3.2	3.2	3.2	3.0	0.0	0.0	0.0
Flow-rate (ml/min)	0.4	0.5	0.5	0.3	0.3	0.5	0.5	0.3	0.3
Time prep/injection (h)	0.5	18	18	0	0	0	0	18	18
Particle diameter (μm)	3	5	3	5	3	3	5	3	5
Column temperature (°C)	20	35	25	25	35	35	25	25	35
% AcCN	30	32	28	28	32	32	28	28	32
Band width	8	12	4	4	12	4	12	12	4

[a] The experimental 0 corresponds to the optimized method.

chromatogram. Relationship of absorbance at three different wavelengths (269, 310 and 410 nm) was then calculated.

3. Results and discussion

3.1. Chromatographic separation

Final elution conditions are shown in Table 2. Representative chromatograms are presented in Fig. 2. Optimization was corroborated by means of the desirability coefficients. In any case, where optimization of multiple responses is the sole objective, use of desirability coefficients provides a simple method of transforming any number of responses into a single dimensionless value which may be compared with any other such value. Response variables may be expressed in dimensional or non-dimensional units and only very rarely can a set of response variables be transformed into a common dimension. However, a combination of values for n responses may be expressed as a dimensionless quantity, known as the desirability coefficient [10]. To use this approach it is necessary to numerically set a weight (relative rank) for each response on a scale of zero to one. The weight ranks the responses from the most to least important in terms of achieving the response target. The weight is independent of the desirability, which is graphically set for each response on a scale of zero to one, where zero corresponds to the value obtained when the response is wholly undesirable and one to the value obtained when it is wholly satisfactory. A graphical desirability profile is set for each response.

In the present case, the curves shown in Fig. 3 were used to transform the response values of k', α and R into desirability coefficients. k', α and R for each experiment are shown in Table 3.

The k' value was selected for the first compound eluted; the optimal value was considered as 2, minimum value for the optimal interval, as reported in the literature [15]. α and R were considered for each pair of adjacent peaks. The optimal α was taken from between the 1.7 and 2.0 interval. Lower values indicate poor separation, higher values have little contribution to the resolution theoretical value [15]. Optimal R was taken as 1.7, a value at which the

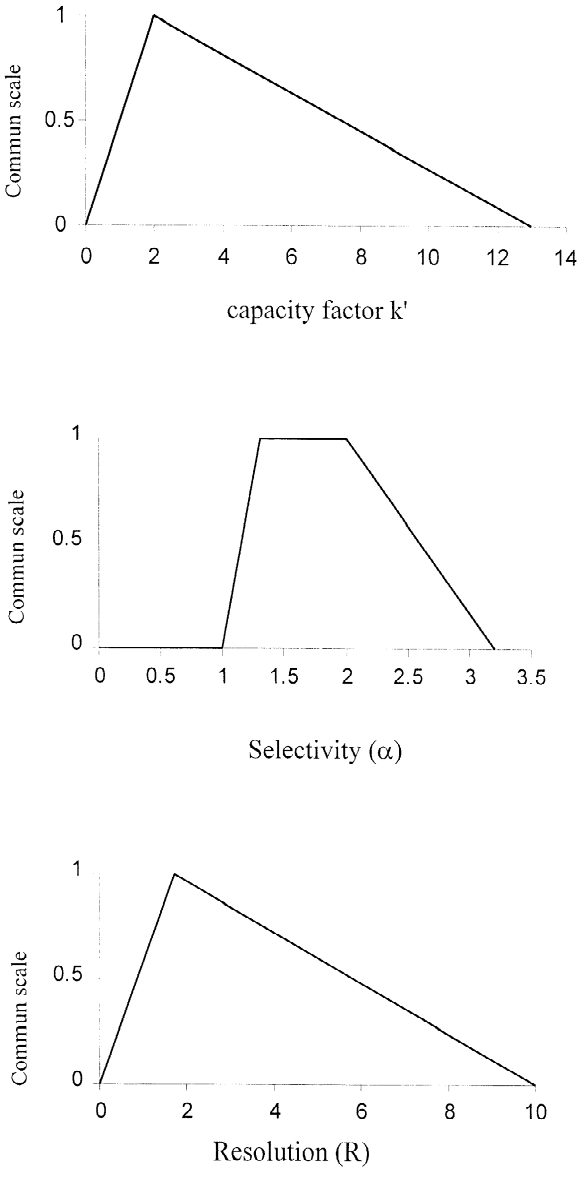

Fig. 3. Graphics used for the derivatization of the desirability coefficients, taken from the data in Table 3. Values taken as optimal for R, k' and α are explained in the text.

signals are totally resolved. Higher values indicate large elution times, which was considered inappropriate. The desirability coefficient was thus calculated for each chromatogram, by means of the sum of R, k' and α standardized values. For example, for experiment 3, k' value for the first peak was 1.86, which represents, in the common scale (normalized)

Table 3
Calculation of desirability coefficients

Exp.	k'		α								R								DC
	*	**	1		2		3		4		1		2		3		4		
			*	**	*	**	*	**	*	**	*	**	*	**	*	**	*	**	
A	1.20	0.20	3.08	0.05	1.21	0.57	1.21	0.57	0	0[a]	1.04	0.69	0.37	0.22	0.42	0.26	0	0[a]	2.56
B	2.07	0.99	1.91	1.00	1.18	0.47	1.13	0.33	0	0[a]	3.17	0.80	0.96	0.60	0.68	0.40	0	0[a]	4.59
C	1.86	0.92	1.93	1.00	1.16	0.42	1.11	0.30	1.24	0.64	3.16	0.81	0.95	0.63	0.70	0.45	1.86	0.95	6.12

Exp. A, pre-established method [6]. Exp. B, optimized method (5 μm column). Exp. C, optimized method (3 μm column). DC is the sum of the desirability values obtained for α, R and k' in each experiment. *Values obtained experimentally. **Values calculated in the common scale. 1, 2, 3, and 4 correspond to adjacent consecutive peaks

[a] 0 values indicate completely overlapped peaks.

a value of 0.92; similarly, α for peaks 1 and 2 was 1.93 corresponding to a normalized value of 1; resolution for the same pair of peaks was 3.16 corresponding to a 0.81 value in the common scale.

Analytical separation was enhanced with the method developed; the desirability coefficient in chromatogram b resulted in a value of 4.59, compared with that obtained with the previously reported method of 2.56 (chromatogram a). Using the 3 μm column (Fig. 3c), this value was even higher: 6.12, which means an optimization of 140% with respect to the reference method. In this system, the resolution was greatly increased as can be seen. It is noteworthy that only in the new chromatographic condition (Fig. 2c), isoperoxisomicine A2 could be resolved and quantified; in the other two conditions (Fig. 2a and b) it coelutes with peroxisomicine A3.

Peak identification in each chromatogram was achieved by means of t_R as well as spectrum analysis. Peroxisomicines show almost identical spectra, whereas in the spectra obtained from isoperoxisomicines the maxima at 269 and 410 nm are shifted to longer wavelengths; furthermore, isoperoxisomicines present a third maximum at 310 nm, not present in the spectra of peroxisomicines.

3.2. Peak purity

Selectivity was tested by means of purity analysis of the chromatographic peak by means of spectra superposition and absorbance relationship at three different wavelengths, selected as explained before, through each chromatographic signal [16]. In the present case, impurities that might be found, could be the same compounds tested, arising from the extraction procedure. For this reason, the results of the purity test for each chromatographic peak in the mixture were compared with those obtained with the individual components. Peaks were considered pure when matched factors were superior to 99%.

3.3. Precision

The precision of the system expressed as coefficient of variation was lower than 1.17% for retention times. The C.V. for areas and heights range from 0.76 to 1.06 and from 0.07 to 1.47, respectively. This is an acceptable precision.

The C.V.s obtained for the analytical method were in the range of 5.70–9.15 for areas and 4.58–10.48 for heights (Table 4), which lies under the recommended value, namely 11, for the concentrations used in this research [17].

3.4. Linearity

A linear relationship (correlation coefficients ranging between 0.998 and 1.000) was found between

Table 4
Precision of the developed method; $n=4$

Compound	Area (mAU s) C.V.	Height (mAU) C.V.
PA2	9.15	10.48
PA1	9.10	10.78
IsoPA1	5.70	5.98
PA3	7.55	4.58
IsoPA2	8.84	6.46

Table 5
Regression equations for each compound tested

	Wavelength detection (nm)		
	269	310	410
PA1	$y = -14.0 + 10.35x$	$y = -0.70 + 1.26x$	$y = -1.10 + 2.26x$
PA2	$y = -0.10 + 10.37x$	$y = -2.70 + 1.24x$	$y = 2.40 + 2.28x$
PA3	$y = -11.20 + 8.88x$	$y = -1.60 + 0.93x$	$y = -4.60 + 1.91x$
IsoPA1	$y = -9.70 + 6.27x$	$y = -5.70 + 1.86x$	$y = 4.20 + 1.90x$
IsoPA2	$y = 0.50 + 3.77x$	$y = -1.60 + 0.93x$	$y = -3.50 + 1.10x$

y, Response; x, mass.

area and concentration in a range of 5 to 200 ng of injected mass at 269, 310 and 410 nm for all the compounds analyzed. In Table 5 the regression analysis for each compound can be seen. Taking into account the spectral characteristics of the compounds under study, three wavelengths were selected for the regression.

3.5. D_L and Q_L

In order to calculate D_L and Q_L, the method recommended by IUPAC does not take into account the method precision factor; the capacity of the instrument to produce instrumental noise is the unique factor taken into account; the values thus calculated are very small, due to the low noise level in the instrument used (Table 6). On the other hand, the values determined by means of the method based on the calibration curves, although high, are more realistic because the precision of the method is being considered (Table 7).

3.6. Robustness

The parameter chosen to evaluate robustness experiments was t_R, as it is involved with resolution

Table 6
Detection and quantification limits obtained from the standard deviation of the blank

Compound	Detection limits			Quantification limits		
	269 nm	310 nm	410 nm	269 nm	310 nm	410 nm
PA1	0.11	0.05	0.09	0.36	0.16	0.31
PA2	0.11	0.05	0.09	0.36	0.16	0.31
PA3	0.12	0.06	0.11	0.42	0.21	0.37
IsoPA1	0.18	0.03	0.11	0.59	0.11	0.37
IsoPA2	0.29	0.05	0.19	0.98	0.18	0.64

Table 7
Detection and quantification limits obtained from the residual deviation of the regression lines

Compound	Detection limits (ng)			Quantification limits (ng)		
	269 nm	310 nm	410 nm	269 nm	310 nm	410 nm
PA1	2.80	1.20	3.00	9.20	6.30	11.30
PA2	3.20	5.30	4.70	12.10	18.40	16.20
PA3	4.50	1.10	4.80	16.40	5.60	18.60
IsoPA1	0.90	0.70	1.80	10.50	5.50	3.80
IsoPA2	4.20	3.80	0.40	14.20	14.30	10.20

Table 8
Retention times obtained from experiments for robustness

Compound	Experiments								
	0	1	2	3	4	5	6	7	8
PA2	2.146	1.315	2.136	2.830	2.434	1.438	1.921	3.249	2.326
IsoPA1	3.443	2.141	3.163	5.418	3.092	2.203	3.768	5.434	3.997
PA1	3.889	1.895	3.225	4.521	3.891	2.200	3.118	5.769	4.039
IsoPA2	4.234	2.357	3.750	6.257	3.520	2.521	4.382	6.565	4.732
PA3	5.068	2.310	4.166	5.629	4.885	2.795	4.016	7.946	5.270

Experiment $0 = t_R$ obtained with the optimized method, C_{18} (3 μm). Wavelength, 410 nm.

and separation parameters. Changing some parameters, such as acetic acid concentration, results in a slight modification in t_R, these modifications were not significant. With regard to particle diameter, although alterations in the elution orders were previously shown (Fig. 2), the mathematical approach used here did not show significant difference; this could be explained because all the variables are taken into consideration at the same time. However, a significant difference change could be obtained when applying the following variables: flow-rate, AcCN amount and column temperature (Table 8).

4. Conclusions

This study describes the optimization and validation of a HPLC method, which allows for the first time the simultaneous quantification of minor components present in samples of the peroxisomicine A1, at present in use in a phase I clinical trial.

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