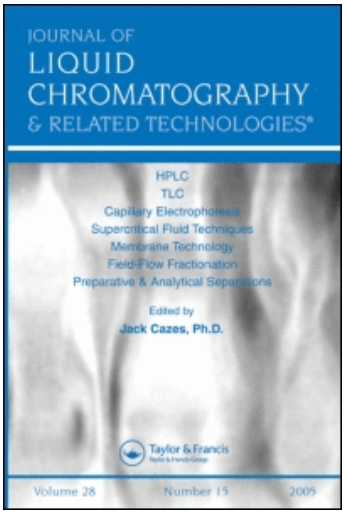

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A REVERSE PHASE HPLC METHOD FOR QUANTIFICATION OF PEROXISOMICINE AND OTHER ANTHRACENONIC COMPOUNDS

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ABSTRACT

An HPLC analytical system is described for the separation, identification, and quantification of Peroxisomicine A_1 (T-514) and related anthracenonic compounds.

Peroxisomicine is regarded as a potential antineoplastic agent. The system employed uses reverse phase (both C_8 and C_{18}) and diode array detection. The resolution, sensitivity, and reproducibility achieved make the system suitable to be employed for the analysis of plant extracts as well as biological fluids.

INTRODUCTION

Peroxisomicine A_1 (3,3'-dimethyl-3,3',8,8',9,9'-hexahydroxy-3,3' ,4,4'-tetrahydro-(7,10')-bianthracen-1,1'-(2H,2'H)dione) also known as T-514 (Fig.1) is a dimeric anthracenonic compound isolated from plants of genus *Karwinskia.*[1] The plants from this genus are characterized by the toxicity of its fruit upon ingestion.[2,3,4] Peroxisomicine A_1 exhibits selective *in vitro* toxicity on tumor cells,[5] suggesting that this compound could have a potential antineoplastic effect, and is currently under preclinical screening.

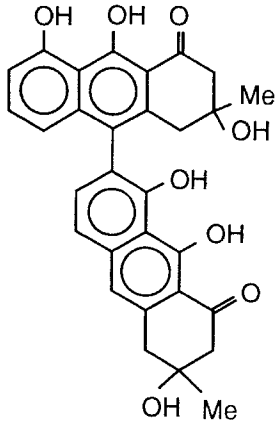


Figure 1. Structure of Peroxisomicine or T-514.

Peroxisomicine A_1 is found in the seeds of some species of the genus, especially in *K. humboldtiana* and K *parvifolia*.[6] From these plants, other secondary metabolites were isolated and identified. All of them are dimeric 9,10- dihydroxy anthracenones, which have been named according to their respective molecular weights. In *K. parvifolia* we reported five dimeric anthracenones besides Peroxisomicine A_1, namely: T-496 and T-516, previously described in *K. humboldtiana,* a diastereoisomer of Peroxisomicine A_1 (Diast T-514 or Peroxisomicine A_2), and, more recently, a third stereoisomer and a positional isomer of Peroxisomicine A_1, called Y and X respectively in this work. Details of the structures of these two compounds will be published elsewhere.

In order to solve the problems inherent to the production and quality control of this substance, we have considered necessary to have an analytical technique, with resolution, precision, sensitivity, and reproducibility. The proposed technique would be useful in investigating the pharmacokinetic studies of Peroxisomicine A_1.

Previous to this work, we reported the use of TLC (silica gel) and quantification by means of a densitometer,[7] taking advantage of the strong absorbance, in the visible range, of these type of compounds. Although this method proved to be useful for quantification of this and other anthracenones present in seeds and biological fluids,[8,9] we found severe difficulties when we tried to separate Peroxisomicine A_1 from other substances with similar Rf,

present also in the extracts. The errors inherent to quantification were not acceptable for our analytical requirements; the sensitivity was not adequate for studies on the mechanism of action of Peroxisomicine A_1; detection limit was 0.1 μg and calibration curves were linear in the range of 0.2-2 μg.

A spectroscopic method has also been reported to quantify another hydroxyanthracenone isolated from *K. humboldtiana* (the so called T-544 or tullidinol). This procedure has many interferences and the linearity reported is between 0.5 and 5 mM.[10]

Based on the physical properties, as well as spectral characteristics of Peroxisomicine A_1, we propose, in the present work, the use of the HPLC to solve the analytical problem. There is no previous report in the literature about the use of HPLC to analyze or separate dimeric anthracenonic compounds of this type.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol, and distilled water were HPLC grade from Aldrich Co. Acetic acid was purchased from Merck. Standards of Peroxisomicine A_1 and other anthracenonic compounds were isolated, purified, and identified in our laboratory by the procedures previously described.[6,7] Purity and identity was checked by chromatography, melting points, and spectroscopic data. Standards from these substances were prepared by diluting stock solutions of each compound (1 mg/mL in methanol) with methanol, HPLC grade. Due to instability of the compounds under investigation, the stock solutions were frozen at -20 °C and the dilutions were prepared just prior to the analysis.

Apparatus and Chromatographic Conditions

The HPLC system used was a Hewlett-Packard HP-1090 Series II/L. Samples of 5 μL were injected by an autoinjector onto one of the columns and conditions detailed as follows: a) Silica column (5μm, 300 x 0.4 mm); eluent: benzene/acetone (80:10),0.1% acetic acid; Flow 1 ml/ min.; b) bonded-phase CN-propyl column (5μm, 300 x 0.4 mm); eluent: CCl_4/ACN (98:2),0.1% acetic acid. Flow 1 mL/min; c) ODS-Hypersil column (5μm, 100 mm x 2.1 mm I.D.). Elution was accomplished as follows: 4 minutes isocratic with 35% solvent A, consisting of acetonitrile/water/acetic acid (30:70: 1.6) and 65% solvent B

(methanol); then a linear gradient was applied for 2 minutes to reach 100% B; during 2 minutes was maintained in 100% B and then return to the initial conditions in 2 minutes; an additional minute was left before applying the next injection. The flow was 0.4 mL/min; d) MOS Hypersil column (5 μm, 100 x 2.1 mm). Solvent systems were the same as in the ODS column, but the initial conditions were 50% B. All other parameters were similar. The temperature of the column was kept at 23° C in all cases.

All the mobile phases were filtered prior to use through compatible membrane filter (0.45 μm, Millipore). Signals were monitored by an HPLC 1090 photodiode-array detector with a main sample wavelength of 410 nm and a bandwidth of 10 nm. Signals were also recorded at 269 nm and 310 nm. with the same bandwidth. The reference wavelength was 550 nm with a bandwidth of 50 nm. Data were collected and analyzed on a HP HPLC 3D Chem Station, DOS series.

Preparation of Samples

For analysis of plant extracts, air dried ground fruits from *K. parvifolia* (0.5 g), were successively extracted with petroleum ether and ethyl acetate at room temperature by means of a stirrer, until the solutions appeared colorless. The ethylacetate extract containing the dimeric anthracenones was evaporated to dryness under N_2 (to prevent decomposition) and redissolved in methanol.

Total blood samples obtained from drug-free volunteers with EDTA were spiked with the adequate volume of a solution of Peroxisomicine A_1 (1 mg/mL), in order to obtain final concentrations of 5, 10, and 20 μg/mL; 0.3 mL of acetonitrile was added to 0.2 mL of these solutions, mixing thoroughly during 1 minute and centrifuged at 3000 rpm for 1 minute. The supernatant was isolated and filtered through Millipore filter, porosity 0.45 μm.

Analysis of Data

Results are expressed as mean ± SD and were statistically evaluated using the program "Mystat" for Macintosh. Linearity was assessed by means of regression square analysis for the calculation of correlation coefficients, slopes and intercepts.

Table 1

Retention Times of Dimeric Anthracenones from Genus *Karwinskia*

Compound	C_8 Column	C_{18} Column
Peroxisomicine A_2	1.66	1.64
Peroxisomicine A_1	2.65	2.43
T-516	2.85	2.42
X	2.55	2.85
Y	3.00	3.17
T-496	6.92	6.99

RESULTS

Analytical Separation

In order to choose an appropriate chromatographic system, we tested different stationary phases. Separation of the analyses required stationary phases that exhibited good resolution for qualitative and quantitative determination of the compounds of interest. Using silica, good resolution was obtained, but after several experiments, asymmetry in the peaks was observed, appearing from an excessive retention of compounds. With the normal bonded-phase (CN-propyl), good resolution was also obtained, both under analytical and in saturation conditions. The toxicity and cost of the solvents used were an inconvenience for their further use. With the reverse phase (Fig. 2) resolution, both under analytical and saturation conditions, was good and the time of analysis was only 10 minutes. We chose this system to continue our work; however, in order to identify some components in the plants, a C_8 phase was also needed, run under the same system of eluents as the C_{18}, but in different proportions. Retention times of the diverse anthracenonic compounds under analysis in both phases, are shown in Table 1.

Detection

The λ_{max} for these compounds lies between 260 and 270 nm, but we used another maximum at 410 nm to avoid interferences present in the biological matrix.

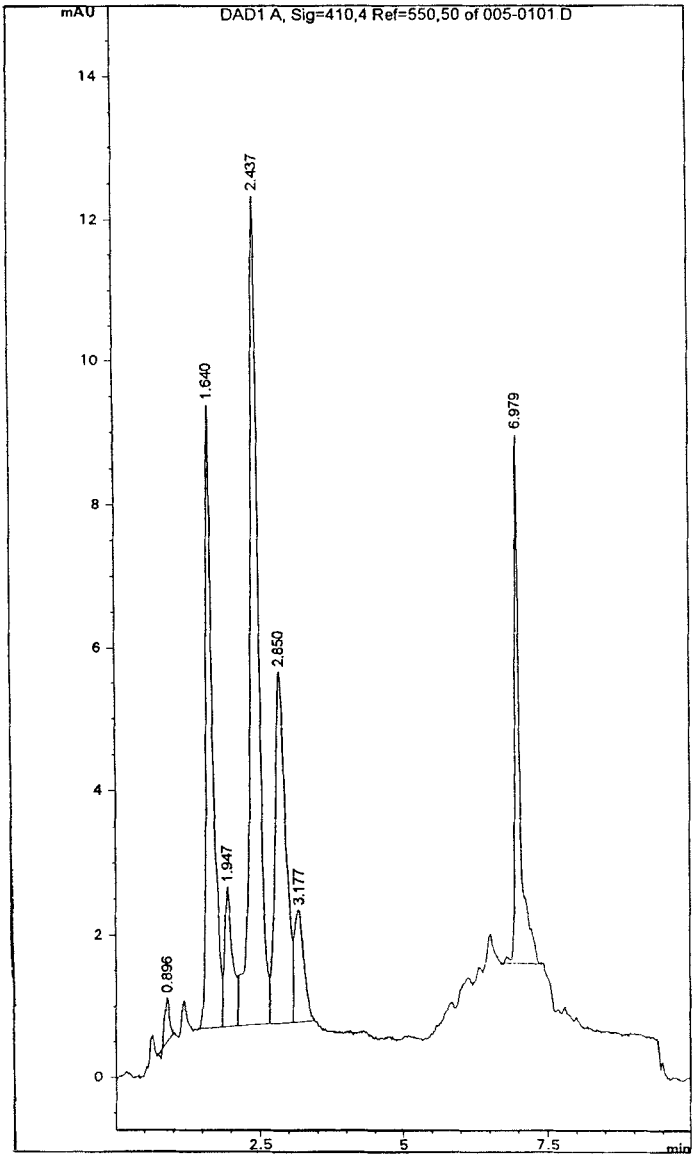
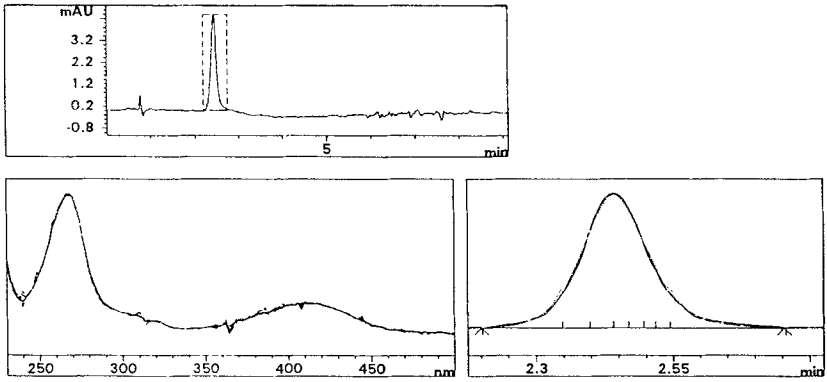


Figure 2. Chromatogram of an extract from seed of *K. parvifolia;* column ODS; peroxisomicine A_1 appears at retention time 2.43 min., peroxisomicine A_2 at 1.64 min; compound X at 2.85 min; compound Y at 3.17 min and T-496 at 6.98 min. Elution conditions are in the text.

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--> Spectral Data contains no impurity <--

Purity level   : 997.569  (mean, threshold 990)
Reference      : Nearest Peak baseline spectra (integrated)
PeakStart/End  : BaseLine / BaseLine
Spectra        : 7 (Range 5.4/21.3 mAU) (Selection set by user, 7)
Spectra        : 0 % below threshold of 990
```

Figure 3. Spectral purity analysis of peroxisomicine A_1, using diode array detector. Spectral range: 220-600 nm. Absorbance ratios were displayed at 269, 310 and 410 nm. The level of purity obtained for this fraction was 99.97%

In order to verify the purity of the standards used, the spectral purity was tested by a diode array system, by superposition of spectra in 7 different points of the chromatogram and calculating the absorbances ratios at 269, 310 and 410 nm. Fig. 3 shows the spectral purity analysis of the Peroxisomicine A_1.

In some instances, we complemented this analysis with the use of the C_8 system (Fig 4).

Precision

The precision of the chromatographic system was evaluated by injecting constant volumes (5 μL) of standards of Peroxisomicine A_1 in concentrations ranging from 1 μg/mL to 250 μg/mL. The coefficient of variation (CV) of retention times, areas, and heights are summarized in Table 2.

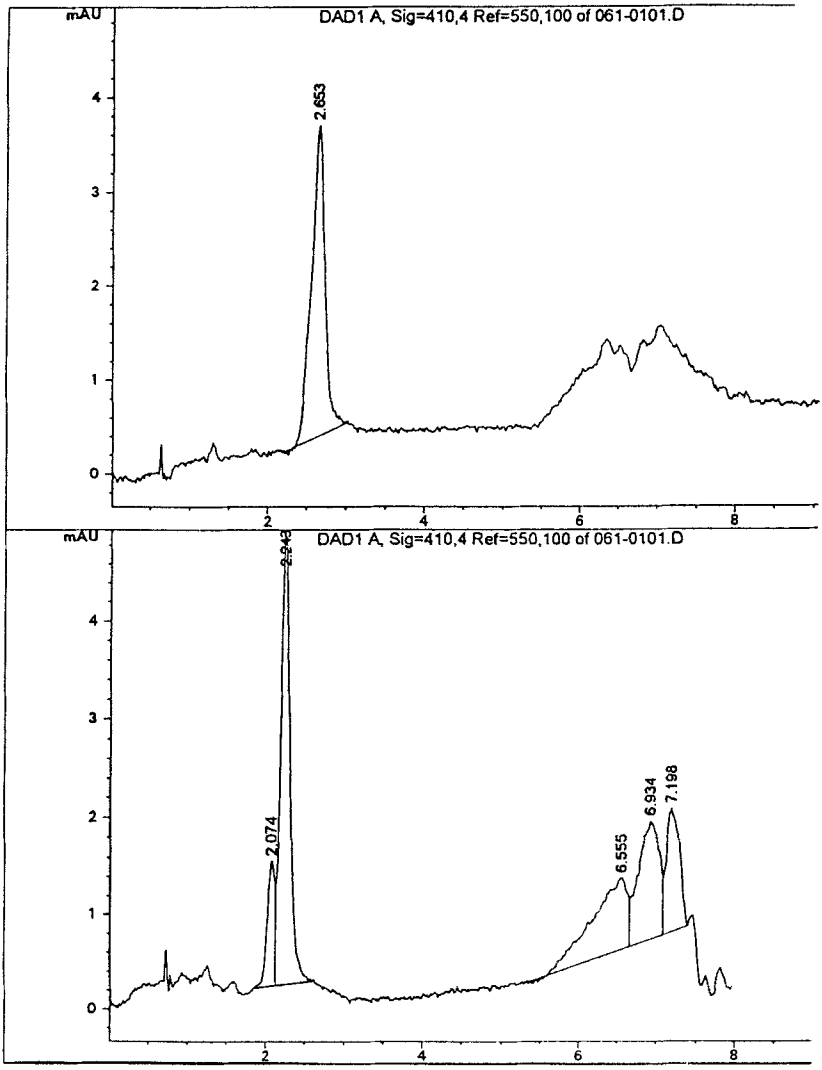

Figure 4. Chromatograms of the same fraction in C_{18} (upper) and C_8 (lower). Conditions as in text.

Linearity and Sensitivity

A linear relationship was obtained between area and concentration, within a range of 5-250 ng of injection. The assay linearity was checked by linear regression analysis of independent calibration curves. Using peak areas,

Table 2

Precision of Areas, Heights and Retention Times for Standards of Peroxisomicine A_1

	Mass	Area		Height		Ret. Time (min)	
n	(ng)	X	CV	X	CV	X	CV
6	5	11.82	10.8	--	--	2.41	0.2
10	30	54.13	8.8	5.96	10.4	2.41	0.1
9	50	71.78	5.2	8.10	6.0	2.39	0.2
10	100	126.62	1.8	14.16	2.4	2.38	0.1
6	400	791.56	1.4	76.29	1.1	2.41	0.1
6	1000	2132.49	1.0	167.43	0.5	2.43	0.1

Table 3

Recovery Rate of Peroxisomicine A_1 Added to Blood Samples

Conc (μg/mL)	n	Recovery (%)	S.D.	C.V.
5	5	81.84	4.38	6.56
10	3	83.58	2.55	3.05
20	3	82.60	2.87	3.51

correlation coefficients were computed as ranging between 0.998 and 0.999. The curves intercepts were -2.75 ± 1.05; slopes were 0.478 ± 0.02.

Recovery

Samples of blood, spiked with peroxisomicine A_1 in concentrations of 5, 10, and 20 μg/mL, were processed as described in Experimental. Percents of recovery obtained are shown in Table 3.

DISCUSSION

At present, there are no previous reports about HPLC analysis of hydroxyanthracenones. The method herein reported has the aim of achieving the requirements of sensitivity and resolution required for the analysis of the

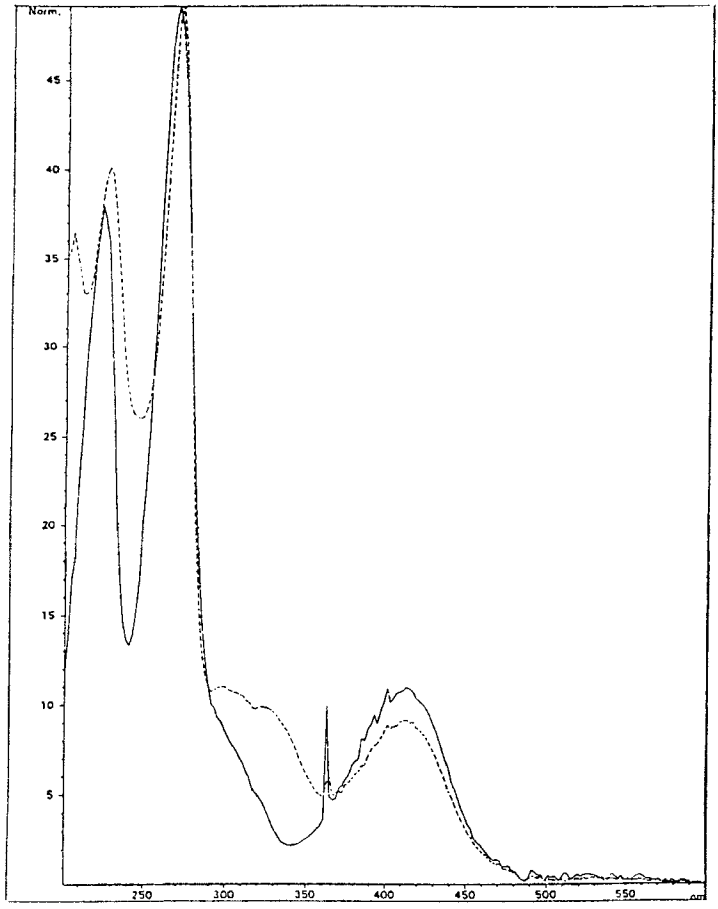

Figure 5. Spectra taken at two sites of the peak appearing in the upper chromatogram of Figure 4 (C_{18}) show the impurity present.

compounds of interest, as well in biological fluids as in plant extracts. It is also rapid, simple, accurate, and robust in application.

Separation

During the development of the methodology, we tried different solvent systems as well as different columns. Only the reverse phase systems (both C_{18} and C_8) exhibited satisfactory properties. It was necessary to use gradients for

the analysis of plant extracts because some of the compounds showed long retention times when the elution was isocratic. This is the case for one of the main components in seeds, the so called T-496, which can be eluted only when we achieve 100% methanol.

Symmetry, as well as width of the peaks, were significantly improved with the use of acetonitrile and acetic acid. The lengths of the columns used and the low flow rates (0.4 mL/min) increased sensitivity by using less solvent.

We also emphasize the importance of using two different chromatographic systems in order to separate all the components present. As is shown in Table 1, the compound X appears between Peroxisomicine A_1 and compound Y in C_{18}, while, in C_8, it appears before Peroxisomicine A_1; the order of elution of Y does not change. Similarly, T-516 is not well resolved from Peroxisomicine A_1 in C_{18}, but, in C_8, both compounds are properly separated.

Detection

Although satisfactory results can be obtained at a fixed λ, the diode array system presents the advantage of detecting at various λ simultaneously, helping us to assure identity and purity of the peaks. The relationships of areas and heights of the peaks at three different λ (410, 269 and 310 nm) as well as the analysis in two different systems, allowed the detection of impurities which elute sometimes near the compounds of interest (Figs. 4 and 5).

Quantification

We only report the results of quantification for the Peroxisomicine A_1. This is the compound of more interest in our laboratory due to its biological activity.

We tried to use an internal standard, and for that purpose we thought that Peroxisomicine A_2 was a good option, but the recovery was substantially less when we used this compound; for this reason we decided to use the external standard method. The lower limit of quantification was 5 ng for the Peroxisomicine A_1. If necessary, this limit can be easily improved by using UV detection or fluorescence instead of visible light. However, in this work we prefer the wavelength 410 nm because there are no interferences from the biological material.

A high recovery extraction procedure was a prerequisite to achieve the results required. We observed that the method proposed for the preparation of the sample prior to analysis and precipitation of proteins is advantageous due to its low cost, rapidity, small sample volume, and precision in the results.

We examined mixtures of acetonitrile/sample of 2:1 and 1.5:1. The results obtained by both methods were not significantly different, so we chose the last one which meant less dilution of the sample.

When applying the steps considered as optimal for sample extraction and chromatographic separation to blood analysis, all blank samples tested revealed no interferences with endogenous substances.

Applicability

The method described here is used currently to analyze plant extracts from genus Karwinskia (aerial and subterranian parts) as well as monitoring the purification procedures of Peroxisomicine A_1 and similar compounds.

The diode array detector, coupled with the chromatography system, is of great utility to assure purity of the different lots of peroxisomicines used in biological and clinical trials (Phase I).

We also propose this methodology as an alternative to the TLC method already described in order to analyze blood from people intoxicated with these plants.

Implementation of this method to the pharmacokinetics and metabolic studies of Peroxisomicine A_1 are currently in progress.

We are currently working on the conditions to scale up the procedure for the isolation and purification of Peroxisomicine A_1.

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