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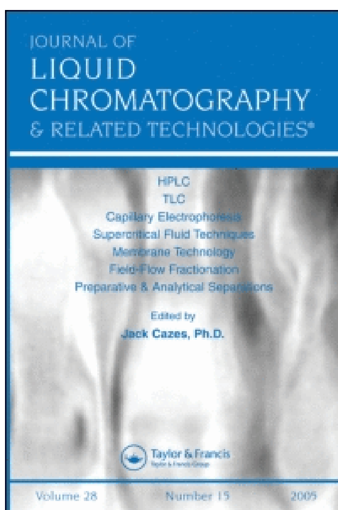

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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PURITY DETERMINATION OF PEROXISOMICINE A1 BY HPLC WITH A DIODE ARRAY DETECTOR

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Online publication date: 24 July 2002

To cite this Article Salazar, Ma. de La Luz , De Alba, Pedro Luis Lopez , López, Alfredo Pineyro and De Torres, Noemi Waksman(2002) 'PURITY DETERMINATION OF PEROXISOMICINE A1 BY HPLC WITH A DIODE ARRAY DETECTOR', Journal of Liquid Chromatography & Related Technologies, 25: 12, 1747 — 1760
To link to this Article: DOI: 10.1081/JLC-120005870
URL: http://dx.doi.org/10.1081/JLC-120005870

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
Vol. 25, No. 12, pp. 1747–1760, 2002

PURITY DETERMINATION OF PEROXISOMICINE A1 BY HPLC WITH A DIODE ARRAY DETECTOR

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ABSTRACT

A simple and reliable method for determination of isoperoximicine in peroxisomicine-contaminated batches was developed. The method consists of mathematical derivatization of zero order chromatograms, plus a standard addition technique required to increase the sensitivity of the method due to the small amounts of impurity present. Combination of both techniques offers a simple generalized methodology for determination of the final quality product, with potential application in the pharmaceutical field.

Key Words: Peroxisomicine; HPLC; DAD; Peak purity; Derivatization

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DOI: 10.1081/JLC-120005870 1082-6076 (Print); 1520-572X (Online)
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INTRODUCTION

HPLC analysis of systems containing compounds of similar physico-chemical characteristics has always been a difficult, expensive, time consuming task to accomplish, due to high peaks overlap. This challenging task becomes even more complicated if the number of compounds to be quantified or identified is considerably small relative to other components in the mixture. Examples of these systems are found everyday in the pharmaceutical industry, making it desirable to develop methodologies that allow a fast, reliable, transferable HPLC analysis. Several chemometric techniques have been applied to resolve partially overlapped chromatographic signals.[1,2,3] One of these methods is based on mathematic derivatization of the chromatograms,[2] producing a new graphic representation given by (d^nA/d^nt), where A represents the detector signal, t the elution time, and n the derivative order. In a first order derivative, the maximum signal points from the zero-order chromatograms will be represented by points where dA/dt is equal to 0, while the inflection points will be the maximum or minimum values in the first order derivative chromatogram. This mathematical tool allows resolving overlapped zero order chromatographic peaks and for quantifying and identifying compounds, which separation is poor by a given method. Such mathematical technique works well when the amounts of components with overlapped peaks are comparable, however, quantification problems arise when the amount of one component becomes comparatively higher relative to the other compound. This can also be the case even when peaks are well resolved. To eliminate such limitation, a standard addition method is applied.

Peroxisomicine A1, (3,3′-dimetil-3, 3′, 8,8′, 9,9′-hexahidroxi-3, 3′, 4,4′-tetrahidro-(7,10′)-biantracen-1, 1′-(2H, 2′H) dione) (Fig. 1), is a dimeric hydroxyanthracenone isolated from fruits of toxic plants from genus *Karwinskia.*[4] The compound belongs to a new class of topoisomerase II inhibitors,[5] which presents a high and selective toxicity toward liver and skin cell cultures, and is currently the subject of preclinical studies as an antitumor drug.[6] Despite all purification steps, final product batches of peroxisomicine A1 present small amounts of a natural impurity named isoperoxisomicine A1 (Fig. 1). Both compounds have similar chemical characteristics and present similar retention times in most HPLC methods studied[7] (Fig. 2), making the quantification and identification of isoperoxisomicine very difficult; consequently, the peroxisomicine purity analysis. In the present work, we applied derivative techniques with the standard addition approach to develop a quantification method for the determination of isoperoxisomicine A1 in batches of peroxisomicine A1. Additionally, chromatographic peak purity assessment was performed by means of the evaluation and optimization of analytical parameters.

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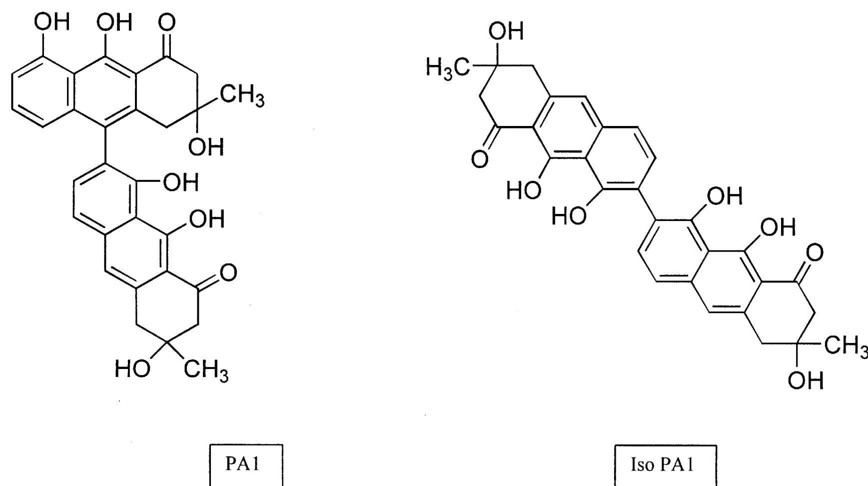

Figure 1. Structure of Peroxisomicine A1 (PA1) and Isoperoxisomicine A1 (Iso PA1).

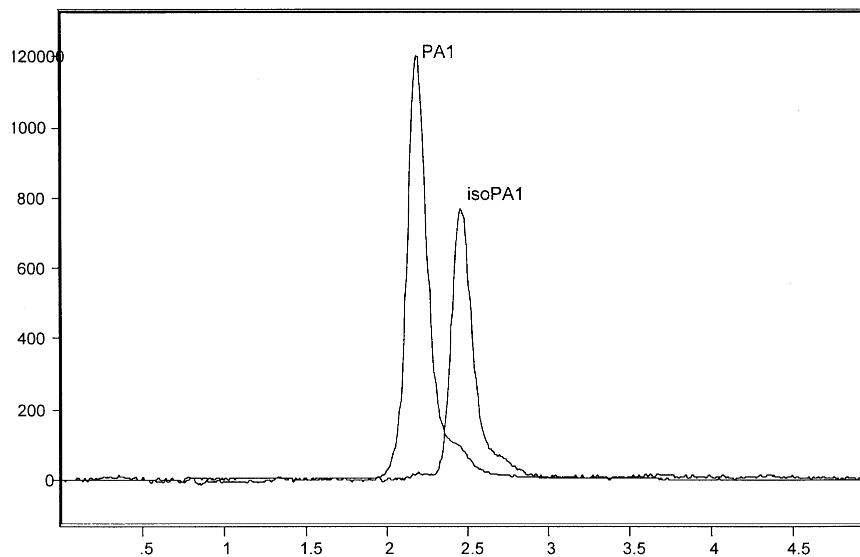

Figure 2. Overlaid chromatograms of PA1 and Iso PA1. Conditions are described in text.

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EXPERIMENTAL

Reagents

Peroxisomicine A1, peroxisomicine A2, and isoperoxisomicine A1 standards were isolated, purified, and characterized by NMR, HPLC, and UV-Vis spectrophotometry in our laboratory as previously described.[8] All solvents used were HPLC grade (Merck, Darmstadt)

Equipment

HPLC analysis was carried out on a HPLC, HP 1090 system with diode array detector in a Reverse Phase Hypersil ODS column 5 μm (100 × 2.1 mm).

Software

Peak purity analysis was carried out using HP HPLC-3D Chem Station DOS series software. Mathematical treatment of data such as derivatization, was done with Galactic Grams 32 v5 software (Salem, NH).

HPLC Separation Method

Mobile Phase A, methanol; Mobile Phase B, water: acetonitrile: acetic acid (60 : 40 : 1.6). Flow: 0.4 mL/minute.

Gradient elution method:

Time (min)	%B
0	40
4	0
6	0
8	40

All samples and mobile phases were filtered through Millipore HVLP filters with 0.45 μm pore size.

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Purity Analysis of Peroxisomicine

Peak purity experiments by HPLC were done by analyzing the presence of one major chromatographic peak, thus eliminating the possibility of interferences due to sample degradation or contamination, followed by signal spectral analysis using a diode array UV-Vis detector.

Prior to purity analysis of the peak, contour graphics were obtained from 220 to 600 nm. Peak purity analysis was done by three different methods: first, doing spectral matching at seven points along the chromatographic peak, three spectra on the upslope, one at the apex, and three on the downslope; second, by comparing the absorbance ratios between four different established wavelengths (269, 280, 310, and 410 nm); and third, by normalized overlaid chromatograms at 310, 410, and 269 nm. Samples were injected six times.

All spectra were corrected with references set at the baseline before and after peak integration. Method sensitivity was determined by comparing % purity results between unspiked and spiked samples with 2, 4, 6, 8, and 10% of isoperoxisomicine.

Purity optimization was done by analysis of experimental conditions, such as eluent solvent composition, pH and amount of sample, as well as of instrumental variables. The latter were optimized by following a Placket and Burman experimental design, and monitoring percent of purity as a response. Selected variables, as well as the evaluated values are shown in Table 1.

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Table 1. Parameters Considered for Ruggedness Evaluation in Chromatographic Peak Purity Analysis

Parameter	Present Value	High Value	Low Value
Wavelength range (L/l)	230–500	550	220
Spectra/peak (E/e)	7	11	5
References spectra (R/r)	Baseline	Near baseline	Apex
Spectra smoothing (S/s)	5 points	10 points	0 points
Reference spectrum (N/n)	Apex	All spectra peaks	Average Peak Spectra
Spectra to calculate threshold (U/u)	7	11	5
Time of sampling (C/c)	4	8	1

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IsoPA1 Quantitation by Internal Standard Method

Peroxisomicine A2 was used as the internal standard. To accomplish this, a calibration curve was constructed with variable concentrations of standard isoperoxisomicine (2, 4, 6, 8, 10%) added to a contaminated peroxisomicine A1 solution, with a final concentration of 100 μg/mL in methanol grade HPLC; to each sample was added 20 μL of a 100 μg/mL PA2 solution.

IsoPA1 Quantitation by Addition Standard Method

The addition standard method was applied to quantitate isoperoxisomicine in contaminated peroxisomicine A1 batches. To accomplish this, a calibration curve was constructed with variable concentrations of standard isoperoxisomicine (2, 4, 6, 8, 10%) added to a contaminated peroxisomicine A1 solution, with a final concentration of 100 μg/mL in methanol grade HPLC. Contaminant isoperoxisomicine A1 concentration was determined by the intersection at the *x*-axis after extrapolation of the fitted data to a straight.

Detection and quantification limits were calculated on the basis of the standard deviation of the response and the slope from the calibration curve data, as follows:

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

Where

σ: standard deviation of the response

s: slope of the calibration curve

Precision was calculated by means of variations in response peak after four consecutive injections from a methanolic solution of PA1 containing different proportions of isoPA1.

To improve resolution, contaminant quantitation was done by applying the addition standard technique to the first derivative chromatograms in the wavelength domain. To intensify the signal, an increment factor (5*x*) was applied.

RESULTS AND DISCUSSION

Due to the mixture complexity of peroxisomicine crude product, a final purification step by HPLC is required and final product quality is followed by means of melting point determination, normal and reverse phase thin layer chromatography, HPLC, and UV-Vis spectrophotometry. Specifically, spectrophotometric analysis is done by monitoring the compound absorbance at four different wavelength ratios. These techniques, as actually applied, allow for

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quality control performance over peroxisomicine batches; however, no appropriate quantitation of isoperoxisomicine A1 in peroxisomicine batches had been achieved up to now.

To overcome this problem, several techniques have been proposed focusing on resolving and increasing detection limits for isoperoxisomicine A1 when present as an impurity in PA1 batches. Among those techniques[9,10] are: spectral subtractions, derivative spectra matching, normalized spectra matching, time-dependant spectrophotometric analysis of the chromatographic peak, chromatogram comparison at different wavelengths, and comparison with spectral libraries that compare spectra of a given with a standard reference.

Purity Analysis of Peroxisomicine

Under given experimental conditions, peroxisomicine A1 and isoperoxisomicine A1 retention times were 2.23 and 2.52, respectively, with a 0.7 resolution factor. UV-Vis spectra of both analytes are shown in Fig. 3, where spectral differences can be identified, such as differences in the maxima signal (~ 270 and 410 nm) plus a 310 nm signal in the case of Isoperoxisomicine.

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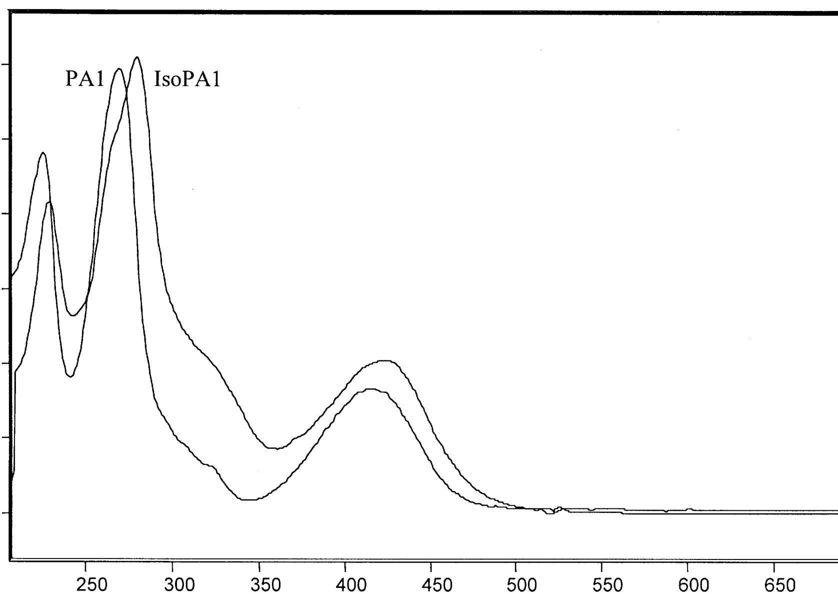

Figure 3. Overlaid spectra of PA1 and IsoPA1 standards captured from the apex in chromatographic peak.

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The contour plot did not show the presence of the impurity in the peroxisomicine A1 batch, however, it was detected by using spectra matching, as well as by absorbance ratios between different wavelengths at 269, 280, 310, and 410 nm (Fig. 4b) with major spectral differences observed at 260–310 nm (Figs 4a and 4c). Chromatograms matching at these same wavelengths showed a variation in the retention time and chromatographic peak shape at 310 nm, which is an indication of the presence of an impurity.[1] Analysis of the variables that affect method robustness showed that the amount of sample injected has statistically significant impact in the purity analysis over other experimental parameters (Table 2). On the other hand, the Placket and Burman design showed that the wavelength range included in the spectra, the number of spectra per peak, and the wavelength reference value are the major factors with the most weight in purity analysis (Table 3).

Although the presence of isoPA1 could be detected in concentrations as low as 0.7% in PA1 batches, no direct correlation could be obtained between % of spectral purity and isoPA1 added. Therefore, this method detects the presence of the impurity, but is without reliability in its quantitation (Table 4).

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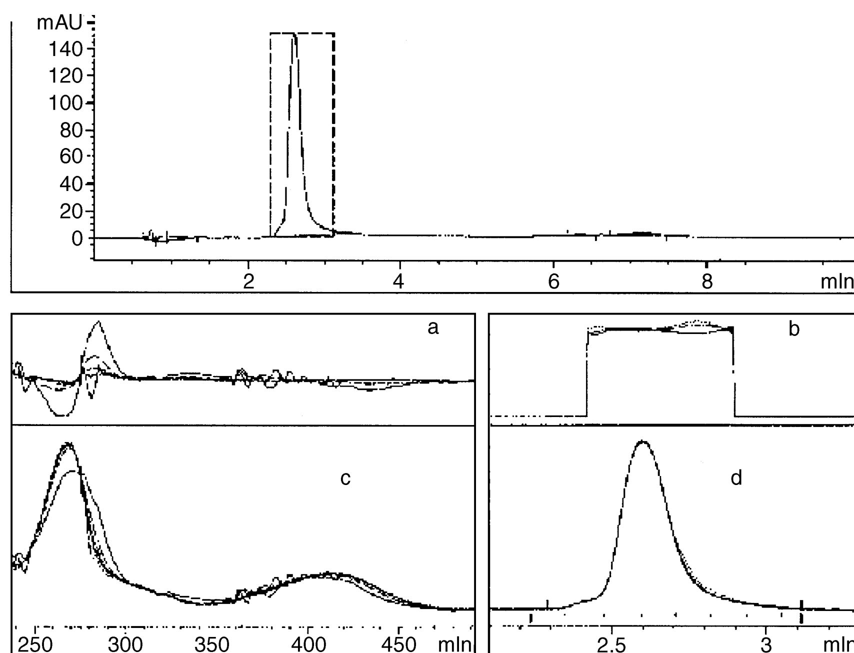

Figure 4. Purity analysis of a PA1 batch.

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Table 2. External Factors Affecting PA1 Chromatographic Peak Purity Analysis

Factor	Condition	Average Spectral Purity Level	%RSD
Injected mass	500 micrograms	97.9	2.7
	100 micrograms	95.5	4.6
pH of aqueous phase	3.2 (3.2% acetic acid)	97.2	3.5
	5.8 (0% acetic acid)	97.6	5.4
Mobile phase composition	65% methanol	97.3	3.0
	60% methanol	97.6	4.6

$N = 5$

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Isoperoxisomicine A1 Quantitation

Among the quantification methods here evaluated, the addition standard method showed the best results using derivative chromatograms. Isoperoxisomicine quantitation was first attempted by means of the internal standard method. For that purpose, peroxisomicine A2 was selected, as it has the requirements for being used as an internal standard, namely: similar response to the detector than the compounds under investigation and good resolution (t_R under experimental conditions 1.9 min). However, the results obtained by this procedure were not

Table 3. Response Differences for the Parameter Under Evaluation in Spectral Purity Analysis

	Higher	Lower	Difference
Wavelength	98.42	93.71	4.71
Spectra/peak	94.21	97.92	−3.71
References spectra	94.06	98.07	−4.01
Spectra smoothing	98.25	93.88	4.36
Reference spectrum	97.15	94.98	2.16
Threshold	94.88	97.25	−2.36
Time of spectral sampling	97.02	95.11	1.91

Precision average = 96.92.
$s = 1.75$.
If the difference between the higher and lower value obtained for each parameter is higher than $s*2^{1/2}$, the difference is significative.[11]

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Table 4. Correlation Between IsoPA1 Added and Spectral Purity of PA1 Chromatographic Peak

% IsoPA1 Added	% Purity
0	97.0
2	96.4
4	88.4
6	88.0
8	88.6
10	85.0

Threshold = 10 mAU

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satisfactory; therefore, isoperoxisomicine quantification was further accomplished by using the addition standard method and first derivative chromatograms, giving results comparable to those obtained by other reference techniques (0.7% by ^{1}HNMR, unpublished results) when applied to contaminated peroxisomicine batches (Table 5). Zero-order chromatograms showed an overvaluation of the results, probably due to the poor resolution of the peaks under investigation. The derivatization of the chromatograms showed a marked increase in peak resolution (Fig. 6) when compared to zero order chromatograms (Fig. 5). D_L and Q_L were 0.6 and 2.0 ng, respectively. %RSD obtained from the precision analysis were 18.4 and 4.6 for contaminated batches containing 0.78% and 6.6% isoPA1, respectively; the first sample being below the calculated QL, which accounts for the high %RSD found.

Table 5. Regression Analysis of IsoPA1 in PA1 Batches by HPLC. Standard Addition Method

Peak Integration	Regression Equation	Correlation Coefficient	% PA1
Zero-order spectra (height)	$Y = 3.552x + 9.604$	0.998	2.70
First derivative from 0 to maximum	$Y = 184.2x + 164.35$	0.998	0.89
First derivative from 0 to maximum	$Y = -160x + -316.4$	0.998	1.27
Maximum–minimum	$Y = 345x + 152$	0.996	0.44

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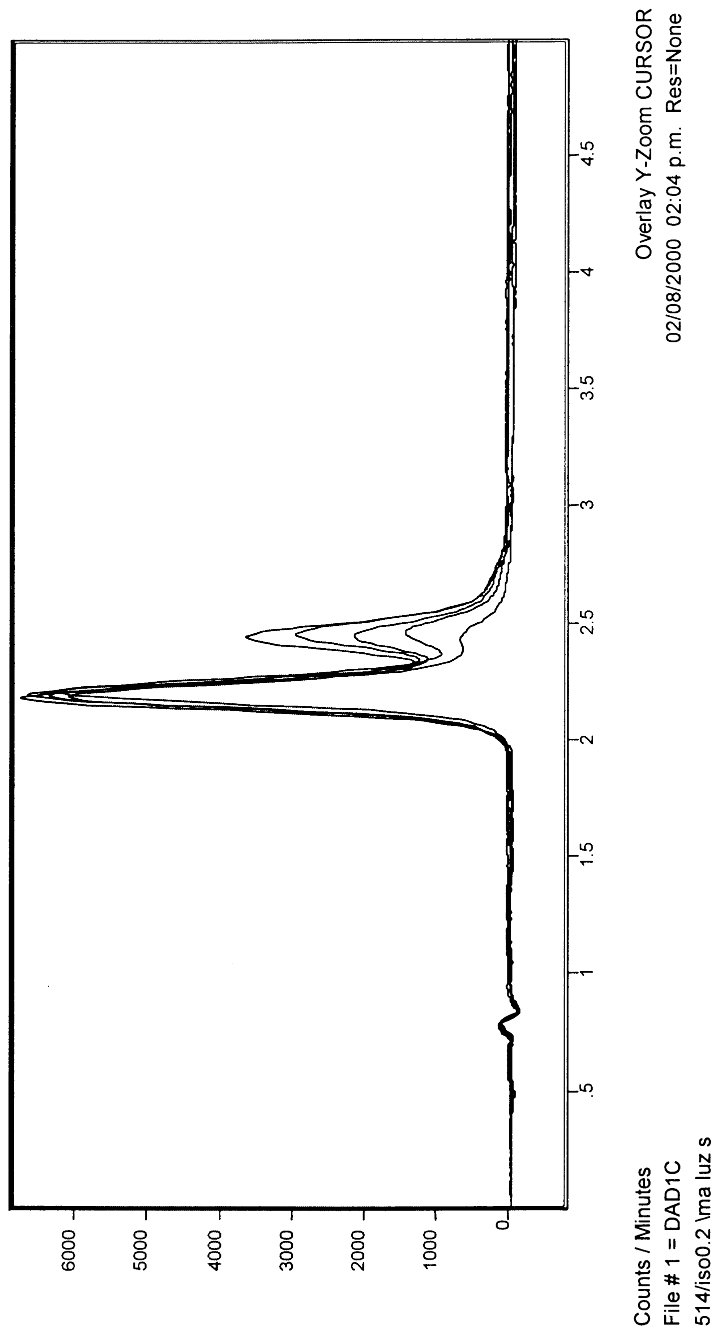

Figure 5. Overlaid chromatograms of IsoPA1 standards in PA1 matrix.

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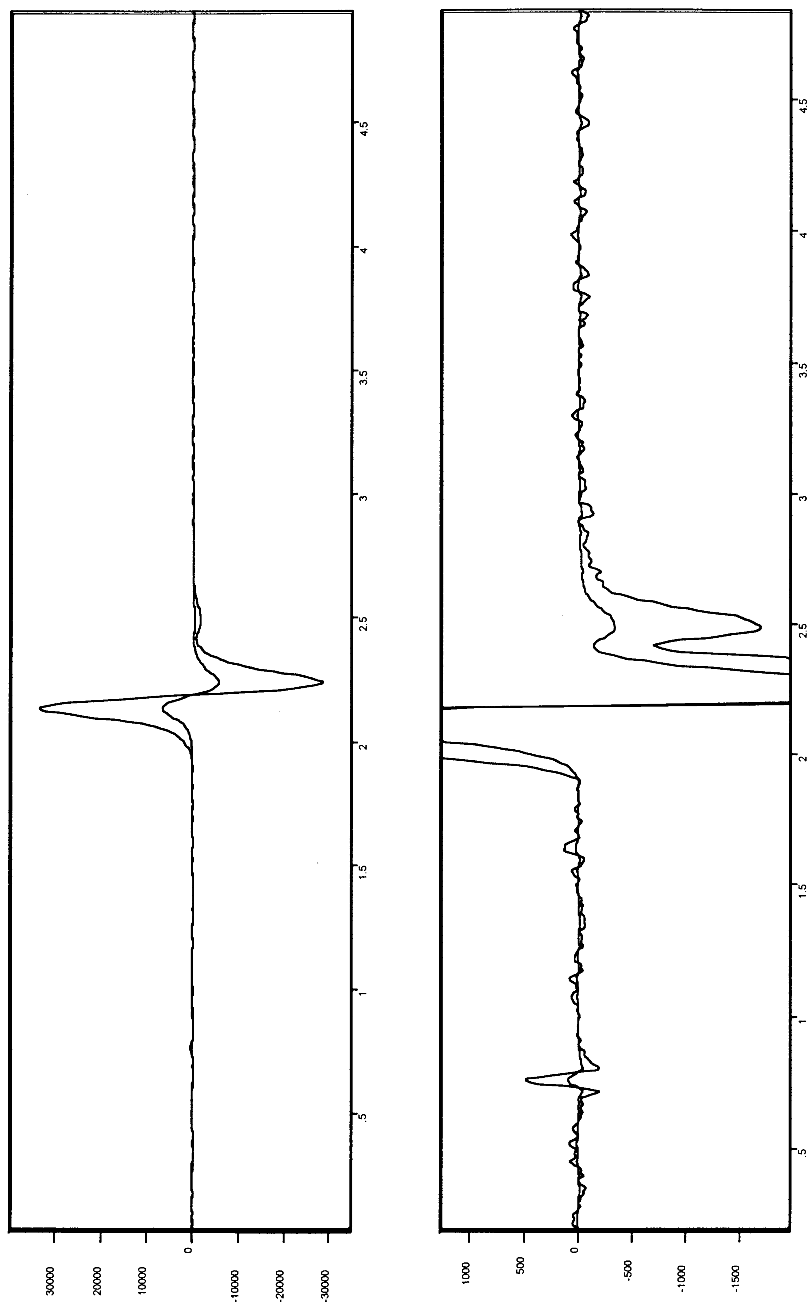

Figure 6. First derivatives chromatograms of IsoPA1 standards in PA1 matrix.

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CONCLUSIONS

It was possible to identify isoPA1 at relatively low concentration in PA1 batches through different spectral analysis methodologies, such as spectral matching, normalized chromatogram matching, and by absorbance ratio at 269, 280, 310, and 410. However, impurity quantitation was not accomplished due to the big difference in the concentration ratio between analyte and impurity. The addition standard method, when using derivative chromatograms, was successfully applied in the quantitation of the contaminant showing analytical characteristics comparable to those of standard methods.

ACKNOWLEDGMENTS

We thank Lucia Ceniceros for technical assistance, Prof. R.M. Chandler-Burns for the critical reading of MS, PAICYT for partial financial support through grant number CA-375-00, and CONACYT for financial support in the form of a fellowship for M.S. and grant number 26497-N.

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Received December 3, 2001
Accepted January 8, 2002
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