Current Chromatography, 2018, *5*, 104-111

RESEARCH ARTICLE

Development and Validation of a Novel HPLC Method for the Analysis and Quantification of Dehydroleucodine in Plant Extracts

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ARTICLE HISTORY

Received: September 27, 2018
Revised: November 07, 2018
Accepted: November 08, 2018

DOI:
10.2174/2213240605666181109154409

Abstract: ***Background*:** Dehydroleucodine is a sesquiterpene lactone isolated from *Artemisia douglasiana* Besser. Distinctive biological and pharmacological activities have been shown extensively for this molecule. Only one analytical method has been described for the quantification of dehydroleucodine in biological experimental systems. However, this assay involves fluorescent detection that is normally not recommended for routine analysis.

***Objective*:** The goal of this study was to develop and validate a novel methodology for rapid detection and quantitation of dehydroleucodine by HPLC with UV detection.

***Method*:** The method involved the use of a C_{18} separation column, an acetonitrile/water (80:20, v/v) solution as a mobile phase in an isocratic mode at a flow rate of 1 ml/min, and UV detection at 254 nm.

***Results*:** In the selected experimental conditions, dehydroleucodine exhibited a well-defined chromatographic peak with a retention time of 3.51 min. The chromatographic signal shows a linear dependence with the dehydroleucodine concentration. Correlation coefficient: 0.99. LOD: 1.5 ng/mL. LOQ: 15 ng/mL.

***Conclusion*:** The current method is simpler, faster, and cheaper than the previously reported one, and besides it could be useful to quantify this lactone and related pharmacological compounds. Furthermore, quality control of medicinal extracts of plant origin could be analyzed, and the concentration of the active constituent could be certified by this method.

Keywords: Dehydroleucodine, HPLC, matico, plant extracts, *Pseudomonas aeruginosa,* UV detection.

1. INTRODUCTION

A large variety of medicinal plants and bioactive compounds have been used extensively to treat various diseases since ancient times [1]. One of the most well-known preparations in Argentine folk medicine is an infusion of the leaves of *Artemisia douglasiana* Besser (Asteraceae family), popularly known as "matico" [2-6].

Dehydroleucodine, a sesquiterpene lactone of the guaianolide type (Fig. **1**), was isolated after a phytochemical study of this plant [3, 4]. Many animal models and *in vitro* experimental designs have been used in dehydroleucodine research. Distinctive biological and pharmacological activities have been shown for this lactone, including gastrointestinal cytoprotective effects [7-11], mast cell stabilizer action [12, 13], aromatase inhibitory activity [14], antioxidant properties [15, 16], adipocyte differentiation effects [17] and antimicrobial activities against *Pseudomonas aeruginosa* [18]. DhL also induces the activation of matured oocytes of *Rhinella arenarum in vitro* [19, 20] and the expression of TP73 and TP53 in human astrocytoma cells [21].

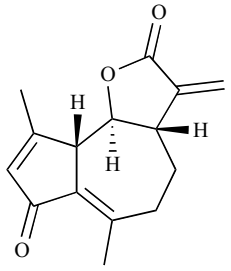

Fig. (1). Chemical structure of dehydroleucodine.

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2213-2414/18 $58.00+.00

Table 1. Regression data and analytical parameters for pure dehydroleucodine y = (3.16 ± 0.66) + (24.03 ± 0.18)x.

Parameter	Value
Slope (peak area)	24.03 ± 0.17
Intercept (peak area)	3.16 ± 0.65
Number of data points	8
Correlation coefficient (*r*) (peak area)	0.99
Limit of detection (LOD, ng/ml)	1.50
Limit of quantitation (LOQ, ng/ml)	15
Within-day reproducibility (retention time, R.S.D., %)	2.19
Within-day reproducibility (peak area, R.S.D., %)	3.29
Inter-day reproducibility (retention time, R.S.D., %)	2.27
Inter-day reproducibility (peak area, R.S.D., %)	4.18

Literature review reveals that only one analytical method has been described for the quantitative measurement of dehydroleucodine in biological experimental systems and plant extracts. This lactone can be measured by high performance liquid chromatography (HPLC) with postcolumn *o*-phthalaldehyde derivatization and fluorescence detection [22]. However, this assay involves fluorescent detection that is normally not recommended for routine analysis.

The goal of this study was to develop and validate a novel methodology for rapid detection and quantitation of dehydroleucodine by HPLC with UV detection in order to be applied to quantify this lactone in biological fluids and plant extracts.

2. RESULTS AND DISCUSSION

2.1. Chromatograms

Dehydroleucodine showed a well-defined chromatographic peak with a retention time of 3.51 min. Fig. (**2**) shows the chromatograms obtained from injection of standards at low (A), average (B), and high (C) concentrations of dehydroleucodine.

2.2. Method Validation

2.2.1. Precision

Repeatability (intra-day variation) and reproducibility (inter-day variation) were determined according to ICH (International Conference on Harmonization of Technical Requirements) and USP (United States Pharmacopeia) recommendations.

The within-day and inter-day assays were determined by injecting 6 replicate samples of dehydroleucodine standard at a 10 µg/ml level for over one year, and expressed as the relative standard deviation (R.S.D.): R.S.D.(%) = (standard deviation/mean of the peak areas) x 100 (Table **1**). Results from these assays demonstrated that signals were adequately reproducible to develop analytical applications.

2.2.2. Limit of Detection (LOD) and Limit of Quantification (LOQ) Values

The limit of detection (LOD) and limit of quantitation (LOQ) were defined for a signal/noise ratio ≥ 3 and ≥ 10, respectively. These were determined by analyses of an extensive calibration curve in the low concentration range (n=6). These parameters are shown in Table **1**.

2.2.3. Linearity

Several standard curves were prepared over a concentration range of dehydroleucodine as indicated. Data from HPLC peak area *versus* sesquiterpene lactone concentration plots were treated by linear least square regression analysis. Standard curves were evaluated for intra-day and inter-day reproducibility. Each experiment was repeated in triplicate.

The chromatographic peak showed a linear dependence with the concentration of dehydroleucodine, thus enabling the quantitation of this sesquiterpene lactone. The linear regression equation was y = (3.16 ± 0.66) + (24.03 ± 0.18)x; *r* = 0.99; y: peak area; x: concentration of dehydroleucodine.

For quantitation, the calibration plot method for a 10 mg/ml to 1 ng/ml concentration range of dehydroleucodine was used. Regression analysis showed a good linear relationship (r^2 = 0.99) over wide concentration ranges of 0.05 µg/ml to 1 mg/ml for dehydroleucodine; LOD was 1.5 ng/mL and LOQ of 15 ng/mL in less than 4 min with high accuracy. The regression parameters of the calibration curve are shown in Table **1**.

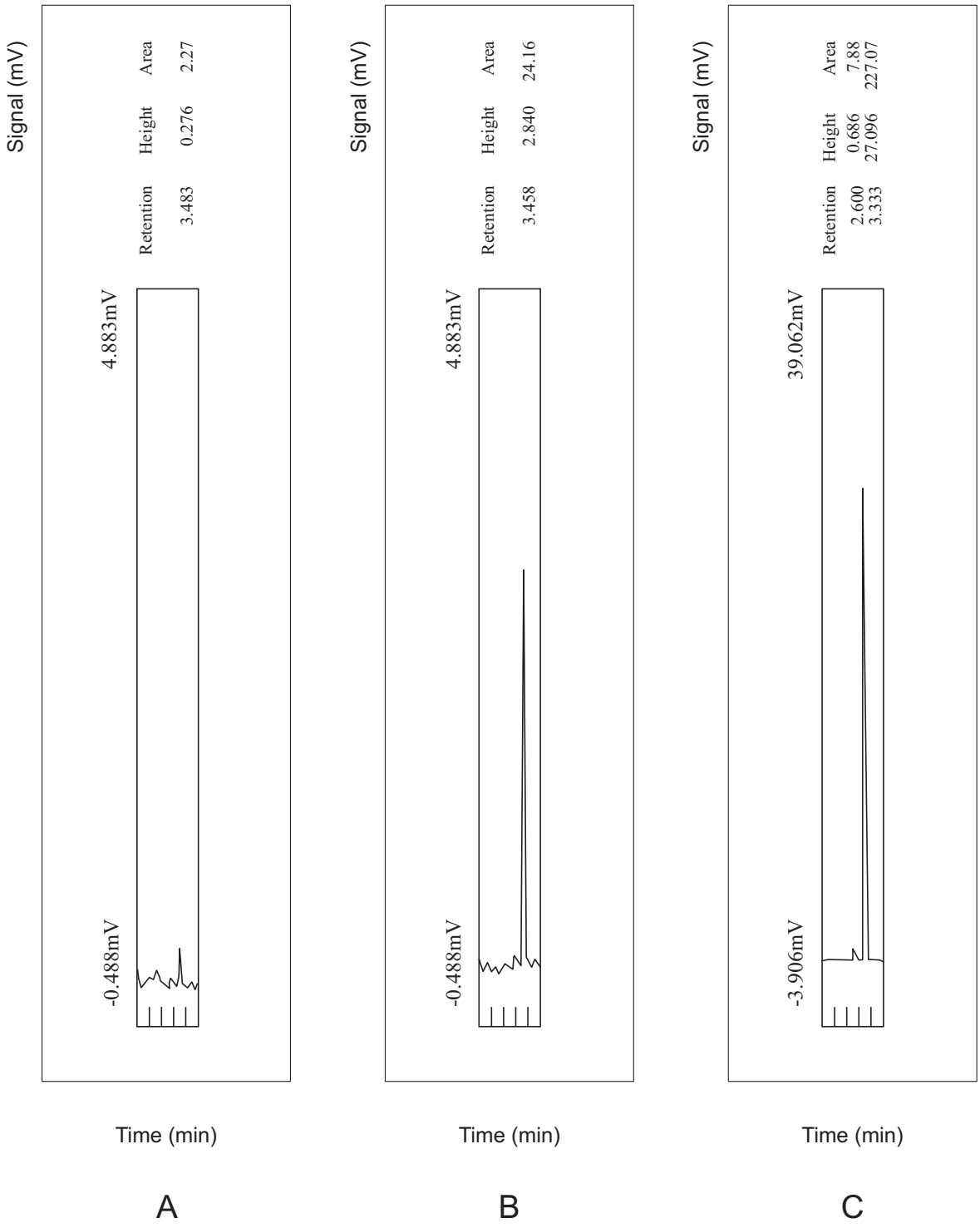

Fig. (2). Chromatographic profiles from dehydroleucodine standards. (**A**) 0.1 µg DhL/ml; (**B**) 1 µg DhL/ml; (C) 10 µg DhL/ml.

2.2.4. Stability Tests

When dehydroleucodine solutions were stored at -20ºC for 40 h, 4ºC for 40 h, 24ºC for 40 h, 24ºC for 40 h in constant darkness, and at 37°C for 10 min, no changes in the chromatograms were observed, evidencing that the compound was not affected under such conditions. A significative increase (50%) of the UV absorbance was registered when dehydroleucodine solutions were treated at high power level (100%) in a microwave oven for 10 sec. The effect of pH on dehydroleucodine stability was also evaluated in dehydroleucodine standard solutions (6.67 mg/ml). Dehydroleucodine samples were treated with: (1) 6 N HCl (pH < 1) for 10 min, or (2) 2 N NaOH (pH > 10). The effect of pH on

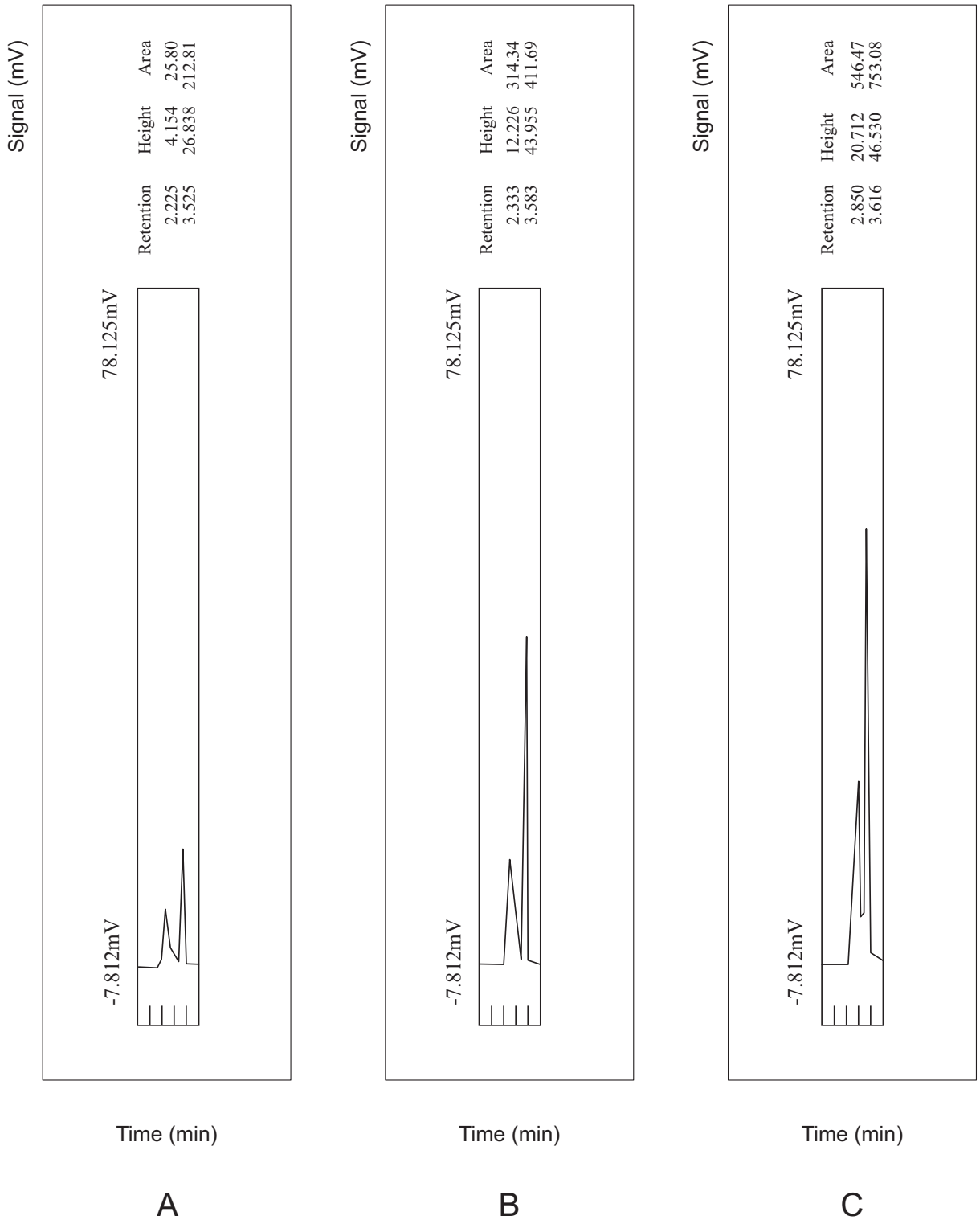

Fig. (3). Chromatographic profiles from *Artemisia douglasiana* Besser extracts. (**A**) 7.5 mg air-dried material/ml; (**B**) 15 mg air-dried material/ml; (**C**) 30 mg air-dried material/ml.

stability was assessed in dehydroleucodine standard solutions (6.67 mg/ml). Absorbance from dehydroleucodine samples treated with either 6 N HCl (pH < 1) for 10 min or 2 N NaOH (pH > 10) was significantly decreased (100%).

2.2.5. Application: Analysis of Herbal Extracts

Extracts from *Artemisia douglasiana* Besser were analyzed in order to demonstrate the applicability of the method. Extracts were processed and analyzed by HPLC as described

above. Chromatograms obtained by injection of samples containing low (A), average (B), and high (C) extract concentrations are shown in Fig. (**3**). Dehydroleucodine from the extracts exhibited a well-defined chromatographic peak with a retention time of 3.50 min. No endogenous compounds interfered with the detection of the sesquiterpene lactone. The absolute recovery of dehydroleucodine from plant extracts was found to be 95% (high accuracy).

When extract solutions were stored at 4ºC for 40 h, 24ºC for 40 h, and 24ºC for 40 h in constant darkness, no changes in dehydroleucodine peaks were observed, evidencing that the sesquiterpene lactone was not affected under such conditions.

2.2.6. Comparison of HPLC Methods

In 2003, we first reported the analysis of dehydroleucodine by HPLC using post column derivatization and fluorescence detection [22]. The method involved an efficient extraction procedure, the use of a strong cation exchange column, a 200 mM KH_2PO_4 solution as a mobile phase in an isocratic mode at a flow rate of 0.55 mL/min, post column *o*-phthalaldehyde automatic derivatization, and fluorescence detection (excitation at 360 nm and emission at 450 nm) [22]. Under the experimental conditions, dehydroleucodine showed a well-defined chromatographic peak with a retention time of 7.73 min. Linearity ranged from 100 to 500 µg/mL, LOD was 1.71 ng/mL, and LOQ 17 ng/mL in less than 8 min with high accuracy.

The current approach is now faster [t_R (fluorescence) *ca.* 8 min, and t_R (UV detection) *ca.* 3 min]. Furthermore, sample preparation is easier because of deleting sample derivatization required for fluorescence detection.

This approach uses UV detection that is more accessible for laboratories than fluorescence detection and post column derivatization. The method is highly sensitive with a LOD of 1.50 ng/mL and LOQ of 15 ng/mL in a run of less than 4 min with high accuracy.

Regression analysis showed a good linear relationship over wide concentration ranges of 0.05 µg/ml to 1 mg/ml for dehydroleucodine.

3. EXPERIMENTAL

3.1. Instrumentation and Analytical Conditions

The HPLC analyses were carried out on a Series III solvent delivery system (Konik Instruments, USA), a C6W Valco manual injector with a 10-µl sampling loop (Isco, USA), and a V^4 UV-absorbance detector (Isco, USA) at 254 nm. A 10-µl sample was injected onto a Microsorb-MV C_{18} column (4.6 × 250 mm ID, 5 µm particle size; Varian, USA) using a solvent composition of acetonitrile/water (80:20, v/v) at a flow rate of 1 mL/min. The mobile phase was filtered through a 0.22 µm Millipore filter under vacuum, and degassed before use. All chromatograms were recorded and analyzed using the Peaksimple II computer program (Isco, USA). Immediately after preparation, vials containing standard or extract solutions were refrigerated and covered with aluminum foil to protect from light. Total run time was 5 min.

3.2. Reference Compounds and Solvents

A standard of dehydroleucodine was kindly provided by Prof. Carlos Tonn [INTEQUI, Química Orgánica, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis (UNSL), San Luis, Argentina]. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was distilled, deionized, and further purified *via* a Milli-Q system (Millipore, Milford, MA, USA). All solutions were filtered through a 0.22 µm Millipore filter before injecting into the HPLC equipment.

3.2.1. Extracts of Artemisia Douglasiana

Artemisia douglasiana Besser was collected in the mountains of San Luis province, Argentina. This species was identified by Prof. Luis A. Del Vitto (voucher specimen UNSL no.55, Herbarium of the UNSL). The air-dried material (3 g) was extracted with 100 ml of hot water for 10 min (extract stock solution: 30 mg/ml). Extract solutions were obtained by diluting the extract stock solution with Milli-Q water (15 mg/ml and 7.5 mg/ml). Aliquots of the stock solution were stored at -80ºC for up to one year. All solutions were filtered through a 0.22 µm Millipore filter before injecting into the HPLC apparatus.

3.2.2. Isolation and Purification of Dehydroleucodine

Dehydroleucodine was isolated from extracts of *A. douglasiana* as previously described [3,12]. Briefly, the air-dried material was soaked in $CHCl_3$ at room temperature [3,12]. The extracts were evaporated in vacuum, and dissolved in 95% ethanol. After addition of 4% aqueous lead tetraacetate solution, the aqueous cloudy solution was filtered through a celite pad, and the filtrate was concentrated under vacuum [3, 12]. The mixture was extracted three times with $CHCl_3$ and the solution was concentrated under vacuum [3, 12]. The final residue was chromatographed in a medium-pressure chromatography system using ethyl acetate/*n*-hexane (1:9, v/v) as eluent. Dehydroleucodine (100% purity) [mp. 128-130°C (colorless crystals; EtOAc); $[\alpha]^{25}_D$ +48.98º (*c* 1.42, CH_2Cl_2)] was identified by ^{1}H- and ^{13}C-NMR (nuclear magnetic resonance), and mass spectrometry [3, 12].

3.2.3. Stock and Standard Solutions

A stock solution of dehydroleucodine (10 mg/ml) was prepared by sonication of dehydroleucodine in dimethyl sulfoxide (DMSO) at room temperature for 2 min. No signal was detected when injecting DMSO into the HPLC chromatograph under the chromatographic conditions used. Aliquots (10 µl) of the stock solution were stored at -80ºC, and were stable (less than 5% loss) for at least 1 year. Freshly prepared standard solutions were obtained by diluting the stock solution with mobile phase (100 µg/ml to 1 ng/ml). All solutions were filtered through a 0.22 µm Millipore filter before injecting into the HPLC equipment.

Table 2. Comparison of the proposed method (UV detection) to other published method (fluorescence detection) [22].

Parameter	UV	Fluorescence
Retention time (min)	3.51 ± 0.01	7.73 ± 0.04
Slope (peak area)	24.03 ± 0.17	103209.88
Intercept (peak area)	3.16 ± 0.65	2305943.4
Number of data points	8	8
Correlation coefficient (*r*) (peak area)	0.99	0.99
Limit of detection (LOD, ng/ml)	1.50	1.71
Limit of quantitation (LOQ, ng/ml)	15	17
Within-day reproducibility (retention time, R.S.D., %)	2.19	0.52
Within-day reproducibility (peak area, R.S.D., %)	3.29	2.05
Inter-day reproducibility (retention time, R.S.D., %)	2.27	0.96
Inter-day reproducibility (peak area, R.S.D., %)	4.18	7.95

3.2.4. Calibration Curves

Calibration curves were constructed at the following concentration range of dehydroleucodine: 1000, 1000, 100, 10, 6.67, 1, 0.1, 0.05, 0.01, and 0.001 µg/ml. A 10 µl volume of each standard solution was injected (n=8) onto the HPLC column. Calibration curves were constructed by plotting the peak area *versus* the concentration of dehydroleucodine. Data for standard curves were subjected to linear regression analysis. The squared correlation coefficient was used to evaluate the linearity of the calibration curves.

3.2.5. Assay Validation

To determine the intra-assay and inter-assay precision, the retention time and peak area for dehydroleucodine were analyzed in 6 replicates of dehydroleucodine standards at a 10 µg/ml level for over one year.

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the ratio of signal/noise ≥ 3 and ≥ 10, respectively. These were determined by analyses of an extensive calibration curve in the low concentration range (n=6).

3.2.6. Stability Tests

The stability of the sesquiterpene lactone in solution was tested by standing standard samples (6.67 mg/ml) at -20ºC over 40 h, 4ºC over 40 h, 24ºC over 40 h, 24ºC over 40 h in constant darkness, 37°C for 10 min and high power level (100%) in a microwave oven for 10 sec. The effect of pH on dehydroleucodine stability was also assessed in standard solutions (6.67 mg/ml). Dehydroleucodine samples were treated with: (1) 6 N HCl (pH < 1) for 10 min, or (2) 2 N NaOH (pH > 10) for 10 min.

3.2.7. Application: Analysis of Herbal Extracts

To demonstrate the applicability of the method, extracts from *Artemisia douglasiana* Besser were analyzed. The stability of dehydroleucodine in these extracts was tested by storing extract samples (30 mg/ml) at 4ºC for 40 h, 24ºC for 40 h, and 24ºC for 40 h in constant darkness.

The absolute recovery of dehydroleucodine from plant extracts was estimated by comparing the peak height obtained from injections of standard solutions with those ob-

tained from injection of plant extracts spiked with known concentrations of dehydroleucodine.

CONCLUSION

HPLC has been selected for the analysis of dehydroleucodine because as a sesquiterpene lactone is known to have low volatility and thermolability. Therefore, reversed HPLC is the method of choice. Furthermore, UV is a suitable detection for dehydroleucodine because of having *alpha*,*beta*-unsaturated carbonyls in a cyclic ketone and a lactone group as chromophores.

The excellent sensitivity, wide linearity range, and good overall reproducibility may be useful for the detection and reliable determination of dehydroleucodine in biological matrices, even at very low concentrations.

The current method is simpler, faster, and cheaper than the previously reported one, and besides can be used to quantify this sesquiterpene lactone and related pharmacological compounds (Table **2**). Furthermore, quality control of medicinal extracts of plant origin can be analyzed, and the concentration of the active constituent certified by this method.

LIST OF ABBREVIATIONS

HPLC	=	High Performance Liquid Chromatography
DhL	=	Dehydroleucodine
UV	=	Ultraviolet
OPA	=	*o*-phthalaldehyde
DMSO	=	Dimethyl Sulfoxide
LOD	=	Limit of Detection
LOQ	=	Limit of Quantitation
RSD	=	Relative Standard Deviation
r	=	Correlation Coefficient

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) and Universidad Nacional de Cuyo (Argentina). We thank Prof. Dra. Alicia B. Pomilio and Arturo Vitale (Universidad de Buenos Aires, Argentina) for their careful reading of our manuscript and their many insightful comments and suggestions. Thanks are also due to the Ministerio de Ciencia y Técnica (Mincyt, Argentina) for electronic bibliography facilities. AB Penissi is a Research Member of CONICET.

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