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Short communication

Trace quantification of 1-octacosanol and 1-triacontanol and their main metabolites in plasma by liquid–liquid extraction coupled with gas chromatography–mass spectrometry

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ABSTRACT

A method for the simultaneous determination of 1-octacosanol and 1-triacontanol and their main metabolites in rat plasma was developed. The procedure involved ethanolic NaOH saponification of the sample, acidification, liquid–liquid extraction, and derivatization of the analytes to its trimethylsilylether/ester, followed analysis by gas chromatography–mass spectrometry (GC–MS) in selected ion monitoring (SIM) mode. Quantification was performed by the internal standard method using betulin. The method had a good linearity over the range 8.4–540 ng/ml ($r \geq 0.998$) and showed an excellent intra-day (R.S.D. = 0.59–3.06%) and inter-day (R.S.D. = 2.99–5.22%) precision according to the acceptance criteria. The detection limits ranged between 1.32 and 3.47 ng/ml. The method was applied successfully to study the total plasmatic concentration of 1-octacosanol, octacosanoic acid, 1-triacontanol, and triacontanoic acid, after an oral dose of policosanols mixture, using plasma samples of 100 μl.

1. Introduction

Policosanols are a mixture of primary aliphatic alcohols mainly derived from the waxy coating of sugarcane (*Saccharum officinarum* L.), being 1-octacosanol and 1-triacontanol the major components (Fig. 1A) [1]. The utilization of policosanols in the treatment and prevention of cardiovascular diseases seems to be an important pharmacological action, as shown by the hypocholesterolemic effects reported in studies in animal models [2], healthy human subjects [3], and patients with either type II hypercholesterolemia [4], type II diabetes [5], post-menopausal women [6], or with liver dysfunction [7]. Although recent studies have been unable to reproduce the hypocholesterolemic effects of these mixtures [8,9], there is general agreement that policosanols are safety drugs, without deleterious side effects [10].

In vivo, the dominant route of policosanol metabolism is oxidation to fatty acids [11], in which octacosanoic acid and triacontanoic acid (Fig. 1A) are the main metabolites of 1-octacosanol and 1-triacontanol, respectively. In agreement with this view, the formation of octacosanoic acid and other saturated fatty acids has been detected in plasma of monkeys fed orally with octacosanol [12].

The developed techniques to determine policosanols in plasma include measurements of 1-octacosanol by an analytical procedure of solvent extraction and gas chromatography after its intravenous infusion, validated in the range of 50–2000 ng/ml [13], and a solid phase extraction coupled with gas chromatography, in the low nanogram range [14]. The above studies present the disadvantages that (a) the procedure allows only the quantification of free octacosanol in plasma, assuming that this alcohol is not esterified with fatty acids in blood circulation; (b) policosanol metabolism includes the assembly of their components into lipoprotein particles to reach blood [11], therefore 1-octacosanol recuperation from plasma could be affected by subjecting the sample to protein precipitation [14]; and (c) the use of gas chromatography with flame ionization detector (GC-FID) for complex biological samples [13,14] requires an exhaustive isolation of the trace analytes.

According to the above considerations, this work describes a simple and reliable method for the simultaneous determination of 1-octacosanol and 1-triacontanol and their main metabolites octacosanoic acid and triacontanoic acid in rat plasma. This involves saponification to hydrolyse the ester linkages in the biological matrix, similar to the procedure described for measuring cholesterol and its precursors in rat plasma [15], acidification of the samples, followed by liquid–liquid extraction and quantification by gas chromatography–mass spectrometry (GC–MS) using selected ion monitoring (SIM) mode. GC–MS avoids the uncertainty of the identification of the compounds of interest and provides adequate

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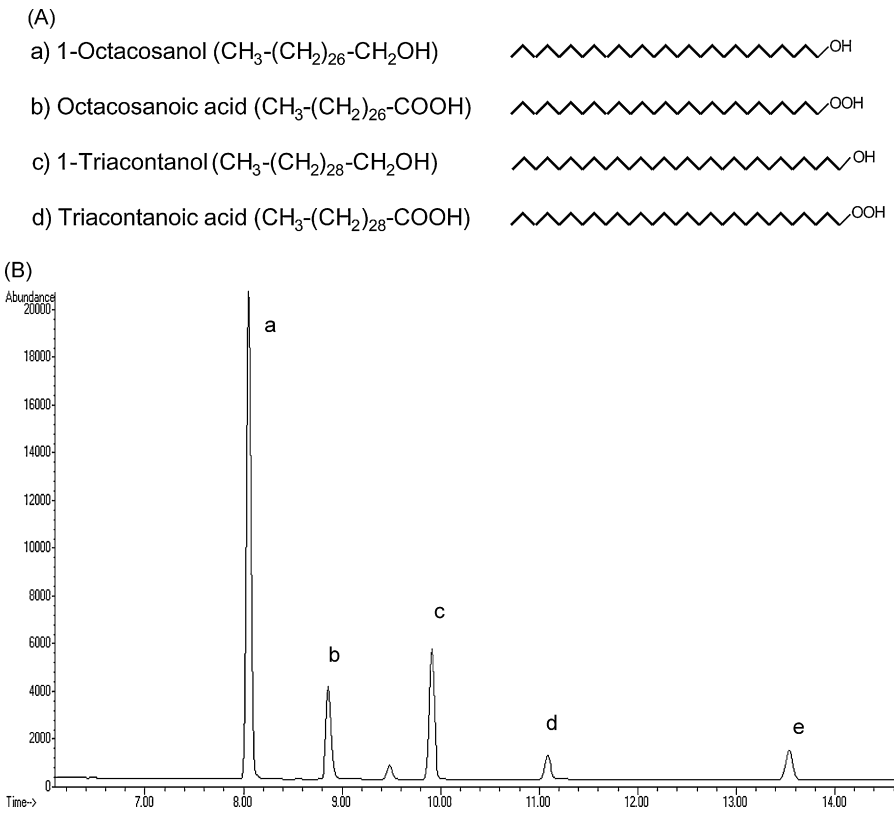

Fig. 1. (A) Structures of (a) 1-octacosanol, (b) 1-octacosanoic acid, (c) 1-triacontanol, and (d) triacotanoic acid. (B) GC–MS/SIM chromatogram of rat plasma after a single oral dose of policosanols (100 mg/kg), and spiked with the internal standard betulin (e).

sensibility using one tenth (100 μl) of the plasma volume (1 ml) needed in GC-FID [13,14].

2. Experimental

2.1. Reagents

The standards 1-octacosanol, 1-triacontanol, octacosanoic acid, triacontanoic acid, the internal standard betulin, and the silylation reagent N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (99% GC purity), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Grade p.a. sodium hydroxide, hydrochloric acid (HCl), ethanol, and heptane were obtained from Merck KgaA (Darmstadt, Germany). Policosanol mixture 96% purity (62% 1-octacosanol, 19% 1-triacontanol) was purchased from Pharmapia International (Shanghai, China).

2.2. Preparation of standard solutions

The development and validation of the procedure was carried out in model samples subjected to the procedure described in the section below. Betulin was used as internal standard, whereas 1-octacosanol, 1-triacontanol, octacosanoic acid, and triacontanoic acid were the target analytes. Stock solutions were prepared by dissolving 10 mg of the analytes in 100 ml of heptane, and aliquots were taken and further diluted to yield concentrations in the range of 8.4–540 ng/ml in order to obtain the calibration curves.

2.3. Sample preparation

Plasma (100 μl, at room temperature) was spiked with 100 μl of betulin solution 50 ng/ml in heptane (internal standard) and 1 ml of ethanolic NaOH solution (1N NaOH dissolved in ethanol/distilled water (80/20, v/v)) was added. The mixture was mixed by vortex for 30 s, subjected to saponification for 1 h at 80 °C, and acidified with 300 μl of 5N HCl. The phase separation was carried out by employing liquid–liquid separation, adding 2 ml of heptane, and mixing by vortex 1 min. Samples were subjected to 70 °C for 10 min and then the organic phase was removed and evaporated to dryness under nitrogen stream. This extraction step was repeated 3 times. The extract obtained was washed twice with 2 ml distilled water. After exhaustive evaporation of the solvent obtained from the above extractions, the residue was reconstituted in 500 μl of the derivatizing agent BSTFA during 20 min at 80 °C. Finally, 0.5 μl was injected automatically into the GC–MS for quantitative analysis.

2.4. Chromatographic conditions

Plasma concentrations were determined by GC model 7890A (Agilent Technologies, California, USA) coupled with an inert mass spectrometry detector model 5975C. The column used was a HP-5 MS (30 m × 250 μm I.D., 0.25 μm film thickness) from Agilent Technologies (California, USA). The oven temperature was programmed as follows: initial temperature 200 °C, a 20 °C min^{-1} ramp to 300 °C where it was held for 10 min. Helium was used as carrier gas at 1 ml/min flow. Splitless injection was performed at 300 °C. The interface was heated at 300 °C. The mass spectrometer operating conditions were ion source temperature at 230 °C, electron energy of 70 eV, and in selected ion monitoring mode. Solvent delay set at 6 min.

2.5. Qualitative and quantitative determinations

Analysis was performed in SIM mode based on the use of target ions. The target and qualifier abundances were determined by injection of individual derivatized analytes and internal standard under the same chromatographic conditions in full scan from *m/z* 40 to 600. One target ion was selected from the spectrum of each com-

pound to quantify the response in the SIM mode (1-octacosanol, 467; 1-triacontanol, 495; octacosanoic acid, 481; triacontanoic acid, 509). Analytes were identified according to target ions and retention times.

2.6. Application of the method

The applicability of the method was evaluated by a pharmacokinetic study, determining 1-octacosanol, 1-triacontanol, octacosanoic acid, and triacontanoic acid concentrations in rat plasma after a single oral dose of policosanols (100 mg policosanols/kg body weight equivalent to 60 mg 1-octacosanol/kg body weight used in other studies)[14]. Male Sprague–Dawley rats weighing 180–220 g (Animal Facility of the Institute of Biomedical Sciences, Faculty of Medicine, University of Chile) were used. Blood samples were obtained by cardiac puncture in anesthetized animals (1 ml/kg zolazepam chlorhydrate [25 mg/ml] and tiletamine chlorhydrate [25 mg/ml]; Zoletil 50, Virbac S/A, Carros, France) and plasma was separated by centrifugation at 3000 × *g* for 15 min at 4 °C. Blank samples were collected before the policosanols administration and post-dose plasma were collected at 30, 60, 90, 120 and 180 min, and frozen at −20 °C until analysis (assays were carried out in triplicate). The study protocol was approved by the Bioethics Committee of the Faculty of Medicine, University of Chile (CBA UCH 0269).

2.7. Statistical analyses

Values shown represent the mean ± standard error of the mean (S.E.M.) for the number of separate experiments indicated. One-way ANOVA test assessed the statistical significance of differences between mean values. A *P* value of less than 0.05 was considered significant.

3. Results and discussion

The present article describes the simultaneously measurement of alcohols and fatty acids in plasma after an oral administration of policosanols mixture, allowing the trace quantification of the major components found in the mixture (1-octacosanol and 1-triacontanol). The reported investigations used hexacosanol as internal standard [13,14]; however, the presence of this alcohol in the policosanols mixture makes necessary the use of an alternate molecule as internal standard, when the alcohols mixture is administered. This molecule cannot be aliphatic alcohols with chain lengths varying from 24 to 34 carbon atoms also present in the policosanols mixture, their respective fatty acids produced by the metabolism in vivo, or any other compound found naturally in blood circulation. For these reasons, betulin was chosen as the internal standard, a lipophilic high molecular weight compound that allows to be spiked prior to the saponification step quantifying possible losses of analytes during the sample treatment.

The developed method allowed obtaining the alcohols and the fatty acids in the same sample. Moreover, a non-polar capillary column (HP-5 MS) was appropriate for separating the target analytes. Consequently, the experimental assay developed reduces to the half the number of injection samples into the GC–MS and avoids the utilization of different capillary columns, diminishing considerably the operation time and cost of the method.

Among the advantages of using GC–MS (SIM mode) instead GC-FID are the high chromatographic sensitivity of alcohols with respect to other homologous substances, the low nanogram detection limit, the elimination of the compound interference, and the certainty of the identification of the analyte, which was confirmed by comparing the mass spectrum of each analyte with plasma spiked with policosanols, in full scan mode. The mass spectra of the trimethylsilyl derivatives of 1-octacosanol (Fig. 2A), octacosanoic acid (Fig. 2B), 1-triacontanol (Fig. 2C), triacontanoic acid (Fig. 2D), and betulin (Fig. 2E) obtained from pure standards are shown.

Under the chromatographic conditions indicated above, the maximum deviation from the average retention time, in 15 repetitive injections, was less than 5 s for all analytes. The chromatogram in Fig. 1B shows the target analytes of the study and the internal standard, confirming that alcohols and acids are adequately separated from each other.

3.1. Optimization of extraction process

In order to obtain the highest recovery of the analytes in the liquid–liquid extraction process, different solvents and diverse volumes were assayed. The most efficient solvent was heptane allowing recoveries up to 95% with 4 successive extractions of 2 ml each. The analysis demonstrated that phase separation was appropriated and multiple extractions with little volumes gave better results than one extraction with 8 ml. Aqueous washings of the extract to eliminate trace acid avoid the possible degradation of organic compounds at high temperatures. The saponification and silanization time were determined after several separate analyses, confirming that both reactions were complete. Furthermore, saponification of synthesized policosanol esters, dissolved in heptane and subjected to the same conditions outlined in sample preparation (Section 2.3), showed hydrolysis efficiencies greater than 98% for 1-octacosanol and 1-triacontanol.

3.2. Quantitation procedure, detection limits, precision, and linearity

Calibration graphs were constructed for all the analytes in the concentrations ranges 8.4–540 ng/ml, using the internal standard method. Therefore, response ratios were calculated as the area of the native analyte ion divided by the area of the internal standard. The linear equations for 1-octacosanol, octacosanoic acid, triacontanol, and triacontanoic acid were: $y = 0.212x + 0.231$ (correlation coefficient, $r = 0.999$ ($n = 7$)), $y = 0.0429x + 0.447$ ($r = 0.998$ ($n = 7$)), $y = 0.2182x - 0.427$ ($r = 0.998$ ($n = 7$)), $y = 0.0437x + 0.1032$ ($r = 0.999$ ($n = 7$)), respectively.

In order to check the precision of the developed method, the intra-day relative standard deviations (R.S.D.s) of the assays for 5 consecutive extractions at two different levels of concentrations were performed under the selected conditions. The same samples were analyzed over a period of 7 days ($n = 5$). As can be observed in Table 1 the R.S.D. values were less than 6% in all cases, reflecting an appropriate behaviour of the analytical process and chromatographic separation, in accordance with the acceptance criteria (15%) [16]. The recovery of the analytes in rat plasma at three different concentrations is shown in Table 1. The limits of detection (LOD) determined as a signal to noise ratio of 3:1 ranged from 1.32 ng/ml (1-octacosanol) to 3.47 ng/ml (1-triacontanol).

3.3. Application of the method

The developed method allowed to study the bioavailability of policosanols and their main metabolites in vivo, by assaying plasma samples from the pharmacokinetic study between 0 and 180 min. Fig. 3A shows 1-octacosanol and octacosanoic acid concentration–time profile after oral administration of a single dose of policosanols mixture (100 mg/kg), whereas Fig. 3B shows 1-triacontanol and triacontanoic acid concentration–time profile after the same dose.

Furthermore, the process utilized in related articles [13,14] and employed in a pharmacokinetic study in rats and monkeys [12] for measuring 1-octacosanol in plasma, permits only the quantification of free 1-octacosanol. It is possible that fatty alcohols may

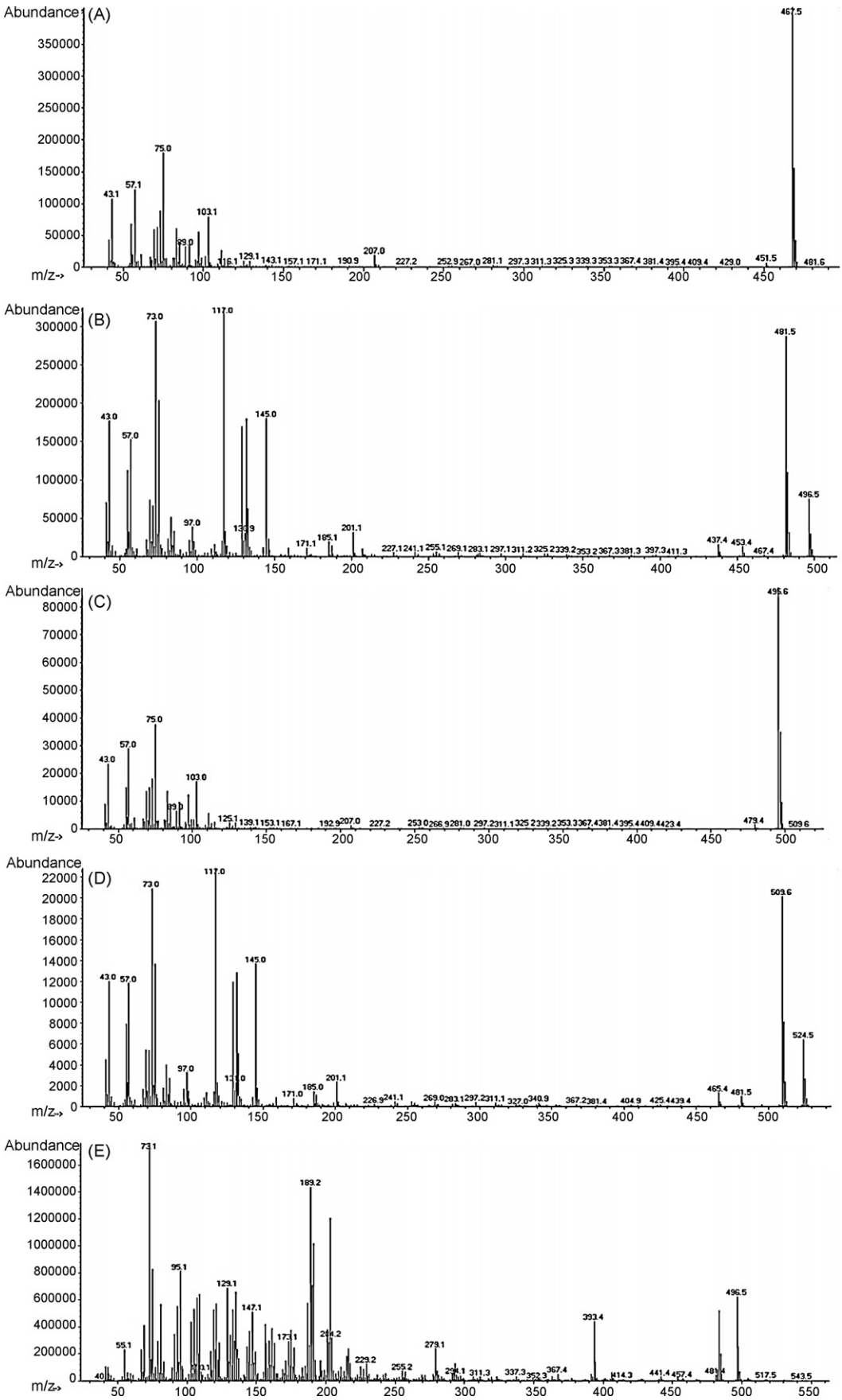

Fig. 2. Mass spectra of derivatized 1-octacosanol (qualifier ions 468, 469) (A), octacosanoic acid (496, 497) (B), 1-triacontanol (482, 496) (C), triacontanoic acid (510, 524) (D), and betulin (393, 411) (E).

Table 1
Intra-day and inter-day S.D. and recovery of the analytes in the 8.4–540 ng/ml concentration range ($n = 5$).

	Intra-day R.S.D. (%)		Inter-day R.S.D. (%)		Recovery (%)		
	8.4 ng/ml	540 ng/ml	8.4 ng/ml	540 ng/ml	20 ng/ml	250 ng/ml	500 ng/ml
1-Octacosanol	3.21	0.59	4.33	2.99	90.2 ± 2.8	93.5 ± 3.3	95.3 ± 1.9
Octacosanoic acid	3.11	1.76	4.75	3.50	89.5 ± 3.2	93.3 ± 2.6	93.1 ± 1.4
1-Triacontanol	2.10	3.74	4.38	4.63	90.8 ± 2.7	92.2 ± 2.1	93,9 ± 2.4
Triacontanoic acid	3.06	2.74	5.22	4.88	87.6 ± 3.1	91.1 ± 1.9	90.6 ± 3.0

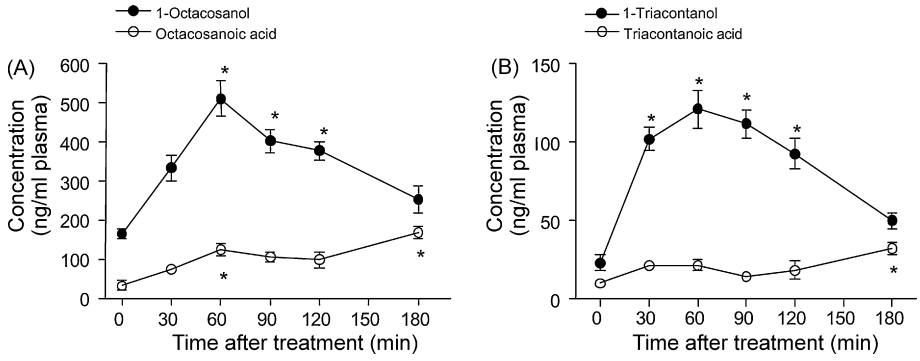

Fig. 3. Plasma concentration–time profile of 1-octacosanol and octacosanoic acid (A) and of 1-triacontanol and triacontanoic acid (B) in rats after a single oral dose of policosanols (100 mg/kg) given at time zero. Data correspond to means ± S.E.M. for three separate experiments per point. $^{*}P < 0.05$ compared to time zero, assessed by one-way ANOVA followed by Bonferroni's multiple comparison test.

be esterified in the enterocyte or in bloodstream, as other alcohols such as cholesterol are. Our results show that 1-octacosanol in plasma, after a single oral dose of policosanols, is at significant higher concentrations than reported in the previous investigations [12,14]. The reason for such difference is probably caused by the hydrolysis of the ester linkage between alcohols and fatty acids (saponification step), which allows the quantification of total policosanols in plasma.

4. Conclusion

An optimized liquid–liquid extraction procedure coupled with GC–MS is proposed as a sensitive and accurate method for quantifying the main alcohols and their metabolites in rat plasma. It is the first study determining total policosanols (free and esterified) by including saponification of the sample in the protocol. The developed method achieves very low limits of detection (1.32–3.47 ng/ml) and excellent intra-day and inter-day standard deviations (0.59–5.22%). This procedure is linear in the concentration range assayed, reproducible, and useful for pharmacokinetic studies of alcohols and their respective long chain fatty acids after policosanols oral administration to rats.

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References

[1] M. Janikula, Altern. Med. Rev. 7 (2002) 203.
[2] M.L. Arruzazabala, M. Noa, R. Menéndez, R. Más, D. Carbajal, S. Valdés, V. Molina, Braz. J. Med. Biol. Res. 33 (2000) 835.
[3] F. Hernández, J. Illnait, R. Más, G. Castano, L. Fernandez, M. Gonzalez, N. Cordovi, J.C. Fernandez, Curr. Ther. Res. Clin. Exp. 51 (1992) 568.
[4] G. Castaño, R. Más, L. Fernández, J. Illnait, R. Gámez, E. Alvarez, Int. J. Clin. Pharmacol. Res. 21 (2001) 43.
[5] N. Crespo, J. Illnait, R. Más, L. Fernández, J. Fernández, G. Castaño, Int. J. Clin. Pharmacol. Res. 19 (1999) 117.
[6] A. Mirkin, R. Más, M. Martinto, R. Boccanera, A. Robertis, R. Poudes, A. Fuster, E. Lastreto, M. Yañez, G. Irico, B. McCook, A. Farré, Int. J. Clin. Pharmacol. Res. 21 (2001) 31.
[7] R. Zardoya, L. Tula, G. Castaño, R. Más, J. Illnait, J.C. Fernández, E. Díaz, L. Fernández, Curr. Ther. Res. Clin. Exp. 57 (1996) 568.
[8] M.F. Dulin, L.F. Hatcher, H.C. Sasser, T.A. Barringer, Am. J. Clin. Nutr. 84 (2006) 1543.
[9] H.K. Berthold, S. Unverdoeben, R. Degenhardt, M. Bulitta, I. Gouni-Berthold, JAMA 295 (2006) 2262.
[10] L. Fernández, R. Más, J. Illnait, J.C. Fernandez, Curr. Ther. Res. 59 (1998) 717.
[11] D. Haim, L.A. Videla, AgroFOOD Ind. Hi-tech. 19 (2008) 60.
[12] R. Menéndez, D. Marrero, R. Más, I. Fernández, L. González, R.M. González, Arch. Med. Res. 36 (2005) 113.
[13] L. Gonzalez-Bravo, J. Magraner-Hernandez, P.C. Acosta-Gonzalez, N. Perez-Souto, J. Chromatogr. B 682 (1996) 359.
[14] D. Marrero Delange, L. Gonzalez Bravo, J. Chromatogr. B 762 (2001) 43.
[15] M.J. Paik, J. Yu, M.B. Hu, S.J. Kim, K.R. Kim, Y.H. Ahn, S. Choi, G. Lee, Clin. Chim. Acta 396 (2008) 62.
[16] H.T. Karnes, C. March, Pharm. Res. 10 (1993) 1420.