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Journal of Chromatography B, 762 (2001) 43–49

JOURNAL OF CHROMATOGRAPHY B

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Trace determination of 1-octacosanol in rat plasma by solid-phase extraction with Tenax GC and capillary gas chromatography

David Marrero Delange*, Luis González Bravo

Center of Natural Products, CNIC, Ave. 25 and 158, Playa, P.O. Box 6414, Havana, Cuba

Received 13 February 2001; received in revised form 9 July 2001; accepted 9 July 2001

Abstract

1-Octacosanol is the major component of policosanol, a new natural lowering-cholesterol agent. A sensitive solid-phase extraction with a Tenax GC capillary gas chromatography method for determining the proportion of this fatty alcohol in plasma after its denaturation with trichloroacetic acid was developed. The trimethylsilyl ether derivative of the target analyte obtained from the organic extract showed excellent chromatographic properties and was detectable in the low nanogram range (1 ng/ml). Adequate separation from plasma's extract was achieved with a fused-silica capillary column (30 m×0.25 mm I.D.) with SPB-5 (0.5 μm film thickness) and operated with temperature programming from 100 to 200°C at 40°C/min and from 200°C increased at 10°C/min to 320°C, then held for 30 min, the carrier gas flow-rate (argon) was 1 ml/min. Quantification was performed by the internal standard method using 1-hexacosanol. The reliable relative retention parameters and the mass response factors values, and their confidence levels, ensure a proper GC sensitivity, necessary for the determination of the alcohol being analyzed. The method was evaluated to a concentration range from 6 to 47.6 ng/ml of plasma obtaining recoveries from 95 to 98%. The correlation between the theoretical concentration values and the corresponding experimental values was appropriate ($y=0.9718x-0.0915$; $r^2=0.9998$). The method showed a good within-day (RSD=4.3%) and between-day (RSD=6.0%) precision according to the acceptance criteria (<10%). This procedure was successfully applied to the study of 1-octacosanol in rat plasma samples after a single oral administration (40 mg/kg) of policosanol. © 2001 Published by Elsevier Science B.V.

Keywords: 1-Octacosanol

1. Introduction

1-Octacosanol ($C_{28}OH$) is the major component of policosanol, a mixture of high-molecular-mass primary fatty alcohols obtained from sugar cane (*Saccharum officinarum* L.) wax [1–3]. Oral treatment with policosanol reduced serum cholesterol levels in several experimental models [4,5], in healthy volunteers [6] and in patients with type II hypercholesterolaemia [7,8]. Also, this drug has shown antiplatelet properties in humans [9]. Data obtained from short and long-term clinical studies have proven that it is very safe and well tolerated and no drug-related adverse effects have been demonstrated up to date [10,11].

A previously validated solvent extraction and capillary gas chromatography (GC) analytical procedure for the determination of $C_{28}OH$ in plasma

*Corresponding author.

E-mail address: david_delange@yahoo.com (D. Marrero Delange).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00331-0

following intravenous fast infusion of policosanol to rat, swine and Beagle dogs has been reported [12]. However, this method was precarious and not sensitive enough for isolating and quantifying this alcohol in plasma after an oral administration, because of the low plasmatic concentration level found, among other drawbacks inherent to the liquid–liquid extraction (LLE) technique employed.

An elegant and successful approach is the use of solid-phase extraction (SPE). SPE is a well-established technique for isolating drug and its metabolites from biological fluids because of its high selectivity, trace enrichment, sensitivity and matrix simplification [13,14]. Inorganic adsorbents, siloxane-bonded silica materials, non-polar and macroreticular resins are most commonly employed in such a way. Tenax GC (poly 2,6-diphenyl-*p*-phenylene oxide), is a linear porous polymer, which lacks interest for the outlined purposes due in large measure to its low specific surface area (19–30 m^2/g) [15]. In spite of this limitation, its use for extracting neutral substances from non-biological aqueous systems [16] has been demonstrated and it is also suitable for trapping of volatile constituents of human breath and urine [17,18]. Moreover, it has been used in preparative GC due to its separation character and high oxidative and temperature resistance [19]. It is also known, that Tenax has a molecular-interaction specificity, which determines a higher retention of hydrocarbons than alcohols and acids with the same boiling point [20]. Furthermore, favorable early results have been described by Vreuls et al. [21], where quantitative recoveries (>95%) for C10 through C26 *n*-alkanes were reached.

There are a small number of publications that use GC techniques for the analysis of $C_{28}OH$ in various biological matrixes [22,23].

Taking into account the above discussion, the aim of the present paper was the development of a new capillary GC and SPE method based on Tenax's quantitative adsorption capacity for the traces of $C_{28}OH$ in plasma after an oral administration of policosanol and to find an alternative method to LLE that would provide excellent recoveries and sensitivity in the low-nanogram range. Here, was also followed a well-defined validation criteria for pharmacokinetic studies [24]. In this sense, a new application for Tenax in the extraction of a new natural drug from plasma was found.

2. Experimental

2.1. Materials and reagents

The standards 1-hexacosanol ($C_{26}OH$) and 1-octacosanol were purchased from Sigma (St. Louis, MO, USA), 99.0% GC purity. Acetone and methanol GR dried, max. 0.01 and 0.005% of water, respectively, were obtained from E. Merck (Darmstadt, Germany). Sylon-CT, silylation reagent for glassware was supplied by Supelco (Bellefonte, PA, USA), *N*-methyl-*N*-(trimethylsilyl)trifluoracetamide (MSTFA) was supplied by Sigma, 99.0% GC purity. Trichloroacetic acid (TCA) was from Fluka (Buchs, Switzerland) analytical-reagent grade >99.5%. Water was deionized and glass distilled. Tenax GC, 60–80 mesh was purchased from Supelco. All glassware was rigorously silanized and rinsed thoroughly with HPLC-grade methanol and dried before used.

2.2. Capillary gas chromatographic analysis

The plasma concentrations were determined by GC (Shimadzu GC 14A) using a flame ionization detection (FID) system with an SPB-5 fused-silica capillary column (30 m×0.25 mm I.D., 0.5 μm film thickness) from Supelco. A 1 m×0.32 mm I.D. uncoated and deactivated retention gap was coupled by a glass fitting to the column. The conditions for the gas chromatograph were: injector temperature, 300°C; detector temperature, 300°C; oven temperature gradient, from 100 to 200°C at 40°C/min, then increased by 10°C/min from 200 to 320°C and subsequently kept at 320°C for 30 min. In the optimized system, the hydrogen and air flow-rates were 30 and 300 ml/min, respectively. Argon was used as a carrier gas at a flow-rate of 1 ml/min. Injector parameters: split–splitless mode, septum purge flow-rate 5 ml/min and a spilt-vent flow-rate at 45 ml/min and closed for 60 s. Determination of the areas of the chromatographic peaks were made by a computing integrator (C-R4A Chromatopac, Shimadzu, Japan). Aliquots of 1–2 μl were manually fast-injected with a 10 μl Hamilton syringe into the injection chamber. All the analysis were made in the same way as described previously.

Peak identification of 1-octacosanol in plasma samples was performed by GC–MS analysis in a GC 8000 gas chromatograph (Fisons Instruments, Italy),

equipped with a mass-selective detector Model MD800, on-line coupled to Lab-base software (VG, Masslab, UK). The split–splitless introduction and working conditions were the same as described above, it also employed the same column.

GC–MS parameters were as follows: ion source and interface temperatures were 250 and 200°C, respectively. Ionization energy was 70 eV. The mass spectrum was continuously acquired from m/z 40 to 600 with a scan speed of 1 s/decade in full scan mode. Inner carrier gas pressure (He)=1.2 p.s.i. (1 p.s.i.=6894.76 Pa).

2.3. Preparation of standard solutions

The development and validation of the procedure was carried out in model samples. In this respect, were used very-long-chain fatty alcohols, $C_{26}OH$ as internal standard and $C_{28}OH$ as the target analyte. Stock solutions were prepared, by dissolving 5 mg of each in 50 ml dried acetone. From these solutions were taken several aliquots and further diluted yielding concentrations in the range of 6 to 47.6 ng/ml for making the calibration curve.

2.4. SPE of plasma extract

Plasma (1 ml, at room temperature) sample diluted 1:2 with water was spiked with $C_{28}OH$ and mixed by vortex for 20 s. A 700-μl volume of TCA (20%) for protein precipitation was employed, then centrifuged for 10 min at 3000 *g* to effect phase separation. The supernatant was subjected directly to SPE using a Tenax GC column (Fig. 1) as described below. A laboratory-made glass column (10.4×0.6 cm) filled with a Tenax bed (about 100 mg) was fitted between two small plugs of silanized glass wool. Just prior to sample application, Tenax was conditioned by passing through it a moderate nitrogen flow with simultaneously heating at 300°C. Them, the supernatant was slowly forced through the adsorbent bed by positive pressure at a 1 ml/min flow-rate. A washing step with 2 ml of water to remove polar interferences was performed and subsequently the wetted adsorbent was dried with a nitrogen flow. Afterwards, the trapped $C_{28}OH$ among others endogenous analytes were eluted into a reacti-vial with 4 ml of dry methanol. The methanolic extract was then spiked with $C_{26}OH$ and reduced to the volume approximately

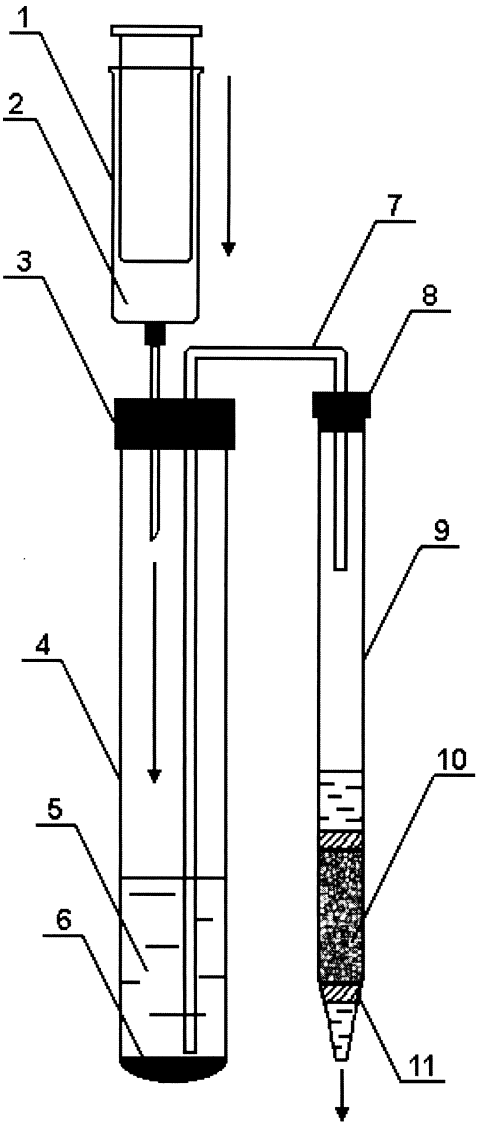

Fig. 1. Solid-phase extraction device. 1, Hypodermic syringe; 2, air; 3, screw-cap with two holes and silicone seal; 4, assay tube; 5, supernatant; 6, protein pellet; 7, glass capillary; 8, silicone septa; 9, glass column; 10, Tenax GC adsorbent and 11, glass wool.

50–60 μl by blowing nitrogen across the surface of the solvent at 70°C. Then, dichloromethane is added to form an azeotropic mixture with the eluted remanent water and thus yields a dry solid residue after evaporation of the liquid. The residue was derivatized with 20 μl of MSTFA during 15 min at 70°C. Aliquots of 1–2 μl were injected onto the GC

capillary columns for qualitative and quantitative analysis.

2.5. Withdrawing and pouring of supernatant

First of all, care was taken to reduce quantitative losses of $C_{28}OH$ during manipulation. For such a way, the arrangement illustrated in Fig. 1 was employed. The columns were kept under nitrogen until use. Once the column is coupled to the centrifugation tube (12×1.5 cm) by means of the bent glass capillary (15×0.1 cm), the supernatant was slowly forced through the adsorbent bed by positive pressure at a 1 ml/min flow-rate depressing the syringe plunger. Blanks of the column after the SPE procedure were carried out to check the presence of $C_{28}OH$ and remanent impurities.

2.6. Linearity, accuracy and precision

For calibration, plasma standards spiked with a fixed amount of the internal standard and $C_{28}OH$ concentrations ranging between 6.1 and 47.6 ng/ml were prepared and assayed as described above. A calibration curve was obtained by correlating the theoretical (nominal) concentration values and the corresponding experimental (calculated) ones, adjusting the curve by single linear regression. The model systems were prepared from the drug-free rat plasma samples. The accuracy (expressed as recovery) of the method was evaluated by assaying plasma samples spiked with known concentrations of $C_{28}OH$. The intra- and inter-day precision data were estimated by measuring plasma standards in the whole studied range on day one ($n=5$) and on the 3 following days.

2.7. Qualitative and quantitative determinations

Identification of $C_{28}OH$ in the chromatographic profile of plasma samples was done by direct comparison with the added pure I.S. to the sample and by its relative retention (r_{is}). In order to confirm the presence of $C_{28}OH$ in plasma samples after an oral administration of policosanol, the samples were subjected to GC–MS analysis, monitoring the most abundant M−15 ion at m/z 467, and other characteristic fragments at m/z 57, 73, 75, 89, 97, 103, 111 and 125 for the trimethylsilyl (TMS) alcohol derivatives.

Replicates of four different concentration 6.1, 12.3, 24.5 and 47.6 ng/ml ($n=5$) of the alcohols with the internal standard added were prepared and analyzed as described previously (Section 2.3).

The quantitative determination of $C_{28}OH$ for recovery studies and calibration curve of the method was carried out by the internal standard technique, calculating the relative response factors (RRFs).

2.8. Application of the method

The developed method was evaluated during several pharmacokinetic studies where trace levels of $C_{28}OH$ in rat plasma samples after a single oral dose administration (40 mg/kg) of policosanol were determined. Young adult Sprague–Dawley rats weighing 250 to 300 g from the National Center for Laboratory Animals (CENPALAB, Havana, Cuba) were used for obtaining the drug-free plasma. Blood samples were obtained in the presence of heparin from the abdominal artery and plasma was separated by centrifugation at 3000 *g*.

Blank plasma samples ($n=3$) were collected before the drug administration and post-dose plasma samples were collected at 10, 15, 30, 45, 60, 75, 90, 120, 180 and 240 min and were frozen at −20°C in the dark until analysis.

3. Results and discussion

Endogenous compound interference, the relatively low chromatographic sensitivity of alcohols with respect to the other homologous substances, delicate manipulation of the biological matrix and its organic extract among other methodological aspects, made the determination of very-long-chain fatty alcohols a difficult task [12].

Taking into account these findings, it was firstly necessary to make an optimization of the injector–column–detector system conditions to carried out the trace analysis by capillary GC. Several septum purge (top split) and split-vent ratios and introduction times to the closed valve in the injection port were proven to adequate these parameters for calculating the RRF and the r_{is} of $C_{28}OH$. In this sense, the

obtained RRF (1.057±0.066; RSD=5.1%) and r_{is} (1.113±0.003; RSD=0.3%) values, ensured an appropriate sensitivity and selectivity of the capillary GC procedure.

The capillary column with bonded stationary phase (SPB-5), was appropriate for separating the target analyte giving good resolution and low background from the plasma components (Fig. 2). The retention gaps (were changed after 30 injections) together with the solvent flush introduction technique and the high injection temperature, allow us to obtain sharp peaks, diminish the discriminative effect and increase the precision of the split–splitless introduction.

Several attempts were made in order to obtain higher recoveries of C_{28}OH, using various solvents and different volumes of them for optimal elution efficiency of C_{28}OH. It was noticed that neither *n*-hexane nor acetone gave poor recoveries, not exceeding 40%, even for a volume greater than 4 ml. The most efficient solvent was methanol (4 ml) allowing the best recoveries and giving the cleanest blank of Tenax column, making it possible to reuse the column up to 15 times and reducing the cost of the method.

One of the advantages of Tenax for SPE is that it is easier to condition without solvents ensuring little chromatographic background. Consequently, the Tenax reduces the time and costs of the analysis. The washing step with 2 ml of deionized water was found to be suitable for removing polar contaminants present in the supernatant.

One of our goals with the employment of SPE was to remove a large quantity of impurities with subsequently optimize the recovery, the selectivity and sensitivity of the method. Protein precipitation with TCA allowed the an increase of around 27% of the recovery of C_{28}OH due to its high protein-binding and hydrophobicity and contributed to avoid the clogging of the Tenax columns and deterioration of the GC column. Highly cleaned fingerprints were also obtained. However, it was impossible to eliminate other endogenous lipids.

It was also noted that at least 9% of C_{28}OH could

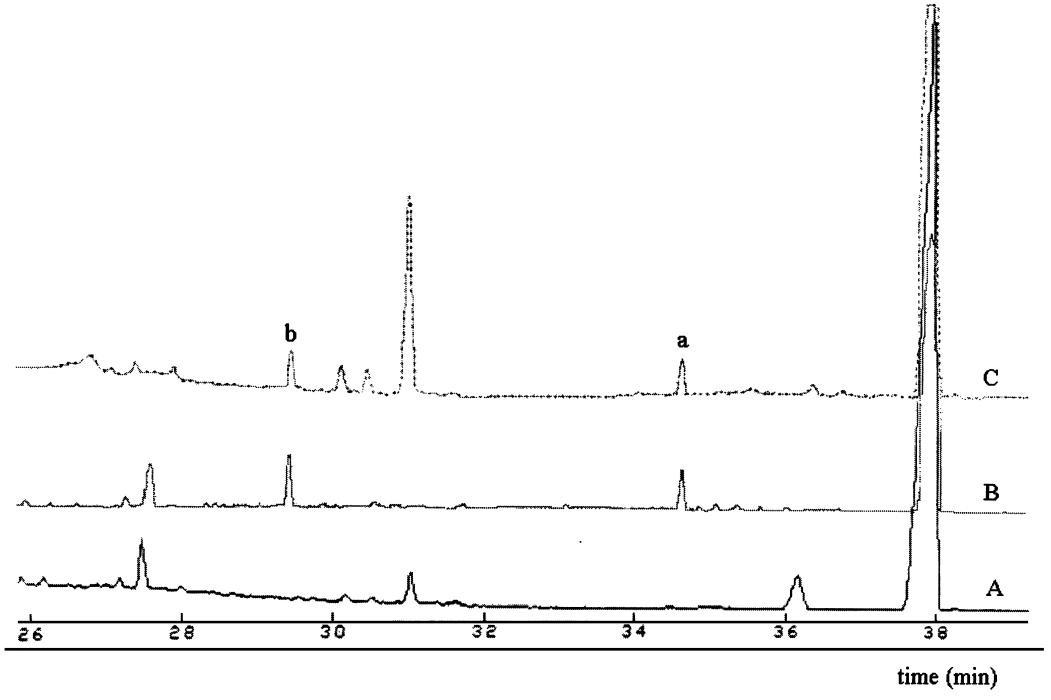

Fig. 2. Partial multi-overlay chromatograms from: the control plasma rat (A), spiked plasma containing 12.3 ng/ml of each standard (B) and the rat plasma sample taken 30 min after an oral dose of policosanol (C), observing a prominent peak (a) at 35.2 min corresponding to 14 ng/ml of C_{28}OH and peak (b) is the 1-hexacosanol at a concentration of 12.3 ng/mg.

be lost by adsorption onto the glassware surface, because of this all glassware was rigorously silanized.

Representative chromatograms of plasma extracts (control, spiked and oral post-dose) analysed by capillary GC are presented in Fig. 2.

3.1. Validation study

The linearity of the method was quite good, observing a great correlation ($y=0.9718x-0.0915$, $r=0.9999$) between the nominal and measured amounts of C_{28}OH over the whole concentration range (6.1 to 47.6 ng/ml, $n=5$), which cover the therapeutic range. The limit of quantification was 6 ng/ml, and the limit of detection based on a signal-to-noise ratio of 3:1 for the spiked plasma was 0.3 ng/ml.

Higher recoveries for C_{28}OH from spiked plasma were obtained ranging from 95 to 98% (Table 1).

The intra-day precision and accuracy was high, as shown by the low relative standard deviation (RSD) between 3.3 and 6.9% and relative error for the whole range concentration (Table 1). The between-day precision and accuracy are shown in Table 2.

These results reflect an appropriate behavior of the whole analytical process (extraction, concentration, desorption and chromatographic separation) in accordance with the acceptance criteria (15%) [25].

3.2. Application of the method

The applicability of the method was demonstrated by assaying a number of plasma samples originating from a pharmacokinetic study. Fig. 3 shows the 1-octacosanol concentration–time profile in rat plasma after oral administration of policosanol as single dose (40 mg/kg).

Table 1
Intra-day precision[a] and accuracy[b] data of C_{28}OH in spiked plasma

Concentration (ng/ml)		RSD (%)	Accuracy (%)
Nominal	Calculated, mean±SD		
6.1	6.0±0.2	3.3	98.3
12.3	11.9±0.4	3.4	96.7
24.5	23.4±0.9	3.8	95.3
47.6	46.3±3.2	6.9	97.2

[a] Expressed as RSD (%); $n=5$, $\alpha=0.05$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)]×100.

Table 2
Inter-day precision[a] and accuracy[b] data of the method for the whole concentration range over a period of 3 days

Concentration (ng/ml)		RSD (%)	Accuracy (%)
Added	Found±SD		
6.1	5.8±0.5	8.6	95.1
12.3	11.7±0.6	5.1	95.1
24.5	23.2±1.2	5.2	94.7
47.6	46.1±3.5	7.6	96.8

[a] Expressed as RSD (%); $n=5$, $\alpha=0.05$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)]×100.

4. Conclusion

A non-polar fused-silica capillary column (SPB-5) together with the SPE procedure forms an efficient team for the quantitative GC analysis of 1-octacosanol in plasma samples. For the first time the combination of Tenax GC as adsorbent and TCA as denaturing agent was used, providing a more sensitive and selective way of sample preparation than with traditional liquid–liquid extraction procedures. The developed method is linear in the concentration range assayed, reproducible and suitable for phar-

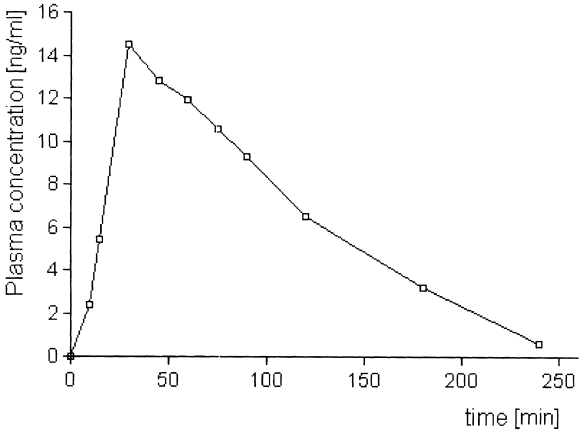

Fig. 3. Mean plasma concentration–time profile of 1-octacosanol in rats after a single oral dose of policosanol (40 mg/kg, $n=3$).

macokinetic studies of policosanol after its oral administration to rats.

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