Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 850 (2007) 177–182
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Gas chromatographic–mass spectrometric determination of brain levels of α-cholest-8-en-3β-ol (lathosterol)

Berta Luzón-Toro, Alberto Zafra-Gómez*, Oscar Ballesteros

Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avda. Fuentenueva s/n, E-18071 Granada, Spain

Received 22 August 2006; accepted 15 November 2006
Available online 1 December 2006

Abstract

A gas chromatographic–mass spectrometric (GC–MS) method is proposed for the detection and quantification of lathosterol in rabbit brain. This compound is one of the most important precursors of the cholesterol synthesis. The interest in brain cholesterol metabolism is growing nowadays since it was described to play an important role in some neurodegerative disorders such as Alzheimer's disease and Multiple Sclerosis. The analytical methodology proposed involves a liquid–liquid extraction procedure (LLE) followed by a silylation step previous to the GC–MS analysis. The chromatographic separation was performed by using a low bleed HP5-MS fused silica capillary column. A clean up is not necessary when using single-ion monitoring (SIM) mode. The molecular ion appears at 458 *m/z*; being as well the base peak. α-Naphtol was used as an internal standard. The detection limit obtained was 0.09 μg mL^{-1}. The method was applied to the determination of brain lathosterol levels in rabbits fed with different types of diets (control and atherogenic, supplemented or not with natural polyphenolic antioxidants). The quantification of the compound in samples showed a reduction, after 1 month, of this precursor of cholesterol synthesis in groups fed with antioxidant supplemented diets.

Keywords: Lathosterol; Cholesterol precursor; GC–MS analysis; Polyphenolic antioxidants; Brain

1. Introduction

High concentrations of cholesterol have been implicated as a possible risk factor for some neurodegerative disorders such as Alzheimer's disease (AD) and Multiple Sclerosis [1–4]. These levels have been linked to pathological processing of the amyloid precursor protein (APP) and formation of β-amyloid in the brain, a hallmark of the AD pathology [4–9]. The central nervous system (CNS) is very rich in cholesterol. It has been estimated that about 25% of the total cholesterol contained in the body is present in the brain, while the whole body cholesterol just makes up about 2% [10,11,15]. Studies in patients with AD showed that the plasma and CSF levels of this oxysterol and its ratio to cholesterol were significantly affected due to neurodegeneration which enhanced the release of cholesterol in the brain [12–14,16].

Cholesterol synthesis comprises at least 19 steps from the first sterol. Sterols such as lanosterol and other methyl sterols and demethylated cholestanol, desmosterol, as well as lathosterol, are directly related to natural synthesis [17,18]. In fact, lathosterol is one of the most important precursors, and its ratio to cholesterol has been used as a marker for whole body cholesterol synthesis [19,20].

The quantification of lathosterol is complicated by the presence of numerous other lipids and the low concentration of the compound relative to cholesterol (ratio lathosterol/cholesterol is approximately 1/100). It is well known that sample preparation is one of the most critical steps in the analysis of biological fluids and compounds in biological matrices. Since methods, previously proposed in the bibliography, lack sufficient details about preparation of samples, the chromatographic conditions and the analytical sensitivity, they are not easily reproducible and sample preparation is expensive and time consuming. In addition, flame ionization detection (FID) is a poor technique for adequate identification of compounds in gas chromatography [21,22]. Sterols have been analysed by capillary gas chromatography in biological fluids, mainly serum, liver or kidney after a laborious sample

* Corresponding author. Tel.: +34 958 243 326; fax: +34 958 243 328.
E-mail address: azafra@ugr.es (A. Zafra-Gómez).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.11.022

preparation procedure including saponification [23–30]. Solid phase extraction (SPE) and solid phase microextraction (SPME) followed by gas chromatography-flame ionization detector (GC-FID) have also been proposed [30,31]. Lütjohann et al. propose a very good GC–MS method for the determination of lathosterol in serum and brain samples [32]; however, the main drawback of the method is that they involve extensive, time-consuming and expensive procedures for sample preparation and derivatization. Long sample preparation times are obviously disadvantageous, and multistep procedures are prone to analyte loss.

In this paper, a simple and rapid method for a sensitive and precise determination of lathosterol in brain is proposed. After optimization, the method was applied to study the effects of natural polyphenolic antioxidants on brain cholesterol metabolism by measuring lathosterol levels in rabbit brain. An atherogenic rabbit model fed with different types of diets is studied. This model was selected as an easy method to increase brain and serum cholesterol levels in a short period of time. The supplementation of diets with a natural antioxidant showed its efficiency as a potential neuroprotector. The experimental model of diet-induced atherosclerosis in rabbits has been extensively used to evaluate the effects of fat in the development of lesions and as biomarkers of the disease [33,34].

2. Experimental

2.1. Reagents and standards

All reagents were of analytical grade unless stated otherwise. Water was purified with a Milli-Q plus system (Millipore, Bedford, USA). Methanol, trichloromethane, ethanol and ethyl acetate, were supplied by Panreac (Barcelona, Spain). Tris–HCl buffer, ethylendiaminotetracetic acid (EDTA), *R*,*R*-dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma–Aldrich (St. Louis, USA).

Stock solutions of 5α-cholest-8-en-3β-ol (lathosterol), supplied by Sigma–Aldrich (St. Louis, USA), containing 1000 μg mL^{-1} were prepared in ethanol 99% (v/v) (Panreac, Barcelona, Spain). The solutions were stored in dark bottles at 4 °C, remaining stable for at least 3 months. These solutions were used to obtain calibration curves.

A standard solution of 1000 μg mL^{-1} of α-naphtol (Sigma–Aldrich, St. Louis, USA) in ethanol was used as internal standard after adequate dilution to a final concentration of 1.0 μg mL^{-1}.

N,*O*-Bis(trimethylsilyl) acetamide (TMSA), *N*-trimethylsilyl imidazole (TMSI), trimethylchlorosilane (TMCS), the mixture formed by TMCS, hexamethyldisilazane (HMDS) and pyridine (Sigma Sil A) and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were supplied by Sigma–Aldrich (St. Louis, USA).

2.2. Apparatus

Gas chromatographic analysis was performed using a Varian CP3400 GC System (Varian Inc., Palo Alto, USA) fitted with a splitless automatic injector CP8200 for a low background HP5-MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) supplied by Agilent (Palo Alto, USA). A silylated injector liner split/splitless (2 mm i.d.) was used. Detection was carried out with a MS/MS 2000 ion-trap detector (Varian, Inc., Palo Alto, USA) and the GC–MS operation control and the data process were carried out by Saturn 5.5.1 software package (Varian Inc., Palo Alto, USA).

The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0 mL min^{-1}. The samples were injected in the splitless mode, with the split closed for 2.0 min. The sample volume in the direct injection mode was 1 μL.

The conditions used for electron impact ionization (EI) were: ion energy of 70 eV and a mass range from 140 to 465 *m*/*z* were used. The MS was tuned everyday to *m*/*z* 69, 219 and 502 with perfluorotributylamine (PFTBA) as a calibration standard. Single-ion monitoring (SIM) acquisition mode (dwell time 100 ms/ion) was selected.

2.3. Preparation of animal and diets

Thirty-five male New Zealand rabbits (weight 2.5–3.0 kg) were obtained from Harlan Interfauna Iberica SA (Barcelona, Spain). The animals were randomly distributed into five experimental groups ($n = 7$) and individually housed under standard conditions of lighting (12 h day:12 h night cycles), temperature (18 ± 1 °C) and humidity (65.0%). Drinking water was available ad libitum throughout the study and food intake for each animal was standardised to 150 g day^{-1}. The diets used in the study were as follows: 97.0% standard chow diet (Panreac, Barcelona, Spain), 3.0% sunflower oil (control diet); 96.0% standard chow diet, 1.3% of cholesterol and 3.0% of lard to induce atherosclerosis (atherogenic diet); 97.0% standard chow diet, supplemented with 5.6 mg kg^{-1} of a natural antioxidant. The three diets used contained 6.0% of total fat, excluding cholesterol. The composition of the standard chow diet was 13.5% protein, 3.0% fat, 50.0% carbohydrates, 15.5% fibre, 7.0% minerals and 11.0% water. The nomenclature used was, initial control (IC), atherogenic (AT 30 and 60 days) and natural antioxidant (AO 30 and 60 days). Table 1 shows an outline of diets used in this study.

At the end of the experimental period, the rabbits were anesthesized with a solution of sodium pentothal in ultrapure water (1/50) and then exanguinated with a carotid cannula. In order to avoid sample alteration, the head of the rabbit was sectioned between the tegmentum and the first cervical, and the skin and

Table 1
Summary of the diets used in the study

Components	Diets		
	Control diet (IC)	Atherogenic diet (AT)	Antioxidant diet (AO)
Chow diet[a] (%)	97	96	97
Sunflower oil (%)	3	0	0
Cholesterol (%)	0	1.3	0
Lard (%)	0	3	0
Natural antioxidant (mg kg^{-1})	0	0	5.6
Total fat (excluding cholesterol) (%)	6	6	6

[a] Standard chow diet: 13.5% protein, 3% fat, 50% carbohydrates, 15.5% fibre, 7% minerals and 11.0% water.

muscles were removed from back of neck towards nose. The dissection started sectioning the skull from foramen in an anterior manner, to take it away carefully. A little press with forceps in the medulla oblongata region under the brain was applied to remove the brain. After collection, samples were stored at −80 °C until use.

The brain samples were accurately weighed and homogenized with a pH 7.4 buffer (1 mL buffer per gram of tissue) using a Potter homogenizer. The buffer was a mixture of 20 mM tris–HCl buffer, 2 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM benzamidine, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4. EGTA, EDTA (metal chelators) and DTT (reducing agent) were used to reduce the oxidation damage. Benzamidine and PMSF are used as protease inhibitors in order to avoid the cerebral proteins degradation.

2.4. Extraction procedure

A 4 mL of the homogenate was extracted with 10 mL of a mixture trichloromethane/methanol (2:1, v/v, in a 50 mL Falcon tube). The mixture was strongly mixed before centrifugation 10.0 min at 2500 rpm. The underlying organic phase was transferred to a spherical flask and the process was repeated twice. After the combination of all three trichloromethane/methanol phases, they were dried in a rotary vacuum pump at 45 °C. The dried samples were suspended again in a total volume of 7 mL of trichloromethane/methanol (2:1, v/v) mixture and 10 μL of α-naphtol (1000 μg mL^{-1}) was added to 0.5 mL of this extract and was then evaporated under nitrogen. Finally, 250 μL of ethyl acetate was added and shaken until completely dissolved.

2.5. Derivatization and gas chromatography analysis

The 25 μL of a mixture containing *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, pyridine and ethyl acetate (3:1:1, v/v/v) was added to 25.0 μL of the extract. The mixture was mechanically shaken for 1.0 min at room temperature. At this point, the sample was ready to be injected into the GC–MS. It was checked that the silylated compound were stable for at least 3 days.

Gas chromatographic analysis was carried out in the splitless mode, with the split closed for 2.0 min. The injector temperature was set at 280 °C (25.0 min). The oven temperature was held at 150 °C for 3.0 min and then linearly increased to 290 °C at a heating rate of 30 °C min^{-1}, remaining constant for 17.33 min. The total running time was 25.0 min. The transfer line, manifold and trap temperatures were at 260, 40 and 210 °C, respectively.

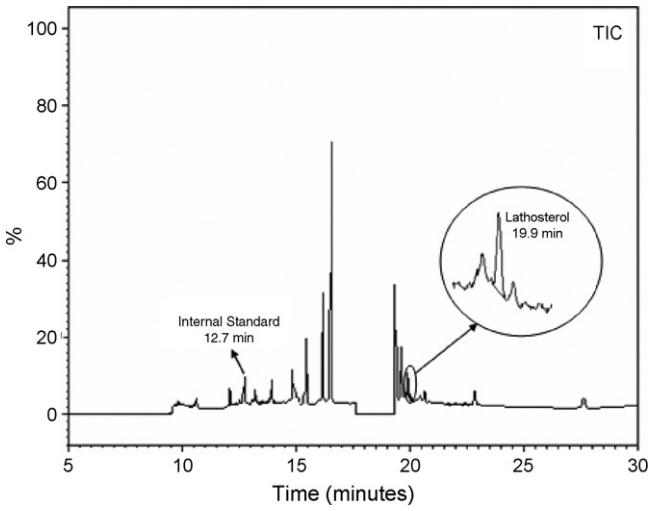

Fig. 1. Representative total ion chromatogram (TIC) of a brain sample, spiked with α-naphtol.

3. Results and discussion

3.1. Derivatization of the analytes

As silylation reagents, *N*,*O*-bis(trimethylsilyl) acetamide (TMSA), *N*-trimethylsilyl imidazole (TMSI), trimethyl chlorosilane (TMCS), a mixture formed by TMCS, hexamethyldisilazane (HMDS) and pyridine as polar solvent in a ratio 1:3:9 named Sigma Sil A and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) in presence of pyridine as base were tested. Reaction time and temperature were simultaneously modified. BSTFA in presence of pyridine was selected as the most adequate silylation reagent for lathosterol, and it was observed that 1 min at room temperature using the reagent was enough to produce the complete derivatization of compounds.

The effect of varying the concentration of BSTFA in ethyl acetate, the volume of pyridine and the reaction time was studied.

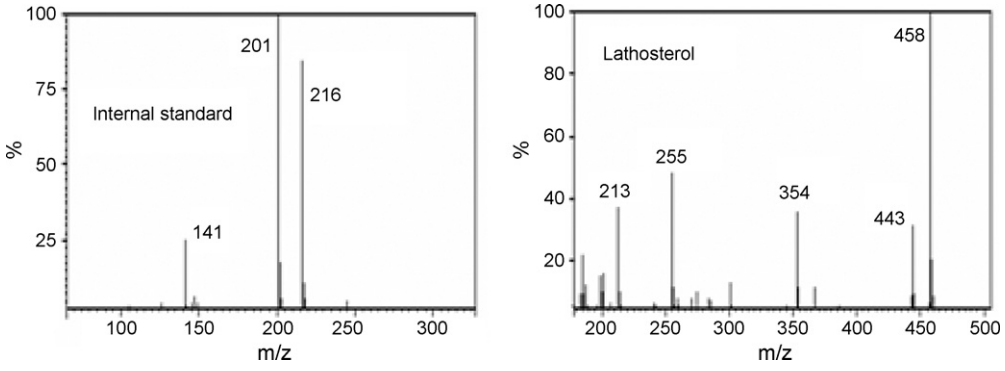

Fig. 2. Mass spectra of silylated lathosterol and α-naphtol.

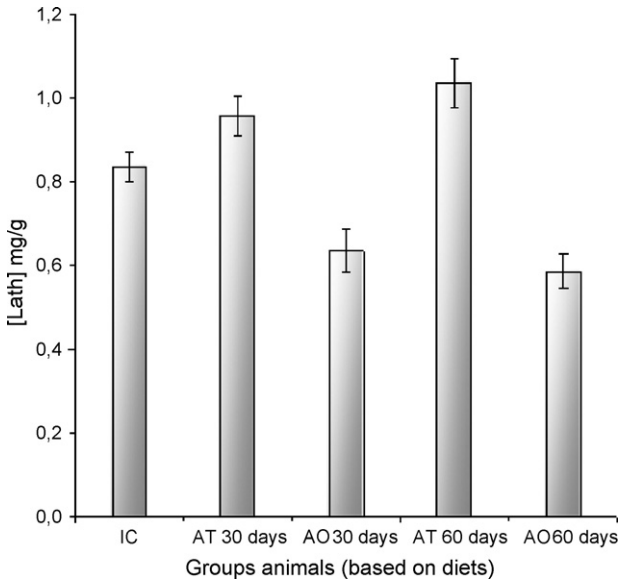

Fig. 3. Rabbit brain lathosterol levels measured by GC–MS (AT: atherogenic diet, AO: antioxidant diet, IC: initial control).

The optimum result for derivatization of 25 μL of the rabbit brain extract was obtained using a mixture of *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, pyridine and ethyl acetate 3:1:1 (v/v/v).

3.2. Gas chromatographic–mass spectrometric analysis

Fig. 1 shows a characteristic chromatogram obtained for a silylated sample extract. Note that the mass recorder was closed between 17.6 and 19.4 min to remove the high signal due to the cholesterol present in samples and to avoid filament damage. Silylated lathosterol eluted at 19.9 min while the internal standard (α-naphtol) eluted at 12.7 min.

SIM mode was used for quantification. The selection of high mass fragments as quantification ions is of great interest, particularly when complex matrices are to be analysed, due to the decreased likelihood of interferences. The mass spectra obtained in scan mode are shown in Fig. 2.

Table 2
Analytical parameters

Parameter	Value
n	7
b (mL μg^{-1})	8.56×10^{-2}
S_b	6.82×10^{-4}
a	3.30×10^{-2}
S_a	3.43×10^{-3}
Determination coefficient (%)	99.68
Linear dynamic range (μg mL^{-1})	0.1–10.0
$CC_{\alpha,0.05}$ (μg mL^{-1})	0.090
$CC_{\beta,0.05}$ (μg mL^{-1})	0.150
Relative standard deviation (%)	5.2
$S_{y/x}$	8.15×10^{-3}
Lack-of-fit test P_{lof} (%)	10.5

n: Calibration levels; a: intercept; S_a: intercept standard deviation; b: slope; S_b: slope standard deviation; $CC_{\alpha,0.05}$: decision limit; $CC_{\beta,0.05}$: detection capability; S_{rc}: regression standard deviation; P_{lof}: P-value for lack of fit test.

Silylated lathosterol shows the base peak at 458 *m*/*z* corresponding to the molecular ion and it was used as target ion. The peak corresponding to a loss of a methyl group at *m*/*z* 443 [M−15] was used as qualifier ion. For the silylated internal standard, α-naphtol, the ions used were those corresponding to *m*/*z* 216 (molecular ion) and 201 (loss of a methyl group).

3.3. Analytical performance

Calibration graphs for samples treated according to the analytical procedure described above were made using SIM mode. The calibration graphs are linear at least for the concentration range 0.1–10.0 μg mL^{-1}. Linearity of the calibration graphs was tested according to the Analytical Methods Committee [35]; the lack-of-fit test was applied to six replicates of each standard.

Table 3
Lathosterol concentrations measured in rabbit brains

C (μg mL^{-1})	m_{tissue} (g)	$mg_{Lath}\ g^{-1}_{tissue}$	Mean (mg g^{-1})	S.D. (R.S.D.)
Control				
6.822	4.24	0.805	0.837	0.036 (4.3%)
3.892	2.90	0.871		
7.870	4.70	0.837		
7.798	4.67	0.835		
5.319	3.33	0.795		
6.181	3.80	0.819		
8.419	4.70	0.896		
Atherogenic 30 days				
9.195	4.45	0.944	0.957	0.48 (5.0%)
4.457	2.38	0.936		
8.726	4.58	0.953		
8.662	4.88	0.888		
7.780	4.25	1.029		
9.512	4.54	1.009		
8.702	4.59	0.940		
Atherogenic 60 days				
5.208	4.46	1.064	1.036	0.058 (5.6%)
5.344	4.38	0.915		
4.771	4.13	1.030		
6.225	4.47	1.091		
5.597	4.27	1.072		
5.185	4.12	1.055		
6.920	5.02	1.024		
Antioxidant 30 days				
10.012	4.75	0.580	0.635	0.050 (7.9%)
8.688	4.75	0.596		
7.914	3.84	0.578		
11.590	5.31	0.696		
9.233	3.94	0.655		
10.189	4.83	0.651		
9.259	4.52	0.690		
Antioxidant 60 days				
3.780	3.34	0.566	0.585	0.041 (7.0%)
6.787	5.29	0.641		
5.551	4.89	0.568		
4.179	3.61	0.579		
5.617	5.22	0.534		
3.780	3.34	0.566		
6.787	5.29	0.641		

Table 4
ANOVA and Bonferroni's Multiple Comparison post hoc test

ANOVA	SS	d.f.	MS
Treatment (between columns)	1.082	4	0.2704
Residual (within columns)	0.067	30	0.0022
Total	1.149	34	

Bonferroni's test	Mean diff	*t*-Value	*P*-value	95% CI of diff
Control vs. AT 30	−0.1201	4.766	<0.05	−0.1965 to −0.0438
Control vs. AT 60	−0.1990	7.894	<0.05	−0.2754 to −0.1226
Control vs. AO 30	0.2017	8.002	<0.05	0.1253 to 0.2781
Control vs. AO 60	0.2519	9.991	<0.05	0.1755 to 0.3282
AT 30 vs. AT 60	−0.07886	3.128	<0.05	−0.1552 to −0.0025
AO 30 vs. AO 60	0.05014	1.989	>0.05	−0.0262 to 0.1265

SS: sum of squares; d.f.: degrees of freedom; MS: mean square; CI: confidence interval.

Two instrumental replicates of three standards at each calibration level were performed. The results for the intercept (a), slope (b), correlation coefficient (R^2) and probability level of the lack-of-fit test (P_{lof} (%)) are summarized in Table 2. Thus, the data yield shows good linearity within the stated ranges. The precision, determined as relative standard deviation (R.S.D.), was measured for a concentration of 1.0 μg mL^{-1} of lathosterol by performing ten independent determinations.

A fundamental aspect which needs to be examined in the validation of any analytical method is its limit of detection in order to determine whether an analyte is present in the sample. In this paper, the criteria for method performance – that include the decision limit, CC_α, and the detection capability, CC_β [36] – have been proposed. The decision limit defines the limit from which it can be decided that a sample is contaminated with an error probability of α. The detection capability is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of β. The decision limit and the detection capacity which are better adjusted to a statistical evaluation are implemented. Thus, CC_α and CC_β were calculated and the results obtained are also summarized in Table 2 (probability 5%).

3.4. Lathosterol determination

An atherogenic model in rabbits was established by dietary manipulation. Brain from rabbits fed with different types of diet (control, atherogenic and antioxidant) for 30 and 60 days were collected at initial time (control group), and treated according to Section 2.3 described above. The concentration of lathosterol was determined by interpolation from the standard calibration curve within its linear dynamic range. Table 3 shows the data comparison of lathosterol content depending on the diet and on the period of application (30 and 60 days). Fig. 3 shows the evolution of lathosterol levels in rabbit brain samples.

A statistical analysis using Graph Pad Prism, Version 4.0 software of data represented in Fig. 3 was carried out. ANOVA followed by a Bonferroni's Multiple Comparison post hoc test for comparing pair of groups was performed. The results are shown in Table 4.

From Table 4, it can be concluded that there is a significant difference ($P < 0.05$) between control and each one of the atherogenic groups (AT 30 and AT 60), as well as among control group and the groups fed with antioxidant, at both times (AO 30 and AO 60).

On the contrary, when groups AT 30 and AT 60 were compared, a significant increase in brain lathosterol concentration ($P < 0.05$) was detected. However, when AO 30 and AO 60 were compared, P-value is larger than 0.05 and the confidence interval includes the value 0, which means that there is no significant difference between both the groups compared.

After the application of Bonferroni Multiple Comparison test for correction, it has been demonstrated that brain lathosterol increase in atherogenic group and reduction in antioxidant group are both statistically significant.

4. Conclusions

A simple, sensitive, precise and rapid method to evaluate the level of lathosterol in brain samples is proposed. The method utilized GC–MS determination of this precursor of cholesterol synthesis in rabbit brain; the data obtained were used as markers of the evolution of brain cholesterol levels thus making the periods of study shorter. The data in Table 4 and Fig. 3 demonstrate that animals supplemented with natural antioxidant have a notable reduction of the brain lathosterol levels after 30 days; however, no further changes are observed within 60 days compared to 30 days. The group fed with an atherogenic diet showed a high increase in lathosterol content after 1 month, as expected. The presence of polyphenolic antioxidants in the diet affected the production of lathosterol and, therefore, of cholesterol production in the brain.

Acknowledgement

This work was supported by the Spanish Interministerial Commission of Science and Technology (CICYT) in its "Juan de la Cierva" program.

References

[1] I.L. Notkola, R. Sulkava, J. Pekkanen, Neuroepidemiology 17 (1998) 14.
[2] G.P. Jarvik, E.M. Wijsman, W.A. Kukull, G.D. Schellenberg, C. Yu, E.B. Larson, Neurology 45 (1995) 1092.
[3] M. Kivipelto, E.L. Helkala, M.P. Laakso, A. Mannermaa, J. Tuomilehto, H. Soininen, A. Nissinen, BMJ 322 (2001) 1447.
[4] K.M. Thelen, P. Falkai, T.A. Bayer, D. Lütjohann, Neurosci. Lett. 403 (2006) 15.
[5] S. Locatelli, D. Lütjohnan, H. Hartmut, J. Schmidt, O. Carsten, U. Deisiegel, K. von Bergmann, Arch. Neurol. 59 (2002) 213.
[6] L.M. Refolo, M.A. Pappolla, B. Malester, J. LaFrancois, R. Bryant-Thomas Wang, K. Sambamurti, K. Duff, Neurobiol. Dis. 7 (2000) 321.
[7] H.M. Abdul, G.L. Wenk, M. Gramling, B. Hauss-Wegrzyniak, D.A. Butterfield, Neurosci. Lett. 368 (2004) 148.
[8] E.R. Frears, D.J. Stephens, C.E. Wallters, H. Davies, B.M. Austen, NeuroReport 10 (1999) 1699.
[9] M. Simons, P. Keller, B. de Strooper, K. Beyreuther, C.G. Dotti, K. Simons, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6460.
[10] M. Andersson, P.O. Elmberger, C. Edlund, K. Kristensson, G. Dallner, FEBS Lett. 269 (1990) 15.
[11] I. Björkhem, S. Meaney, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 806.
[12] R.E. Botti, J. Triscari, H.Y. Pan, J. Zayat, Clin. Neuropharmacol. 14 (1991) 256.
[13] L. Bretillon, A. Sidén, L.O. Wahlund, D. Lütjohann, L. Minthon, M. Crisby, J. Hillert, C.G. Groth, U. Diczfalusy, I. Björkhem, Neurosci. Lett. 293 (2000) 87.
[14] J. Davignon, Circulation 109 (2004) 1139.
[15] J.M. Dietschy, S.D. Turley, Curr. Opin. Lipidol. 12 (2001) 105.
[16] A. Papassotiropoulos, D. Lütjohann, M. Bagli, S. Locatelli, F. Jessen, M.L. Rao, W. Maier, I. Björkhem, K. von Bergmann, R. Heun, NeuroReport 11 (2000) 1959.
[17] A.A. Kandutsch, A.E. Russell, J. Biol. Chem. 235 (1960) 2256.
[18] K. Bloch, R.B. Clayton, P.B. Schneider, J. Biol. Chem. 224 (1957) 175.
[19] G.S. Tint, G. Salen, Metabolism 26 (1977) 721.
[20] H.J.M. Kempen, J.F.C. Glatz, J.A. Gevers Leuwn, H.A. van der Voort, M.B. Katan, J. Lipid. Res. 29 (1988) 1149.
[21] T.A. Miettinen, Clin. Chim. Acta 124 (1982) 245.
[22] T.A. Miettinen, P. Koivisto, in: G. Paumgartner, A. Stiehl, W. Gerok (Eds.), Bile Acids and Cholesterol in Health and Disease, MTP Press Limited, Boston, MA, 1983, p. 183.
[23] B. Lindhental, A. Holleran, T. Aldaghlas, B. Ruan, G. Schoroepfer, W. Wilson, J. Kelleher, FASEB 15 (2001) 775.
[24] J. Parcerisa, G. Richardson, M. Rafecas, R. Codony, J. Boatella, J. Chromatogr. A 805 (1998) 259.
[25] EC Annexe V, Off. J. Eur. Commun. EC, Brussels (5-9–91) L248/15.
[26] M.A. Kaluzny, L.A. Duncan, M.V. Merritt, D.E. Epps, J. Lipid. Res. 26 (1985) 135.
[27] G. Salen, G. Xu, G. Tint, A. Batta, S. Shefer, J. Lipid. Res. 41 (2000) 1983.
[28] K. Berge, K. Bergmann, D. Lutjoham, R. Guerra, S. Grundy, H. Hobbs, J. Cohen, J. Lipid. Res. 43 (2002) 486.
[29] T. Miettinen, T. Strandberg, H. Gylling, Artherioscler. Thromb. Vasc. Biol. 20 (2000) 1350.
[30] K.H. Phillips, D.M. Ruggio, J.A. Bailey, J. Chromatogr. B 732 (1999) 17.
[31] C. Domeño, B. Ruiz, C. Neri, Anal. Bioanal. Chem. 381 (2005) 1576.
[32] D. Lütjohann, M. Stroick, T. Bertshc, S. Kühl, B. Lindenthal, K. Thelen, U. Andersson, I. Björkhem, K. von Bergman, K. Fassbender, Steroids 69 (2004) 431.
[33] S.A. Wiseman, J.N. Mathot, N.J. de Fouw, L.B. Tijburg, Atherosclerosis 120 (1996) 15.
[34] J.P. De La Cruz, M.A. Villalobos, J.A. Carmona, M. Martin-Romero, J.M. Smith-Agreda, F.S. de la Cuesta, Thromb. Res. 100 (2000) 305.
[35] Analytical Methods Committee, Analyst 119 (1994) 2363.
[36] Commission decision laying down performance criteria for the analytical methods to be used for certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC, Draft SANCO/1805/2000, 2000. Rev. 1.