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Simultaneous reversed-phase high-performance liquid chromatographic separation of non-polar isoprenoid lipids and their determination

Marco Vincenzo Piretti[a,*], Giampiero Pagliuca[a], Giorgio Tarozzi[b]

[a] *Dipartimento di Biochimica, Sezione di Biochimica Veterinaria, Università di Bologna, via Tolara di Sopra 50, 40064 Ozzano dell'Emilia, Bologna, Italy*

[b] *Dipartimento di Scienze Biomediche, Sezione di Fisiologia, Università di Modena, via G. Campi 287, 41100 Modena, Italy*

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Abstract

A procedure for the rapid identification and determination of non-polar isoprenoid lipids from animal tissues was developed. The complete determination can be carried out by reversed-phase HPLC of just two samples. The first, extracted from unaltered tissues and suitably processed by column chromatography, provides information about free cholesterol, cholesteryl esters, coenzymes Q, free dolichols and dolichyl esters. The second, obtained from saponified tissues, can be used to detect both total cholesterol and total dolichols. Specific calibration graphs were constructed for the determination of the different constituents.

1. Introduction

The end-products of the mevalonate pathway play several primary biological roles. They either act in the regulation of the fluidity of the cell membranes (cholesterol, Chol), or take part in the respiratory chain (ubiquinones, Ubi) or behave as essential intermediates in the formation of asparagine-linked glycoproteins (dolichyl phosphates, Dol-P). Moreover, in recent years, particular attention has been paid to dolichols (Dol) and Dol-esters because of their implication in normal ageing and certain diseases [1–4]. The investigation of isoprenoid lipids is usually performed with two different approaches, depending on the information desired. When, besides free Chol and Dol, Chol-esters and Dol-esters, together with squalene (Squal) and Ubi, are to be measured, the total lipid directly extracted from the untreated tissues constitutes the object of the analysis. On the other hand, when only total Chol and Dol, or Dol-P, are to be measured, the tissues are usually reacted with a strongly alkaline reagent before extracting the lipid residue.

The analytical procedures generally carried out require complex preliminary separations of the various lipid classes, which are normally determined individually by reversed-phase HPLC [5–7]. The analysis we propose introduces considerable simplification since it makes it possible to separate and determine all the constituents of polyisoprenoid systems in just one step. This means that the lipid extract from the untreated tissues and the corresponding extract

* Corresponding author.

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from the saponified tissues can be analysed by means of two single injections into the HPLC apparatus after simple treatments to prepare the samples. Moreover, the use of suitable calibration graphs to determine the various constituents reduces the time for the whole analysis and minimizes the cost of the procedure.

2. Experimental

2.1. Chemicals

Organic solvents and reagents were of analytical-reagent or HPLC grade. Silica gel 60 plates were purchased from Merck (Darmstadt, Germany) and aluminium oxide, silicic acid and Celite for column chromatography were purchased from J.T. Baker (Deventer, Netherlands). Chol, Squal, Ubi-9, Ubi-10 and Dol-19 standards and oleyl chloride were supplied by Sigma (St. Louis, MO, USA).

2.2. Synthesis of standard cholesteryl oleate (Chol-Ol)

Oleyl chloride (300 μl) was added to 75 mg of Chol dissolved in anhydrous pyridine (1 ml). The mixture was stirred overnight at room temperature, 0.25 *M* H_2SO_4 (25 ml) was then added and diethyl ether (25 ml) was used to extract the reaction products. The organic solution was washed again with 0.25 *M* H_2SO_4 (25 ml) and twice with water (25 ml). Finally, it was dried over anhydrous Na_2SO_4. After evaporating the solvent, the crude residue, possibly containing oleic anhydride [8], free oleic acid and unreacted Chol, was purified as follows. A 1 *M* methanolic solution of KOH (3 ml) was added to the residue and the mixture was stirred for 10 min at 40°C to hydrolyse the anhydride. After evaporation of methanol under a stream of nitrogen, the residual material was dissolved in diethyl ether (25 ml) and washed twice with 25 ml of water in a separating funnel. After drying the ether solution over anhydrous Na_2SO_4 and evaporating the solvent, the solid material was dissolved in chloroform (500 μl) and passed through a small chromatographic alumina column (4 × 0.6 cm I.D.), which retains the free fatty acids, by eluting with chloroform (5 ml). Finally, after evaporation of the chloroform, the recovered material was purified by column chromatography to separate the unreacted Chol from the Chol-Ol. For this purpose a glass column (50 × 2 cm I.D.) packed with silicic acid–Celite (5:1) was used. The lipid mixture was dissolved in *n*-hexane–diethyl ether (8:2) (500 μl) and loaded on the top of the column. Elution was carried out with the same solvent and the fractionation was monitored by TLC. By evaporating the solvent from the combined corresponding fractions, 73 mg (86% yield) of pure (≥99% by HPLC) Chol-Ol was recovered.

2.3. Synthesis of standard Dol-19-Ol

The procedure used for the synthesis and purification of the ester was substantially as described for Chol-Ol. By starting from 15 mg of Dol-19, 4 mg (22% yield) of pure (≥97% by HPLC) Dol-19-Ol was obtained.

2.4. Animal tissues

Isoprenoid lipids from rat brain and bovine liver were used to set up the HPLC separation. The corresponding tissue samples were supplied by the Veterinary Surgery Clinic of the University of Bologna and the Department of Biomedical Sciences of the University of Modena.

2.5. TLC determinations

Silica gel plates were developed using *n*-hexane–diethyl ether–acetic acid (80:20:1) at 4°C [9]. After development, the plates were sprayed with H_2SO_4–aqueous formaldehyde (9:1, v/v) and heated at 150°C until coloured spots appeared. Phosphorylated compounds were detected using Dittmer reagent [10].

2.6. HPLC conditions

All measurements were made using a Kontron 325 S ternary liquid chromatograph equipped

with a 10-μl loop, combined with a Spectra-Physics SP 8440 UV–Vis variable-wavelength detector. Retention times (t_R) and quantitative parameters were determined with a Spectra-Physics Model 4270 integrator. The data system output was fixed at 1.0 absorbance unit/V. A 5-μm Ultracarb 5 ODS-20 column (150 × 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA) was used, coupled with a precolumn (100 × 4.6 mm I.D.), packed with Partisil-10 PAC (Whatman, Clifton, NJ, USA).

Two solvent systems were employed: methanol–water (85:15) (A) and 2-propanol (B). The following elution programme was used: time 0, A–B (60:40); 10 min, A–B (60:40); 50 min, A–B (15:85); 75 min, A–B (15:85). The chromatograms were systematically recorded by injecting 10-μl aliquots of each sample dissolved in *n*-hexane–2-propanol (1:1), using a flow-rate of 1 ml/min. Peaks were monitored at 210 nm.

2.7. Regeneration of column activity

When the column performance deteriorated, i.e., the separations were no longer optimum, the original activity was regenerated by washing the column with 100% solvent A (flow-rate 0.5 ml/min) for 1 h and storing it in the same solvent at room temperature for about 1 week.

2.8. Quantitative analysis

All the quantitative measurements were performed with the aid of suitable calibration graphs that had been constructed under the same detector and data system recording conditions. For each reference standard, ten solutions of suitable concentration in *n*-hexane–2-propanol (1:1) were prepared. They were checked under the above HPLC conditions used for the separation of the isoprenoid mixtures. The average values of the area counts of the chromatographic peak, after three 10-μl injections of every standard solution, were plotted against the corresponding concentration value to construct the calibration graphs. Table 1 shows the concentration ranges of the various standard solutions and the parameters used for constructing the calibration graphs.

2.9. Extraction of total lipids from untreated tissues and their purification for HPLC analysis

Wet material, cut into small pieces, was treated with 20 volumes (ml per g of tissue) of $CHCl_3$–CH_3OH (2:1) containing 0.3% (w/v) of butylated hydroxytoluene (BHT). The mixture was homogenized with an Ultraturrax apparatus and extracted according to Folch et al. [11]. After drying the organic phase over anhydrous Na_2SO_4, the solvent was evaporated and the residue was immediately purified for HPLC analysis. For this purpose, up to 500-mg aliquots of raw lipid extract were loaded on the top of a glass column (20 × 1.5 cm I.D.) packed with silicic acid–Celite (5:1). Step fractionation was carried out by eluting initially with *n*-hexane–diethyl ether (95:5) until triacylglycerols were eluted. Chloroform was then passed through the column to collect the remaining non-polar constituents. By checking the fractionation by TLC, all the fractions containing triacylglycerols were discarded. The remaining fractions were combined and the residue obtained after evaporating the solvent was systematically weighed into a 1-ml flask and dissolved in *n*-hexane–2-propanol (1:1). The solution was used directly for TLC and HPLC analyses.

2.10. Recovery of isoprenoid constituents through the silicic acid–Celite chromatographic column

A reference solution was prepared by dissolving appropriate amounts of non-polar isoprenoid standard constituents in a defined volume of *n*-hexane–2-propanol (1:1). The corresponding chromatogram was recorded and the amount of each constituent in the mixture was determined with the aid of the corresponding calibration graphs. The solvent was then evaporated and the residue was passed through a silicic acid–Celite column as described above. The eluted material was collected and dissolved in the same volume of *n*-hexane–2-propanol (1:1) as before and the solution obtained was again analysed by HPLC. By comparing the peak-area counts recorded for the various constituents before and after process-

ing the standard mixture, we evaluated the corresponding recoveries, which were found to be as follows: Chol 93%, Squal 92%, Ubi-9 94%, Ubi-10 96%, Chol-Ol 94%, Dol-19 97% and Dol-Ol 98%.

2.11. Extraction of isoprenoid lipids after alkali hydrolysis of the tissues

About 1 g of wet tissue was placed in a 10-ml Sovirel screw-capped tube and 3 ml of methanol and 2 ml of 15 *M* KOH [7] were added. The tube was sealed with a Teflon-lined plastic cap and heated at 100°C in a silicone-oil bath for 60 min. After cooling to room temperature, the resulting liquid mixture was extracted twice with 25 ml of diethyl ether. The ether extract (50 ml) was washed in sequence with 50 ml each of distilled water, 5% acetic acid, 5% sodium hydrogencarbonate and distilled water. After drying over anhydrous sodium sulphate, the organic phase was filtered and the solvent was evaporated under vacuum using a rotary evaporator (bath temperature 40°C). The lipid residue was immediately treated three times with diazomethane [12] to convert the free fatty acids contained therein into the corresponding methyl esters. The raw material so obtained, without further purification, was systematically weighed into a 1-ml flask and dissolved in *n*-hexane–2-propanol (1:1). The solution was used directly for TLC and HPLC analyses.

3. Results and discussion

Because of the common initial pathway, the regulation and interdependence of biosynthesis of Squal, Chol, Chol-esters, Ubi, Dol, Dol-esters and Dol-P have been studied intensively in recent years. They are normally distributed in all animal tissues and can be easily extracted with proven procedures. The total lipid extract obtained according to Folch et al. [11] contains all the above isoprenoid lipids. Current analytical procedures for determining the total amount of Chol and Dol only, however, involve strong alkaline hydrolysis of the tissues to release Chol and Dol from the corresponding esters, which causes the partial destruction of Ubi [13]. It follows that, depending on the objective of the study, two different systems are usually analysed, i.e. the total isoprenoid lipid mixture collected from the untreated tissues and/or the partial mixture extracted after saponification of the tissues.

HPLC provides precise identification and quantification of the different isoprenoid constituents. As far as we know, so far only Greenspan et al. [14] have proposed a procedure to measure all the constituents of complex isoprenoid lipid mixtures simultaneously. In this case, however, the separation of the isoprenoid constituents was performed with a cyanopropyl column, a normal phase that does not allow the different lipid species to be separated. Hence cholesteryl esters, ubiquinones and dolichols appear as single peaks. Moreover, dolichyl esters were not included in this study. Hence we can conclude that so far appropriate methods to measure all the constituents of complex isoprenoid lipid mixtures simultaneously do not exist. On the other hand, rather lengthy procedures are normally adopted to isolate the various components that are to be measured separately. Undoubtedly the use of prepacked cartridges such as Millipore Sep-Pak or similar mini-columns facilitates such procedures [7,15], even if the isolation of the various constituents requires a tedious stepwise elution process. Moreover, every determination involves adding to the sample a suitable amount of a reference standard, which increases the cost of the analysis.

Our proposed method significantly simplifies the current procedures because, in any case, a single treatment allows us to prepare the sample for the analysis. Fig. 1 shows the excellent separations obtainable using the HPLC conditions we have suggested for analysing the two typical non-polar isoprenoid lipid mixtures generally investigated.

Fig. 1a shows the separation of the more complex mixture, which contains all the non-polar isoprenoid lipids present in animal tissues. Before recording the corresponding chromatogram, both phospholipids and triacylglycerols

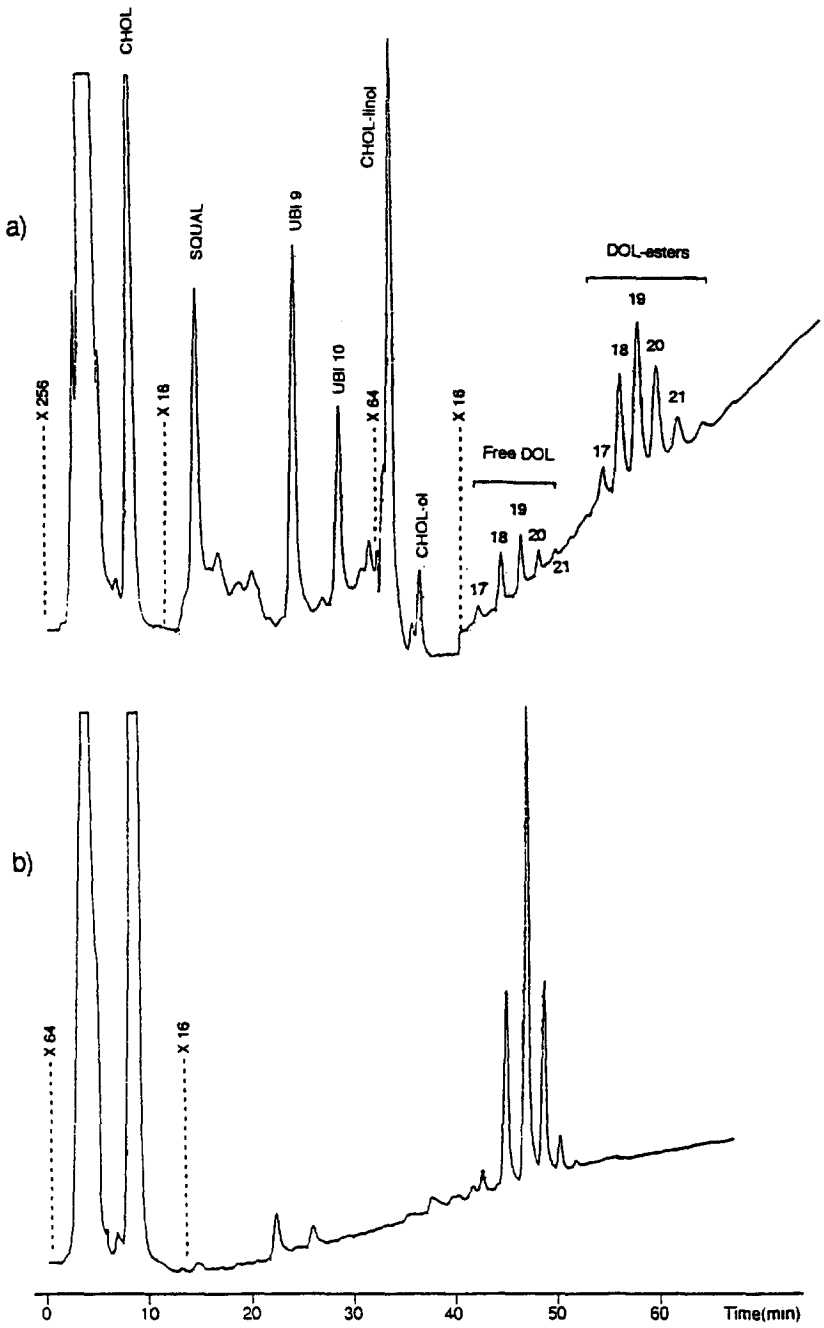

Fig. 1. HPLC profile of non-polar isoprenoid lipids from rat brain. (a) Total mixture after silicic acid–Celite column chromatography; (b) after alkali hydrolysis and methylation. Numbers reported on Dol and Dol-ester peaks refer to the number of corresponding isoprenoid units.

that are systematically contained in the total lipid extract from untreated tissues must be removed. In the case of phospholipids, this is because their presence in the sample can mislead the evaluation of Chol and, in the case of triacylglycerols, because their position in the chromatogram would partially overlap Chol-esters (see Fig. 2). To this end, the total lipid extracted according to Folch et al. [11] can be fractionated by silicic acid–Celite column chromatography. By performing the elution with moderately polar solvents, guaranteeing an excellent recovery of the non-polar constituents without removing the polar ones from the phase, triacylglycerols can be isolated and discarded. We ascertained that the lipid mixture obtained by combining the remaining fractions contains around 95% of the total non-polar isoprenoid constituents. Fig. 1b shows the separation of the mixture containing Chol and Dol extracted from the saponified tissues. For the determination of total Chol and Dol only, the lipid material extracted from the hydrolysed tissues could be directly analysed under the same HPLC conditions that allow us to separate the whole isoprenoid mixture. However, to avoid possible interference owing to the presence in the extract of some free fatty acids, originating from hydrolysis of the corresponding potassium salts during the diethyl ether–water extraction [9], before carrying out the HPLC separation we preferred to react the extract with diazomethane [12] to convert them into the corresponding methyl esters, since this treatment does not result in a loss of material. Fig. 3 shows the effect of methylation on the HPLC separation.

According to the literature, Dol has generally been determined by using Dol-23 as an internal standard, Chol has been measured by reference to ergosterol, whereas Ubi-6 has been used for the evaluation of Ubi-9 [7]. In spite of their biological significance, however, Chol- and Dol-esters have often been neglected and only a small

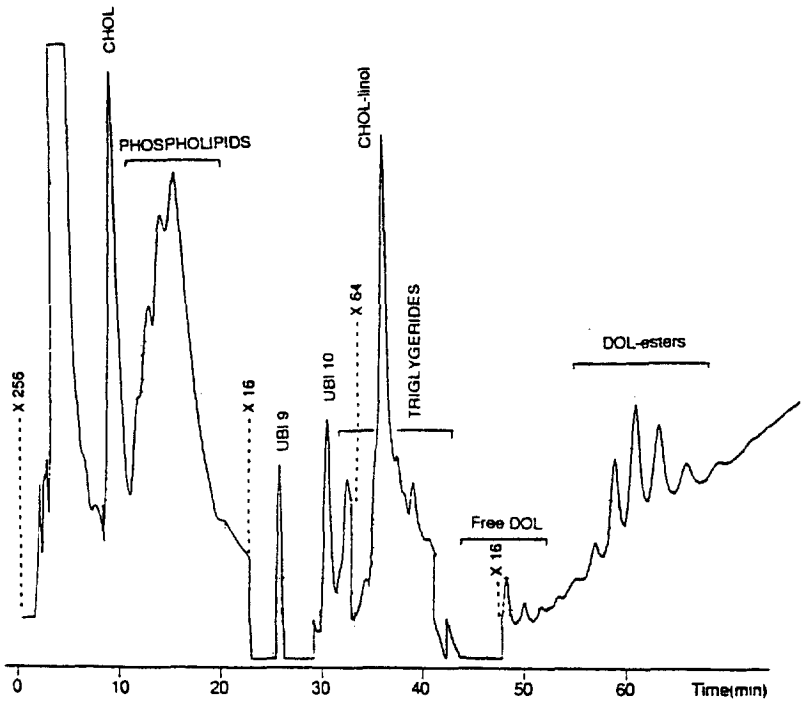

Fig. 2. HPLC profile of non-polar isoprenoid lipids from rat brain before silicic acid–Celite column chromatography. The chromatogram clearly shows the necessity to remove phospholipids and triacylglycerols from the extract.

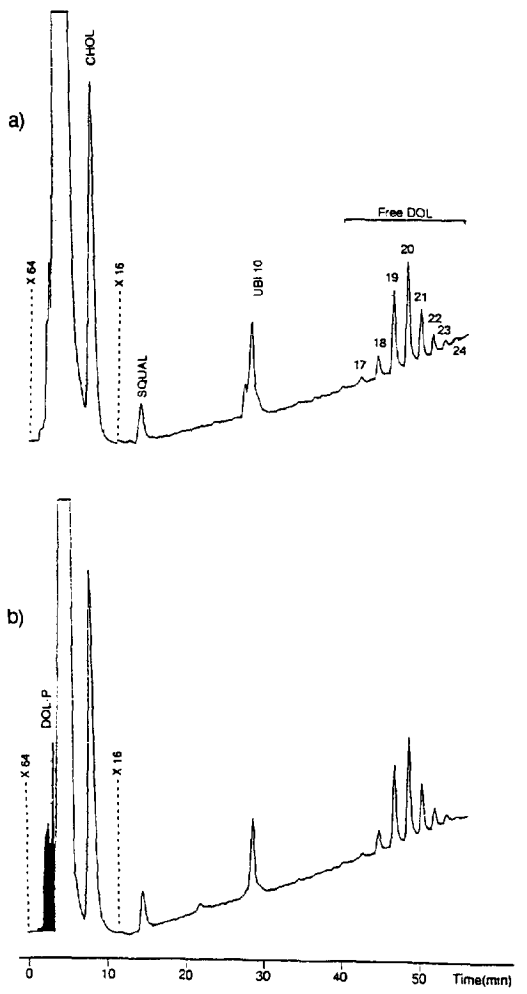

Fig. 3. HPLC profile of the isoprenoid lipid mixture from bovine liver after alkali hydrolysis: (a) before and (b) after methylation. Numbers reported on Dol peaks refer to the number of corresponding isoprenoid units.

amount of data on their determination has been reported [6,16].

As far as quantification is concerned, our position is different to the conventional practice. After ascertaining the very limited loss of every isoprenoid constituent by processing the whole lipid extracts by silicic acid–Celite column chromatography, we found quantification with the aid of suitable calibration graphs, to be faster and less expensive. Consequently, standard Chol, Squal, Ubi-9 and Ubi-10 were used to construct the calibration graphs. Standard Dol-19 was preferred for the calibration graph of Dol, and Chol-Ol and Dol-19-Ol were especially synthesized for the quantification of Chol-esters and Dol-esters. Underlying this choice there is the consideration that Dol-19 and Dol-19-esters are the main constituents of this class of polyprenols in almost all animal tissues, whereas Chol-Ol and Dol-Ol are very common among Chol- and Dol-esters [6,16–18], even if sometimes, as in rat brain, we detected mainly Chol-linoleate.

Table 1 lists the slopes (m) and the intercepts (q) together with the correlation coefficients (r) for the calibration straight lines ($y = mx + q$) in the range of concentrations corresponding to the amounts of the isoprenoid lipid constituents usually contained in animal tissues. The peak-area counts ($\times 10^{-3}$) on the ordinate are plotted against the concentration of the reference standard (mg/l) on the abscissa. The high linearity of the graphs guarantees the accuracy of the quantitative determinations. By using the above calibration graphs, all the individual constituents can be properly quantified, whereas the measurements of Chol-esters (except for Chol-Ol, which in our chromatograms produces a well resolved peak), Dol homologues (except for Dol-19) and Dol-esters were accurate, albeit approximate.

Nevertheless, since in this research area quantitative determinations are not generally important for their intrinsic value, but are normally useful in comparing the amounts of various isoprenoid lipid constituents in different tissues of the same animal, or in comparing their contents in analogous tissues of different animals (as, for instance, in evaluating the status of ageing), we consider it preferable to have a way of correctly evaluating the differences in the amounts of each constituent instead of trying to determine the exact amount, sometimes by tentative calculation [7]. This is the basis of our proposed procedure with, as further advantages, a considerable saving of time and generally lower operational costs in comparison with the procedures proposed up to now. To show its usefulness we report in Table 2 the non-polar isoprenoid lipid composition of a rat brain, one hemisphere of which was directly extracted

Table 1
Calibration graphs for the determination of non-polar isoprenoid lipids: slope (m), intercept (q) and correlation coefficient (r) of the calibration straight lines $y = mx + q$

Reference standard	Concentration range (mg/l)	t_R (min)	m	q	r
Chol	$2.5 \cdot 10^3$–$25.1 \cdot 10^3$	8	2265.68	522.13	0.999
Squal	2–100.5	14.5	35.81	−10.23	0.999
Ubi-9	1–60	24	56.55	−8.67	0.999
Ubi-10	1–60	28.5	37.93	−19.09	0.999
Chol-Ol	26.3–$5.6 \cdot 10^3$	36	1.29	−50.14	0.999
Dol-19	2.6–350	45	31.06	−31.90	0.999
Dol-19-Ol	3.1–307	62	34.14	−54.30	0.999

In the graphical representations we chose as units 1 mg/l on the abscissa and 10^{-3} peak-area counts on the ordinate. The parameters m, q and r were determined according to the least-squares calibration mode. For this purpose, only the experimental data were used, while the points 0;0 were never considered. A 10-μl aliquot of each standard solution was injected three times to match the corresponding peak-area counts.

Table 2
Non-polar isoprenoid lipid composition of rat brain

Constituent	Untreated tissue[b]	Literature[c]	Hydrolysed tissue[b]	Literature[c]
Cholesterol[a]	16.1 mg	14.3[6] 13.4[7] 12.5–9.4[19]	17.6 mg	3.8–9.8[5]
Squalene	40.9 μg	9.4[6]		
Ubiquinone 9	12.7 μg	21.3[d][7]		
Ubiquinone 10	11.0 μg	23.3–12.8[d][19]		
Cholesteryl esters[a]	5.4 mg	9.9[6]		
Free dolichols	12.6 μg	17.7[6] 19.2[7] 17.7–24.1[19]	60.5 μg	9.4–27.9[5] 45.3[20]
Dolichyl esters	58.3 μg	No data		

[a] The values were determined after suitably diluting the sample.
[b] The data reported refer to 1 g of wet tissue.
[c] Corresponding data available in the literature.
[d] Data refer to "ubiquinone".

whereas the other underwent alkali hydrolysis, and analysed in accordance with our procedure. The amounts of the different constituents we obtained agree with the corresponding values available in the literature (see Table 2). In addition, since they were determined all together, with just one HPLC injection of the respective sample, they may be considered homogeneous and comparable to one another.

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