Optimisation of the separation of four major neutral glycosphingolipids: application to a rapid and simple detection of urinary globotriaosylceramide in Fabry disease

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Abstract

A simple method for the separation of the four major neutral glycosphingolipids, present in all human tissue, was developed. This gradient normal phase-HPLC method utilises a polyvinyl alcohol bonded stationary phase and an evaporative light-scattering detection (ELSD). Screening pure solvents in a binary gradient elution mode allowed, in a first step, to assess the behaviour of the studied solutes and to select the solvents for further mobile phase optimisation. The proportion of the remaining solvents was defined to reach a maximal resolution. The reduction of the analysis time and the enhancement of the signal were obtained by optimising the gradient slope and the flow-rate. Optimal levels of triethylamine and formic acid (TEA-FA) for the enhancement of the evaporative light scattering detector response were established at 0.1% (v/v). Thus, the optimal conditions for the separation of the four glycosphingolipids was obtained with a gradient elution from a 100% chloroform to a 100% acetone:methanol (90:10 (v/v)) mobile phase at 0.2 ml min^{-1}, using a 10% min^{-1} gradient slope. Finally, this method was applied to detect the excess of one of the neutral sphingolipids, namely globotriaosylceramide (Gb$_3$) in the urine of patients affected with Fabry disease. A liquid–liquid extraction of the sediments obtained from an aliquot of only ten ml of urine proved sufficient to detect the excess of Gb$_3$ present in both hemizygote and heterozygote patients. In all, the ability of our method to detect abnormal amounts of Gb$_3$ in urinary sediments could allow the diagnosis of weakly symptomatic Fabry patients in large screening programs
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1. Introduction

Neutral glycosphingolipids, which are present in tissues and various body fluids, are amphiphilic molecules composed of a ceramide backbone linked to, one osidic chain [1]. Physiologically, four major neutral glycosphingolipids called glucosylceramide (Gb$_1$), galactosylglucosylceramide (Gb$_2$), globotriaosylceramide (Gb$_3$) and globoside (Gb$_4$) have been identified [2]. They possess one, two, three and four sugar units or osidic moieties, respectively.

The accumulation of one or more of these lipids in tissues and/or fluids is called a sphingolipid-storage disease. These sphingolipidoses are genetic diseases due to a mutation in the genes encoding lysosomal acid lipid hydrolases, leading to the progressive lysosomal accumulation of endogenous lipids. One of them, Fabry disease, is caused by a deficiency of the α-D-galactosidase A, which leads to the accumulation of the neutral glycosphingolipid Gb$_3$ [3].

In hemizygous males, the unambiguous diagnosis of Fabry disease is based on the enzymatic assay of α-galactosidase A activity in plasma or leucocytes. In heterozygous females, this enzymatic diagnosis can be misleading underlying the need for genotyping or additional diagnostic procedures. The demonstration of the Gb$_3$ excess in biological fluids is more used for screening purposes. In this case the detection method of Gb$_3$ generally consists of a separation method for the different glycosphingolipids followed by an appropriate mode of detection. The most

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rapid method utilises flow injection analysis with electrospray ionization-tandem mass spectrometry, it requires only a simple 50-fold dilution of biological samples [11]. All other reported methods are more complex, requiring a lipid extraction of the biological matrix, followed by a glycosphingolipid separation (using HPLC or HPTLC) and/or isolation (fractionation with solid-phase extraction cartridge) and finally coupled with a direct or indirect detection method. For instance, TLC with colorimetric revelation [4–6], HPLC with UV detection after benzoylation [7–9] and more recently LC MS/MS [10–12] or TLC with ELISA [13] are used.

Our objective is to confirm the diagnosis for patients presenting evocative clinical signs of Fabry disease by separating the four neutral glycosphingolipids, with a special focus on the Gb_3, using a rapid and simple HPLC method with evaporative light-scattering detection (ELSD). Such detection mode is widely used for the lipid analysis [14,15] and avoids time consuming derivatisation steps. ELSD allows the detection of a solute provided that it is less volatile than the mobile phase. It is particularly attractive for the analysis of solutes which do not absorb UV radiation and it is compatible with gradient elution unlike the refractive index detector [16]. The major limitation of this technique is the relatively high limit of detection. However, in the case of lipids, its response can be significantly enhanced by the addition of equal amounts of triethylamine and formic acid (TEA-FA) in the mobile phase [17–19].

With respect to the HPLC separation of neutral glycosphingolipids, due to the different length of their osidic chain, silica stationary phases are generally used [7,9,20,21]. Other, different polar stationary phases, such as diol [22] or cyano [23], and more recently polymerized polyvinyl alcohol bonded on silica particles (PVA-Sil®) [24,25], were reported to be an alternative to silica for the analysis of lipid classes. PVA-Sil® was preferred because it allows obtaining more reproducible separations and requires shorter conditioning times for separations performed with elution gradient [25].

An optimisation of the separation of the four physiologically neutral glycosphingolipids (Gb_1, Gb_2, Gb_3 and Gb_4) with a PVA-Sil® stationary phase and an enhancement of the signal with the ELSD were thus performed. Next, the method was assessed by analysing urine samples taken from healthy volunteers and Fabry patients

2. Materials and methods

2.1. Chemicals

All solvents were HPLC grade. Methanol (MeOH) and acetonitrile (ACN) were purchased from Prolabo (Fontenay-sous-Bois, France), propan-1-ol, propan-2-ol, heptane, acetone and formic acid (FA) from Fisher Scientific (Elancourt, France). Chloroform ($CHCl_3$) stabilized

with about 0.75% (v/v) ethanol came from Carlo Erba (Val de Reuil, France) and TEA from Sigma (Saint-Quentin Fallavier, France).

Neutral glycosphingolipids were purchased from Sigma, for the glucosylceramide, lactosylceramide and globoside, and from Biovalley (Marne la Vallée, France) for globotriaosylceramide. Stock solutions containing $1\,\mathrm{mg\,ml^{-1}}$ of each neutral glycosphingolipid were prepared in chloroform/methanol (2/1 (v/v)).

2.2. Instrumentation and chromatographic set-up

The HPLC instrumentation consisted of a Ultra-Plus II pump (Micro-Tech Scientific Inc., Havard Apparatus Inc, Holliston, USA), a Valco INJ-P4 manual injector (Valco Instruments Co. Inc., Houston, USA) with a 5 μl loop and Kroma 2000 software (Bio-Tek, Saint-Quentin en Yvelines, France). Separation of the glycosphingolipids was carried out on a polyvinyl alcohol-bonded stationary phase, PVA-Sil® (5 μm, 150 mm × 2.0 mm i.d., YMC Europe, Schermbeck, Germany) and a guard column of the same material (10 mm × 2.0 mm). Detection was accomplished with a Cunow DDL 11 evaporative light scattering detector (Eurosep, Cergy Saint-Christophe, France). The detector settings were kept constant in all experiments using an evaporation temperature of 30 °C, an air-pressure of 1.0 bar and a gain setting of 6.

2.3. Extraction methods

A liquid–liquid extraction of Gb_3 was performed on urinary samples according to Folch's method [26]. The samples were homogenized with a chloroform-methanol mixture (2/1 (v/v)) to a final dilution 20-fold the volume of tissue sample, considering that 1 g of samples was 1 ml. The obtained monophasic solution contained crude extract of lipids. The solution was then mixed with a volume of water corresponding to 20% of the volume of organic solvents. After homogenisation and centrifugation (10 min at 2150 g), the solution was separated into two phases. The upper phase was carefully removed and the lower phase, containing lipids, was evaporated at room temperature under gentle air stream. The obtained residue was reconstituted with 100 μl of chloroform–methanol (2/1 (v/v)) and analysed.

To obtain urinary sediment and supernatant, a centrifugation was performed during 10 min at 2150 g, before the extraction procedure.

3. Results and discussion

3.1. Optimisation of the separation

The separation of neutral glycosphingolipids with HPLC was pioneered by McCluer and associates [7–9,21]. In these studies, the separation of benzoylated derivates of neutral

Table 1

Structure of the osidic chain of the four neutral glycosphingolipids (Glc = glucose; Gal = galactose; GalNac = n-acetylgalactose; Cer = ceramide)

Glycosphingolipid	Structure of osidic chain
Glucosylceramide (Gb$_1$)	Glc-β-(1 → 1')-Cer
Lactosylceramide (Gb$_2$)	Gal-β-(1 → 4)-Glc-β-(1 → 1')-Cer
Globotriaosylceramide (Gb$_3$)	Gal-α-(1 → 4)-Gal-β-(1 → 4)-Glc-β-(1 → 1')-Cer
Globoside (Gb$_4$)	GalNAc-β-(1 → 4)-Gal-α-(1 → 4)-Gal-β-(1 → 4)-Glc-β-(1 → 1')-Cer

Table 3

Evolution of the time corrected resolution criterion (t_{ne}) according to the composition of the strong solvent system of the gradient

Acetone/methanol (v/v)	t_{max} (min)	$R_{S min}$	t_{ne} (min)
0/100	8.88	<0.1	>1950
25/75	10.28	<0.1	>1400
50/50	12.97	0.7	26.5
75/25	20.03	1.5	8.90
90/10	28.79	2.7	3.95

glycosphingolipids was performed on silica columns. Separation experiments of glycosphingolipids were also carried out on TLC using silica plates [4–6]. However, the disadvantages of silica (i.e. poor reproducibility and peak asymmetry induced by the trace amounts of water either in solvents or in samples) led us to select PVA-Sil®. This polyvinyl alcohol phase is chemically bonded on silica particles and its separation properties are similar to silica. Moreover, the absence of free silanols on PVA-Sil® allows the addition of triethylamine-formic acid for detection purposes in the mobile phase without spoiling the retention properties of the stationary phase for non ionisable species [25].

The separation of neutral glycosphingolipids on a polyvinyl alcohol stationary phase was not reported in the literature but our group already reported on the systematic mobile phase development on PVA-Sil® for lipid materials in general [25]: In reference [25], a solvent screening in binary and tertiary gradient elution mode to distinguish the more apolar (triacylglycerols, diacylglycerols and cholesterol) from to the more polar (zwitterionic phospholipid) was described. Here however the polarity range of the studied compounds was more reduced due to the fact only the osidic chain varied between 1 and 4 osidic residues (Table 1).

The behaviour of the neutral glycosphingolipids was explored by applying a binary elution gradient from 100% weak solvent to 100% strong solvent, with a gradient slope at 2.5% min^{-1} and a flow-rate at 0.5 ml min^{-1}. According to the eluotropic strength scale established on silica [27], two weak solvents (heptane and chloroform) were associated to five strong solvents (acetonitrile, acetone, propan-2-ol,

propan-1-ol and methanol). Thus, seven binary elution gradients were performed.

Retention times, analysis durations, minimal resolution ($R_{S min}$) and selectivity (α) of the pair of peaks with the minimal resolution were reported in the Table 2. As expected, the retention order followed the number of sugar units linked to the ceramide moiety: Gb$_1$, Gb$_2$, Gb$_3$ and Gb$_4$. According to the retention times of solutes, the eluotropic strength of the tested solvents on PVA-Sil® can be classified as: heptane < chloroform < acetonitrile < acetone < propan-2-ol < propan-1-ol < methanol. This order was consistent with previous work [25].

For each tested mobile phase, the smallest resolution concerned the pair of peaks Gb$_3$–Gb$_4$. Their selectivity increased with the decrease of elution strength. Therefore, there was a minor contribution of strong solvents on the selectivity and the choice of solvents, constituting the mobile phase, was essentially based on their eluotropic strength.

Some binary gradients were also no longer considered for technical reasons. With propan-1-ol and propan-2-ol an important operating back pressure at the end of the gradient slope occurred and with acetonitrile a poor signal/noise ratio was encountered. Two weak (heptane and chloroform) and two strong (acetone, methanol) solvents now remained as possible components of the mobile phase. Concerning the strong solvents, the eluotropic strength of acetone was not sufficient for the elution of the most polar solute, Gb$_4$. So, the strong solvent had to be replaced by methanol. As a weak solvent, chloroform was preferred over heptane because of its miscibility with methanol and its bigger elution strength (Table 3). Nevertheless when using a

Table 2

Retention behaviour of the four neutral glycosphingolipids in binary elution gradient systems

Weak solvent	Strong solvent	t_R Gb$_1$ (min)	t_R Gb$_2$ (min)	t_R Gb$_3$ (min)	t_R Gb$_4$ (min)	Analysis duration (min)	$R_{S min}$ (Gb$_3$–Gb$_4$)	α (Gb$_3$–Gb$_4$)
Chloroform	Acetone	11.5	23.5	36.2	>40.0	>40.0	ND	ND
	Acetonitrile	10.6	18.2	24.5	>40.0	>40.0	ND	ND
	Propan-2-ol	7.3	11.0	13.1	14.2	16.3	0.73	1.09
	Propan-1-ol	6.9	10.5	12.6	13.2	14.7	0.54	1.05
	Methanol	6.1	7.8	9.1	9.2	10.3	<0.1	1.01
Heptane	Propan-2-ol	11.0	16.9	22.1	24.9	26.0	1.85	1.13
	Propan-1-ol	10.7	16.4	21.1	24.1	24.8	1.97	1.15
	Methanol			Solvents not miscible in all proportions				

ND: not determined.

Table 4
Evolution of the analysis run time, relative response of Gb$_3$ and minimal resolution according to value of flow-rate and gradient slope ($n = 3$)

Flow-rate (ml min^{-1})	Gradient slope (% min^{-1})	Total run time (min)	Relative response of Gb$_3$[a]	$R_{S\,min}$
0.5	2.5	28.0	1	3.5
	5	16.6	1.2	2.3
	10	11.2	2.0	1.5
0.2	2.5	31.2	2.9	3.9
	5	19.7	3.8	3.1
	10	12.5	7.2	2.4

[a] Defined as the ratio of peak area at the selected flow-rate and gradient slope and of Gb$_3$ peak area over the flow-rate of 0.5 ml min^{-1} and a gradient slope of 2.5% min^{-1}.

binary gradient from chloroform to methanol, the elution strength of methanol appeared too high to permit a sufficient resolution between Gb$_3$ and Gb$_4$. The eluotropic strength of methanol can however be counterbalanced with another solvent, with a smaller eluotropic strength, such as acetone. Consequently, ternary gradients, from chloroform to an acetone-methanol mixture, were envisaged. To find out the appropriate acetone-methanol ratios, an optimisation was performed using the time corrected resolution criterion, t_{ne}. The time corrected resolution criterion, t_{ne}, allowed to take into account the duration of analysis and the resolution [28]. The values of t_{ne} were obtained from the Eq. (1):

$$t_{ne} = \frac{t_{max}}{R_{S\,min}^2} \qquad (1)$$

where t_{max} is the retention time of the last eluted solute and $R_{S\,min}$, the minimum peak resolution. The t_{ne} values were calculated for different volume fractions of acetone–methanol and reported in the Table 3. As seen, lower eluotropic strength's implied lower t_{ne} values and higher resolutions, indicating a better separation.

An acetone–methanol (75/25 (v/v)) mixture permitted the separation of the four neutral glycosphingolipids in about 21 min, with a sufficient minimal resolution ($R_{S\,min} = 1.5$). Although these conditions appeared satisfactory, the acetone–methanol (90/10 (v/v)) composition leading to a greater minimal resolution ($R_{S\,min} = 2.7$) and offering more flexibility to adapt the chromatographic conditions (gradient slope, flow-rate) to improve the ELSD response, was finally chosen.

3.2. Optimisation of the detection

As already stated, an advantage of using PVA-Sil® instead of silica, was its compatibility with response modifiers such TEA-FA in the mobile phase. During the mobile phase optimisation, all tested mobile phases contained a constant amount of triethylamine (0.1% (v/v)) and an equimolar amount of formic acid already previously determined [17,18].

According to Deschamps et al. [19], for a mobile phase containing a constant TEA-FA concentration, the monitored signal increases when the flow-rate is reduced. Decreasing the flow-rate increases the analysis duration, which can be

compensated by an increase of the gradient slope. Therefore two flow-rates, 0.2 and 0.5 ml min^{-1}, and three gradient slopes, 2.5, 5 and 10% min^{-1}, were tested with regard to the ELSD response.

Results concerning the total analysis run time and the peak area for a same injected amount of Gb$_3$ were summarised in the Table 4. The signal intensity is increased about seven times with a 0.2 ml min^{-1} flow-rate and a 10% min^{-1} gradient slope compared to 0.5 ml min^{-1} and 2.5% min^{-1} gradient conditions. In ELSD detection, the intensity of the scattered light is proportional to the size of the solutes particles. This particle size is influenced by the solute concentration along the peak profile. Thus, narrowing the elution band profile using high gradient slope contributed to the higher observed sensitivity.

Finally, the influence of the TEA-FA concentration in the mobile phase was assessed by testing four concentrations: 0, 0.05, 0.1 and 0.2% (v/v) of triethylamine and the stoichiometric amount of formic acid (1/1). An enhancement of the recorded signal due to TEA-FA was seen, as illustrated in Fig. 1. However no significant increase of the signal was observed by a change from 0.1 to 0.2% (v/v) TEA-FA. These observations were in agreement with a previous study [17] where the ELSD response enhancement reached a plateau at about 0.1% (v/v) TEA-FA. With 0.1% TEA-FA (v/v) added in the mobile phase, the gain factors were 5 for Gb$_2$, 17 for Gb$_4$, 11 for Gb$_3$ and 16 for Gb$_1$. According to Deschamps et al. [18], the origin of this increase is assumed to
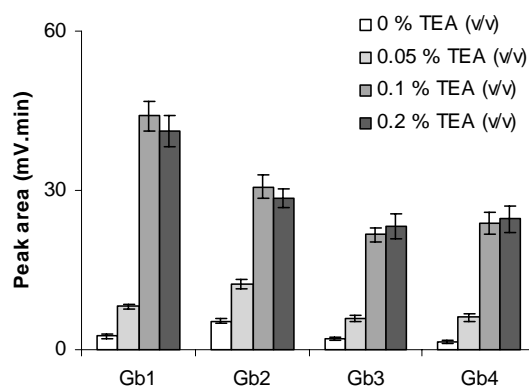

Fig. 1. Effect of the percentage of TEA-FA in the mobile phase on the response enhancement of the ELSD, $n = 3$ measurements. Concentration of each neutral glycosphingolipid = 0.25 mg ml^{-1}.
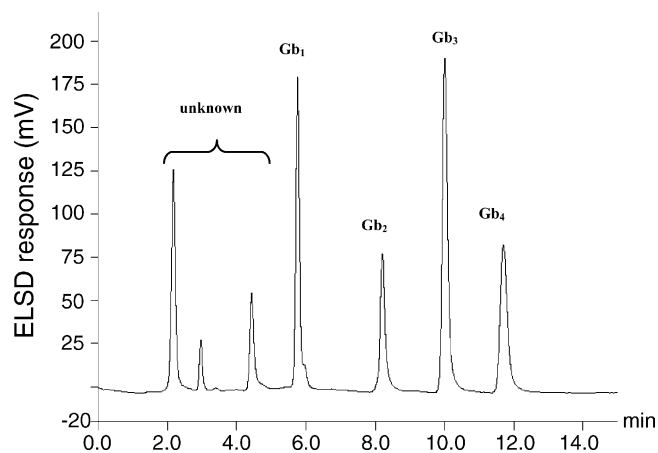
Fig. 2. Separation of the four neutral glycosphingolipids. Chromatographic conditions: Flow-rate $0.2\,ml\,min^{-1}$, elution gradient from 100% chloroform to 100% acetone–methanol (90/10 (v/v)) at a $10\%\,min^{-1}$ slope followed by a plateau during 5 min. $P_{DEDL} = 1$ bar, $T_{DEDL} = 30\,°C$.

result from the formation of complex association between TEA-FA and solutes. This association implies an increase of the diameter of the irradiated particles that provokes a higher scattered light intensity. Therefore, as the analysis was performed with an elution gradient, the difference in response enhancement between the different glycolipids was expected to come from the changes in mobile phase composition during the analysis. Moreover, the non-linear response of the ELSD is described as:

$$y = am^b \qquad (2)$$

where m represents the amount of substance injected, and a and b are numerical coefficients. The addition of 0.1% TEA-FA (v/v) in the mobile phase contributed to the increase of the values of the numerical coefficients [19] and thus the observed enhancement of the peak area.

Fig. 2 illustrates the final and optimal separation of the four neutral glycosphingolipids. Three peaks at 2.1, 3.0 and 4.8 min were not identified, possibly they are impurities of different standards of glycosphingolipids and solvents used during the injection.

The limit of detection (LOD), defined as 3 standard deviations of the blank, was about 80 ng of injected Gb_3. Previous HPLC separations of neutral glycosphingolpids reported slightly lower LOD e.g. with UV detection set at 230 nm is was 30 ng for Gb_4 [21]. The ELSD detector is often considered as less sensitive and providing higher LOD's compared to the UV detector, when UV absorbing compounds are considered. Since the benzoyl group is a strong chromophore, this lower LOD achieved by the HPLC method with derivatization was expected. However this lower LOD is obtained after a long derivatisation step of 4 [9] to 16 h [7]. Looking at the required sensitivity for the biological analysis, as demonstrated in the next section such derivatisation was not necessary here.

The analysis duration varied from 7 to 15 min in previous works [4,7–9], which was comparable with the analysis times on PVA-Sil® of about 12.5 min.

3.3. Assessment of the detection of Gb_3 in real urine samples

Prior to the detection of the presence of neutral glycosphingolipids in an urine sample, an extraction of the solutes was necessary because of the complexity and the variability of the biological matrix. The classical method was a liquid–liquid extraction [26,29], but a further step can be envisaged using a chromatographic approach to reach a higher level of purity [4,9,30–32].

In order to achieve an acceptable throughput for the method, a simple liquid–liquid extraction was assessed. Gb_3, present in urine, essentially reflects the amount of Gb_3 deposited in renal cells. Some studies used homogenised urines called "total urines" [6] but other were only performed on the urinary sediments after filtration [9,33] since the main amount of Gb_3 appeared to be in urinary sediments [34].

To investigate the application of our method to the total urine, the supernatant and the sediment, three aliquots of urines from healthy volunteers (male and female) were
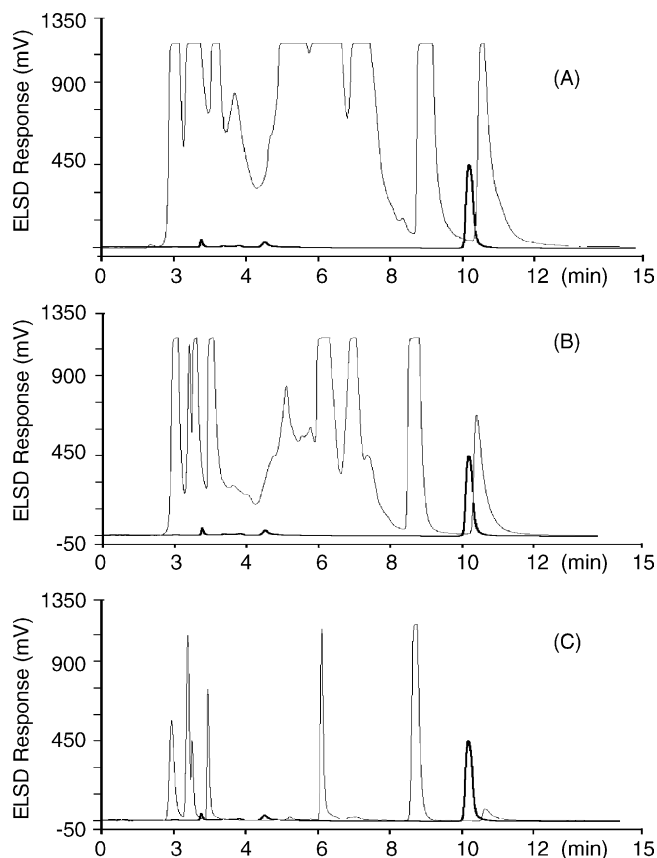

Fig. 3. Chromatographic profile corresponding to the extraction of 10 ml total urines (A), corresponding supernatant (B) and sediments (C) from a healthy volunteer—comparison with Gb_3 standard (thick chromatogram).
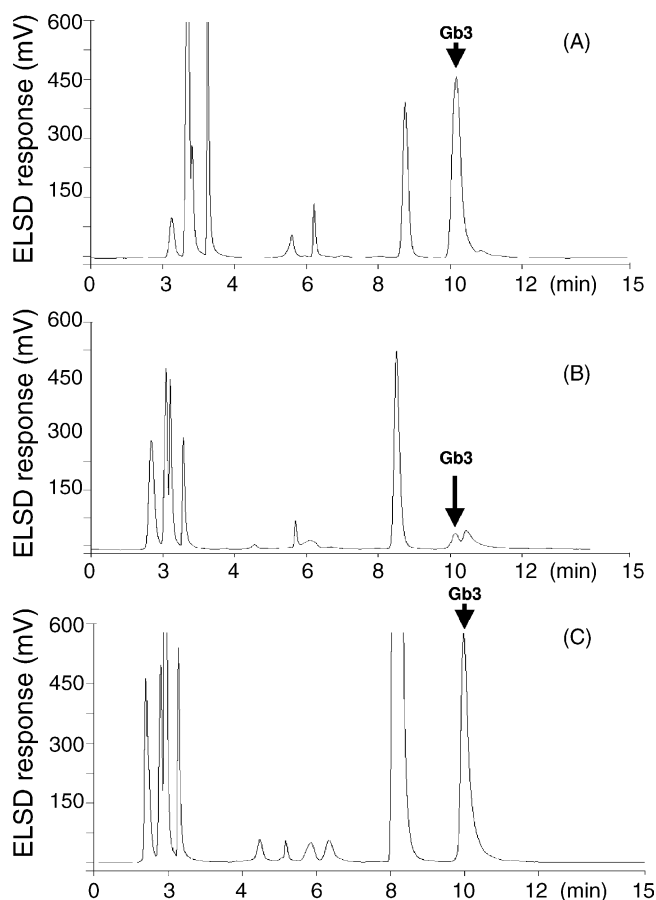
Fig. 4. Treatment of urinary 10-ml sediments from different populations of patients affected with Fabry disease (A: hemizygote patient; B: heterozygote patient; C: 11-years old hemizygote boy).

extracted and analysed (Fig. 3). The weakest interference was observed with the urinary sediment. The amount of interfering peaks increased for the supernatant and was maximum for the sample of total urine.

Next, the presence of Gb_3 in urine samples from hemizygote patients affected by Fabry disease, was researched. This population of patients contained only males developing important clinical signs of the disease due to a very reduced enzymatic activity [3]. Therefore, these patients should present important amounts of Gb_3 in their urine due to the renal failure induced by the disease [35]. Using our technique, the presence of Gb_3 was easily observed in sediments (Fig. 4A) but also in supernatant, with more important amounts in the sediment compared to the supernatant.

Finally, the urine samples of heterozygote and young hemizygote patients were tested. Lower Gb_3 concentrations should be observed with the heterozygote patient because of a residual enzymatic activity of α-galactosidase A [33]. Experimentally, the elimination of Gb_3 was detected in the urine sediments for the heterozygote patient (Fig. 4B). We also observed a chromatographic peak following the Gb_3 signal comparable to healthy volunteers (Fig. 3). With the

ELSD being nearly an universal detector, the risk of interfering signal can not be negligible.

For a young hemizygote patient, the Gb_3 amount in urine may be lower as Fabry disease is a storage pathology accumulating Gb_3 over the years. However, the Fig. 4C illustrates indeed that the Gb_3 signal recorded in the case of an 11-year old boy affected by the disease, is significant.

4. Conclusions

The method for the determination of the excretion of urinary Gb_3 presented here was simple, rapid and accessible by routine laboratories. The ELSD allowed to perform a direct detection without the use of an expensive and/or complicate mode of detection. With the addition of an equimolar mixture of TEA-FA, the major drawback of this detector, its low sensitivity, was controlled, permitting the detection of Gb_3 from limited volumes of urine. Moreover, the main Gb_3 amount was recorded in sediments which required only a light sample treatment procedure.

As this approach allowed revealing the accumulation of the enzyme substrate prior to the assessment of the enzymatic activity. The ability to detect abnormal amounts of Gb_3 in deposition in urinary sediments provides a useful tool to screen a population of patients presenting one specific clinical sign, such as a left ventricular hypertrophy, revealing an unknown Fabry disease. Furthermore, since the proposed method provides the separation of four physiologically glycosphingolipids, it may be useful to explore other sphingolipid storage diseases such as Gaucher disease (accumulation of Gb_1 due to a β-glucosidase deficiency), providing that an appropriate sample treatment is realised.

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