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Journal of Chromatography B, 720 (1998) 9–14

JOURNAL OF CHROMATOGRAPHY B

Quantitative analysis of free sphingoid bases in the brain and spinal cord tissues by high-performance liquid chromatography with a fluorescence detection

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Received 11 August 1998; received in revised form 21 September 1998; accepted 22 September 1998

Abstract

The *o*-phthaldialdehyde precolumn derivatives of psychosine, sphinganine and sphingosine extracted from brain and spinal cord tissues were determined by high-performance liquid chromatography–fluorescence detection. This method was developed with the purpose of detecting an endogenous amount of psychosine, sphingosine and sphinganine using small aliquots of brain tissues and spinal cord in rats. These sphingolipid bases were extracted in various ratios of chloroform–methanol and several pH values. Recovery of the method is about 81% in 12 ng/tube (final volume, 320 μl), 90–95% in 45 ng/tube of sphingosine and sphinganine within 2–12% relative standard deviation. Detection limits of these sphingoid bases were about 0.05 pmol/mg brain tissue. In the forebrain, brainstem and spinal cord of rats at three different ages of postnatal days (PND) 1, PND 13 and 6 months old, the endogenous concentrations of psychosine, sphingosine and sphinganine were determined. From these results, this method is suitable for the determination of sphingoid bases in small aliquot of brain and spinal cord tissues.

Keywords: Psychosine; Sphinganine; Sphingosine

1. Introduction

Sphingolipids are ubiquitous constituents of cell membranes in organisms. The backbone components of sphingolipids are the long chain bases, where C_{18} sphingosine is a major type that predominantly exists in mammalian species and is considered playing a role as the second messenger involved in the sphingomyelin signal transduction pathway [1–3]. Other major sphingoid bases contain sphinganine, psychosine, and 4-hydroxysphinganine. Sphinganine (dihydrosphingosine) is recently thought to be the intermediate in the biosynthetic pathway of sphingolipids, and sphingosine appears to be produced in a degrading step of sphingolipid. An accumulation of psychosine was documented in the brains of globoid cell leukodystrophy patients and the nervous tissues of a twitcher mouse [4,5]. Free sphingosine has been reported to exist in quail intestine [6], rabbit skeletal and cardiac muscle [7], several murine tissues [8], human epidermis [9], and rodent liver [10].

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0378-4347/98/$19.00
PII: S0378-4347(98)00449-6

The sphingosine contents were analyzed by various methods, including gas–liquid chromatography [9,11], high-performance liquid chromatography (HPLC)–fluorescence detection [10,12], HPLC–UV [6,13], and enzymatic assay [14]. Kobayashi et al. [8] developed a HPLC–fluorescence method for detection of sphingoid bases by solid–liquid extraction. The addition of a further step after derivatization, however, gave less recovery. This method was sensitive but required time-consuming and labour-intensive purification as well as hydrolysis step. In addition, the large scale of solvents used to measure sphingosine and sphinganine in liver and other tissues after base hydrolysis by HPLC–fluorescence technique needs to be modified, particularly for the application to brain tissue in developing rats.

A sensitive and simple method without hydrolysis is indispensable for the measurement of free form of sphingoid bases (specifically, sphingosine and sphinganine), using the small amount of the brain or spinal cord tissues in the central nervous system. The purpose of the present work, therefore, was focused on developing a simple, simultaneous and sensitive method for measuring sphingosine analogues from brain and spinal cord tissues.

2. Experimental

2.1. Chemicals

DL-*threo*-Dihydrosphingosine, DL-*erythro*-dihydrosphingosine, *trans*-D(+)-*erythro*-sphingosine, and psychosine (1-β-D-galactosylsphingosine) were obtained from Sigma (St. Louis, MO, USA). C_{20} dihydrosphingosine was generous gift from Dr. Merrill (Atlanta, GA, USA). *o*-Phthaldialdehyde was purchased from Sigma. Methanol and chloroform of HPLC grade were obtained from J. T. Baker (Phillipsburg, NJ, USA). The other agents used for sphingoid bases were of analytical grade. Purified water from Milli-Q water system (Millipore, MA, USA) was used for the preparation of mobile phase buffers.

2.2. Equipment

HPLC analysis was performed with a Waters' model M-6000A pump connected to a 16-μl flow cell of a fluorescence detector (Waters 470, Waters, Milford, MA, USA). A Waters 746 integrator was used. A C_{18} Nova-Pak column (60 Å, 4 μm, 150× 3.9 mm) was obtained from Waters. The mobile phase used for sphingoid base analysis consisted of methanol–5 m*M* potassium phosphate buffer (9:1, v/v) at the flow-rate of 1.1 ml/min, and the detector was set at excitation and emission wavelength of 340 and 440 nm, respectively. Attenuation of the integrator was fixed at 16.

2.3. Brain and spinal cord dissection

Rats were sacrificed by decapitation. Whole brain and spinal cord were removed rapidly and placed on dry ice after dissection. Whole brain was dissected into brain stem and cerebellum; the remaining portions of brain were divided into forebrain and hindbrain. The dissected regions were stored at −70°C until analysis.

2.4. Sphingoid extraction in the brain and spinal cord tissue

Two regions of brain and spinal cord were homogenized by ultrasonification (Cell Disruptor W-350 Sonifier, Branson Ultrasonic, Danbury, CT, USA) for 10 s in 4 vol of 50 m*M* potassium phosphate buffer (pH 7.0). One hundred μl of the forebrain and brainstem homogenates and 80 μl of spinal cord homogenate were used. C_{20}-sphinganine (10 μ*M*, 20 μl) was added to the homogenate in a screw-cap culture tube (13×100 mm, with screwed Teflon cap) and vortex-mixed. One-half ml of borate buffer (0.4 *M*, pH 13.1) and 4 ml of chloroform–methanol (9:1, v/v) were added. The layer was agitated on a shaker (Eberbach, Ann Arbor, MI, USA) for 20 min, and centrifuged (Hermle Z360K, Woodbridge, NJ, USA) for 8 min at 600 *g* (2000 rpm) at 4°C. The chloroform layer was evaporated in an evaporator (Savant, Savant Instruments, Farmingdale, NY, USA). The culture tubes were shaken for 5 min and centrifuged after the addition of 0.5 ml of borate buffer (0.4 *M*, pH 4.4) and 3 ml of *n*-hexane to the residue. The hexane layer was removed and discarded by suction. Forty μl of 10 *M* potassium hydroxide followed by 4 ml of chloroform–methanol (2:1, v/v) was added to the aqueous layer, and the

mixture was shaken. The organic layer was rinsed with 3 ml of borate buffer (0.4 *M*, pH 10.5) and evaporated.

2.5. The o-phthaldialdehyde (OPA) derivatization of sphingoid bases

Samples were derivatized with *o*-phthaldialdehyde (OPA) and were analyzed by a fluorescence detector [15]. The residue was dissolved in 200 μl of methanol and 100 μl of borate buffer (0.4 *M*, pH 10.5). Twenty μl of OPA solution (10 mg OPA solution dissolved in 1 ml of methanol that contained 100 μl of 0.4 *M* borate buffer (pH 10.5) and 10 μl of 2-mercaptoethanol; total volume, 1.11 ml; freshly prepared) was added. After 10 min, the solution was filtered by centrifuging with a 0.2-μm nylon membrane (Bioanalytical System, West Lafayette, IN, USA), and 5 μl of the filtered solution was injected onto the HPLC.

3. Results and discussion

3.1. Stability of OPA-sphingoid derivatives

The retention time of psychosine (peak 1), *trans*-D(+)-*erythro*-sphingosine (ESo, C_{18}, peak 2), two isomers (*erythro*- and *threo*-) of DL-dihydrosphingosine (peak 3; ESa, and peak 4), C_{20}-dihydrosphingosine (peak 5) as internal standard were 5.2, 6.3, 7.7, 8.5 and 15.0 min, as the chromatograms shown in Fig. 1A. Each peak showed good resolutions and no interfering peaks were observed in chromatograms obtained from forebrain (Fig. 1B), brainstem (Fig. 1C) and spinal cord (Fig. 1D). The stability of ESo- and ESa-OPA was measured for 3 days at each three different concentration of 1.5, 3.0 and 6.0 ng added into tubes (final volume, 320 μl) after refrigerated storage in the dark, and were represented in Fig. 2. ESo and ESa at 6.0 ng both showed a little increase of these peak areas at day 3. The ratio of these peaks to internal standard, however, was quite consistent

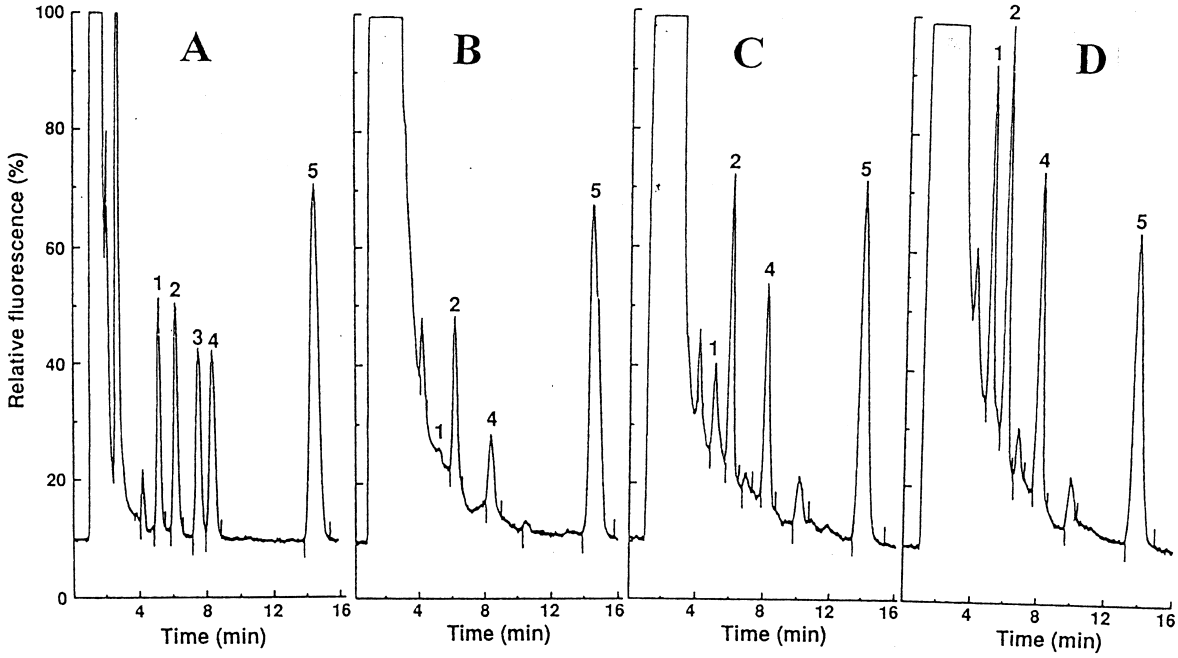

Fig. 1. Typical HPLC chromatograms of spiked authentic sphingoid bases (A) and of brain or spinal cord tissues extracted; (B) forebrain; (C) brainstem; (D) spinal cord. Psychosine (peak 1), *trans*-D(+)-*erythro*-sphingosine (peak 2), DL-*threo*-dihydrosphingosine (peak 3), DL-*erythro*-dihydrosphingosine (peak 4), and C_{20}-dihydrosphingosine (internal standard) are shown on the chromatograms. All chromatograms were obtained after extraction as described in Section 2. DL-*threo*-Dihydrosphingosine (peak 3) was not detected in brain and spinal cord tissues measured.

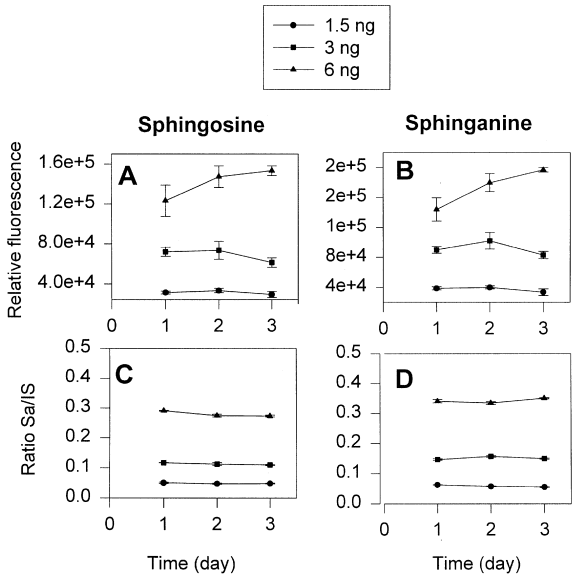

Fig. 2. Stability of sphingosine- and sphinganine-*o*-phthaldialdehyde derivatives between days at each three different concentrations. The boxes A and B indicate the absolute peak areas of sphingosine and sphinganine, respectively. The ratios of sphingosine and sphinganine to internal standard were shown in boxes C and D, respectively. Standards containing sphinganine and sphingosine were derivatized with *o*-phthaldialdehyde at room temperature and stored at 4°C ($n=3$, mean±S.E.).

over 3 days with a coefficient of variation of 1.5–13.3%. Also, this method is sensitive in capability of detecting 1.5 ng (final 4.7 ng/ml) of ESo and ESa.

3.2. Effect of pH and solvent on the extraction of sphingoid bases

The effect of pH on extraction of sphingosine and sphinganine is shown in Fig. 3. The highest detector response of sphingosine and sphinganine was observed at pH 13 (Fig. 3A). When the optimal ratios of chloroform and methanol mixture were measured (Fig. 3B), the ratios of chloroform–methanol of 3:1, 4:1 and 9:1 were represented relatively a similar amount of extraction of sphingosine and sphinganine. Chloroform only resulted in the lowest extraction of sphingosine and sphinganine.

3.3. The standard curves of the sphingoids

Regression of the standard curves of extracted sphingosine and sphinganine were linear between 1.5

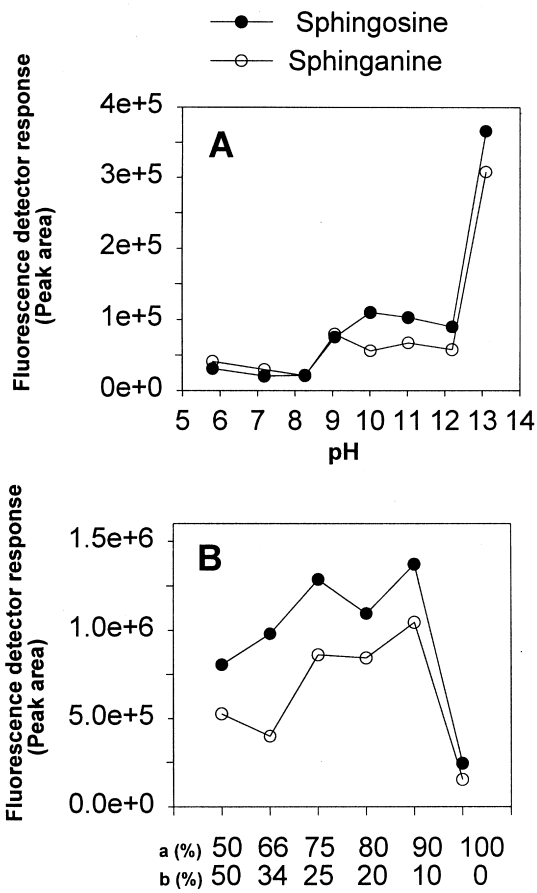

Fig. 3. Effects of pH (Box A) and solvent components (Box B) on extraction of sphinganine and sphingosine. Each point is represented as mean value in duplicate. (A) Standards of sphingosine and sphinganine to be spiked in phosphate buffer were adjusted to each pH indicated using 0.4 *M* borate buffer solution with 10 *M* KOH or 2 *N* HCl solutions and extracted with 4 ml of chloroform–methanol (2:1, v/v) by shaking for 20 min. The tubes were centrifuged at 600 *g* for 10 min. Organic layer was transferred and evaporated. After derivatization with *o*-phthaldialdehyde solution, 5 μl of the solution was injected on the HPLC. (B) Using the optimal pH of 13.1 obtained from the experiment above, sphingosine and sphinganine spiked to the phosphate buffer were extracted with various ratios of chloroform (a)–methanol (b; v/v) indicated and were prepared as described in A.

and 60 ng/100 μl with a relative coefficient of variation of 0.989 (8 points) and the slopes (0.0174 and 0.0172, respectively) and *y*-intercepts (0.003 and 0.008, respectively) of two curves were very similar between 1.5 and 60 ng/100 μl concentrations spiked in tubes. Regression of the extracted psychosine standard curve (between 5 and 230 ng/100 μl; 8

points) was linear with its slope of 0.020 and *y*-intercept of 0.068 (coefficient of variation=0.988).

3.4. The recoveries and accuracy of sphingoid bases

The recoveries of psychosine, sphingosine and sphinganine were investigated using either phosphate buffer or homogenate brain tissues (forebrain was used) at two different concentrations. These results were indicated in Table 1. Phosphate buffer was used because psychosine, sphingosine and sphinganine are endogenous compounds in brain tissues. Recoveries of psychosine were 94 and 91% at 18.4 and 46 ng authentic psychosine spiked in phosphate buffer, respectively. The recovery of psychosine in brain tissues was 104%. Sphingosine was recovered by 81% at 11.98 ng sphingosine spiked in phosphate buffer and by 89% at 44.9 ng sphingosine spiked in phosphate buffer. Recoveries of sphinganine were 81% at 12 ng sphinganine spiked in phosphate buffer and 94% at 45 ng sphinganine spiked in phosphate buffer. Recoveries of sphinganine and sphingosine added to the brain tissue homogenate resulted in about 219 and 131%, respectively. Accuracy of these sphingoid bases was ranged from 2.1 to 13.6%.

3.5. Determination of sphingoid bases in various ages of rat brain and spinal cord

In the forebrain, brainstem and spinal cord of rats at three different ages of postnatal days (PND) 1, PND 13 and 6 months old, the endogenous concentrations of psychosine, sphingosine and sphinganine were determined, and summarized in Table 2. The levels of sphingosine in forebrain and brainstem in 6-month-old rats were significantly ($P<0.05$) higher than those at PND 1 and PND 13. The increases of sphinganine levels in brainstem were age-dependent. Sphingosine levels of 6-month-old rats in forebrain and brainstem were significantly different from those of PND 1 or PND 13. The concentrations of psychosine in forebrain at PND 1 and PND 13, and brainstem at PND 1 were not detected. The lowest limit for psychosine is 0.05 pmol/mg tissue. The concentrations of psychosine in forebrain between PND 1 and PND 13 and brainstem at PND 1, therefore, will be located at a level lower than this limit. The levels of sphingosine at PND 13 in spinal cord were significantly different as compared to those of forebrain and brainstem at the same age. In PND 13, the levels of sphinganine in brainstem and spinal cord were significantly higher than forebrain (Table 2). Psychosine, sphingosine and sphinganine levels were relatively variable depending on brain regions and on the type of organ tissues [8], which supports our result obtained in brain and spinal cord tissues of rats. Based on the comparison of three different brain and spinal cord tissues, sphingosine and sphinganine levels may be abundant to the caudal region of brain (spinal cord> brainstem>forebrain). Sphingosine levels in lung extracted from large scale of tissue in 5-week-old C57BL/6J mice were reported to be 20 pmol/mg tissue, which was the highest among the measured tissues, including spinal cords with sphingosine of

Table 1
Recovery of psychosine (Ps), sphingosine (So), and sphinganine (Sa) in potassium phosphate buffer (PPB) and brain tissues[a]

Compounds	Added (ng)	Found (ng)	Recovery (%)	C.V. (%)
Ps, PPB	18.4	17.3±1.4	94.0±7.4	13.6
Ps, PPB	46.0	41.7±1.8	90.5±4.0	7.7
Ps, Brain	46.0	47.9±3.2	104.0±7.0	11.7
So, PPB	12.0	9.7±0.5	80.7±4.3	9.2
So, PPB	44.9	40.1±2.2	89.3±4.9	9.5
So, Brain	44.9	98.3±3.7	218.8±8.2	6.5
Sa, PPB	12.1	9.8±0.3	81.1±2.9	6.1
Sa, PPB	45.2	42.7±0.5	94.3±1.2	2.1
Sa, Brain	45.2	59.3±4.0	131.1±9.0	11.8

[a] Data were shown as mean±S.E. of three tubes. Potassium phosphate buffer (50 m*M*, pH 7.0) was used. Extraction procedure is the same as mentioned in Section 2.

Table 2
Concentrations of psychosine (Ps), sphingosine (So), and sphinganine (Sa) in various ages of rat brain and spinal cord

	Forebrain	Brainstem	Spinal cord
Psychosine (pmol/mg tissue)			
PND 1 (n=5)	ND	ND	1.05±0.007[a]
PND 13 (n=5)	ND	5.65±2.88[a]	6.58±2.04[a]
Six months (n=10)	9.93±3.74	10.39±1.43[a]	NA
Sphingosine (pmol/mg tissue)			
PND 1 (n=5)	2.75±0.6[a]	2.03±0.19[a]	2.69±0.22[a]
PND 13 (n=5)	3.75±0.51[a]	5.14±0.39[a]	7.32±0.68[b,**]
Six months (n=10)	15.50±0.87[b]	20.38±1.59[c,*]	NA
Sphinganine (pmol/mg tissue)			
PND 1 (n=5)	ND	0.50±0.03[a]	0.52±0.07[a]
PND 13 (n=5)	2.84±1.66[a]	3.94±1.42[b]	7.36±1.35[b]
Six months (n=10)	2.53±0.15[a]	6.96±0.48[c,*]	NA

PND, postnatal days. ND, not detected. NA, not available.
a, b, and c are significantly different each other between the ages in the same region of brain or spinal cord ($P<0.05$).
*Significantly different from forebrain within the same age group ($P<0.05$).
**Significantly different from forebrain or brainstem within the same age group ($P<0.05$).

10.5 pmol/mg tissue [8]. Also, the present method takes advantage of determining free levels of sphingosine, sphinganine and psychosine compared to the reported hydrolysis methods [10,13].

In conclusion, this method is simple and sensitive to measure sphingosine, sphinganine and psychosine with relatively high recoveries (81–94%), requiring the very small amount (16–20 mg) of brain or spinal cord tissues. When determined the endogenous concentrations of these sphingoid bases at three different ages in rat central nervous system, the levels of sphingoid bases were age- and tissue region-specific.

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