Journal of Chromatography, 555 (1991) 267–271
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 386

Short Communication

Use of N-acetylpsychosine as internal standard for quantitative high-performance liquid chromatographic analysis of glycosphingolipids

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(First received January 30th, 1991; revised manuscript received April 15th, 1991)

ABSTRACT

The use of N-acetylpsychosine as an internal standard for the quantitative high-performance liquid chromatography (HPLC) of *p*-nitrobenzoyl derivatives of glycosphingolipids is described. It is suitable because the chromogen reacts on equimolar basis with both N-acetylpsychosine and sample glycosphingolipids. The use of N-acetylpsychosine as an internal standard was validated by determining the glycosphingolipid content of a system of metastatic variants selected from a murine fibrosarcoma line (T3 cells). Reproducible results were obtained throughout several quantitative analyses of cellular glycosphingolipids and it was possible to determine the glycosphingolipid content of as few as $5 \cdot 10^6$ cells.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for determination of glycosphingolipids (GSLs) gives a better resolution and recovery than techniques based on column or thin-layer chromatography (TLC). However, the spectrophotometric detection of GSLs eluted from HPLC columns is precluded by the strong UV absorbance of the organic solvents (*e.g.*, chloroform recommended for the separation of GSLs. This difficulty has been only partially overcome by the conversion of GSLs into UV-absorbing derivatives [1–5], owing to the incompleteness of the derivatization reactions. Although this problem could be solved by including an internal standard in the derivatization reaction, there are no reports on the use of an appropriate molecule for the HPLC determination of GSL derivatives.

This paper reports the use of N-acetylpsychosine as an internal standard for the HPLC determination of *p*-nitrobenzoyl derivatives [2,3] of GSLs from a system of metastatic fibrosarcoma cell lines, *viz.*, the T3 cells and T3a1:p clone. GSLs of these cell lines were composed of glucosyl-, lactosyl- and globotriaosylceramides [6]. The use of N-acetylpsychosine was considered particularly advantageous for this purpose

0021-9673/91/$03.50

because the chromogen reacts with both the internal standard and sample GSLs on an equimolar basis as both GSLs and N-acetylpsychosine contain a common reactive group, the amidic linkage. In addition, N-acetylpsychosine exhibits the same physicochemical properties as those of the GSLs under study, and can therefore be added to the sample before starting the reaction.

EXPERIMENTAL

Preparation of N-p-nitrobenzoyl derivatives of N-acetylpsychosine and glycolipid standards

Psychosine was obtained from rat brain galactosylceramide, which was hydrolysed with butanolic 1 *M* potassium hydroxide under reflux for 2.5 h. Psychosine was freed from hydrolysis products by partition in an equal volume of water and then purified by silica gel column chromatography [7]. Psychosine was converted into N-acetylpsychosine by acetylation in pyridine–acetic anhydride (3:2) followed by *O*-deacetylation with sodium methoxide [8]. N-Acetylpsychosine and standards of glucosylceramide (GlcCer), lactosylceramide (LacCer), globotriaosylceramide (Gb3ose) and globoside (Gb4ose) were used to construct calibration graphs of peak area ratio *versus* concentration. GlcCer, LacCer and Gb3ose were prepared from a fibrosarcoma grown in CBA mice and Gb4ose was prepared from human erythrocyte ghosts. Total lipids were extracted according to Folch *et al.* [9] and then submitted to Florisil column chromatography [8] in order to separate GSLs from other lipid classes. GSLs were fractionated by TLC on 0.25-mm silica gel H plates (Merck, Darmstadt, Germany) in chloroform–methanol–water (60:25:4). After detection with primuline spray reagent [10], individual GSLs were identified by the gas chromatographic analysis of their carbohydrate groups [11].

N-Acetylpsychosine and GSL standards were acetylated according to Saito and Hakomori [8] and converted into *p*-nitrobenzoyl derivatives by reaction with *p*-nitrobenzoyl chloride (70 mg/ml) in pyridine at 60°C for 6 h [4]. These derivatives were recovered from the reaction mixture by solvent partitioning followed by solid-phase extraction on Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, USA) [5].

HPLC conditions

p-Nitrobenzoyl derivatives were dissolved in 50 μl of carbon tetrachloride and injected into a Perkin-Elmer silica B5 column (25 × 0.46 cm I.D.) mounted in a Perkin-Elmer Series 3B HPLC apparatus equipped with a Model LC75 spectrophotometric detector set at 254 nm. Chromatography was performed at a flow-rate of 1.1 ml/min with a linear gradient from 1% to 5% 2-propanol in hexane–chloroform (2:1) over 26 min, then from 5% to 10% 2-propanol in hexane–chloroform (2:1) over 8 min, and finally with the final solvent mixture for 10 min.

Analysis of glycolipid composition of murine fibrosarcoma cell lines

T3 cells and T3a1:p clone were cultured as described [6]. Cells from subconfluent cultures were harvested by means of a "rubber policeman", washed in phosphate-buffered saline and sonicated. Proteins were assayed by the method of Lowry *et al.* [12]. Lipids were extracted according to Folch *et al.* [9] and the extracts were acetylated together with 5 μg of N-acetylpsychosine and submitted to Florisil column

chromatography [8] in order to separate the GSLs from the other lipid classes. Acetylated GSLs were converted into their *p*-nitrobenzoyl derivatives and analysed as described above.

RESULTS AND DISCUSSION

Fig. 1 shows that the calibration graph for *p*-nitrobenzoyl-N-acetylpsychosine is linear between 0.5 and 55 nmol of the standard. This line differed only by 15% from that obtained after converting weighed amounts of N-acetylpsychosine into its *p*-nitrobenzoyl derivative, indicating an almost quantitative conversion of the standard into its derivative. Quantitative conversion of GlcCer and Gb4ose into *p*-nitrobenzoyl derivatives was also reported by Suzuki *et al.* [3].

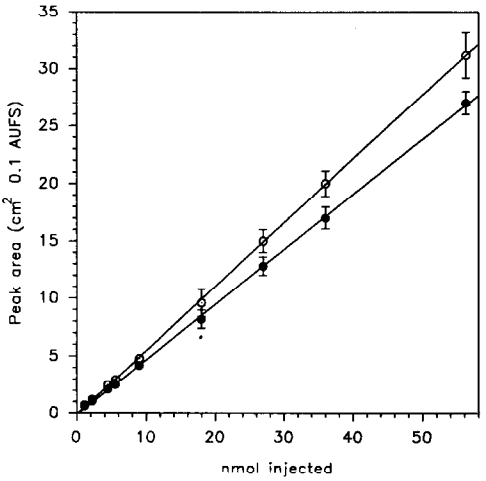

Fig. 1. Recovery of N-acetylpsychosine after conversion into its *p*-nitrobenzoyl derivative. The top line (○) was constructed with the peak areas produced with increasing amounts of *p*-nitrobenzoyl-N-acetylpsychosine. The molar response factor was 0.556 cm^2/nmol (r = 0.999). The bottom line (●) was obtained by converting known amounts of N-acetylpsychosine into its *p*-nitrobenzoyl derivative, then measuring the resultant peak areas after HPLC. The molar response factor was 0.479 cm^2/nmol (r = 0.998). For both lines, the experimental points represent the mean ± S.D. of eight determinations. Peak areas are expressed as cm^2 at 0.1 a.u.f.s. (25 cm) and at a chart speed of 0.5 cm/min.

As shown in Fig. 2, the determination of known amounts of GlcCer, LacCer and Gb3ose standards together with N-acetylpsychosine (54 nmol) gave similar peak-area ratio calibration graphs, indicating that, under the experimental condition used, there was no difference in the recoveries of all three of the GSLs. A value close to unity for the slopes of these curves demonstrates that the recovery of the GSL standards is identical with that of N-acetylpsychosine. The slope for Gb4ose was twice those for GlcCer, LacCer and Gb3ose, which is accounted for by the two groups present in Gb4ose, *i.e.*, the amidic bond of the ceramide and the acetamidic bond of the terminal N-acetylgalactosamine, which can react with the chromogen.

The use of N-acetylpsychosine as an internal standard in the HPLC of GSLs was validated by determining the GSL content of two fibrosarcoma cell lines grown in

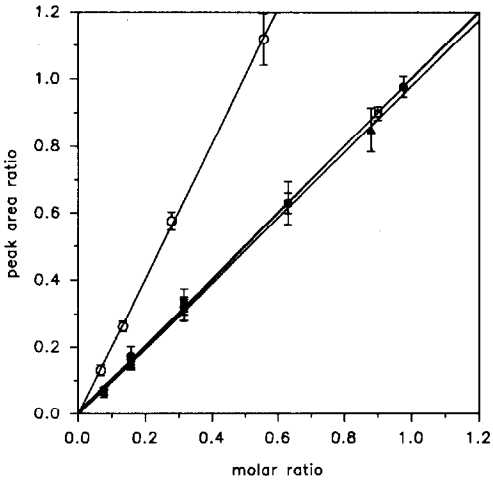

Fig. 2. Peak-area ratio calibrations for GlcCer, LacCer, Gb3ose and Gb4ose with N-acetylpsychosine as internal standard. Known amounts of an equimolar mixture of glycolipid were derivatized in the presence of 54 nmol of N-acetylpsychosine and determined. The lines were obtained by plotting the peak-area ratio *versus* the molar ratio between each glycolipid standard and N-acetylpsychosine. Slopes of the lines: (●) GlcCer = 0.993; (□) LacCer = 1.003; (▲) Gb3ose = 0.981; (○) Gb4ose = 2.023. For lines, the experimental points represent the mean ± S.D. of eight determinations.

tissue culture: T3 cells and T3a1:p clone. Gb3ose was the prevalent GSL in T3 cells (10.6 nmol/mg protein), followed by LacCer (2.5 nmol/mg protein), while the major GSL component of T3a1:p clone was LacCer (13.9 nmol/mg proteins) (Table I). Fairly reproducible results were obtained throughout several quantitative HPLC analyses of cellular GSLs, and it was possible to determine the GSL content of as few as $5 \cdot 10^6$ cells, *i.e.*, 0.5 nmol in the case of the least represented GSLs, *i.e.*, GlcCer.

TABLE I

GLYCOSPHINGOLIPID CONTENT OF T3 CELLS AND T3a1:p CLONE AS DETERMINED BY HPLC WITH N-ACETYLPSYCHOSINE AS INTERNAL STANDARD

Cell line	Glycosphingolipid[a]		
	GlcCer	LacCer	Gb3ose
T3	1.2 ± 0.3	2.5 ± 0.4	10.6 ± 1.4
T3a1:p	1.3 ± 0.3	13.9 ± 0.6	3.1 ± 0.4

[a] Values, reported as nmol/mg proteins, are the means ± S.D. of nine determinations. The protein content per 10^6 cells averaged 130 and 110 μg in T3 cells and T3a1:p clone, respectively.

ACKNOWLEDGEMENTS

This study was supported by grants from the Consiglio Nazionale delle Ricerche (Special Project: Oncologia), from the Associazione Italiana per la Ricerca sul Cancro and from the Ministero della Pubblica Istruzione (40 and 60%). The authors thank Dr. A. Fonnesu, Chairman of the Institute of General Pathology, University of Florence, for his interest in this research.

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