Article

Metabolomic study of polyamines in rat urine following intraperitoneal injection of γ-hydroxybutyric acid

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Abstract: 1) Background: Recently, illegal abuse of γ-hydroxybutyric acid (GHB) has increased in drug-facilitated crimes, but determination of GHB exposure and intoxication is difficult due to rapid metabolism of GHB. Its biochemical mechanism has not been completely investigated. And metabolomic study by polyamine profile and pattern analyses was not performed in rat urinefollowing intraperitoneal injection with GHB. 2) Methods: Polyamine profiling analysis by gas chromatography-mass spectrometry combined with star pattern recognition analysis was performed in this study. Multivariate statistical analysis was used to evaluate discrimination between control and GHB administration groups. 3) Results: Six polyamines were determined in control, single and multiple GHB administration groups. Star pattern showed distorted hexagonal shapes with characteristic and readily distinguishable patterns for each group. N^1-Acetylspermine ($p < 0.001$), putrescine ($p < 0.006$), N^1-acetylspermidine ($p < 0.009$), and spermine ($p < 0.027$) were significantly increased in single administration group but were significantly lower in the multiple administration group than in the control group. N^1-Acetylspermine was the main polyamine for discrimination between control, single and multiple administration groups. Spermine showed similar levels in single and multiple administration groups. 4) Conclusions: The polyamine metabolic pattern was monitored in GHB administration groups. N^1-Acetylspermine and spermine were evaluated as potential biomarkers of GHB exposure and addiction.

Keywords: Metabolomics; γ-Hydroxybutyric acid; Polyamine profiling analysis, Gas chromatography-mass spectrometry; Star pattern recognition analysis; Multivariate analysis

1. Introduction

γ-Hydroxybutyric acid (GHB) is an endogenous short chain fatty acid that acts as a neurotransmitter and neuromodulator in the mammalian brain [1]. Synthesized GHB has been used to treat ethanol withdrawal and cataplexy associated with narcolepsy and for its anesthetic effect [2-4]. However, it has been used illegally in drug-facilitated rapes or other crimes [5,6]. In recent decades, GHB has been used as a recreational drug in Europe and the Netherlands [7,8]. The use of GHB in date rape has also been increasing over the years in clubs [9].

GHB intoxication causes central nervous system depression, hypoventilation, and seizures [10], while GHB overdose can cause coma and death [11]. GHB is synthesized from γ-aminobutyric acid (GABA), which acts as GHB-specific and $GABA_B$ receptors in neurotransmission systems such as GABA and dopamine [12-20]. However, the pharmacological and neurobiological mechanisms of

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GHB are still not entirely clear in biological systems. Our research group reported altered organic acid (OA) profiles including GHB and 2-hydroxyglutaric acid (2-HG) as major metabolite of GHB in single and multiple GHB-treated groups [21]. In this study, the biochemical mechanism of GHB exposure and intoxication was not completely understood although most OAs including GHB and 2-HG were increased compared with those of control in rat urine samples after single and multiple administration of GHB [21].

Molecular researches on CNS including depression have focused on the monoamine system [22]. Originally, polyamines (PAs) were studied with a focus on cell growth, cell proliferation, and synthesis of protein and nucleic acids by regulating acetylation-deacetylation according to biochemical condition changes [23-27]. Recently, a PA-mediated stress-response signaling system, and the possibility of diagnostic markers of disease associated with the CNS, has been reported [10,28]. In previous reports, endogenous PAs were analyzed for the diagnosis of CNS injury and several diseases including cancer [29]. Our research group reported altered PA metabolism in urine of rats exposed to radiofrequency [30], in cerebrospinal fluid of Parkinson's disease patients [31], and in urine of Alzheimer's disease patients [32]. Altered PA levels are closely related to potential biomarkers of various cancers and CNS metabolic disorders. Also, altered PA levels were related to stress response in rat models [33], and administration of putrescine was associated with behavioral changes in the depressed state [34]. These findings explain the importance of PAs in the CNS. Thus, PA metabolism in the CNS has become an important research topic. As such, the endogenous PA profile analysis is important for understanding the biochemical events following treatment with GHB. In previous reports, PA profiling analysis of *N*-ethoxycarbonyl-*N*-pentafluoropropionyl (EOC/PFP) derivatives by gas chromatography-mass spectrometry (GC-MS) was useful for monitoring various disease states [30-32,35]. Thus, in this study, urinary PA profiling analysis in rat following intraperitoneal injection with GHB once per day for one day or 10 consecutive days was performed for understanding of biochemical events and monitoring of GHB exposure and intoxication.

2. Materials and Methods

2.1. Animals

Six male Sprague-Dawley rats (Orient Bio, Seoul, Korea), seven weeks old, were used after a one-week acclimation period in the laboratory animal facility. The rats were provided with distilled water and a commercial diet ad libitum for this period. The animal room was maintained at a temperature of 24 ± 2ºC and a relative humidity of 50 ± 20%, with a 12-h light/dark cycle [21].

2.2. Animal study

All procedures were approved by the Animal Care and Use Committee at the National Forensic Service. Before GHB administration, each animal was housed in a metabolic cage. On the first day, drug-free urine was collected into a dry-ice-chilled container for 12 h. Then 2600 mg/kg body weight of GHB dissolved in distilled water was intraperitoneally administered to the rats once per day for ten consecutive days. Urine was collected on day 2 (single administration) and 11 (multiple administration) into a dry-ice-chilled container for 12 h. The rats were fasted during urine collection. All urine samples were stored at -80ºC until analyzed [21].

2.3. Chemicals and reagents

Putrescine, cadaverine, spermidine, N^1-acetylspermidine, N^1-acetylspermine, spermine, 1,6-diaminihexane, ethyl chloroformate (ECF) and pentafluoropropionyl anhydride (PFPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether, ethyl acetate, toluene and sodium chloride of pesticide grade were purchased from Kanto Chemical (Tokyo, Japan). Sodium hydroxide was supplied by Duksan (Ansan, Gyeonggi-do, Republic of Korea). All other chemicals were of analytical grade.

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2.4. Gas chromatography–mass spectrometry

The GC-MS analysis in selected ion monitoring (SIM) mode was performed with an GCMS-TQ8040 interfaced to a triple quadrupole mass spectrometer (Shimadzu Corp., Kyoto, Japan) in electron impact mode at 70 eV equipped with the Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase; 25 m × 0.20 mm i.d., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Palo Alto, CA, USA). Injector, interface, and ion source temperatures were 260, 300, and 230ºC, respectively. The carrier gas was helium at a flow rate of 0.5 ml/min in linear velocity mode. Samples (1.0 μL) were introduced using the AOC-20i auto-injector and AOC-20s auto-sampler in split-injection mode (10:1). The oven temperature was initially set at 180ºC for 1 min and programmed to 300ºC at a rate of 30ºC/min with a holding time of 5 min. The mass range scanned was 45–750 u at a rate of 10000 u/sec. In the SIM mode, one quantitative ion and two qualitative ions for each PA were used for peak identification and quantification.

2.5. Sample preparation for urinary PA profiling analysis

PA profiling analysis was performed with urine from control and GHB-treated rats. The urine samples (equivalent to ca. 0.1 mg of creatinine) and 1,6-dimainohexane as internal standard (IS) were individually spiked to an aliquot of distilled water (ca. 1 mL) and adjusted to pH ≥ 12 with 5.0 M NaOH. The two-phase extractive EOC reaction in the aqueous phase was performed for primary amine groups of PA by vortex-mixing for 10 min with ECF (50 μL) present in the dichloromethane phase (ca. 1 mL). The mixture was saturated with NaCl and extracted with diethyl ether (3mL), ethyl acetate (2 mL) and a mixture of diethyl ether (2 mL) and dichloromethane (1 mL) in sequence. The combined extracts were evaporated to dryness under a gentle stream of nitrogen at 40ºC with subsequent PFP derivatization at 60ºC for 60 min with PFPA (20 μL), toluene 10 (μL), and ethyl acetate 20 (μL). The reacted mixture was then evaporated to dryness under a gentle stream of nitrogen at 40ºC and dissolved in toluene (20 μL) for GC–MS analysis.

2.6. Star pattern recognition analysis and statistical analysis

The quantitative levels of six PAs in each urine sample were determined based on the calibration curves. The PA levels in each rat urine sample were then normalized to the corresponding control mean values and used as the variables for star pattern recognition analysis using MS Excel. A nonparametric Kruskal-Wallis test was used to detect statistical differences in normalized metabolite between the control and GHB-treated groups using IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY). Multivariate statistical data analysis and classification analysis were performed on the log10-transformed data, with mean-centering, pareto-scaling and application of 6-fold cross validation for supervised learning. Principle component analysis (PCA) of the unsupervised learning algorithm and partial least squares-discriminant analysis (PLS-DA) of the supervised learning algorithm were applied for multivariate analysis using the R statistical system (http://www.R-project.org/, version 3.5.0) with *ropls* [PCA, PLS(-DA) and OPLS(-DA) for multivariate analysis and feature selection of omics data, 30 October 2017] package.

3. Results

3.1. PA profiles and univariate statistical analysis

The six PAs of putrescine, cadaverine, spermidine, spermine, N^1-acetylspermidine, and N^1-acetylspermine were quantified in rat urine samples of single and multiple GHB administration groups by GC-MS-SIM mode. The quantified value was corrected with creatinine (0.1 mg). Among the six PAs, N^1-acetylspermine ($p < 0.001$), putrescine ($p < 0.006$), N^1-acetylspermidine ($p < 0.009$), and spermine ($p < 0.027$) were significantly different in the Kruskal-Wallis test. The False discovery rate (FDR) by Benjamini-Hochberg procedure showed that N^1-acetylspermine, putrescine, N^1-acetylspermidine, and spermine were adjusted to $p < 0.0013$, $p < 0.021$, $p < 0.021$, and $p < 0.0045$ (Table 1).

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Table 1. The levels of six PAs, Kruskal-Wallis test, PCA loading score, and PLS-DA VIP score in rat urine

No	Analytes	Amount (μg/ creatinine of 0.1 mg) in urine (Mean ± SD)			Normalized values [1]		Kruskal-Wallis	FDR [2]	PCA loading score		PLS-DA
		Control (n=6)	Day 1 [3] (n=6)	Day 10 [4] (n=5)	Day 1	Day 10	p-value	p-value	p1	p2	VIP score
1	Putrescine	5.05±1.37	6.87±1.13	3.94±0.78	1.36	0.78	0.006	0.021	0.342	0.322	0.871
2	Cadaverine	0.69±0.39	0.80±0.31	0.65±0.35	1.16	0.93	0.594	0.669	0.182	0.622	0.823
3	Spermidine	4.88±1.51	5.41±2.29	4.13±1.19	1.11	0.85	0.683	0.683	0.306	-0.056	0.860
4	N^1-Acetylspermidine	1.44±0.56	2.32±0.87	0.94±0.20	1.62	0.65	0.009	0.021	0.280	0.494	1.077
5	N^1-Acetylspermine	0.05±0.03	0.37±0.26	0.14±0.04	7.10	2.71	0.001	0.013	0.736	-0.241	1.390
6	Spermine	0.06±0.01	0.12±0.07	0.11±0.03	1.85	1.70	0.027	0.045	0.369	-0.451	0.857
7	PA SUM	10.69±2.79	13.20±3.49	8.83±1.91	1.24	0.83	0.075	0.096			
8	Acetylated PA SUM	1.49±0.57	2.70±0.84	1.08±0.19	1.81	0.73	0.007	0.021			
9	Total PA SUM	12.28±3.07	15.99±3.60	10.01±1.99	1.30	0.82	0.030	0.045			

[1] Values normalized to the corresponding control composition values, [2] False discovery rate by Benjamini-Hochberg method, [3] Day 1; single GHB administration group, [4] Day 10; multiple GHB administration group

3.2. Star pattern recognition analysis

In control and single GHB administration groups, putrescine was most abundant, followed by spermidine and N^1-acetylspermidine, while in the multiple GHB administration group, spermidine was most abundant, followed by putrescine and N^1-acetylspermidine among the six quantified PAs (Table 1). Alteration of all PAs varied, ranging from 0.65 to 7.10. In particular, N^1-acetylspermine, spermine, N^1-acetylspermidine, and putrescine were significantly increased by 610, 85, 62, and 36% in the single administration group compared with those of control, respectively. And, in the multiple administration group compared with control, N^1-acetylspermine and spermine were significantly increased by 171 and 70%, while N^1-acetylspermidine and putrescine were significantly decreased by 35 and 22%, respectively. PA SUM (sum of putrescine, cadaverine, spermidine, and spermine), acetylated PA SUM (sum of N^1-acetylspermidine and N^1-acetylspermine), and total PA SUM (sum of six PAs) in the single administration group were higher than those in the multiple administration group (Fig. 1). This metabolic pattern of PA profiling was similar to the metabolic pattern of organic acid profiling in our previous report [21].

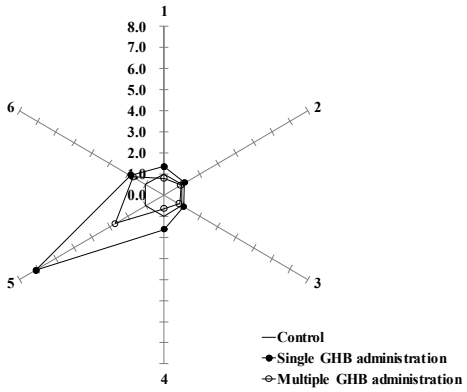

Figure 1. Star symbol plots of mean data from the rat urine of control and GHB administration groups drawn on the basis of the level of the PAs after normalization to the corresponding control mean values. The numbers on the rays correspond to those in Table 1.

3.3. Univariate statistical analysis

PCA and PLS-DA, as one of the powerful unsupervised and supervised learning, respectively, were performed using the values converted to transform log10-transformed, mean-centering and

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pareto-scaling. The PCA score plot with the first two components showed unclear separation between the three groups, explaining 79.2% of the total variances with p1 and p2 (Fig. 2a and 2b). The PLS-DA was calculated with four PLS components (Fig 2c) that performed a 6-fold internal cross validation and overfitting confirmed that overfitting did not occur because R2X and Q2Y increased with the number of components. The PLS-DA score plot showed better separation than PCA, but R2X (0.973), R2Y (0.783), and Q2Y (0.590) were not clearly separated (Fig 2d). For discrimination between the three groups, PCA loading score was evaluated with p1 and p2. In the loading score of p1, N^1-acetylspermine (0.7363) had the highest score, followed by spermine (0.3691) and putrescine (0.3415). The loading score of p2 showed cadaverine (0.6219) with the highest score, followed by N^1-acetylspermidine (0.4942) and spermine (-0.4513). In PLS-DA, N^1-acetylspermine (1.390) and N^1-acetylspermidine (1.077) were selected above 1.0 in the VIP score and the other four PAs also obtained a VIP score of over 0.823. Information for all PAs scores is shown in Table 1.

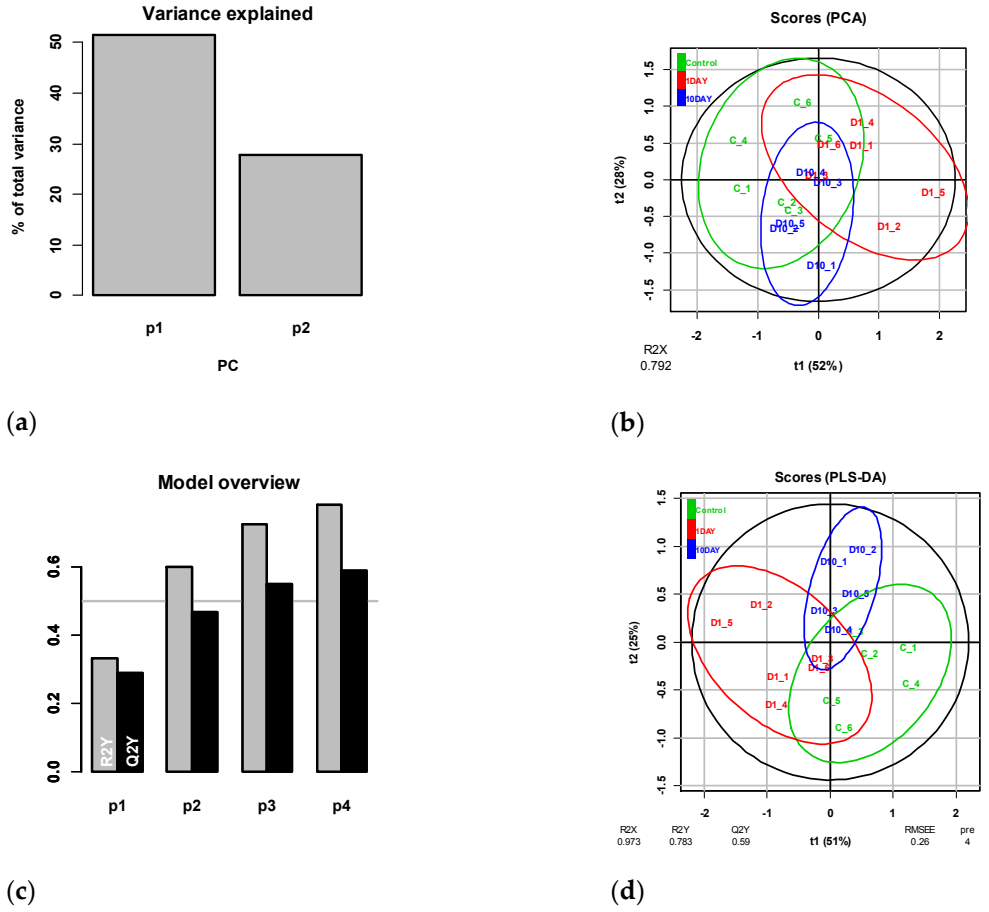

Figure 2. Multivariate statistical analysis of rat urine of control and GHB administration groups. C_, D1_, and D10_ indicate control, single GHB administration, and multiple GHB administration, respectively: (**a**) PCA variance explained; (**b**) PCA score plot; (**c**) PLS-DA model overview; (**d**) PLS-DA score plot

4. Discussion

Putrescine derived from ornithine and/or arginine is a precursor of spermidine and spermine. PAs are positively charged at physiological pH and interact with negatively charged molecules such as DNA, RNA, and proteins and can affect various cell activities such as proliferation, differentiation, and apoptosis [23-25]. Acetylation reduces the positive charge to minimize the effect on cell activities, and acetylated derivatives of overproduced PAs are released outside of the cell or transferred to other needed places [26,27]. All PAs investigated in this study were increased in the single GHB administration group, but most were trended to reduce to control levels in the multiple GHB administration group. These results show that the PA metabolic alteration following a single

administration is much larger than that after multiple administration. In the first exposure to GHB (single GHB administration groups), the cellular stress caused by excessive GHB was greatly increased, and the overproduced PAs were excreted in the urine. In the multiple GHB administration group, it can be expected that the PAs in the urine decreased to control levels due to adaptation to the cellular stress. In particular, N^1-acetylspermidine (62%) and N^1-acetylspermine (610%) were significantly increased in the single GHB administration group. Also, PA SUM, acetylated PA SUM, and total PA SUM increased after single GHB exposure (Table 1). The variation in acetylated PA SUM was larger than PA SUM because the overproduced PAs were converted into acetylated PAs. It was not reported why PA was increased by GHB administration. Previous reports on increased spermidine/spermine acetyltransferase (SSAT) in various cellular stresses including inflammation could support our results [36-40]. In unsupervised and supervised multivariate analyses, score plots of PCA and PLS-DA were not clearly separated but can be considered to have high reliability because the formation distributions are similar [41]. In PCA loading score and PLS-DA VIP score, N^1-acetylspermine was evaluated as the most important metabolite; the quantitative level also showed a significant difference. Spermine did not obtain a satisfactory score in multivariate analysis, but it maintained a similar level in GHB single and multiple administration groups. Thus, N^1-acetylspermine shows a very large change upon first exposure to GHB, while spermine maintained increased levels after single and multiple administrations. Finally, in the present study, PA metabolism was monitored in response to GHB administration. N^1-Acetylspermine and spermine were evaluated as potential biomarkers of GHB exposure and addiction.

Author Contributions: Conceptualization, M-JP and SL; Methodology, H-SL; Software, H-SL; Validation, H-SL, CS and MJ; Formal Analysis, H-SL and Y-AK.; Investigation, MJ and SC; Resources, MP and BC.; Data Curation, M-JP and H-SL; Writing-Original Draft, H-SL; Writing-Review & Editing, M-JP and H-SL; Visualization, H-SL; Supervision, M-JP and SL; Project Administration, M-JP and SL; Funding Acquisition, M-JP and SL.

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Conflicts of Interest: The authors declare no conflict of interest.

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