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Determination of urinary 5-hydroxytryptophol glucuronide by liquid chromatography–mass spectrometry

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Abstract

5-Hydroxytryptophol glucuronide (GTOL) is the major excretion form of 5-hydroxytryptophol (5-HTOL), a minor serotonin metabolite under normal conditions. Because the concentration of 5-HTOL is markedly increased following consumption of alcohol, measurement of 5-HTOL is used as a sensitive biomarker for detection of recent alcohol intake. This study describes the development and evaluation of a liquid chromatography–electrospray ionization mass spectrometry (LC–MS) procedure for direct quantification of GTOL in human urine. Deuterium labelled GTOL (GTOL-$^2H_4$) was used as internal standard. GTOL was isolated from urine by solid-phase extraction on a C_{18} cartridge prior to injection onto a gradient eluted Hypurity C_{18} reversed-phase HPLC column. The detection limit of the method was 2.0 nmol/L and the measuring range 6–8500 nmol/L. The intra- and inter-assay coefficients of variation were <3.5% ($n = 10$) and <6.0% ($n = 9$), respectively. The new LC–MS method was highly correlated with an established GC–MS method for urinary 5-HTOL ($r^2 = 0.99$, $n = 70$; mean 5-HTOL/GTOL ratio = 1.10). This is the first direct assay for quantification of GTOL in urine. The method is suitable for routine application.

Keywords: Alcohol biomarker; Ethanol; 5-Hydroxytryptophol glucuronide; LC–MS

1. Introduction

Alcohol consumption leads to an increased synthesis of the serotonin (5-hydroxytryptamine, 5-HT) metabolite 5-hydroxytryptophol (5-HTOL) and a concomitant decreased synthesis of 5-hydroxyindole-3-acetic acid (5-HIAA), resulting in an increased 5-HTOL/5-HIAA ratio [1–6]. The increase in the urinary 5-HTOL level following alcohol intake is dose dependent, and the levels remains elevated for several hours after ethanol itself is no longer measurable [3,7]. Based on the prolonged detection window for 5-HTOL compared with ethanol, determination of 5-HTOL in samples of urine has been used in different clinical and forensic settings as a sensitive biomarker for recent alcohol consumption [4–10].

Unlike 5-HIAA, which is excreted from the human body in free form, urinary 5-HTOL is mainly excreted as glucuronide or sulphate conjugates [11]. Thus, to determine the total excretion of 5-HTOL, both free and conjugated amounts should be measured. However, free 5-HTOL accounts for only <5% of the total elimination whereas the majority (about 80%) is excreted as 5-HTOL glucuronide (GTOL) [11], making GTOL an attractive single target for direct quantification [4].

The most common analytical approach for glucuronide conjugates has been enzymatic hydrolysis followed by determination of the free substance. Methods used for measurement of 5-HTOL involve an initial hydrolysis of GTOL with β-glucuronidase, followed by quantification of free plus liberated 5-HTOL by HPLC [12,13], liquid chromatography–mass spectrometry (LC–MS) [14], or gas chromatography–mass spectrometry (GC–MS) [4], of which the latter has been most widely used to date. These methods either suffer from insufficient sensitivity or specificity

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(HPLC), or involve time-consuming and laborious derivatization and/or solvent extraction steps (GC–MS), which make them less suitable for use in the routine clinical or forensic laboratory. The advent of modern liquid-phase ionization techniques, such as electrospray and direct coupling of liquid chromatography to mass spectrometry has made it possible to apply LC–MS methods for polar molecules without the need to resort to elaborate sample preparation procedures [15]. The LC–MS technique has already been used for a wide array of substances, e.g., ethyl and morphine glucuronides [16–18] and should also enable direct measurement of GTOL in urine.

The aim of this study was to develop a sensitive and specific LC–MS method for direct quantification of GTOL in urine.

2. Materials and methods

2.1. Urine samples

Urine samples were collected at random from patients undergoing treatment for alcohol and drug abuse in an outpatient treatment program in Stockholm (Sweden). Urine samples were also obtained from healthy individuals (social drinkers) who had not consumed any alcoholic beverages in the past 10 days, according to self-report. GTOL control samples were collected from one healthy person without previous intake of alcohol (low control = endogenous level) and at 2 h after drinking ∼7 g ethanol (high control), respectively. The urine specimens were stored at −20 °C until analysis. The study was approved by the local ethics committee.

2.2. Chemicals

5-HTOL was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and 5-hydroxy-(α,α,β,β-$^2H_4$)-tryptophol (5-HTOL-$^2H_4$) was synthesized as described previously [19]. GTOL (molecular weight, 353.3 g/mol) and tetradeuterated GTOL (GTOL-$^2H_4$, internal standard; molecular weight, 357.3 g/mol) were prepared enzymatically from 5-HTOL and 5-HTOL-$^2H_4$, respectively, by reaction with UDPGA in the presence of UDP glucuronosyltransferase (Sigma). The mixture was dissolved in 0.1 mol/L Tris–HCl (pH 7.4) (Merck, Darmstadt, Germany) and 13 mmol/L $MgCl_2$ (Merck) was added. After incubation by gentle shaking for ∼24 h at 37 °C, the solution was treated with ice-cold acetonitrile (J.T. Baker, Deventer, Holland) and any insoluble matter removed by centrifugation. The resulting supernatant was evaporated to dryness and the residue dissolved in methanol. Further purification of GTOL was achieved by preparative HPLC and the identity of the final product was confirmed by MS and by elementary analysis. Levels of prepared GTOL standards were assigned by comparison with 5-HTOL standards (correction factor −22%). Using three different urine samples, the enzymatic hydrolysis of GTOL to 5-HTOL using *Escherichia coli* β-glucuronidase [4] was demonstrated to be >96%.

Stock solutions of GTOL and GTOL-$^2H_4$ were prepared in methanol and stored at −20 °C until use and were found to be stable for at least 12 months. All other chemicals were of analytical grade and all solutions were prepared in ultra-pure water (>18 MΩ/cm). Solid-phase extraction (SPE) cartridges, (C_{18}, 100 mg/1 mL) were obtained from Thermo Hypersil-Keystone (Runcorn, UK).

2.3. LC–MS system

The LC–MS system was a Perkin-Elmer series 200 LC system consisting of a vacuum degasser, a ternary pump, and an autosampler, connected to a Sciex API 2000 MS with PE Sciex Analyst Software version 1.1 (Applied Biosystems, Ontario, Canada). The electrospray interface was used with the instrument operating in the negative ion mode. The following conditions were used: interface temperature, 450 °C; corn voltage, −4100 V; multiplier voltage, 2500 V; curtain gas pressure, 10 psi; nebulizer gas pressure, 30 psi; auxiliary gas pressure, 55 psi.

Separation of GTOL was performed on a 5 μm 100 mm × 2.1 mm (i.d.) Hypurity C_{18} column (Thermo Hypersil-Keystone), equipped with a 10 mm × 2.0 mm Hypurity C_{18} guard column, by acetonitrile gradient elution at a flow rate of 200 μL/min (Table 1). Buffer A consisted of 2% acetonitrile in 50 mmol/L formic acid and buffer B of 50% acetonitrile in 50 mmol/L formic acid.

2.4. Analytical procedure for GTOL

A 250 μL aliquot of urine or standard specimen was mixed with 250 μL of 2% trifluoroacetic acid containing 20 μmol/L of the internal standard (GTOL-$^2H_4$). The C_{18} SPE cartridges were assembled on a Multi-Prep extraction device (Vac Master, International Sorbent Technology Ltd., Mid Glamorgan, UK) and conditioned with 1.0 mL methanol followed by 1.0 mL deionised water. The specimens were passed through the columns by applying a vacuum and the columns were then washed with 0.5 mL deionised water. The extraction cartridges were dried under vacuum for 5 min and the analytes eluted with 1.0 mL methanol–water (50:50, v/v). All

Table 1
Gradient profile used for the separation of urinary GTOL with the LC–MS method

Time (min)	Mobile phase	
	Buffer A[a] (%)	Buffer B[a] (%)
0	100	0
2.0	100	0
6.0	50	50
7.0	0	100
15.0	0	100
16.0	100	0
25.0	100	0

[a] Buffer A consisted of 2% acetonitrile in 50 mmol/L formic acid, and buffer B of 50% acetonitrile in 50 mmol/L formic acid.

samples were taken to dryness under a stream of dry nitrogen at 40 °C during 2 h and the final content dissolved in 100 μL deionised water and transferred to autosampler vials. The injection volume was 15 μL. LC–MS analysis was performed using selected-ion monitoring (SIM) of the predominating pseudomolecular ions for GTOL (*m/z* 352.3) and GTOL-$^2H_4$ (*m/z* 356.3). A calibration curve covering 6.4–8500 nmol/L GTOL was prepared by serial dilution of the stock solution with deionised water. The GTOL concentration of unknown samples was determined from the peak area ratio between GTOL and GTOL-$^2H_4$, by reference to the calibration curve.

2.5. Methods comparison

The GTOL results obtained with the LC–MS method were compared with the corresponding 5-HTOL results obtained with an established GC–MS method, which involves enzymatic hydrolysis with β-glucuronidase prior to quantification [4]. The GC–MS method for 5-HTOL has a measuring range up to 10,000 nmol/L and the intra- and inter-assay coefficients of variation (CV) are 3% and 7%, respectively [4,20]. The GTOL calibration standards were analysed to secure similar calibration levels of both methods.

2.6. Urinary 5-HIAA determination

The urinary concentration of 5-HIAA was determined by HPLC with electrochemical detection, as previously described [21].

3. Results

3.1. Solid-phase extraction conditions

Initial experiments revealed that direct analysis of urine was not feasible for LC–MS determination of GTOL, because the target limit of detection (<25 nmol/L) [12] was not achieved and chromatographic interference sometimes occurred. Endogenous concentrations of GTOL were, however, detected with good linearity following SPE of samples on C_{18} or strong anion-exchange (SAX) cartridges. The C_{18} phase was chosen over SAX, because it gave a higher sensitivity and more reproducible results (data not shown). Moreover, elution of GTOL with 50% methanol in water was more selective and gave less interference than using 100% methanol. Finally, the analytes in the eluate were concentrated to increase the sensitivity of the method. The absolute recovery was documented using GTOL-$^2H_4$. Urine samples were spiked with 2.0 μmol/L GTOL-$^2H_4$ before and after SPE and, by comparing the GTOL/GTOL-$^2H_4$ responses, the recovery was found to range from 94% to 104% ($n = 3$).

3.2. HPLC conditions

A Hypurity C_{18} analytical column showed a suitable retention time for GTOL with a capacity factor of about 4.8 (Fig. 1). A Hypercarb column with polar graphite material, as was used for ethyl glucuronide [18] was found not to be suitable because of longer retention times and poor chromatography due to peak tailing. With the Hypurity C_{18} column, gradient elution was preferred over isocratic elution, because of shorter retention time and improved peak shape, resulting in better separation and less interference. In routine use, a total analysis time of 25 min was used, to avoid interference from late eluting compounds and to obtain column equilibration. The mobile phase consisted of acetonitrile which provided better chromatographic selectivity in comparison to methanol. In addition, methanol caused higher back pressure and decreased the sensitivity.

3.3. Method validation

A linear correlation between the calculated GTOL concentration and the area ratio to the internal standard was obtained throughout the range 0–8500 nmol/L GTOL (Fig. 2). The limits of detection (LOD) of the LC–MS method for GTOL in water and urine were 1.2 and 2.0 nmol/L, respectively (signal-to-noise ratio of 3), and the limit of quantification (LOQ) in urine was set at 6.4 nmol/L (signal-to-noise ratio of 10). The intra- and inter-assay CV were <3.5% and <6%, respectively (Table 2). The carry-over in the LC–MS system was less than 0.003%, as determined after injection of a urine sample spiked with a very high concentration of GTOL (>500 μmol/L). The methods comparison between the present LC–MS method for GTOL and the GC–MS method for 5-HTOL (free plus liberated from GTOL) included a total of 70 urine samples, 21 of which were obtained from healthy abstinent volunteers and the remaining 49 from patients undergoing treatment for alcohol and drug abuse. The methods were highly correlated ($r^2 = 0.99$) with no outliers noted (Fig. 3). The mean ratio for GC–MS (5-HTOL) over LC–MS (GTOL) for all samples, covering the range 10–6000 nmol/L, was 1.10 (S.D., 0.16).

Stability of GTOL during storage at −20 °C was documented for the control samples for >6 months. The stability of extracts on the autosampler was documented for at least 48 h at room temperature.

Table 2
Reproducibility of the LC–MS method for urinary GTOL

	n	GTOL concentration (mean nmol/L)	S.D.	CV (%)
Repeated injection	10	101	1.3	1.3
Intra-assay	10	151	4.9	3.3
	10	1510	49	3.2
Inter-assay[a]	9	76	4.5	5.8
	9	1840	95	5.2

[a] Analyses were performed in nine batches over a 6-month period using the low and high controls stored at −20 °C.

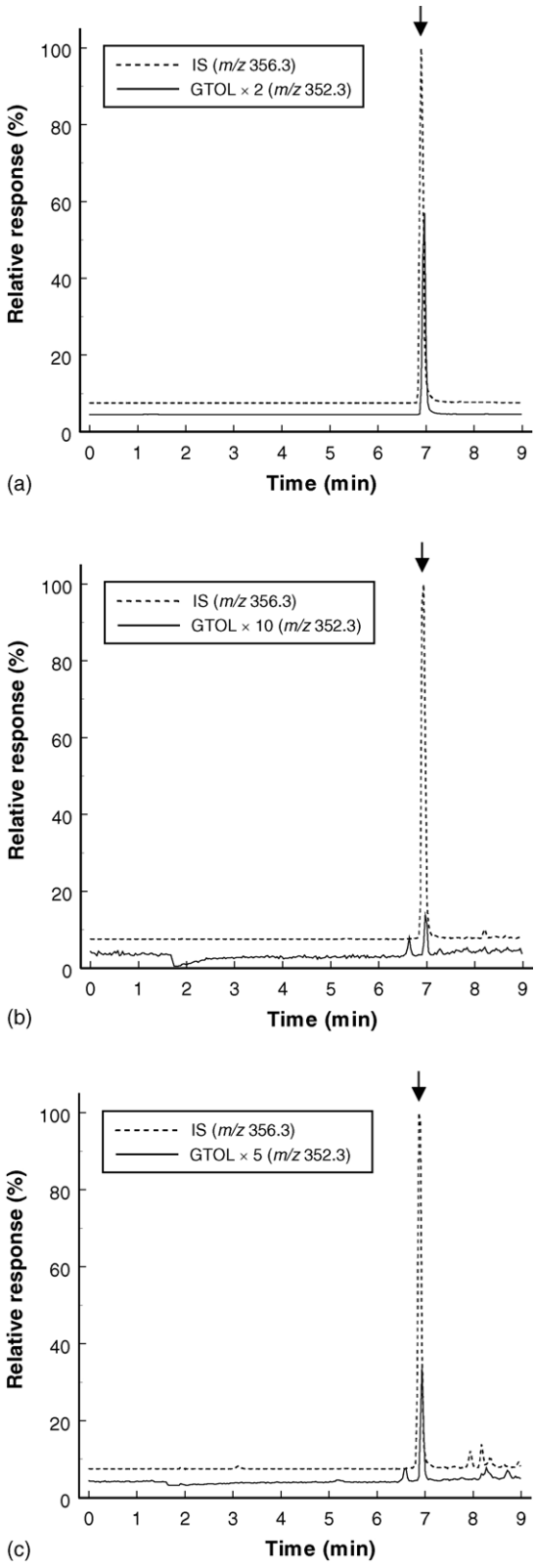

Fig. 1. LC–MS chromatograms showing the peaks for GTOL (*m*/*z* 352.3) and GTOL-$^2H_4$ (*m*/*z* 356.3, internal standard) injected on the Hypurity C_{18} analytical column for (a) a water calibrator containing 5000 nmol/L GTOL, (b) a human urine sample containing 120 nmol/L GTOL (endogenous level), and (c) a human urine sample containing 1000 nmol/L GTOL (previous alcohol intake).

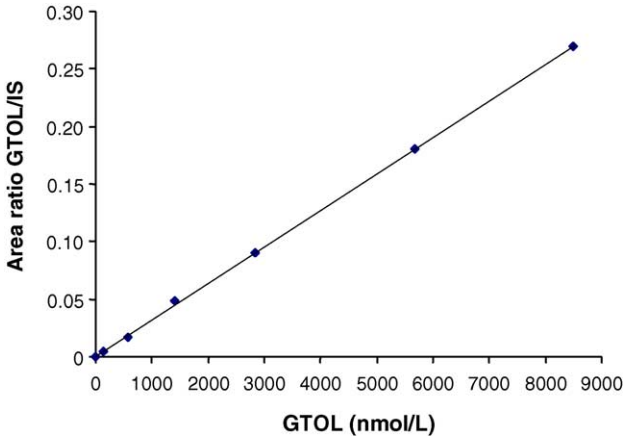

Fig. 2. A calibration curve for GTOL, prepared in water and covering 0–8500 nmol/L, by the LC–MS method ($r^2 = 0.9991$).

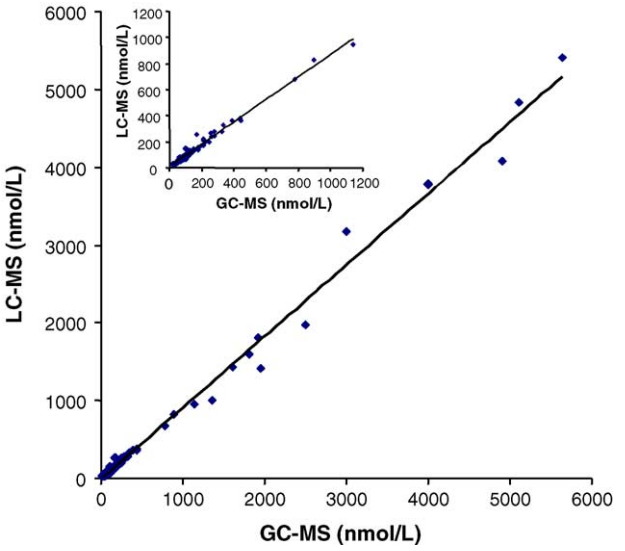

Fig. 3. Correlation between urinary GTOL values obtained with the LC–MS method and the corresponding 5-HTOL values (sum of free and glucuronide conjugated 5-HTOL) obtained by the GC–MS method for 70 human urine samples. *Inset*: Data for the subset of 59 urine samples with a GTOL level <1200 nmol/L.

3.4. Concentrations of GTOL in clinical samples

The endogenous GTOL concentrations in urine samples obtained from 13 healthy volunteers who had abstained from ethanol for several days prior to sampling, according to self-report, are given in Table 3, together with the corresponding GTOL/creatinine and GTOL/5-HIAA ratios. The

Table 3
Data for 13 human urine samples without previous intake of ethanol

	GTOL (nmol/L)	GTOL/creatinine (nmol/mmol)	GTOL/5-HIAA (pmol/nmol)
Range	38–327	4.8–20.2	2.6–12.0
Mean	109	12.1	6.2
Median	83	11.5	6.6
S.D.	65	4.4	2.5

chromatogram obtained from a urine sample containing an endogenous level of GTOL is presented in Fig. 1b.

The concentration of GTOL in clinical samples (with and without prior alcohol consumption) ranged from 21 to 5640 nmol/L (Fig. 3). This concentration range is in agreement with those observed previously for 5-HTOL.

4. Discussion

This study demonstrates for the first time that direct quantification in urine of GTOL, a 5-HT metabolite and the major excretion form of 5-HTOL [11], can be performed by LC–MS. This represents an analytical improvement over previous indirect methods, including HPLC [12,13], GC–MS [4], and LC–MS [14], in that enzymatic hydrolysis of GTOL into free 5-HTOL, or derivatization, is not required. The development of an immunoassay (ELISA) for direct measurement of GTOL will make this sensitive biomarker for acute alcohol consumption even more attractive for routine clinical use [22]. The LC–MS method can then be used for confirmatory analysis.

Because urine contains high biological noise/background at low masses, which reduces the selectivity and sensitivity of the LC–MS system, a simple SPE sample purification procedure was used for quantification of low, endogenous concentrations of GTOL (<25 nmol/L) which can occur in dilute urines. In this way, the limit of quantification was reduced from ~25 nmol/L with the GC–MS method to ~6 nmol/L by LC–MS. The GTOL concentrations observed in urine samples collected from healthy volunteers who had abstained from ethanol for at least 10 days prior to sampling was in the same range as the total 5-HTOL levels previously reported [11] for control individuals. The 5-HTOL concentrations obtained with the GC–MS method were slightly higher than the GTOL concentrations by LC–MS, but this was expected as the GC–MS method measures the sum of free and glucuronide conjugated 5-HTOL. In a previous study [11], the free level of 5-HTOL in urine, determined by excluding the enzymatic treatment in the GC–MS method, normally accounted for less than 5% of the total 5-HTOL concentration.

When using urinary 5-HTOL or GTOL as an alcohol biomarker for detection of recent drinking [4], or for forensic purposes to confirm or rule out artifactual ethanol formation [23–26], the result is reported as the ratio to 5-HIAA. This is used to compensate for variations in 5-HT turnover and in urine dilution, resulting in an overall improved specificity for alcohol. To compensate for urine dilution, the 5-HTOL(GTOL)/creatinine ratio may also be used, but this will not compensate for fluctuations in 5-HT metabolism due to dietary intake of 5-HT [11,27] or treatment with antidepressant drugs [28]. The GTOL/5-HIAA and GTOL/creatinine ratios observed without previous intake of ethanol were in agreement with the previously reported levels [2,8,11–13]. A study on post-mortem urines showed a slightly lower ratio for 5-HTOL/5-HIAA [14].

Until now, measurement of the urinary 5-HTOL/5-HIAA ratio has involved two separate analytical methods; GC–MS for 5-HTOL and HPLC for 5-HIAA. Recently, a sensitive and accurate LC–MS method for the simultaneous determination of both compounds was described [14]. This method represents an improvement in terms of simplicity, because only one analytical procedure is required. However, the method still involves enzymatic hydrolysis of GTOL to 5-HTOL, and derivatization of both compounds was also included. The development of a direct method for GTOL and 5-HIAA in urine, without the need for enzymatic hydrolysis and derivatization, would therefore represent a further improvement, and make the routine use of this ratio as a sensitive biomarker for recent alcohol intake even more feasible. Although LC–MS provides an efficient technology for sensitive and specific metabolite analysis, selectivity of the system may still be a difficult task. Thus, to improve selectivity, future use of tandem mass spectrometry (LC–MS/MS) may be preferable, although this may result in loss of sensitivity. LC–MS/MS has become used in bioanalysis due to its speed, sensitivity and specificity [29,30] with applications for a number of conjugated metabolites of ethanol and illicit drugs [31–36].

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