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Short Communication

Determination of urinary 5-hydroxytryptophol by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A high-performance liquid chromatographic method for the routine determination of elevated urinary levels of the serotonin metabolite 5-hydroxytryptophol (5-HTOL) is described. Urine samples were treated with β-glucuronidase, and 5-HTOL was isolated by solid-phase extraction on a small Sephadex G-10 column prior to injection onto an isocratically eluted C_{18} reversed-phase column. Detection of 5-HTOL was performed electrochemically at +0.60 V *vs.* Ag/AgCl. The limit of detection was *ca.* 0.05 μM, and the intra-assay coefficients of variation were below 6% with urine samples containing 0.2 and 2.1 μM 5-HTOL and a standard solution of 2.0 μM ($n = 5$). The recovery of 5-HTOL after the sample clean-up procedure was close to 100%. A good correlation ($r^2 = 0.97$; $n = 12$) was obtained between the present method and a sensitive and specific gas chromatographic–mass spectrometric method. The total (free plus conjugated) 5-HTOL levels in urine were normally below 0.2 μM, but after an acute dose of alcohol they increased to 0.5–15 μM.

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INTRODUCTION

The catabolic pathway of serotonin (5-hydroxytryptamine; 5-HT) begins with oxidative deamination to 5-hydroxyindole-3-acetaldehyde (5-HIAL) by the action of monoamine oxidase (EC 1.4.3.4). 5-HIAL is then either oxidized to 5-hydroxyindole-3-acetic acid (5-HIAA) by aldehyde dehydrogenase (EC 1.2.1.3) or reduced to 5-hydroxytryptophol (5-HTOL) by alcohol dehydrogenase (EC 1.1.1.1) or aldehyde reductase (EC 1.1.1.2).

In humans, the major portion of 5-HT is ultimately converted into 5-HIAA, which is excreted in the urine [1]. The 5-HTOL level in human urine is normally less than 1% of the 5-HIAA level, and it is excreted predominantly in conjugated form [2,3]. Although 5-HTOL is normally a minor metabolite, it becomes more important quantitatively during alcohol intoxication, when a shift in the 5-HT catabolism occurs from 5-HIAA toward increased formation of 5-HTOL [3,4]. This effect has been attributed to either competitive inhibition of aldehyde dehydrogenase by acetaldehyde, the first product of ethanol oxidation, or to the increased ratio of NADH to NAD^+, both of which favour reduction of 5-HIAL to 5-HTOL [5]. Shortly after ingestion of alcohol, a marked and dose-dependent increase in the urinary excretion of 5-HTOL is observable, and the level remains elevated several hours after ethanol itself is no longer detectable [2]. Based on these findings, an elevated urinary level of 5-HTOL is presently considered as a biochemical marker indicative of recent alcohol consumption [6]. However, ingestion of foods rich in 5-HT (*e.g.* banana, pineapple, kiwi, and walnut) [7] greatly enhances the urinary excretion of both 5-HTOL and 5-HIAA [8], and might thus yield false-positive results unless 5-HTOL is expressed as a ratio relative to 5-HIAA [8].

So far, the normally very low urinary concentrations of 5-HTOL have been determined by a sensitive and specific gas chromatograpic–mass spectrometric (GC–MS) method [2]. However, this technique is unsuitable when samples are to be analysed routinely. Since no alternative methods for measuring 5-HTOL in urine have been published to our knowledge, the aim of the present study was to develop a high-performance liquid chromatographic (HPLC) method for the routine quantitative determination of elevated urinary levels of total (free plus conjugated) 5-HTOL.

EXPERIMENTAL

Chemicals

5-Hydroxytryptophol, sulphatase (from *Helix pomatia*; type H-1) and sodium octyl sulphate were purchased from Sigma (St. Louis, MO, USA). β-Glucuronidase [β-D-glucuronide glucurunosohydrolase, EC 3.2.1.31, from *Escherichia coli*; water solution in 50% (v/v) glycerol] was obtained from Boehringer Mannheim (Mannheim, Germany). The enzymatic preparation did not contain detectable amounts of 5-HTOL. All other chemicals used were of analytical grade from Merck (Darmstadt, Germany). All solutions were prepared in deionized Milli-Q water (Millipore, Bedford, MA, USA).

Apparatus and chromatographic conditions

The HPLC system consisted of a Milton Roy Model 396 minipump (LDC, Riviera Beach, FL, USA) and a Model LC-3 electrochemical detector with a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, USA). The potential was maintained at +0.60 V *vs.* an Ag/AgCl reference electrode, and the detector sensitivity range was usually set at 0.5 or 1.0 nA/V. The analytical column was a Nucleosil-120 C_{18} reversed-phase column (75 mm × 4.0 mm I.D., particle size 3 μm) from Skandinaviska GeneTec (Kungsbacka, Sweden). The column was eluted isocratically at a flow-rate of 1.0 ml/min at ambient temperature with a mobile phase consisting of 105 m*M* citric acid, 10 ml/l methanol, 50 μ*M* EDTA and 25 μ*M* sodium octyl sulphate (pH 2.2). The mobile phase was vacuum-filtered through a 0.45-μm type HA filter (Millipore) before use.

Urine samples

Samples of urine were obtained from healthy subjects and from patients participating in an alcohol-dependence treatment programme. Morning urine samples were collected in plastic tubes and stored at −80°C until analysis. Most of the samples were collected from subjects who had been drinking unknown amounts of alcohol at different times before sampling.

Analytical procedures

After thawing at room temperature, a 200-μl aliquot of the urine sample was mixed with 200 μl of 50 m*M* sodium phosphate buffer (pH 6.5) and 20 μl of the β-glucuronidase solution and incubated in capped plastic tubes for 1 h at 37°C. The samples were then centrifuged at 13 000 *g* for 5 min and stored on ice until analysis.

5-HTOL was isolated by a one-step sample clean-up procedure on a small "disposable" Poly-Prep column (Bio-Rad Labs., Kent, UK) packed with Sephadex G-10 (0.6 ml bed volume; Pharmacia, Uppsala, Sweden). Of the hydrolysed urine sample, 250 μl were applied onto the resin equilibrated with 0.1 *M* sodium acetate buffer (pH 5.5). The column was then washed with 4.0 ml (in 1.0-ml portions) of the sodium acetate buffer, after which 5-HTOL was eluted with 2.5 ml (in 0.5-ml portions) of a 1:1 (v/v) mixture of NaCl (1.5 *M*) and methanol. The eluate was vortexed and a 20-μl aliquot injected into the HPLC system.

The Sephadex columns were regenerated by washing the resin with 3 ml of 0.1 *M* NH_4OH, followed by 3 ml of 0.1 *M* acetic acid, and were re-equilibrated with the sodium acetate buffer. Trapped air bubbles were removed by stirring the resin with a wooden stick.

Alternatively, a centrifuged 20-μl aliquot of the hydrolysed urine sample was diluted ten- or twenty-fold in ice-cold deionized water and injected directly into the chromatographic system.

The recovery of 5-HTOL after the Sephadex G-10 sample clean-up procedure was determined by comparing the 5-HTOL level in the 2.5-ml eluate collected with that of the originally applied sample, as determined by the direct injection procedure. Correction was made for the ten-fold dilution obtained with the Sephadex procedure.

The GC–MS analyses were performed as described in detail elsewhere [2,6]. The urinary creatinine concentration was determined according

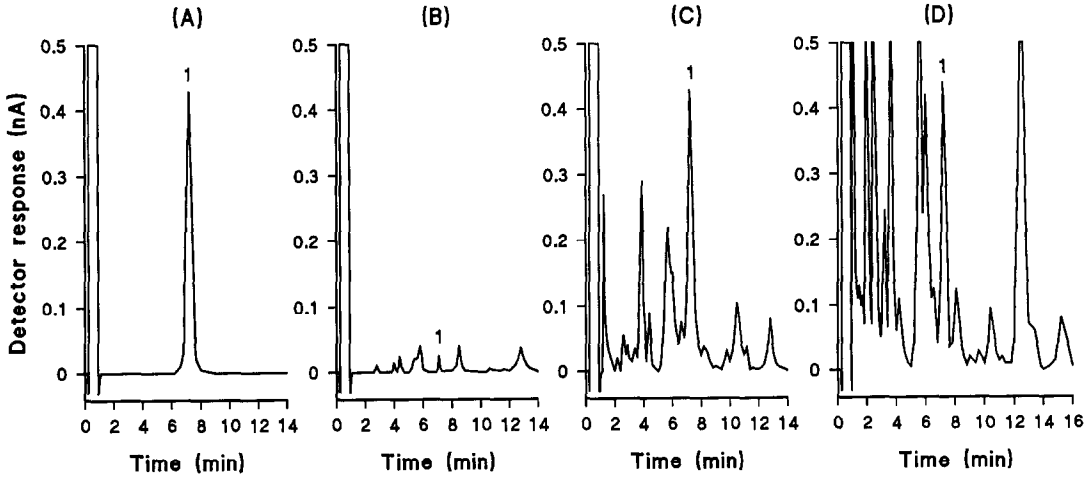

Fig. 1. Determination of 5-HTOL (free plus conjugated form) in human urine. Authentic urine samples were treated with β-glucuronidase, and 5-HTOL was analysed by HPLC with electrochemical detection (+ 0.60 V *vs.* Ag/AgCl). The detector sensitivity range was set at 0.5 nA/V. (A) A standard solution containing 5.0 μ*M* 5-HTOL; (B) a urine sample containing *ca.* 0.2 μ*M* 5-HTOL, injected after a one-step sample clean-up procedure on Sephadex G-10; (C) a urine sample containing 4.6 μ*M* 5-HTOL, injected after Sephadex G-10 clean-up; (D) the same urine sample as in C, but instead diluted in ice-cold water and injected directly into the HPLC system. Peak 1 = 5-HTOL.

to the Jaffé reaction [9], using a Monarch 1000 Chemistry System (Instrumentation Laboratory, Lexington, MA, USA).

RESULTS

Methodology

The HPLC elution profiles obtained after injection of a 5-HTOL standard solution and authentic urine samples containing normal and elevated 5-HTOL levels are shown in Fig. 1. The retention time for 5-HTOL was *ca.* 7 min. After the Sephadex G-10 sample clean-up procedure, an improved separation of 5-HTOL from the background was obtained (Fig. 1).

The liberation of 5-HTOL from the conjugated form was studied as a function of time and the amount of β-glucuronidase, using urine samples containing elevated levels of 5-HTOL. In assays carried out under standard conditions with a urine sample containing 1.6 μ*M* 5-HTOL, the amount of free 5-HTOL had reached its maximum after only 10 min incubation, and remained stable during the 2 h studied. The amount of enzyme could be reduced to 10% of the standard without significantly affecting the final analytical result, as studied with a urine sample containing 5.9 μ*M* 5-HTOL. However, when the β-glucuronidase solution was diluted 100-fold, only *ca.* 60% of 5-HTOL was recovered in the free form. The 5-HTOL levels obtained with the present hydrolysis procedure did not differ significantly from the ones obtained with the previous procedure [2], which used a sulphatase preparation containing β-glucuronidase activity and a 16-h incubation period (r^2 = 0.99).

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD FOR DETERMINATION OF URINARY 5-HYDROXYTRYPTOPHOL

	5-HTOL concentration (μ*M*)	C.V. (*n* = 5) (%)
Authentic urine samples		
Intra-assay	0.2	5.5
Intra-assay	2.1	4.6
Inter-assay[a]	2.1	8.4
Standard		
Intra-assay	2.0	3.8

[a] Assays were carried out on separate days.

A calibration curve covering 0.1–5.0 μ*M* was prepared with water solutions of 5-HTOL treated according to the standard procedure. A good linear relationship (r^2 = 0.99) between the peak height and the amount of 5-HTOL was observed in the concentration range used. The 5-HTOL level of unknowns was determined from the peak height in the chromatogram by reference to the standard curve.

After the Sephadex G-10 sample clean-up procedure, 98 ± 3% (mean ± S.D. for eight authentic urine samples) of 5-HTOL was recovered in the 2.5-ml eluate collected, whereas less than 1% was found in the fractions collected before and after this volume. This implies that analyses can be performed without using an internal standard. The limit of detection for the clean-up procedure was *ca.* 0.05 μ*M*, assuming a signal-to-noise ratio of at least 3:1, and it was in the range 0.3–0.5 μ*M* when diluted samples were injected directly into

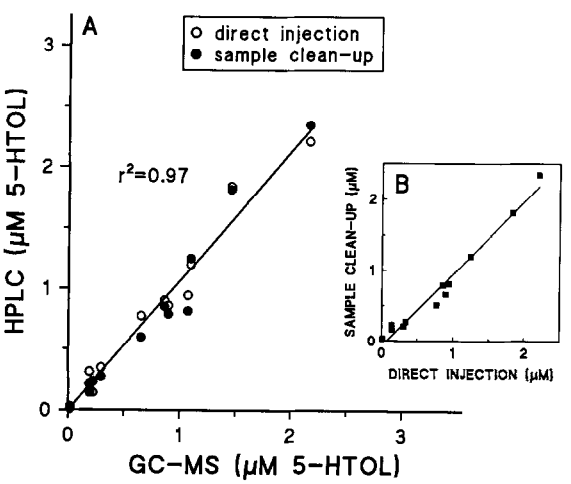

Fig. 2. (A) Correlation between the present HPLC method (both using direct injection of a diluted sample and injection after a one-step sample clean-up on Sephadex G-10) and a sensitive and specific GC–MS method [2] for the quantitative determination of urinary 5-HTOL. (B) Correlation between the direct injection and sample clean-up procedures for HPLC determination of urinary 5-HTOL (r^2 = 0.97; *n* = 12).

the HPLC system. The intra- and inter-assay coefficients of variation (C.V.) of the method are given in Table I.

A good correlation between the present HPLC method and the GC–MS method was obtained (equation: HPLC = 1.06GC–MS − 0.01), when analyses were performed on urine samples containing up to 2.2 μM 5-HTOL (Fig. 2A). This also confirmed the identity of the 5-HTOL peak in the HPLC chromatogram. Moreover, there was a good correlation between the direct injection and Sephadex G-10 procedures (Fig. 2B).

Untreated samples of urine could be stored for one week at 4, 25 and 30°C, and for several months at −80°C, without detectable decline in 5-HTOL levels. After the solid-phase extraction procedure, 5-HTOL was stable in the eluate for several days at 4°C.

Levels of 5-HTOL in urine

The level of 5-HTOL in urine normally ranged between 0.03 and 0.25 μM or, if expressed relative to the creatinine concentration, between 3 and 10 nmol 5-HTOL per mmol creatinine. After alcohol consumption, the levels increased to 0.5–15 μM (50–800 nmol/mmol creatinine). Elevated 5-HTOL levels (greater than 0.5 μM) were also observable following ingestion of bananas, a food rich in 5-HT.

DISCUSSION

So far, the low 5-HTOL levels that normally occur in human urine have been determined by a sensitive and specific GC–MS method [2]. The present study shows that elevated urinary 5-HTOL levels, which occur after ingestion of alcohol or 5-HT-rich foods, are also measurable by HPLC with electrochemical detection, using either direct injection of a diluted sample or, preferably, a solid-phase extraction procedure using Sephadex G-10 prior to injection. Similar sample clean-up procedures using small Sephadex G-10 columns have previously been used for the isolation of indoleamines and catecholamines from various biological sources [10–13].

Although the sample preparation makes the 5-HTOL method more time-consuming, injections into the HPLC system can be made more often, since late-eluting peaks occur occasionally when diluted crude urine samples are injected directly. Moreover, it was usually impossible to quantitate 5-HTOL concentrations reliably in the low normal range using the direct injection procedure, since interfering peaks often occurred in the chromatograms of concentrated urine samples containing high levels of creatinine. Elevated 5-HTOL levels (greater than 0.5 μM) were, however, detectable with equal reliability with either of the two analytical procedures.

In humans 5-HTOL is excreted in urine predominantly in the conjugated form [2,3]. It was previously reported that 5-HTOL appeared in roughly equal amounts as glucuronide and sulphate conjugates following oral administration of 5-HT substance [3]. However, in the present study, no difference in the total (free plus conjugated) levels of 5-HTOL was observed when the enzymatic hydrolysis was performed with either a sulphatase preparation containing β-glucuronidase activity or with β-glucuronidase only. Since the 5-HTOL measured originated from endogenous sources of 5-HT, this indicated that glucuronidation followed by urinary excretion is normally the predominant route for the elimination of peripheral 5-HTOL. The cause of these discrepant results may be that the glusulase used by Davis *et al.* [3] contained enough β-glucuronidase activity to hydrolyse the 5-HTOL glucuronide present.

In conclusion, the present HPLC method permits rapid quantitative determination of elevated urinary levels of total 5-HTOL. The method might be of value as a screening method for routine monitoring of recent (*i.e.* within 24 h) alcohol consumption, for example in the treatment of alcohol dependence. If desired, positive results can subsequently be verified by GC–MS [2].

REFERENCES

1 S. Udenfriend, E. Titus and H. Weissbach, *J. Biol. Chem.*, 216 (1955) 499.
2 O. Beck, S. Borg, L. Eriksson and A. Lundman, *Naunyn-Schmiedeberg 's Arch. Pharmacol.*, 321 (1982) 293.

3 V. E. Davis, H. Brown, J. A. Huff and J. L. Cashaw, *J. Lab. Clin. Med.*, 69 (1967) 132.
4 A. Feldstein, H. Hoagland, H. Freeman and O. Williamson, *Life Sci.*, 6 (1967) 53.
5 J. M. Walsh, *Adv. Mental Sci.*, 3 (1973) 233.
6 A. Voltaire, O. Beck and S. Borg, *Alcohol. Clin. Exp. Res.*, 16 (1992) 281.
7 J. M. Feldman and E. M. Lee, *Am. J. Clin. Nutr.*, 42 (1985) 639.
8 A. Helander, T. Wikström, C. Löwenmo, G. Jacobsson and O. Beck, *Life Sci.*, 50 (1992) 1207.
9 M. D. D. McNeely, *Gradwohl's Clinical Laboratory Methods and Diagnosis*, C. W. Mosby, St. Louis, MO, 1980, pp. 507–509.
10 S. F. Contractor, *Biochem. Pharmacol.*, 15 (1966) 1701.
11 B. H. C. Westerink and J. Korf, *J. Neurochem.*, 29 (1977) 697.
12 C. J. Early and B. E. Leonard, *J. Pharmacol. Methods*, 1 (1978) 67.
13 J. Odink, H. Korthals and J. H. Knijff, *J. Chromatogr.*, 424 (1988) 273.