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Determination of 5-hydroxytryptophol in urine by high-performance liquid chromatography: application of a new post-column derivatization method with fluorometric detection*

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Abstract: The aim of the present study was to develop a high-performance liquid chromatographic (HPLC) method for determination of the serotonin metabolite 5-hydroxytryptophol (5HTOL) in human urine. 5HTOL was liberated from its conjugated form by enzymatic hydrolysis and isolated by a sample clean-up procedure on a small Sephadex G-10 column. The eluate was injected onto an isocratically eluted C_{18} reversed-phase column and 5HTOL was converted into a fluorescent oxazole derivative by on-line post-column reaction with benzylamine in the presence of potassium hexacyanoferrate(III). The limit of detection was about 10 nM and the intra-assay coefficients of variation were below 4% with urine samples and standard solutions. The results indicate that the method can be used as a screening method to discriminate between normal and elevated levels of total (free + conjugated) 5HTOL in urine.

Keywords: *5-Hydroxytryptophol; serotonin; urine; HPLC; post-column derivatization; benzylamine; fluorometric detection.*

Introduction

5-Hydroxytryptophol (5HTOL) occurs naturally in animals as a metabolite of serotonin (5-hydroxytryptamine, 5HT). Serotonin is inactivated metabolically by oxidative deamination to 5-hydroxyindole-3-acetaldehyde (5HIAL) by the action of monoamine oxidase. 5HIAL is further degraded by aldehyde dehydrogenase to form 5-hydroxyindole-3-acetic acid (5HIAA), and by alcohol dehydrogenase or aldehyde reductase to form 5HTOL. In man, 5HIAA is the predominant end metabolite and the concentration of 5HIAA in body fluids is an often used measure of serotonin functional turnover. The 5HTOL level is normally only about 1–5% of the 5HIAA level in brain and CSF [1, 2], and less than 1% in urine [3].

Although 5HTOL is normally a minor metabolite of serotonin, the formation is increased dramatically after the ingestion of ethanol while 5HIAA is concomitantly decreased [4]. This metabolic shift is manifested in urine, and the 5HTOL excretion will not normalize until several hours after the blood and urinary ethanol levels have reached zero [5]. Based on this observation, and elevated urinary level of 5HTOL has been used as a biochemical marker to indicate recent alcohol consumption [6]. The urinary level of 5HTOL is also increased after the ingestion of serotonin-rich foods [7] and in patients with carcinoid tumours (unpublished observations).

Measurement of 5HTOL in urine is rather complicated, since it occurs in low concentrations and predominantly in conjugated form with glucuronic acid and, to a lesser extent, sulphuric acid [3, 4, 8]. 5HTOL is assumed to be conjugated at the phenolic 5-hydroxyl group [9]. So far, 5HTOL has been analysed using a specific and reliable gas chromatographic–mass spectrometric (GC–MS) method following the liberation of conjugated 5HTOL by enzymatic hydrolysis [3, 6]. However, a more simple method would be desired for routine screening.

The aim of the present study was to develop

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a high-performance liquid chromatographic (HPLC) method for routine determination of 5HTOL in urine by applying a new principle for detection of 5-hydroxyindole compounds based on post-column derivatization and fluorometric detection [10].

Materials and Methods

The urine specimens were initially treated with β-glucuronidase (from *Escherichia coli*; Boehringer Mannheim, Germany) for 1 h at 37°C after which free plus glucuronide conjugated 5HTOL was isolated by a one-step sample clean-up procedure on a small Sephadex G-10 column (0.6-ml bed volume; Pharmacia, Uppsala, Sweden) as described in detail elsewhere [8]. The recovery of 5HTOL after the sample clean-up procedure was close to 100%. Alternatively, the enzymatic hydrolysis can be carried out with a sulphatase preparation which also contains β-glucuronidase activity (type H-1 from *Helix pomatia*; the Sigma Chemical Co., St Louis, MO, USA). This procedure is much more time-consuming (16 h), but will also liberate the sulphate conjugated 5HTOL. However, this form accounts for only a minor part of total 5HTOL in urine [8].

Analysis of 5HTOL was carried out using a new principle for detection of 5-hydroxyindoles based on post-column derivatization with benzylamine in the presence of potassium hexacyanoferrate(III) to form a highly fluorescent oxazole derivative [10] (Fig. 1).

The HPLC system consisted of a Pharmacia-LKB Model 2248 pump and a Rheodyne Model 7125 injector (Cotati, CA) with a 20-μl sample loop. Chromatography was carried out at ambient temperature on a 3-μm Nucleosil 120 C_{18} column (75 × 4.0 mm, i.d.). The mobile phase consisted of 10 mM sodium acetate buffer (pH 4.7) and acetonitrile (95:5, v/v) and was eluted at 0.8 ml min^{-1}. The eluate from the column was mixed with the reagent solution using an Upchurch Model U-466 (Oak Harbor, WA) low dead-volume mixing chamber. The reagent solution consisted of 20 mM benzylamine and 3 mM potassium hexacyanoferrate(III) in a mixture of 25 mM borate buffer (pH 10) and acetonitrile (1:1, v/v) and the flow rate was 0.4 ml min^{-1}. The mixture was passed through a 30-m knitted reaction coil (0.3 mm, i.d.; ICT, Frankfurt, Germany) at ambient temperature and, finally, into a Shimadzu Model RF-551 fluorescence detector using excitation and emission wavelengths of 345 and 481 nm, respectively. The 5HTOL concentration was determined from the peak-height in the chromatogram by reference to a standard calibration curve. The standards were subjected to the same procedure as the urine samples.

Results

The derivatization reaction was tested at two different temperatures. The 5HTOL peak-height was almost twice as high if the reaction coil was kept at room temperature (20–25°C) instead of at 70°C, the temperature recommended in the original publication [10]. In addition, the back-ground fluorescence was higher at 70°C. If the preliminary purification step was omitted, interfering peaks sometimes occurred in the chromatogram when assays were carried out with urine samples. The 5HTOL peak was about 25–30% higher when the reaction coil length was increased from 20 to 30 m. Thus, for the standard procedure, a 30-m reaction coil was used and kept at room temperature.

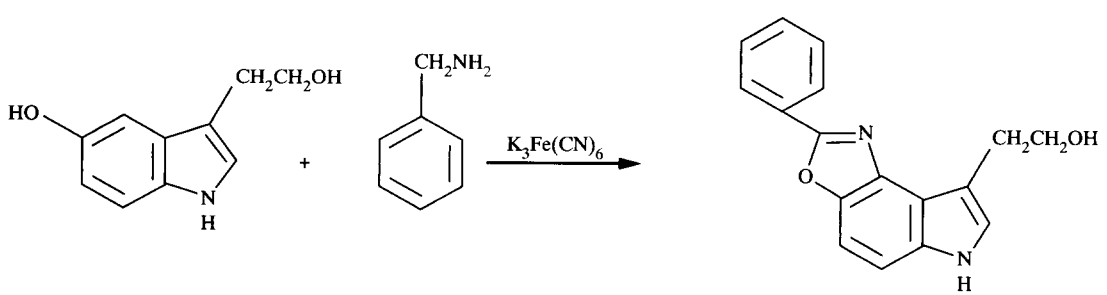

Figure 1
Proposed mechanism for the reaction of 5-hydroxytryptophol (5HTOL) with benzylamine in the presence of potassium hexacyanoferrate(III) to form a fluorescent oxazole derivative [8].

The HPLC elution profiles obtained after injection of 5HTOL standard solutions (100 and 1000 nM) and authentic urine samples are shown in Fig. 2. The retention time for 5HTOL was about 10.5 min and no major interfering peaks were observed in the chromatogram (Fig. 1b). The detector response was linear in the concentration range 0–5000 nM (r^2 = 0.99). A 5HTOL standard curve covering 8–500 nM is shown in Fig. 3.

The detection limit of the method (signal-to-noise ratio = 3) was about 10 nM for a 5HTOL standard solution and the intra-assay relative standard deviations (RSDs) were 3.4 and 3.8% (n = 10) with a standard and an authentic urine sample containing about 500 nM, respectively.

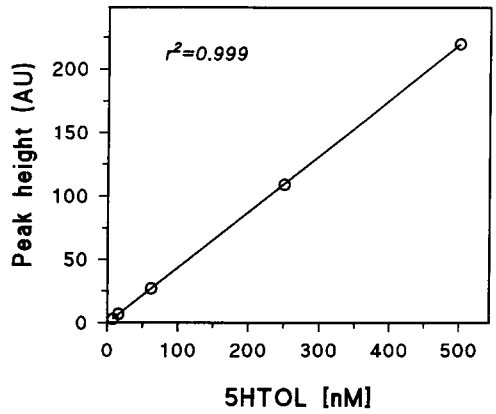

Figure 3
Calibration curve with standard solutions of 5-hydroxytryptophol (5HTOL) determined by HPLC with post-column derivatization and fluorimetric detection.

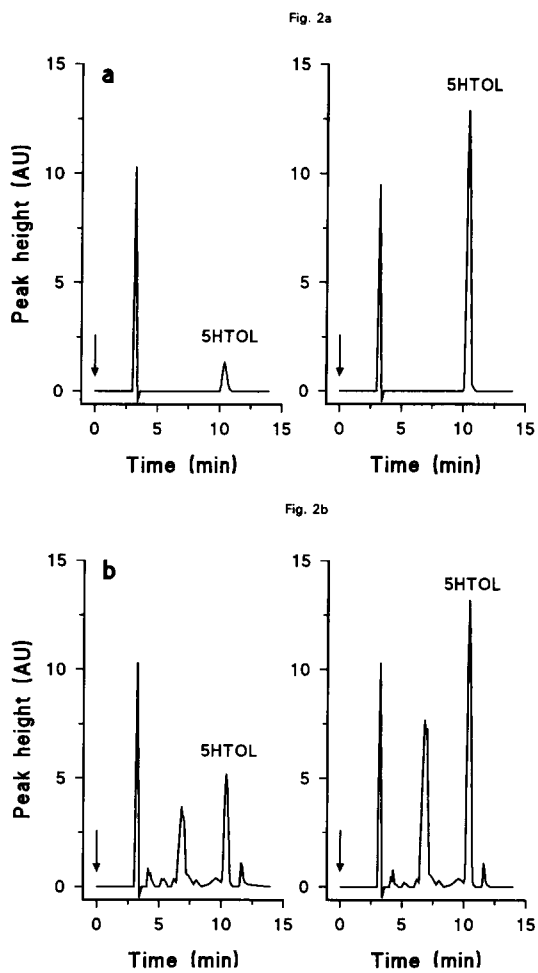

Figure 2
HPLC determination of free plus glucuronide conjugated 5-hydroxytryptophol (5HTOL) in human urine after post-column derivatization with benzylamine and potassium hexacyanoferrate(III) and fluorimetric detection: (a) 5HTOL standard solutions containing 100 and 1000 nM 5HTOL, respectively; (b) Authentic urine samples containing approximately 400 and 1000 nM 5HTOL, respectively. The urine samples were treated with β-glucuronidase prior to analysis to liberate conjugated 5HTOL.

Discussion

Numerous HPLC methods have been described for the determination of 5HIAA in urine [see 11 for review]. However, the measurement of 5HTOL is more complicated since it occurs in much lower concentrations and predominantly in conjugated form. So far, the analytical strategy has been to liberate 5HTOL from its conjugated form by enzymatic hydrolysis and the total (free + conjugated) 5HTOL is subsequently determined using GC–MS [3, 6]. The GC–MS method involves solvent extraction and formation of a tri-pentafluoropropionyl derivative. However, even though this method is specific and reliable, a more simple method is desired for routine work. We, therefore, developed an alternative HPLC method for screening of elevated levels of 5HTOL [8]. The method utilizes electrochemical detection to obtain sensitivity and specificity, but, sometimes, problems with chromatographic interference occurred when it was applied for routine clinical use. Recently, a new principle for detection of 5-hydroxyindoles based on post-column reaction and fluorometric detection has been introduced and applied to the measurement of 5HIAA in human urine [10]. The method utilizes the specific reaction of benzylamine with the phenolic 5-hydroxyl group to form a highly fluorescent oxazole derivative [12].

In the present method, the post-column fluorescence derivatization method was combined with the preliminary purification procedure used in the previous HPLC method and applied for determination of urinary 5HTOL. The chromatographic interference due to endogenous fluorescent compounds was markedly reduced as compared to detection by native fluorescence, and the derivatization reaction appears to be rather seletive for 5-hydroxyindoles. For example, the glucuronide conjugate of 5HTOL, where glucuronic acid is attached to the phenolic 5-hydroxyl group, was not detected by the derivatization method (unpublished observations). The sensitivity of the new method was found to be in the same range as with electrochemical detection, which indicates that it can be used for routine determination of the low concentrations of 5HTOL normally occurring in human urine (<250 nM) [8].

In summary, the present results indicate that a new principle for detection of 5-hydroxyindole compounds based on post-column reaction with benzylamine in the presence of potassium hexacyanoferrate(III) and fluorometric detection allows for quantitative determination of total (free + conjugated) 5HTOL levels in human urine.

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