Quantitative Determination of 2-Hydroxy-3-Methoxy-6β-Naltrexol (HMN), Naltrexone, and 6β-Naltrexol in Human Plasma, Red Blood Cells, Saliva, and Urine by Gas Liquid Chromatography

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Abstract

Two gas liquid chromatographic methods differing mainly in sensitivity are described for the quantitative determination of 2-hydroxy-3-methoxy-6β-naltrexol (HMN), a minor metabolite of naltrexone (NT) in human body fluids. The methods also incorporate simultaneous determinations of naltrexone and its major human metabolite, 6β-naltrexol (3-OL), in urine, serum (or plasma), red blood cells (RBC), and saliva. Flame ionization detection of the bis-(trimethylsilyl) trifluoracetamide (BSTFA) derivatives provided sufficient sensitivity for quantification of the bases in urine. However, lower limits in serum, RBC and saliva necessitated the use of more sensitive electron capture detection of the perfluoropropionyl (PFPA) derivatives of the bases. Because HMN and 6β-naltrexol PFPA derivatives have nearly identical gas chromatographic retention times, their separation was achieved by differential extraction based on their different partition characteristics between the aqueous and organic solvents in the plasma of 4 subjects. In 16 and 24 hrs after 2200 mg NT doses, the relative percentages were 22.1% HMN, 84% NT, and 73.5% 3-OL. In urine samples collected at the same time, the relative percentages were 14.4% HMN, 80% NT, and 76.8% 3-OL. The nonpolar nature of HMN and the greater polarity of 3-OL may have influenced their differential distribution into RBCs and saliva. In the RBCs, 95.1% HMN and no significant amount of 3-OL was found; in saliva, 92.3% of 3-OL and no HMN was found.

Introduction

2-Hydroxy-3-methoxy-6β-naltrexol (HMN) is a minor metabolite of naltrexone in man (1-4) (Figure 1). It was isolated from human red blood cells and urine of subjects taking naltrexone (1). A methylated 2,3-catechol type metabolite of naltrexone with the same mass unit as HMN was also reported (2). The assignment of the methyl group in the 3-position was initially based on the fact that this metabolite is excreted only in the free form. Naltrexone, 6β-naltrexol and various similar molecules are being glucuronidated in the 3 position and large percentages of such molecules are excreted into the urine in the conjugated form. If the 3 position is blocked by methylation, no glucuronide can form, as it was observed with HMN (1). More recently, the structure of HMN was proven by synthesis (3) and by studies utilizing nuclear magnetic resonance spectra (4). In an earlier pharmacokinetic study, HMN was quantitated in urine and expressed as 6β-naltrexol equivalents (5). The recent availability of synthetic HMN allowed development of new and specific quantitative methodology. In this communication we report on clinically applicable methods for the determination of HMN, naltrexone and its major metabolite 6β-naltrexol in plasma (or serum), RBC, saliva, and urine.

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Materials and Methods

Chemicals and Reagents

2-Hydroxy-3-methoxy-6β-naltrexol, 6β-naltrexol and naltrexone were a gift from Dr. R. Willette of NIDA (Bethesda, MD), while nalorphine was obtained from Merck & Co., Inc. (Rahway, NJ). The solvents used in this study were all glass distilled, G.C. grade purchased from Burdick and Jackson (Muskegon, MI). All aqueous solutions, buffers and dilute acids were prepared in glass distilled water, and all glassware was siliconized using 5% Dri-Film SC-87 in toluene. Pentfluoropropionic anhydride (PFPA) and Dri-Film SC 87R were purchased from Pierce Chemical Co. (Rockford, IL), and bis(trimethylsilyl) trifluoroacetamine (BFSTA) was purchased from Regis Chemical Co. (Morton Grove, IL). Glusulase® was purchased from Endo Laboratories (Garden City, NY).

Subjects

The samples for this study were kindly provided by Professor Volavka, The Missouri Institute of Psychiatry, University of Missouri School of Medicine. The subjects were all male chronic schizophrenic patients who received incremental oral doses from 50 to 400 mg naltrexone in the first week and 400 mg naltrexone daily in the second week. The 100 mg doses and above were split and given at 8:00 AM and 4:00 PM. Since all the samples were collected just prior to the 8:00 AM dose, they represent sample 24 hours past the first and 16 hours past the second half of the daily dosage. Two blood, saliva, and spot urine samples were collected from each patient on the 7th day of the 1st and 2nd weeks. Thus, in both cases the patients were already receiving 2X200 mg naltrexone per day.

Extraction Procedure for the Recovery of Naltrexone and its Metabolites From Urine

Urine (2.5 mL) and 20 μg of etorphine (the internal standard) were added to test tubes containing 2.5 mL Glusulase® solution (4.5 mL Acetate buffer pH 5.2 plus 1 mL stock Glusulase® solution) and incubated overnight at 37°C. After incubation, the samples were saturated with NaHCO₃ to attain pH = 8-8.5 and 0.5 mL of the hydrolysate solution was extracted with 7 mL chloroform for determination of β-naltrexol and 3.0 mL was extracted with 7 mL of benzene for the quantitation of naltrexone and HMN. Both the benzene and chloroform fractions were back-extracted by shaking with 5 mL of .5 N HCl. The organic phase was discarded, and the aqueous phase was saturated with NaHCO₃, extracted with 7 mL chloroform and the chloroform fraction evaporated to dryness using an N-EVAP® analytical evaporator. The extraction procedure with chloroform yielded recovery of naltrexone of 97 ±1% of HMN, 84.2 ±0.9%, and of β-OL 87 ±1.2%, as compared to absolute standards. Extraction utilizing benzene provided recoveries of HMN of 54 ± 1.2%, naltrexone 55 ± 1.7%, and β-OL was not recovered.

Derivatization and Gas Chromatographic Conditions

Complete dryness was assured by capping the tubes with rubber stoppers and flushing each tube with nitrogen gas for 2-5 minutes using flushing (in) and a vent (out) needle. After removal of the needles, 25 μL BSTFA was injected through the rubber stopper, and the tubes were heated for 15 minutes at 70°C. After cooling, 1 to 2 μL aliquots were injected into a Perkin Elmer 900 gas chromatograph. The column was a 2 m × 2 mm I.D. glass spiral packed with 3% OV-17 on Gas-Chrome Q 80-100 mesh. The temperature of the detector and flash heater was 285°C. The column oven temperature was 230°C. The flow rates of hydrogen and air were 30 mL/minute and 300 mL/minute, respectively, while the carrier flow rate was 30 mL/minute. The relative retention times of 6β-naltrexol, HMN, naltrexone and etorphine were 4.8, 6.2, 7.4 and 9.2 minutes, respectively (Figure 2). The reliable limit of quantitation for this method is 10 to 20 ng/mL of urine.

Extraction Method for the Recovery of HMN and Naltrexone From Plasma, Serum, RBC or Saliva

One mL of plasma, serum, packed RBC suspension or saliva, 25 ng nalorphine (internal standard), and 1.0 mL of .2 M tris-maleate buffer (pH 7.5) were shaken with 10 mL of benzene for 5 minutes on an automatic shaker, followed by 2 minute centrifugation. The benzene layer was transferred into a clean centrifuge tube containing 5 mL of benzene for 5 minutes on an automatic shaker, followed by 2 minute centrifugation. The benzene layer was discarded and 4.5 mL of the acid phase was transferred into a clean centrifuge tube and neutralized with 1N NaOH. The pH was adjusted to 7.5 with .2 M tris buffer. The free bases were extracted from the aqueous phase with 7 mL of benzene. After shaking and centrifugation, the benzene layer was transferred into a clean 15 mL test tube and evaporated to dryness using a rotary flash evaporator. The recovery of HMN from plasma using this
method was 51 ± 1.4%, and that of naltrexone 54 ± 2.3%; β-OL was not recovered under these conditions.

Derivatization and Gas-Chromatographic Conditions

To the dry residues 150 μL of PFPA were added; the tubes were tightly stoppered and placed in a heating block at 110°C for 40 minutes. After cooling, the anhydrides were evaporated under a stream of nitrogen. The dried samples were taken up in 25 to 50 μL of ethyl acetate, and 0.5-1.0 μL aliquots were injected into a Hewlett Packard 5830A gas chromatograph equipped with a N\textsubscript{5} electron capture detector. The column was a 2 m × 2 mm I.D. glass spiral packed with 3% OV-22 on Supelcoport 80/100 mesh. The temperature of the detector was 300°C; the flash heater 240°C and the column oven 220°C. The flow rate of the carrier (90% argon 10% methane) was 25 mL/minute. The relative retention times of 6β-naltrexol, HMN, naltrexone, and nalorphine were 3.38, 3.48, 3.94, and 6.16 minutes, respectively (Figure 3). The lower limit of reliable quantitation of this procedure is between 0.5 to 1 ng/mL of biofluid.

Results and Discussion

The method of quantitation for HMN in urine is similar to the one reported earlier for naltrexone (6). Utilizing the synthetic HMN standard, a calibration curve was prepared for amounts between 5.0 and 30.0 μg of HMN. This concentration was within range of HMN present in the urine of subjects taking up to 400 mg naltrexone daily. In Figure 2, the BSTFA derivatives of HMN, naltrexone, 6β-naltrexol, and etorphine gas chromatographic tracings are presented. The separation is well defined among the bases for simultaneous quantitation. However, when 400 mg or larger daily doses of naltrexone are administered, the excessive amount of 6β-naltrexol may interfere with the quantitation of HMN and naltrexone. Under such conditions, the separate extraction system should be utilized, one for 6β-naltrexol and another for HMN and naltrexone as described in the methods section.

Naltrexone, 6β-naltrexol, and HMN are in substantially lower concentration in blood and saliva than in urine. For this reason, more sensitive methodology was needed. The PFPA
derivatives of naltrexone and of 6β-naltrexol has been used for quantitation of these bases in plasma(7). Using this method, it was observed that the retention time of 6β-naltrexol occasionally shifted as much as 3 to 4 seconds while the retention times of naltrexone and the internal standard remained essentially the same. The answer to this phenomenon was clarified only recently. Figure 3 shows the gas chromatographic tracings of the PFPA derivatives of HMN, naltrexone, 6β-naltrexol, and the internal standard nalorphine. It is apparent from the tracings (panel A & B) that only 0.1 minute or 6 seconds separate 6β-naltrexol (3.38 minutes) and HMN (3.48 minutes). When both of these substances are present in a mixture, no separation of the two substances is accomplished. However, the magnitude and direction of the retention time shift between HMN and β-OL depends on the amount of each substance present. In Figure 3, panel C, β-OL and HMN are present in approximately equal amounts, indicated by the retention time of 3.42 minutes, median between the two pure standards. In all previous studies when PFPA was used as the derivatizing agent, the values reported for 6β-naltrexol are over-estimated because they also include HMN.

Separation of HMN and 6β-naltrexol PFPA derivatives was unsuccessful on various chromatographic stationary liquid phases. For this reason, another approach was utilized. The non-polar nature of HMN and the greater polarity of 6β-naltrexol provided physico-chemical differences sufficient to separate the two metabolites during extraction. In addition, it was observed that 6β-naltrexol recovery increased with increase of pH from 7 to 9.4. To achieve exclusion of 6β-naltrexol, the pH was lowered to 7.5, and a nonpolar solvent benzene was used. Under these conditions, HMN recovery was 51 ± 1.4%, while 6β-naltrexol was not recovered at all. Using chloroform at pH 9.4 resulted in the recovery of both metabolites, thus for 6β-naltrexol determination the HMN levels must be determined using the benzene extracts, and then the values subtracted from the combined 6β-naltrexol and HMN values which are determined from the chloroform extracts. Naltrexone can be quantitated equally well in both systems.

To verify the usefulness of these methods, samples of blood (plasma and RBC), saliva and urine were collected from subjects taking 400 mg naltrexone. (See exact doses and sample collection conditions under subjects). The data derived from the analysis of urine samples is presented in Table I. Since it was not a continuous 24-hour urine collection, only the relative abundance of the bases present at the time of the collection can be assessed. 6β-Naltrexol accounted for 76.6% of the bases, followed by HMN (14.4%) and naltrexone (9.0%). In the plasma samples the order of relative abundance was the same as in urine with similar proportion of 6β-naltrexol (73.5%), significantly more HMN (23.1%) and slightly less naltrexone (3.4%) present (Table II).

Differential distribution of HMN and 6β-naltrexol into RBC and saliva (Table II) also demonstrated the physico-chemical differences between the two metabolites. In RBCs only the less polar HMN distributed in the extent of 96.1%, while 3.9% was naltrexone and the 6β-naltrexol levels were not detectable. Conversely, in saliva HMN was absent; the more polar 6β-naltrexol comprised 92.3% of the bases, and naltrexone made up the remaining 7.7%.

### Table I. Urinary Levels of Naltrexone, 6β-Naltrexol and 2-Hydroxy-3-Methoxy-6β-Naltrexol (HMN) in a Single Spot Sample 16 and 24 Hours After 2X200 mg Naltrexone

<table>
<thead>
<tr>
<th>Base µg/mL</th>
<th>Subject</th>
<th>Naltrexone</th>
<th>6β-Naltrexol</th>
<th>HMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.3</td>
<td>152.0</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>62.8</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>59.7</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.4</td>
<td>49.4</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
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<td>6</td>
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<td>262.3</td>
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<td>8</td>
<td>18.1</td>
<td>184.5</td>
<td>14.6</td>
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Mean ± SD 14.3 ± 13.0 122.4 ± 74.8 23.0 ± 16.8

% of Total Base 9.0 76.6 14.4

### Table II. Plasma, RBC, and Salivary Levels of Naltrexone, 6β-Naltrexol and 2-Hydroxy-3-Methoxy-6β-Naltrexol (HMN) After 2X200 mg Naltrexone Given 24 & 16 Hours Prior to Sample Collection

<table>
<thead>
<tr>
<th>Base ng/mL</th>
<th>Subject</th>
<th>Plasma</th>
<th>6β-Naltrexol</th>
<th>HMN</th>
<th>RBC</th>
<th>Naltrexone</th>
<th>6β-Naltrexol</th>
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<tr>
<td>Subject</td>
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<td></td>
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<tr>
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<td>105*</td>
<td>572.2*</td>
<td>151.3*</td>
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<td>115.0*</td>
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<td>10.0</td>
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<td>9.0</td>
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<td>53.2</td>
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<td>7.3</td>
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<tr>
<td>8</td>
<td>6.9</td>
<td>231.5</td>
<td>62.8</td>
<td></td>
<td></td>
<td>5.8</td>
<td>112.5</td>
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</table>

Mean ± SD 9.4 ± 5.2 200.6 ± 39.1 62.9 ± 14.4 6.8 ± 1.9 166.5 ± 47.1 13.9 ± 17.1 166.7 ± 184

% of Total Base 3.4 73.5 23.1 3.9 96.1 7.7 92.3

*Sample was drawn after naltrexone dose; values not included in the calculations. N.S. = no samples available.
The first blood sample from Subject 3 (Table 1) was taken shortly after naltrexone administration. Thus, the levels of drug and metabolites were substantially higher than in the 24-hour samples. The high naltrexone, 6β-naltrexol, and HMN plasma levels indicate excellent absorption and rapid first pass biotransformation of naltrexone, concuring with data reported in an earlier pharmacokinetic study (5).

In future studies, the differential distribution of drugs into RBC vs. plasma vs. saliva may become a useful system to evaluate relative polarities among metabolites and parent compounds of other drugs as well. Based on these data, for toxicological purposes 6β-naltrexol in plasma, saliva, urine, and HMN in whole blood, RBC and urine are better indicators of naltrexone use than the parent compound itself in any of the body fluids, especially when the samples are collected long after naltrexone ingestion.

Conclusion

In conclusion, clinically useful methods were developed for the specific and quantitative measurement of naltrexone and its metabolites in various body fluids. The results indicate that 6β-naltrexol and HMN remain in larger concentrations and for a longer period of time in the body than the parent compound. The physico-chemical differences in solubility between HMN and 6β-naltrexol were demonstrated in their extraction characteristics and their preferential distribution into RBC and saliva.

References


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Gas Chromatography-Chemical Ionization Mass Spectrometry of Cocaine and Its Metabolites in Biological Fluids

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Abstract

A gas chromatographic-chemical ionization mass spectrometric (GC-CIMS) method is described for the determination of cocaine, benzoylecgonine, and norcoca. The procedure uses stable isotopes as internal standards and a mixture of methane-ammonium as chemical ionization reagent gas. Run-to-run and within-run coefficients of variation (%) are less than 10% and the method has a sensitivity of less than 5 ng/mL from 1 mL or 1 gram of sample. The procedure has been applied to a number of cases involving cocaine intoxication and analytical data from these are described.

Introduction

Cocaine is a naturally occurring stimulant drug which is extracted from the leaves of the coca plant (Erythroxylon coca). The leaves of this South American, Andean shrub have been chewed by Bolivian and Peruvian Indians since antiquity for

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