Hydrocodone combined with acetaminophen is commonly used for moderate pain. Hydrocodone is metabolized by cytochrome P450 (CYP) 2D6 into hydromorphone and by CYP3A4 into norhydrocodone. This was a retrospective study evaluating hydrocodone, hydromorphone and norhydrocodone distributions in urine. Urine specimens (n = 76,924) were obtained from patients on chronic opioid therapy during their first or single visit and were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS-MS). The patients were at least 16 years of age and had documented hydrocodone use via a medication list. There were 48,710 specimens that were positive for all three analytes. Mean hydrocodone, hydromorphone and norhydrocodeone mole fractions (95% confidence interval) were 0.39 (0.38–0.39), 0.12 (0.11–0.12) and 0.49 (0.48–0.49), respectively. Hydromorphone fractions were lower in women compared with men (0.11 versus 0.13; P < 0.0001). Hydrocodone mole fractions were higher in the 65-year and older age group compared with the 16- to 39-year age group (0.4 versus 0.36; P ≤ 0.005). Concurrent use of a CYP2D6 and/or CYP3A4 inhibitor altered hydromorphone and norhydrocodeone mole fractions, compared with the control group. Patient factors affect hydrocodeone and metabolite mole fractions and suggest increased awareness of their contribution when attempting to interpret urine drug testing results.

Introduction

Hydrocodone is a semisynthetic opioid that is structurally related to codeine. Hydrocodone stimulates the mu-opioid receptor, inhibits the release of transmitters from sensory neurons and alters pain transmission. In 2008, the American Association of Poison Control Centers’ National Poison Data System (NPDS) reported 11,726 poisoning cases that involved hydrocodone alone, 26,166 poisoning cases that involved hydrocodone in combination with other drugs and 21 hydrocodone-associated deaths (1). Hydrocodone is more potent than codeine (minimum adult lethal dose ~100 mg) and has a greater addiction liability (2). Adverse or toxic effects of overdose include stupor, muscle flaccidity, respiratory depression, hypotension and coma (2). Hydrocodone is marketed as an extended release oral formulation and as a combination product with acetaminophen or ibuprofen. The combination products are classified as Schedule III controlled substances.

After administration of hydrocodone, urinary excretion of hydrocodone is 6–20%, norhydrocodeone 2–14% and hydromorphone 5–6% (3, 4). Other hydrocodeone metabolites detected in human urine include dihydrocodeine, isodihydrocodeine, dihydromorphone and isodihydromorphone (3, 4). Hydrocodeone undergoes cytochrome P450– (CYP–) dependent oxidation to the O- and N-demethylated products, thus forming hydromorphone via CYP2D6 and norhydrocodeone via CYP3A4 (5, 6). Genetic polymorphisms exist for CYP2D6 and CYP3A4. Genetic polymorphisms of CYP2D6 result in a phenotypic classification ranging from poor to ultra-extensive metabolizers (7). About 2–10% of the population are homozygous for non-functional CYP2D6 mutant alleles resulting in a poor metabolizer phenotype (7). Patients who are CYP2D6 poor metabolizers may experience variable hydrocodeone side effects, efficacy and dependence (8, 9).

Methods

Subject selection

Urine specimens were collected at pain physician practices from patients on opioid therapy for routine drug monitoring purposes. This study received Institutional Review Board exempt status by the University of California, San Diego Human Research Protection Program. Between January 2011 and August 2011, 517,775 random, de-identified urine specimens were collected and analyzed by Millennium Laboratories (San Diego, CA, USA). Inclusion criteria were specimens from a subject’s first or single visit, subjects 16 years of age or older, detectable concentrations (>50 ng/mL) of hydrocodone, hydromorphone and norhydrocodeone analytes, and documented hydrocodeone use via a medication list (e.g. physician-reported hydrocodeone use). Exclusion criteria were as follows: specimens with urine creatinine...
concentrations of <20 mg/dL, missing patient demographic data (e.g. date of birth, sex), physician-reported hydromorphone, codeine and morphine use, as well as detectable concentrations of morphine, codeine and heroin metabolite (6-monoacetylmorphine). Morphine, codeine and 6-monoacetylmorphine concentrations were detected via liquid chromatography–tandem mass spectrometry (LC–MS-MS) with the lower limit of quantitation of <50 ng/mL for each analyte. Subjects with urine creatinine concentrations of <20 mg/dL were excluded because this indicated a diluted specimen (15, 16). For the evaluation of CYP2D6 and CYP3A4 inhibitor use, physician-reported CYP2D6 inducer \( (n = 14) \) use and physician-reported CYP3A4 inducer use \( (n = 1,629) \) were additional exclusion criteria.

**Analytical procedure**

Urinary concentrations were analyzed by LC–MS-MS (12, 17, 18). In brief, an Agilent 1200 series binary pump SL LC system, well-plate sampler and thermostatted column compartment paired with an Agilent Triple Quadrupole Mass Spectrometer and Agilent Mass Hunter software were used for the analysis of hydrocodone, hydromorphone and norhydrocodone. Chromatographic separation was performed using an acetonitrile–formic acid–water gradient running at 0.4 mL/min and a 2.1 × 50 mm², 1.8-μm Zorbax SB C 18 column. Mobile phase A = 0.1 percent formic acid in water, B = 0.1 percent formic acid in acetonitrile and column temperature was set to 50°C. Samples were prepared for injection by incubating 25 μL of urine with 50 Units of β-Glucuronidase Type L-II (keyhole limpet) Sigma Product number G 8132 (Sigma-Aldrich Corp., Saint Louis, MO, USA) in 50 μL of 0.4 M acetate buffer (pH 4.5) for 3 h at 45°C. Five microliters of the solution was injected for each sample.

All spectra were collected using positive electrospray ionization. The optimized instrumental parameters were as follows: gas temperature, 350°C; drying gas, 12 L/min; nebulizer gas (nitrogen), 35 psi \( (\sim 24,100 \text{ Pa}) \); capillary voltage, 3,000 V; and fragmenter voltage, 60 V. Multiple reaction monitoring (MRM) mode was used for quantitation. Scan time was set to 500 ms. In the MRM mode, two transitions were used to identify and quantitate a single compound. A quantitative transition was used to calculate concentration based on the quantifier ion, and a second transition was used to ensure accurate identification of the target compound based on the ratio of the qualifier ion to the quantifier ion. The ion criteria were used for the qualitative identification with ±20% of the average ion ratios for calibrators.

HPLC grade water, acetonitrile, methanol and formic acid were obtained from VWR (Westchester, PA, USA). Hydrocodone, hydromorphone and normorphine were obtained from Cerilliant Corp. (Round Rock, TX, USA). At the beginning of the analysis, the internal standard solution contained 1,500 ng/mL of deuterated standard and enzyme β-glucuronidase. About 100 μL of this solution was added to 25 μL of patient’s sample. The final concentration of the internal standard was 1,200 ng/mL.

A four-point calibration curve was created by using a linear fit and forcing the line to go through the origin. Accepted accuracy for calibrators was ±20% of the target value, and the coefficient of determination \( (R^2) \) was required to be ≥0.99 as verification of linearity and goodness of fit. The lower limit of quantitation for the hydrocodone, hydromorphone and norhydromorphone was 50 ng/mL. The upper limit of linearity for both the hydrocodone and hydromorphone assays was 100,000 ng/mL. The inter-assay coefficient of variation for all the analytes at the low and high ends of the quantitation curve was <10% based on the analysis of >100 runs.

**Data and statistical analysis**

Specimens were creatinine normalized to mg/g creatinine to account for subject variability due to muscle mass and hydration status (19). In order to compare the parent drug and metabolite in urine, creatinine normalized data were converted into moles (20). Mole fractions of hydrocodone, hydromorphone and norhydrocodone were then determined by multiplying the molecular weight and dividing by 1,000 (20). Mole fractions were used for data analysis purposes since there is less interindividual variability versus urine concentrations (20), and to determine whether the proportions of each metabolite change with total exposure or with concurrent use of CYP2D6 and/or CYP3A4 inhibitors.

For the age analysis, specimens were divided into three groups: 16–39 years, 40–64 years and 65 years and older (21). For the evaluation of urinary pH, specimens were divided into three groups: 3.7–6.8, 6.9–7.9 and 8–11. For the evaluation of CYP2D6 and CYP3A4 inhibitor use, specimens were separated based on physician-reported medication use. Specimens that did not include CYP2D6 and CYP3A4 inhibitors represented the control group. A list of known CYP2D6 and CYP3A4 inhibitors were obtained from the University of Indiana, School of Medicine (22). The medication history for each individual was evaluated for the listed 36 CYP2D6 inhibitors and 31 CYP3A4 inhibitors. Mole fractions based on the presence of CYP2D6 and/or CYP3A4 inhibitor use were compared with mole fractions from the control group.

Descriptive statistics were conducted with Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA), and all statistical analysis was completed with OriginPro v8.1 (OriginLab, Northampton, MA, USA). Mole fractions were log-transformed to achieve a normal distribution. Mole fractions are presented as means and 95% confidence intervals (95% CI). A one-way ANOVA and two-sample unpaired \( t \)-test were performed. Statistical significance, with a Bonferroni adjustment due to multiple comparison testing, was set at \( P \leq 0.005 \) (23).

**Results**

The sample population is summarized in Figure 1. A total of 76,924 subjects were positive for at least one or more analytes. There were 1,145 specimens (1.5%) that were positive for only hydrocodone, 388 (0.5%) specimens for hydromorphone and 2,614 (3.4%) specimens for norhydrocodone. There were 48,710 specimens positive for all the three analytes. Mean hydrocodone, hydromorphone and norhydrocodone mole fractions (95% CI) were 0.39 (0.38–0.39), 0.12 (0.11–0.12) and 0.49 (0.48–0.49), respectively.

**Sex analysis**

Hydrocodone, hydromorphone and norhydrocodone mole fractions were compared between women \( (n = 27,622) \) and men...
In men, mean hydrocodone, hydromorphone and norhydrocodone mole fractions (95% CI) were 0.41 (0.40–0.41), 0.13 (0.12–0.13) and 0.46 (0.45–0.46), respectively. In women, mean hydrocodone, hydromorphone and norhydrocodone mole fractions (95% CI) were 0.38 (0.37–0.38), 0.11 (0.1–0.11) and 0.52 (0.51–0.52), respectively. Hydromorphone mole fractions were higher in men compared with women ($P < 0.0001$). Norhydrocodone mole fractions were higher in women ($P < 0.0001$).

Age and urinary pH analysis

Mean ± SD age was 51 ± 13 years. Data are summarized in Table I. Significant differences in hydrocodone, hydromorphone and norhydrocodone mole fractions were observed. Hydrocodone mole fractions were highest in the 65-year and older age group compared with the other age groups. The hydromorphone mole fractions were similar across the evaluated age groups, whereas norhydrocodone mole fractions were highest in the 16- to 39-year age group. There were 38,618, 7,685 and 2,407 specimens with urine pH of 3.7–6.8 (acidic), 6.9–7.9 (neutral) and 8–11 (basic), respectively (Table II). Hydromorphone and norhydrocodone mole

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**Table I**

Mean (95% CI) Hydrocodone, Hydromorphone and Norhydrocodone Mole Fractions Based on Age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Mole fractions</th>
<th>Hydrocodone</th>
<th>Hydromorphone</th>
<th>Norhydrocodone</th>
</tr>
</thead>
<tbody>
<tr>
<td>16–39 years</td>
<td>9,774</td>
<td>0.36 (0.35–0.36)$^{a,b}$</td>
<td>0.12 (0.12–0.13)$^{a,b}$</td>
<td>0.52 (0.51–0.53)$^{a,b}$</td>
<td></td>
</tr>
<tr>
<td>40–64 years</td>
<td>30,743</td>
<td>0.39 (0.38–0.39)$^{b}$</td>
<td>0.11 (0.11–0.12)$^{b}$</td>
<td>0.49 (0.48–0.49)$^{b}$</td>
<td></td>
</tr>
<tr>
<td>65 years and older</td>
<td>7,426</td>
<td>0.4 (0.4–0.41)</td>
<td>0.11 (0.11–0.12)</td>
<td>0.48 (0.47–0.48)</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$P-value ≤ 0.005 compared with the 46- to 64-year age group.

$^{b}$P-value ≤ 0.005 compared with the 65-year and older age group.

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**Table II**

Mean (95% CI) Hydrocodone, Hydromorphone and Norhydrocodone Mole Fractions Based on Urinary pH

<table>
<thead>
<tr>
<th>Urinary pH</th>
<th>N</th>
<th>Mole fractions</th>
<th>Hydrocodone</th>
<th>Hydromorphone</th>
<th>Norhydrocodone</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7–6.8</td>
<td>38,618</td>
<td>0.41 (0.4–0.41)$^{a,b}$</td>
<td>0.11 (0.1–0.11)$^{a,b}$</td>
<td>0.48 (0.47–0.49)$^{a,b}$</td>
<td></td>
</tr>
<tr>
<td>6.9–7.9</td>
<td>7,685</td>
<td>0.31 (0.3–0.31)$^{b}$</td>
<td>0.15 (0.14–0.15)$^{b}$</td>
<td>0.54 (0.53–0.54)$^{b}$</td>
<td></td>
</tr>
<tr>
<td>8–11</td>
<td>2,407</td>
<td>0.3 (0.29–0.3)</td>
<td>0.15 (0.15–0.16)</td>
<td>0.54 (0.54–0.55)</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$P-value ≤ 0.005 compared with the 6.9–7.9 urinary pH group.

$^{b}$P-value ≤ 0.005 compared with the 8–11 urinary pH group.
fractions were higher in the neutral and basic urinary pH groups (Table II).

Concurrent hydrocodone and CYP2D6 and/or CYP3A4 inhibitor use

Subjects (n = 21,177) lacking a physician-reported medication that was a CYP2D6 and CYP3A4 inhibitor (control group) observed hydrocodone, hydromorphone and norhydrocodone mean mole fractions of 0.39, 0.11 and 0.49, respectively. There were 3,787 patients with a physician-reported CYP2D6 inhibitor medication. The most commonly reported CYP2D6 inhibitors were duloxetine (n = 1,700), sertraline (n = 723) and bupropion (n = 503). There was no difference in hydrocodone mole fractions between the CYP2D6 inhibitor group and the control group. In contrast, hydromorphone and norhydrocodone mole fractions were significantly different for the CYP2D6 inhibitor group (Table III).

There were 497 patients with a physician-reported CYP3A4 inhibitor medication. The most commonly reported CYP3A4 inhibitors were verapamil (n = 119) and diltiazem (n = 69). Hydrocodone, hydromorphone and norhydrocodone mean mole fractions during concurrent use of a CYP3A4 inhibitor were 0.46, 0.12 and 0.42, respectively. There was no difference in hydromorphone mole fractions between the CYP3A4 inhibitor group and the control group. In contrast, hydrocodone and norhydrocodone mole fractions were significantly different for the CYP3A4 inhibitor group (Table III). Upon dual inhibition with a CYP2D6 and CYP3A4 inhibitor (n = 3,819), hydrocodone and norhydrocodone mole fractions increased, while hydromorphone decreased (P < 0.005; Table III).

Table III

<table>
<thead>
<tr>
<th>Condition</th>
<th>N</th>
<th>Mole fractions</th>
<th>Hydrocodone</th>
<th>Hydromorphone</th>
<th>Norhydrocodone</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CYP2D6 and CYP3A4 inhibitors (control group)</td>
<td>21,177</td>
<td>0.39 (0.38–0.39)</td>
<td>0.11 (0.11–0.13)</td>
<td>0.49 (0.48–0.49)</td>
<td></td>
</tr>
<tr>
<td>CYP2D6 inhibitor</td>
<td>3,787</td>
<td>0.39 (0.38–0.39)</td>
<td>0.08 (0.07–0.08)</td>
<td>0.53 (0.52–0.53)</td>
<td></td>
</tr>
<tr>
<td>CYP3A4 inhibitor</td>
<td>497</td>
<td>0.46 (0.44–0.48)</td>
<td>0.12 (0.11–0.13)</td>
<td>0.42 (0.41–0.44)</td>
<td></td>
</tr>
<tr>
<td>CYP2D6 and CYP3A4 inhibitors</td>
<td>3,819</td>
<td>0.41 (0.39–0.41)</td>
<td>0.07 (0.06–0.07)</td>
<td>0.53 (0.51–0.53)</td>
<td></td>
</tr>
</tbody>
</table>

*P-values determined upon comparison with the control group.

Sex effects on hydrocodone and metabolites

Hydromorphone and norhydrocodone mole fractions were significantly different based on sex. Hydromorphone mole fractions were higher in men, whereas norhydrocodone mole fractions were higher in women (P < 0.0001). CYP3A4 activity has been observed to be higher in female liver microsomes, whereas CYP2D6 activity is higher in male liver microsomes. This may explain the observed differences in hydromorphone and norhydrocodone mole fractions observed in the current study and the reported differences regarding opioid analgesia and side effect profiles between men and women (26). However, it should be noted that numerous mechanisms have been suggested to explain the observed sex differences and should not be discounted. These include, but are not limited to, higher estrogen concentrations in women, the relationship between analgesic and antianalgesic properties, psychological factors and genetic influences (26).

Age effects on hydrocodone and metabolites

Physiologic factors such as hepatic function, renal function, liver mass and hepatic blood flow are known to decrease with age (27). In the current study, hydrocodone mole fractions were higher in the 65-year and older age group compared with the 16- to 39-year age group. This seems to suggest an age-related reduction in metabolic capacity of hydrocodone, such as decreased CYP2D6 activity, since the extent of hydromorphone metabolite formation was low. However, age-related changes due to decreased CYP2D6 and/or CYP3A4 activity may not be contributory factors as one study reported a lack of age-dependent changes in CYP2D6 and CYP3A4 (28). Regardless of which physiologic factor impacted hydrocodone and hydromorphone mole fractions, it is of practical value to closely monitor a patient’s response to opioid medication use. In the older adult, cognitive function and functional mobility may be compromised, which place these individuals at a higher fall risk.
**Urinary pH effects on hydrocodone and metabolites**

Data are limited describing the relationship between the amount excreted of opioid and corresponding urinary pH. Methadone is one example whereby excretion is urinary pH-dependent, while the metabolite EDDP is not dependent on pH (29). A significant difference in the excretion pattern of the parent drug and metabolites based on urinary pH was observed in this study (Table II). The urine specimens used for the analysis had a variable pH range. These results suggest a need to minimize urinary pH variations by several approaches, including, but not limited to, maintaining specimen integrity at the collection site and during laboratory analysis (30).

**Concurrent hydrocodone and CYP2D6 and/or CYP3A4 use**

Interindividual variability of hydrocodone pharmacokinetics and pharmacodynamics is due, in part, to CYP2D6- and CYP3A4-mediated drug–drug interactions (5, 31). Concurrent administration of a CYP2D6 inhibitor would be expected to lower hydromorphone metabolite formation due to competition for binding sites on the CYP2D6 enzyme between hydrocodone and the CYP2D6 inhibitor. Our results are in agreement, as hydromorphone mole fractions during concurrent use of a CYP2D6 inhibitor were lower compared with the control group (Table III). Norhydrocodone mole fractions were also higher and suggest a compensatory metabolic pathway. Hydrocodone mole fractions did not change during concurrent CYP2D6 inhibitor use. This may be due to the contribution of an alternative metabolic pathway (i.e. ketoreduction) by which hydrocodol epimers are formed (24).

Concurrent administration of a CYP3A4 inhibitor would be expected to lower norhydrocodone metabolite formation due to competition for binding sites on the CYP3A4 enzyme between hydrocodone and the CYP3A4 inhibitor. Our results are in agreement, as norhydrocodone mole fractions during concurrent use of a CYP3A4 inhibitor were lower compared with the control group (Table III). A compensatory metabolic pathway would also be anticipated and result in higher hydromorphone mole fractions. However, this was not observed as there was no statistically significant difference in hydromorphone mole fractions during concurrent CYP3A4 inhibitor use compared with the control group (Table III). Rather, hydrocodone mole fractions were higher in the CYP3A4 inhibitor group, which is likely due to parent drug accumulation.

Dual CYP2D6 and CYP3A4 inhibitor use affected hydrocodone, hydromorphone and norhydrocodone mole fractions, compared with the control group (Table III). Upon comparison between CYP2D6 and CYP3A4 inhibitor use versus CYP2D6 inhibitor use, the extent of differences in hydrocodone, hydromorphone and norhydrocodone is similar (Table III). We speculate that CYP2D6 inhibitor use plays a more prominent role in contributing to hydrocodone and metabolite mole fraction differences compared with CYP3A4 inhibitor use.

**Study limitations**

The current study lacked dosing administration of hydrocodone. One study has suggested that peak urine concentrations occur 3–9 h after administration, with detection times of 28, 26 and 40 h for hydrocodone, hydromorphone and norhydrocodone, respectively (32). There may also be variation in the timing of hydrocodone relative to CYP2D6 and/or CYP3A4 inhibitor administration. The extent of contribution of these factors impacting hydrocodone, hydromorphone and/or norhydrocodone mole fractions is unknown. Another study limitation was the presumption of medication use from physician-reported medication lists. Subjects may have been taking hydromorphone, morphine and/or codeine with unreported use. Attempts to limit this confounding variable included exclusion of specimens with detectable concentrations of morphine and codeine. Additionally, subjects who were taking hydrocodone may have been study exclusions on the basis of unreported use.

No data were available regarding a subject’s CYP2D6 and CYP3A4 genotype and qualitative and/or quantitative analysis of medicaments that were CYP2D6 and/or CYP3A4 inhibitors. However, CYP2D6 and CYP3A4 genotyping of all patients prior to starting an opioid is not routinely performed in clinical practice. Rather, CYP genotyping is generally performed on a reactive basis, in an effort to possibly explain a challenging clinical scenario (e.g. excessive sedation with low starting dose and lack of analgesia after escalating doses). Even with medications such as antidepressants and tamoxifen, with known alterations based on CYP2D6 genotype (33, 34), formal recommendations are lacking to genotype all patients prior to initiating therapy. In addition, qualitative and/or quantitative analysis of all medications listed on the physician-reported medication list is unlikely.

**Conclusions**

This was a retrospective study evaluating hydrocodone, hydromorphone and norhydrocodone distributions in urine. There were 48,710 specimens that were positive for all the three analytes. Norhydrocodone had the highest mole fraction, followed by hydrocodone and hydromorphone. Women had lower hydrocodone and hydromorphone, but higher norhydrocodone mole fractions. Hydrocodone mole fractions were higher in the 65-year and older age group compared with the 16- to 39-year age group. However, the extent of hydromorphone metabolite formation was low. Hydrocodone and metabolites are affected by urinary pH variations. Increased awareness of concurrent hydrocodone and medications that are metabolized by CYP2D6 and/or CYP3A4 is recommended, in lieu of the observed differences in hydrocodone and metabolite mole fractions during CYP2D6 and/or CYP3A4 inhibition. Research is underway examining the combination of these patient factors (age, sex and urinary pH), the extent of CYP2D6 and/or CYP3A4 inhibition, and the specific medication that is a CYP2D6 and/or CYP3A4 inhibitor on affecting hydrocodone, hydromorphone and norhydrocodone mole fractions.

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