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Impact of β-Glucuronidase Mediated Hydrolysis on Haldol® Urinalysis

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Abstract

Reports have suggested that patients with mental health disorders including major depressive disorder and schizophrenia have dramatically low adherence levels to prescribed medications. Patients on haloperidol (Haldol®) therapy, regardless of their disease, were found to have higher adherence levels—though still strikingly low. This work shows that high levels of the glucuronidated form of haloperidol are present in patient urine samples. Time-of-Flight (TOF) mass spectrometry experiments are consistent with both the presence of haloperidol glucuronide and that hydrolysis of haloperidol patient urine samples leads to significantly increased concentrations of free haloperidol. Urine samples collected from patients prescribed haloperidol were tested with and without hydrolysis revealing a significant increase in the number of patients testing positive when the samples were hydrolyzed before analysis. These data demonstrate that hydrolysis greatly improves the sensitivity and consistency of results for patients on haloperidol therapy resulting in positivity data that strongly correlates with the dosage form administered.

Introduction

Haloperidol (Haldol®) is a "typical" antipsychotic drug prescribed for the treatment of acute symptoms of schizophrenia and many other mental health disorders including Tourette syndrome and delirium (1, 2). Overall, potential drug adherence has been reported to be particularly low in patients with major depressive disorder (MDD) followed by bipolar disorder (BP) and less so in patients with schizophrenia at 19% adherence (3). Another report suggested that haloperidol patients are ~63.5% adherent overall regardless of disease or dosage form (4). While therapeutic assessment of drug adherence requires blood testing, urine drug testing can provide information on patient usage for chronic drug paradigms (5). Haloperidol has been reported to be present in human urine at <1% of a single oral dose with "no evidence found for the formation of haloperidol glucuronide" (6). Other reports have detailed the metabolism of haloperidol in plasma demonstrating glucuronidation as the primary metabolic pathway (7, 8). This work details the results of both liquid chromatography time of flight mass spectrometry (LC/TOF) and enzyme hydrolysis followed by liquid chromatography mass spectrometry mass spectrometry (LC/MSMS) that are consistent with haloperidol glucuronide as the major metabolite in urine and that hydrolysis of urine drug samples can significantly improve the sensitivity of urine drug testing for haloperidol.

Experimental

All specimens that were used in this analysis were de-identified. Ameritox is accredited by the CAP and abides by CAP, Clinical Laboratory Improvement Amendments (CLIA), and Health Insurance Portability and Accountability Act (HIPAA) requirements. Due to the secondary analysis nature of this work and the absence of clinical conclusions, neither US Food and Drug Administration (FDA) nor other clinical trial review/approval was obtained by Ameritox. Writing this manuscript did not involve human subjects as defined by the US Code of Federal Regulations (45 CFR 46.102); thus, an IRB approval of these specific research activities was not necessary.

The LC/MSMS method described here was validated for haloperidol as per an internal Standard Operating Procedure (SOP) described by Enders and McIntire (9) based upon College of American Pathologist requirements, Clinical Laboratory Improvement Amendments (CLIA)

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guidelines, and a few other notable sources (10–14). A summary of validation results is presented in Table I. The details of the validation are the same as previously described for quetiapine in Strickland *et al.* (15).

Materials

The internal standard for haloperidol, haloperidol-D4, was purchased from Cerilliant (Round Rock, TX). The reference standard for all LC/TOF measurements, (hydrocodone D6) was purchased from Cerilliant (Round Rock, TX). All solvents, including methanol (Optima grade), acetonitrile (Optima grade), isopropanol (Optima grade), and formic acid (88%) were purchased from VWR (Radnor, PA). Drug-free urine was obtained from Utak Laboratories (Valencia, CA). A recombinant β-glucuronidase enzyme, IMCSzyme™, was purchased from IMCS (Columbia, SC). Standards were either not available or were not certified for 4-fluorobenzoylpropionic acid, 4-fluorobenzoylacetic acid, reduced haloperidol, haloperidol glucuronide, or reduced haloperidol glucuronide. Identification of these analytes was through TOF library matching to the chemical formula as described later.

Sample sets

Determination of the metabolite distribution in patients' urine was completed initially using LC/TOF analysis of 11 samples from patients who were prescribed haloperidol. Following identification of the haloperidol glucuronide in these samples, 5 of the 11 samples were analyzed by LC/TOF before and after enzyme hydrolysis using 2,000 Fishman units of enzyme at 60°C for 60 min to further support identification of haloperidol glucuronide in urine (pre-hydrolysis) and haloperidol (post-hydrolysis). All subsequent samples were analyzed by the validated LC/MSMS method using a Waters Acquity UPLC® Xevo TQ-MS. 151 patient samples with haloperidol prescriptions were analyzed pre-hydrolysis and post-hydrolysis to determine the impact of hydrolysis on haloperidol positivity. All samples were stored at 4°C prior to analysis.

Hydrolysis

After identification of the presence of haloperidol glucuronide, a hydrolysis method for haloperidol testing was further developed and validated. Earlier work by Morris *et al.* (16) determined 60°C as the optimal incubation temperature for the IMCSzyme™ used for the haloperidol hydrolysis. All hydrolyzed patient samples included in this study were assessed after 15, 30, 45, 60, 90 and 120 min incubation at 60°C.

LC/TOF analysis

Patient urine specimens (100 μL) were diluted 5× with 400 μL of reference standard (RS), 0.25 μg/mL of hydrocodone-D6 in water. Hydrocodone D6 is used as an internal reference standard for all LC/TOF injections to guarantee successful injection of the sample. Prepared samples were injected (5 μL) and separated on a Phenomenex Kinetex® Phenyl-Hexyl, 2.1 × 50 mm, 2.6 μm column at 50°C and analyzed on an Agilent 6530 Q-TOF (in TOF mode) with an Agilent 1290 LC system. The LC/TOF method conditions are detailed in Strickland *et al.* (15). A haloperidol control (300 ng/mL) was run along with the patient samples to assist in positive identification.

Sample data were processed using Agilent Mass Hunter Qualitative Analysis and PCDL (Personal Compound Database and Library) Manager Software. A database of haloperidol and 5 of its possible metabolites' chemical formulas was compiled and used to search against the samples. The software matched compounds based on retention time (if available), mass accuracy (<20 parts per million (ppm)), the isotopic distribution pattern, and the isotopic spacing theoretically derived from the chemical formula. To be identified as positive, a compound had to have consistent retention times across patient samples when a known retention time was lacking, otherwise the retention times had to be within ±0.05 min of a control; the mass accuracy had to be <20 ppm; and the composite score of the mass accuracy and isotopic features had to be ≥70 (out of a possible 100). The raw area counts of the chromatographic peak for the analyte were divided by the raw area counts for the hydroxycodone-D6 (metabolite discovery samples) reference standard to give an idea of relative abundance. This was then converted to a % relative abundance by dividing each relative abundance by the sum of the relative abundances of the metabolites present.

LC/MSMS analysis

Urine samples (100 μL) were diluted 5× with 400 μL of a Master Mix solution (contains ≥3,625 U/mL of β-glucuronidase, 0.2 μg/mL Haloperidol-D4, and 67 mM phosphate buffer, pH 7.5) prior to incubation in a VWR® Gravity Convection oven at 60°C for 60 min. Since the calibrators did not include any glucuronides, they were not submitted to the incubation, but were prepared in the same manner with Master Mix solution. Enzyme hydrolysis was completed using ≥1,450 Fishman units/sample of IMCSzyme™ and incubating at 60°C for 60 min. These conditions were selected based on work by Morris *et al.* (16) and the hydrolysis efficiency study detailed herein.

Prepared samples were injected (5 μL) and separated on a Waters Acquity UPLC® CSH Phenyl-Hexyl 2.1 × 50 mm, 1.7 μm column with an Acquity inline filter at 50°C and analyzed on the Waters

Table I. Validation data for haloperidol hydrolysis confirmation method.

	Linearity			Carryover	Precision and accuracy						Matrix	Interference
	LOQ/LOD[a] (ng/mL)	ULOL[b] (ng/mL)	R^2	Avg. conc. ng/mL ($N = 5$)	Avg. % target ($N = 30$)			Avg. % CV[c] ($N = 30$)			% Matrix effect	Interfering compounds
					30 ng/mL	300 ng/mL	3,000 ng/mL	30 ng/mL	300 ng/mL	3,000 ng/mL		
Haloperidol	5	5,000	0.9997	0.0	93.9	94.8	104.1	3.0	3.0	3.8	42.6	None

[a]LOQ, limit of quantification; LOD, limit of detection.
[b]ULOL, upper limit of linearity.
[c]CV, coefficient of variation.

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Acquity UPLC® Xevo TQ-MS system. The LC/MSMS method conditions are detailed in Strickland *et al.* (15) Analyte and internal standard mass transitions, cone voltages, and collision energies are listed in Table II.

Results and discussion

Investigation of metabolite distribution in urine was initiated with 11 patient samples that had tested positive on the existing method, which excludes hydrolysis, being diluted and run on the Agilent LC/TOF system. A total of five potential metabolites of haloperidol were added to a database to be searched for their presence in urine: reduced haloperidol, 4-fluorobenzoylpropionic acid, 4-fluorobenzoylacetic acid, reduced haloperidol glucuronide and haloperidol glucuronide (6). The summary of the relative abundance of these metabolites in patient samples can be found in Table III. While significant amounts of reduced haloperidol were detected, the haloperidol glucuronide appeared to be present on average at a higher concentration than the reduced haloperidol, based on % relative abundances. Interestingly, the reduced haloperidol glucuronide was not present in any significant amount nor were the proposed metabolites, 4-fluorobenzoylpropionic acid and 4-fluorobenzoylacetic acid. While the use of the reduced haloperidol for haloperidol monitoring seems attractive, commercial standards are not available thus making this more difficult than the chosen hydrolytic pathway.

As there was not a haloperidol glucuronide standard readily available, five of these patient samples were analyzed on the LC/TOF with and without enzyme hydrolysis to confirm the identified haloperidol glucuronide. The updated % relative abundances can be seen in Table IV. A representative sample from the set of 5, as shown in Figure 1, has a significant haloperidol glucuronide peak and smaller haloperidol parent peak present pre-hydrolysis. As discussed in the methods section, a haloperidol standard was used to demonstrate consistency with the putative haloperidol peak in the LC/QTOF experiment. Coupled with the exact mass nature of the QTOF instrument, this leads to strong agreement of the assignment of haloperidol and haloperidol glucuronide to the peaks observed in Figure 1. Post-hydrolysis, the putative glucuronide peak disappears while a significant peak for haloperidol is apparent. The hydrolytic conversion of haloperidol glucuronide to haloperidol is strongly supported by these data.

The small peak eluting at 2.25–2.3 min is consistent with the ^{13}C isotope of reduced haloperidol (see Table III) having the same exact mass as haloperidol itself. Post-hydrolysis, the free reduced haloperidol is observed at that retention time (Table III) under the conditions used in this work albeit at a different mass. Hence, the ^{13}C isotope of reduced haloperidol renders it "visible" when looking for the exact mass of haloperidol itself.

A hydrolysis efficiency study was conducted to determine the minimum time required to achieve complete hydrolysis. The data shown in Figure 2 were gathered from positive haloperidol patient samples. The data in Figure 2 show 60 min as the optimal time required for complete haloperidol glucuronide hydrolysis at 60°C using the IMCSzyme™ at >1,450 Fishman units (U)/sample. The relative percentage increase of the concentration of haloperidol is shown in Figure 2 instead of the absolute concentration so that data

Table II. Haloperidol and haloperidol-D4 LC/MSMS confirmation transitions

	Transition	Cone voltage	Collision energy (CE, V)
Analyte			
Haloperidol	376.0 → 94.8[a]	30	66
	376.0 → 165.0[b]	32	27
Internal Standard			
Haloperidol-D4	380.0 → 169.0[a]	32	27
	380.0 → 194.0[b]	32	23

[a]Quantifier ion.
[b]Qualifier ion.

Table III. % Relative abundance of haloperidol metabolites in human urine as determined by LC/TOF analysis.

	Haloperidol (mass = 375.1401)	Haloperidol glucuronide (mass = 551.1722)	Reduced haloperidol (mass = 377.1558)	Reduced haloperidol glucuronide (mass = 553.1879)
Retention time (min)	2.43	2.06	2.24	1.89
1	[a]9.6%	19.0%	71.4%	ND
2	10.1%	36.2%	50.5%	3.2%
3	17.8%	66.3%	10.7%	5.2%
4	13.4%	68.3%	15.7%	2.5%
5	29.3%	36.4%	34.3%	ND
6	5.3%	31.2%	60.1%	3.4%
7	3.6%	85.4%	7.2%	3.8%
8	4.6%	58.4%	20.8%	16.2%
9	15.9%	72.1%	10.1%	1.9%
10	17.0%	64.0%	16.4%	2.6%
11	6.5%	19.1%	74.3%	ND
Range	3.6–29.3%	19.0–85.4%	7.2–74.3%	1.9–16.2%
Average	12.35%	50.58%	33.77%	4.85%

% Relative abundance was with the following formula: $\frac{\frac{Abundance\ of\ Haloperidol\ Metabolite}{Abundance\ of\ Hydrocodone-D6}}{Sum\ of\ All\ Haloperidol\ Metabolite\ Relative\ Abundances} \times 100$. A control containing haloperidol (300 ng/mL) was run to assist in the confirmation haloperidol. All other metabolites were identified based on TOF matching criteria to the chemical formula for each compound (mass accuracy, isotopic spacing, and isotopic distribution). ND = not detected; [a] = a peak was detected, but scored <70 based on the software criteria (mass accuracy, isotopic pattern, isotopic distribution and retention time) and was excluded from the average calculations. 4-Fluorobenzoylpropionic acid and 4-fluorobenzoylacetic acid were not detected in any sample.

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Table IV. % Relative abundance of haloperidol metabolites pre-hydrolysis and post-hydrolysis in human urine as determined by LC/TOF analysis.

	Pre-hydrolysis				Post-hydrolysis			
	Haloperidol	Haloperidol glucuronide	Reduced haloperidol	Reduced haloperidol glucuronide	Haloperidol	Haloperidol glucuronide	Reduced haloperidol	Reduced haloperidol glucuronide
A	5.10%	85.63%	4.15%	5.11%	87.63%	0.04%	12.28%	[a]0.05%
B	[a]3.08%	90.11%	[a]0.97%	5.84%	89.58%	ND	10.42%	ND
C	[a]20.94%	35.60%	43.46%	ND	64.61%	ND	35.39%	ND
D	3.85%	84.19%	4.57%	7.38%	85.55%	0.33%	13.92%	0.20%
E	[a]1.65%	92.13%	[a]1.09%	5.13%	89.93%	ND	10.07%	ND

% Relative abundance was calculated with the following formula: $\frac{\frac{\text{Abundance of Haloperidol Metabolite}}{\text{Abundance of Hydrocodone} - D6}}{\text{Sum of All Haloperidol Metabolite Relative Abundances}} \times 100$. A control containing haloperidol (300 ng/mL) was run to assist in the confirmation haloperidol. All other metabolites were identified based on TOF matching criteria to the chemical formula for each compound (mass accuracy, isotopic spacing, and isotopic distribution). ND = not detected; [a] = a peak was detected, but scored <70 based on the software criteria (mass accuracy, isotopic pattern, isotopic distribution, and retention time) and was excluded from the average calculations. 4-Fluorobenzoylpropionic acid and 4-fluorobenzoylacetic acid were not detected in any sample.

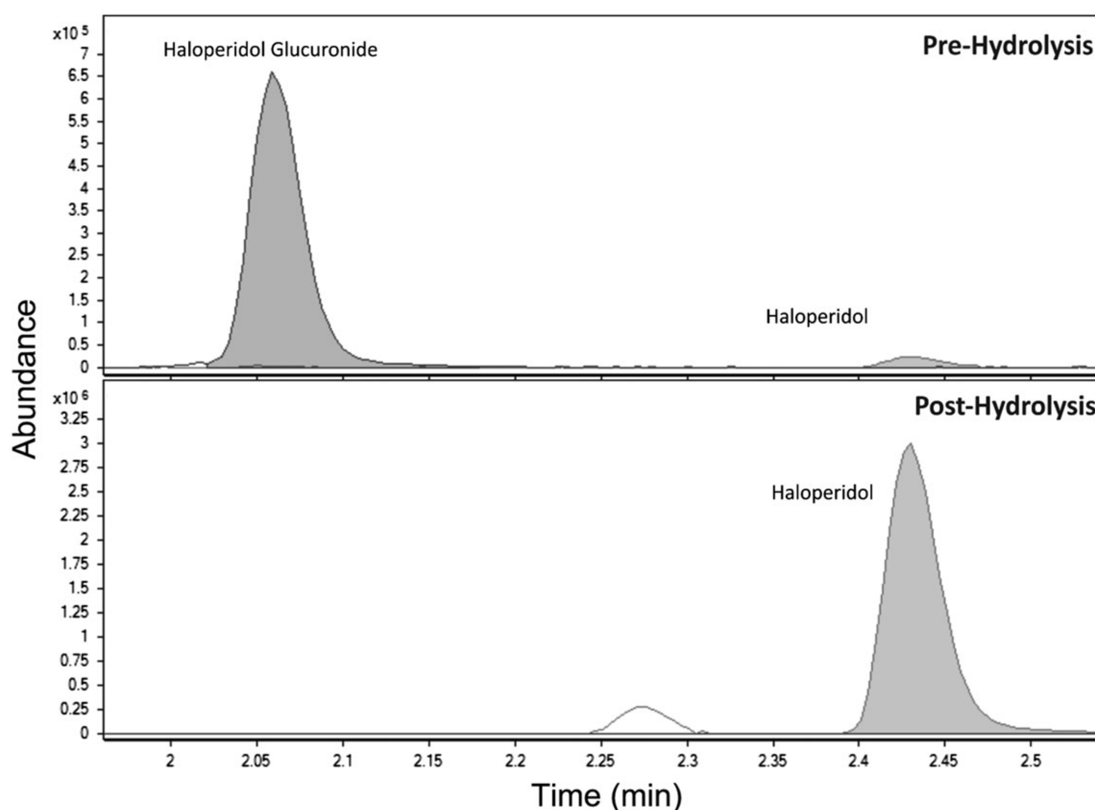

Figure 1. LC/QTOF extracted ion chromatograms for haloperidol glucuronide (mass = 551.99) and haloperidol (mass = 375.86) pre-hydrolysis and post-hydrolysis.

from all the patient samples could be presented on the same relative scale.

The LC/MSMS method used in this work was validated as described in Enders and McIntire (9). This method was assessed for linearity (the reproducible regression or fit of the calibration curve compared to expected concentration values), limits/sensitivity (the lower and upper concentration limits at which the method could accurately identify and quantify analytes), precision and accuracy (the capability of the method to yield reproducibly accurate results over a period of multiple days at concentrations spanning the concentration range of interest), carryover (the highest level of analyte present which ideally does not produce a concentration level above 50% of the lower limit in a following blank injection), interference/selectivity (the ability of the method to be unaffected by the presence of other medications/compounds including antipsychotics, opiates, benzodiazepines, opioids, drugs of abuse, antidepressants and tricyclic antidepressants), and matrix effects (the suppression or enhancement of analyte signals of interest due to the presence of matrix—in this case urine). The last validation study was the patient comparison which is used to confirm whether a new method

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accurately quantitates actual patient samples when compared to the currently in-use and accepted method for this particular class of compounds. A summary of the validation results can be found in Table I.

Of note, the matrix effect study shows an average 42% positive enhancement of the haloperidol signal. Trufelli *et al.* (17) pointed out that while a great deal of work has been reported to explain negative matrix effects, positive matrix effects are less well characterized. A possible explanation involves the amphiphilic nature of ionized haloperidol resulting in concentration of the analyte on the droplet surface during ESI ionization which would artificially enhance the mass spectrometry signal. The matrix would therefore enhance this surface process perhaps through ionic strength within the droplet itself. Whatever the mechanism, such matrix enhancements are not that unusual and have minimal impact on day to day operation.

The effects of hydrolysis were studied using the validated LC/MSMS method and 151 samples from patients who were prescribed the medication. This method has a limit of quantification (LOQ) and reporting cutoff of 5 ng/mL for haloperidol. Results pre-hydrolysis and post-hydrolysis show that for almost every sample, the haloperidol concentrations are substantially higher post-hydrolysis (Table V). It is difficult to calculate an average % increase in the amount of haloperidol post-hydrolysis inasmuch as many samples that were "0" pre-hydrolysis increased dramatically (i.e., from negative to positive). There are patients who demonstrated a "0" pre-hydrolysis who remained "0" post-hydrolysis demonstrating that true negative patients will remain negative post-hydrolysis.

These data are in contrast to reports in the literature (6) wherein conjugation of the parent drug in urine "has not been reported" yet consistent with reports of haloperidol glucuronide in plasma (7, 8). Table V suggests that the percent of patients testing positive (a mass spectrometer confirmation positive result with matching prescription) is closer to 80% for those prescribed haloperidol compared to an earlier report of 63.5% (4). It should also be noted that without hydrolysis this set of 151 patients is ~50% positive showing the impact of hydrolyzing haloperidol on the reduction of false negatives.

A small sample of each dose type reveals the data presented in Table VI, wherein patients using the injectable version of haloperidol (i.e., haloperidol solution and haloperidol decanoate derivative solution) exhibit the highest correlation of positive results consistent with route of administration, as expected. Those on tablets have a lower correlation of positive results, nevertheless still a higher correlation than the case where hydrolysis is not utilized. However, those patients taking the oral solution have the lowest correlation of positive consistent with medication rate indicating a possibly high non-adherence rate among this population.

Analysis of a data set comprised of historical samples prior to hydrolysis implementation is shown in Table VI and suggests that without hydrolysis, a significant number of "false negatives" are observed no matter what the dosage pathway. The impact of hydrolysis for adequate detection of this drug is demonstrated in Table VI where the injectable haloperidol solution is detected in 91% of patients tested and the injectable haloperidol decanoate which is usually administered because of its extended effect (2) is detected in 100% of the patients tested.

Conclusion

Overall, these data demonstrate that hydrolysis before analysis of haloperidol in urine samples provides a greater level of sensitivity and

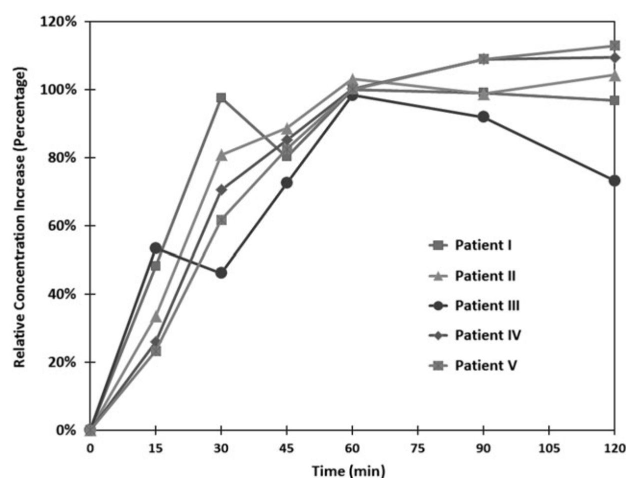

Figure 2. Results of hydrolysis time study using patient positive samples

Table V. Summary of 151 patient samples % positive results including all haloperidol dosage forms

Parameter	Pre-hydrolysis	Post-hydrolysis
Number of specimens	151	151
Minimum concentration (ng/mL)	<5.0	<5.0
Maximum concentration (ng/mL)	414.0	16,805.6
Mean	30.7	982.5
Standard deviation	66.7	1,907.3
Total positive	75	119
Total negative	76	32
% Positive	49.67	78.81

Table VI. Impact of hydrolysis on % positive patients by dosage form

Dose type	Pre-hydrolysis				Post-hydrolysis			
	Negative	Positive	% Positive	Failed SVT[a]	Negative	Positive	% Positive	Failed SVT[a]
Injectable Haloperidol Decanoate	17/53	36/53	68%	3	0/20	20/20	100	2
Injectable Haloperidol Solution	59/178	119/178	67%	14	3/35	32/35	91	3
Haloperidol Tablets	284/646	362/646	56%	45	26/181	155/181	86	8
Haloperidol Oral Solution	N/A	N/A	N/A	N/A	73/106	33/106	31	3

[a]Specimen validity testing (acceptable pH range 4.5–9.0, acceptable specific gravity range 1.003–1.040, acceptable creatinine concentration range 5–400 mg/dL, and no oxidant(s) present).

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consistency among subjects on haloperidol therapy, and therefore provides a superior urine analyte for evaluation of a subject's potential adherence with a haloperidol therapeutic regimen. The presence of the glucuronidated form of haloperidol in urine is significant, consistent with earlier reports about haloperidol metabolism in plasma, and represents an improved way to monitor potential adherence to this drug.

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