A Simple High-Performance Liquid Chromatography Assay for the Major Cisapride Metabolite, Norcisapride, in Human Urine

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Abstract
A simple high-performance liquid chromatography assay using fluorescence detection for the major metabolite of the gastric prokinetic drug cisapride, norcisapride, is presented. Analysis is performed using an Alltech Platinum EPS C8 column with a mobile phase made up of methanol and 0.02M sodium dihydrgen phosphate (45:55, v/v) containing triethylamine (1 g/L). Complete resolution is achieved among norcisapride, the internal standard (metoclopramide), and endogenous urinary components. The assay is linear over the range 50–2000 ng/mL with a mean recovery of 71.2% across the analytical range following solvent extraction with toluene–isoamyl alcohol (95:5, v/v). Intraday coefficients of variation (precision) determined at 200 and 1000 ng/mL are 6.0 and 9.8%, respectively, and interday coefficients of variation are 8.8 and 6.6%, respectively. Intra- and interassay accuracy (as mean relative error) determined at the same concentrations is within 10% in all cases. An analysis of urine samples from a healthy volunteer following the administration of a single 10-mg oral dose of cisapride is shown.

Introduction

Cisapride undergoes extensive metabolism in humans and dogs, and at least 30 metabolites have been identified following its administration to rats (4). Following oral dosing in humans, the renal excretion of cisapride is extremely low (0.2% of the dose) (5), rendering its detection by conventional chromatographic analysis difficult. In contrast, excretion of the major metabolite, norcisapride (Figure 1), which is produced via oxidative N-dealkylation at the piperidine nitrogen, accounts for 41–45% of the initial dose (5). Norcisapride has only one-sixth the pharmacological activity of cisapride (6).

A reliable assay for urinary norcisapride was required by our department as part of an investigation into the disposition of cisapride and the possible inhibition of its metabolism. No previous high-performance liquid chromatography (HPLC) assays for norcisapride in urine could be found in the literature. Gladziwa et al. (6) used an unpublished gas chromatography method for the analysis of norcisapride in plasma, urine, and dialysate, and the retention time of norcisapride was identified in the radio-HPLC method used in the cisapride disposition studies by Meuldermans et al. (4,5). In addition, Preechagoon and Charles (7) found that norcisapride eluted in 2 min with the solvent front in their HPLC method for cisapride in neonatal plasma.

This paper describes a simple HPLC assay for urinary norcisapride requiring only 0.5-ml samples, and the method has a linearity of response between 50 and 2000 ng/mL.

Experimental

Chemicals
Norcisapride was supplied by Janssen-Cilag Pty. Ltd. (Sydney, Australia), and metoclopramide was a gift from Beecham Laboratories (Melbourne, Australia). Methanol and toluene (HPLC grade) were obtained from Mallinkrodt (Paris, KY), triethylamine

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hydrochloride was purchased from Sigma Chemical Company (St. Louis, MO), and isoamyl alcohol was obtained from Ajax Chemicals (Sydney, Australia). All chemicals were of analytical reagent grade or higher and were used without further purification.

**Apparatus**

The HPLC system consisted of a Waters (Sydney, Australia) model 510 pump combined with an ETP Kortec (Sydney, Australia) model K65B automated sample injector and a Hitachi (Tokyo, Japan) model F1000 fluorescence spectrophotometer operated at 295 nm (excitation) and 300 nm (emission). Separation was performed at ambient temperature on a Platinum EPS C_3 column (150 mm x 4.6-mm i.d., 5-µm particle size, Alltech Associates, Sydney, Australia) with a mobile phase flow rate of 1.3 mL/min. Data was recorded and analyzed using Millenium 2020 chromatography software (Waters).

**Mobile phase**

The mobile phase consisted of methanol and 0.02M sodium dihydrogen phosphate (45:55, v/v) containing triethylamine (1 g/L). The final pH of the mobile phase was adjusted to 7.0 using 1.0M sodium hydroxide, and the solution was filtered under vacuum through a 0.45-µm membrane (Millipore, Sydney, Australia).

**Standard solutions**

Stock solutions of norcisapride (10 µg/mL) and metoclopramide (2 mg/mL) were prepared in methanol. The stock solutions were stored in amber glass vials at –20°C and appeared to be stable for at least 2 months. Appropriate dilutions of the norcisapride stock solution were made in methanol and stored at 4°C. Urinary standards were prepared by the addition of aliquots (100 µL) of the diluted standards to 15-mL borosilicate glass tubes followed by the removal of the solvent under a gentle stream of air at 35°C. The residue was reconstituted in mobile phase (100 µL) of the diluted standards to 15-mL borosilicate glass tubes followed by the removal of the solvent under a gentle stream of air at 35°C. Drug-free urine (0.5 mL) was added to the tubes to provide final norcisapride concentrations of 50, 100, 200, 500, 1000, and 2000 ng/mL. A working solution of metoclopramide (2 µg/mL) was prepared in water and stored at 4°C. The final pH of the mobile phase was adjusted to 7.0 using 1.0M sodium hydroxide, and the solution was filtered under vacuum through a 0.45-µm membrane (Millipore, Sydney, Australia).

**Sample collection**

Urine samples were collected immediately prior to dosing and over 12 h intervals for 36 h following the administration of a 10-mg tablet of cisapride (Prepulsid, Jannsen-Cilag) to a healthy adult volunteer. Samples were stored at –20°C until analysis.

**Sample assay**

Urine samples (0.5 mL) were pipetted into 15-mL borosilicate glass tubes with teflon-lined caps and alkalized with sodium hydroxide (1.0M, 100 µL). Metoclopramide working solution (100 µL) was added to each tube and vortex mixed for 15 s. Toluene–isoamyl alcohol (95:5, v/v, 2 mL) was then added, and the tubes were capped and rotary mixed for 5 min. Following centrifugation at 2000 x g for 5 min, the organic layer was transferred to a clean tube, and the solvent was evaporated under a gentle stream of air at 35°C. The residue was reconstituted in mobile phase (100 µL) with vortex mixing, and a 50-µL aliquot was injected into the HPLC. Standards (prepared as previously described) and appropriate blanks were assayed in an identical manner.

**Assay validation**

Linearity of the assay was demonstrated on 6 separate occasions by processing urine standards at the 7 concentrations over the range 50–2000 ng/mL listed previously. Peak-area ratios (norcisapride/metoclopramide) were plotted against the norcisapride concentration and analyzed using weighted (1/concentration) least-squares linear regression. Interassay precision and accuracy were determined over 5 separate occasions using duplicate spiked urine samples at 2 concentrations (nominally 200 and 1000 ng/mL), whereas 8 replicates at the same nominal concentrations were analyzed on a separate occasion to determine intra-assay results. Precision was assessed in terms of the relative standard deviation of the measured concentrations in a replicate set, and accuracy was determined from the mean relative error in a replicate set (i.e., the difference between measured and nominal concentrations of the spiked samples).

The recovery of norcisapride from urine was assessed by comparing the slopes of the calibration curves for norcisapride from extracted samples versus non-extracted samples at the nominal concentrations of 50, 200, and 1000 ng/mL. All samples were assayed in triplicate, and the internal standard was not extracted in both sample sets. The specificity in relation to endogenous compounds was demonstrated by the analysis of a series of randomly selected drug-free urine samples (n = 10).

**Results and Discussion**

Figure 2 shows the chromatograms obtained following the analysis of drug-free urine, drug-free urine containing norcisapride (500 ng/mL) and metoclopramide (400 ng/mL), and urine containing 605 ng/mL norcisapride from a sample collected between 12 and 24 h following a single 10-mg dose of cisapride to a healthy adult volunteer. The retention times for norcisapride and metoclopramide were approximately 9.8 and 12.5 min, respectively. Under the chromatographic conditions described, complete resolution was achieved between norcisapride and metoclopramide and between these analytes and the endogenous compounds in the urine samples. The use of acetonitrile as the
The organic component of the mobile phase resulted in the coelution of norcisapride and metoclopramide, although this combined peak was still well resolved from the endogenous urine components.

Metoclopramide proved to be a suitable internal standard because of its similar fluorescence properties and elution characteristics. The use of metoclopramide as an internal standard posed no problems in these disposition studies because they were conducted in healthy, nonmedicated volunteers. Care would need to be taken to exclude co-medication with metoclopramide should this assay be used to measure urinary norcisapride from a population of treated patients.

The assay was linear for norcisapride between 50 and 2000 ng/mL, with a typical calibration curve over this range producing a regression of \( y = 0.0014198x - 0.03504 \) with a correlation coefficient \( r^2 \) of 0.9994 (where \( y \) is the peak area ratio and \( x \) is the concentration of analyte). This range was found to be adequate for the concentrations observed from the analysis of urine samples following a 10-mg dose of cisapride to adult volunteers (data presented elsewhere).

Intrabatch precision and accuracy were evaluated from assays of spiked samples (\( n = 8 \)) at three concentrations (Table I). Interbatch precision and accuracy were assessed from assays of duplicate samples analyzed on 5 separate occasions (Table II); both precision and accuracy were within 9%.

The limit of quantitation (LOQ), defined as the lowest concentration at which both accuracy and precision are within 20%, was deemed to be 50 ng/mL during the use of this assay for clinical studies. The data obtained for triplicate 50-ng/mL standards measured on 6 separate occasions (precision, 3.1%; accuracy, 4.8%) provided results that easily satisfied these criteria, indicating that a somewhat lower LOQ may have been achievable if required.

The mixture of toluene and isoamyl alcohol (95:5, v/v) used to extract the norcisapride from urine provided an extraction efficiency of 71.2% across the analytical range. Relatively clean extracts were generated with the use of this solvent mixture with no interfering peaks in the area of elution of norcisapride and metoclopramide observed from the analysis of 10 drug-free urine samples. The use of this solvent mixture obviated the use of benzene (with its associated problems) as used by Meuldermans et al. (5). Toluene without the added isoamyl alcohol provided a lower extraction efficiency for norcisapride, and the mixture of 2-propranolol (10%, v) in chloroform utilized by Preechagoon and Charles for the extraction of cisapride from plasma (7) proved unsuitable because of the coextraction of a large number of endogenous compounds from urine.

**Conclusion**

A simple and reliable HPLC method for the analysis of norcisapride in urine samples using fluorescence detection has been described. The assay provided adequate recovery with good precision and accuracy. Norcisapride eluted with good resolution and separation from the endogenous components. The method has been used in our laboratory for the analysis of urinary norcisapride in samples collected from a number of healthy volunteers following the oral administration of cisapride.

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**Table I. Intrabatch Precision and Accuracy for the Assay of Norcisapride**

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Measured concentration (ng/mL)</th>
<th>Precision (%)(^*)</th>
<th>Accuracy (%)(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>185.2</td>
<td>6.0</td>
<td>7.2</td>
</tr>
<tr>
<td>1000</td>
<td>999.8</td>
<td>9.8</td>
<td>7.6</td>
</tr>
</tbody>
</table>

\(^*\) Assessed in terms of the relative standard deviation of the measured concentrations in a replicate set (\( n = 8 \)).

\(^*\) Determined from the mean relative error in a replicate set (\( n = 8 \)).

**Table II. Interbatch Precision and Accuracy for the Assay of Norcisapride**

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Measured concentration (ng/mL)</th>
<th>Precision (%)(^*)</th>
<th>Accuracy (%)(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>200.3</td>
<td>8.8</td>
<td>7.0</td>
</tr>
<tr>
<td>1000</td>
<td>990.9</td>
<td>6.6</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\(^*\) Assessed in terms of the relative standard deviation of the measured concentrations in 5 duplicate sets (\( n = 10 \)).

\(^*\) Determined from the mean relative error in 5 duplicate sets (\( n = 10 \)).
References


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