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# Extended application of an LC–MS/MS method for the analysis of vesnarinone and its metabolites in human urine and dialysate fluid

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**Abstract**

Vesnarinone, a positive inotropic drug developed for congestive heart failure, and its metabolites (OPC-8230, OPC-18136, OPC-18137) were analyzed in human dialysate and urine (plus an additional metabolite: OPC-18692 in urine) samples using a modification to a previously published LC–MS/MS assay for the analysis of human plasma and urine samples. OPC-8192, a structural analogue of vesnarinone, was used as the internal standard. The analytes of interest were extracted from human dialysate or urine by a solid phase extraction method using a pre-conditioned C-18 extraction column. The analytes were then resolved by a 7 min gradient elution on a reverse phase high performance liquid chromatographic column. Vesnarinone and metabolites were detected on a PE/Sciex API III+Biomolecular Mass analyzer in MS/MS mode using a Turbo IonSpray interface. The linear range of quantitation in dialysate was 2.00–100.00 ng/ml for vesnarinone and 0.50–25.00 ng/ml for each metabolite. In urine, the linear range was of 0.50–25.00 μg/ml for vesnarinone and 0.10–5.00 μg/ml for the metabolites. This method was used to support the analysis of urine and dialysate samples from renally impaired patients who are on vesnarinone treatment.


*Keywords:* LC–MS/MS; Vesnarinone; Metabolites; Human dialysate; Precursor ion; Product ion

## 1. Introduction

Vesnarinone (3,4-dihydro-6-[4-3,4-dimethoxybenzoyl)-1-piperazinyl]-2(1H)-quinolinone) is a positive inotropic drug [1]. Vesnarinone can form several metabolites either by the cleavage of the piperazine moiety at the amide bond or by hydroxylation of the piperazinyl ring [2]. A High Performance Liquid Chromatographic (HPLC) method was first developed for measuring vesnarinone and its metabolites quantitatively [3,4]. Subsequently, a more specific and sensitive method [5] was developed for measuring vesnarinone and its metabolites. The concentrations of vesnarinone and its metabolites were expected to be lower in dialysate than in human plasma.

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Therefore, a more sensitive method was required for measuring vesnarinone and its metabolites in dialysate samples. The existing Liquid Chromatographic–Mass Spectrometric/Mass Spectrometric (LC–MS/MS) method was modified for measuring vesnarinone and metabolites OPC-8230, OPC-18136, and OPC-18137, in human dialysate samples. In addition, the urine method was modified to include OPC-18692, a newly isolated metabolite of vesnarinone.

Two independent HPLC methods were developed for measuring vesnarinone and its metabolites in plasma. The first method analyzed vesnarinone, OPC-8230 and OPC-18136 using gradient elution chromatography with fluorescent detection. In the second method vesnarinone and OPC-18137 were measured using isocratic elution chromatography with ultraviolet detection. A third HPLC method was developed for measuring vesnarinone and six metabolites (OPC-8230, OPC-18136, OPC-18137, OPC-8931, OPC-8982, and OPC-1533) in urine. These methods were not useful for routine sample analysis, due to their long analytical run times and interferences from co-administered drugs and endogenous substances. Therefore, a new LC–MS/MS method was developed for measuring vesnarinone and three primary circulating metabolites (OPC-18136, OPC-18137, and OPC-8230) in plasma and urine [5]. In order to measure vesnarinone and its metabolites (OPC-18136, and OPC-18137, OPC-8230) in dialysate samples the LC–MS/MS method was improved to achieve higher sensitivity. For measuring an additional analyte in urine the method was modified to include the additional analyte (OPC-18692). OPC-8192, a structural analogue of vesnarinone was used as the internal standard.

## 2. Experimental

### 2.1. Materials

All reagents used were of analytical or HPLC grade except where noted. All solvents and analytical reagents were supplied by standard suppliers such as Fisher Scientific, Inc., (Fair Lawn, NJ 07140), Baxter Scientific Products Division (1118, Clay St., North Kansas City, Missouri 64116) or JT Baker, Inc., (Philipsburg, NJ 08865). Vesnarinone, OPC-18136, OPC-18137, OPC-8230, and OPC-8192 (Internal Standard) were synthesized and provided by Otsuka Pharmaceutical Co. Ltd., 224-18, Ebisuno, Hirashi, Kawauchi-Cho, Tokushima, Japan.

### 2.2. Instrumentation

A Shimadzu LC-10AD (or equivalent) pump, with a Varian Star 9100 injector and a Shimadzu SCL-10A controller along with a Beckman Ultrasphere ODS (4.6, 45 mm, 5 μ) (or equivalent) column were used for the HPLC system for sample analysis. A PE/Sciex API III+Analyzer with Turbo IonSpray interface was used as the Mass spectral Detector.

### 2.3. Preparation of standard solutions

#### 2.3.1. Dialysate

Primary solutions for vesnarinone, OPC-8230, OPC-18136, OPC-18137 and OPC-8192 were prepared in methanol. To prevent degradation, 1.0 ml of 10 mM L-ascorbic acid solution prepared in methanol was added while making up the total volume of the primary solutions. All primary solutions were 300 μg/ml except for vesnarinone at 1000 μg/ml and OPC-8192 at 100 μg/ml. Serial dilutions of primary stock solutions were used to generate a calibration curve from 0.50 to 25.00 ng/ml for the metabolites and 2.00–10.00 ng/ml for vesnarinone. All stock solutions were protected from Ultra violet (UV) light exposure and processed under subdued light. The primary and secondary stock solutions were stored in amber bottles at 4 °C for a maximum of 3 months.

#### 2.3.2. Urine

Primary solutions in for vesnarinone, OPC-8230, OPC-18136, OPC-18137, OPC-18692 and OPC-8192 were prepared in methanol. To prevent degradation 1.0 ml of 10 mM L-ascorbic acid solution prepared in methanol was added while making up the total volume of the primary solutions. All primary solutions were 400 μg/ml

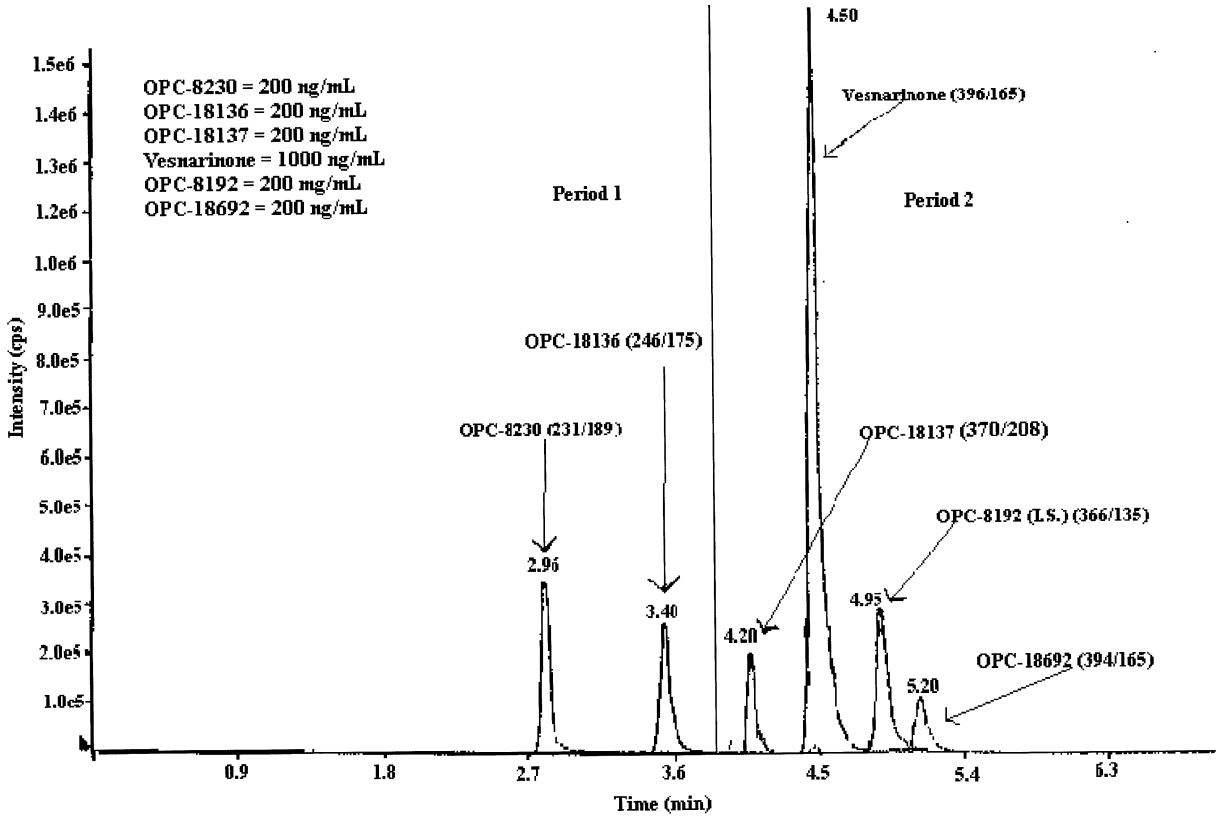


Fig. 1. Overlaid total product ion chromatograms for vesnarinone, metabolites and internal standard.

Table 1
Time program for chromatography

| Dialysate | | Urine | | Volume (ml/%) |
|---|---|---|---|---|
| Time (min) | Flow | Time (min) | Flow | |
| 0.01 | T.FLOW | 0.01 | T.FLOW | 0.3 |
| 0.01 | B.CONC | 0.01 | B.CONC | 0.0 |
| 3.00 | B.CONC | 3.10 | B.CONC | 45.0 |
| 3.65 | T.FLOW | 3.80 | T.FLOW | 0.3 |
| 3.70 | T.FLOW | 3.85 | T.FLOW | 0.5 |
| 4.50 | B.CONC | 4.50 | B.CONC | 45.0 |
| 4.55 | B.CONC | 4.55 | B.CONC | 100.0 |
| 5.50 | B.CONC | 6.00 | B.CONC | 100.0 |
| 5.55 | B.CONC | 6.05 | B.CONC | 0.0 |
| 6.20 | T.FLOW | 6.20 | T.FLOW | 0.5 |
| 6.25 | T.FLOW | 6.25 | T.FLOW | 0.3 |
| 7.00 | STOP | 7.00 | STOP | |

Table 2
Regression parameters for each analyte in human dialysate samples

| Analyte | Linearity range (ng/ml) | Mean slope | Mean intercept | Mean correlation coefficient |
|---|---|---|---|---|
| Vesnarinone | 2.00–100.00 | 106.13 | −0.929 | 0.999 |
| OPC-8230 | 0.50–25.00 | 229.04 | −0.336 | 0.997 |
| OPC-18136 | 0.50–25.00 | 398.47 | −0.155 | 0.997 |
| OPC-18137 | 0.50–25.00 | 158.74 | −0.157 | 0.998 |

except for vesnarinone at 1000 μg/ml and OPC-8192 at 100 μg/ml. Serial dilutions of primary stock solutions were used to generate a calibration curve from 0.10 to 5.0 μg/ml for the metabolites and 0.50 to 25μg/ml for vesnarinone.

### 2.4. Chromatographic conditions

An aqueous mobile phase stock solution was prepared by pipetting 1.0 ml of glacial acetic acid into a 1000 ml volumetric flask containing 200 ml of deionized water and diluted to volume with more deionized water. Mobile Phase 'A' (2.5% acetonitrile/97.5% aqueous) was prepared using 25 ml of acetonitrile diluted to 1000 ml with mobile phase aqueous solution. Mobile phase 'B' (80% acetonitrile/20% aqueous) was prepared using 800 ml of acetonitrile diluted to 1000 ml with mobile phase aqueous solution.

The gradient elution mobile phase consisted of varying proportions of mobile phase 'A' and mobile phase 'B'. A 7 min gradient elution was used to separate the analytes (A total ion chromatogram is shown in Fig. 1). The time program used for dialysate and urine methods are given in Table 1.

### 2.5. Sample preparation

Dialysate (500 μl) or urine (50 μl) sample was pipetted into a culture tube and 50 μl of internal standard solution (OPC-8192) was added. To this mixture, 1 ml of 50 mM ammonium acetate solution was added and the tube was covered and vortexed. A UCT, C18, (200 mg, 3 ml) solid-phase extraction column was pre-conditioned using 2 ml of methanol, 2 ml of 10 mM L-ascorbic acid and 2 ml of 50 mM ammonium acetate. The sample was then applied to the column. The column was washed with 2.0 ml of 50 mM ammonium acetate and 2 ml of *n*-butyl chloride. A vacuum of $\sim 5$ psi was applied for a minute to dry the column. Vesnarinone, its metabolites, and the internal standard were eluted with $3 \times 1.0$ ml 6 N acetic acid/10 mM L-ascorbic acid/methanol (2/3/95) solution. A vacuum was again applied for 30 s to remove all of the solvent from the column. The eluant was evaporated under nitrogen and reconstituted in 100 μl of mobile phase 'A' for dialysate samples or reconstituted in 1.0 ml for urine samples and vortex mixed for 30 s. The sample was transferred to an amber autosampler vial and 10 μl of the sample was injected onto the HPLC column.

### 2.6. LC–MS/MS analysis

A Perkin Elmer/Sciex API III+ was used for the mass spectral analysis. The spectrometer was equipped with a Turbo Ionspray interface in the MS/MS mode. Liquid nitrogen was used as the nebulizer and auxiliary gas, ultra high purity nitrogen and argon were used as curtain and collision gas, respectively. The analytes were identified and quantified based on their retention time from the HPLC system and their response in the mass spectrometer (Table 2). System calibration for the analytes was established by weighted regression ($1/x$) of the peak area ratio (analyte/internal standard) versus the concentration of analytes in the calibration standards.

### 2.7. Acceptance criteria for the validation

The acceptance criteria for the calibration standards are that the error relative to nominal

Table 3
Regression parameters for each analyte in human urine samples

| Analyte | Linearity range (μg/ml) | Mean slope | Mean intercept | Mean correlation coefficient |
|---|---|---|---|---|
| Vesnarinone | 0.50–25.00 | 1.24 | −0.217 | 0.997 |
| OPC-8230 | 0.10–5.00 | 1.15 | −0.015 | 0.997 |
| OPC-18136 | 0.10–5.00 | 1.27 | −0.10 | 0.998 |
| OPC-18137 | 0.10–5.00 | 2.42 | −0.022 | 0.998 |
| OPC-18692 | 0.10–5.00 | 2.20 | 0.039 | 0.996 |

value will be no more than 15%, except at lower limit of quantitation (LOQ), where $\leq 20\%$ is acceptable. The precision value will be limited to a %coefficient of variation (%CV) of 15%, except at LOQ where $\leq 20\%$ is acceptable. Accuracy of calculated concentrations for QC samples will be limited to $\leq 15\%$ error relative to nominal. The precision limit for QC sample will be %CV of $\leq$ 15%. All QC sample concentration values within the precision and accuracy of the method will be reported and included in the descriptive statistics.

## 3. Results

### 3.1. Dialysate

The LOQ was established as 0.50 ng/ml for OPC-8230, OPC-18136, and OPC-18137 and at 2.00 ng/ml for vesnarinone in dialysate samples. The linear range for the metabolites in urine was 0.10–5.0 μg/ml, and 0.50–25 μg/ml for vesnarinone using 50 μL of human urine. Three standard curves provided correlation coefficients greater

Table 4
Precision, accuracy and sensitivity for each analyte in human dialysate

| Analyte | Statistical variable | Within batch precision and accuracy | | | | Among batch precision and accuracy | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | A | B | C | D |
| Vesnarinone | *N* | 6 | 14 | 14 | 14 | 6 | 14 | 14 | 14 |
| | Mean | 1.86 | 3.58–3.74 | 34.48–37.2 | 83.29–89.26 | 1.86 | 3.7 | 35.0 | 86.23 |
| | %CV | 11.8 | 5.0–16.0 | 0.4–4.1 | 1.8–8.0 | 11.8 | 10.8 | 3.9 | 6.3 |
| | %Accuracy | 93.0 | 89.5–93.5 | 104.5–112.7 | 101.6–108.9 | 93.0 | 92.5 | 106.1 | 105.2 |
| OPC-8230 | *N* | 6 | 14 | 14 | 14 | 6 | 14 | 14 | 14 |
| | Mean | 0.46 | 0.99–1.0 | 8.98–9.48 | 21.90–23.4 | 0.46 | 0.99 | 9.08 | 22.61 |
| | %CV | 4.3 | 4.0–21.0 | 4.2–17.0 | 4.3–12.8 | 4.3 | 9.1 | 7.0 | 6.3 |
| | %Accuracy | 92.0 | 99.0–100.0 | 108.8–114.9 | 106.8–114.1 | 92.0 | 99.0 | 110.1 | 110.3 |
| OPC-18136 | *N* | 6 | 14 | 14 | 13 | 6 | 14 | 14 | 13 |
| | Mean | 0.53 | 0.99–1.07 | 8.70–9.45 | 22.04–23.1 | 0.53 | 1.03 | 9.0 | 22.56 |
| | %CV | 5.7 | 5.8–13.1 | 6.2–15.6 | 2.9–16.7 | 5.7 | 10.7 | 9.2 | 8.2 |
| | %Accuracy | 106.0 | 99.0–107.0 | 105.5–114.5 | 107.5–112.7 | 106.0 | 103.0 | 109.1 | 110.0 |
| OPC-18137 | *N* | 6 | 14 | 14 | 14 | 6 | 14 | 14 | 14 |
| | Mean | 0.54 | 0.88–1.01 | 8.29–8.61 | 20.04–21.61 | 0.54 | 0.94 | 8.45 | 20.46 |
| | %CV | 14.8 | 3.4–12.4 | 0.9–5.3 | 1.9–13.0 | 14.8 | 10.6 | 4.3 | 6.5 |
| | %Accuracy | 108.0 | 88.0–101.0 | 100.5–104.4 | 97.8–105.4 | 108.0 | 94.0 | 102.4 | 99.8 |

(A) 0.5 ng/ml for OPC-8230, OPC-18136 and OPC-18137 and 2.0 ng/ml for vesnarinone. (B) 1.0 ng/ml for OPC-8230, OPC-18136 and OPC-18137 and 4.0 ng/ml for vesnarinone. (C) 8.25 ng/ml for OPC-8230, OPC-18136, and OPC-18137, and 33.0 ng/ml for vesnarinone. (D) 20.0 ng/ml for OPC-8230, OPC-18136, and OPC-18137, and 822.0 ng/ml for vesnarinone.

Table 5
Precision, accuracy and sensitivity for each analyte in human urine

| Analyte | Statistical variable | Within batch precision and accuracy | | | | Among batch precision and accuracy | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | A | B | C | D |
| Vesnarinone | *N* | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| | Mean | 0.41–0.47 | 1.25–1.27 | 12.01–12.05 | 18.65–19.21 | 0.45 | 1.26 | 12.03 | 18.86 |
| | %CV | 8.5–17.4 | 4.8–10.2 | 3.6–5.2 | 3.2–5.3 | 13.3 | 7.1 | 4.2 | 4.2 |
| | %Accuracy | 82.0–94.0 | 104.2–105.8 | 100.1–100.4 | 93.3–96.1 | 90.0 | 105.0 | 100.3 | 94.3 |
| OPC-8230 | *N* | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| | Mean | 0.09–0.11 | 0.24–0.25 | 2.43–2.53 | 3.94–3.99 | 0.10 | 0.24 | 2.47 | 3.96 |
| | %CV | 10.0–18.2 | 4.2–12.5 | 1.6–7.9 | 3.5–5.1 | 20.0 | 8.3 | 5.7 | 4.3 |
| | %Accuracy | 90.0–110.0 | 100.0–104.2 | 101.3–105.4 | 98.5–98.8 | 100.0 | 100.0 | 102.9 | 99.0 |
| OPC-18136 | *N* | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| | Mean | 0.09–0.11 | 0.24–0.25 | 2.36–2.39 | 3.92–3.94 | 0.10 | 0.24 | 2.37 | 3.93 |
| | %CV | 10.0–18.2 | 4.2–8.3 | 3.0–5.4 | 2.3–5.4 | 20.0 | 8.3 | 3.8 | 4.3 |
| | %Accuracy | 90.0–110.0 | 100.0–104.2 | 98.3–99.6 | 98.0–98.5 | 100.0 | 100.0 | 98.8 | 98.3 |
| OPC-18137 | *N* | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| | Mean | 0.09–0.11 | 0.24–0.27 | 2.65–2.72 | 4.33–4.53 | 0.11 | 0.26 | 2.69 | 4.46 |
| | %CV | 11.1–18.2 | 3.7–20.8 | 4.0–7.5 | 3.5–7.2 | 18.2 | 11.5 | 5.2 | 5.6 |
| | %Accuracy | 90.0–120.0 | 100.0–108.3 | 110.4–113.3 | 108.3–113.3 | 110.0 | 108.3 | 112.1 | 111.5 |
| OPC-18692 | *N* | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| | Mean | 0.11–9.1 | 0.23–0.27 | 2.58–2.82 | 4.62–4.86 | 0.12 | 0.25 | 2.67 | 4.77 |
| | %CV | 7.1–9.1 | 7.4–11.1 | 5.3–13.6 | 5.6–7.6 | 16.7 | 12.0 | 9.7 | 6.9 |
| | %Accuracy | 110.0–140.0 | 95.8–112.5 | 107.5–117.5 | 115.5–121.5 | 120.0 | 104.2 | 111.3 | 119.3 |

(A) 0.1 μg/ml for OPC-8230, OPC-18136, OPC-18137 and OPC-18692, and 0.5 μg/ml for vesnarinone. (B) 0.24 μg/ml for OPC-8230, OPC-18136, OPC-18137 and OPC-18692, and 1.2 μg/ml for vesnarinone. (C) 2.4 μg/ml for OPC-8230, OPC-18136, OPC-18137, and OPC-18692, and 12.0 μg/ml for vesnarinone. (D) 4.0 μg/ml for OPC-8230, OPC-18136, OPC-18137, and OPC-18692, and 20.0 μg/ml for vesnarinone.

than or equal to 0.995 for all analytes. The mean regression parameters for vesnarinone and metabolites in human dialysate and urine samples are given in Table 2. The day-to-day repeatability of the method is shown by the performance of the QC samples. Freeze-thaw stability of dialysate samples was investigated over three cycles using QC samples (Table 4). The relative recoveries (accuracy) ranged from 101.9 to 116.1% with %CVs ranging from 0.5 to 6.8. The high recovery was not seen in all samples and therefore it was speculated that it was an anomaly during sample preparation. Dilution of the samples 10 times did not affect accuracy or repeatability, thus allowing dilution of samples that were above quantifiable limits. QC samples had a percent coefficient of variation (%CV) between 0.4 and 16.0% for vesnarinone and between 0.9 and 21% for the metabolites within batch. Similarly, the among batch %CV ranged between 3.9 and 11.8% for vesnarinone and between 4.3 and 14.8% for the metabolites. Interferences from endogenous substances were not a factor in this method.

### 3.2. Urine

The published method for analysis of vesnarinone and metabolites has been extended to include a new metabolite, OPC-18692. The LOQ was established as 0.10 μg/ml OPC-8230, OPC-18136, OPC-18137, and OPC-18692 and at 0.50 μg/ml for vesnarinone. The linear range for the metabolites in urine was 0.10–5.0 μg/ml, and 0.50–25 μg/ml for vesnarinone using 50 μl of human urine. Five standard curves run over five days had correlation coefficients of 0.996 or greater. The mean regression parameters for vesnarinone and metabolites in human urine

samples are given in Table 3. The QC samples provided evidence for the repeatability of the method (Table 5). Dilution of the QC samples by 10-fold did not affect the accuracy or precision. In addition, three freeze-thaw cycles did not affect the stability of the analytes in urine as seen by the relative recoveries (85.1–102.9%) and %CVs (1.3–17.5) for the samples. The samples were found stable for four hours at room temperature prior to extraction and were stable for 15 days when stored at −20 °C. The within batch %CV values for the QC samples was between 3.2 and 17.4% for vesnarinone and 1.6–20.8% for the metabolites. The among-batches %CV value for vesnarinone was between 4.2 and 13.3%, whereas for the metabolites it was between 3.8 and 20.0%. Interferences from endogenous substances were not a factor in this method.

## 4. Discussion

The current method has improved sensitivity for the metabolites (400-fold lower) and for vesnarinone (50-fold lower) in human dialysate as compared to the previous published plasma LC–MS/MS method [5]. The method developed in urine is similar to the dialysate method described except that an additional metabolite, OPC-18692, was quantified. In addition, the present method shortens the run time to 7 min compared to a 30 min run time for the previously published methods [3,4].

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