ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1999) 1037–1045

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

LC/MS/MS analysis of vesnarinone and its principal metabolites in plasma and urine

Prasad N.V. Tata [a], Karl S. Schorno [b], T. Douglas Cowart [a], Steven L. Bramer [a,*]

[a] *Department of Clinical PK/PD and Metabolism, Otsuka America Pharmaceutical, 2440 Research Boulevard, Rockville, MD 20850, USA*

[b] *Kansas City Analytical Services, 12700 Johnson Drive, Shawnee, KS 66216, USA*

Received 3 May 1998; received in revised form 1 August 1998; accepted 17 August 1998

Abstract

An LC/MS/MS assay was developed and successfully used to quantitate vesnarinone and its principal metabolites (OPC-8230, OPC-18136, and OPC-18137) in human plasma and urine. Samples were pre-treated with liquid–solid extraction followed by simultaneous monitoring of primary and daughter ions which were used for the identification and quantitation of the analytes on LC/MS/MS. This assay offers advantages of specificity, speed and greater sensitivity over the previously developed HPLC-UV assay. The lower limit of quantitation is 500 ng ml^{-1} for vesnarinone and 20 ng ml^{-1} for OPC-8230, OPC-18137, and OPC-18136 in plasma. Methodology is similar for the estimation of these analytes in urine with the lower limit of quantitation being 500 ng ml^{-1} for vesnarinone and 100 ng ml^{-1} for each metabolite. Ascorbic acid was added to stabilize the analytes from degradation. This LC/MS/MS method was developed to overcome many practical problems associated with the HPLC method. The LC/MS/MS method offers the flexibility of analyzing additional metabolites and changing the linearity range to accommodate the differences in linear range (200–10 000 ng ml^{-1} for vesnarinone and 20–1000 for metabolites) for the analytes.

Keywords: LC/MS/MS; Vesnarinone; Metabolites; Parent ion; Product ion

1. Introduction

Vesnarinone (OPC-8212) is an orally active positive inotropic agent with moderate vasodilatory effect. It was reported to be a selective inhibitor of phosphodiesterase found in the human kidney and myocardial tissue [1]. Vesnarinone (3,4-dihydro-6-[4-3,4-dimethoxybenzoyl) -1-piperazinyl]-2(1H)-quinolinone (mol. wt. 395.45) is metabolized to at least nine biotransformation products and their conjugates: OPC-8230 (mol. wt. 231.29), OPC-18136 (mol. wt. 245.48), OPC-18137 (mol. wt. 369.42), OPC-8677 (mol. wt. 427.45), and OPC-8757 (mol. wt. 381.43), are the principal metabolites [2], and are formed either by

* Corresponding author. Tel.: +1-301-9900030, Ex. 2160; Fax: +1-301-7217160; e-mail: steveb@mocr.oapi.com.

0731-7085/99/$ - see front matter
PII: S0731-7085(98)00194-0

cleavage of the amide bond through the piperazine moiety or hydroxylation of the piperazinyl ring. Reported analytical work include HPLC methods for the quantitative estimation of vesnarinone [3,4]. For monitoring vesnarinone and trace quantities of metabolites during non clinical and clinical studies, a more sensitive assay needed to be developed and successfully validated.

Initially, the HPLC methodology was developed for measuring vesnarinone and its metabolites; OPC-8230, OPC-18136 and OPC-18137 in plasma (unpublished data, 1989). This method is somewhat complicated, requiring gradient elusion and fluorescent detection for measuring OPC-8230 and OPC-18136 using atenolol as internal standard and an isocratic elusion and ultraviolet detection for measuring vesnarinone and OPC-18136 using OPC-8192 as internal standard. Another HPLC method was developed for the detection and quantitative estimation of vesnarinone and its metabolites OPC-18136, OPC-18137, OPC-8230, OPC-8931, OPC-8982 and OPC-1533(6-HQ) in urine (unpublished data, 1990). Due to the long analytical run times and interference from the coadministered drugs and other endogenous substances while using the developed methods, a specific and rapid LC/MS/MS method was needed for measuring vesnarinone and its metabolites OPC-18136, OPC-18137 and OPC-8230 in plasma and urine. The LC/MS/MS method uses OPC-8192—a structural analogue of vesnarinone—as an internal standard.

2. Materials and method

All solvents and chemicals used were of either HPLC grade or of known analytical purity. All solvents and analytical reagents were supplied by standard suppliers such as Fisher Scientific (Fair Lawn, NJ), Baxter Scientific Products Division (McGaw Park, IL), or JT Baker (Phillipsburg, NJ). Ascorbic acid (99.9%) was purchased from Sigma (St. Louis, MO). Vesnarinone, OPC18136, OPC-8230, OPC-18137, OPC-18692 and OPC-8192 (internal standard; mol wt. 365.43), ($\sim$ 99.5% purity) were synthesized and provided by Otsuka Pharmaceutical, Tokushima, Japan.

3. Extraction of the analytes from plasma and urine

All solutions were protected from direct UV light exposure and processed under subdued light. All of the primary reference standard solutions were dissolved in methanol. Ascorbic acid was added to all of the solutions, resulting in a 400 nM final concentration of ascorbic acid.

Human plasma (100 μl) or urine (50 μl) was transferred into a culture tube to which 50 μl each methanol and internal standard (OPC-8192) solution (1000 ng ml^{-1}) was added. The mixture was then vortexed. To this mixture, 1 ml of 50 mM ammonium acetate was added, vortexed for 30 s, and centrifuged at 3000 rpm for 10 min. The contents were then subjected to a solid-phase extraction using a UCT C-18 column (200 mg, 3 ml), which was preconditioned prior to loading the samples with successive elusions of 2 ml of methanol, 10 mM of ascorbic acid, and 50 mM of ammonium acetate solutions. Onto this preconditioned solid-phase extraction columns supernatant of the centrifuged plasma or urine extracts were loaded. Initially the column was washed with 2.0 ml each of ammonium acetate (50 mM) and *n*-butyl chloride. The column was dried by applying a light vacuum to remove any previous solvents. Vesnarinone, its metabolites, and the internal standard were extracted by eluting (3×1 ml) with a solvent mixture of 6 N acetic acid–10 mM ascorbic acid–methanol (2:3:95) and all of the solvent mixture was removed from the column by applying a slight vacuum. The elute from the column was evaporated to dryness at room temperature under a thin stream of nitrogen. The residue was reconstituted with 500 μl of mobile phase and transferred into an amber colored autosampler vial.

4. LC/MS/MS analysis of plasma and urine samples

The LC system was composed of a Shimadzu LC-1OAD solvent delivery pump, a Shimadzu SCL-1OA system controller, and a Varian Star 9100 injector. This system was connected to an

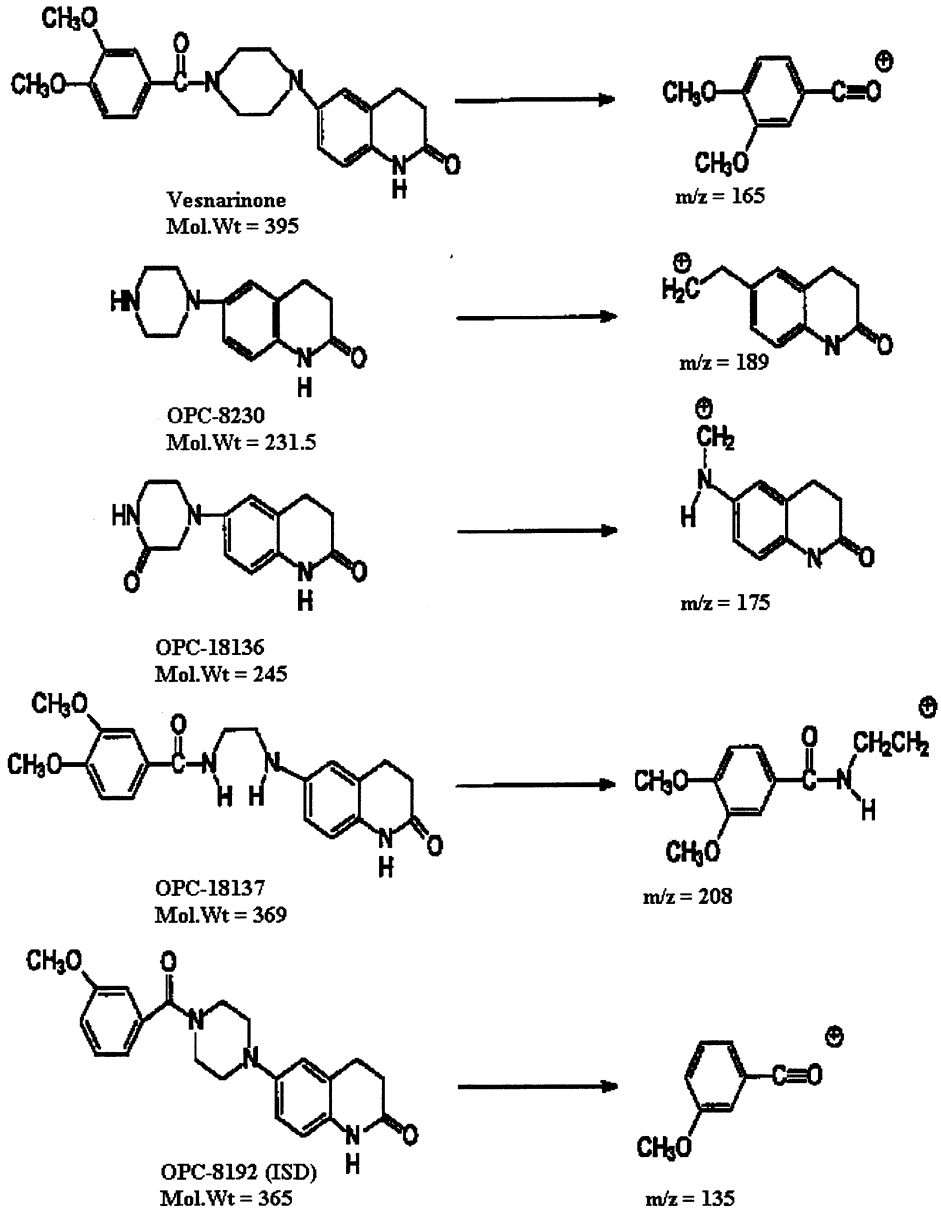

Fig. 1. Formation of parent and productions of vesnarinone and its principal metabolites.

Ultrasphere ODS (450 × 4.6 mm, 5 μ) column for separating the components and analyzed by a PE/Sciex API III + Mass spectrometer equipped with a turbo ion spray interface in MS/MS mode. Liquid nitrogen was used as a nebulizer and auxiliary gas, UHP nitrogen and UHP argon were used as curtain and collision gas. Components of the extract were separated during a 7-min gradient elusion by mobile phase A (acetonitrile: 0.1% acetic acid; 2.5:97.5) and mobile phase B (acetonitrile: 0.1% acetic acid; 80:20). The components of the eluent were identified and quantified by the retention time and response of precursor and product ions (Fig. 1; Table 1) produced in the

Table 1
Identification of vesnarinone and its metabolites by LC/MS/MS

Compound	Retention time (min)	Precursor ion	Product ion
Vesnarinone	4.50	396	165
OPC-18137	4.20	370	208
OPC-18136	3.48	246	175
OPC-8230	2.96	232	189
OPC-8192	4.95	366	135

MS/MS detector. Standard linear curves were established for each analyte by plotting a weighted regression line between 1/concentration and ratio of the response of the analyte and internal standard.

5. Results

This method is based on simultaneously monitoring the mass spectral response of parent and product ions for each analyte (Fig. 2), after establishing the identity by relative retention of each analyse in reference to the retention time of the internal standard (OPC-8192). The lower limit of quantitation for OPC-8212 is 200 ng ml^{-1} in plasma and 500 ng ml^{-1} in urine. The corresponding recovery at the lower limit of quantitation (LLOQ) is $\sim$94 and 96%, respectively. The LOQ for the metabolites is 20 ng ml^{-1} for plasma and 100 ng ml^{-1} for urine. The percentage recovery for the metabolites at LOQ was $\sim$89.9 and 100% for plasma and urine, respectively. The percentage recovery in plasma for OPC was

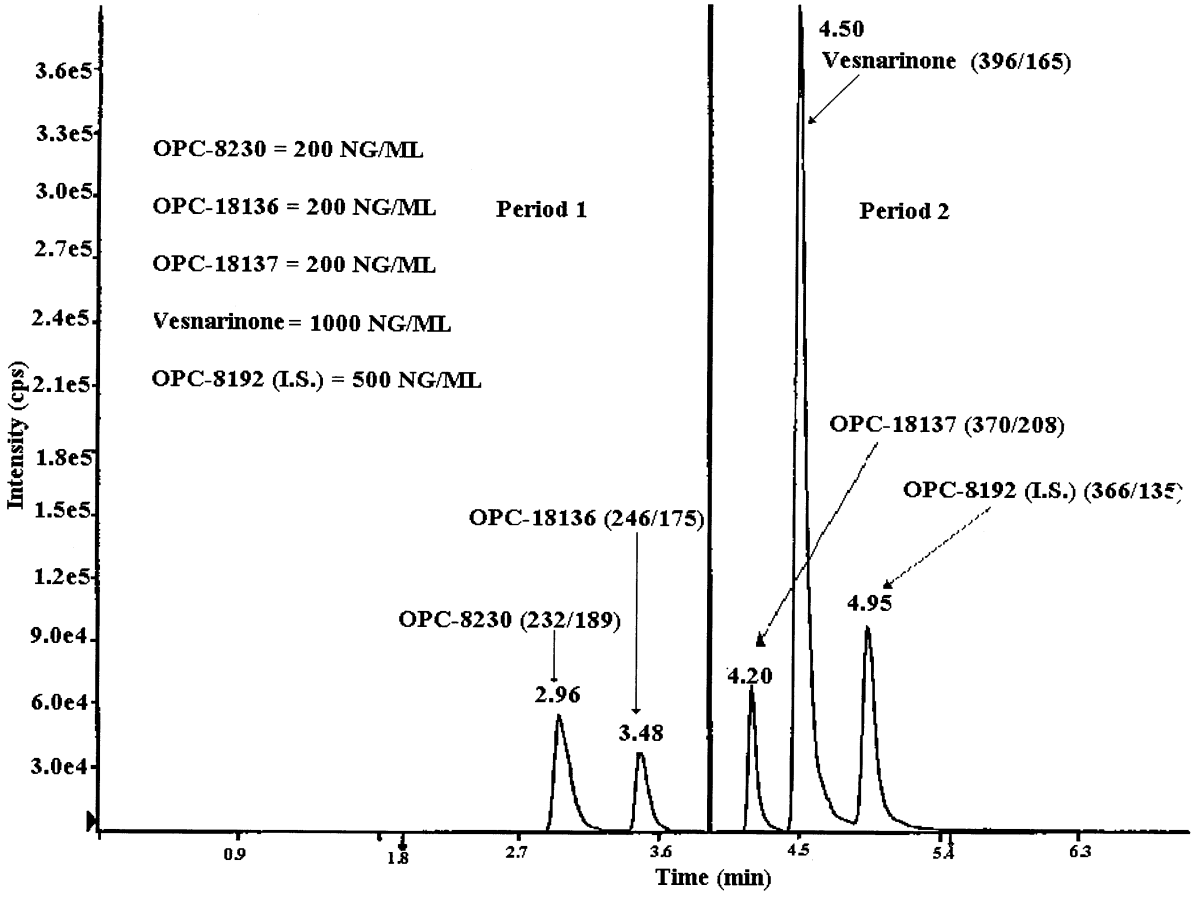

Fig. 2. Resolutions of vesnarinone and its principal metabolites.

18136, 98.35%; for OPC-18137, 101.8%; and for OPC-8230, 98.15%. In urine, the percentage recovery for all of the metabolites was 100%. The plasma assay was linear in the range 500–10 000 ng ml^{-1} of vesnarinone. Using a split standard curve, LLOQ was improved to 200 ng ml^{-1} with linear ranges of 200–5000 ng ml^{-1} (low curve) and 500–10 000 ng ml^{-1} (high curve). A linear response was observed for the metabolites OPC-18136, OPC-18137, and OPC-8230 in the range 20–1000 ng ml^{-1}. For urine samples, a linear relationship was observed in the range 100–5000 ng ml^{-1} for metabolites OPC-8230, OPC-18136, and OPC-18137. The range for OPC-8212 in urine was 500–25 000 ng ml^{-1}. The mean regression parameters for each analyte for both plasma and urine during the three day method validation is summarized in Table 2. A set of quality assurance samples were analyzed on different days to test the reproducibility and accuracy of the analytical method (Tables 3 and 4). This LC/MS/MS method was found suitable for analyzing vesnarinone and the mentioned metabolites in the presence of famotidine, AZT, distilfiram, quinidine and erythromycin. Normal endogenous substances present in plasma and urine did not interfere with the assay.

6. Discussion

The present method offers the advantage of a 7-min run time compared with the previous HPLC run time of ~30 min. The LC/MS/MS method was developed for monitoring vesnarinone, OPC-18136, OPC-18137 and OPC-8230 as they were known to be the primary circulating metabolites. The LC/MS/MS method uses identical extraction and analytical conditions for both plasma and urine. The LC/MS/MS method was developed based on the monitoring of the parent and product ions for each analyte.

This method is accurate and precise for all of the analytes in plasma and urine and as seen from the inter-day and intra-day precision, it is reproducible. All analytes were found to be stable during three free-thaw cycles. The autosampler stability of the extracted sample was found to be stable for at least 12 h. When stored below −20°C, OPC-8212, OPC-18136, OPC18137 and OPC-8230 were found to be stable in urine for a period of 5 months and in plasma, the analytes were stable for 7 months as indicated by a recovery of ~90.0%. Diluting plasma or urine sample by 2–5 fold with a blank matrix, did not show any effects on the assay values, which will allow analysis after dilution for the samples which show values greater than the quantifiable limits.

Regression statistics presented in Table 2 list variations in the correlation coefficient of vesnarinone and its metabolites in plasma ($>$ 0.997) and urine (>0.994). Day-to-day reproducibility is demonstrated by the performance of the quality control standards within-and between-batch performance as listed in Tables 3 and 4. The percent coefficient of variation for the quality control standards for the within-the-batch precision for low, mid and high levels range from 1.5 to 8.5% (plasma) and 0.6 to 14.0 (urine) for vesnarinone and 1.2–24.8% (plasma) and 0.0–20.0% (urine) for the metabolites. The percentage coefficient of variation for the quality control standards for the among-batch-precision for low, mid and high levels range from 2.6 to 5.8% (plasma) and from 1.9 to 8.5% (urine) for vesnarinone and 2.7–17.3% (plasma) and 3.3–10.3% for the metabolites. To achieve reproducibility and accurate results for both the analytes in low and high concentrations, the voltage on lens going in the collision cell (L-7) was optimized in two periods. The metabolites are present in low concentrations in the low nanogram range (20–100 ng ml^{-1}) and the parent drug is present in a high nanogram range (5000–20 000 ng ml^{-1}). The two metabolites (OPC-8230 and OPC-18136) of primary interest eluted early, where as vesnarinone, internal standard and the metabolite OPC-18137 eluted at a later time. Due to the considerable differences in retention times, it was possible to tune the voltage applied on the lens going into the collision cell (L-7) in each period for optimum sensitivity. This change allowed us to analyze the drugs in both concentration ranges without re-

Table 2
Mean regression parameters of the analytes in plasma and urine

Analyte	Plasma				Urine			
	Linearity range (ns ml^{-1})	Slope	Intercept	Correlation coefficient	Linearity range (μg ml^{-1})	Slope	Intercept	Correlation coefficient
Vesnarinone	200–5000	459.4	−50.78	0.999	–	–	–	–
Vesnarinone	500–10 000	503.2	−166.98	0.999	0.5–25.00	0.74	−0.11	0.995
OPC-8230	20–1000	694.6	−3.768	0.999	0.1–5.00	1.10	−0.012	0.996
OPC-18136	20–1000	1427.5	−3.075	0.997	0.1–5.00	2.29	−0.007	0.994
OPC-18137	20–1000	1075.1	−3.395	0.998	0.1–5.00	1.71	−0.003	0.997

Table 3
Precision, accuracy and sensitivity of vesnarinone and its metabolites in plasma

Analyte	Parameter	Within batch ($n = 6$)				Between batch ($n = 24$)			
		a	b	c	d	a	b	c	d
Vesnarinone (200–5000 ng ml^{-1})	Mean	172.6–183.5	425.5–447.7	3031.2–3088.2	7561.1–7825.9	178.8	438.1	3065.4	7671.1
	%C.V.	1.5–8.5	2.9–4.2	2.0–3.2	2.2–4.1	5.8	3.9	2.6	3.4
	%Recovery	86.3–90.1	95.0–99.9	94.7–96.5	85.9–88.9	89.4	97.8	95.8	87.2
Vesnarinone (500–10 000 ng ml^{-1})	Mean	–	355.3-380.8	3209.8-3254.8	8167.5–8484.4	–	369.3	3251.9	8304.8
	%C.V.	–	4.0–5.5	2.1–3.3	2.2–4.1	–	5.2	2.7	3.5
	%Recovery	–	79.3–80.6	100.3–102.8	92.8–96.4	–	82.4	101.6	94.4
OPC-8230 (20–1000 ng ml^{-1})	Mean	18.2–22.1	36.3–38.6	254.9–259.5	704.2–713.8	19.2	37.0	258.0	709.2
	%C.V.	3.2–24.8	4.0–17.9	1.7–5.7	2.6–6.8	17.3	9.9	4.4	4.4
	%Recovery	88.1–106.6	93.8–99.7	92.2–93.9	92.7–93.9	92.9	95.6	93.3	93.3
OPC-18136 (20–1000 ng ml^{-1})	Mean	15.9–19.7	39.4–41.6	275.5–298.3	714.1–832.6	18.1	40.9	288.4	782.1
	%C.V.	4.5–17.9	4.3–18.4	4.9–18.4	5.5–14.9	14.3	12.6	10.5	11.6
	%Recovery	79.6–98.5	87.9–92.9	86.1–93.2	81.1–94.6	90.5	91.3	90.1	88.9
OPC-18137 (20–1000 ng ml^{-1})	Mean	16.6–18.3	40.5–42.9	293.9–304.8	798.9–834.0	17.1	41.4	300.3	822.2
	%C.V.	4.4–11.7	4.4–9.0	1.2–4.7	2.0–4.5	9.3	6.3	3.2	3.3
	%Recovery	83.1–91.3	90 4–95.8	91.8–95.2	90 8–94 8	93.8	92.3	93.8	93.4

[a] 20.72 ng ml^{-1} for OPC-8230, 20.00 ng ml^{-1} for OPC-18136 and OPC-18137 and 200 ng ml^{-1} for vesnarinone.
[b] 38.69 ng ml^{-1} for OPC-8230, 44.00 ng ml^{-1} for OPC-18136 and OPC-18137 and 448 ng ml^{-1} for vesnarinone.
[c] 276.34 ng ml^{-1} for OPC-8230, 320.00 ng ml^{-1} for OPC-18136 and OPC-18137 and 3200 ng ml^{-1} for vesnarinone.
[d] 759.2 ng ml^{-1} for OPC-8230, 880.00 ng ml^{-1} for OPC-18136 and OPC-18137 and 8800 ng ml^{-1} for vesnarinone.

Table 4
Precision, accuracy and sensitivity of vesnarinone and its metabolites in urine

Analyte	Parameter	Within batch ($n = 6$)				Between batch ($n = 24$)			
		a	b	c	d	a	b	c	d
Vesnarinone (0.50–25.00 μg ml^{-1})	Mean	0.4–0.5	1.6–1.6	3.0–3.2	18.5–19.1	0.5	1.6	3.1	18.8
	%C.V.	2.0–14.0	0.6–2.6	1.2–10.8	1.2–4.8	8.5	1.9	6.4	3.5
	%Recovery	86.0–98.0	103.3–104.7	99.0–107.7	86.8–88.6	94 0	104.0	103 7	87.2
OPC-8230 (0.1–5.0 μg ml^{-1})	Mean	0.1–0.1	0.3–0.3	0.6–0.6	4.0–4.1	0.1	0.3	0.6	4.1
	%C.V.	0.0–11.0	3.2–6.5	3.2–12.0	2.9–9.1	10.0	6.7	6.6	5.7
	%Recovery	100.0–103.3	100.0–103.3	98.3–103.3	91.9–95.1	100.0	100.0	101.7	94.0
OPC-18136 (0.1–5.0 μg ml^{-1})	Mean	0.1–0.1	0.3-0.3	0.6–0.6	3.8–4.2	0.1	0.3	0.6	4.0
	%C.V.	10.0–20.0	3.4–14.3	3.3–14.0	6.0–10.8	10.0	10.3	8.6	8.3
	%Recovery	90.0–100.0	93.3–103.3	95.0–101.7	88.2–96.3	100.0	96.7	96.7	91.9
OPC-18137 (0.1–5.0 μg ml^{-1})	Mean	0.1–0.1	0.3–0.3	0.6–0.6	3.8–4.1	0.1	0.3	0.6	822.2
	%C.V.	10.0–10.0	3.3–6.7	3.3–12.3	3.6–13.0	10.0	3.3	6.8	3.3
	%Recovery	100.0–100.0	100.0–100.0	95.0–101.7	87.0–94.0	100.0	100.0	98.3	93.4

[a] 0.10 μg ml^{-1} for OPC-8230, OPC-18136 and OPC-18137 and 0.5 μg ml^{-1} for vesnarinone.
[b] 0.30 μg ml^{-1} for OPC-8230, OPC-18136 and OPC-18137 and 1.5 μg ml^{-1} for vesnarinone.
[c] 0.60 μg ml^{-1} for OPC-8230, OPC-18136 and OPC-18137 and 3.0 μg ml^{-1} for vesnarinone.
[d] 4.32 μg ml^{-1} for OPC-8230, OPC-18136 and OPC-18137 and 21.60 μg ml^{-1} for vesnarinone.

injection, with a high degree of accuracy and reproducibility.

In conclusion, the assay described is specific for vesnarinone, OPC-18136, OPC-18137, and OPC-8230. The LC/MS/MS method offers advantages of rapid analysis, flexibility to monitor additional metabolites, and improved sensitivity. This method was successfully used for measuring vesnarinone and the mentioned metabolites in plasma and urine samples collected during the various ongoing clinical studies [5,6].

Acknowledgements

The authors wish to acknowledge Dr Suresh Mallikaarjun, Assistant Director, for his critical comments and Helena Matos, Senior Administrative Assistant for her support in the preparation of this manuscript.

References

[1] H. Masuoka, M. Ito, M. Sugioka, H. Kozeki, T. Konishi, T. Tanaka, T. Nakano, Biochem. Biophys. Res. Commun. 190 (1993) 412–417.
[2] G. Miyamoto, H Sasabe, N Tominaga, N. Uegaki, M. Tominaga, T. Schimizu, Xenobiotica 18 (1988) 1143–1155.
[3] G. Miyamoto, H. Sasabe, M. Odomi, T. Schimizu, J. Chromatogr. 338 (1985) 450–455.
[4] S. Wu, C. Wu, J. WCUMS 23 (1992) 106–109.
[5] S.L. Bramer, V. Garg, C. Lee, W. Smith, A. Grant, V. Alicknavitch, T.D. Cowart, Pharm. Res. 14 (1997) 508S.
[6] V. Garg, T.D. Cowart, M. Noorisa, K. C. Lasseter, S.C. Dilzer, S.L Bramer, Pharm. Res. 14 (1997) 508S.

.