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Liquid chromatography-electrospray tandem mass spectrometric assay suitable for quantitation of halofuginone in plasma

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Abstract

An LC-MS/MS method was developed to quantitate the potential antitumor agent halofuginone in plasma. The assay uses 0.2 ml of plasma; chlorohalofuginone internal standard; acetonitrile for protein precipitation; a Phenomenex SYNERGI 4 μ Polar RP 80A (4 μm, 100 mm × 2 mm) column; an isocratic mobile phase of methanol:water:formic acid (80:20:0.02, v/v/v); and positive-ion electrospray ionization with selective reaction monitoring detection. Halofuginone eluted at approximately 2.4 min, internal standard eluted at approximately 2.9 min, and no endogenous materials interfered with their measurement. The assay was accurate, precise, and linear between 0.1 and 100 ng/ml. Halofuginone could be quantitated in dog plasma for at least 24 h after an i.v. dose of 0.1 mg/kg. The assay is being used in ongoing pharmacokinetic studies of halofuginone.

Keywords: Halofuginone

1. Introduction

Halofuginone (Fig. 1) is a quinazolinone alkaloid that is approved to prevent coccidiosis in poultry [1,2] and to treat cryptosporidiosis [3–5] and theileriosis in cattle [6,7]. The ability of halofuginone to inhibit both Type 1 collagen synthesis [8–14] and matrix metalloprotease gene expression [15,16] is potentially useful in reducing tumor growth, because angiogenesis in a growing tumor utilizes Type 1 collagen [15,17], and matrix metalloproteinases are associated with the ability of a tumor to metastasize [15,16,18,19]. Furthermore, halofuginone has been shown to reduce the excessive amount of collagen (and resulting scar tissue) associated with graft-versus-host disease [8,9], abdominal adhesions [11], scleroderma [8], pulmonary fibrosis [10], urethral stricture formation [12], postoperative peritendinous fibrous adhesions [13], radiation-induced fibrosis [20], and hepatic cirrhosis [14]. Halofuginone has also been shown to decrease extracellular matrix deposition [15,16] and has documented inhibitory effects on angiogenesis [21], glomerular mesangial proliferation [22], and intimal hyperplasia [23]. Consequently, halofuginone is being evaluated in a multi-center clinical trial as a topical treatment for HIV-associated Kaposi's sarcoma and is being developed as a potential systemic treatment for other human malignancies.

As a result of efforts to develop halofuginone as a potential antineoplastic agent, as well as investigate its potential use in other clinical situations, there is the need to evaluate halofuginone pharmacokinetics and plasma concentrations in

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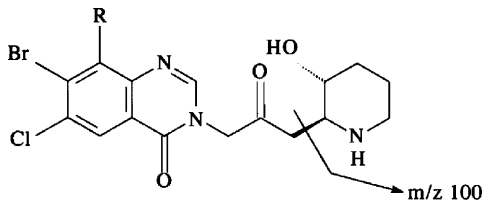

Halofuginone	R = H	m/z 416
Chlorohalofuginone	R = Cl	m/z 450

Fig. 1. Structures of halofuginone and chlorohalofuginone and the *m/z* 100 product ion monitored.

preclinical models and clinical trials. Although LC-MS/MS assays have been developed for quantitating halofuginone in chicken liver and eggs [24,25], they are not well-suited for quantitating halofuginone in plasma because one assay does not employ an internal standard and both assays involve labor-intensive extraction techniques and large sample volumes. In addition, older GLC [26] and HPLC assays [27–33], including one previously developed in our laboratory [27], for quantitation of halofuginone in animal feed or biological matrices are not sufficiently sensitive to quantitate the plasma concentrations of halofuginone anticipated to be produced by the clinical doses of halofuginone being used topically or being proposed for evaluation as systemic therapy. As a result, an HPLC-electrospray tandem mass spectrometric (LC-MS/MS) assay has been developed to quantitate anticipated clinically relevant concentrations of halofuginone in plasma. Its suitability in this regard was subsequently demonstrated by using it to quantitate halofuginone in plasma of a dog treated in a toxicology study that was performed in anticipation of subsequent clinical trials.

2. Experimental

2.1. Reagents and materials

All solvents used were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Control human plasma was prepared by centrifugation of outdated, citrate-anticoagulated blood obtained from the Central Blood Blank (Pittsburgh, PA, USA). Heparinized, control beagle dog plasma was provided by Battelle Toxicology (Columbus, OH, USA) or was purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Medical grade nitrogen, argon, and liquid nitrogen were purchased from Valley National Gases Inc. (Pittsburgh, PA, USA). Halofuginone and chlorohalofuginone (Fig. 1) were graciously provided by Collgard Biopharmaceuticals (Petah Tikva, Israel).

2.2. Sample preparation

A 1 mg/ml stock solution of halofuginone was prepared by dissolving halofuginone in methanol:water (50:50, v/v). This was stored at 4 °C until further dilutions were made for standard curves and quality control samples. A stock solution of chlorohalofuginone internal standard was prepared in a similar fashion except the 1 mg/ml solution was further diluted in methanol to a final concentration of 1 μg/ml. Standard curves were prepared by placing triplicate, 0.2 ml samples of human or dog plasma containing 0.1, 0.3, 1, 3, 10, 30, or 100 ng/ml halofuginone into 1.5 ml microcentrifuge tubes. Quality control samples were prepared at concentrations of 0.5, 5, and 50 ng/ml in human and dog plasma. Ten microliters of 1 μg/ml chlorohalofuginone internal standard and 1 ml of acetonitrile were added sequentially to each tube. The samples were vortexed for 1 min at a setting of eight on a Vortex Genie (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at 12,000 × *g* and room temperature for 6 min. The resulting supernatants were transferred to 12 mm × 75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen at 27 °C. Each dried residue was redissolved in 100 μl of methanol:water:formic acid (80:20:0.02, v/v/v), vortexed briefly, transferred to HPLC autosampler vials, and 25 μl of the redissolved material were injected into the LC-MS/MS system.

2.3. Chromatography

Chromatography was performed on a Waters Alliance 2695 system (Milford, MA, USA) fitted with a SYNERGI 4 μ POLAR-RP 80A (4 μm, 100 mm × 2 mm) column (Phenomenex, Torrance, CA, USA). The isocratic mobile phase consisting of methanol:water:formic acid (80:20:0.02, v/v/v) was pumped at 0.2 ml/min, and overall chromatographic run time was 5 min.

2.4. Mass spectrometry

Mass detection was carried out using a Waters Quattro micro triple-stage, benchtop quadrupole mass spectrometer with electrospray ionization in positive ion, selected reaction monitoring (SRM) mode. For both full scan MS and SRM MS/MS, the settings of the mass spectrometer were as follows: capillary voltage 5.0 kV; cone voltage 45 V; source temperature 120 °C; and desolvation temperature 450 °C. The cone and desolvation gas flows were 110 and 550 l/h, respectively. When performing SRM scans, the collision voltage was 40 V. Quadrupoles 1 and 2 each had low mass resolution and high mass resolution set at 12.0. The dwell time was 0.5 s and interscan delay was 0.1 s. Halofuginone and internal standard ions monitored in SRM scans were *m/z* 416 > 100 and *m/z* 450 > 100, respectively. The LC system and mass spectrometer were controlled by Waters MassLynx software (version 4.0), and data were collected with the same software. The analyte-to-internal standard ratio was calculated for each standard by dividing the halofuginone peak area by the peak area of the internal standard. Standard curves of halofuginone were constructed by plotting the analyte-to-internal standard

ratio versus the known concentration of halofuginone in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, followed by back calculation of concentrations.

2.5. Analysis of dog plasma samples

Samples used to demonstrate the applicability of the LC-MS/MS method for quantitation of halofuginone in plasma were provided by Battelle Toxicology (Columbus, OH, USA). Because clinical trials of systemically administered halofuginone had not begun when the assay was developed and validated, plasma samples were obtained from a purebred, female beagle dog treated with 0.1 mg/kg halofuginone as 1 h i.v. infusions as part of an animal care and use committee-approved, good laboratory practices toxicology study performed in preparation for subsequent clinical studies. Blood samples were collected in heparinized tubes before halofuginone administration; at 30 min into the 1 h infusion; at the end of the infusion; and at 2, 5, 10, 20, and 30 min after completion of the infusion, as well as at 1, 1.5, 2, 4, 8, 12, 16, 20, and 24 h after completion of the infusion. Plasma was prepared by centrifuging each blood sample at approximately $1000 \times g$ for 10 min, and the resulting plasma layers were stored at −70 °C until analyzed with the procedure described above.

3. Results

Although halofuginone and chlorohalofuginone have nominal molecular masses of 413 and 443, respectively, the presence of chlorine ($^{35}Cl/^{37}Cl$) and bromine ($^{79}Br/^{81}Br$) in their structure resulted in the most abundant precursor ions in the ESI positive mode being *m/z* 416 and *m/z* 450 for halofuginone and chlorohalofuginone, respectively (Fig. 2A and B). The CID fragmentation of halofuginone, wherein three product ions are produced (Fig. 3A), has been documented. The *m/z* 100 product ion (Fig. 1) was monitored because it was the most abundant and stable ion for both halofuginone and internal standard (Fig. 3A and B). With the chromatography conditions described, halofuginone eluted at approximately 2.4 min, and chlorohalofuginone eluted at approximately 2.9 min (Fig. 4). There was near-baseline separation of halofuginone and chlorohalofuginone, and no endogenous materials in human or beagle dog control plasma interfered with measurement of either compound.

3.1. Linearity

Three triplicate standard curves were performed in human and beagle plasma on three sequential days. The assay proved to be linear and acceptable, as the regression coefficients were ≥0.99 for each of the three human and beagle standard curves. The slopes of the lines describing the three standard curves in human plasma were 0.026, 0.021, and 0.020, respectively,

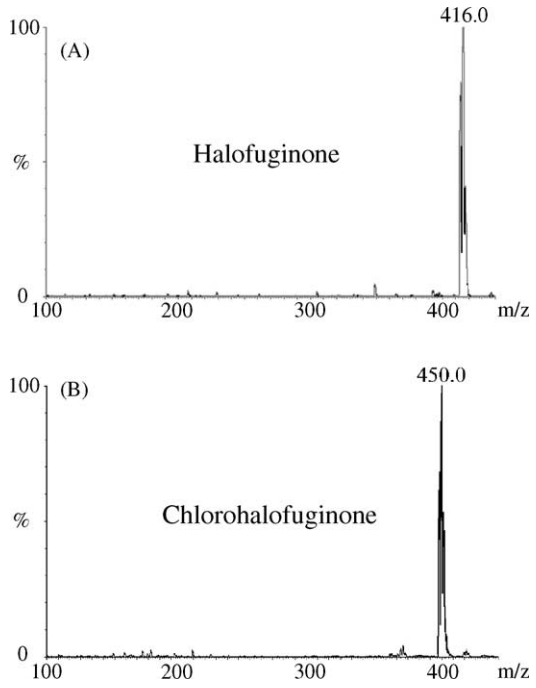

Fig. 2. Electrospray ionization mass spectra of (A) halofuginone and (B) chlorohalofuginone.

producing a mean slope of 0.022, a standard deviation of 0.0032 and coefficient of variation of 14.4%. The slopes of the lines describing the three standard curves in beagle plasma were 0.033, 0.031, and 0.031, respectively, producing a mean slope of 0.032, a standard deviation of 0.001 and coefficient of variation of 3.6%. The individual values for mean and

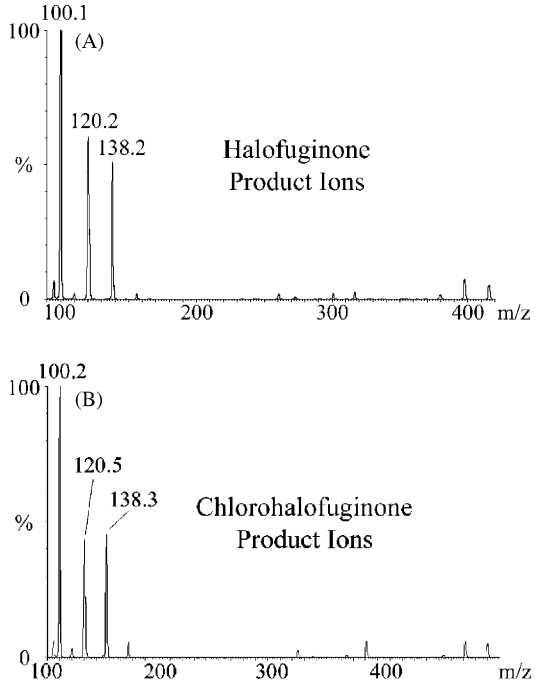

Fig. 3. CID product ion spectra from (A) *m/z* 416 of halofuginone and (B) *m/z* 450 of chlorohalofuginone.

Table 1
Accuracy and precision of back-calculated calibration samples for halofuginone

Plasma	Nominal concentration (ng/ml)	Mean (n = 9) concentration (ng/ml)	S.D.	CV (%)	Accuracy (%)
Human	0.1	0.100	0.009	8.7	100
	0.3	0.300	0.022	7.3	100
	1	1.02	0.064	6.3	102
	3	3.03	0.16	5.2	101
	10	10.32	0.72	6.9	103.2
	30	29.65	1.56	5.3	98.8
	100	98.57	4.66	4.7	98.6
Dog	0.1	0.103	0.005	4.8	103
	0.3	0.304	0.014	4.7	101
	1	0.992	0.070	7.1	99
	3	2.866	0.089	3.1	96
	10	10.352	0.607	5.9	103
	30	31.101	1.554	5.0	104
	100	101.136	5.884	5.8	101

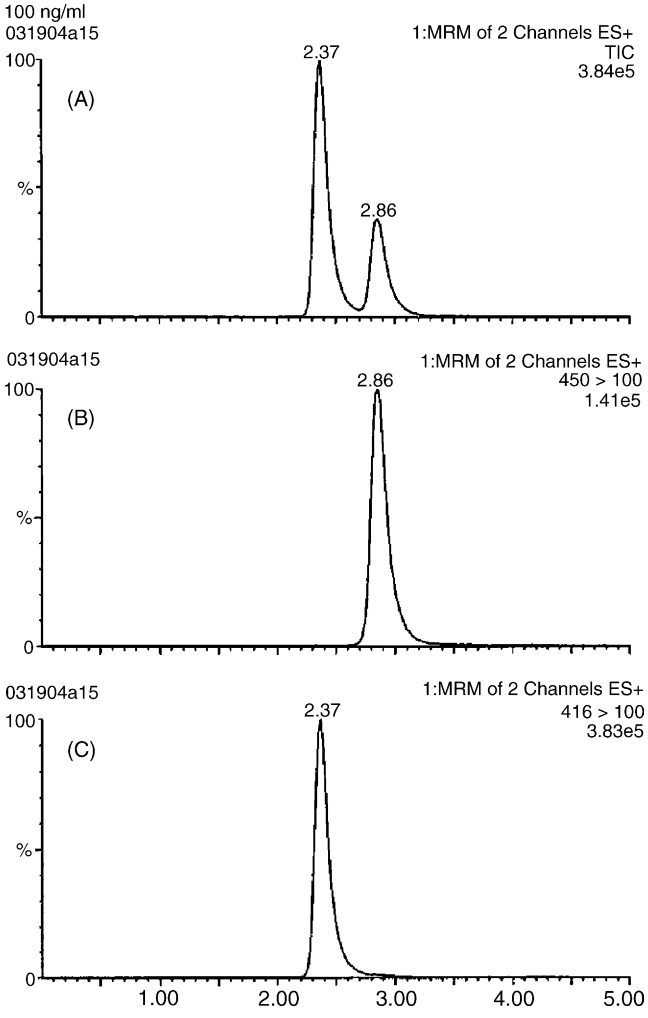

Fig. 4. LC-MS/MS chromatogram of 100 ng/ml halofuginone and 50 ng/ml chlorohalofuginone internal standard isolated from human plasma. (A) Total ion current, (B) SRM of m/z 450 > 100 for chlorohalofuginone internal standard, and (C) SRM of m/z 416 > 100 for halofuginone.

standard deviations of back-calculated values at each nominal concentration used in the human and beagle standard curves are displayed in Table 1, as is the accuracy calculated from those values.

3.2. Accuracy and precision

In order to assess the accuracy and precision of the assay in human and beagle plasma, quality control samples at concentrations of 0.5, 5, and 50 ng/ml (five samples at each concentrations) were analyzed on three sequential days. These concentrations were chosen because they represented the range of concentrations anticipated in animals treated as part of the preclinical testing of halofuginone and in patients eventually treated with halofuginone. The inter- and intra-day back-calculated concentrations with standard deviations, coefficients of variation, and accuracies resulting from these analyses are displayed in Table 2. The coefficient of variation for both the inter- and intra-day variability were less than 15% at each concentration studied. The mean accuracy for both the intra- and inter-day evaluations was between 87 and 99% over the three days on which quality control samples were tested.

3.3. Recovery and stability

The recovery of halofuginone was tested by using the LC-MS/MS assay described to quantitate the amount of halofuginone in human plasma and comparing that value to the assay value for a known amount of halofuginone prepared and assayed in mobile phase. This was done in triplicate using halofuginone concentrations of 0.3, 3, and 30 ng/ml. The mean percentage recovery at 0.3 ng/ml was 92%, while the mean recoveries at 3 and 30 ng/ml were 62 and 73%, respectively. In order to test the feasibility of using an auto-sampler as part of the assay, a solution containing 30 ng/ml halofuginone and 100 ng/ml internal standard was prepared in mobile phase, sealed in autosampler vials, and repeatedly injected by autosampler over 24 h. There was no

Table 2
Accuracy and precision of halofuginone QC samples

Plasma	Nominal concentration (ng/ml)		Intra-day			Inter-day mean (n = 15)
Human	0.5	Mean (n = 5), accuracy (%)	90.3	86.7	91.1	89.4
		S.D.	1.3	9.6	13.2	9.0
		CV (%)	1.5	11.1	14.5	10.0
	5	Mean (n = 5), accuracy (%)	95.1	92.6	92.2	93.3
		S.D.	4.9	2.5	2.9	3.6
		CV (%)	5.1	2.7	3.2	3.8
	50	Mean (n = 5), accuracy (%)	95.0	93.9	98.4	96.8
		S.D.	2.6	2.1	1.5	2.4
		CV (%)	2.7	2.2	1.5	2.5
Dog	0.5	Mean (n = 5), accuracy (%)	97.1	96.0	97.9	97.0
		S.D.	3.6	2.0	1.5	2.5
		CV (%)	3.7	2.1	1.5	2.6
	5	Mean (n = 5), accuracy (%)	96.8	94.5	98.0	96.5
		S.D.	2.6	2.7	1.4	2.6
		CV (%)	2.7	2.9	1.4	2.7
	50	Mean (n = 5), accuracy (%)	99.0	93.5	97.3	96.6
		S.D.	1.3	4.1	0.89	3.4
		CV (%)	1.3	4.4	0.91	3.5

evidence of decomposition of either halofuginone or internal standard during that time.

3.4. Freeze-thaw testing

Triplicate human plasma samples containing 0.3, 3, or 30 ng/ml halofuginone were sequentially frozen at −70 °C and thawed three times during a 24 h period. In addition, human plasma containing halofuginone was maintained at 22 °C for 8 h. The 22 °C samples, freeze-thaw samples, and freshly prepared samples were processed according to the above LC-MS/MS method. The mean percentage recoveries of 0.3, 3, and 30 ng/ml freeze-thaw samples were 88, 85 and 85%, respectively; whereas, the mean percentage recoveries for the same three concentrations stored for 8 h at 22 °C were 94, 85, and 84%, respectively.

3.5. Specificity

The assay was characterized for specificity by analyzing 10 different control human plasma sources. There was no evidence of interference at SRM m/z 416 > 100 or 450 > 100.

3.6. Demonstration of applicability to biological samples

In order to demonstrate the applicability of the LC-MS/MS method to plasma samples from a pharmacokinetic study, it was used to quantitate concentrations of halofuginone in the plasma of a dog that received 0.1 mg/kg of halofuginone as a 1 h infusion on two separate occasions (days 1 and 25) (Fig. 5). As demonstrated in Fig. 5, the 0.1 mg/kg halofuginone infusion produced peak plasma halofuginone concentrations between 8.7 and 11.2 ng/ml, and on both days of study, plasma samples obtained at 24 h after the 1 h halofuginone infusion contained halofuginone concentrations three times greater than the 0.1 ng/ml lower limit of quantitation of the assay.

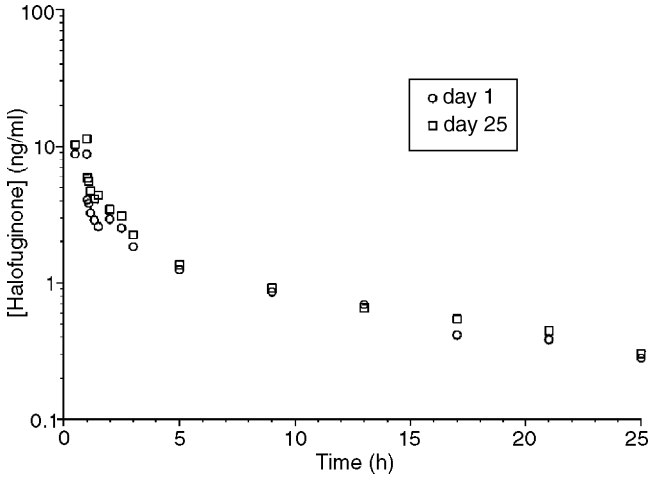

Fig. 5. Concentrations of halofuginone in plasma of a beagle dog given two 1 h infusions of halofuginone at a dose of 0.1 mg/kg.

4. Discussion

The LC-MS/MS method described in the current manuscript is suitable for ongoing preclinical studies of halofuginone and anticipated subsequent clinical studies of halofuginone because of its sensitivity and the lack of interference from endogenous materials. In addition, the 0.2 ml sample volume required for the assay is reasonable, the lower limit of quantitation of the assay is approximately 300-times

less than that of our previous HPLC/absorbance-detection assay of halofuginone [27], and the acetonitrile precipitation is much less labor intensive than those of currently described LC-MS/MS assays. Finally, the 5 min run time is suitable for reasonable throughput of samples. The overall applicability of this method is evidenced by its having proven suitable for preclinical toxicology studies of halofuginone [34], and its currently being employed to support a recently activated clinical trial of topically administered halofuginone.

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