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Development and validation of a liquid chromatography/tandem mass spectrometry method for the determination of DMXAA in human and mouse plasma

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Abstract

A rapid, sensitive, and specific LC/MS/MS-based method was developed for determining the concentration of DMXAA in human and mouse plasma. Sample preparation involved a single protein precipitation step using acetonitrile. Separation of DMXAA and 6-isopropoxy-9-oxoxanthene-2-carboxylic acid, the internal standard, was achieved on a Waters X-Terra™ C_{18} (50 mm × 2.1 mm i.d., 3.5 μm) analytical column using a mobile phase consisting of acetonitrile/10 mM ammonium acetate (55:45, v/v) containing 0.1% formic acid and isocratic flow at 0.2 mL/min for 3 min. The analytes were monitored by tandem mass spectrometry with electrospray positive ionization. Linear calibration curves were generated over the range of 5–3000 ng/mL. The values for precision and accuracy were <9.6%, except at the LLOQ (5 ng/mL) level, which was within 16.8%. Recovery of DMXAA in mouse plasma was >65%. DMXAA was stable through 2 freeze/thaw cycles, to 2 h in mouse plasma or 50% acetonitrile, and on the autosampler to 5.1 h. This method was subsequently used to measure concentrations of DMXAA in mice following intraperitoneal administration.

Keywords: DMXAA; LC/MS/MS; Pharmacokinetics

1. Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a cytokine-inducing, small molecule vascular-disrupting agent that has recently completed Phase I clinical trials in New Zealand and UK [1–3]. DMXAA was originally synthesized as a more potent analogue of flavone-8-acetic acid (FAA) [4]. Despite the excellent preclinical activity, FAA was ineffective in clinical trials, stimulating construction of derivatives [5,6]. DMXAA has activity in a wide variety of murine tumors alone and in combination with radiation and other anticancer agents [4,7–16]. The antitumor mechanisms of DMXAA have been hypothesized to include the direct induction of endothelial cell apoptosis and the indirect induction of cytokines in situ within the tumor microenvironment. In mice, DMXAA selectively inhibits tumor blood flow and induces TNF-α, nitric oxide and serotonin production, causing extensive vascular collapse and hemorrhage necrosis [17,18]. Phase I clinical trials have shown the ability of DMXAA to both decrease tumor blood flow and increase the plasma concentrations of 5-hydroxyindole-3-acetic acid (5HIAA), a serotonin metabolite [1–3].

In recent years, several analytical methods for DMXAA based on reversed-phase HPLC with fluorescence detection have been reported [3,19,20]. However, these methods have some disadvantages, including the necessity for relatively complex sample preparation or long chromatographic run times. A recent clinical trial involving the clinical evaluation of DMXAA in cancer patients used a validated LC/MS/MS methods for pharmacokinetic evaluations [1]. However, this manuscript did not describe the analytical methodology or the calibration range utilized. Here, we describe a rapid and sensitive analytical method for the determination of DMXAA concentrations in

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mouse plasma based on LC/MS/MS with electrospray positive ionization.

2. Experimental

2.1. Chemical and reagents

DMXAA and the internal standard, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid were purchased from Sigma Co. (St. Louis, MO, USA). Formic acid (98%, v/v, in water), methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was obtained from a Milli-Q-UF system (Millipore, Milford, MA, USA) and used in all aqueous solutions. Drug-free (blank) mouse plasma was from Innovative Research (Southfield, MI, USA). Drug-free (blank) human plasma from healthy donors originated from Pittsburgh Blood Plasma, Inc. (Pittsburgh, PA, USA).

2.2. Stock solutions, calibration standards, and quality control samples

Stock solutions of DMXAA at a concentration of 1 mg/mL were prepared by dissolving 10 mg in 10 mL of acetonitrile and stored in glass vials at −20 °C. The stock solutions were diluted in blank human or mouse plasma on each day of analysis to prepare seven calibration standards containing DMXAA at the following concentrations: 5, 10, 50, 100, 500, 1000, and 3000 ng/mL. Quality control (QC) samples were prepared independently in blank plasma at least four different concentrations for DMXAA. These included 5 ng/mL, the lower limit of quantification (LLOQ) QC; 15 ng/mL, the low QC; 800 ng/mL, the medium QC; 2000 ng/mL, the high QC; and 800 ng/mL diluted 1:10 with blank plasma, the dilution QC.

2.3. Sample preparation

Prior to extraction, frozen plasma samples were thawed in a water bath at ambient temperature. A 0.1 mL aliquot of thawed plasma was added to a borosilicate glass test tube (13 mm × 100 mm) containing 0.3 mL of acetonitrile solution and 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (50 ng/mL), which was used as internal standard. The tube was mixed vigorously for 30 sec on a vortex-mixer, followed by centrifugation at 2000 rpm for 5 min at ambient temperature. A volume of 100 μL of the top organic layer was transferred to a 250-μL polypropylene autosampler vial, sealed with a Teflon crimp cap, and 10 μL was injected onto the HPLC instrument for quantitative analysis using a temperature-controlled autosampling device operating at 10 °C.

2.4. Chromatographic and mass-spectroscopic conditions

Chromatographic analysis was performed using a Waters Model 2690 separations system (Milford, MA, USA). Separation of the analytes from potentially interfering material was achieved at ambient temperature using a Waters X-Terra MS column (50 mm × 2.1 mm i.d.) packed with a 3.5-μm C_{18} stationary phase, protected by a guard column packed with 3.5 μm RP18 material (Milford, MA, USA). The mobile phase used for the chromatographic separation was composed of acetonitrile containing 0.1% formic acid–10 mM ammonium acetate (55:45, v/v), and was delivered isocratically at a flow rate of 0.2 mL/min. The total run time was 3 min. The column effluent was monitored using a Micromass Quattro LC triple-quadrupole mass spectrometric detector (Beverly, MA, USA). The instrument was equipped with an electrospray interface and controlled by Masslynx version 3.5 software (Micromass). The samples were analyzed using an electrospray probe in the positive ionization mode operating at a cone voltage of 45 V for DMXAA and 20 V for the internal standard. Samples were introduced into the interface through a heated nebulized probe (350 °C). The spectrometer was programmed to allow the $[MH]^+$ ion of DMXAA at *m/z* 283.0 and that of the internal standard at *m/z* 299.0 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 25 eV for DMXAA and 18 eV for the internal standard. The daughter ions for DMXAA (*m/z* 237.0) and the internal standard (*m/z* 257.0) were monitored through the third quadrupole (Q3) (Fig. 1). Argon was used as collision gas at a pressure of 0.0027 mBar, and the dwell time per channel for data collection was 0.5 s.

2.5. Calibration curves

Calibration curves for DMXAA were computed using the ratio of the peak area of analyte and internal standard by using a weighted (1/[nominal concentration]) least-squares linear regression analysis. The parameters of each calibration curve were used to compute back-calculated concentrations and to obtain values for the QC samples and unknown samples by interpolation.

2.6. Method validation

Method validation runs for human and mouse plasma calibrator standards and QCs were performed on three separate occasions and included calibration curves processed in duplicate and QC samples at five different concentrations, each in triplicate. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precisions, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV}_{(\mathrm{DMXAA})} = 100 \times \left\{ \frac{[\mathrm{DMXAA}]_{\mathrm{mean}} - [\mathrm{DMXAA}]_{\mathrm{nominal}}}{[\mathrm{DMXAA}]_{\mathrm{nominal}}} \right\}$$

Estimates of the between-run precisions were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated using the JMP™ statistical discovery software version 4 (SAS Institute, Cary, NC, USA). The between-run precision

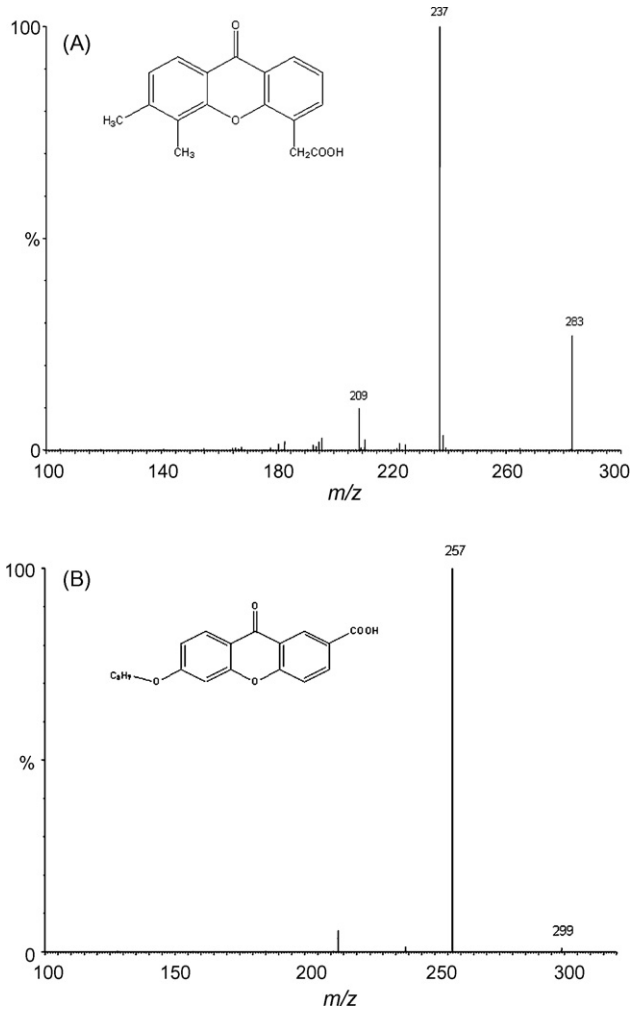

Fig. 1. Full-scan product ion spectrum and chemical structure of DMXAA (A) with monitoring at *m/z* 283.0 → 237.0, and 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (B) with monitoring at *m/z* 299.0 → 257.0.

(BRP), expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = 100 \times \left(\frac{\sqrt{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}}{\mathrm{GM}} \right)$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = 100 \times \left(\frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \right)$$

The specificity of the method was tested by visual inspection of chromatograms of extracted human and mouse plasma samples from six different control individuals that had not been treated with DMXAA for the presence of endogenous or exogenous interfering peaks. It was required that the peak areas in the samples from the control plasma be less than 20% of the peak areas of samples containing 5 ng/mL DMXAA diluted in plasma.

The extraction efficiency of the assay was measured by comparison of the peak area ratio of DMXAA extracted from plasma

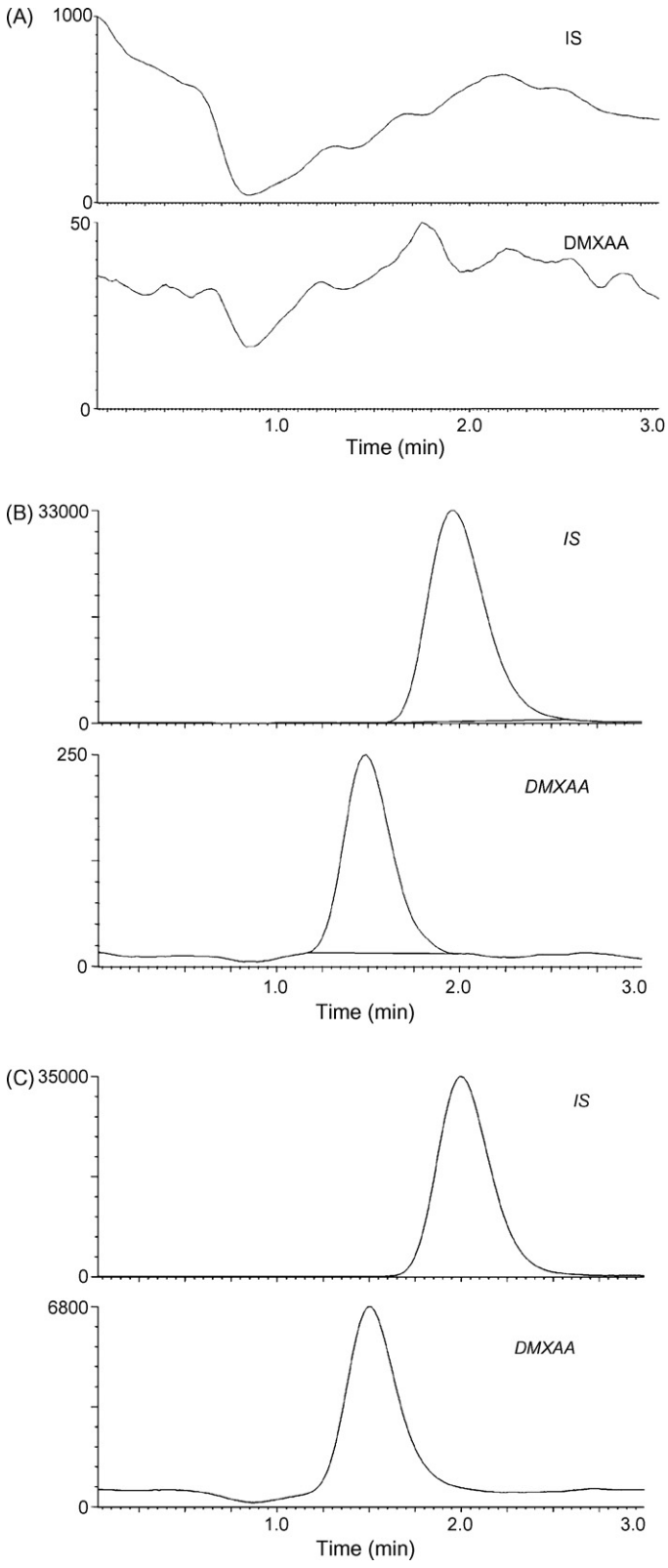

Fig. 2. Selected ion chromatograms of blank plasma (A), plasma spiked with DMXAA (5 ng/mL) and the internal standard (IS) (50 ng/mL) (B), and a selected mouse sample obtained at 20 h after the intraperitoneal administration of 240 μg of DMXAA (C). The retention times for DMXAA and the internal standards were 1.47 ± 0.01 and 1.95 ± 0.01 min, respectively.

and an aqueous solution in triplicate at concentrations of the low, medium, and high QCs. The stability of DMXAA in plasma was tested at concentrations of the low and high QCs in triplicate after 2 freeze–thaw cycles at −20 °C. The short-term stability of DMXAA in 50% acetonitrile and in plasma was assessed in triplicate at room temperature (on the benchtop) for 4 h. Stability of drug in neutral extracts was assessed on the autosampler at 10 °C.

2.7. Mouse specimens

Six- to eight-week-old BALB/c mice, purchased from Harlan Breeders (Indianapolis, IN), were used for tumor implantation. Five million CT26 cells were injected subcutaneously into the right flank of each mouse. Tumors were allowed to grow to a size of ∼500 mm^3, at which point mice were treated with DMXAA. Immediately before use, DMXAA was dissolved in sterile 5% sodium bicarbonate under subdued lighting to prevent photolytic degradation. A single dose of 240 μg DMXAA was given to each mouse intraperitoneally. Groups of two or three mice were euthanized at 0.5, 3, 6, 9, and 20 h following drug administration. Blood was collected in heparinized tubes, centrifuged, and the plasma removed and stored at −20 °C and protected from light until analysis. All animal experiments were overseen and approved by the Animal Welfare Committee of Johns Hopkins University and were in compliance with University standards.

Mean plasma concentrations at each sampling point were calculated for DMXAA. Pharmacokinetic variables were calculated from mean DMXAA concentration–time data using non-compartmental methods as implemented in WinNonlin version 5.0 (Pharsight Corp., Mountain View, CA). The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were the observed values from the mean data. The plasma concentration–time curve to the last quantifiable point (AUC_{last}) was calculated using the linear trapezoidal method.

3. Results and discussion

3.1. Detection and chromatography

The mass spectrum of DMXAA showed a protonated molecular ion ([MH^+]) at *m/z* 283.0. The major fragment observed was at *m/z* 237.0, which was selected for subsequent monitoring in the third quadrupole (Fig. 1A). The mass spectrum of the internal standard, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid, showed a [MH^+] at *m/z* 299.0, and the high collision energy gave one major product ion at *m/z* 257.0 (Fig. 1B).

No peaks were observed in the chromatograms of blank plasma from six donors (data not shown). Representative chromatograms of blank mouse plasma and plasma spiked with internal standard and DMXAA are shown in Fig. 2. The mean (±standard deviation) retention times for DMXAA and internal standard under the optimal conditions were 1.47 ± 0.01 and 1.95 ± 0.01 min, respectively, with an overall chromatographic run time of 3 min. The selectivity for the analysis was indicated by symmetrical resolution of the peaks. Furthermore, in drug-free specimens there were no significant chromatographic interferences at the retention times of the analyte or internal standard.

3.2. Linearity of detector responses

The calculated peak area ratios of DMXAA to 6-isopropoxy-9-oxoxanthene-2-carboxylic acid versus the

Table 1
Construction of standard curves for DMXAA in plasma

Nominal concentration (ng/mL)	Concentration (ng/mL)[a]	Accuracy (%)	Precision (%)		No. of samples
			Within-run	Between-run	
Mouse plasma					
5	4.5 ± 0.4	89.3	4.4	9.7	6
10	10.1 ± 0.6	100.7	4.1	5.6	6
50	50.4 ± 3.4	100.7	6.7	–[b]	6
100	100.0 ± 5.6	100.0	6.4	–[b]	6
500	523.6 ± 5.8	104.7	1.4	–[b]	6
1000	1079.8 ± 34.6	108.0	3.6	–[b]	6
3000	2896.7 ± 49.4	96.6	2.1	–[b]	6
Human plasma					
5	5.4 ± 0.1	107.1	2.9	–[b]	6
10	10.2 ± 0.5	101.6	5.7	–[b]	6
50	49.5 ± 1.9	99.0	3.9	–[b]	6
100	95.9 ± 2.2	95.9	2.3	0.4	6
500	497.0 ± 11.7	99.4	2.8	2.4	6
1000	950.9 ± 28.1	95.1	3.6	–[b]	6
3000	3056.2 ± 64.5	101.9	2.7	–[b]	6

Performed in duplicate on three separate occasions.
[a] Values are means ± standard deviations.
[b] No significant variations were observed as a result of performing the assays in different runs.

Table 2
Assessment of accuracy, precision, and recovery

Nominal concentration (ng/mL)	Accuracy (%)	Precision (%)		Recovery (%)	No. of samples
		Within-run	Between-run		
Mouse plasma					
5	83.2	4.8	5.2	–[b]	9
15	100.8	6.1	–[a]	75.7	9
800	100.6	5.7	–[a]	65.6	9
2000	97.6	2.4	2.3	75.7	9
80 (1:10)	102.8	4.3	3.5	–[b]	9
Human plasma					
5	109.6	2.9	–[a]	–[b]	9
15	90.9	1.2	1.0	–[b]	9
800	90.6	2.4	1.3	–[b]	9
2000	99.0	0.1	1.9	–[b]	9

Performed in triplicate on three separate occasions.
[a] No significant additional variation was observed as a result of performing the assay in different runs.
[b] Not performed.

nominal concentrations of the analyte displayed a linear relationship in the tested range of 5–3000 ng/mL. A weighting factor inversely proportional to the variance at the given concentration level was employed. This weighting factor was chosen by comparison to uniform weighting after evaluation of goodness-of-fit by assessment of the R^2 value, intercept closest to a zero value, % recovery of calibrators and QCs, and assessment of residuals. After applying the peak area ratio in combination with a weighting factor of $1/x$, a mean least-squares linear-regression correlation coefficient of greater than 0.99 was obtained in all analytical runs. The statistical evaluation of the coefficients of the ordinary least-squares line indicated little bias in the slope and intercept, further supporting the conclusion that there were minor matrix effects and blank problems, respectively.

For each point on the calibration curves for DMXAA, the concentration back-calculated from the equation of the regression analysis was always within 8.0% of the nominal value, except at 5 ng/mL, where the accuracy was within 10.7% of the nominal value (Table 1). A linear regression of the back-calculated concentrations versus the nominal values yielded a unit slope and an intercept not significantly different from zero (data not shown). The distribution of the residuals showed random variation, was normally distributed, and centered at zero (data not shown).

The LLOQ for DMXAA was established at 5 ng/mL for human and mouse plasma. This concentration was associated with a mean signal-to-noise ratio of 127 based on nine observations.

3.3. Accuracy, precision, and recovery

For QC samples prepared by spiking human or mouse plasma with DMXAA, the within-run and between-run variability (precision) was less than 6.1%. Likewise, the mean predicted concentration (accuracy) was within 9.6% of the nominal value, except at 5 ng/mL, where the accuracy was within 16.8% of the nominal value (Table 2). The relative recovery of DMXAA from mouse plasma was greater than 65% at low QC, medium QC, and high QC concentrations.

3.4. Analyte stability

QC samples prepared in human plasma undergoing two freeze–thaw cycles showed no significant degradation (<2%) for DMXAA. Plasma spiked with DMXAA and DMXAA stock solution stored at room temperature was stable for up to 2 h (Table 3). In neutral extracts, DMXAA was stable up to 5.1 h on the autosampler without any significant degradation, allowing for approximately 100 samples to be analyzed simultaneously within a single chromatographic run.

Table 3
Assessment of stability

Condition	DMXAA	
	15 ng/mL	2000 ng/mL
Freeze–thaw stability in mouse plasma (−20 °C)[a]		
Cycle 1	102.0	97.7
Cycle 2	99.2	98.0
Short-term stability in mouse plasma (room temperature)[a]		
Time = 1 h	93.8	90.8
Time = 2 h	86.4	85.8
Time = 4 h	78.6	78.4
Short-term stability in 50% acetonitrile (room temperature)[a]		
Time = 0.5 h	92.7	98.9
Time = 1 h	87.5	95.7
Time = 2 h	85.8	94.0
Time = 4 h	73.8	85.6
Autosampler stability (10 °C)[b]		
Time = 2.5 h	100.1	102.2
Time = 5.1 h	97.9	103.2

Expressed as the mean percentage change from time zero (nominal concentration).
[a] Performed in triplicate.
[b] Performed repeatedly for 7 h with 1 sample.

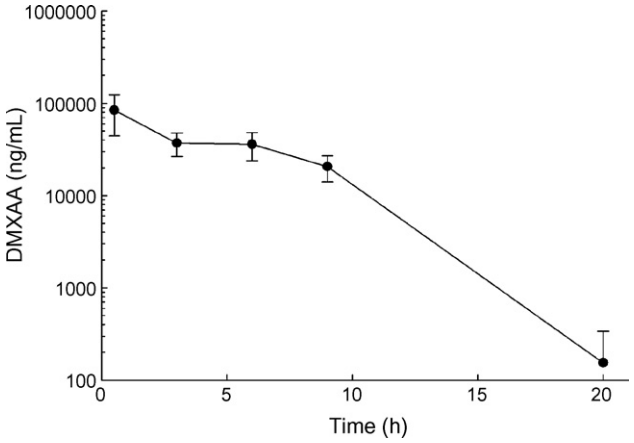

Fig. 3. Mean (±standard deviation) DMXAA plasma concentration–time profile in mice receiving a single dose of 240 μg via the intraperitoneal route.

3.5. Plasma concentration–time profiles

The present LC–MS–MS method was successfully applied to study the pharmacokinetics of DMXAA in mice receiving a single dose of 240 μg of DMXAA via the intraperitoneal route. Fig. 3 shows the mean DMXAA plasma concentration–time profiles collected up to 20 h. Following a single IP dose of 240 μg of DMXAA, the maximum plasma concentration achieved was 83935 ng/mL, which occurred at 0.5 h; the terminal half-life was 1.6 h. These results are largely consistent with those reported previously [3,20], though differences in doses and dose routes preclude an exact comparison.

4. Conclusion

An assay for measuring DMXAA in human and mouse plasma has been developed and validated. In comparison to published methods, the current assay is about three times more sensitive. Moreover, it uses less sample volume and the sample preparation procedure is simpler [3,19,20]. These characteristics, combined with an overall chromatographic run time of 3 min, allow the assay to be easily applied to the quantification of DMXAA in a large number of plasma samples. The method was quantitative and accurate over concentrations ranging from 5 to 3000 ng/mL, sufficient for measuring plasma pharmacokinetics in mice after a single intraperitoneal administration of DMXAA.

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