Journal of Chromatography B, 878 (2010) 2796–2802

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A general HPLC–UV method for the quantitative determination of curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore in rat biomatrices

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ARTICLE INFO

Article history:
Received 29 April 2010
Accepted 24 August 2010
Available online 20 September 2010

Keywords:
Curcumin analogues
HPLC
Microsomal stability
Piperidones
Rat biomatrices
Validation

ABSTRACT

Curcumin and its derivatives generally display favorable cytotoxic activities against a number of cancer cell types. We focus our rational antineoplastic drug design program on curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. Favorable outcomes from pharmacological screens of this series demanded further pharmacokinetic evaluations to determine their suitability as effective compounds *in vivo*. To allow such evaluations and to provide a general, sensitive, rapid and simple method for the analysis of compounds containing the 1,5-diaryl-3-oxo-pentadienyl scaffold, we developed an HPLC method with ultraviolet detection for their detection in various biological matrices of a relevant preclinical species, i.e. the rat. Our HPLC method is specific for the analysis of many members in this series in rat blood, plasma, serum and hepatic microsomes following liquid–liquid extraction with TBME (1:30, v/v). The assay procedure involves chromatographic separation on a Zorbax-Eclipse C-18 column under isocratic conditions with the mobile phase consisting of acetonitrile and ammonium acetate buffer (pH 5.0, 10 mM) in different ratios depending upon the compound. The method was validated for NC 2083 in rat serum and rat liver microsomes, a potential lead compound, to demonstrate its applicability. The standard curve was linear ($r^2 \geq 0.997$) from 50 to 5000 ng/mL. Intra- and inter-day precision and accuracy of the method were within USFDA specified limits. The stability of NC 2083 was established in an auto-injector, on bench-top, during freeze–thaw cycles and long-term stability at −80 °C for 40 days. The method is suitable for a number of compounds containing the 1,5-diaryl-3-oxo-pentadienyl scaffold with divergent log *P* values with only minor adjustments in the buffer to acetonitrile ratio of the mobile phase.

1. Introduction

Many naturally occurring chalcones and flavones contain an aryl ring conjugated with an alpha, beta unsaturated keto group (Ar–CH=CH–CO) [1,2]. Such molecules display various important therapeutic actions [3] and, therefore, these compounds have received considerable interest. Recently, Dimmock and co-workers extended this 3-aryl-2-propenoyl group to a 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (Fig. 1) [4]. The literature refers to this pharmacophore as a curcumin analogue [5], and a substantial number of these compounds have excellent antineoplastic properties [2,6–10]. Other laboratories are also examining the biological potential of this pharmacophore [5,11–13]. In order to initiate some *in vitro* and *in vivo* studies on a rational basis, a robust analytical method is mandatory. In particular, pharmacokinetic evaluations have become necessary to advance promising compounds further in the discovery process and to provide feedback to the rational drug design process. Quantitative determination of this series of compounds will require the availability of a versatile bioanalytical method.

To the authors' best knowledge, no bioanalytical method has been reported for curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group. Therefore, we developed a general HPLC method for the compounds containing this pharmacophore. Due to its widespread accessibility, HPLC with UV detection was selected as the instrument of choice.

We selected 13 structurally diverse compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group with varying log *P* values (1.93–6.40), different substituents (with varying sigma and pi values) in both aryl rings, and different linker groups between C-2 and C-4 (Fig. 1; Table 1). We tested the specificity of the method in different rat biomatrices including plasma, serum, blood and hepatic microsomes. Further, we selected a novel promising cytotoxin in this series, NC 2083 [3,5-bis(4-chlorophenylmethylene)-1-[4-{2-

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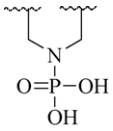
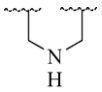
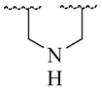
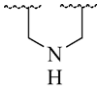
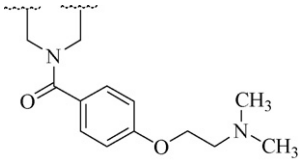
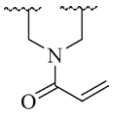
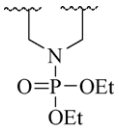
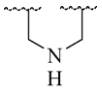
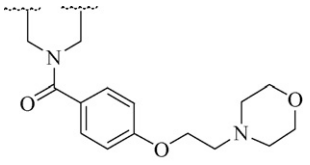
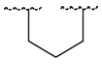
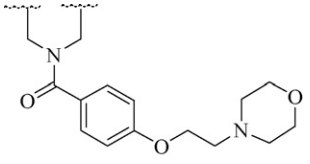
doi:10.1016/j.jchromb.2010.08.034

Table 1
HPLC mobile phase conditions and analyte retention times for the analysis of 1,5-diaryl-3-oxo-pentadiene containing cytotoxic compounds (see Fig. 1).

Code	R^1	R^2	R^3	mi log *P* value	Retention time	ACN:AAB
NC 2324	3,4,5-$(OCH_3)_3$	3,4,5-$(OCH_3)_3$		1.93	6.72	40:60
NC 2443[a]	2-F	2-F		3.23	14.95	40:60
NC 1831	H	H		3.36	9.53	40:60
NC 1834	4-OCH_3	4-OCH_3		3.47	7.49	40:60
NC 2067[b]	H	H		4.60	7.12	40:60
NC 2288	H	H	H	4.18	9.72	60:40
NC 1845	4-OCH_3	4-OCH_3		4.03	5.75	60:40
NC 2313	4-OCH_3	4-OCH_3		4.09	6.59	60:40
NC 1876	4-Cl	4-Cl		4.71	9.71	60:40
NC 2081[b]	H	H		4.45	5.38	60:40
NC 2095	4-NO_2	3,4,5-$(OCH_3)_3$		4.56	14.00	60:40
NC 2164	3,4,5-$(OCH_3)_3$	3,4,5-$(OCH_3)_3$		4.23	9.52	60:40
NC 2083[b]	4-Cl	4-Cl		6.40	11.52	60:40

ACN: acetonitrile; AAB: ammonium acetate buffer (10 mM, pH 5.0); mi log *P*: molinspiration log *P*.
[a] Referred as EF24 in the literature [5,12,22].
[b] Obtained as hydrochloride salt.

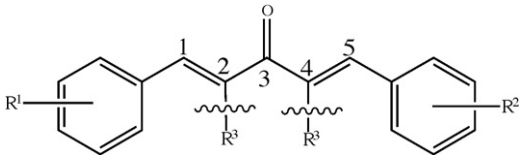


Fig. 1. Basic structure of the 1,5-diaryl-3-oxo-1,4-pentadienyl group containing compounds (see Table 1).

(4-morpholinyl)ethoxy}phenylcarbonyl]-4-piperidone hydrochloride], to validate this method. NC 2083 is under development at the University of Saskatchewan as an antineoplastic agent. This compound has an IC_{50} values of <0.005 and 1.45 μM toward the HCC-2998 and SW620 human colon cancer cells, respectively, *in vitro* [14,15]. The present communication reports a versatile HPLC method for the analysis of compounds that contain the 1,5-diaryl-3-oxo-1,4-pentadienyl substructure, such as NC 2083, in biological fluids. This method can be directly adopted after partial validation for pharmacokinetic screening of the compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group.

2. Materials and methods

2.1. Chemicals and reagents

Compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group were synthesized at the College of Pharmacy and Nutrition, University of Saskatchewan using reported procedures [2,6–9,14]. Tris (tris(hydroxymethyl)aminomethane) and HPLC grade acetonitrile were purchased from Fisher Scientific (Ottawa, ON, Canada). Tris hydrochloride, tert-butyl methyl ether (TBME) and EDTA (ethylenediaminetetraacetic acid) were purchased from EM Sciences (Merck, Darmstadt, Germany). All other chemicals were purchased from Sigma–Aldrich (Oakville, ON, Canada). Milli-Q water with a resistance value of 18.2 MΩ was obtained from a Milli-Q Synthesis Water System (Millipore, MA). Drug free serum, blood and rat liver microsomes (RLM) samples were collected from adult, healthy male Wistar rats. Rat liver microsomes were prepared as described previously [16,17], and the protein content was estimated with Lowry's method using bovine serum albumin as standard [18]. All protocols employed in the collection of serum and hepatic microsomal preparations were in accordance with the Canadian Council of Animal Care (CCAC) guidelines and approved by the University Committee on Animal Care and Supply.

2.2. Method development and validation

2.2.1. Instrumentation and HPLC conditions

The HPLC system consisted of an Agilent series 1200 quaternary pump with an online degasser, auto sampler and diode array detector (Agilent Technologies, Mississauga, ON, Canada). Sample aliquots (50 μL) were injected onto an Eclipse XDB-C_{18} column (4.6 mm × 150 mm, 5 μm) (Agilent Technologies, Mississauga, ON, Canada) and absorbance was monitored at 330 nm. The system was run in an isocratic mode with a mobile phase consisting of acetonitrile:ammonium acetate buffer (pH 5.0, 10 mM) in the ratio of 60:40 (v/v) (for NC 2083) and was delivered at a flow rate of 1 mL/min. An ammonium acetate stock solution (50 mM) was prepared and diluted with Milli-Q water as necessary to achieve a final concentration of 10 mM. The mobile phase was filtered through a 0.22 μm Nylon filter (Pall Scientific, Ville St. Laurent, QC, Canada) and degassed in an ultrasonic bath for 30 min prior to use. The column was maintained at room temperature during the run and subsequently washed with water followed by acetonitrile after every use. The carry over into the autosampler was negated by the injection of blank mobile phase following injection of the highest standard curve concentration. No carryover was detected.

2.2.2. Preparation of stock and working solutions

All stock and working solutions were prepared under low light conditions. Primary stock solutions (1 mg/mL) of analytes and the internal standard (NC 2288) were made by dissolving them first in DMSO and subsequently diluting with acetonitrile such that DMSO in the stock solution was ≤25% (v/v). The stock solutions were diluted with acetonitrile to prepare secondary working stock solutions of 0.5, 1, 2, 5, 10, 20 and 50 μg/mL for NC 2083 and 40 μg/mL for the internal standard. The primary stock solution for the internal standard was also diluted with acetonitrile to prepare a working stock solution (40 μg/mL). Quality control (QC) samples were prepared similarly with stock solutions at 0.5, 1.5, 25 and 40 μg/mL, which were diluted 10-fold with rat blank serum to give the LLOQ (lowest limit of quantitation), low QC (LQC; 0.15 μg/mL), middle QC (MQC; 2.5 μg/mL) and high QC (HQC; 4.0 μg/mL). All stock and working solutions were stored at −20 ± 5 °C and used to prepare the standard curve on the day of analysis.

2.2.3. Preparation of calibration standards and quality control (QC) samples

Calibration standards were prepared with each analysis run by spiking 10 μL of the individual working stock solutions to 90 μL pooled blank rat serum or heat inactivated rat liver microsomes (55 °C for 5 min; 0.4 mg/mL) [16] with vortex mixing for 30 s. A separate analyst prepared quality control samples, which were spiked in bulk at four concentration levels (LLOQ, LQC, MQC and HQC) on the first day of validation. These quality control samples were aliquoted into polypropylene microcentrifuge tubes and stored at −80 ± 5 °C in the dark until analysis. All calibration standards and samples were prepared under low light conditions.

2.2.4. Recovery

The recovery of NC 2083 and NC 2288 was determined following liquid–liquid extraction with tert-butyl methyl ether (TBME). For NC 2083, the recovery was determined at LQC, MQC and HQC, whereas recovery of NC 2288 was determined at 4 μg/mL. The recovery was assessed by comparison of post-extracted serum samples with unextracted samples (in the reconstituting solvent) at the same concentrations. Post-extracted samples were prepared by the addition of 10 μL of appropriate QC working solutions into 90 μL of rat blank serum or rat liver microsomes and processed as described in Section 2.2.5. Unextracted samples were prepared by the addition of 10 μL of working stock solutions of QC samples into 140 μL of the mobile phase.

2.2.5. Sample preparation

Ten μL of the internal standard solution (40 μg/mL) was added into the calibration standards, QC samples, and samples in other rat biomatrices (blood, plasma and liver microsomal suspensions). To each sample, 3 mL of TBME was added and vortex-mixed for 5 min and centrifuged at 778 × *g* for 5 min at 4 °C (5804 R, Eppendorf, Mississauga, ON, Canada). The aqueous layer containing serum was snap frozen using liquid nitrogen and the organic layer was transferred into a glass tube and evaporated to dryness under vacuum at 40 °C in a CentriVap concentrator (LABCONCO, Kansas City, Missouri, USA). The residue was reconstituted in 150 μL of mobile phase and vortex-mixed for 90 s. The reconstituted samples were transferred to amber colored HPLC vials and placed in the autosampler.

2.2.6. Method validation

The full method validation of NC 2083 was performed in accordance with USFDA guidelines for the assay of NC 2083 in rat serum

and partial validation (precision, accuracy, autosampler stability and bench-top stability) was conducted in heat inactivated rat liver microsomes (0.4 mg/mL). The specificity of the method was also established for all analytes in rat serum, plasma, blood and rat liver microsomes.

Specificity was evaluated by analysis of six different rat biomatrix samples to detect any potential interference with co-eluting endogenous substances. Linearity was determined by plotting the ratio of the peak area associated with the analyte concentration of seven calibration standards that ranged from 0.5 to 5 μg/mL to the peak area associated with the internal standard. A linear least square analysis was conducted with $1/\chi^2$ as weighting factor, and the slope, intercept and coefficient of determination (r^2) were determined to establish linearity. The limit of detection (LOD) was defined as the lowest detectable concentration with a signal to noise ratio of 3. The lowest limit of quantification was defined at the lowest concentration that gives precision and accuracy within ±20% of the nominal value.

The intra- and inter-day precision and accuracy of the method was established by analysis of six replicates of samples at four different concentrations (LLOQ, LQC, MQC, HQC) on three different days. Single assay runs were accepted only when the relative standard deviation (RSD) was found to be less than ±15% at all other concentrations except at LLOQ, which allowed ±20%. The criteria for accuracy was set at ±15% of the nominal concentration of the QC samples except at LLOQ, where it was set at ±20%. In no case did more than one third of the QC samples violate these criteria.

Stability studies involving freeze–thaw stability, bench-top stability and long-term stability were undertaken at LQC, MQC, and HQC, whereas autosampler stability was tested at LLOQ also. Freeze–thaw stability was tested after three freeze–thaw cycles spaced at least 24 h apart with sample storage at −80 ± 5 °C between sample thawing. Bench-top stability was established at room temperature and in an ice-bath for 6 h. For autosampler stability, processed and reconstituted samples were kept in an autosampler for 24 h before injection. Predicted concentrations were calculated using newly prepared calibration standards. Samples were stored at −80 ± 5 °C for 30 days before analysis to establish long-term stability. Samples were considered stable when the criteria for precision and accuracy were met.

Dilution integrity was assessed by dilution of 100 μg/mL spiked serum samples to 1 and 0.5 μg/mL with blank serum. These diluted samples were processed and analyzed with criteria for accuracy and precision as described previously.

2.3. Applications of the method

2.3.1. Serum protein binding

Blood was collected from healthy male adult Wistar rats (average weight 300 ± 25 g) via cardiac puncture under isoflurane anaesthesia. After a 30-min coagulation period at room temperature, serum was collected by centrifugation at 3200 rpm for 5 min. NC 2083 in DMSO (0.5 mg/mL) was diluted ten times with serum to yield serum samples ($N = 3$) at a concentration of 50 μg/mL. Serum was equilibrated at 37 °C for 20 min. One mL of serum samples were transferred into a micropartition kit (Millipore, MA, USA) and spun using a fixed angle (25°) rotor (TA-14-50) in a Beckman Coulter Allegra 25R centrifuge (Beckman Coulter, Mississauga, ON, Canada) for eight minutes at 1000 × *g*. The filtrate was quantified for the presence of NC 2083 using reverse phase HPLC–UV method.

2.3.2. Hepatic microsomal stability study

Application of the method involved assessment of the metabolic stability of NC 2083 in rat liver microsomes. The rate of metabolism of NC 2083 was assessed in triplicate using the substrate depletion approach [19] with the following conditions: NC 2083 (5 μg/mL), protein concentration (0.4 mg/mL), phosphate buffer pH 7.4 (50 mM), and $MgCl_2$ (2 mM). The mixture was pre-incubated at 37.0 ± 0.2 °C for 15 min before initiation of the reaction by addition of NADPH (1 mM). The reaction was continued for up to 2 h at 37.0 ± 0.2 °C with orbital shaking at 80 rpm. The reaction was stopped at 0 (pre-incubation), 5, 10, 15, 30, 60, 90 and 120 min by addition of 50 μL of diluted (1:1) phosphoric acid. The samples were analyzed for NC 2083 using the method described above. The amount remaining to be metabolized was calculated and plotted on a log-normal scale to calculate the slope of the initial part of the curve [20]. The metabolic half-life was calculated by dividing 0.693 by the slope.

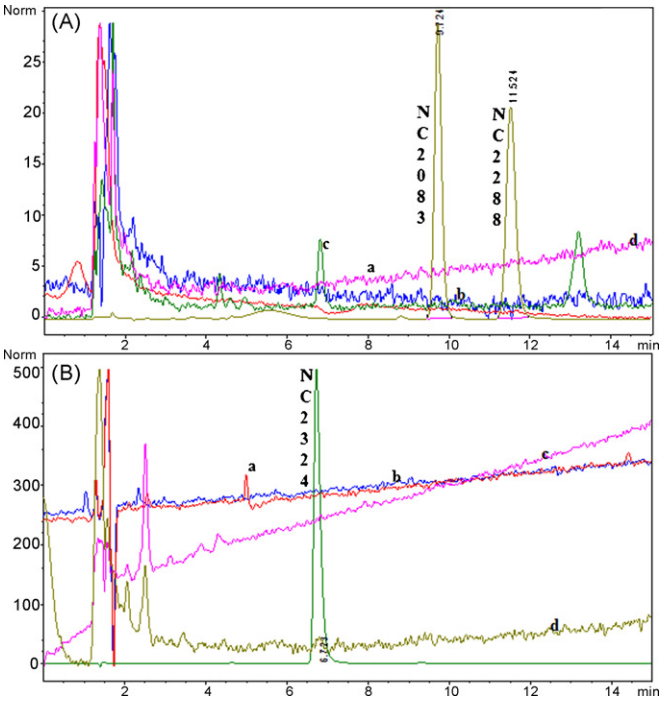


Fig. 2. Chromatogram of (A) NC 2083 and (B) NC 2324 overlaid with chromatograms of blank rat serum (a), plasma (b), blood (c), and liver microsomes (d). (A) 60:40 acetonitrile:ammonium acetate buffer mobile phase composition; (B) 40:60 acetonitrile:ammonium acetate buffer mobile phase composition.

3. Results

3.1. Recovery

Analyte recovery was consistent at different concentration levels. The mean ± SD recoveries of NC 2083 from rat serum were 53.0 ± 6.5, 55.8 ± 3.9 and 55.3 ± 3.2% at the LQC, MQC and HQC, respectively, whereas mean ± SD recoveries from rat liver microsomes were 50.9 ± 2.7, 47.1 ± 3.2 and 50.2 ± 4.4, respectively. The recovery of internal standard at 4 μg/mL concentration from rat serum and rat liver microsomes was found to be 56.8 ± 4.1 and 58.6 ± 4.7%, respectively.

3.2. Method validation

The method is specific for the determination of all the compounds in Table 1. Fig. 2A and B shows representative chromatograms from blank serum, plasma, blood and rat liver microsomes spiked with NC 2083 (with NC 2288 as internal standard) and NC 2324, respectively, as illustrations of specificity at two diverse mobile phase conditions. Adjustment in the ratio of acetonitrile and ammonium acetate buffer allowed chromatographic separation of other compounds containing the 1,5-diaryl-3-

Table 2
Intra-day assay precision and accuracy for NC 2083 determination by HPLC–UV detection in rat serum ($N = 6$).

Quality control (QC)	Analysis day	Observed concentration (mean ± S.D., ng/mL)	Precision[a]	Accuracy[b]
LLOQ (50 ng/mL)	1	48.7 ± 2.3	4.8	97.4
	2	46.4 ± 2.8	6.0	92.8
	3	50.6 ± 2.3	4.5	101.1
LQC (150 ng/mL)	1	148.4 ± 7.0	4.7	98.9
	2	136.7 ± 3.2	2.3	91.1
	3	135.6 ± 5.0	3.7	90.4
MQC (2500 ng/mL)	1	2564.8 ± 68.3	2.7	102.6
	2	2611.5 ± 68.2	2.6	104.5
	3	2504.1 ± 29.0	1.2	100.2
HQC (4000 ng/mL)	1	4058.6 ± 206.2	5.1	101.5
	2	4166.8 ± 103.8	2.5	104.2
	3	4109.7 ± 95.0	2.3	102.7

[a] Expressed as % R.S.D. ((S.D./mean) × 100%).
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

Table 3
Intra-day assay precision and accuracy for NC 2083 determination by HPLC–UV detection in rat liver microsomes ($N = 6$).

Quality control (QC)	Analysis day	Observed concentration (mean ± S.D., ng/mL)	Precision[a]	Accuracy[b]
LLOQ (50 ng/mL)	1	52.8 ± 3.8	7.1	105.6
	2	49.8 ± 3.4	6.9	99.7
	3	51.1 ± 4.5	8.8	102.4
LQC (150 ng/mL)	1	148.2 ± 9.5	6.4	98.8
	2	156.7 ± 10.4	6.6	104.4
	3	147.6 ± 6.3	4.3	98.4
MQC (2500 ng/mL)	1	2784.2 ± 33.2	1.2	111.4
	2	2717.5 ± 44.1	1.6	108.7
	3	2600.5 ± 67.1	2.6	104.0
HQC (4000 ng/mL)	1	4386.4 ± 84.8	1.9	109.7
	2	4241.1 ± 91.1	2.2	104.1
	3	4441.0 ± 75.1	1.7	110.1

[a] Expressed as % R.S.D. ((S.D./mean) × 100%).
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

oxo-1,4-pentadienyl pharmacophore with varying log P values (log P = 1.93–6.40) (Table 1), and specificity was established for these compounds in all rat biomatrices evaluated (Fig. 2). For all analogues, the chromatographic run time was less than 15 min.

The validation results of this method for the quantitative determinations of NC 2083 in rat serum and rat liver microsomes satisfied the criteria specified by USFDA. The validation of other analogues was not carried out at this point in time; however, the laboratories adopting this method need to perform a partial validation for individual compounds.

The limit of detection and limit of quantification based on signal to noise ratios of 3 and 10 were 18.4 and 61.5 ng/mL, respectively. The method was linear in the concentration range of 50–5000 ng/mL with average coefficient of determination values of ≥0.997. The accuracy across different concentration levels of the calibration curve in rat serum and inactivated rat liver microsomes, varied from 90.5 to 108.4% while the average percent CV varied from 0.1 to 5.5 (data not shown). Calibration curves gave reliable reproducibility at different concentration levels on different occasions.

Intra-day and inter-day precision and accuracy in rat serum and liver microsomes is shown in Tables 2–4. Intra-day accuracy in rat serum and liver microsomes ranged from 90.4 to 104.5% and 98.4 to 111.4%, respectively, while precision ranged from 1.2 to 6.0% and 1.2 to 8.8%, respectively. Inter-day accuracy in rat serum and liver microsomes ranged from 93.7 to 102.8% and 100.6 to 108.1%, respectively, while precision ranged from 2.8 to 5.8% and 3.1 to 7.6%, respectively. Dilution integrity of the method in rat serum

Table 4
Inter-day assay precision and accuracy for NC 2083 determination by HPLC–UV detection in rat serum and rat liver microsomes (RLM) ($N = 18$).

	Nominal concentration (ng/mL)	Observed concentration (mean ± S.D., ng/mL)	Precision[a]	Accuracy[b]
Serum	50	48.7 ± 2.9	5.8	97.4
	150	140.5 ± 7.8	5.6	93.7
	2500	2560.1 ± 70.9	2.8	102.4
	4000	4111.7 ± 142.8	3.5	102.8
RLM	50	51.3 ± 3.9	7.6	102.5
	150	150.8 ± 9.4	6.2	100.6
	2500	2700.7 ± 91.2	3.4	108.0
	4000	4322.5 ± 135.4	3.1	108.1

[a] Expressed as %R.S.D. ((S.D./mean) × 100%).
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

Table 5
Stability of NC 2083 in rat serum and rat liver microsomes (RLM) under various storage conditions ($N = 6$).

Quality control (QC)	Storage condition	Concentration[a] (mean ± SD, ng/mL)	Precision[b]	Accuracy[c]
LQC (150 ng/mL)	0 h	148.4 ± 7.0	4.7	98.9
	24 h (AIS)	147.7 ± 6.3	4.3	98.5
	6 h (BT)	119.5 ± 14.1	17.7	79.6
	6 h (BT in dark)	117.3 ± 22.6	19.3	78.2
	6 h (BT on ice in dark)	131.2 ± 4.5	3.4	88.7
	F/T-3rd cycle	136.7 ± 1.8	1.3	91.1
	40 days at −80 °C	133.0 ± 2.9	2.2	88.7
	0 h-RLM	148.2 ± 9.5	6.4	98.8
	24 h (AIS)-RLM	162.1 ± 8.1	5.2	104.0
	6 h (BT on ice in dark)-RLM	150.4 ± 11.7	7.8	100.3
MQC (2500 ng/mL)	0 h	2564.8 ± 68.3	2.7	102.6
	24 h (AIS)	2550.8 ± 52.9	2.1	102.0
	6 h (BT)	1975.1 ± 41.1	2.1	79.0
	6 h (BT in dark)	1999.5 ± 91.5	4.6	80.0
	6 h (BT on ice in dark)	2408.0 ± 52.5	2.2	96.3
	F/T-3rd cycle	2493.7 ± 34.3	1.4	99.8
	40 days at −80 °C	2369.3 ± 40.6	1.7	94.8
	0 h-RLM	2784.2 ± 33.2	1.2	111.4
	24 h (AIS)-RLM	2572.6 ± 141.7	5.5	102.9
	6 h (BT on ice in dark)-RLM	2608.1 ± 129.2	5.1	101.5
HQC (4000 ng/mL)	0 h	4058.6 ± 206.2	5.1	101.5
	24 h (AIS)	4044.7 ± 198.2	4.9	101.1
	6 h (BT)	3211.3 ± 90.9	2.8	80.3
	6 h (BT in dark)	3276.2 ± 168.4	5.1	81.9
	6 h (BT on ice in dark)	3826.6 ± 97.9	2.6	95.7
	F/T-3rd cycle	3914.7 ± 67.5	1.7	97.9
	40 days at −80 °C	3551.4 ± 80.6	2.3	88.8
	0 h-RLM	4386.4 ± 84.8	1.9	109.7
	24 h (AIS)-RLM	4620.4 ± 90.4	2.1	110.2
	6 h (BT on ice in dark)-RLM	4187.4 ± 128.2	3.1	104.7

AIS: auto-injector stability; BT: bench-top stability; F/T: freeze–thaw stability.
[a] Back calculated plasma concentrations.
[b] Expressed as % R.S.D. ((S.D./mean) × 100%).
[c] Calculated as (mean determined concentration/nominal concentration) × 100%.

was assessed following 100–200-fold sample dilutions. The average percent accuracy values were 102.1 ± 2.5 and 95.6 ± 2.8, while precision values were within 2.5% and 2.9% at 100- and 200-fold dilution, respectively, indicating good dilution integrity. Dilution integrity in rat liver microsomes was not established because the microsomal stability was conducted at 5 μg/mL, which was within the linearity range.

Given NC 2083's sensitivity to light, autosampler stability (24 h), bench-top stability (6 h) under light and dark conditions and on ice under dark, freeze–thaw stability (three cycles), and long-term storage stability at −80 ± 5 °C (40 days) of NC 2083 in serum samples were determined. Autosampler stability (24 h) and bench-top stability (6 h; on ice in dark) of NC 2083 in rat liver microsomes were established; however, the freeze–thaw stability and long-term stability studies were not carried out because the microsomal stability study samples were processed immediately after the experiment. NC 2083 was stable under all conditions except on bench-top under light and dark conditions for 6 h (Table 5). These results indicated a need to use an ice-bath under low light conditions during sample preparation.

3.3. Application of the HPLC–UV method to assess

3.3.1. Serum protein binding

More than 99.9% of NC 2083 exists as bound to rat serum proteins. Only free form of the drug exhibits its pharmacological effect, and thus only 0.1% of the compound in systemic circulation will be responsible for its action. Since serum protein-drug complexes are usually reversible, the high protein binding characteristics may contribute to a longer duration of action.

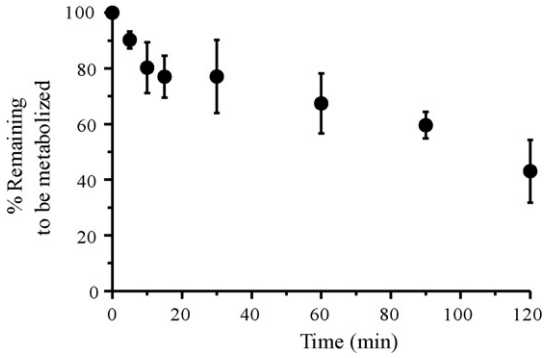


Fig. 3. Mean ± SD of percent remaining to be metabolized of NC 2083 in rat liver microsomes ($N = 3$) using 0.4 mg/mL of protein concentration at different time intervals up to 2 h with incubation at 37 ± 0.2 °C.

3.3.2. Metabolic stability of NC 2083 in rat liver microsomes

Fig. 3 illustrates the metabolic stability of NC 2083 in rat liver microsomes (0.4 mg/mL). Substantial depletion of NC 2083 was observed within 2 h of initiation of the incubation with more than half of the compound metabolized by this time. Substrate depletion was linear up to 10 min on log-normal scale with a coefficient of determination of 0.9985 and a slope of −0.0219 (data not shown). The calculated *in vitro* half-life of NC 2083 in rat liver microsomes was 31.6 min. Based on the classification of McNaney et al. [20], this compound has intermediate systemic clearance suggesting its suitability for further pharmacokinetic and pharmacodynamic evaluations.

4. Discussion

Currently, curcumin analogues containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group are highly promising compounds in antineoplastic drug research. Curcumin itself is poorly bioavailable and unstable at different pH values [21]. Poor bioavailability of the compound is attributed to poor absorption characteristics and/or first pass metabolism (mostly by the liver). Liver microsomal stability screening may identify the potential for extensive first pass metabolism, and such information is critical in the early phases of drug discovery to inform the rational drug design process. Therefore, early identification of curcumin analogues with similar pharmacokinetic problems is necessary. Rapid pharmacokinetic screening (*in vitro* and *in vivo*), though, requires a simple and versatile analytical method for the quantification of different curcumin analogues in an appropriate preclinical species. This experimentation necessitates the development of a general HPLC–UV method that could quantify a large number of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore within reasonably short chromatographic run times and with minimal alterations to chromatographic conditions.

Dimmock and co-workers synthesized a wide range of curcumin analogues possessing highly variable *c* log *P* values (range 1.93–6.40). We chose 13 compounds within the series that demonstrated good cytotoxicity against colon cancer cell lines and widely different log *P* values to develop a simple and versatile HPLC–UV method of analysis. The compound set included 4-piperidone analogues (Fig. 1; $R^3 = -CH_2-NH-CH_2-$) and cyclohexanone analogues (Fig. 1; $R^3 = -CH_2-CH_2-CH_2-$). This method requires minor modifications in the ratio of acetonitrile to ammonium acetate buffer to elute all compounds in a chromatographic run time of less than 15 min.

During the method development, a 60:40 acetonitrile: ammonium acetate buffer mobile phase resulted in analyte retention times that were directly proportional to the polarity of compounds, whereas this trend was reversed when the acetonitrile composition was reduced to 40%. This outcome is difficult to explain as the decrease in elution strength of the mobile phase should increase the retention times of non-polar compounds more than the polar ones; however, the observed trend was reversed. Nevertheless, we could achieve suitable and specific chromatographic separation of all chosen compounds in all rat biomatrices evaluated.

We subsequently validated this method for a promising compound, NC 2083, in rat serum and liver microsomes to allow for further pharmacokinetic evaluations. The method was accurate, precise and specific. NC 2083 was stable under most sample preparation, analytical and storage conditions but was unstable at bench-top for 6 h. Sample preparation under low light conditions with use of an ice-bath provided assurance of NC 2083 stability. Given its putative affinity for thiol-containing proteins, we investigated its ability to bind covalently to albumin to explain the apparent loss of NC 2083 in rat serum during storage at room temperature. Our studies indicated minimal covalent binding with serum albumin (unpublished data) and the cause for this apparent loss of NC 2083 remains unknown.

5. Conclusion

Given the growing interest in 1,5-diaryl-3-oxo-1,4-pentadienyl containing curcumin analogues as a potential class of highly cytotoxic and selective antineoplastic compounds, we describe a versatile, simple, rapid and specific HPLC–UV method for the quantification of different compounds containing this pharmacophore in various rat biomatrices. With minor modification in the mobile phase composition, this method accommodates compounds with a very wide range of *c* log *P* values (1.93–6.40) without losing specificity. Therefore, this method can be directly adopted for preclinical pharmacokinetic studies of this class of compounds; however, a partial validation for suitability of this method to individual compounds in laboratories adopting this method will be necessary.

Acknowledgements

The Canadian Institutes of Health Research (CIHR) and the Forge Ahead Fund from the Industry Liaison office at the University of Saskatchewan funded this project. We would like to thank Jatinder Kaur Mukker for her assistance during the method development process. R.S.P. Singh was supported by a Dean's scholarship, College of Graduate Studies and Research, University of Saskatchewan.

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