DEVELOPMENT AND VALIDATION OF A NEW LC/MS/MS ANALYTICAL METHOD FOR THE DETERMINATION OF THE CURCUMIN IN HORSE PLASMA SAMPLES

Alessia Ceriani, Daniela Chiereghin, Alessio Donatini and Sergio Pengo

R&D Labs S.r.l.
via Gaudenzio Ferrari 21 Saronno - VA

INTRODUCTIONS

Curcumin (CU), a derivative of the plant *Curcuma longa*, is used extensively in the food industry. It is a major component of curry powder, and research has shown that curcumin may prevent cancer and other chronic diseases (1).
A robust, sensitive and accurate analytical method for the LC-MS-MS determination of Curcumin (CU) in equine plasma was developed in our laboratories based on information taken from the literature (2, 3, 4).
This communication describes the experimental conditions used and the results obtained during the validation of the LC-MS-MS method. The Guidance for Industry "Bioanalytical Method Validation" issued by FDA on May 2001 and the GLP recommendations were taken in account as a base for the validation plan.

MATERIALS AND METHODS

TEST ARTICLE

Curcumin (CU), Batch 040309101, Indena, Retesting Date 02.2008, HPLC purity 89.96%.
Naproxene, (IS), batch 075K1030 Sigma-Aldrich, assay 99.8% expiry date September 2008.

MATERIALS

Methanol, HPLC grade
Acetonitrile, HPLC gradient grade
Formic acid 98-100% analytical grade
Blank equine plasma for calibration standards, control samples and QCs
SPE Strata-X 33 µm Polymeric Sorbent 30 mg/1 mL (Phenomenex)
Purified water, HPLC grade, Milli-Q apparatus (Millipore)

CHROMATOGRAPHIC SYSTEM AND CONDITIONS

- 1200L LC-MS-MS (Varian) constituted of:
  Binary pump: Model ProStar 210
  Autosampler: Model ProStar 410 working at 10°C temperature
  Column oven: 30°C
  Detector: Mass spectrometer 1200L, turbo ion-spray source.
- Mode: positive
- Precursor ions: 369.3 for CU; 231.0 for IS
- Mass transition ion-pair: 369.3 → 245.0 for CU
- Mass transition ion-pair: 231.0 → 185.0 for IS
- Collision energy: -6 V for CU; -8 V for IS
- Capillary: 20 V
- Detector Voltage: 1900 V
- Drying gas: 300°C, 24 psi
- Volume injected: 20 µL
- Flow rate: 0.2 – 0.5 mL/min according to the gradient (Table 1)
- Mobile phase: A: Purified water – Acetonitrile - Formic Acid (50:50:0.1 v/v)
  B: Purified water – Acetonitrile - Formic Acid (10:90:0.1 v/v)
- Run time: 6 min
- Acquisition time: 1.6 min (from 2.3 to 3.9 min)
- Guard column: Gemini C18 4x2 mm (Phenomenex)
- Column: Gemini C18 5 µm 100x2 mm (Phenomenex)

REAGENT SOLUTIONS

Methanol 20%: 40 mL of Methanol were transferred into a 200-mL volumetric flask and diluted to volume with purified water.
Formic acid 0.1 M: 0.4 mL of Formic acid were transferred into a 100-mL volumetric flask, diluted to volume with purified water.

MOBILE PHASES

Mobile phase A: Purified water – Acetonitrile - Formic Acid (50:50:0.1 v/v).
250 mL of purified water, 250 mL of Acetonitrile, 0.5 mL of Formic Acid.
Mobile phase B: Purified water – Acetonitrile - Formic Acid (10:90:0.1 v/v).
50 mL of purified water, 450 mL of Acetonitrile, 0.5 mL of Formic Acid.

EXTRACTION PROCEDURE

To each sample, 10 µL of ISWS and 300 µL of Formic acid 0.1 M were added. The sample was loaded on a SPE tubes previously activated with 1 mL of methanol and washed with 1 mL of water. After sample loading, the SPE tubes was washed with 700 µL of methanol 20% and the analytes were eluted with 700 µL of Methanol in a conical glass tube. The organic phase was evaporated at approximately 40°C under a gentle stream of nitrogen. The residue was reconstituted with 150 µL of mobile phase A and transferred into a micro-vial. A 20-µL aliquot was injected into the chromatographic system.

CALIBRATION CURVES AND CALCULATIONS

Calibration data were calculated using CU/IS peak area ratios. Regression analysis was performed by the method of weighed least square regression analysis ($1/x^2$) as defined during the set up of the method.
The observed concentrations in calibration standards and quality control samples were calculated from the regression equation:

$y = a + bx$ x = Curcumin concentration
y = peak area Curcumin/IS ratio
a = intercept, b = slope

Table 1

Step	Time (min:sec)	Flow (mL/min)	A (%)	B (%)
1	Initial	0.2	100	0
2	0:30	0.2	100	0
3	1:30	0.2	0	100
4	3:55	0.2	0	100
5	3:56	0.5	100	0
6	4:55	0.5	100	0
7	4:58	0.2	100	0
8	6:00	0.2	100	0

VALIDATION PROGRAM

The method was validated with regard to the following parameters:

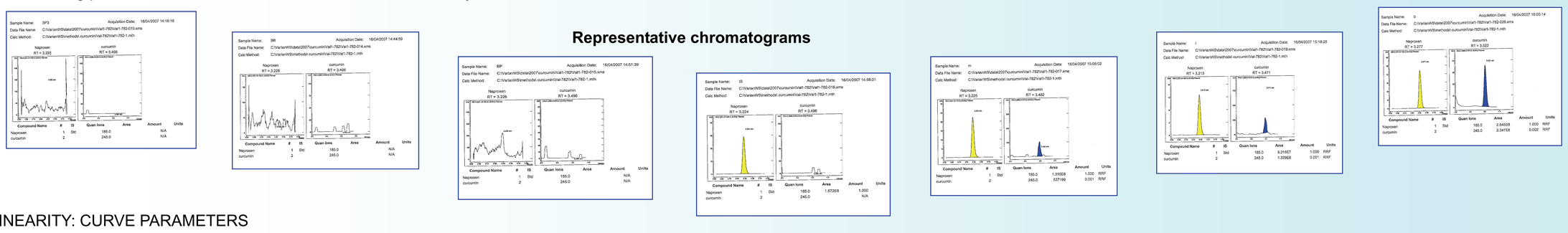
- Selectivity
- Linearity
- Lower Limit of quantification
- Limit of detection
- Precision
- Accuracy
- Recovery
- Post-Preparative Stability
- Short-Term Temperature Stability
- Freeze/Thaw Stability
- Long-Term Stability
- Working Standard Solutions Stability

RESULTS

SELECTIVITY
Selectivity was assessed by checking chromatograms of control blanks (plasma and reagent), of pure and extracted plasma standards to determine whether any interfering peak was present at the retention time of each analyte.
Selectivity included: Blank plasma, blank reagent, blank plasma and zero-level sample (IS).

Representative chromatograms included blank plasma sample (BP3), a blank reagent (BR), a blank plasma (BP), a zero-level sample (IS), a plasma standard at 5.275 ng/mL (LLOQ) (m), 15.825 ng/mL (i) and 527.500 ng/mL (b).
No interfering peaks were observed at the retention time of the analytes.

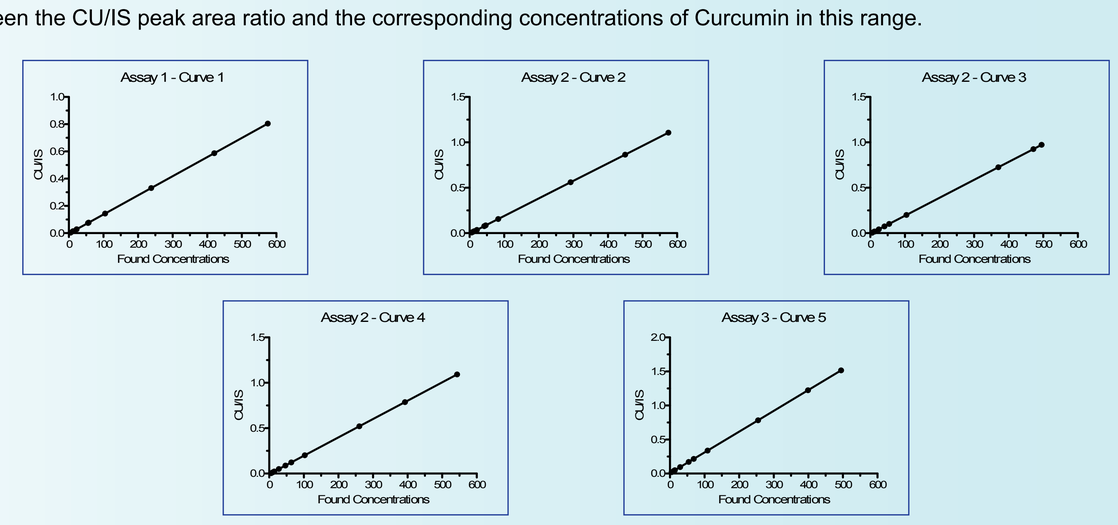
Representative chromatograms



LINEARITY: CURVE PARAMETERS
The concentration range studied was from 5.275 to 527.500 ng/mL. A linear correlation was found between the CU/IS peak area ratio and the corresponding concentrations of Curcumin in this range.
Date of the analysis of the calibration curves and curve parameters are in the Table 2.

Table 2

Assay no.	Curve no.	Initial date	Initial time	Final date	Final time	Curcumin Curve parameters		
		dd.mm.yy	hh.mm.ss	dd.mm.yy	hh.mm.ss	Slope	Intercept	R^2
1	1	16.04.07	15.05.02	16.04.07	16.05.14	0.001404	-0.003060	0.993027
2	2	17.04.07	15.29.21	17.04.07	16.29.28	0.001933	-0.004775	0.999467
2	3	17.04.07	17.43.13	17.04.07	18.43.28	0.001976	-0.006037	0.988528
2	4	17.04.07	19.57.08	17.04.07	20.57.16	0.002022	-0.006388	0.996451
3	5	19.04.07	16.58.22	19.04.07	17.58.33	0.003051	0.005934	0.999630



R-square values were >0.988. This result indicates an acceptable degree of linearity for Curcumin.

QUANTIFICATION (LLOQ) AND DETECTION LIMIT (LOD)
LLOQ was set at 5.275 ng/mL. LOD was of 1.25 ng/mL.

PRECISION AND ACCURACY
The following QCs were studied: LQC: 15.825 ng/mL (3 times the LLOQ) - MQC: 52.750 ng/mL - HQC: 422.000 ng/mL (80% of the ULOQ)
Intra-assay precision was ≤11.78%, intra-assay accuracy ranged 93.30-109.81%
Inter-assay precision was ≤8.52%, inter-assay accuracy ranged 99.84-100.29%

RECOVERY
Recovery of Curcumin resulted to be 74.45% (RSD 6.47%) and that of Internal Standard 80.94% (RSD 8.96%), values considered well acceptable. At all level studied, precision was within 15%.

POST-PREPARATIVE STABILITY
Stability was confirmed by comparing the concentration between Time-0 and 33 h. The mean percent difference was of 5%.

SHORT-TERM TEMPERATURE STABILITY
For each sample, percent difference versus the mean value of Time-0 samples did not exceed 15%, indicating that the analyte was stable when kept for at least 5 hours at room temperature after thawing.

FREEZE/THAW STABILITY
For each sample, percent difference versus the mean value of Time-0 samples did not exceed 15%, as required by the acceptance criteria, indicating that the analyte was stable to at least three freeze/thaw cycles.

LONG-TERM STABILITY
The analysis of blank plasma samples spiked with 14.85 ng/mL and 396 ng/mL of Curcumin on February 2, 2007 was performed on April 24, 2007 after 81 days of storage at -80°C.
R-square value was >0.993 indicating an acceptable degree of linearity.

WORKING STANDARD SOLUTION STABILITY
Working standard solutions of Curcumin and IS were prepared on April 11, 2007 and stored at -80°C. On April 26, 2007, pure standards at concentration corresponding to LQC, MQC and HQC were prepared and injected into the system. The data indicate that the period of validity for WSS of Curcumin and IS is at least 15 days.

CONCLUSIONS

The results of this study indicate that:
Specificity was acceptable as no interferences were present in blank equine plasma samples; the calibration curves resulted to be linear in the range 5.275-527.500 ng/mL with a r^2 of >0.988; intra-assay precision (RSD%) of quality control samples was ≤11.78%; intra-assay accuracy ranged from 93.30 to 109.81%; inter-assay precision (RSD%) of quality control samples was ≤8.52%; inter-assay accuracy ranged from 99.84 to 100.29%; the lower limit of quantification was set at 5.275 ng/mL with a precision of 4.51 and an accuracy of 99.76%; recovery of Curcumin resulted to be 74.45% (RSD 6.47%) and that of Internal Standard 80.94% (RSD 8.96%).
Post-Preparative Stability studied for a period of 33 h did not indicate any degradation of the analytes; short-term and Freeze/Thaw Stability study did not indicate any degradation of the analyte; the analysis of Long-Term Stability samples indicates that Curcumin was stable for 81 days at -80°C; WSS resulted to be stable for at least 15 days.
The results of this study indicate that the method showed an acceptable degree of linearity, accuracy and precision when applied to equine plasma samples spiked with appropriate concentrations of Curcumin and IS.

REFERENCES

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