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Utilization of UPLC-tandem mass spectrometry for quantitation of colouring matters in dietary formulation

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ABSTRACT

A simple and sensitive ultra-performance liquid chromatography with Tandem Mass Spectrometric detection (UPLC-MS/MS) method was developed and validated for the simultaneous quantitation of two food colouring matters, Curcumin (CUR) and Riboflavin (RIB). Chromatographic separation was done on Hypersil gold 50×2.1 mm (1.9 µm) column, with gradient programing of mobile phase starting with aqueous 0.1% formic acid and increasing the percentage of 0.1% formic acid in acetonitrile. The utilization of multiple reaction monitoring (MRM) improved the selectivity of detection and decreased the matrix effect. The method was linear in the range of 5-500 ng/mL for CUR and RIB. Intra- and inter-day reproducibility were within the accepted criteria. The method was successfully applied for the determination the laboratory prepared mixtures of the two selected colouring matters. The use of mass spectrometry enhanced the selectivity and sensitivity of detection which allows the robust use of the method in routine quality control tests of the two colouring matters (CUR and RIB).

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1. Introduction

Food additives are currently used in many infant, dietary and pharmaceutical formulations. They are used to improve the taste, colour or appearance of a processed food. Food additives are defined as substances with or without a little nutritive importance which are used in food industries during processing [1]. Colouring matters are the most commonly used food additives. Curcumin and riboflavin colouring matters are widely used colouring matters in infants, dietary and pharmaceutical products (Figure 1).

There are several methods of analysis of the selected colouring matters in food products using different analytical techniques either alone or in combination with other food additives. For colouring matter, the intrinsic colour allowed to use visible and florescence spectrophotometric techniques [2-4]. Chromatographic techniques are the most commonly used for analysis of the selected food additives using different methods of detection for example; UV-Visible, fluorescence and photodiode array detectors [5-8].

Recently in the last two decades, tandem mass spectrophotometry became the most useful and reliable technique to overcome the poor sensitivity and selectivity of other tools. Also, the use of multiple reaction monitoring decreases the matrix interference [9,10].

To our knowledge, there are few analytical methods using LC-MS/MS in quantitation of the selected colouring matters [9-11]. There is a big gap in analysis of the selected food additives using this sensitive technique in infant, dietary and pharmaceutical formulations. The aim of this work is to cover this gap and provide a validated and robust ULPC-tandem mass spectrometry method that can be used for the analysis of the selected food additives (CUR and RIB).

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of analytical grade and solvents were of HPLC grade. Curcumin (CUR) (98%), riboflavin (RIB) (98%), diphenhydramine (IS) (98%), methanol, acetonitrile

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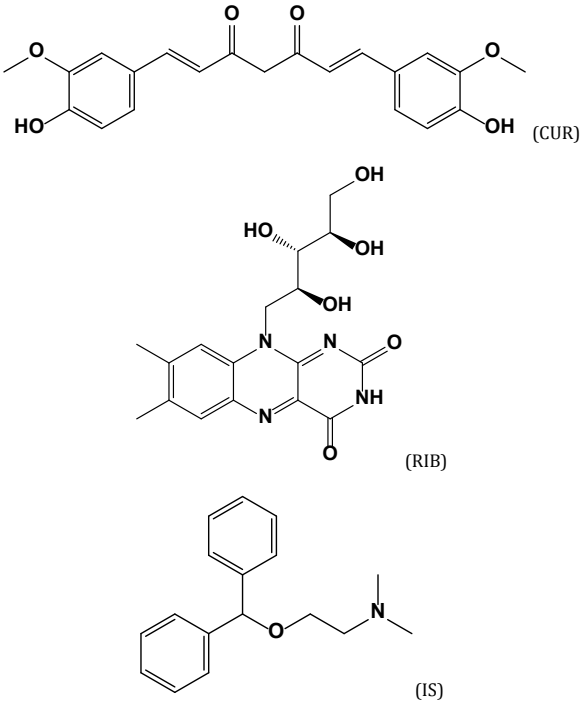

Figure 1. Chemical structures of curcumin, riboflavin and diphenhydramine.

and formic acid were purchased from Sigma-Aldrich, Germany. MiliQ water from Elga Labwater, Prima 7 (UK) was used during the experiment.

2.2. Instrumentations

The assay was done using a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer (Thermo Scientific, New York, USA) equipped with an electrospray ionization (ESI) source and connected to Accela U-HPLC system which was composed of Accela 1250 quaternary pump and Accela open autosampler, New York, USA (operated at 25 °C). Acquisition and processing of the data were performed using Xcalibur software version 2.2.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved on Hypersil Gold column (C18-bonded ultrapure silica based column) 50×2.1 mm (1.9 µm). Binary mixture of aqueous 0.1% formic acid and 0.1% formic acid in acetonitrile was used following gradient elution with flow rate of 0.3 mL/min, oven temperature was adjusted at 40 °C. Gradient programing started from: 20% mobile phase B at zero time then ramped to 90% mobile phase B from 0.0-1.5 min, hold at 90% mobile phase B till 3 min, back to 20% mobile phase B from 3.0-5.0 min. The injection volume was 2 µL and the total run time for each sample was 5 min. Mass spectrometer was run in positive-ion mode for all analytes and eluate was introduced to mass scanner using electrospray ionization (ESI).

Quadrupole mass spectrometer used multiple reaction monitoring mode. The optimized parameters are: auxiliary gas of 5 psi, sheath gas of 25 psi, capillary temperature of 270 °C, turbo ion spray temperature of 400 °C and ion spray voltage of 3600 V. The transition of molecular ions to the product ions for CUR (m/z) 369.1→ 177.2, RIB (m/z) 377.18→ 243.2, and IS (m/z) 256.20 → 167.16. The collision energies were 23, 24 and 19 V for CUR, RIB, and IS, respectively.

2.4. Standard solutions

Stock standard solutions of 1 mg/mL of each analyte and IS were prepared in methanol and stored at 4 °C. Appropriate dilution of each standard solution was done using methanol to obtain the required working standard solutions and were also stored at 4 °C.

2.5. Procedures

2.5.1. Linearity and calibration range

Each calibrant was prepared from the working standard solution of each analyte using the required dilution. The concentrations of the calibrants were ranging from 5-500 ng/mL for RIB and CUR. IS (50 ng/mL) is added to each calibrant solution. A volume of 2 µL of each solution was injected into the LC-MS/MS system. The response of each calibrant was expressed as ratio of its peak area to IS peak areas and was plotted versus the corresponding concentrations.

2.5.2. Laboratory prepared mixtures

The working standard solutions of each analyte were mixed in different ratios to obtain binary solutions of CUR and RIB in the concentration range of 5-500 ng/mL, respectively, using the same procedures in Section 2.5.1.

3. Results and discussion

3.1. Method development

CUR contains hydroxyl group (OH), connected to an aromatic ring which enhance the formation of $[M+H]^+$ and it is easily ionized in ESI. RIB contains aliphatic amino group (NH) which is easily ionized to form $[M+H]^+$. Different mass parameters were optimized to improve the intensity of peaks for example: optimizing sheath gas that facilitate the introduction of ions through the orifice of mass analyser, in

Table 1. Regression parameters for CUR and RIB by the proposed LC-MS/MS method.

Item	CUR	RIB
Linearity range (ng/mL)	5-500	5-500
Slope (b)	0.0416	0.0218
Intercept (a)	0.0044	0.0021
LOD	1.2	1.8
LOQ	3.6	5.5
r^2	0.999	0.999

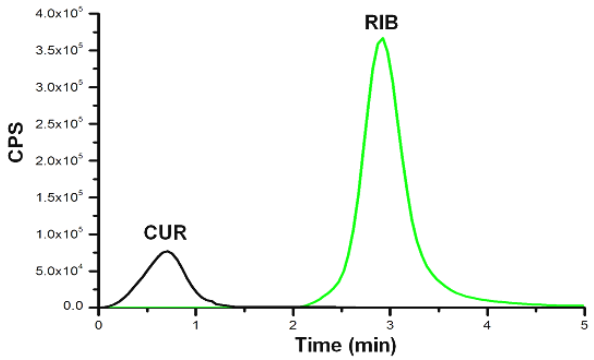

Figure 2. Chromatogram of CUR and RIB at their upper limit of quantitation (ULOQ).

which overusing of gas flow decreased the intensity due to dispersion of the ions. Also a spray voltage (the voltage that is responsible for attracting the ion toward the cone of mass analyser) of less than 3600 V for the positive mode is not enough to attract the ions toward mass analyser. Collision energy voltage (CE) is one of the critical parameters in MRM and its optimization will improve the S/N (Signal/ Nosie) to decrease ion suppression or enhancement of matrix. Under the optimized mass parameters, different mobile phases (Water with acetonitrile or methanol) containing neutral modifiers (Ammonium formate and acetate) and acidic modifiers (formic acid and acetic acid) on HILIC (Hydrophilic Interaction Chromatography), biphenyl and C18 columns were used to select the most efficient system. Under the optimized mass and chromatographic parameters for the two analytes were separated from each other with good resolution as shown in Figure 2. Different washing solvents were tested to avoid carryover. The addition of formic acid in the mobile phase helps to improve peak sharpness. Peak shape was satisfactory for quantitative work even at very low concent-rations.

3.2. Method validation

Method was validated in terms of linearity, ranges, limits of detection, and limits of quantification, accuracy and precision. The method was validated according to the International Council for Harmonisation (ICH) guidelines [12].

3.2.1. Linearity and range

Using the optimized mass and chromatographic parameters, the good linear relationships were obtained between concentration and peak area ratio for analytes. The calibration curve was found to be linear in the concentration ranges of 5-500.0 ng/mL for CUR and RIB. The regression parameters are listed in Table 1. Linear regression analysis of the data gave the following equations:

$$CUR: R = 0.0044 + 0.0416\ C\ (r^2 = 0.999) \quad (1)$$

$$RIB: R = 0.0021 + 0.0218\ C\ (r^2 = 0.999) \quad (2)$$

where R is the peak areas ratio and C is the concentration of drug in ng/mL and r^2 is the regression coefficient. The high values of the correlation coefficients (>0.999) indicate good linearity of the calibration graphs.

3.2.2. Limit of quantitation and limit of detection

The limit of detection (LOD) is the lowest concentration of analyte that can be detected, while the limit of quantitation (LOQ) is the lowest concentration of analyte that can be quantified by the method. Calculations of LOD or LOQ were done base on standard deviation (S.D.) of the response and slope of calibration curve (Table 1).

$$LOD = 3.3\ \sigma/s \quad (3)$$

$$LOQ = 10\ \sigma/s \quad (4)$$

where s = Slope of calibration curve and σ = residual standard deviation of response.

Residual standard deviation (S.D.) of response could be calculated from S.D. of blank response or residual standard deviation of the regression line (*y*-residual) or S.D. of *y*-intercept of the regression line $S_{y/x}$, (Standard error of estimate) [13]. In the proposed method calculation was done based on S.D. of the intercept. The results were listed in Table 1.

3.2.3. Accuracy

Evaluation of the accuracy of the proposed method was made by the analysis of five concentrations of the standard solution of each drug each concentration repeated three times. The recovery % was calculated and results of the proposed method were statistically tested for accuracy (Table 2).

3.2.4. Precision

Evaluation of the intra-day precision was made by replicate assay of the standard solutions of the studied drugs on the same day, while the inter-day precision was evaluated through replicate the assay of standard solutions of the studied drugs on three successive days (Table 2).

Table 2. Data of accuracy and precision obtained by the proposed method and the reported ones [3] for the analysis of CUR and RIB in pure form *.

Item	CUR	RIB
Mean ± S.D.	99.85±0.58	99.92±0.72
% R.S.D.	5.8	7.2
n	5	5
% Error (% R.S.D./√n)	2.59	3.22
Intra-day accuracy	99.96±0.64	99.46±0.34
Inter-day accuracy	99.70±0.33	99.80±0.93

* S.D.: Standard deviation; %R.S.D.: Percent relative standard deviation.

Table 3. Results of system suitability of the proposed method.

Compound	Retention time (min)	Capacity factor (k)	Selectivity (α)	Resolution	Tailing factor	Theoretical plates	HETP *
CUR	0.8	0.43	-	-	1.65	3756	0.006
RIB	3.2	4.71	4.00	1.37	1.45	2280	0.035

* High equivalent to theoretical plate.

Table 4. Determination of CUR and RIB in laboratory prepared mixtures by the proposed LC-MS/MS method.

Concentration (ng/mL)		Area ratio		Concentration found (ng/mL)		Recovery percentage	
CUR	RIB	CUR	RIB	CUR	RIB	CUR	RIB
5	5	0.451	0.052	4.9	4.8	97.6	96.6
25	25	0.626	0.098	24.7	24.4	98.7	97.7
50	50	1.173	0.074	48.4	48.9	96.8	97.8
100	100	1.351	0.144	99.0	98.0	99.0	98.0
200	200	2.053	0.119	194.3	192.3	97.1	96.2
400	400	0.984	0.229	385.1	388.9	96.3	97.2
Mean						97.6	97.3
S.D.						1.0648	0.7336
% RSD						1.09	0.75

The value of standard deviation (S.D.) was small what indicates that the repeatability of the proposed method is good.

3.2.5. System suitability

System suitability applied to confirm the suitability of chromatographic system for analysis with high degrees of accuracy and precision. The suitability of method was done by determination of analytes concentration using external method (Table 3).

3.2.6. Robustness of the method

The robustness of an analytical method measures the capacity of the method to restrain minute but deliberate changes in method parameters [4]. Evaluation of the robustness of the proposed method was done for the chromatographic parameters as well as, the mass parameters, e.g. flow rate of mobile phase (±10 µL/min), vaporizer temperature or transfer capillary temperature (±5 °C), collision energy (±2 V) and sheath gas pressure (±5 psi). The changes in theses parameters did not show significant changes in the values of peak areas.

3.3. Application of the proposed method

The proposed method was applied for analysis laboratory mixture of CUR and RIB in different proportions. Satisfactory results were obtained. The concentration of each drug was calculated from its regression equation (Table 4).

4. Conclusion

We developed and validated UPLC-MS/MS method for simultaneous determination of CUR and RIB in dietary formulation. The utilization of UPLC improves peak resolution and separation in short time to save time and solvents. The method is simple, rapid, selective and sensitive. The proposed method was suitable for routine analysis and quality control testing of combined mixtures of the analytes.

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Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

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