Direct UPLC–MS–MS Validated Method for the Quantification of 5-Aminolevulinic Acid: Application to in-vitro Assessment of Colonic-Targeted Oral Tablets

Ibrahim A. Alsarra[1,2,*], Alaa Eldeen B. Yassin[2], Magdi Abdel-Hamid[3], Fars K. Alanazi[2,3], and **Ibrahim A. Aljuffali[2,4]**

[1]Center of Excellence in Biotechnology Research, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; [2]Department of Pharmaceutics, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia; [3]Kayyali Chair for Pharmaceutical Industry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia; and [4]Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, 250 Green Street, Athens, GA 30602, USA

Abstract

A reliable, sensitive, specific, and rapid ultra-performance liquid chromatography–tandem mass spectrometric (UPLC–MS–MS) method was developed for the determination of 5-aminolevulinic acid (5-ALA) in orally-administered colonic delivery system. The prepared system is a compression-coated tablet using granulated chitosan as the coat layer. L-Tyrosine (TYR) was used as an internal standard with no need for derivatization. The chromatographic system consisted of Acquity UPLC BEH C18 column and isocratic mobile phase composed of acetonitrile and 0.1% formic acid with a flow rate of 2.5 min. The assay was based on ESI+ mode in a multiple reaction monitoring (MRM) transitions at *m/z* 132.08 > 86.0 and *m/z* 132.08 > 114.0 and *m/z* 182.1 > 91.2 for 5-ALA and TYR, respectively. Limit of quantification was 5.0 ng/mL and the calibration curve was linear (r^2 = 0.994). Within-run precision and between-run repeatability were expressed as relative standard deviation and were lower than 2.5%. The recoveries from control samples were > 95%. The method was successfully applied for evaluation in assay and release profile of 5-ALA colon targeted tablets media containing suspended rat cecal contents pH 6.8 medium (colonic) for colonic delivery.

Introduction

5-Aminolevulinic acid hydrochloride (ALA) (Figure 1A) is a small water soluble precursor of porphyrin in the first step of the heme biosynthesis in the body (1,2). Porphyrins are water-soluble biological pigments occurring widely in animal and plant tissues. They play important biological functions, such as metal-binding cofactors in hemoglobin of animal blood, chlorophyll in plants for photosynthesis, and certain enzymes for cell respiration (3,4).

Photodynamic therapy (PDT) is a binary therapeutic modality, which is currently under investigation for the treatment of several kinds of malignancies (5). It relies on the interaction of two individually harmless components: a photosensitizer and an external source of radiation (6). PDT with topical 5-ALA had been used successfully in the treatment of a number of neoplastic lesions or pre-maliganat disorders, including basal cell carcinoma, actinic keratosis, Bowen's disease, vulval Page's disease as well as non-neoplastic diseases (5,6).

5-ALA elicits synthesis and accumulation of fluorescent porphyrins (protoporphyrin IX) in epithelia and neoplastic tissues, such as malignant gliomas (7). It is used to visualize tumorous tissue in neurosurgical procedures. Studies have shown that the intraoperative use of this guiding method may reduce the tumor residual volume and prolong progression-free survival in patients (8).

5-ALA belongs to a class of alpha-amino ketones with a weak chromophoric carbonyl group and, consequently, is unsuitable for quantification by conventional ultraviolet (UV) absorption spectroscopy (4), and under certain conditions, dimerization may result in formation of cyclic pyrazin derivatives (9,10). 5-ALA also suffers from instability, especially in buffered solutions at physiological pH, when administered orally or intravesically into the bladder (9).

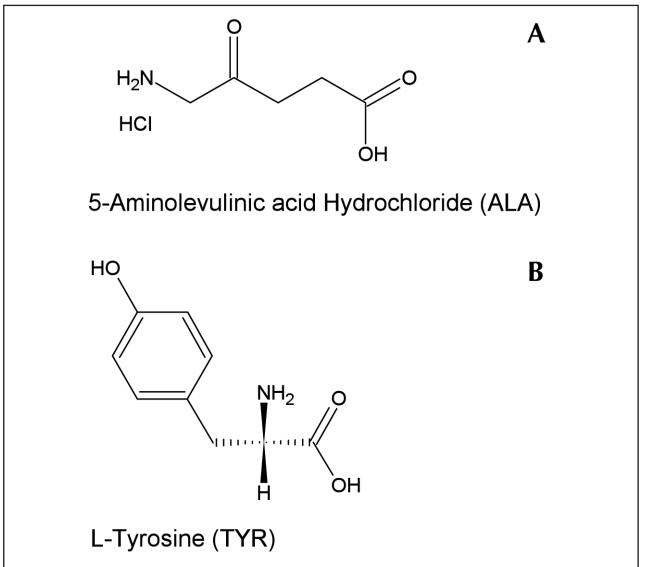

Figure 1. Chemical structures of 5-Aminolevulinic acid (ALA) (A) and L-Tyrosine (B).

*Author to whom correspondence should be addressed: email ialsarra@ksu.edu.sa.

A number of approaches have been used for quantification of 5-ALA in biological fluids including spectrophotometric (2,11,12), fluorimetric (13), fourier transform infrared spectroscopy for detection of 5-ALA and its hexyl ester (14), and capillary electrophoresis (15). HPLC is one of the most routine tools for pharmaceutical analysis in both industrial and university laboratories (4). Consequently, analytical methods using chromatography with chemical derivatization are most commonly utilized in the quantification of 5-ALA.

A drawback of the reported chemical derivatization methods includes the instability of the o-phthaldehyde 5-ALA complex resulting in decreasing fluorescence intensity with time (16). A method involving fluorenylmethyloxycarbonylchloride (FMOC) for 5-ALA derivatization was reported to be more robust than the standard o-phthaladehyde method. The FMOC/ALA-complex was stable with no change in its fluorescence chromatography after 1 week storage at room temperature in daylight (17). The 2-amino-3-hydroxyl naphthalene was also used to derivatize 5-ALA in biological samples (18). These long derivatization methods are unapplicable for release monitoring in different aqueous media with varying pH values containing very low 5-ALA concentrations, as expected for delayed release formulation.

The detection and quantification of 5-ALA in cells and biological fluids is of considerable significance, not only in PDT, but also in many other fields, especially in the treatment and diagnosis of colon cancer. Tandem mass spectrometry (MS–MS) is a powerful technique for the quantitative analysis of small molecules. It has been extensively used as a quantitative technique in the analysis of drugs in biological matrices with high sensitivity, selectivity, and speed (19). The technique is based on physicochemical characteristics of analytes in an electrical field at near vacuum and subsequent collision induced fragmentation (20). In general practice, a tandem mass spectrometer is operated in a combination with high-performance liquid chromatography (HPLC), which greatly increases its analytical performance (21). Ultra-performance liquid chromatography (UPLC) offers greater analytical power because it allows down-scaling of sample volume and high throughput sample handling. Therefore, it supersedes HPLC–MS–MS in analytical performance.

The purpose of this work was to develop an efficient and reproducible analytical technique for quantifying 5-ALA in rat caecal contents medium as a standard in-vitro test for the evaluation of the colon selectivity for colonic targeted oral systems utilizing UPLC–MS–MS.

Experimental

Materials

5-Aminolevulinic acid hydrochloride (5-ALA) (purity approximately ≥ 97) and chitosan (high molecular weight) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). L-Tyrosine (TYR) (Figure 1B), formic acid (98%), hydrochloric acid, and acetonitrile were purchased from BDH Laboratory Supplies (BDH Chemicals Ltd., Poole, UK). Tribasic ammonium phosphate was purchased from Riedel Dehaën (Darmstadt, Germany). Deionized water was obtained by passage distilled water through ELGA (a trade name of Vivendi Water Systems Ltd., Wycombe, Bucks, UK) and was further filtered through a 0.2 µm membrane filter (Millipore, Bedford, MA). All solvents used were of HPLC-grade, while other chemicals and reagents were of analytical-grade, and were obtained from BDH Laboratories Supplies (BDH Chemical Ltd., Poole, UK).

Preparation of standard solutions

No isotopically labeled internal standard was commercially available for 5-ALA. Alternatively, TYR was used as internal standard (IS) and the stock standard solution for 5-ALA was prepared at a concentration level of 1000 ppm, then serially diluted to give 12 concentrations ranging from 5.0 to 2500 ppb. To each concentration, TYR solution was added at 250 ppb. Four quality control (QC) solutions at different levels were chosen to encompass the whole working range at 10.0, 100, 1000, and 2500 ppb levels.

LC–MS–MS method

Liquid chromatography was performed on Acquity (UPLC) system, using Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm × 50 mm) obtained from Waters (Waters Inc., Bedford, MA). Nitrogen was generated using Peak NM30LA-MS generator. Analyses were performed in an isocratic elution mode using an aqueous 0.1% formic acid–acetonitrile (9:1, v/v), with a flow rate of 0.5 mL/min, and the injection volume was 5 µL. The mobile phases were filtered through a 0.2 µm filter before use. The mass spectrometer was a Waters TQD, equipped with ESCI interface operated in positive electrospray ionization mode (ESI^+) using multiple reactions monitoring (MRM) method. Nitrogen gas was used as desolvation gas and cone gas with a flow rate of 650 and 50 L/h, respectively, while argon gas was used as collision gas with flow rate 0.2 mL/min. The source was kept at 120°C, while the desolvation temperature was 450°C. Capillary extractor and RF lens were set at 3.00 kV, 3.00 V, and 0.1 V, respectively.

Validation experiments

The validation experiments were designed to test several performance parameters, such as linearity range, sensitivity, limit of detection, limit of quantitation, accuracy, intra- and inter-day precision, sample thermal stability, matrix effects, and carry over effect. Sextet analyses of all 12 calibrator solutions constituted a working linear range experiment. Before each validation experiment, the highest concentration calibrator was injected into the column until a reproducible response was obtained. A blank mobile phase was injected in triplicates to determine the carry-over effect.

The intra-day precision was determined by six replicate analyses of quality control (QC) samples at the four concentrations, while the inter-day precision was determined by repeated QC analysis performed on six different days repeatedly as sextets. The concentration in each sample was determined using fresh calibration standards prepared on the same day, and the precision was calculated as the relative standard deviation (RSD).

Thermal stability was assessed by subjecting QC solutions to two different temperatures 5°C and 20°C (room temperature) for 6 h. Solutions of 5-ALA with the same QC levels prepared in an ammonium phosphate buffer (pH 6.8) were also evaluated. Aliquots (1 mL) were withdrawn every 30 min and subjected to

analysis after suitable dilution. For estimation of carry-over effect, a blank solvent was injected in triplicates after the analysis of the highest calibration sample over three validation days. The area of 5-ALA and TYR peaks at their expected retention times in blank solvent were calculated.

Formulation of 5-ALA compression-coated tablets

The compression-coated tablets were prepared using the same formula and procedure introduced by Yassin et al. (22). Firstly, the core tablets were prepared with a formula containing 25 mg 5-ALA, 0.8 mg starch 1500, 0.6 mg magnesium stearate, and 27 mg Avicel PH 101 by direct compression using Erweka tablet compression machine (Erweka-Type AR400, Erweka Apparatebau, Heusenstamm, Germany). Each of the prepared core tablets were subjected to compression coating with 100 mg 10% PVP granulated chitosan (high molecular weight) using a suitable larger compression set. Simply, half the amount of the coating material was placed in the die and the tablet was positioned in the center, while the remaining of the coating material was added and the compression was applied.

In-vitro evaluation of the colon selectivity of the prepared tablets

Yassin et al. (22) showed that the prepared coated tablets was found to be completely resistant to both the in-vitro gastric representative medium (0.1 N HCl, pH 1.2) and intestinal representative medium (phosphate buffer, pH 6.8). The colon selectivity of the system was evaluated by monitoring the release profile in medium containing rat caecal contents.

Drug release in medium containing rat caecal contents

Male rats of mixed breeds (Wistar and albino) weighing 200–300 g were used throughout this study. The rats were killed while under ether anesthesia and the caeci were exteriorized, legated at the two-ends, and cut-loose. The experimental protocols were approved by the Animal Care and Use Committee and were in accordance with the recommendations in the University Guide for the Care and Use of Laboratory Animals (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia). The contents of the formed caecal bags were individually weighed, pooled, and suspended in a chilled ammonium phosphate buffer medium (pH 6.8) to give a final dilution of 3% (w/v). Three tablets were incubated in 100 mL of the suspension at 37°C ± 0.5 and shaken at 80 rpm using a thermostatic shaking water bath. The experiments were performed under nitrogen atmosphere to simulate anaerobic conditions. Samples (1 mL) were withdrawn at different time intervals and centrifuged at 6000 rpm for 20 min. Aliquots of 500 µL were taken from the supernatant, diluted to 25 mL, spiked with 250 ppb of TYR, and were replaced with fresh media to maintain the sink conditions. Incubating of a tablet in the buffer without rat caecal contents was used as a control experiment.

Statistical analysis

The significance of difference between the cumulative percentage release of 5-ALA from tablets incubated in medium containing rat caecal content was statistically compared with control samples using paired t-test using a statistical software package (Statistical Analysis System, SAS Institute, Inc., Cary, NC). Differences between each two related parameters were considered statistically significant for p-value equal to or less than 0.05.

Results and Discussion

MRM transitions

Two transitions were monitored for each 5-ALA (*m/z* 132.08) and TYR (*m/z* 182.04) parent ions to ensure selectivity and specificity. Each transition has its specific cone and collision voltages. The 5-ALA transitions were *m/z* 132.08 > 86.0 and *m/z* 132.08 > 114.0 with cone voltage of 20.0 V and collision energies of 12.0 and 10.0 V, respectively, while the cone voltage was set at 22.0 V for TYR transitions (*m/z* 182.04 > 91.2 and *m/z* 182.04 > 136.0), and their specific collision energies were 26.0 and 15.0 V, respectively. The dwell time was set at 0.1 s.

LC chromatogram parameters

During 1.0 min as chromatogram life, 5-ALA and TYR were eluted at 0.35 and 0.64 min, respectively, at the highest calibration level. The selectivity (α) of 5-ALA was 0.54 with respect to TYR peak. After smoothing of peaks (mean, 2 × 3), the resolution (R) between the two peaks, was 1.6, indicating a complete base to base separation, although in the LC–MS–MS method, the separation is not required for selectivity to be ascertained (Figure 2). Peak symmetry expressed as tailing factor was 1.05 for both peaks after smoothing.

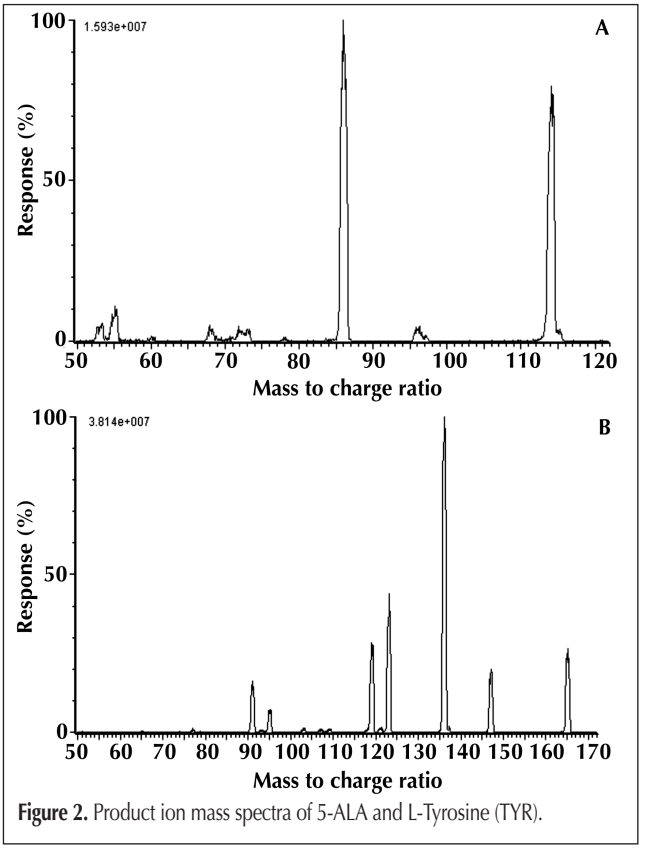

Figure 2. Product ion mass spectra of 5-ALA and L-Tyrosine (TYR).

Method validation

Sensitivity

Limit of quantitation (LOQ), of 5-ALA assay was 5.0 ppb that expresses 1.0% of release of 5-ALA 25 mg tablets in 1.0 L and after diluting 100 µL with 5 mL internal standard solution. LOQ was assessed by inter-day accuracy and precision of six replicate injections of QC samples at level of 5.0 ppb. Inter-day accuracy and precision were 98.7% and 1.9%, respectively.

Table I. Accuracy and Precision

	QC level (ppb)	Intra-day			
		Day 1	Day 2	Day 3	Mean
RSD (%)	10.0	1.7	2.3	1.8	1.9
Recovery (%)		99.3	95.3	101.4	98.7
RSD (%)	100.0	1.1	1.7	2.1	1.6
Recovery (%)		102.2	99.3	103.1	101.5
RSD (%)	1000.0	1.5	2.2	2.1	1.9
Recovery (%)		101.3	98.6	102.6	100.8
RSD (%)	2500.0	2.2	2.5	2.3	2.3
Recovery (%)		104.5	103.5	102.5	103.5

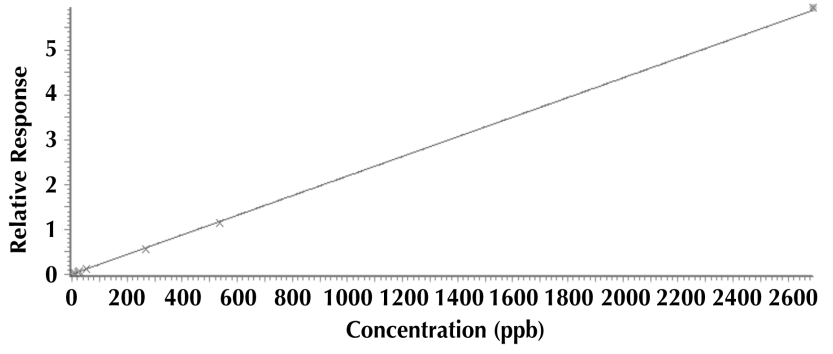

Figure 3. Typical calibration curve.

Linear calibration range

The calibration curve was generated using Waters Quanlynx Application Manager for Masslynx Software by plotting the peak area ratio of 5-ALA to TYR for each calibration solution. The linear regression was constructed from 5 to 2500 ppb, and a weighting of 1/x was applied. For all validation days, the calibration curves had an $r^2 > 0.998$ (Figure 3).

Accuracy and precision

Intra-day accuracy and precision was assessed by the analysis of six replicate injections of the four QC levels on three different days (Table I).

Carryover

The carryover effect of this UPLC method was assessed in triplicate runs of blank solution after run of highest calibrator solution. The noise signals in each blank chromatogram (at retention times of both 5-ALA and TYR), were expressed as the percentage of the absolute peak areas in the highest calibrator. These levels are negligible when compared with LOQ.

Stability of QC solution

Analysis of samples incubated at both 5 and 20°C did show a significant reduction in the concentration of 5-ALA. Moreover, the release study results confirmed the considerable stability of 5-ALA in solutions at 37°C (Table II) over the specified study period (6 h).

In-vitro release study

In order to avoid ion suppression of 5-ALA transitions and formation of stable sodium adduct, ammonium phosphate buffer was used instead of sodium phosphate buffer. Release study showed the capability of the method to detect very low concentrations of 5-ALA in medium containing 3% rat caecal contents suspended in ammonium phosphate buffer (pH 6.8).

Table II. Stability of QC Solutions

	Time interval (h)												Temp
QC level	0.5	1	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	(°C)
10 ppb	+2.0	+1.0	–2.2	–1.6	+1.3	–3.2	–2.3	+1.4	–2.2	–2.4	+2.2	–0.4	5
Residuals	–1.4	+2.3	+2.2	–1.7	+1.2	–0.1	+2.5	–2.0	+2.4	–3.1	–2.2	+4.4	20
(%)	–2.1	–3.2	+2.4	–2.2	–0.1	+1.6	+2.4	+1.3	+3.3	–1.4	–0.3	+1.2	37
100 ppb	–0.3	–2.6	+2.1	–3.6	–0.5	+1.7	+0.7	+1.2	–2.5	–4.2	+2.1	–3.2	5
Residuals	–2.5	–4.4	+3.5	–3.2	+0.9	–2.4	+3.1	–2.2	–3.4	+2.1	–2.0	–2.1	20
(%)	–0.4	+2.1	–2.1	–4.5	–0.7	+1.2	–0.3	+2.1	–3.2	+1.2	+1.6	–2.3	37
1000 ppb	+2.1	–0.5	+2.3	+1.3	+2.7	–2.1	–0.6	–1.8	–2.4	+1.5	+1.2	–4.3	5
Residuals	–3.2	–4.3	–0.5	+1.2	–4.5	–0.9	+2.3	–3.2	–3.4	–1.9	+2.1	+1.4	20
(%)	–4.2	–2.6	–5.2	–3.4	–3.2	+1.3	–5.6	–4.7	–2.8	–3.2	–2.5	–4.8	37
2500 ppb	–1.2	–2.1	+1.6	–3.7	–2.3	–3.1	–2.6	–2.5	–3.4	–2.3	+1.4	–2.3	5
Residuals	–2.3	–3.1	–0.9	–3.1	+2.3	–2.8	–2.7	+2.7	–4.5	–2.3	–0.7	+2.1	20
(%)	–2.4	–3.2	–4.1	–3.5	–4.2	+2.4	–3.2	–2.2	–1.4	–3.2	–2.2	–4.1	37

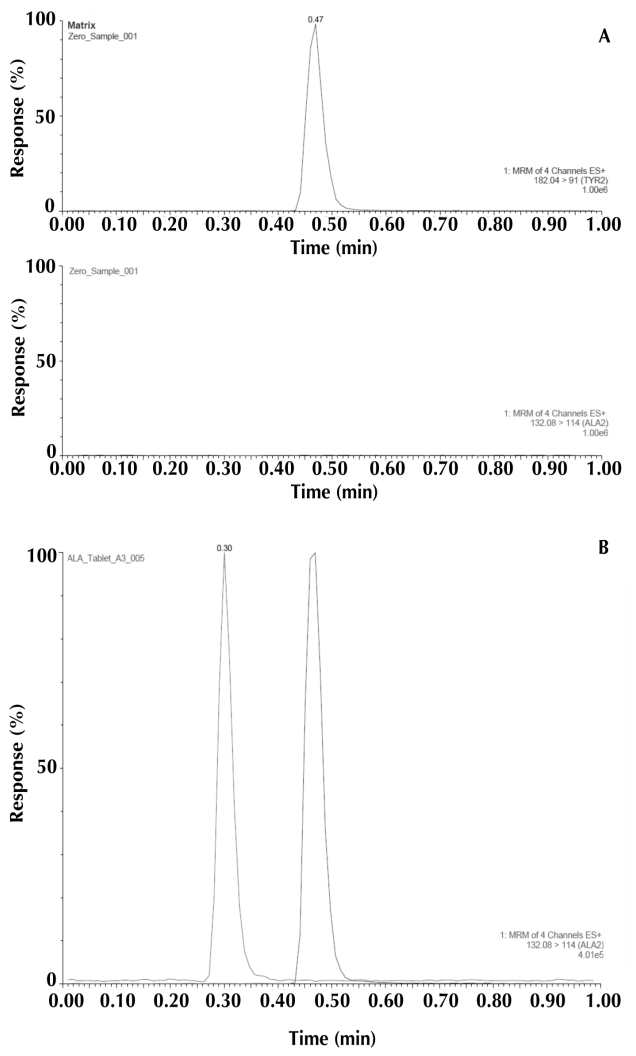

Figure 4. chromatogram of rat cecal medium at zero time after spiking with TYR (IS) (A) and ALA tablet chromatography after three-hours dissolution in rat cecal medium (B).

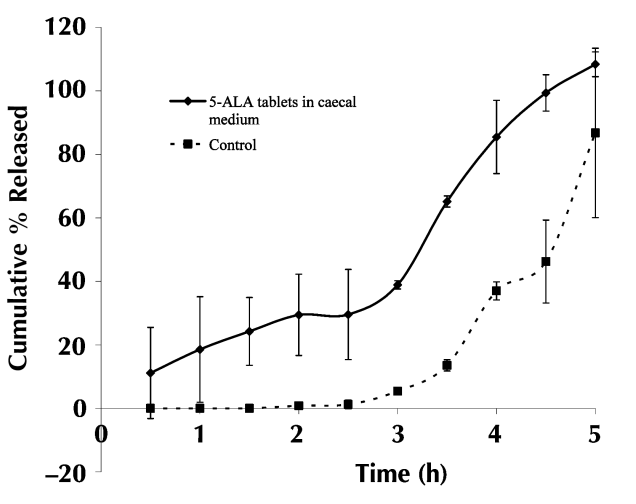

Figure 5. The release profile of 5-ALA from a colonic tablet in phosphate buffer containing 3% rat caecal contents under anaerobic condition compared with the release in a medium free of rat caecal contents under the same conditions as control.

All the peak characteristics remained unchanged compared with that of the standard curve medium. Figure 4 showed the chromatogram of rat cecal medium at zero time after spiking with IS (Figure 4A) and the 3 h sample (Figure 4B). It is clear that the rat cecal medium did not show any peaks and almost appeared as a plain line. In addition, the medium did not affect all the characteristics of the IS peak especially no significant change in the peak area. All the other samples showed the presence of the characteristic 5-ALA peak with increasing concentration as the sampling time increases (Figure 4B).

Figure 5 presents a comparison between the release profile of 5-ALA from the prepared colonic tablets in presence and absence of rat caecal contents. It is clear that in presence of rat caecal contents, a biphasic release profile was exhibited showing a slow release in the first period (up to 2.5 h) followed by a faster release rate till the end of study. This can be attributed to the slow diffusion of 5-ALA through the coat layer in first period while the partial disintegration of the coat layer as a result of enzymatic degradation led to the formation of wide pores in the coat layer and consequently a faster release rate. The release from the control tablets showed different profile with lag time of 2.5 h followed by a gradual release with incomplete release during the 5 h course of study. The difference was statistically significant at all time points; expect samples taken at 0.5, 1.0, and 5.0 h ($p \leq 0.05$). This indicates that the release of 5-ALA from prepared colonic system is highly triggered by the local environment of the colon.

Conclusion

The validated assay method using UPLC–MS–MS provides an efficient means for the determination of the in-vitro release profile of 5-ALA in medium containing suspended rat caecal contents under anaerobic conditions as the standard medium used for in-vitro evaluation of colon drug delivery systems. The developed method provides a quick, simple, reproducible, and sensitive assay for quantifying 5-ALA at low concentration with high accuracy. The method can also be readily adapted to routine quality control analysis with no need to further derivatization procedures. Comparison with previously reported assay method, this method is simple, faster and more specific than existing methods for analyzing 5-ALA. The overall efficiency and simplicity of the method, plus the absence of interference encourage the application of this method as a powerful tool for evaluating 5-ALA in medium containing suspended rat caecal contents.

Acknowledgements

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